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RESEARCH ARTICLE

Generation of CRISPR/Cas9-mediated gene-targeted pigs via somatic cell nuclear transfer

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Abstract The domestic pig has been widely used as an important large animal model. Precise and efficient genetic modification in pig provides a great promise in biomedical research. Recently, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system has been successfully used to produce many gene-targeted animals. However, these animals have been generated by co-injection of Cas9 mRNA and single-guide RNA (sgRNA) into one-cell stage embryos, which mostly resulted in mosaicism of the modification. One or two rounds of further breeding should be performed to obtain homozygotes with identical genotype and phenotype. To address this issue, gene-targeted somatic cells can be used as donor for somatic cell nuclear transfer (SCNT) to produce gene-targeted animals with single and identical mutations. In this study, we applied Cas9/sgRNAs to effectively direct gene editing in porcine fetal fibroblasts and then mutant cell colonies were used as donor to generate homozygous gene-targeted pigs through single round of SCNT. As a result, we successfully obtained 15 tyrosinase (*TYR*) biallelic mutant pigs and 20 *PARK2* and *PINK1* double-gene knockout (KO) pigs. They were all homozygous and no off-target mutagenesis was detected by comprehensive analysis. $TYR^{-/-}$ pigs showed typical albinism and the expression of parkin and PINK1 were depleted in $PARK2^{-/-}/PINK1^{-/-}$ pigs. The results demonstrated that single- or double-gene targeted pigs can be effectively achieved by using the CRISPR/Cas9 system combined with SCNT without mosaic mutation and detectable off-target effects. This gene-editing system provides an efficient, rapid, and less costly manner to generate genetically modified pigs or other large animals.

Keywords Albinism · Animal model · Genome engineering · Parkinson's disease

Abbreviations

Cas	CRISPR-associated nuclease
CRISPR	Clustered regularly interspaced short palindromic repeats
KO	Knockout
OTS	Off-target site
PFF	Porcine fetal fibroblast
PINK1	PTEN-induced putative kinase 1
SCNT	Somatic cell nuclear transfer
sgRNA	Single-guide RNA
TYR	Tyrosinase
WT	Wild type

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Introduction

Genetically modified pigs have many applications in biomedical and agricultural research. They have been commonly used to model various human diseases and develop therapeutic strategies. CRISPR/Cas9 system as a new gene-editing technique, found in bacteria and archaea, has been successfully used to create many gene-targeted animals, such as mouse, rat, and monkey, because of its high efficiency and availability [1–8]. Its worth noting that these animals have been generated by co-injection of Cas9 mRNA and sgRNA into one-cell stage embryos. Double-strand break and non-homologous end joining mediated by Cas9 mRNA and sgRNA occur in the target locus probabilistically; as a result, mosaic mutation and numerous alleles in target site are formed, which would complicate subsequent studies. Therefore, many of the resulting founder animals are chimeric with multiple mutations. To establish animals with single mutation, one or two rounds of further breeding and selection need to be performed among the offspring, but this procedure is not easily applicable in large animals, such as pigs, because they have long gestation cycles and entail high recipient costs. In addition, the founders without germline mutation or mosaicism would be useless in further breeding and therefore related research. Hence, we aimed to target genes in somatic cells by applying CRISPR/Cas9 technology and then perform SCNT to produce knockout pigs in this study. CRISPR/Cas9 system-mediated gene editing in porcine fetal fibroblasts (PFFs) has been reported by Tan et al. [9]. The efforts that used mutant cells as donor nuclei to generate gene-targeted pigs carrying stable and identical mutations have not been followed.

Here, we aimed to investigate the feasibility of generating gene-targeted pigs by targeting genes in somatic cells via CRISPR/Cas9 system and SCNT approach. We initially targeted a gene, *TYR*, encoding tyrosinase, which is a key enzyme involved in melanin biosynthetic pathway. The genetic deficiency of *TYR* gene in human can cause oculocutaneous albinism type 1 (OCA1), an autosomal recessive disorder characterized by reduced melanin pigment in the hair, skin, and eyes [10, 11]. CRISPR/Cas9 system can also be used to target multiple genes in cells and embryos with high efficiency [2, 5, 12]. As such, we also aimed to target two genes in the same somatic cell simultaneously. We chose *PARK2* and *PINK1* genes as the loci of interest. *PARK2* gene encodes a protein called parkin, a component of multiprotein E3 ubiquitin ligase complex. *PINK1* gene encodes PTEN-induced putative kinase 1, a mitochondrial serine/threonine-protein kinase. The mutation of either of these two genes would lead to autosomal recessive early-onset Parkinson's disease in humans [13, 14].

Materials and methods

Animals

The animal use protocol complied with the guidelines of Institutional Animal Care and Use Committee of Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences (Animal Welfare Assurance #A5748-01). All surgical procedures were performed under anesthesia, and all efforts were made to minimize animal suffering.

Construction of Cas9 and sgRNA vectors

CMV promoter-driven Cas9 (41815) and Cas9-nickase (Cas9n, 41816) plasmids were purchased from Addgene. U6-sgRNA cloning vector was constructed by introducing 2 *Bbs*I restriction sites to the downstream region of U6 promoter of plasmid gRNA_GFP-T1 (41819, Addgene). Targeting sgRNA was designed by GN20GG rule [15, 16]. Two complementary oligo DNA of sgRNA were synthesized and then annealed to a double-strand DNA, ligated to the *Bbs*I sites of U6-sgRNA cloning vector to form sgRNA expressing plasmid. These constructs were confirmed by sequence analysis (BGI, Guangzhou, China).

PFFs cell culture, transfection and selection

Porcine fetal fibroblasts were isolated from 35-day-old fetuses of two Chinese indigenous strains, called the Banna mini-pig and Bama mini-pig. The fetuses, removed heads, tails, limbs and viscera, were digested in cell culture medium containing 0.32 mg/mL collagenase IV and 2,500 IU/mL DNase I for 3 h at 39 °C. Isolated PFFs were then cultured in 10 cm dishes for 12 h and frozen in fetal bovine serum (FBS) containing 10 % dimethylsulfoxide. A day before transfection, PFFs were thawed and cultured to sub-confluency. Then, approximately 1×10^6 PFFs were electroporated with vectors of Cas9 and sgRNA at 230 μV and 500 μF using a Gene Pulse Xcell electroporator (Bio-Rad, Hercules, CA, USA). After 24 h of recovery, the electroporated cells were selected with G418 at appropriate concentration for about 10 days. Individual cell colonies were picked up and cultured in 48-well plates. When confluency was reached, the cell colonies were subcultured and 10 % of each colony was lysed individually in 15 μL of lysis buffer (0.45 % NP-40 plus 0.6 % Proteinase K) for 90 min at 56 °C and then 10 min at 95 °C. The lysate was used as template for PCR. The primers were designed to amplify across the target sites. For *TRY* gene, the forward primer was 5′-CCTGATGGAGAAGGAATGCTGC-3′ and reverse primer was 5′-TTGGCCATAGGTGCCTGTG-3′. For *PINK1* and *PARK2* gene, PCR were performed using

PINK1 and *PARK2* specific primers, respectively, forward primer 5′-GTTTGCCTGGTAGCCAAGGA-3′ and reverse primer 5′-CTTCACTCGTCAGCGACCTCA-3′ for *PINK1*; forward primer 5′-GGTGAGGGGTAAATCAGT TCA-3′ and reverse primer 5′-CAGCAGAAGCAGCATA AGCA-3′ for *PARK2*. The PCR conditions were 94 °C for 5 min; 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, for 35 cycles; 72 °C for 5 min; hold at 16 °C. The PCR products were sequenced to identify the existence of the mutation. Some PCR products were selected to ligate into a pMD18-T vector (Takara, Dalian, China) and sequenced to determine the exact mutant sequences. The positive cell colonies were expanded and then cryopreserved. These cells were thawed and cultured to reach sub-confluency in a 24- or 12-well plate before SCNT.

SCNT and production of mutant piglets

Methods used for porcine oocyte collection, in vitro maturation and SCNT were similar to our previous studies [17–19]. Briefly, cumulus oocyte complexes (COCs) were selected and cultured in maturation medium for 42–44 h at 39 °C. Matured oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm with a glass pipette in a manipulation medium of HEPES-buffered M199 plus cytochalasin B (7.5 μg/mL). The cells identified as gene knockout by sequencing were used as donor cells to be injected into the perivitelline space of the oocytes. Fusion and activation were performed with two successive DC pulses at 1.2 kV/cm for 30 μs using an electrofusion instrument. The reconstructed embryos were cultured in PZM3 at 39 °C overnight and surgically transferred to the oviducts of the surrogates that were observed in estrus the day before. The pregnancy status was monitored by ultrasonography 24 days after embryo transfer. Once detected, the pregnancy status was monitored weekly until delivery. The cloned piglets were delivered by natural birth. The genomic DNA that was isolated from the ear skin biopsy of the newborn cloned piglets was assessed for mutagenesis at the targeted site by PCR-based assays.

Histology and immunofluorescence staining

The tissues of skin and eyes from $TYR^{-/-}$ pigs, brains from $PARK2^{-/-}/PINK1^{-/-}$ pigs were fixed with 4 % buffered paraformaldehyde for 48 h. The entire tissue was then dissected, embedded in paraffin wax, and exhaustively sectioned at 3 μM to a slide. Sections were deparaffinized in xylene and rehydrated in a graded series of alcohol, followed by dH_2O. Sections of skin and eyes from $TYR^{-/-}$ pigs were stained with hematoxylin and eosin, then differentiated, and coverslipped. Immunofluorescence staining was performed on sections of brain cortex from $PARK2^{-/-}/PINK1^{-/-}$ pigs. Briefly, antigen retrieval was performed in a 1 M citrate buffer (pH 6.0) bath for 20 min. Tissues were incubated with primary antibodies (rabbit anti-Parkin polyclonal, AB9244, Millipore, Temecula, CA, USA; anti-PINK1 antibody, ab23707, Abcam, Cambridge, UK) overnight in a humid chamber at 4 °C. The tissues were washed thrice with PBS and incubated for 1 h with a Alexa Fluor 594-labeled goat anti-rabbit IgG (H + L) secondary antibody (A-11012, Invitrogen, Eugene, OR, USA) and the slides were analyzed by fluorescence microscopy (Olympus, Tokyo, Japan).

Off-target analysis

Off-target cleavage sites were predicted and searched as previously described [2]. All potential off-target sites (OTS) with homology to 23 bp sequence (sgRNA + PAM) were retrieved by a base-by-base scan of the whole pig genome, allowing for ungapped alignment with up to 6 mismatches in the sgRNA target sequence. All the potential OTS were PCR amplified and sequenced to confirm the off-target effects, respectively. The primers for amplifying the off-target sites are listed in Supplemental Table 1.

Results

CRISPR/Cas9-mediated gene targeting in PFF cells

For *PARK2* and *PINK1* genes, sgRNAs were designed based on the original Cas9 approach [15, 16]. *PARK2*-sgRNA and *PINK1*-sgRNA target the first exon region following the start codon, respectively (Fig. 1a). For the *TYR* gene, a method based on modified Cas9 called Cas9-nickase was used to design sgRNAs [20]. Cas9-nickase harbors a D10A mutation and can only nick one strand of double-stranded DNA. Double nicks are created when a pair of sgRNA functions with Cas9-nickase, consequently cutting the double strands of the target DNA. Cas9-nickase-based approach could reduce the risk of off-target and inflict less damage to the integrity of a genome [20]. Figure 1a shows the sgRNA pair targeting the *TYR* gene exon 1 region.

To target *PARK2* and *PINK1* genes, we co-transfected Cas9, *PARK2*-sgRNA, and *PINK1*-sgRNA expressing vectors into PFFs derived from a 35-day-old fetus by electroporation. To target the *TYR* gene, we co-transfected Cas9-nickase and two *TYR*-sgRNAs encoding vectors into PFFs. After viable cells were selected using G418 for approximately 10 days, surviving cell colonies were collected and then analyzed individually by sequencing PCR products covering the target locus. The mutation rates among the selected cell colonies at the target loci were

A

TYR locus (Chr 9)

sgRNA2

GCACCCCTGGGACCTCAGTTCCCCTTCACCGGGGTGGATGAACGGGAGTCTTGGCCCT
CGTGGGGACCCTGGAGTCAAGGGGAAGTGGCCCCACCTACTTGCCCTCAGAACCGGGA

sgRNA1

PARK2 locus (Chr 1)

sgRNA

CCATTTGCGTGTGATTTTCGCAGGGAAGGAGCTTAGGAATGACTTGACAGTGCAG
GGTAAACGCACACTAAAAGCGTCCCTTCCTCGAATCCTTACTGAACTGTCACGTC

PINK1 locus (Chr 6)

sgRNA

TGCCTGGCGTCTGCCACACACAGCCAGGGGCTGCTCCAGGGCCTGCTGCGGATGT
ACGGACCGCAGACGGTGTGTGTCGGTCCCCGACGAGGTCCCGGACGACGCCTACA

B

TYR mutant colonies

	CCCTGGGACCTCAGTTCCCCTTCACCGGGGTGGATGAACGGGAGTCTTGG	WT
T5	CCCTGGGACCTC----------TTGG CCCT----------TGG	Δ34/Δ43
T12	CCCT----------TCACCGGGGTGGATGAACGGGAGTCTTGG	Δ17/Δ17
T47	CCCT----------//- ACATggta//tcag----------GTCTTGG	Δ50/Δ60,+102
T65	CCCTGGGACC----------GGGAGTCTTGG CCCTGGGACCTCAGT----------CTTGG	Δ29/Δ30
T70	CCCTGGGACCa----------GGGTGGATGAACGGGAGTCTTGG	Δ17,+1/Δ17,+1
T79	CCCTGGGACCTC----------GATGAACGGGAGTCTTGG AGGA----------//-	Δ20/Δ67

PARK2/*PINK1* double mutant colonies

Colonies	*PARK2*	Indel	*PINK1*	Indel
	CGTGTGATTTTCGCAGGGAAGGAGCTT	WT	GGCGTCTGCCACACACAGCCAGGGGCT	WT
P3	CGTGTGATTTTCGCAGG-AAGGAGCTT CGTGTGATTTTCGCAGG-----AGCTT	Δ1/Δ5	GGCGTCTGCCACACAC-tGCCAGGGGCT	Δ1,+1/Δ1,+1
P14	CGTGTGATTTTCGC-GGGAAGGAGCTT CGTGTGATTTTCGCA--GAAGGAGCTT	Δ1/Δ2	GGC----------GCCAGGGGCT	Δ14/Δ14
P17	CGTGTGATTTTC-----GAAGGAGCTT	Δ5/Δ5	GGC----------AGCCAGGGGCT	Δ13/Δ13
P19	CGTGTGATTTTCGCA-GGAAGGAGCTT	Δ1/Δ1	GGCGTCTGCCACACACA-CCAGGGGCT	Δ1/Δ1
P24	CGTGTGATTTTC----------CTT CGTGTGA-------AGG--AGGAGCTT	Δ12/Δ9	GGCGTCTGCCACACACA-CCAGGGGCT GGCGTCTGCCACACACA--CA-GGGCT	Δ1/Δ3
P39	CGTGTGATTTTC----GGAAGGAGCTT	Δ4/Δ4	GGCGTCTGCCACACACAAGCCAGGGGCT	+1/+1
P45	CGTGTGATTTTC---GGGAAGGAGCTT CGTGTGATTTTC----------CTT	Δ3/Δ12	GGCGTCTGCCACACACA-CCAGGGGCT GGCGTCTGCCACACACA--CA-GGGCT	Δ1/Δ3
P51	CGTGTGATTTTC-------AGGAGCTT	Δ8/Δ8	GGCGTCTGCC----------AGGGGCT	Δ10/Δ10
P58	CGTGTGATTTTCGCAGGGgAAGGAGCT T -//----------TT	+1/Δ42	GGCGTCTGCCACACAC-tGCCAGGGGCT	Δ1,+1/Δ1,+1
P67	CGTGTGATTTTCGCAGG-AAGGAGCTT CGTGTGATTTTCGCAGG-----AGCTT	Δ1/Δ5	GGCGTCTGCCACACACA-CCAGGGGCT GGCGTCTGCCACA---------GGGCT	Δ1/Δ9
P73	CGTGTGATTTTCGC-GGGAAGGAGCTT	Δ1/Δ1	GGC----------GGGCT	Δ19/Δ19
P80	CGTGTGATTTTCGCAGG-AAGGAGCTT CGTGTGATTTT----------//-	Δ1/Δ16	GGCGTCTGCCACACACaAGCCAGGGGCT	+1/+1
P108	CGTGTGATTTTCGCAGG-AAGGAGCTT C----------TT	Δ1/Δ24	GGCGTCTGCCACACACA-CCAGGGGCT	Δ1/Δ1
P115	CGTGTGATTTTCGCAGG-AAGGAGCTT	Δ1/Δ1	GGCGTCTGCCACACACA-CCAGGGGCT	Δ1/Δ1
P121	CGTGT----------GGGAAGGAGCTT	Δ10/Δ10	-//----------GGCT GG----------AGGGGCT	Δ43/Δ18

Fig. 1 CRISPR/Cas9 mediates gene targeting in PFFs. **a** Schematic of sgRNAs targeting *TYR*, *PARK2* and *PINK1* loci. The target loci are located in the first exon following the start codon of these three genes. sgRNA targeting sites are highlighted in *red*. PAM are highlighted in *blue* and *underlined*. **b** Sanger sequencing of the targeting sites in mutant colonies used in SCNT. For each gene, the wile-type sequence is shown at the *top* with the target sites highlighted in *red*. The net change in length caused by each mutation is to the *right* of each sequence (+, insertion; Δ, deletion). *Lowercase letters* denote inserted base pairs. PAM is highlighted in *blue*. *Indel* insertion or/and deletion

49.4, 66.7, and 69.9 % for *TYR*-sgRNAs, *PARK2*-sgRNA and *PINK1*-sgRNA, respectively (Table 1). Cas9 and Cas9-nickase can both be used efficiently to generate KO cell colonies. Cas9-nickase was slightly less efficient in targeting genes possibly because of the lower co-transfection efficiency of three plasmids when compared with using Cas9. Mutation patterns remarkably varied among the positive colonies (data not shown). The percentage of cell colonies containing double homozygous indels of sequences in *PARK2* and *PINK1* genes was up to 38.1 % (4 mutant alleles shown in Table 1).

Generation of gene-targeted pigs via SCNT

Six $TYR^{-/-}$ cell colonies and 15 $PARK2^{-/-}/PINK1^{-/-}$ double-KO cell colonies, containing deletions from 1 to 60 bp and insertions from 1 to 102 bp (Fig. 1b), were chosen as donors for SCNT. We usually pooled 3–6 colonies in one nuclear transfer and the reconstructed embryos from the pooled cells were transferred to a surrogate simultaneously (Table 2). A total of 1,705 reconstructed embryos derived from $TYR^{-/-}$ cells were introduced to 7 surrogate mothers. Among these surrogate mothers 4 developed to term and gave birth to 18 female cloned piglets (Fig. 2a; Table 2). Among the 18 piglets, 8 were weak at birth and died in 2 weeks. The remaining piglets appeared healthy (Table 3). A total of 1,729 reconstructed embryos derived from $PARK2^{-/-}/PINK1^{-/-}$ double-KO cells were introduced to 10 surrogate mothers. Among these surrogate mothers, 4 developed to term and gave birth to 20 cloned piglets (Fig. 2a; Table 2). Among these 20 piglets, 2 were stillborn, 7 were weak at birth and died in 2 weeks. Five piglets were born healthy but also died 1 month after birth. The remaining piglets developed normally (Table 3).

DNA sequencing analysis results revealed that 15 of the $TYR^{-/-}$ newborns and 20 $PARK2^{-/-}/PINK1^{-/-}$ newborns carried the expected homozygous mutations at the target loci (Fig. 2b). Among the $TYR^{-/-}$ piglets and $PARK2^{-/-}/PINK1^{-/-}$ piglets, various types of mutations corresponding to the donor cells were found at the target loci, including deletions from 1 to 60 bp and insertions from 1

Table 1 CRISPR/Cas9-mediated gene targeting in PFFs

Target genes	Mutant alleles per colony/total colonies tested				
	4	3	2	1	0
TYR	–	–	18/87 (20.7 %)	25/87 (28.7 %)	44/87 (50.6 %)
PINK1	–	–	82/126 (65.1 %)	6/126 (4.8 %)	38/126 (30.2 %)
PARK2	–	–	80/126 (63.5 %)	4/126 (3.2 %)	42/126 (33.3 %)
PINK1 + *PARK2*	48/126 (38.1 %)	34/126 (27 %)	6/126 (4.8 %)	4/126 (3.2 %)	34/126 (27.0 %)

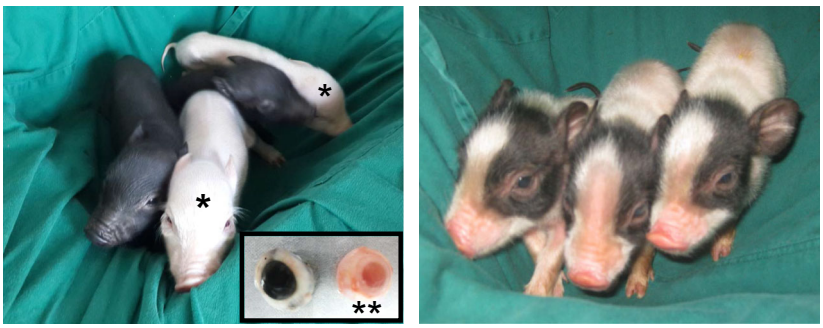
Plasmids encoding Cas9 and sgRNAs targeting *TYR*, *PINK1* and *PARK2* genes were transfected separately (single targeting) or in a pool (double targeting) into PFFs. The number of total alleles mutated in each PFF cell colony is listed from 0 to 2 for single-targeting test experiment, and 0 to 4 for double-targeting test experiment

Table 2 Somatic cell nuclear transfer results for the generation of knockout cloned pigs

Target gene	Cell pool	Transferred embryos	No. recipients	No. (%) pregnancies	No. born	No. (%) mutants
TYR	T65, T70, T79	266	1	0	–	–
	T5, T12, T47	423	2	2	10	10
	T5, T70, T79	415	2	1	4	2
	T5, T47, T65, T70	359	1	1	4	3
	T12, T65, T70, T79	242	1	0	–	–
Total		1,705	7	4 (57.1)	18	15 (83.3)

Target genes	Cell pool	Transferred embryos	No. recipients	No. (%) pregnancies	No. born	No. (%) *PARK2* mutants	No. (%) *PINK1* mutants
PARK2/*PINK1*	P3, P14, P39, P67, P121	348	2	1	4	4	4
	P3, P17, P19, P39, P24, P45	275	2	1	5	5	5
	P3, P14, P45, P115	294	2	0	–	–	–
	P45, P51, P58, P73, P115	419	2	1	4	4	4
	P19, P80, P108, P115	393	2	1	7	3	3
Total		1,729	10	4 (40)	20	20 (100)	20 (100)

A



B *TYR*$^{-/-}$ piglets

Piglet ID	Genotypes	Indel
	CCCTGGGACCTCAGTTCCCCTTCACCGGGGTGGATGAACGGGAGTCTTGG	WT
TYR-1	CCCTGGGACCTCAGTTCCCCTTCACCGGGGTGGATGAACGGGAGccg//ctcGT ACATggta//tcag----------GTCTTGG	+188/Δ60,+102
TYR-2, 3, 4, 7, 10, 12, 13, 15	CCCTGGGACCTC----------TTGG	Δ34/Δ34
TYR-5, 6, 11	CCCTGGGACCTC----------TTGG CCCT----------TGG	Δ34/Δ43
TYR-14	CCCT----------//-	Δ50/Δ50
TYR-16	CCCTGGGACC----------GGGAGTCTTGG CCCTGGGACCTCAGT----------CTTGG	Δ29/Δ30
TYR-17	CCCTGGGACCA----------GGGTGGATGAACGGGAGTCTTGG	Δ17,+1/Δ17,+1

PARK2$^{-/-}$/*PINK1*$^{-/-}$ piglets

Piglet ID	*PARK2* genotypes	Indel	*PINK1* genotypes	Indel
	CGTGTGATTTTCGCAGGGAAGGAGCTT	WT	GGCGTCTGCCACACACAGCCAGGGGCT	WT
PD-1	CGTGT----------GGGAAGGAGCTT	Δ10/Δ10	-//----------GGCT GG----------AGGGGCT	Δ43/Δ18
PD-2	CGTGTGATTTTCGCAGG-AAGGAGCTT CGTGTGATTTTCGCAGG-----AGCTT	Δ1/Δ5	GGCGTCTGCCACACAC-tGCCAGGGGCT	Δ1,+1/Δ1,+1
PD-3	CGTGTGATTTTCGC-GGGAAGGAGCTT CGTGTGATTTTCGCA--GAAGGAGCTT	Δ1/Δ2	GGC----------GCCAGGGGCT	Δ14/Δ14
PD-4	CGTGTGATTTTCGCAGG-AAGGAGCTT CGTGTGATTTTCGCAGG-----AGCTT	Δ1/Δ5	GGCGTCTGCCACACACA-CCAGGGGCT GGCGTCTGCCACA---------GGGCT	Δ1/Δ9
PD-5	CGTGTGATTTTCGCA-GGAAGGAGCTT	Δ1/Δ1	GGCGTCTGCCACACACA-CCAGGGGCT	Δ1/Δ1
PD-6	CGTGTGATTTTC----------CTT CGTGTGA-------AGG--AGGAGCTT	Δ12/Δ9	GGCGTCTGCCACACACA-CCAGGGGCT GGCGTCTGCCACACACA--CA-GGGCT	Δ1/Δ3
PD-7,9	CGTGTGATTTTC----GGAAGGAGCTT	Δ4/Δ4	GGCGTCTGCCACACACaAGCCAGGGGCT	+1/+1
PD-8	CGTGTGATTTTC---GGGAAGGAGCTT CGTGTGATTTTC----------CTT	Δ3/Δ12	GGCGTCTGCCACACACA-CCAGGGGCT GGCGTCTGCCACACACA--CA-GGGCT	Δ1/Δ3
PD-10,11,12	CGTGTGATTTTCGCAGGGgAAGGAGCTT -//----------TT	+1/Δ42	GGCGTCTGCCACACAC-tGCCAGGGGCT	Δ1,+1/Δ1,+1
PD-13	CGTGTGATTTTCGCAGG-AAGGAGCTT	Δ1/Δ1	GGCGTCTGCCACACACA-CCAGGGGCT GGCGTCTGCCACACACA--CA-GGGCT	Δ1/Δ3
PD-14,15,19	CGTGTGATTTTCGCAGG-AAGGAGCTT	Δ1/Δ1	GGCGTCTGCCACACACaAGCCAGGGGCT	+1/+1
PD-16,17	CGTGTGATTTTCGCAGG-AAGGAGCTT CGTGTGATTTT----------//-	Δ1/Δ16	GGCGTCTGCCACACACaAGCCAGGGGCT	+1/+1
PD-18,20	CGTGTGATTTTCGCAGG-AAGGAGCTT C----------TT	Δ1/Δ24	GGCGTCTGCCACACACA-CCAGGGGCT	Δ1/Δ1

Fig. 2 Generation of gene-targeted piglets via SCNT. **a** Newborn piglets carrying *TYR* gene mutation (*left panel*) and *PARK2*/*PINK1* double mutations (*right panel*). The *left panel* shows four newborn piglets from the same litter; the white piglets are *TYR* deficient exhibiting a typical albinism phenotype (*asterisk*) and the black piglets are wild-type littermates. The *bottom right box* shows the light pink-tinted iris and pupil of the *TYR* KO piglets (*double asterisk*) compared with the dark iris of WT piglets. The *right panel* shows *PARK2*/*PINK1* double-KO piglets. **b** Sanger sequencing of the target sites in all cloned pigs. For each gene, the wild-type (WT) sequence is shown at the *top* in which the target sites are highlighted in *red*. *Right-most column* shows the indel of each sequence. TYR-8, 9 and 18 are WT. Deletion and insertion are denoted as "Δ" and "+" plus the number of base pairs. PAM is highlighted in *blue* and *lowercase letters* indicate inserted base pairs

Table 3 Summary of knockout pigs for their health status and origin donor cells

Piglet ID	Gestation length (days)	Birth weight (g)	Health status	Donor cell
TYR-1	115	560	Dead at 2 days	T47
TYR-2	115	540	Dead at 3 days	T5
TYR-3	115	640	Dead at 33 days	T5
TYR-4	115	610	Dead at 33 days	T5
TYR-5	115	380	Dead at 4 days	T5
TYR-6	116	570	Dead at 2 days	T5
TYR-7	116	510	Dead at 14 days	T5
TYR-10	116	350	Dead at 1 day	T5
TYR-11	115	690	Alive	T5
TYR-12	115	590	Alive	T5
TYR-13	115	610	Alive	T5
TYR-14	115	510	Alive	T47
TYR-15	114	320	Dead at 7 days	T5
TYR-16	114	380	Dead at 7 days	T65
TYR-17	114	430	Dead at 41 days	T70
PD-1	119	1,460	Alive	P121
PD-2	119	870	Dead at 2 days	P3
PD-3	119	930	Alive	P14
PD-4	119	850	Dead at 1 day	P67
PD-5	121	790	Dead at 10 days	P19
PD-6	121	730	Dead at 19 days	P24
PD-7	121	970	Dead at 26 days	P39
PD-8	121	950	Dead at 31 days	P45
PD-9	121	690	Dead at 2 days	P39
PD-10	120	950	Alive	P58
PD-11	120	870	Alive	P58
PD-12	120	790	Alive	P58
PD-13	120	560	Stillborn	ND*
PD-14	122	620	Dead at 2 days	ND*
PD-15	122	940	Alive	ND*
PD-16	122	470	Dead at 29 days	P80
PD-17	122	490	Dead at 20 days	P80
PD-18	122	310	Dead at 6 days	P108
PD-19	122	320	Dead at 9 days	ND*
PD-20	122	230	Stillborn	P108

ND not determined

* These donor cells may account for a very low percentage in some colonies, thus are not able to be distinguished through sequencing

to 102 bp (Figs. 1b, 2b). The mutation pattern in four of the piglets was not found among the donor cell lines, which may be caused by the mixed cells (Table 3). As the same reason, there were three unexpected WT pigs due to impure colonies that contain few WT cells (Table 2).

Phenotype identification of gene-targeted pigs

We further examined whether gene mutations cause a depletion of protein expression or related phenotypes. The *TYR* biallelic KO pigs showed typical albinism. The dark pigment in skin, hair, and eyes were completely lost in these *TYR* biallelic KO pigs; whereas the wild-type (WT) littermates were black in the same tissues (Fig. 2a). Melanin was present in WT piglets but was confirmed absent by histologically analyzing the sections of the skins and irides of dead *TYR* biallelic KO piglets with albinism (Fig. 3a, b). These results indicated that tyrosinase and its downstream products were eliminated completely in *TYR* biallelic KO pigs. For *PINK1* and *PARK2* double-KO piglets, immunofluorescence analysis was performed to determine the presence of the two proteins. PINK1 and parkin proteins were expressed in the brain neurons of WT pigs but were undetectable in

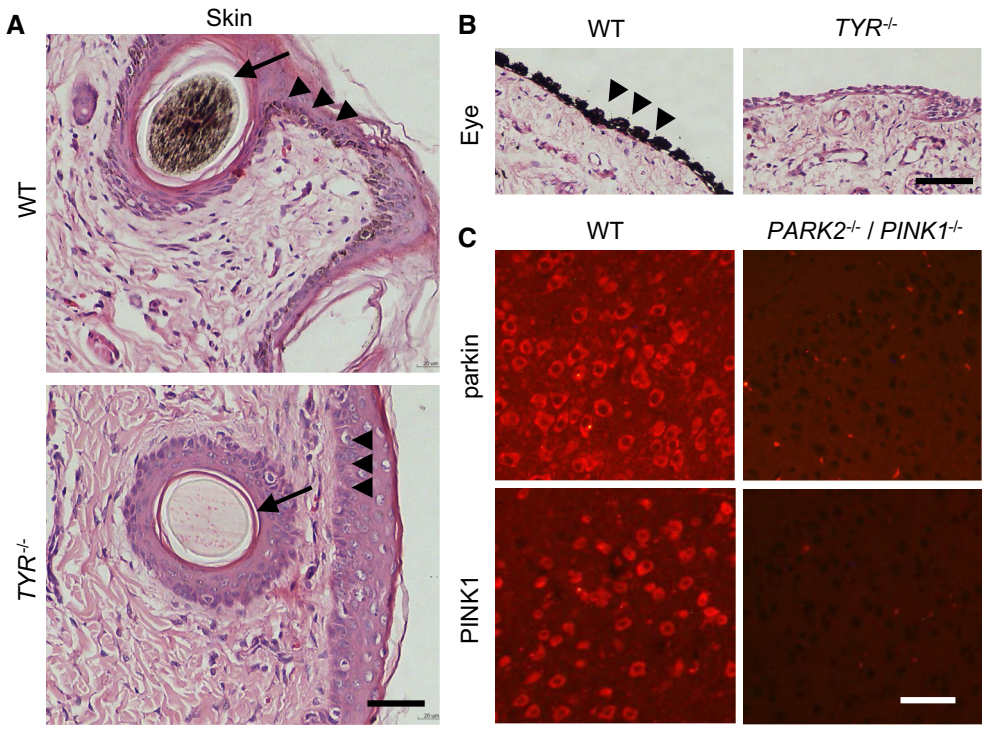


Fig. 3 Phenotype identification of $TYR^{-/-}$ and $PARK2^{-/-}/PINK1^{-/-}$ piglets. **a** H&E staining of the skin of WT and *TYR* KO piglets. The *arrowheads* indicate the melanin in the basal layer of the epidermis. The *arrows* point to the cross-sections of hair strands with different colors from *TYR* KO pig and WT pig. **b** H&E staining of irides of WT and *TYR* KO piglets. The *arrowheads* indicate the melanin in the iris of the WT piglets. **c** Parkin and PINK1 immunofluorescence analysis in the brain cortex sections of WT and *PARK2*/*PINK1* double-KO piglets. The cortex neurons of KO pigs are negative for anti-parkin and anti-PINK1 staining; by contrast, the WT controls show positive staining in the brain neurons. *Scale bars* 50 μm

$PARK2^{-/-}/PINK1^{-/-}$ pigs. This result confirmed that parkin and PINK1 were depleted at a protein level (Fig. 3c). Thus far, typical symptoms of Parkinson's disease, such as shaking, rigidity, slowness of movement, and difficulty in walking, have not been observed in 7-month-old live mutant pigs in this study.

Off-target analysis of gene-targeted pigs

Off-target effect is a major drawback concern of the CRISPR/Cas9 system. To test whether off-target occurred in these genetically modified piglets, we screened the pig genome and predicted 16, 37, 10, and 24 potential OTS for *TYR*-sgRNA1, *TYR*-sgRNA2, *PARK2*-sgRNA and *PINK1*-sgRNA sites, respectively (Supplemental Table 2). Genomic DNA from the ear tissues of the 35 mutant pigs was used to perform off-target analysis. The fragments around the potential off-target loci were amplified by PCR and then sequenced individually. As a result, none of the sequencing reads exhibited mutations, suggesting that no off-target occurred at these 87 potential OTS in any of the 35 piglets derived from Cas9 and Cas9-nickase targeting.

Discussion

In this study, we used Cas9 and Cas9-nickase both and we found the two methods were both efficient in generating knockout pigs. Cas9-nickase showed a bit lower efficiency in targeting that may be caused by lower co-transfection efficiency of three plasmids. Although we did not detect potential off-targeted mutagenesis in our mutant pigs, it surely exists and caused unexpected side effect in many other studies [21–23]. We can consider using Cas9-nickase in case of higher off-target site effect of Cas9, since there is no obvious difference in targeting efficiency between Cas9 and Cas9-nickase. Recently, there was also a report about using truncated sgRNAs (17 or 18 bp) to enhance targeting specificity of Cas9 [24]. These improved CRIPSR/Cas9 versions can reduce off-target mutagenesis in somatic cells and therefore help to increase cloning efficiency.

Previously, generation of gene-targeted animals with CRISPR/Cas9 system was realized through co-injection of Cas9 mRNA and sgRNA together into one-cell stage embryos. Instead, in this study we used plasmids of eukaryotic expressing CRISPR/Cas9 to mutate the genome of somatic cells. Constitutive expression of CRISPR/Cas9

components can lead to higher on-target editing efficiencies. However, extended persistence of these components in the cell might also lead to increased frequencies of off-target mutations, which has been previously reported with ZFNs [25]. Severe off-target side effect would impair the integrity of genome of cell and damage its development in embryo manipulation. Therefore, the clonability of the mutant cells achieved with constitutive expression of CRISPR/Cas9 components needs to be determined. To address this concern, we transferred the mixed embryos derived from different mutant cell lines (3–6) to a single recipient. Table 2 shows that 4 of 6 *TYR* KO cell lines and 12 of 15 *PARK2*/*PINK1* KO cell lines can develop and form cloned pigs; this result indicated that the cloning efficiency of mutant cells was not compromised substantially. We also observed that the number of piglets developed from some particular KO cell colonies (such as T5) was evidently more than that of the other KO cell colonies; hence, these particular KO cell colonies may exhibit better competency to develop to term. All of the fibroblasts subjected to mutation were derived from the same fetus; as such, failure to produce cloned piglets by using some cells may be caused by off-target. Therefore, mixed embryos derived from multiple cell lines could be transferred to a single surrogate, and this procedure could be a practical strategy to increase the chance to achieve cloned gene-targeted pigs via SCNT.

In summary, our study showed that gene-targeted (single- or double-gene targeted) pigs can be effectively achieved by using the CRISPR/Cas9 system combined with SCNT without detectable off-target effects. This gene-editing system provides a convenient and efficient approach to generate genetically modified pigs with agricultural and biomedical applications.

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Conflict of interest The authors have declared no conflict of interest.

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