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A transgene-free method for rapid and efficient generation of precisely edited pigs without monoclonal selection

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Gene-edited pigs for agricultural and biomedical applications are typically generated using somatic cell nuclear transfer (SCNT). However, SCNT requires the use of monoclonal cells as donors, and the time-consuming and laborious monoclonal selection process limits the production of large populations of gene-edited animals. Here, we developed a rapid and efficient method named RE-DSRNP (reporter RNA enriched dual-sgRNA/CRISPR-Cas9 ribonucleoproteins) for generating gene-edited donor cells. RE-DSRNP takes advantage of the precise and efficient editing features of dual-sgRNA and the high editing efficiency, low off-target effects, transgene-free nature, and low cytotoxic characteristics of reporter RNA enriched RNPs (CRISPR-Cas9 ribonucleoproteins), thus eliminating the need for the selection of monoclonal cells and thereby greatly reducing the generation time of donor cells from 3–4 weeks to 1 week, while also reducing the extent of apoptosis and chromosomal aneuploidy of donor cells. We applied RE-DSRNP to produce cloned pigs bearing a deletion edit of the wild-type p53-induced phosphatase 1 (*WIP1*) gene: among 32 weaned cloned pigs, 31 (97%) carried *WIP1* edits, and 15 (47%) were homozygous for the designed fragment deletion, and no off-target event was detected. The *WIP1* knockout (KO) pigs exhibited male reproductive disorders, illustrating the utility of RE-DSRNP for rapidly generating precisely edited animals for functional genomics and disease research. RE-DSRNP's strong editing performance in a large animal and its marked reduction in the required time for producing SCNT donor cells support its application prospects for rapidly generating populations of transgene-free cloned animals.

dual-sgRNA, CRISPR-Cas9 ribonucleoproteins, transgene-free, without monoclonal selection, cloned pig

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INTRODUCTION

Gene editing in pigs has great potential for application in agricultural breeding and for establishing *in vivo* disease models (Du et al., 2021; Fan et al., 2021; Hai et al., 2014; Whitworth et al., 2016; Xu et al., 2020; Yan et al., 2018; Yang et al., 2015; Zheng et al., 2017; Zhou et al., 2015). Co-microinjection of Cas9 mRNA and sgRNA into one-cell stage embryos can potentially produce gene-edited pigs in a single transformation step. However, the resulting pigs often carry genomic mosaicism for the targeted modification, which can confound the rapid generating of a genetically stable population (Zhou et al., 2015). At present, the most

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widely used approach for gene editing in pigs combines plasmid-based editing systems with somatic cell nuclear transfer (SCNT) (Ruan et al., 2018; Zhou et al., 2015). The steps required for gene-editing of donor cells prior to SCNT can decrease their viability (e.g., elevated rates of apoptosis and changes in ploidy among somatic cells), which is a longstanding obstacle in animal cloning that can substantially increase the generation time for edited animals and hinder the production of large populations carrying the desired genomic edits. Specifically, edited donor cells commonly harbor random mutations or have off-target genome edits that result in low rates of recovering accurately edited cells. These issues adversely affect embryo viability, vigor and health of offspring, and can also interfere with the intended functions or effects of a given gene edit (Lamas-Toranzo et al., 2017). The conventional method for overcoming this challenge is by selecting monoclonal cells, which is a time-consuming process requiring a minimum of 3–4 weeks since it requires cell transfection, monoclonal cell culture, amplification of cultures, and genotyping.

It is well-known that successfully isolated monoclonal cell lines cannot be continuingly used as the donor material because long-term *in vitro* culture leads to somatic cell apoptosis and changes in chromosomal ploidy, which reduce the success rates of SCNT for these compromised cells (Lamas-Toranzo et al., 2017; Li et al., 2003; Magnani et al., 2008; Mastromonaco et al., 2006). Thus, current approaches for producing gene-edited livestock use monoclonal cell isolation. Plasmid-based editing systems also have drawbacks that include random integration of foreign genes and unpredictable or hidden off-target genomic effects (Kim et al., 2014; Liang et al., 2017; Liang et al., 2018; Norris et al., 2020; Subburaj et al., 2016; Svitashev et al., 2016; Woo et al., 2015) which can pose a downstream risk to animal health (Li et al., 2019; Liang et al., 2021; Liu et al., 2021). In light of these issues, we hypothesized that circumventing these factors with monoclonal selection-free and transgene-free approach to preparing nuclear donor cells could potentially decrease the generation time while improving the success rates in producing gene-edited pigs.

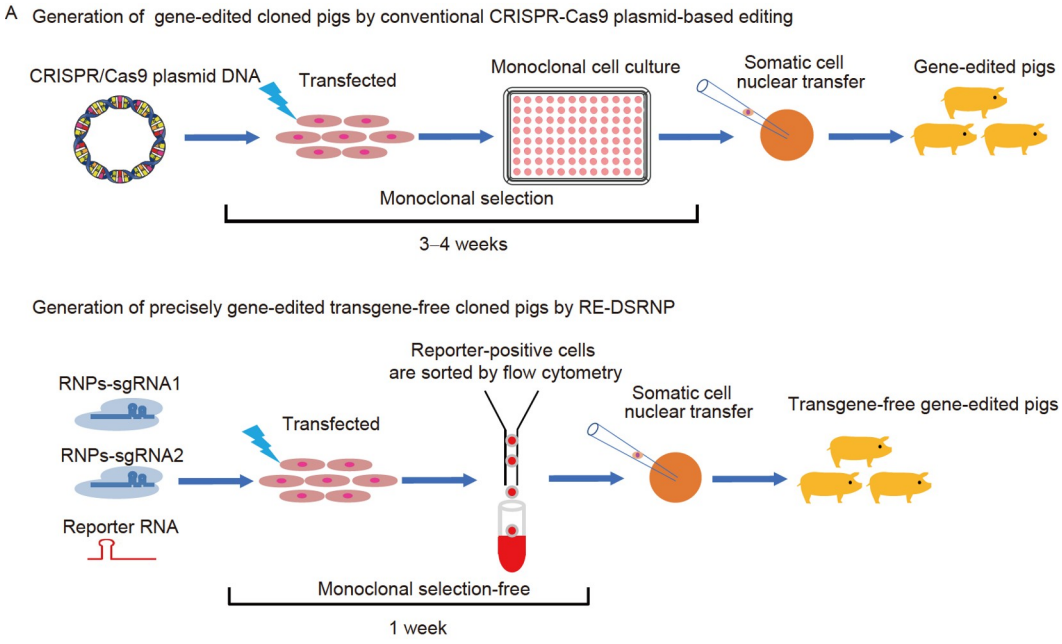
Recent studies have shown that replacing the CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) plasmid with RNPs (CRISPR-Cas9 ribonucleoproteins) can help to prevent the random integration of plasmid DNA (Liang et al., 2017; Liang et al., 2018; Subburaj et al., 2016; Svitashev et al., 2016; Woo et al., 2015), since RNPs can mediate genome editing in mammalian cells and crops without the introduction of transgenes, and with less toxicity and fewer off-target effects than plasmid-based systems (Kim et al., 2014; Liang et al., 2017; Ramakrishna et al., 2014; Woo et al., 2015). However, the adoption of RNPs for genome editing has been limited by the lack of an effective transfection enrichment system, and to our knowledge, RNPs have not yet been used to produce gene-edited non-transgenic clonal livestock. We thus proposed that reporter RNAs, rather than transgene markers, could be an effective potential means of enriching for positively transfected cells to improve the success rates of targeted gene editing.

Here, we describe a relatively straightforward RE-DSRNP (reporter RNA enriched dual-sgRNA/CRISPR-Cas9 ribonucleoproteins) method for rapid generation of gene-edited donor cells. This method incorporates the precise targeting of dual-sgRNA systems with the increased editing rates but reduces off-target effects and cytotoxicity associated with reporter RNA-enriched RNPs, without the need for introducing transgenes. It should be noted that this method does not require selecting monoclonal cells, and directly uses flow cytometry sorting with RNA-based probe to enrich donor cells for SCNT, thereby shortening the production cycle for obtaining cloned pigs from a genetically heterogeneous pool of edited and wild-type embryos. In addition, this method also results in attenuated levels of apoptosis and aneuploidy among donor cells than that of conventional plasmid-based methods (Figure 1A and B). We subsequently applied this RE-DSRNP method to establish a porcine model of male reproductive disease by editing an ortholog of the male-reproduction-related wild-type p53-induced phosphatase 1 (*WIP1*) gene (Cho et al., 2017; Filipponi et al., 2013; Wei et al., 2019). RE-DSRNP facilitated the production of *WIP1* gene-edited cells for SCNT, and the resulting *WIP1* knockout (KO) pigs produced from these cells exhibited obvious male reproductive disorder phenotypes. Based on our collective findings, we thus propose that RE-DSRNP could serve as a potentially reliable approach for generating gene-edited, non-transgenic livestock animals for agricultural breeding or *in vivo* disease modeling.

RESULTS

DSRNP can be used for transgene-free targeted genome editing of PEFs

In order to explore the use of dual-sgRNAs for gene editing in pig embryonic fibroblasts (PEFs), we used a series of dual-sgRNAs to induce fragment deletions at multiple loci, including *pAPN* and multiple sites for *CD163*, with deletion fragments sizes ranging from 120–272 bp. Experiments in which plasmids expressing guide RNAs and Cas9 were transfected into PEFs showed that the use of dual-sgRNAs resulted in higher rates of successful gene editing in PEFs (46%–97%) than that obtained using single sgRNAs (13%–65%). Moreover, the accuracy of these dual-sgRNA-mediated edits was higher than with single-sgRNAs, i.e., the resulting edits were the intended deletion length more frequently with dual-sgRNAs (Table 1; Figures S1 and S2 in



B

	Conventional plasmid-based editing	RE-DSRNP
Transgene-free	No	Yes
Increases editing efficiency by enrichment	No enrichment	Yes, ~1.2–3.2-fold higher than unenriched cells, up to 67%–95%
Generation time of donor cells	3–4 weeks	1 week
Off-target effects screening	Detectable effects	None detected

Figure 1 Overview of transgene-free gene editing in pigs using RE-DSRNP. A, Current methods for generating gene-edited cloned pigs (upper panel) require the use of CRISPR-Cas9 DNA to edit somatic cells and the selection of monoclonal cells. The process of culturing and genotyping of monoclonal cells takes 3–4 weeks, after which the appropriate monoclonal cells are used as donor cells for SCNT. In contrast to this plasmid editing system, the RE-DSRNP method (lower panel) uses dual-sgRNA in combination with reporter RNA enriched RNPs (CRISPR-Cas9 ribonucleoproteins), which incorporates the precise targeting of dual-sgRNA systems with the increased editing rates but reduced off-target effects and cytotoxicity associated with reporter RNA-enriched RNPs, without the need for introducing transgenes, thus eliminating the need for the selection of monoclonal cells and thereby reducing the generation time of donor cells from 3–4 weeks to 1 week. B, Comparison of the methods of using RE-DSRNP to conventional CRISPR-Cas9 plasmids to generate gene-edited cloned pigs.

Supporting Information).

Then, we investigated the direct delivery of DSRNPs to potentially circumvent the need for transgene expression associated with plasmids in PEF gene editing. To this end, we tested the direct delivery of various concentrations of the two RNPs that comprise Cas9 and each of the 2 guide RNAs (together, "DSRNP") targeting the porcine *pAPN* gene in PEFs (Figure 2A). We found that DSRNP delivery by 15 min incubation with 6 μg Cas9 protein and 5 μg of each sgRNA led to the highest editing efficiency (49%) in PEFs at 24 h post transfection (Figure 2B–D).

Moreover, we then compared the editing efficiency, cytotoxicity, and off-target effects between the plasmid (pX330)-based and DSRNP-based editing systems in the deletion of the *pAPN* gene (Figure S3A and B in Supporting Information). The editing efficiency of the DSRNP group (44%) was higher than that of the plasmid group (36%) (Figure 2E). Further, both microscopy and Cell Counting Kit 8 (CCK-8) assays at 24 and 48 h post transfection showed significantly higher cell viability in the DSRNP group compared with that in the plasmid group (Figure 2F and G), indicating that the DSRNP method induced lower cytotoxic effects in PEFs compared with conventional plasmid-based editing. We used CRISPOR online software to predict the most likely off-target binding sites for the two *pAPN*-targeted sgRNAs. Deep sequencing of these three sites detected no off-target effects (0.01% to 0.07%) in reads of PEFs edited by DSRNPs (notes that if proportion of indels was smaller than 0.15%, we considered the "no off-target effects" since this level of indels could be introduced by errors in PCR or sequencing). By contrast, we detected a negligible but distinct level of off-target edits (0.75% of reads) at the *pAPN*-g2-OT1 locus in plasmid-transfected PEFs (Figure 2H; Figure S4 in Supporting Information), indicating that the fewer off-target effects induced by the DSRNP method compared with pX330 plasmid-based editing.

Table 1 Comparison of the efficiency of gene editing mediated by dual-sgRNA and single sgRNA at multiple loci

Loci	gRNA	Size of precise fragment deletion	Mutation efficiency	Precise fragment deletion efficiency
pAPN	gRNA3	–	50% (12/24)	–
	gRNA5	–	30% (6/20)	–
	gRNA3+gRNA5	272 bp	83% (20/24)	38% (9/24)
CD163	gRNA1	–	46% (10/22)	–
	gRNA2	–	31% (9/29)	–
	gRNA3	–	15% (4/27)	–
	gRNA4	–	43% (13/30)	–
	gRNA5	–	65% (20/31)	–
	gRNA8	–	13% (4/31)	–
	gRNA9	–	23% (7/31)	–
	gRNA10	–	24% (9/38)	–
	gRNA1+gRNA8	207 bp	97% (30/31)	29% (9/31)
	gRNA1+gRNA9	207 bp	94% (30/32)	61% (19/31)
	gRNA2+gRNA10	120 bp	46% (16/35)	17% (6/35)
	gRNA3+gRNA10	225 bp	74% (23/31)	45% (14/31)
	gRNA4+gRNA10	195 bp	91% (29/32)	59% (19/32)
	gRNA5+gRNA8	195 bp	71% (22/31)	52% (16/31)

FACS enrichment with a reporter RNA probe provides more edited donor cells than genotyping and expansion of isolate cultures

We next sought to improve the transfection efficiency for DSRNPs in PEFs, which represents a major challenge for producing clonal, gene-edited pigs. To take advantage of the speed and simplicity of fluorescence activated cell sorting (FACS)-based cell sorting, while also avoiding transgene introduction, we tested ATTO^{TM} 550-tracrRNA—an ATTO-labeled fluorescent RNA probe—for enriching PEF cells that were successfully transfected with DSRNP. Specifically, ATTO^{TM} 550-tracrRNA and DSRNPs were simultaneously delivered into PEFs, and ATTO-550 positive cells were sorted by flow cytometry at 24 h post transfection and screened for the targeted edit. We first tested the performance of flow cytometry enrichment of PEFs harboring a *pAPN* deletion edit at different concentrations (from 0.05 to 0.8 nmol) of ATTO^{TM} 550-tracrRNA with low concentrations of each sgRNA (6 μg Cas9 protein, 2 μg sgRNA). The results showed that the highest enrichment was obtained with 0.1 nmol ATTO^{TM} 550-tracrRNA (Figure 3A and B), and that enrichment by ATTO^{TM} 550-tracrRNA resulted in an ~3-fold increase in edited PEFs compared with that in the unenriched controls (Figure 3B).

We also tested effects of ATTO^{TM} 550-tracrRNA enrichment for edited PEFs using the previously determined optimal concentrations of DSRNP and sgRNAs (6 μg Cas9, 5 μg sgRNA) and examined efficiency of targeted editing at other loci in the pig genome. We found that the proportion of PEFs harboring successful deletion edits in the *pAPN*, *ROSA26*, and *MSTN* genes were ~2–3 times higher (*WIP1* was negligibly higher) in the ATTO^{TM} 550-tracrRNA-enriched populations than that in the unenriched control PEFs (Figure 3C–F). In addition, we explored the use of a protein reporter for enrichment (direct delivery of GFP concurrently with DSRNP). Notably, the delivery of 10 and 20 μg of GFP protein resulted in approximately 2-fold higher enrichment for successfully edited cells in the GFP-enriched population than in the unenriched control cells (Figure 3G and H). These results support that GFP protein could also be used for flow cytometry-based enrichment, though resulting in potentially efficiency than ATTO^{TM} 550-tracrRNA at some loci. Our experiments establish that reporter RNA enriched (RE)-DSRNP method can facilitate enrichment for DSRNP-transfected cells to increase the number of PEFs harboring the targeted edits. However, it should be noted that in contrast with methods using genotyping and culture-based enrichment of edited isolates, the higher enrichment is accompanied by genetic heterogeneity (i.e., some wild-type and off-target edits) in the resulting animals, which are screened and culled after birth.

PEFs editing by RE-DSRNP results in lower cytotoxicity and off-target effects than plasmid-based editing

Further, we determined whether the RE-DSRNP method affected cytotoxicity and off-target effects in successfully edited PEFs compared with that in plasmid-edited cells. To this end, we induced targeted deletion edits in the *pAPN* gene of PEFs using four gene editing approaches: the pX330 plasmid without a selection marker, pX330-GFP/pX330-RFP plasmid with fluorescent selection markers, DSRNP without a selection marker, and RE-DSRNP with ATTO^{TM}

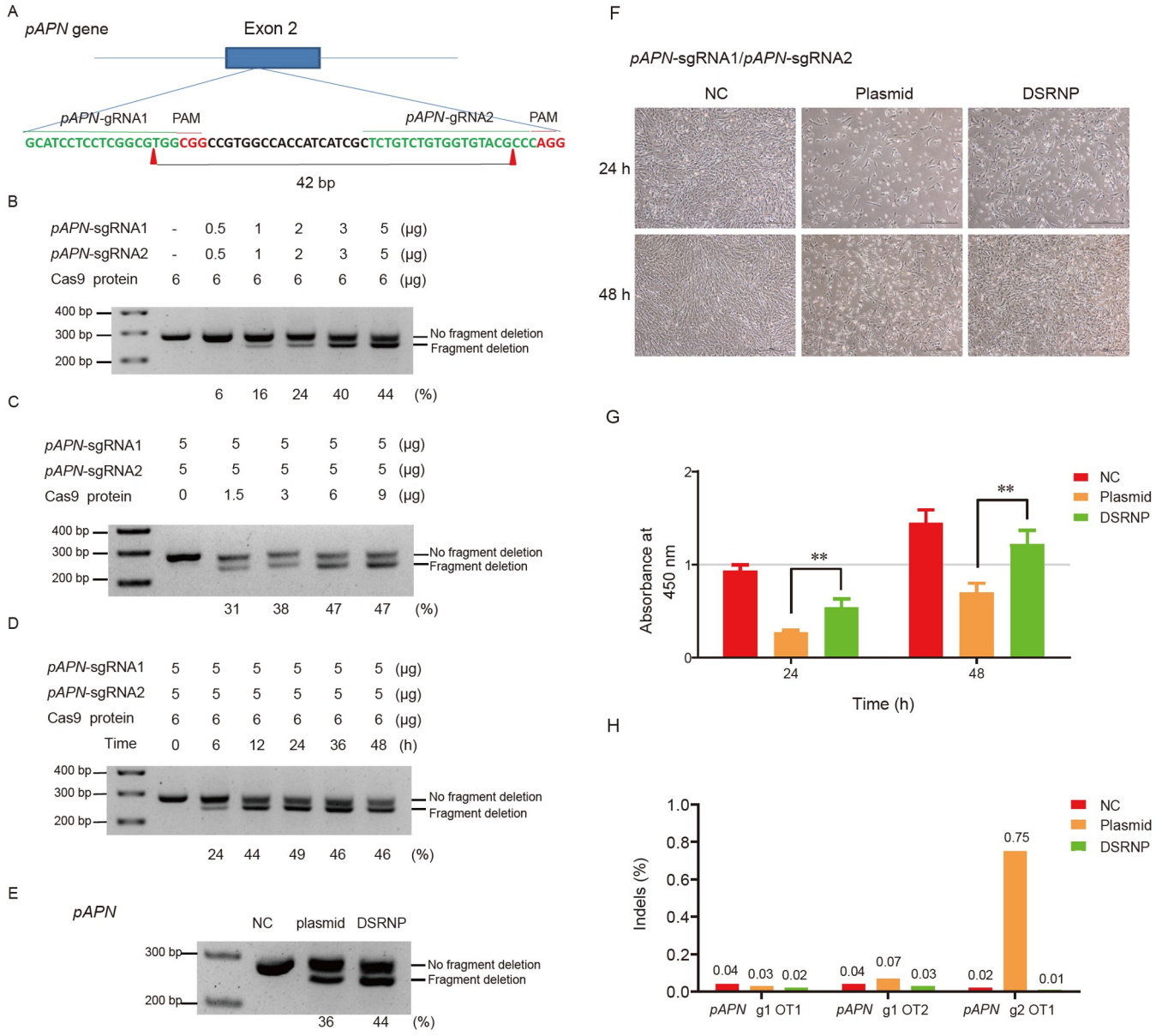


Figure 2 DSRNP can induce edits in PEFs. A, Schematic diagram of the two sgRNA target sites in exon 2 of the pig *pAPN* gene. The exon 2 region is indicated by blue rectangle, the protospacer sequences are shown in green, and the PAM sequences are shown in red. The red triangles represent the predicted cleavage sites of sgRNAs. B, Assessment of optimal sgRNA concentrations for DSRNP-mediated editing of the pig *pAPN* gene. C, Assessment of optimal Cas9 protein concentration for DSRNP-mediated editing of the pig *pAPN* gene. D, Editing time course analyses of DSRNP-mediated gene editing efficiency of the pig *pAPN* gene. E, Comparing the editing efficiency of DSRNP *vs*. a plasmid-based editing system for the pig *pAPN* gene. F and G, Comparing the cytotoxic effects of DSRNP transfection and plasmid transfection in PEFs. Cell viability was examined by (F) microscopy and (G) CCK-8 assays. The untreated PEFs were used as a negative control (NC). Scale bars, 500 μm. Absorbance data at 450 nm was collected for three independent experiments, with 3 replicates per sample group in each experiment. Data are expressed as means±SD. Statistical significance was determined by Student's *t*-test; **, $P<0.01$. H, Comparing the off-target effects of DSRNP *vs*. a plasmid-based editing system in PEFs. For *pAPN*-sgRNA1 and *pAPN*-sgRNA2, three potential off-target sites (predicted by software CRISPOR, the sites with the highest off-target score) were selected to detect the off-target efficiency by deep sequencing.

550-tracrRNA enrichment (Figure S3A–D in Supporting Information). Briefly, both microscopy and CCK-8 assays showed significantly lower cell viability in the two plasmid groups than that in the two DSRNP groups, at both 24 and 48 h (Figure 4A and B). Moreover, we detected no differences in cell viability between the DSRNP and the RE-DSRNP methods (Figure 4A and B), indicating that co-delivery of 0.1 nmol of ATTO^{TM} 550-tracrRNA and DSRNPs could reduce cytotoxicity associated with the editing process. Additionally, the pX330-GFP/pX330-RFP and RE-DSRNP groups subjected to flow cytometry enrichment contained approximately 2-fold higher proportions of successfully edited PEFs than that of the unenriched pX330 and DSRNP approaches (Figure 4C and D).

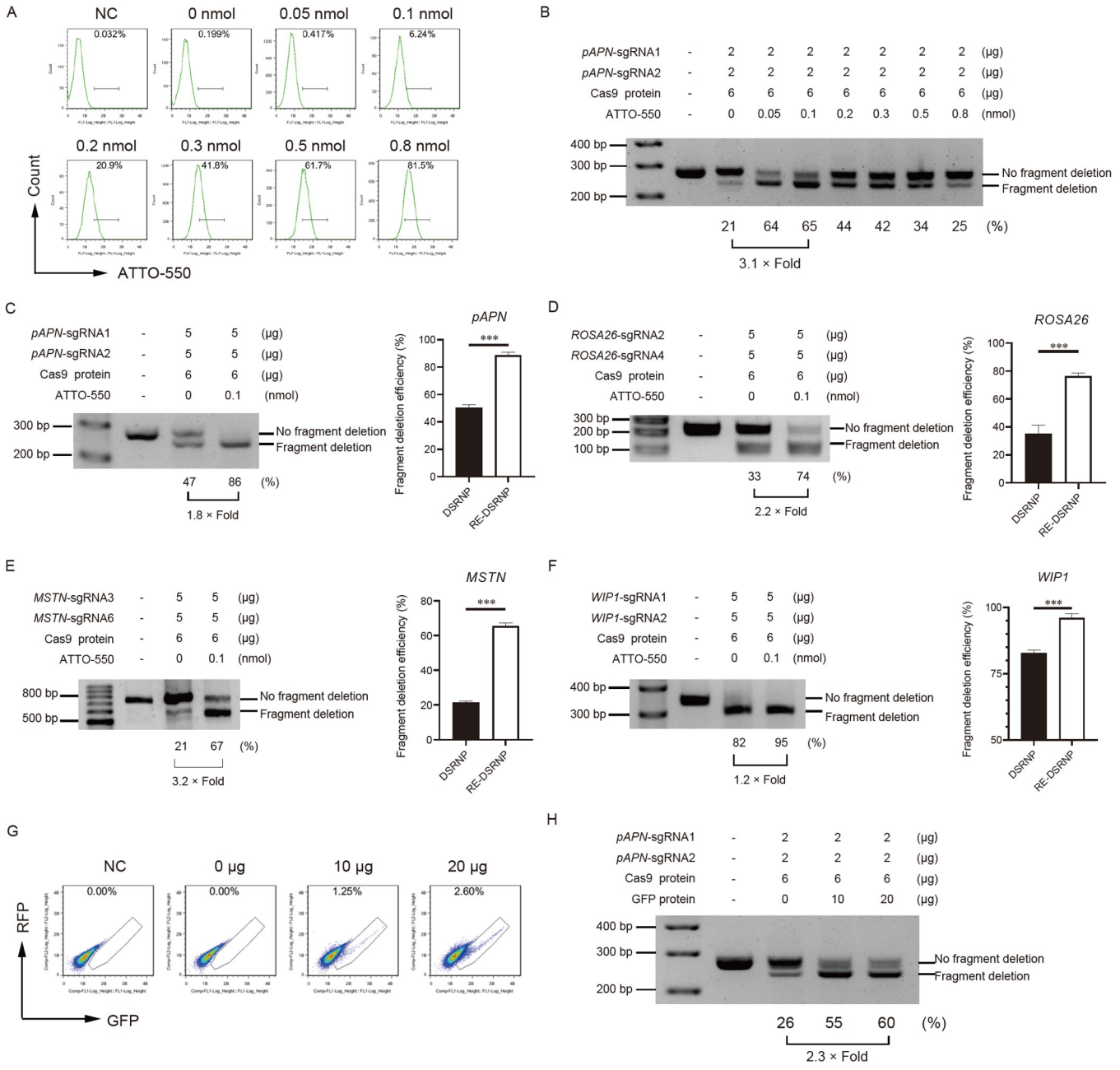


Figure 3 RE-DSRNP can improve editing efficiency in PEFs. A and B, Enrichment effect testing of different concentrations (from 0.05 to 0.8 nmol) of ATTO™ 550-tracrRNA on DSRNP editing efficiency of the pig *pAPN* gene in PEFs transfected with a low concentration of sgRNA (Cas9 protein 6 μg, sgRNA 2 μg). At 24 h after co-transfection of ATTO™ 550-tracrRNA with DSRNP, (A) ATTO-550 positive PEFs were enriched by flow cytometry, and (B) PCR was then used to detect the fragment deletion efficiency. C–F, Testing the enrichment effect of ATTO™ 550-tracrRNA on DSRNP editing efficiency of the pig (C) *pAPN*, (D) *ROSA26*, (E) *MSTN*, and (F) *WIP1* genes in PEFs transfected with a high concentration of sgRNA (Cas9 protein 6 μg, sgRNA 5 μg). Data are expressed as means±SD for n=3. Statistical significance was determined by Student's t-test; ***, P<0.001. G and H, In addition to using ATTO™ 550-tracr RNA, GFP protein was also used to enrich DSRNP-transfected positive PEFs. At 24 h post co-transfected GFP protein with DSRNP, (G) GFP positive PEFs were enriched by flow cytometry and (H) PCR was used to detect the fragment deletion efficiency.

Finally, we assessed the off-target effects of the four approaches using deep sequencing analysis and found that 1.89% and 6.38% of the reads from PEFs edited with either pX330 or pX330-GFP/pX330-RFP approaches harbored off-target indels at the *pAPN*-g2-OT1 locus, respectively. In contrast, no off-target effects (0.11%–0.13% of reads) at this locus were detected in cells edited using the DSRNP or RE-DSRNP approaches. No off-target effects (i.e., 0–0.13% of reads) were detected at neither of the two alternative, predicted, potential off-target sites we examined in any of the edited cells (Figure 4E; Figure S5 in Supporting Information). These results further support that DSRNP-based editing is accompanied by fewer off-target effects than plasmid-based editing in PEFs.

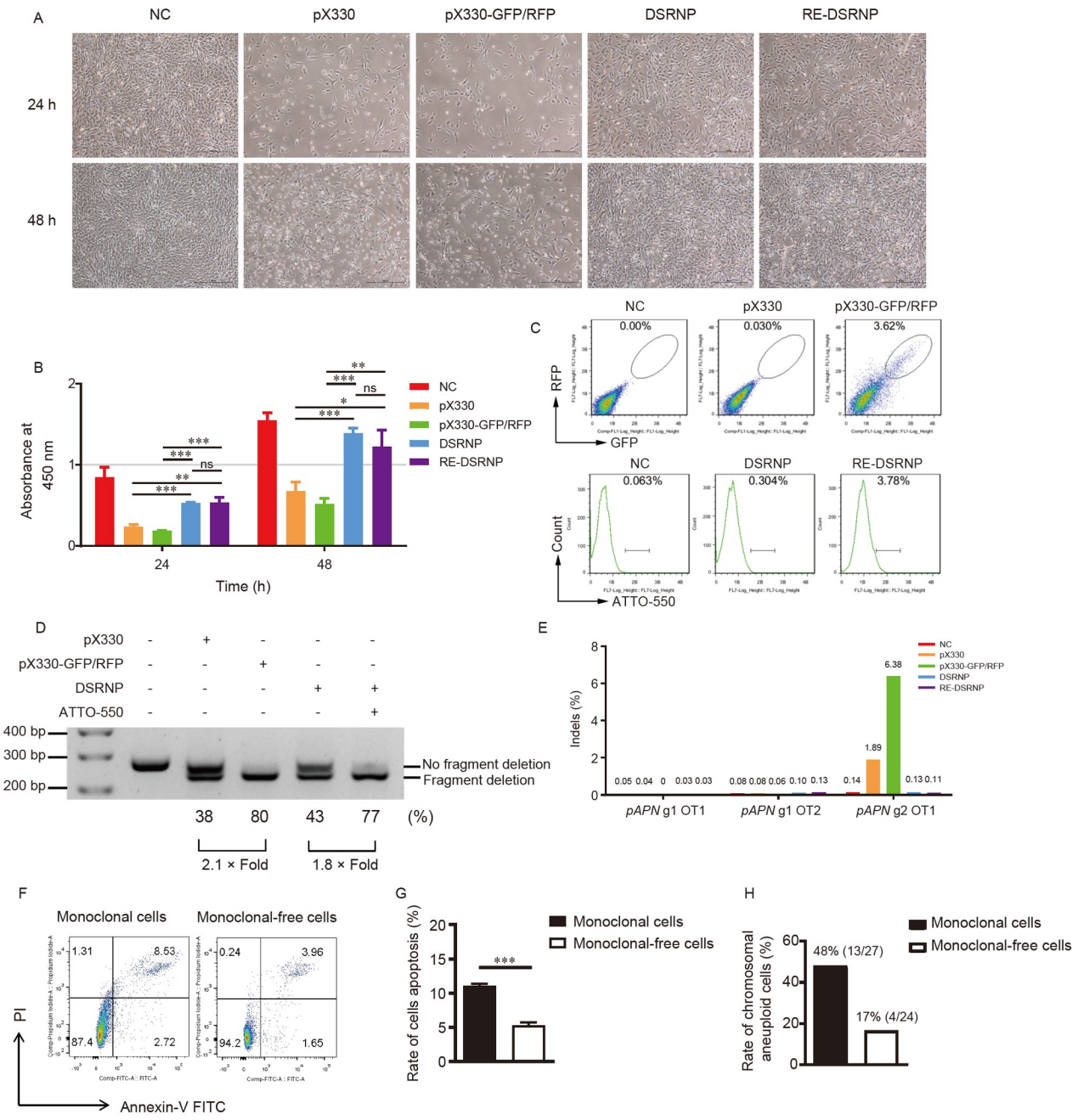


Figure 4 RE-DSRNP results in lower cytotoxicity and off-target effects in PEF donor cells for SCNT than plasmid-based editing by bypassing monoclonal selection and expansion. A and B, Testing the cytotoxicity of the four editing approaches in PEFs. Cell viability was measured by (A) microscopy and (B) CCK-8 assay. The untreated PEFs were used as a negative control (NC). Scale bars, 500 μm. Absorbance data at 450 nm was collected for three independent experiments, with 3 replicates per sample group in each experiment. Data are expressed as means±SD. Statistical significance was determined by Student's *t*-test; ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. C and D, Comparison of the *pAPN* gene fragment deletion efficiencies of the four editing approaches. E, Comparison the off-target effects of the four editing approaches in PEFs. For *pAPN*-sgRNA1 and *pAPN*-sgRNA2, three potential off-target sites (predicted by software CRISPOR, the sites with the highest off-target score) were selected to detect the off-target efficiency by deep sequencing. F and G, Comparison of the extent of apoptosis in the monoclonal cell population and monoclonal-free cell population. Apoptotic cells were defined as annexin-V^+ cells. H, Comparison of the extent of chromosomal aneuploidy for the monoclonal cell population and monoclonal-free cell population. The chromosomal numbers of cells that were not $2n$=38 were considered chromosomal aneuploidy.

RE-DSRNP enrichment shortens SCNT donor cell generation time compared to enrichment by monoclonal selection

To further compare the effects on viability of RE-DSRNP with that of monoclonal selection and culture in enriched edit-positive donor cells for SCNT, we used RE-DSRNP to edit the PEFs, then split the FACS-enriched cells into two populations which we characterized in detail prior to downstream SCNT application. The first population was

examined immediately following enrichment (ready for SCNT at 3–4 d after sorting), while the other was processed using standard isolation and culture methods for obtaining monoclonal cell cultures (ready for SCNT at 21–23 d after sorting) (Figure S6 in Supporting Information). We compared the extent of apoptosis and chromosomal aneuploidy using PI/Annexin-V staining with flow cytometry and karyotype analysis, respectively, in the two cell populations. The monoclonal cell population had significantly higher levels of both apoptotic cells and cells exhibiting chromosomal aneuploidy (Figure 4F–H). This *in vitro* analysis supported our hypothesis that use of RE-DSRNP could not only obviate the need for the labor intensive and time-consuming monoclonal isolation and expansion process, but also provide apparently better quality (i.e., higher viability and lower aneuploidy) donor cells for subsequent SCNT.

WIP1 KO pigs generated by RE-DSRNP editing have impaired reproductive function

To demonstrate the utility of RE-DSRNP in functional genomics research, disease modeling, or agricultural breeding, we targeted the porcine *WIP1* gene, a male reproduction-related gene initially identified in mice (Cho et al., 2017; Filipponi et al., 2013; Wei et al., 2019). First, we used RE-DSRNP to generate knockout Meishan PEFs via *WIP1* deletion edits by co-transfecting 6 μg Cas9 protein, 5 μg each of sgRNA, and 0.1 nmol of ATTO^{TM} 550-tracrRNA into PEFs (Figure 5A). At 24 h post transfection, ATTO-550 positive cells were enriched by FACS, and a portion of the enriched cells were collected for detection of *WIP1* gene editing efficiency. The remaining cells were expanded for 2–3 d prior to use as nuclear donor cells for SCNT. Screening by PCR and gel electrophoresis suggested that as many as 95% of the enriched cells carried the *WIP1* fragment deletion (Figure 5B).

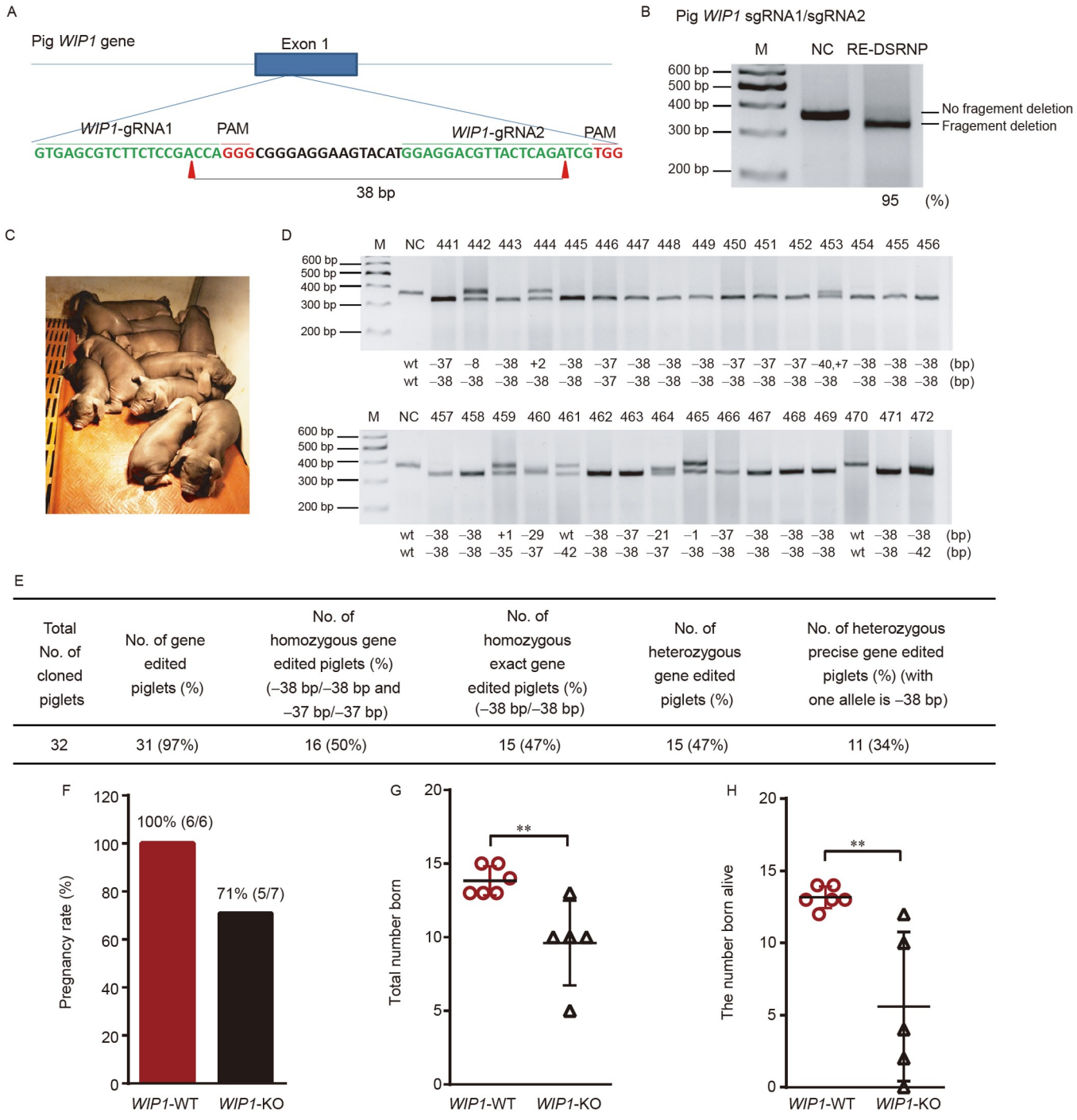
For SCNT, a total of 2,041 reconstructed embryos were transferred into eight recipient sows. Four sows successfully delivered 36 cloned piglets (Figure 5C; Table S1 in Supporting Information). Except for 4 piglets that died before weaning (no sampling and no genotyping) and 1 live *WIP1* wild-type piglet, the remaining 31 weaned piglets all carried the edited *WIP1* gene. The pig cloning efficiency thus reached 1.5% (31/2,041), which was higher than that in previous studies, typically not greater than 0.5% (Ruan et al., 2018). PCR and TA cloning were used to genotype 32 weaned piglets. Among the 32 pigs, 31 (97%) pigs had edits at the *WIP1* locus, 16/31 (52%) of which were homozygous (Figure 5D and E). Notably, only one of the 16 edited pigs had any deviation from the desired −38 bp/−38 bp deletion edit, and this pig differed by only one nucleotide (−37 bp/−37 bp) (Figure 5D and E; Table S2 in Supporting Information). Off-target analysis showed no indels at 20 potential off-target sites (predicted by CRISPOR) in any of the 32 cloned pigs (Table S3 in Supporting Information). In summary, we used RE-DSRNP combined with SCNT to generate clonal *WIP1* KO pigs without isolation and expansion of monoclonal PEFs.

To test whether the *WIP1* edit affected the reproductive performance of Meishan boars, *WIP1* KO and wild type (WT) Meishan boars were mated with WT Meishan sows. We compared the pregnancy rates, total number of piglets born (liveborn and stillborn), and the number born alive (liveborn) between the two groups and found that all six sows bred with WT boars gave birth without obvious defects or impairment, whereas two out of the seven mating sows bred with KO boars had miscarriages, the pregnancy rate of the KO group (71%) was significantly lower than that of the WT group (100%) (Figure 5F). The total number born and the number born alive of the KO group (9.6±1.3 N=5 and 5.6 ±2.3 N=5) were both significantly lower than that of the WT group (13.8±0.4 N=6 and 13.2±0.3 N=6) (Figure 5G and H). These phenotypic analyses supported that *WIP1* deletion by RE-DSRNP-mediated editing led to reduced reproductive performance of boars, and thus validating the suitability of this method for use functional genomics analyses *in vivo*.

DISCUSSION

The recent discovery of unintended integration of plasmid DNA in gene-edited, hornless cattle has caused widespread concern (Norris et al., 2020). While this introduction of foreign genes into cattle may be a one-off event that can be prevented in the future by sequencing the gene-edited animals before establishing production populations, the RE-DSRNP method avoids this possibility because no recombinant DNA is used in the editing process. Moreover, sequencing is conducted after the animals are birthed, so the edited animals are all necessarily screened prior to further breeding or production steps. We therefore anticipate that this method could be used by both academic and private breeding researchers working in the context of regulatory environments that favor transgene-free methods for the husbandry of clonal, gene-edited animals.

As demonstrated by our generation of clonal *WIP1* KO pigs, RE-DSRNP exhibited relatively high editing efficiency (97% of pigs were edited, 47% of pigs were desired homozygous edited allele) with no off-target effects (i.e., none detected in 20 predicted potential off-target sites among 31 edited pigs). To our knowledge, these *WIP1* KO pigs represent the first reported examples of cloning transgene-free, gene-edited large animals. Moreover, our observations of obvious male reproduction disorder phenotypes highlight the utility of this approach for establishing large animal *in vivo* models for gene function or disease. It is worth noting that



E

Total No. of cloned piglets	No. of gene edited piglets (%)	No. of homozygous gene edited piglets (%) (–38 bp/–38 bp and –37 bp/–37 bp)	No. of homozygous exact gene edited piglets (%) (–38 bp/–38 bp)	No. of heterozygous gene edited piglets (%)	No. of heterozygous precise gene edited piglets (%) (with one allele is –38 bp)
32	31 (97%)	16 (50%)	15 (47%)	15 (47%)	11 (34%)

Figure 5 *In vivo* phenotypic analysis of RE-DSRNP edited *WIP1* KO pigs. A, Schematic diagram of the two sgRNA target sites in exon 1 of the pig *WIP1* gene. The exon 1 region is indicated by blue rectangle, the protospacer sequences are shown in green, and the PAM sequences are show in red. The red triangles represent the predicted cleavage sites of sgRNAs. A precise editing with dual-sgRNA results in a 38 bp deletion which caused a frameshift mutation in *WIP1*. B, Fragment deletion efficiency of the *WIP1* gene in PEFs, assessed by PCR. ATTO^{TM} 550-tracrRNA was co-transfected into PEFs with DSRNP, and ATTO-550 positive cells were enriched by flow cytometry at 24 h post transfection. C, ATTO-550 positive PEFs were used as nuclear donor cells for SCNT to generate cloned pigs. D, Detection of genotypes of 32 cloned pigs by PCR and sequencing. E, Assessment of gene editing efficiency and the distribution of cloned pig genotypes. F, Comparison of pregnancy rates of sows after mating with boars of different *WIP1* genotypes. WT group: N=6; KO group: N=7. G, Comparison of the total number of piglets born (liveborn and stillborn) of sows after mating with boars of different *WIP1* genotypes. WT group: N=6; KO group: N=5. H, Comparison of the number born alive (liveborn) from sows after mating with boars of different *WIP1* genotypes. WT group: N=6; KO group: N=5. Data are expressed as means±SD. Statistical significance was determined by Student's t-test; **, P<0.01.

Cas9-based editing can introduce a wide variety of large fragment deletions that cannot be easily detected by conventional PCR-based screens. This issue illustrates the great need for more accurate detection methods or editing tools

that do not rely on DNA double-strand breaks.

Historically, methods for producing gene-edited animals do not employ SCNT, such as co-microinjection of Cas9 mRNA and sgRNA into single-cell-stage embryos, often leads to genetic mosaicism (Ruan et al., 2018; Zhou et al., 2015). Thus, most techniques for producing large animals with genomic edits still rely on SCNT. Here, we show that RE-DSRNP can help reduce the time required to generate edited donor cells for SCNT from 3–4 weeks in conventional methods to 1 week. This rapid turnaround could facilitate model organism production for large-scale experiments in diverse areas of biological, medical, and agricultural research that now require increasingly large population sizes.

It also bears emphasis that the RE-DSRNP method is broadly applicable for targeted editing in most cell types, and in a wide range of organisms (e.g., monkeys, cows, sheep, dogs, cats, etc.). In particular, this method can be used for editing primary somatic cells, which typically cannot be cultured *in vitro* for long durations. It should be noted that a major innovation of this strategy is bypassing the time-consuming step of monoclonal selection and expansion to increase the number of edited cells for SCNT, but that the animals produced for these donor cells include WT and off-target edits that are screened out after birth, when successful editing is already a required step. In conclusion, this study provides a proof-of-concept demonstration that RE-DSRNP can serve as a transgene- and clonal selection-free method to rapidly and efficiently generate targeted edits in PEF donor cells for SCNT for *in vivo* livestock research or molecular breeding applications.

MATERIALS AND METHODS

Vector construction

The CRISPR-Cas9 target sgRNAs were designed and cloned into the vector backbone of different CRISPR-Cas9 systems. Three CRISPR-Cas9 systems were used in this study: pX330 (addgene plasmid #42230), pX330-GFP (Viewsolid, Beijing, China) and pX330-RFP (Viewsolid). The sgRNAs were cloned into the pX330 CRISPR system according to a previously described protocol (Cong et al., 2013), and the pX330-GFP and pX330-RFP CRISPR system were constructed according to the manufacturers' instructions. All primers used for construction of plasmids are listed in Table S4 in Supporting Information.

Cas9 protein and guide RNAs

Cas9 protein tagged with a nuclear localization signal was purchased from New England Biolabs (NEB, USA). PCR products for *in vitro* transcription of sgRNAs were amplified using T7-F and T7-R primers and are listed in Table S5 in Supporting Information. The sgRNAs were transcribed using the HiScribe T7 In Vitro Transcription Kit (NEB) and purified by RNA Clean & Concentrator (ZYMO, USA) according to the manufacturer. The concentration and quality of synthesized RNAs were determined by Nanodrop 2000 and agarose gel electrophoresis, respectively.

PEFs culture, transfection, and sorting

All experimental procedures using animals were conducted in accordance with the Administrative Panel on Laboratory Animal Care (APLAC) protocol and the institutional guidelines provided by the Chinese Academy of Agriculture Sciences. The fetuses of Meishan pigs at 35-days-old were used to isolate PEFs as described by Li et al. (2014). Briefly, the head, limbs, and internal organs were removed from the fetal body and the body was minced and digested with collagenase-DNase in Dulbeccos' modified Eagle's medium (DMEM, GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO), 1% penicillin-streptomycin (GIBCO), 0.5 mg mL^{-1} Collagenase IV (GIBCO), and 100 kU mL^{-1} DNaseI (GIBCO) for 4–5 h at 37°C. The cells were then cultured in DMEM, supplemented with 15% FBS at 38.5°C and 5% CO_2 in a humidified incubator. The PEFs were transfected with the Nucleofector 2b device (Lonza, Switzerland) with an Amaxa kit (Lonza) following the manufacturer's instructions, with program T-016 being selected. The total plasmid amount transfected each time was 10 μg and the total RNPs amount used was from 6 to 19 μg. For plasmid-transfected cells, cells were collected 48 h after transfection for genomic DNA extraction or sorting by flow cytometry. For cells co-transfected with ATTO^{TM} 550-tracrRNA (IDT) and RNPs, we enriched the cells 24 h after transfection. The extracted genomic DNA was used for subsequent experiments.

Detection of genome editing efficiency and genotype identification

In order to determine the efficiency of genome editing, we carried out PCR amplification of the target site using the extracted genomic DNA as a template. The PCR products were separated on a 2% agarose gel, and fragment deletion efficiencies were tested by gray analyses using ImageJ software. At the same time, the obtained PCR products were subcloned into the pMD18-T vector (TaKaRa, Japan), and up to 20 bacterial colonies were picked and sequenced. The primers used for PCR are listed in Table S6 in Supporting Information.

CCK-8 assay

Cell viability was determined following transfection with

either plasmids or RNPs (with or without ATTO) using CCK-8 assays (Dojindo, Japan). The kit used WST-8, a water-soluble tetrazolium salt, to quantify live cells based on colorimetric quantification of orange formazan dye production by enzymatic reduction in the presence of an electron carrier. WST-8 tetrazolium salt is reduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture media. The amount of formazan produced is directly proportional to the number of live cells, measured by absorbance at 450 nm. Transfected cells were seeded in 96-well plates (10^4 cells/well) for 24 h or 48 h, and subjected to CCK-8 assays. Each experiment was independently repeated a minimum of three times.

Deep sequencing

PEFs were collected two days after plasmid transfection or one day after RNPs transfection to extract genomic DNA for deep sequencing. Forward primers with barcodes and common reverse primers (Table S7 in Supporting Information) were used to amplify 3 potential off-target sites (predicted using an online software (CRISPOR, http://crispor.tefor.net/) and the sites with the highest off-target score were selected). Equal amounts of PCR products were mixed as a pool, and the samples were used for Illumina sequencing at LC Sciences (Hangzhou, China). Indels, including insertions and deletions, occurring at the Cas9 cleavage sites were considered as mutations.

Apoptosis detection

For annexin V/PI double staining, cells were harvested and washed twice and resuspended in PBS. Apoptotic cells were identified by double-staining with Annexin V-FITC and PI dye using the Annexin V-FITC Apoptosis Detection kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. After staining and washing, cells were analyzed by BD FACScanto II flow cytometer and further analyzed with FlowJo. Apoptotic cells were defined as annexin-V^+ cells.

Karyotype analysis

PEFs were treated with colchicine (0.025 μg mL^{-1}) for 4 h. After centrifugation, the supernatant was removed, and the cells were placed in 5 mL 0.07 mol L^{-1} KCl and incubated at 37°C for 5 min. Subsequent centrifugation removed the supernatant. Cells were then fixed with 1 mL fixation solution (methyl alcohol:glacial acetic acid=3:1) and left for 15 min; they were then centrifuged to remove the supernatant. An aliquot of 5 mL of fixation solution was then added to cells for 15 min. Cells were centrifuged and fixed again. A further centrifugation removed the supernatant. Then, 0.4 mL of fixation solution was added. Finally, the cell suspension was pipetted onto glass slides, and the number of chromosomes was counted using confocal microscopy. The chromosomal numbers of PEFs that were not $2n$=38 were considered chromosomal aneuploidy.

Generation of gene-edited cloned pigs

The pig oocytes that matured *in vitro* for 40 h were used as nuclear transfer recipient cells, and the obtained PEFs were used as nuclear transfer donor cells. The nuclear transfer donor cells were transferred into enucleated oocytes, and the reconstructed embryos were fused and activated with two successive dendritic cell pulses at 130 V for 30 μs using an electrofusion instrument (BLS, Hungary). Well-developed embryos were surgically transferred into the oviduct of a surrogate the day after observed estrus. After embryo transfer, the pregnancy status of the recipient sows was periodically checked.

Genotyping of cloned pigs

Genomic DNA was extracted from the ear tissues of weaned cloned pigs, and the sequences near the target site were amplified with *WIP1* Sense3/Antisense3 primers (Table S6 in Supporting Information). PCR products were analyzed by agarose gel electrophoresis for the efficiency of fragment deletion, and TA cloning was used to detect the exact genotype of the edited *WIP1* gene.

Off-target analysis of cloned pigs

Potential off-target sites were predicted using online software CRISPOR, and the sites with the highest off-target score were selected. We identified 10 potential off-target sites for *WIP1* gRNA1, and *WIP1* gRNA2. Twenty pairs of primers (Table S8 in Supporting Information) were designed to amplify the potential off-target sites from the genomic DNA isolated from the 32 weaned cloned pigs. Sanger sequencing was performed to determine whether any mutations occurred.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

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