# Adenine Base-Editing-Mediated Exon Skipping Induces Gene Knockout in Cultured Pig Cells

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# Abstract

Gene-knockout pigs have important applications in agriculture and medicine. Compared with CRISPR/Cas9, Adenine base editor (ABE) convert single A·T pairs to G·C pairs in the genome without generating DNA double-strand breaks, and this method has higher accuracy and biosafety in pig genetic modification. However, the application of ABE in pig gene knockout is limited by protospacer-adjacent motif (PAM) sequences and the base-editing window. Alternative mRNA splicing is an important mechanism underlying the formation of proteins with diverse functions in eukaryotes. Spliceosome recognizes the conservative sequences of splice donors and acceptors in a precursor mRNA. Mutations in these conservative sequences induce exon skipping, leading to proteins with novel functions or to gene inactivation due to frameshift mutations. In this study, adenine base-editing-mediated exon skipping was used to expand the application of ABE in the generation of gene knockout pigs. We first constructed a modified “all-in-one” ABE vector suitable for porcine somatic cell transfection that contained an ABE for single-base editing and an sgRNA expression cassette. The “all-in-one” ABE vector induced efficient sgRNA-dependent A-to-G conversions in porcine cells during single base-editing of multiple endogenous gene loci. Subsequently, an ABE system was designed for single adenine editing of the conservative splice acceptor site (AG sequence at the 3’ end of the intron 5) and splice donor site (GT sequence at the 5’ end of the intron 6) in the porcine gene *GHR*; this method achieved highly efficient A-to-G conversion at the cellular level. Then, porcine single-cell colonies carrying a biallelic A-to-G conversion in the splice acceptor site in the intron 5 of *GHR* were generated. RT-PCR indicated exon 6 skipped at the mRNA level. Western blotting revealed GHR protein loss, and gene sequencing showed no sgRNA-dependent off-target effects. These results demonstrate accurate adenine base-editing-mediated exon skipping and gene knockout in porcine cells. This is the first proof-of-concept study of adenine base-editing-mediated exon skipping for gene regulation in pigs, and this work provides a new strategy for accurate and safe genetic modification of pigs for agricultural and medical applications.


# Key Points

1. ABE can triggers efficient single base conversion at multiple gene loci in pig cells.
2. ABE-mediated exon skipping provides an alternative strategy for gene KO in pig cells.

# Introduction

Traditional pig breeding is limited by the long breeding cycle and insufficient genetic resources, highlighting the potential value of genetic modification that can significantly improve a specific heritable production trait in pigs in one generation. Using gene modification techniques, researchers have created a variety of genetically modified pigs with excellent production traits, resulting in significant improvements in feed utilization, lean meat percentage, disease resistance, and healthy fatty acid composition (Petersen 2017; Zhao. et al. 2019; Han et al. 2020; Xu et al. 2020a; Zhu et al. 2020b).

In addition to the potential applications in agriculture, genetically modified pigs have significant biomedical uses as ideal animal models (Petersen 2017; Perleberg et al. 2018; Yan et al. 2018; Zhu et al. 2018; Zhao et al. 2019; Koppes et al. 2020). Pigs have many advantages over other mammals (e.g., rodents and ruminants). Firstly, pigs possess many similarities to humans in terms of body size, physiology, organ development, disease process, gene sequences, and chromosome structure; pigs are thus better models for human diseases. Secondly, pigs are highly fecund, with early sexual maturation (5-8 months), short generation interval (10-12 months), large litters (10-12

piglets per litter on average), and year-round estrus, allowing rapid propagation of experimental materials. Finally, with the use of efficient gene editing techniques represented by CRISPR/Cas9 in pigs, it has become easier and simpler to construct genetically modified pigs that accurately model human diseases (Perleberg et al. 2018; Zhao. et al. 2019).

The mechanism of CRISPR/Cas9 involves Cas9 protein binding to a single guide RNA (sgRNA), cleaving DNA at the locus targeted by the sgRNA and creating a DNA double-strand break (DSB) (Doudna 2020). This activates two DNA repair mechanisms: non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ is a rapid DSB repair mechanism that ligates the ends of broken DNA double strands while inserting or deleting a certain number of base pairs at the break to introduce indels (insertions and deletions). An indel of length other than an integer multiple of 3 induces a frameshift mutation that causes loss of the functional protein (gene knockout). HDR is a more accurate DSB repair mechanism in the presence of a homologous template and introduces foreign gene sequences into the genome (gene knock-in). CRISPR/Cas9 gene editing has enabled efficient genetic modification of pigs and many other large animal species. However, recent studies have shown that the DSB caused by CRISPR/Cas9 may lead to unexpected changes in gene-edited cells such as uncontrolled indels by NHEJ, off-target effects, large genomic deletions or rearrangements, and tumor suppressor p53-mediated DNA damage (Haapaniemi et al. 2018; Ihry et al. 2018; Kosicki et al. 2018).

To avoid the side effects of DSB, researchers have developed gene editing tools that are independent of DSB and that induce single base mutations. Adenine base editor (ABE), for example, fuse Cas9 protein (Nickase Cas9 or nCas9) that cleaves single-stranded DNA with *Escherichia coli* RNA adenine deaminase TadA (Gaudelli et al. 2017). Under the guidance of sgRNA, the A·T base pairs within positions 4-8 are converted to G·C base pairs, enabling targeted repair of genomic mutations. Due to the absence of DSB, ABE effectively avoid the side effects of CRISPR/Cas9 and achieve specific single base conversion. ABE have been shown to have high accuracy in gene editing at the DNA and RNA levels; therefore, they hold significant potential for applications in agriculture and medicine (Anzalone et al. 2020; Porto et al. 2020). The use of ABE in pig gene editing allows target gene modification with minimal DNA changes, and thus high accuracy and biosafety in genetic improvement and disease model construction (Xie et al. 2020).

In the presence of protospacer adjacent motif (PAM) sequences, ABE can be used to convert A·T to G·C at specific positions in the pig genome, thereby modeling genetic diseases caused by single-base mutations (Anzalone et al. 2020; Porto et al. 2020). However, gene knockout requires conversion of the start codon ATG to GTG (or ATG to ACG in reverse complement sequences) to inhibit translation or induce frameshift mutations at the RNA level (Anzalone et al. 2020; Porto et al. 2020). Since ABE depend on NGG PAM and an editing window at positions 4-8 of sgRNA, the probability of a PAM sequence suitable for the start codon ATG is 62.5% (10/16). Specifically, nearly 40% of genes are unsuitable for ABE-mediated gene knockout, and this limits the application of ABE in pig genetic modification.

RNA splicing is a key step in the production of mature mRNAs in eukaryotes. Spliceosome identifies the 5’ end splice donors and 3’ end splice acceptors of introns in mRNAs, removes the introns, and joins exons to form mature mRNAs (Wilkinson et al. 2020). Eukaryotic genes are composed of various numbers of exons. Changes in RNA splicing patterns lead to diverse mature mRNAs and encoded proteins, thereby contributing to eukaryotic genetic diversity. In natural conditions, alternative RNA splicing creates new functional proteins and inactivates genes through frameshift mutation. For example, Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA), which are common genetic diseases in humans, are caused by gene mutations that lead to

abnormal RNA splicing and dysfunction of the encoded proteins (Montes et al. 2019). An important step in exon splicing is the recognition of highly conservative sequences (splice acceptors and donors) in exon-intron-junctions by spliceosome. Studies have shown that splice donors contain highly conservative GT sequence (at the 5' end of the intron), and mutation leads to skipping of the upstream exon; splice acceptors contain highly conservative AG sequence (at the 3' end of the intron), and mutation leads to skipping of the next downstream exon (Huang et al. 2019). Based on the mechanism of exon skipping, exons can be deleted at the mRNA level by base editing and mutation of the conservative bases in splice donors or acceptors. This strategy has been used to repair the coding sequences of the causative genes of DMD in animal models, demonstrating the potential of gene-editing for treating genetic diseases with high accuracy and safety (Long et al. 2018; Yuan et al. 2018).

ABE-mediated exon skipping creates new mRNAs, and if the length of the skipped exon is not an integer multiple of 3, the skipping induces a frameshift mutation and gene knockout (Yuan et al. 2018; Huang et al. 2019). Since eukaryotic genes are composed of several or even tens of exons, ABE-mediated exon skipping holds great promise for gene knockout. Mutation-induced exon skipping has not been used in the construction of genetically modified pigs. In this study, we used the growth hormone receptor (GHR) gene as an example to validate ABE-mediated exon skipping and gene knockout in pigs. This study aimed to provide a foundation for ABE-mediated exon skipping as a means to construct pig models for human diseases and related gene therapies. GHR is a membrane-bound receptor of growth hormones that triggers intracellular signals through binding to GHR to stimulate cell growth and division (Wang et al. 2019). Loss-of-function mutations in human *GHR* trigger Laron syndrome, and loss-of-function mutations in pig *GHR* cause phenotypes such as short stature and stunting that resemble Laron syndrome (Cui et al. 2015; Hinrichs et al. 2018; Yuan et al. 2020). Therefore, *GHR* knockout pigs are an ideal large animal model for human Laron syndrome.

In this study, we first constructed a modified "all-in-one" ABE vector suitable for porcine somatic cell transfection that contained an ABE for single-base editing and an sgRNA expression cassette. The "all-in-one" ABE vector was shown to induce efficient sgRNA-dependent A·T to G·C conversion in porcine cells during single-base editing at multiple endogenous gene loci. The ABE was designed to edit single adenine residues (thymine in reverse complement sequences) in the *GHR* gene at two sites: one was the conservative AG sequence of the splice acceptor at the 3' end of the intron 5, and the other was the conservative GT sequence of the splice donor at the 5' end of the intron 6. Efficient A·T to G·C (or T·A to C·G in reverse complement sequences) conversion was achieved at the cellular level. Then, porcine single-cell colonies carrying a biallelic A-to-G conversion in the splice acceptor in the intron 5 of *GHR* were generated. RT-PCR showed exon 6 skipping at the mRNA level, while Western blotting confirmed the loss of GHR protein, and gene sequencing showed no sgRNA-dependent off-target effects in the genome. These results suggest that ABE-mediated exon skipping led to gene knockout in porcine cells. This work presents the first proof-of-concept study of ABE-mediated exon skipping and gene regulation in pigs, and the results provide a new strategy for accurate and safe genetic modification of pigs for agricultural and medical applications.

## Materials And Methods

# Reagents and chemicals

All of the reagents used in this study were purchased from Sigma-Aldrich Company (St. Louis, MO, USA) unless stated otherwise. Milli-Q ultrapure water (Millipore, Bedford, MA, USA) was used for the preparation of solutions.

Self-made solutions were filtered through a 0.22-μm filter (Millipore) and stored at 4°C or at -20°C until use. Pipette tips, centrifuge tubes, and petri dishes were purchased in aseptic packages and were all disposable.

# Preparation of “all-in-one” modified ABE vector

The sequence information of the modified ABE vector (namely as pCMV-ABEmaxAW) created by was obtained from the Addgene (catalog: #125647) (Rees. et al. 2019). For construction of ABE vector, the ABEmaxAW fragment with *Age*I/*Bgl*II restriction sites was obtained by gene synthesis (BGI, Shenzhen, China), and linked to the PX459 (Addgene catalog: #62988) vector according to our previous study (Wei et al. 2020). The accuracy of molecular cloning was tested by gene sequencing, and the obtained vector was named as PX-ABEmaxAW. Complete sequence information of PX-ABEmaxAW weas provided online with this paper. The gRNA and ABE expression elements were integrated into one vector, namely as “all-in-one” vector. The “all-in-one” vector is very suitable for transfection of pig cells.

# Construction of ABE plasmid for transfection

Design and construction of the ABE plasmid were performed according to our previous studies (Zhu et al. 2018; 2020a; 2020b; Wei et al. 2020; Pan et al. 2021). The sgRNAs used in this study were designed as described below, and were produced by BGI company. PX-ABEmaxAW vector was linearize using *Bsb*I restriction-enzyme digestion, and linked with the annealed sgRNAs using a T4 DNA Ligase (TaKaRa, Dalian, China). The DNA linking products were transduced into competent *Escherichia coli.* cells, were seeded into LB medium (supplemented with 50 μg/ml of ampicillin) and cultured overnight with shaking at 37°C. We confirmed the constructed CBE base-editing plasmid by DNA sequencing (BGI). Plasmids were purified using an EndoFree Maxi Plasmid Kit (Tiangen, Beijing, China) according to the manufacturer’s instructions, and purified plasmids were then stored at -20°C for future use. The DNA concentration was estimated using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Washington, USA).

# Preparation of Bama minipig fibroblast cells

Procedures used for the isolation, cultivation, and transfection of kidney fibroblasts from newborn Bama minipigs were based upon our previous studies (Zhu et al. 2018; 2020a; 2020b; Wei et al. 2020; Pan et al. 2021). After deep anesthetization, the newborn piglet was euthanized and both kidneys were removed and minced in Dulbecco’s phosphate-buffered saline (DPBS; Gibco, Grand Island, NY, USA). Tissue fragments were washed several times with DPBS and digested in a 0.25% (w/v) trypsin-EDTA solution for 30 minutes at 37°C. Isolated cells were cultured for 1 to 2 passages in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 20% (v/v) fetal bovine serum (FBS; Gibco) and then frozen in liquid nitrogen for future use.

# Activity test of adenine base-editing in cultured Bama minipig cells

In human cells, previous studies have shown that the editing window spanning of modified ABE vector (PX-ABEmaxAW) was located at positions 4 to 8 (counting the protospaceradjacent motif (PAM) as positions 21 to 23) (Pan et al. 2021). To assess the base-editing activity of the modified ABE editor on Bama minipig cells, we selected several genome-editing sites, including sites of genes related to economic trait locus (myostatin gene, *MSTN*) and those of genes related to human disease, gene for coding Iroquois homeobox 3 (*IRX3*) and the gene for coding dystrophin (*DMD*), which are being used for gene editing using CRISPR/Cas9 system in our team. All the gene loci contained the expected ABE-editing sites. The sgRNAs for the tested gene sites were listed in Table 1.

Table 1
Target sites used for activity test of adenine base-editing in pig cells

| No. | Gene* | Target sites (sgRNAs)** | Primers | Amplicon size (bp) |
|---|---|---|---|---|
| 1 | *IRX3* | **CCC**CAGCTCGGATACCAGTACAT | PF: AGCAGATCAATAGGCGAACG<br>PR: ccgctaatctactctcgcg | 503 |
| 3 | *MSTN* site1 | GCTGATTGTTGCTGGTCCCG**TGG** | PF: AACCTCTGACAGCGAGATTC<br>PR: tggacatcgtactgatcaatc | 524 |
| 2 | *MSTN* site2 | AAACAACCTGAATCCAACTT**AGG** | PF: GCTGATCTTCTAATGCAAGTG<br>PR: cataggatatgaaactgaacac | 545 |
| 4 | *DMD* | **CCA**CTATTGAAGCACGTAAGTAT | PF: TGAGAGGAATTCTACCCAGG<br>PR: ctttgttgtaggtctaactcc | 476 |

Notes: **IRX3*, gene for coding Iroquois homeobox 3; *MSTN*, gene for coding myostatin; *DMD*, gene for coding Duchenne muscular dystrophy. **The sgRNA sequences are underlined, the protospacer adjacent motif (PAM) sequences are marked in bold fonts. Predicted adenine base editor targeting bases (positions 4 to 8) are shown in red.

For identification of the ABE-mediated base-editing in cultured cells, 100,000 Bama minipig kidney fibroblast cells in passage number 1 to 2 were transfected using Lipofectamine 3000 according to the manufacturer's instruction. One day later, the cells were split into 35-mm cell-culture dishes (NUNC). After 24 h of recovery, the transfected cells were selected with 1.0 μg/mL puromycin (Solarbio, Beijing, China) for 3 days; and after puromycin was withdrawn, we continuously cultured the cells for 5 to 7 days. For each test of these gene loci, three independent cell transfection were performed. The cells from three independent cell transfection were pooled and genomic DNA was extracted by using a TIANamp Genomic DNA Kit (Tiangen, Beijing, China).

Primers were designed to amplify across the gene-editing sites (Table 1). PCR reactions were conducted with 2 μL of genomic DNA, 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), and 10 μL of PrimeSTAR Max (TaKaRa, Dalian, China); and we then added deionized water to a total volume of 20 μL. PCR amplification conditions were as follows: 1 cycle at 95°C for 5 min; 35 cycles at 98°C for 10 sec, 56°C for 5 sec, and 72°C for 5 sec; followed by 72°C for 5 min. We examined the PCR products using 1.5% (w/v) agarose gel electrophoresis containing 0.01% (v/v) Andy Gold™ Nucleic Acid Gel Stain (Applied BioProbes, Davis, CA, USA).

PCR amplicons were purified using universal DNA purification kit (Tiangen) and sent to the GENEWIZ company for Sanger and next generation sequencing according to our previous study (Pan et al. 2021). For next generation sequencing, amplicons were ligated to adapters and sequencing was performed on an Illumina MiSeq 2x300bp sequencing platform. For each test, more than 50k reads were collected. The ABE-mediated base-editing was determined by aligning the reads to the wild type sequences.

ABE-mediated base conversion at the conservative splice sites of the GHR gene in cultured Bama minipig cells

The gene of GHR, a type of cell receptor protein expressed in cell membrane, has been used to validate ABE-mediated exon skipping and gene knockout in pig cells. The exon 6 of porcine *GHR* is 173 nucleotides long (not an integer multiple of 3) and is present in almost all *GHR* transcripts. Furthermore, the transmembrane domain and intracellular domain of GHR are mainly encoded by exon 6 and downstream exons. Therefore, we hypothesized that ABE-mediated exon 6 skipping and subsequent frameshift mutation could disrupt the function of GHR. According to the principle of exon skipping, mutations at the conservative splice sites, such as AG sequence in the splice acceptor of the intron 5 and GT in the splice donor of the intron 6, in *GHR* might induce exon 6 skipping. The sgRNAs targeting conservative AG sequence (sgRNA1) and GT sequence (sgRNA2) were designed and linked to the PX-ABEmaxAW vector according to the method described above (Table 2). ABE-mediated base conversion activity has been tested as described above.

Table 2
Target sites used for conversion of conservative splice sites of GHR gene in pig cells

| No. | Gene* | Target sites (sgRNAs)** | Primer sequences | Amplicon size (bp) |
|---|---|---|---|---|
| 1 | *GHR* site 1 | ATTTCAGGAGCACTCAAGAG**TGG** | PF: TCTGACTGTCAAGCACCTTG<br>PR: GTTCACCAGCTGATCTCATG | 410 |
| 2 | *GHR* site 2 | **CCG**TTGAGGAAATAGGTAAATCA | PF: TCTGACTGTCAAGCACCTTG<br>PR: GTTCACCAGCTGATCTCATG | 410 |

Notes: **GHR*, gene for coding growth hormone receptor. **The sgRNA sequences are underlined, the protospacer adjacent motif (PAM) sequences are marked in bold fonts. Predicted adenine base editor targeting bases (positions 4 to 8) are shown in red. The conservative splice sites of GHR gene are marked with double-underline.

For generation of base-edited single-cell colonies, the ABE plasmid-transfected cells were subcultured in 60-mm culture dishes. After 48 h, the cells were treated with 1.0 μg/ml puromycin for three days to eliminate the non-transfected cells. After removing the screening drug, the remaining drug-resistant cells were further cultured for nine to ten days. Once a single-cell colony with a diameter of 2-3 mm (approximately contain 1,000 cells) was found, the single-cell colony was isolated and inoculated into a 4-well culture clusters (NUNC) for further incubation to confluence. The cell colonies were subcultured and part of them was collected for base-editing identification. Positive base-editing cell colonies were expanded and then cryopreserved. PCR amplicons produced as described above were sent out for Sanger DNA sequencing. The ABE-mediated base-editing was determined by aligning the reads to the wild type sequences.

# Exon skipping and Gene knockout identification

For identification of ABE-mediated exon skipping occurred at the transcription level, a RT-PCR and Sanger sequencing were performed. Briefly, total RNA was isolated from the base-edited single-cell colonies using a MiniBEST Universal RNA Extraction Kit (TaKaRa). cDNA was synthesized from 1 μg of total RNA using a PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa) with 20 μL reaction volumes according to the manufacturer's protocol. The resulting cDNA products were used as the template for amplification by PCR with primers specific to the sequence of porcine *GHR* mRNA (Table 3). The glyceraldehyde-3-phosphate dehydrogenase

gene (*GAPDH*) was served as reference gene. The PCR products were collected for Sanger sequencing to confirm whether the desired exon skipping had been occurred at the transcription level.

Table 3
Primers used for RT-PCR test of *GHR* and *GAPDH*

| Gene No. | Gene name | Primer sequences (5'-3') | Application size (bp) |
|---|---|---|---|
| 1 | GHR | F: CCTAAATTCACCAAGTGCCG | Wild type: 425/Exon skipped: 252 |
| | | R: TACTCCAGGACTATCCATCC | |
| 2 | GAPDH | F: GTTCCAGTATGATTCCACCC | 310 |
| | | R: ATTGCTGACGATCTTGAGGG | |

Notes: **GHR*, gene for coding growth hormone receptor, the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was served as reference gene.

For verification of GHR gene knockout induced by exon skipping, a western blotting test was performed. Briefly, the subcultured cells from the base-edited single-cell colonies were homogenized in cell lysis buffer and 30 μg of isolated total protein were analyzed by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. Then the distributed protein were immuno-blotted onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, USA). The primary mouse monoclonal antibodies against GHR (1:1000; DF8425, Affinity Biosciences) and GAPDH (1:1000; T0004, Affinity Biosciences) were used, and were detected with a horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG secondary antibody (1:1000; sc-358914, Santa Cluz). The immuno-stained membranes were imaged using an Odyssey Fc Imaging system (LI-COR Biosciences, Lincoln, NE, USA) with immunochemiluminescent substrate for detection of HRP. Positive western blotting results for detection of GHR and GAPDH will appear at probably 80 and 36 kDa, respectively.

# Genomic off-target detection in adenine base-edited pig cell colonies

Potential OTSs were predicted by the CRISPR Design Tool (http://crispor.tefor.net) according to our previous studies (Zhu et al. 2018; 2020a; 2020b; Wei et al. 2020; Pan et al. 2021). Four sites with potential genomic off-target effects for *GHR* locus were selected for detection of off-targets occurred in base-edited cell colonies (Table 4). Specific primers were used for PCR reaction, and the products were sequenced to confirm whether genomic off-targeting mutations existed. Genomic off-targets were identified by alignment of sequenced alleles to wild type allele.

Table 4
Off-target sites (OTS) used for analysis of adenine base-edited pig single-cell colonies

| OTS No. | Chr. | Target sites (sgRNAs)* | Score | Primer sequences (5'-3') | Amplicon (bp) |
|---|---|---|---|---|---|
| *GHR* site 1 | Chr15 | ATTTCAGGAGCACTCAAGAG**TGG** | 100 | F: TCTGACTGTCAAGCACCTTG<br>R: GTTCACCAGCTGATCTCATG | 410 |
| OTS 1# | Chr14 | GGTTAAGGAACACTCAAGAG**AGG** | 1.32 | F: CAAACCATGGCATACATGCC<br>R: ATCACTGGCCAGAAATTTCC | 483 |
| OTS 2# | Chr9 | GTTTCAGGAGCACTCAAGGG**AGA** | 1.30 | F: ACATCGTTCATACTCAGGCG<br>R: GCAGTGAATGGAGCTTGAAC | 420 |
| OTS 3# | Chr15 | ACTCAATGAGCACTCAAGAG**TGG** | 0.89 | F: AGGGTTTGGGATGGAAATGC<br>R: GAGAGCCTTTAGTTCTCTGG | 471 |
| OTS 4# | Chr6 | ATCTAAGCTGCACTCAAGAG**AGG** | 0.82 | F: GGACAGATACATACGCTGTC<br>R: TGGTCTTGTAGTTGGTGTCC | 501 |

*The sgRNA sequences are underlined, protospacer adjacent motif (PAM) sequences are labeled with bold fonts. Red letters are the mismatched nucleotides in off-target sequences aligned to sgRNA.

## Results

# Construction of a modified all-in-one ABE plasmid and evaluation of editing activity

An ABE system is composed of an editor for base editing and an sgRNA for target recognition; these are fused into a plasmid to improve cell transfection and editing efficiency. In this study, we first constructed a modified all-in-one ABE vector suitable for porcine somatic cell transfection. The ABE sequence ABEmaxAW modified for reducing off-target effects and improving editing activity was fused with the sgRNA expression cassette in the vector (Fig. 1A). To test the activity of the modified all-in-one ABE plasmid on Bama minipig cells, four endogenous gene loci previously used as the targets of CRISPR/Cas9-mediated gene knockout in Bama minipig cells were selected. These loci included *DMD* and *IRX3* for the construction of a human disease model and the *MSTN* for improvement of pig economic traits (Fig. 1B).

Bama minipig primary fibroblasts were cultured and transfected using Lipofectamine 3000 and treated with 1.0 μg/mL puromycin for three days to delete non-transfected cells. After staining, puromycin was removed, and the

cells were cultured for 5-7 days. Cells were digested for genomic DNA extraction. Primers targeting both ends of the editing targets were used for PCR, and the products were sequenced by Sanger and next generation sequencing. The efficiency of A-to-G conversion (or T-to-C in reverse complementary sequences) was 42.1%, 49.4%, 27.4%, and 28.3% at loci of *IRX3*, *MSTN* site 1, *MSTN* site 2, and *DMD*, respectively (Fig. 1B,C). The ABE plasmid constructed in this study showed high editing efficiency that varied across the editing sites. Subsequent editing window analysis of the base editor revealed efficient adenine editing at 5-7 positions of the sgRNA (Fig. 1D). These results indicate that the modified all-in-one ABE plasmid could be used for the conversion of a single A·T base pair to a G·C base pair in cultured Bama minipig cells.

Adenine base-editing-mediated base conversions at the conservative splice sites of the GHR gene in cultured pig cells

GHR is a membrane-bound receptor of growth hormones, and it plays important roles in cell growth and division. Loss-of-function mutations of the *GHR* in pigs will trigger phenotypes such as short stature and stunting that mimic human Laron syndrome. *GHR*-knockout pigs are an ideal large animal model for human Laron syndrome. Existing *GHR*-knockout pigs are generated by frameshift mutations caused by random indels mediated by CRISPR/Cas9. In this study we verified the feasibility of porcine *GHR* knockout using ABE-mediated exon skipping. The exon 6, which is 173 nucleotides long (not an integer multiple of 3), is present in multiple transcripts of porcine *GHR*. Furthermore, the transmembrane domain and intracellular domain of GHR are mainly encoded by exon 6 and downstream exons. Therefore, we hypothesized that ABE-mediated exon 6 skipping and subsequent frameshift mutation could disrupt the function of GHR.

According to the principle of exon skipping, mutations at the conservative splice acceptor site (AG sequence at the 3’ end of the intron 5) or splice donor site (GT sequence at the 5’ end of the intron 6) in *GHR* might induce exon 6 skipping (Fig. 2A,B). Sequence analysis showed suitable PAM sequences at both sites. The sgRNAs targeting splice acceptor (sgRNA1; Fig. 2C) and splice donor (sgRNA2; Fig. 2D) were designed for the construction of ABE plasmids that were transferred into Bama minipig cells to test the editing activity. Sanger and next generation sequencing showed that both sgRNA1 and sgRNA2 achieved efficient A-to-G conversion (or T-to-C in reverse complement sequence) (Fig. 2C,D).

Given the presence of only single base A in the editing window of sgRNA1 and a base A with bystander effect at position 3 of sgRNA2 (Fig. 2D), sgRNA1 was used to generate cell colonies carrying *GHR* with an ABE-mediated single base mutation. Two rounds of cell transfection were performed in this study, and a total of 27 single-cell colonies were selected, 12 of which were cultured and showed desirable cell activity and morphology. Finally, Sanger DNA sequencing showed that four (33.3%, 4/12) single-cell colonies underwent adenine base-editing-mediated biallelic A-to-G conversions (Fig. 2E; Table 5).

Table 5
Summary of *GHR* site 1 genotypes of adenine base-edited pig single-cell colonies

| Colony No. | *GHR* site1 genotypes* | Summary |
|---|---|---|
| WT control | TTCATCCTTTTTTACCCCCCATTTCAGGAGCACTCAAGAG**TGG**ACTCAAGAATGGAAAGA | WT |
| 1-3# | TTCATCCTTTTTTACCCCCCATTTCGGGAGCACTCAAGAG**TGG**ACTCAAGAATGGAAAGA | Converted |
| | TTCATCCTTTTTTACCCCCCATTTCGGGAGCACTCAAGAG**TGG**ACTCAAGAATGGAAAGA | Converted |
| 1-4# | TTCATCCTTTTTTACCCCCCATTTCGGGAGCACTCAAGAG**TGG**ACTCAAGAATGGAAAGA | Converted |
| | TTCATCCTTTTTTACCCCCCATTTCGGGAGCACTCAAGAG**TGG**ACTCAAGAATGGAAAGA | Converted |
| 2-1# | TTCATCCTTTTTTACCCCCCATTTCGGGAGCACTCAAGAG**TGG**ACTCAAGAATGGAAAGA | Converted |
| | TTCATCCTTTTTTACCCCCCATTTCGGGAGCACTCAAGAG**TGG**ACTCAAGAATGGAAAGA | Converted |
| 2-2# | TTCATCCTTTTTTACCCCCCATTTCGGGAGCACTCAAGAG**TGG**ACTCAAGAATGGAAAGA | Converted |
| | TTCATCCTTTTTTACCCCCCATTTCGGGAGCACTCAAGAG**TGG**ACTCAAGAATGGAAAGA | Converted |

Notes: *The sgRNA1 sequences are underlined, protospacer adjacent motif (PAM) sequences are labeled with bold fonts. ABE-mediated A-to-G conversions are marked in red. WT, wild type.

# Adenine base-editing-mediated exon skipping leads to GHR knockout

To verify that adenine base-editing-mediated mutation in the conservative splice acceptor site (AG sequence) can induce exon skipping, the four single-cell colonies carrying biallelic A-to-G conversions in the target site were subjected to mRNA analysis. After cell culture, total RNA extraction and cDNA synthesis, the gene products were obtained by RT-PCR. Both nucleic acid electrophoresis (Fig. 3A) and Sanger DNA sequencing (Fig. 3B) indicated skipping of the 173-nucleotide exon 6 in the target mRNA sequence.

To further examine the effect of exon 6 skipping on porcine GHR function, total protein were extracted from four single-cell colonies for Western blotting analysis. GHR protein was absent in the four colonies (Fig. 3C), suggesting that adenine base-editing-mediated base conversion of the conservative splice acceptor site (AG sequence) was capable of inducing exon skipping in porcine cells. If the length of the skipped exon is not an integer multiple of 3, exon skipping induces a frameshift mutation and loss of the functional protein (namely is to achieve gene knockout).

# Analysis of sgRNA-dependent off-target effects

Due to the absence of DSB, ABE, especially modified ABE, are expected to have better accuracy and safety than traditional CRISPR/Cas9 system in gene editing. To examine the genomic off-target effects induced by the modified ABE in Bama minipig cells, four single-cell colonies carrying desired base-editing and showing good cell growth and morphology were selected. Four off-target sites with high scores (> 0.8) were predicted by the software (Table 4). Primers were designed for the four sites (Table 4), and PCR was performed based on the genomes of the four single-cell colonies (Fig. 4A), followed by DNA sequencing. As shown in Fig. 4B, neither sgRNA-dependent A-to-G conversion nor deletion/insertion mutations caused by DSB were detected at the four off-target sites. These

findings indicate that the modified all-in-one ABE vector constructed in this study did not induce detectable genomic off-target effects in porcine cells at the selected targets and thus had high accuracy in gene editing.

## Discussion

CRISPR/Cas9 genome-editing holds great promise for genetic modification of pigs, but its application is limited by safety concerns

Gene editing techniques represented by CRISPR/Cas9 have revolutionized the genetic modification of pigs (Petersen 2017; Zhao. et al. 2019). In agricultural practice, a variety of genetically modified pigs with excellent production traits have been developed using CRISPR/Cas9, with significantly improved feed utilization rate, lean meat percentage, disease resistance, healthy fatty acid composition, and sex control (Han et al. 2020; Xu et al. 2020a; Zhu et al. 2020b; Kurtz et al. 2021). In medical practice, CRISPR/Cas9 has been used to create pigs with Huntington's chorea, Parkinson's disease, DMD, and immunodeficiency, thereby providing ideal large animal models for disease pathogenesis and treatment (Perleberg et al. 2018; Yan et al. 2018; Zhu et al. 2018; Koppes et al. 2020).

However, as research progresses, the safety risks associated with CRISPR/Cas9 have attracted increasing attention. In 2018, Kosicki et al. (2018) reported that repair of DSB caused by CRISPR/Cas9 disrupted genome stability in mouse embryonic stem cells, mouse hematopoietic progenitor cells, and human differentiated cell lines in addition to inducing large deletions (kilobases) and complex genomic rearrangements at the targeted sites. In the same year, CRISPR/Cas9 was shown to induce oncogene p53-mediated DNA damage and cell cycle arrest in human epithelial cells, and p53 inhibited CRISPR/Cas9 editing of human pluripotent stem cells (Haapaniemi et al. 2018; Ihry et al. 2018). In 2020, Zuccaro et al. (2020) reported that numerous DSB induced by CRISPR/Cas9 gene editing in early human embryos remained unrepaired, eventually leading to deletions of large regions or even the whole chromosome. In addition, Xu et al. (2020b) recently showed that Cas9 overexpression induced extensive DNA damage and genomic instability and activated the DNA damage response pathway, which in turn triggered the expression of target genes downstream of p53 and eventual cell death. The study also showed that the ability of Cas9 to induce DSB damage was dose-independent, and low-level Cas9 exposure was sufficient to induce DNA damage. These risks will inevitably hinder the application of CRISPR/Cas9 in genetic modification of pigs.

# The adenine base-editing provides a better option for accurate and efficient genetic modification of pigs

The greatest safety risk of CRISPR/Cas9 arises from the induction of DSB, and this can be avoided by using base editors. Base editors do not rely on donor templates and allow conversion of specific single base pairs within the editing window with high accuracy and safety (Gaudelli et al. 2017; Anzalone et al. 2020; Porto et al. 2020). Single-base editing neither creates DSB nor bears the risks of cellular DNA damage or chromosome cleavage.

Cytosine base editor (CBE) was developed earlier and has been more commonly used in pigs (Li et al. 2018; Yu et al. 2018; Xie et al. 2019; Wang et al. 2020). For instance, Li et al. (2018) used BE3 to construct *TWIST2*- and *TYR*-edited porcine fibroblasts. Model pigs with microtia, congenital absent eyelids, ablepharon macrostomia syndrome (AMS), and albinism were generated by somatic cell nuclear transfer from cells carrying edited *TWIST2* and *TYR*. Xie et al. (2019) used BE3 or hA3A-BE3 along with embryo injection and somatic cell cloning to construct pigs with edited *DMD*, *LMNA*, or *RAG1*, *RAG2*, and *IL2RG*. Wang constructed a hA3A-BE3-NG system by

fusing an SpCas9-NG mutant with hA3A-BE3 and achieved C-T conversion at NGN PAM sites, leading to nonsense and missense mutations in several economic trait-related genes (*CD163*, *APN*, *MSTN*, and *MC4R*) in porcine fibroblasts, showing great potential for animal genetic breeding. Yuan et al. (2020) used AncBE4max to edit multiple porcine genes (*GGTA1*, *B4galNT2*, and *CMAH*), and the genetically modified pigs could be used as organ donors for xenotransplantation. These results suggest that base editing tools provide a new option for genetic modification of pigs.

ABE has not yet been widely used in pigs but will play a greater role on the basis of CBE applications. Moreover, existing studies have shown that ABE have lower off-target activity than CBE. In 2019, Zuo et al. (2019) and Jin et al. (2019) in studies using animals and plants, respectively, found that ABE had DNA off-target activity lower than that of CBE and similar to that of CRISPR/Cas9. In addition, with protein engineering and optimization techniques, ABE can be optimized to further reduce off-target activity (Rees. et al. 2019). The ABEmaxAW used in this study has been shown to minimize DNA off-target effects while maintaining the activity of targeting and editing single base pairs, and thus holds great promise in agricultural and medical applications. ABE have been tested for the feasibility of treating genetic diseases at the cellular and animal model levels. An encouraging example is a recent study on ABE treatment of Hutchinson-Gilford progeria syndrome (HGPS) (Koblan et al. 2021). HGPS is caused by a C·G to T·A mutation at the nucleotide position 1,824 of *LMNA* (encoding lamin A) that disrupts the normal splicing of *LMNA* mRNA and leads to the production of a toxic truncated protein, progerin. This protein interferes with the function of normal lamin A encoded by *LMNA*, showing a dominant negative effect, ultimately leading to HGPS in patients harboring this heterozygous mutation in *LMNA*. Koblan et al. (2021) used an AAV9 vector for systemic delivery of the ABE system to HGPS mice and showed that the pathogenic C·G to T·A mutation could be corrected by A·T to G·C conversion mediated by ABE. This restored the normal splicing of *LMNA* mRNA, reduced the symptoms of HGPS, and extended the life span of mice without detectable off-target activity, demonstrating extremely high efficacy and safety in gene therapy. This preclinical proof-of-concept study provided data for the use of ABE in the treatment of diseases resulting from single gene mutations.

In this study, we constructed a modified all-in-one ABE plasmid suitable for transfection of porcine somatic cells. The ABE for single-base editing was fused with an sgRNA expression cassette into the plasmid that induced efficient sgRNA-dependent A·T to G·C conversion at multiple porcine endogenous gene loci. The efficiency of ABE-mediated single-base editing of *DMD* and *IRX3* (genes for human disease models) as well as *MSTN* (a gene for economic trait improvement in pigs) ranged from 27.4–49.4%, values that were slightly lower than indels induced by CRISPR/Cas9 at the same sites (Zhu et al. 2020a; 2020b). This is consistent with the findings of other studies, presumably because the editing efficiency of ABE is dependent on the activity of *E. coli* RNA adenine deaminase TadA, which may be suboptimal in pig cells due to the wide evolutionary divergence (Gaudelli et al. 2017; Xie et al. 2020). The present study also showed that the efficiency of ABE-mediated single-base conversion varied at the same site in different genes and editing windows, in agreement with the study using 293T cells conducted by Rees et al. (2019). Advances in protein engineering and optimization techniques have enabled continuous optimization of ABE and gradual improvement of their editing activities, with the recently developed ABE8e showing better adenine base editing efficiency (Richter et al. 2020).

Despite the relatively low editing efficiency, ABE-medicated gene editing is predictable due to the absence of DSB, and thus has high accuracy and safety (Gaudelli et al. 2017; Anzalone et al. 2020; Porto et al. 2020). In this study, indels and sgRNA-dependent off-target effects induced by DSB were not detected in the porcine single-cell colonies created with ABE, confirming that ABE can perform safe and accurate single base editing in pig cells. The

accuracy and safety of gene editing are very important considerations in pig gene modification for agricultural and medical applications (Zhao. et al. 2019). In this study, single-base editing was carried out in porcine cells, and cloned pigs with edited single bases can be generated by somatic cell nuclear transfer (SCNT). The advantage of SCNT for generating genetically modified pigs is that the genetic modification of donor cells and the generation of cloned pigs can be conducted separately (Perleberg et al. 2018; Ryu et al. 2018; Zhao. et al. 2019). In theory, only one gene-edited cell clone is needed to generate cloned pigs. Thus, the relatively low efficiency of single-base editing in porcine somatic cells has little effect on the generation of cloned pigs and can be compensated by simply increasing the number of cell transfection and single-cell colonies. Therefore, the single-base editing efficiency using the modified all-in-one ABE vector in porcine somatic cells satisfies the need for accuracy and safety in the generation of single-base edited pigs for agricultural and medical applications.

# Exon skipping-induced gene knockout expands the application of adenine base-editing in pig genetic modification

Eukaryotic protein-coding genes are characterized by multiple exons, and alternative mRNA splicing produces various proteins; this is an important factor in eukaryotic gene diversity (Wilkinson et al. 2020). Genetic studies have shown that exon skipping caused by mutations in the conservative splice acceptors and donors is responsible for many human genetic diseases (Montes et al. 2019). For instance, DMD is a common human genetic disease caused by mutations in the conservative splice acceptors and donors in the introns of *DMD* that lead to exon skipping. If the length of an exon is not an integer multiple of 3 (such as the exons 50 and 52 often lacking in DMD patients), the skipping induces a frameshift mutation that disrupts the function of the encoded protein (Montes et al. 2019). Based on the same mechanism, CRISPR/Cas9 can be used to mutate conservative splice acceptor or donor sites in introns to skip adjacent exons and repair the gene coding frame (Min et al. 2019). The disease can be cured by expressing a truncated protein with normal function. The strategy of using CRISPR/Cas9 and base editor to mutate conservative sequences in splice acceptors or donors to induce exon skipping has been demonstrated in a proof-of-concept study with animal models of DMD, showing the potential for a complete cure of this disease with high accuracy and safety (Long. et al. 2018; Yuan et al. 2018).

Single nucleotide mutations are responsible for about two-thirds of human diseases, and they also serve as the genetic basis for variation in agronomically important traits in many crops (Zhao. et al. 2019; Anzalone et al. 2020; Porto et al. 2020). Therefore, it is particularly important to develop techniques for accurate and efficient base substitution. Gene knockout is an important means to generate genetically modified pigs for agricultural and medical applications. For example, *MSTN*-deficient pigs show accelerated muscle growth; *CD163*-deficient pigs are resistant to porcine reproductive and respiratory syndrome virus, and *GGTA1/CMAH/B4GALNT2*-deficient pigs have the great potential for pig-to-human xenotransplantation (Whitworth et al. 2016; Burkard et al. 2017; Xu et al. 2020a; Zhu et al. 2020b; Yue et al. 2021). Limited by PAM sequences and the editing window, the probability that ABE can be used to mutate the start codon ATG for gene knockout is only 62.5% (10/16). Specifically, approximately 40% genes cannot be knocked out by ABE, and this greatly limits the application of ABE in the construction of gene-knockout pigs. Since natural exon skipping may induce loss-of-function mutations, ABE-mediated exon skipping was designed to knock out the porcine gene *GHR*, thereby providing an alternative strategy for use of ABE in the construction of gene-knockout pigs. The exon 6, which is present in all *GHR* transcripts, was selected in this study. Exon 6 is 173 nucleotide long, and its deletion can induce gene frameshift mutations and loss of function in GHR. According to the principle of exon skipping, mutations targeting the conservative splice sites in *GHR* may induce exon 6 skipping. Fortunately, suitable PAM sequences are present at

both sites, and ABE-mediated A-to-G conversion (or T-to-C in reverse complement sequences) was achieved at both loci with high efficiency. These findings establish a foundation for subsequent validation of ABE-mediated exon skipping and *GHR* knockout in porcine cells.

It is generally difficult to generate highly viable genetically modified porcine cells (Ryu et al. 2018; Zhao. et al. 2019). In this study, a modified all-in-one ABE vector suitable for porcine cell transfection and highly active gene editing targets was used to obtain several single-cell colonies. The single-cell colonies had favorable growth and cell morphology. Gene sequencing showed that 33.3% (4/12) of single-cell colonies had biallelic ABE-mediated A-to-G conversions at the target locus. This efficiency is comparable to that of CRISPR/Cas9 in generating gene-knockout pig cells conducted in our previous studies, suggesting that the ABE system is a practical and feasible approach to generating genetically modified porcine cell colonies (Zhu et al. 2018; 2020a; 2020b; Wei et al. 2020; Pan et al. 2021). More importantly, RT-PCR and Western blotting tests showed that ABE-mediated single-base mutations in the conservative splice acceptor in the intron 5 of *GHR* successfully induced exon 6 skipping at the RNA level and gene knockout at the protein level. Therefore, the present study confirmed at the cellular level that gene knockout in pigs can be achieved by ABE-mediated exon skipping. However, whether ABE-mediated exon skipping and frameshift mutation can result in the expected phenotypes of *GHR* knockout needs to be tested in cloned pigs carrying *GHR* with the single-base mutation.

In summary, we constructed a modified all-in-one ABE vector suitable for porcine somatic cell transfection and demonstrated efficient sgRNA-dependent A-to-G conversions at multiple porcine endogenous gene loci. In porcine cells, we confirmed that exon skipping and exon skipping-induced gene knockout can be achieved by ABE-mediated single base mutation in the conservative splice sites of the target gene without inducing detectable sgRNA-dependent off-target effects. This is the first proof-of-concept study of ABE-mediated exon skipping for gene regulation in pigs, and the results provide a new strategy for accurate and safe genetic modification of pigs in agricultural and medical applications.

# Declarations

### Author Contribution

D.S.T., J.H.L and X.X.Z. conceived and designed the experiments. X.X.Z., J.S.P., T.L., Y.C.Y., Q.Y.H., S.P.Y. and Z.X.Q. conducted the experiments and analyzed the results. Z.S.L. and J.C.W. participated in experiment assistant. X.X.Z., J.S.P., T.L., J.H.L and D.S.T. drafted the manuscript. All authors participated in discussions of the results and reviewed the manuscript.

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### Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

**Conflict of Interest:** All authors declare no competing financial and non-financial interests.

**Animal Ethical Approval:** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All of the animal procedures used in this study were carried out in accordance with the *Guide for Care and Use of Laboratory Animals* (8th edition, released by the National Research Council, USA), and were approved by the Animal Care & Welfare Committee of Foshan University (approval no. 2019020). All of the surgical procedures were performed under anesthesia by a veterinarian, and all efforts were made to minimize animal suffering.

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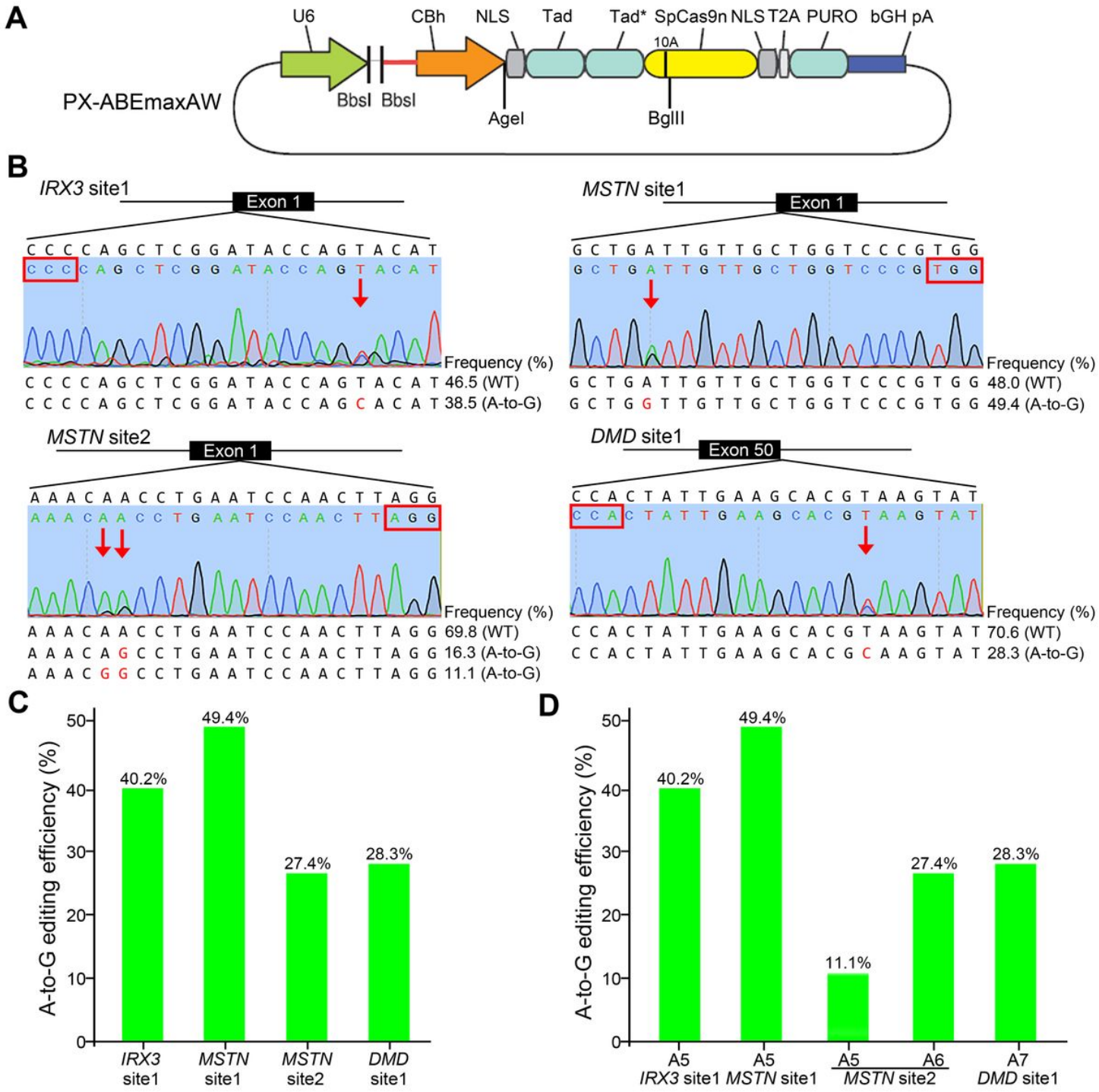
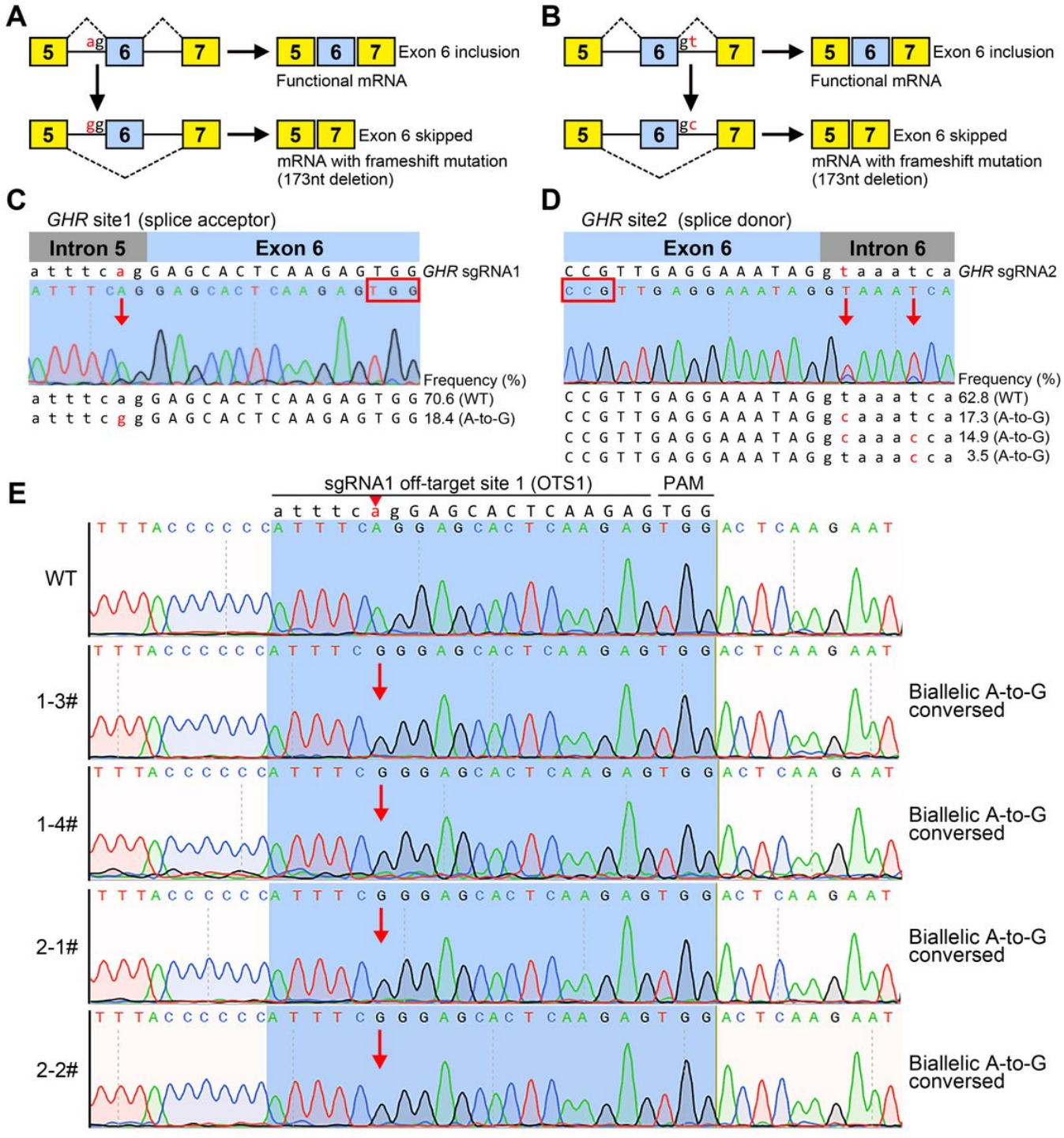
## Figures




## Figure 1

Activity test of the adenine base-editing in cultured Bama minipig cells. (A) The constructed plasmid schematic view of “all-in-one” PX-ABEmaxAW vector constructed in this study. The ABEmaxAW sequence was synthesised and then linked them to the conventional PX459 vector. The expression of sgRNAs was initiated by an U6 promoter. The expression of ABE was controlled by CBh promoter. The PURO (puromycin) resistance gene was used for transfection screening in eukaryotic cells. These vectors could be linearized with BbsI restriction enzyme for linking sgRNAs. (B) ABE-mediated A-to-G conversions at four endogenous gene sites in Bama minipig cells. The gene sites in genome were indicated, the base-editing induced A-to-G (in the complementary chain is T-to-C) conversions were indicated by red arrows, protospacer adjacent motif (PAM) sequences are labeled with red

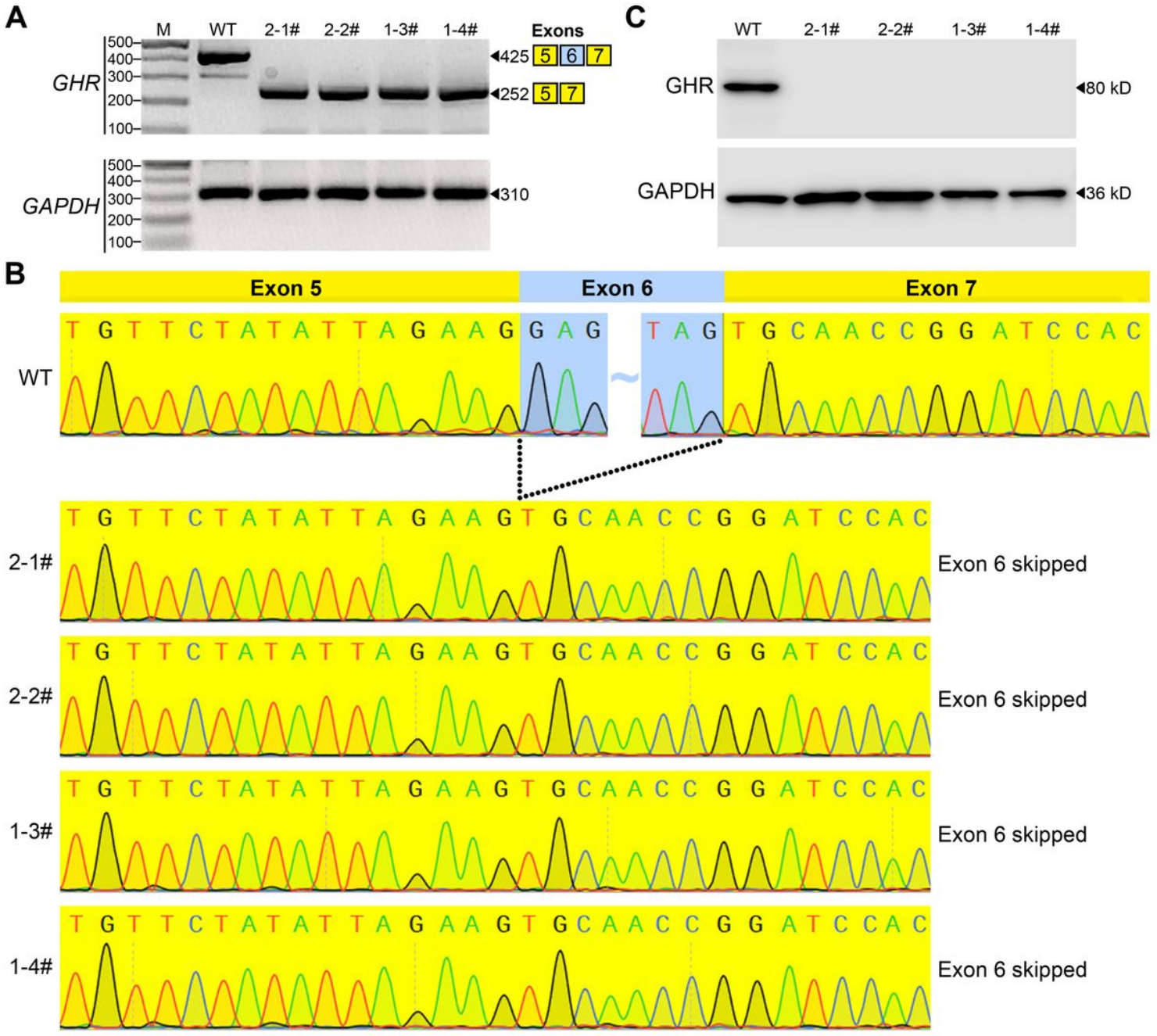
boxes. Representative A-to-G (in the complementary chain is T-to-C) base-editing induced hybrid peaks in Sanger DNA sequencing results were presented. Allele frequencies from deep sequencing are listed to the right. The ABE-mediated A-to-G conversion efficiency and positions at four tested gene sites were presented in panels (C) and (D), respectively.




## Figure 2

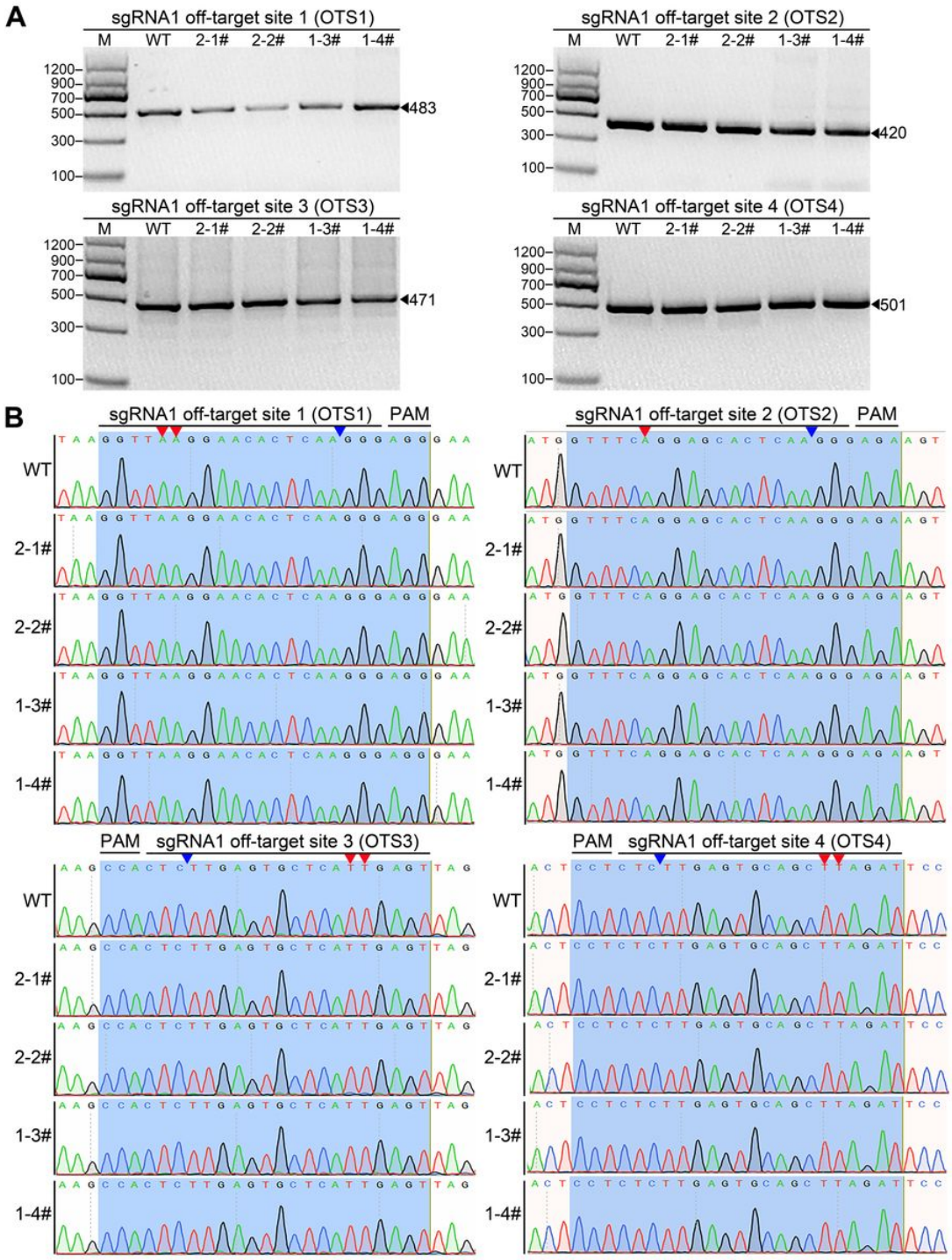
Adenine base-editing-mediated base conversions at the conservative splice sites of the GHR gene in cultured pig cells. The schematic diagrams are showing the designed conversions of the conservative splice sites, namely as AG in splice acceptor of intron 5 (A) and GT in splice donor of intron 6 (B), will induces exon 6 skipping and subsequent frameshift mutation which could disrupt the function of GHR. Panels C and D are showing the

adenine base-editing-mediated base conversions at the splice acceptor and splice donor of the GHR gene in cultured pig cells, respectively. The base-editing induced A-to-G (in the complementary chain is T-to-C) conversions were indicated by red arrows, protospacer adjacent motif (PAM) sequences are labeled with red boxes. Representative A-to-G (in the complementary chain is T-to-C) base-editing induced hybrid peaks in Sanger DNA sequencing results were presented. Allele frequencies from deep sequencing are listed to the right. (E) Sanger DNA sequencing results are showing the obtained four pig single-cell colonies were harboring the ABE-mediated biallelic A-to-G conversions (indicated by red arrows).




## Figure 3

Base editing can alter GHR splicing in cultured pig cells. RT-PCR (A) and Sangger DNA sequencing (B) results are indicating the exon 6 of GHR were skipped in all of the four adenine base-edited pig single-cell colonies. (C) Western blotting test showed the GHR were disrupted in all of the four adenine base-edited pig single-cell colonies. M, DNA marker; WT, wild type.




## Figure 4

Detection of genomic off-target effects in ABE-edited pig single-cell colonies. Four genomic off-target sites (OTS) with potential off-target effects were screened for off-target identification. Specific primers were used for PCR reaction, and the products (A) were sequenced to confirm whether off-targeting mutations existed. The sample collected from wild type (WT) cells was used as a control. (B) Sanger DNA sequencing results are indicating no A-to-G (in the complementary chain is T-to-C) conversions occurred at the four selected OTS and no base deletion/insertion mutations caused by DNA double-strand breaks detected in the tested four CBE-edited single-cell colonies. The OTS targets and protospacer adjacent motif (PAM) are indicating upon the wild type (WT) sequences. The predicted base-editing induced A-to-G (in the complementary chain is T-to-C) conversions were

indicated by red arrowheads. The functional CRISPR/Cas9 induced DNA cleavage sites were indicated by blue arrowheads. Note that the CRISPR/Cas9-induced deletion/insertion mutations usually occur near this site. M, DNA marker.