INTRODUCTION

Transmissible gastroenteritis virus (TGEV) causes an acute and highly contagious enteric disease in pigs, which is characterized by severe enteritis, vomiting, and watery diarrhea. TGEV dramatically affects the porcine industry production. According to reports, the mortality rate in neonatal piglets infected with TGEV is approximately 100%. In spite of vaccine therapy, TGEV infections continue to be a concern in the porcine industry. TGEV enters the host by first attaching its glycoprotein (S protein) to the host cellular surface receptor, porcine aminopeptidase N (pAPN) (Delmas et al., 1992).

Traditional animal breeding is time-consuming, money-consuming and progress is extremely slow. On the other hand, gene knock-out and knock-in strategies are powerful for investigating gene function in livestock and have potential biotechnology applications in farm animal breeding (Lillico et al., 2013; Richt et al., 2007; Whitworth et al., 2016; Wu et al., 2015; Yu et al., 2011). Knock-out of the pathogenic host cellular receptor may represent an effective strategy to investigate the molecular mechanism of the host cellular response and accelerate the livestock resistant breeding progress (Richt et al., 2007; Whitworth et al., 2016).

In this study, we performed site-specific editing of pAPN and produced pAPN site-specific edited swine testis (ST) cells and cloned pigs using TALEN technology. Site-specific editing of pAPN reduced TGEV proliferation in ST cells by 96%–99% at different time periods post-infection. Five pAPN site-specific edited pigs were produced. Our research findings will not only offer a more thorough understanding of the pathogenesis of piglet diarrhea and lay the foundation for breeding TGEV-resistant piglets, but also understanding the molecular mechanisms involved in coronaviral infections.

KEYWORDS

coronaviral infections, pAPN, porcine viral diarrhea, resistance breeding, TGEV

ORIGINAL ARTICLE

Production of porcine aminopeptidase N (pAPN) site-specific edited pigs

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Abstract

Porcine viral diarrhea is an acute and highly contagious enteric disease in pigs which causes huge economic losses in pig industry worldwide. Transmissible gastroenteritis virus (TGEV) is main pathogens responsible for piglets viral diarrhea. Knockout the host cellular surface receptor for TGEV may be an effective way to accelerate the breeding of resistant pigs. In this study, we applied site-specific editing pAPN which is effective in swine testis (ST) cells. Site-specific editing of pAPN reduced TGEV proliferation in ST cells by 96%–99% at different time periods post-infection. Next, the site-specific editing of pAPN porcine fetal fibroblasts were produced, and then the cell colonies were used as donor cells to generate the site-specific editing of pAPN pigs. Our research findings will not only offer a more thorough understanding of the pathogenesis of piglet diarrhea and lay the foundation for breeding TGEV-resistant piglets, but also understanding the molecular mechanisms involved in coronaviral infections.

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In this study, we performed site-specific editing of pAPN and produced pAPN site-specific edited swine testis (ST) cells and cloned pigs using TALEN technology. Site-specific editing of pAPN reduced TGEV proliferation in ST cells by 96%–99% at different time periods post-infection. Five pAPN site-specific edited pigs were produced.

Jianwen Chen and Kaiyuan Pan contributed equally to this work.

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2 | MATERIALS AND METHODS

2.1 | Ethics statement

The animal study proposal was approved by the Institutional Animal Care and Use Committee of the Anhui Agricultural University. All porcine experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the People’s Republic of China.

2.2 | Construction of pAPN TALEN and donor vector

The sketch map of pTALEN-01 and pDonor-D01 constructs was shown in Figure 1 (Gene Copoeia). The pAPN TALEN constructs were assembled by the Golden Gate assembly method using the following TALEN monomer templates, NI for A, NG for T, HD for C, and NN for G. A pair of TALEN constructs was designed to target a region on exon 1 of the pAPN. The TALEN binding target sequences were as follows: left pair tccaaggccctgggcatcct and right pair ggcggccgtggccaccatca. The porcine DNA was as template for amplified the left arm (EcoRI-left arm-LoxP-EcoRI) and the right arm (Xhol-LoxP-right arm-Xhol). The amplified primers were as follows: LF ATgaattcGCAAAACAACTTTATTTCATTCTACTCAG, LR CgaattcATAACTTCGTATAGCATACATTATA CGAAGTATAGGATGCCCGTTGGAA; RF GTActcgagATAACTTCGTATAATGTATGCTATACGAAGTTATGGCGGCCGTGGCCACCATCAG, RR CATctcgagAGGGCTGATTGTCTAGATAGCTTCTC.TA. The right arm and left arm were cloned into pDonor-D01 as a donor vector for pAPN.

2.3 | Evaluate the activity of the TALEN target

To test the availability of the designed TALENs, we used the Gaussia luciferase surrogate reporter assay to detect the activity of the TALEN target (Figure 2 and Supporting Information Table S1). First, Gaussia luciferase (Gluc) reporter gene in CMV-PG03 reporter was disrupted with an in-frame stop codon followed by pAPN TALEN target sequence, then the Gluc function was lost. Second, Gluc donor plasmid, which contains promoter-less intact Gluc, was used for homologous recombination with TALEN-cleaved CMV-PG03-Stop* to restore Gluc function. Third, the pAPN TALEN pair, CMV-PG03-Stop*, and Gluc donor plasmids were co-transfected into 293T cells. Simultaneously, control TALEN pair, CMV-PG03-Stop*, and Gluc donor plasmids were co-transfected into 293T cells. Fourth, following 48 hr post-transfection, both pAPN TALEN-transfected cells and control TALEN-transfected cells were analyzed for Gluc activity using Secrete-Pair™ dual luminescence assay kit (Gene Copoeia: Cat. No. LF031).

2.4 | Evaluate the antiviral ability in gene-editing ST cells

ST cell is a standard cell line that has been used in in vitro TGEV research. A plasmid of pAPN TALEN and its donor were co-transfected into ST cells (2:2:1) by using Neon Transfection System (Life Technologies, Carlsbad, CA, USA). The procedure is 1400 V, 2 ms, 2 plus, 2 × 10^6. cells per reaction in 100 μl Kit. Ten days after puromycin selection, single cell-derived colonies were picked and seeded onto six-well plates until they reached 80%–90% confluency. The cell colonies were screened by PCR and sequenced. The PCR amplification was performed using primer 1 and 2 for left and right arm homologous recombination, and cycling condition as follows: 95°C for 4 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 7 min. The Clone 19 and control ST were infected with TGEV. The RT-PCR amplification was performed using primer 3, and the amplification procedures as mentioned above. To evaluate cellular morphological changes, cell culture supernatants and cDNA from whole cells were collected at 6, 12, 24, and 48 hr post infection (hpi). The qRT-PCR assays were carried out with SYBR-green fluorescence to measure the amount of TGEV(S) RNA (Supporting Information Table S2). The qRT-PCR amplification procedures as mentioned above.

2.5 | Production of gene-editing fibroblast cells

Porcine fibroblast cells isolated from 50–60 day-old pig fetuses were used in this study. Porcine fibroblast cells were grown in DMEM (Gibco, 11965-092) supplemented with 15% FBS (Gibco, 10099-141) at 37°C and 5% CO₂. When the cells reached 70%–80% confluency, a plasmid of pAPN TALENs and its donor were co-transfected into

**FIGURE 1** The map of TALEN-01 and pDonor-D01
these cells by electroporation using Neon Transfection System described as before. Two or three days after transfection, the cells were plated in 10 cm cell plates, 1.6 μg/ml puromycin was added to the cell culture medium for 5 days and cultured as a form of single cell in cell colonies. Next, cell colonies derived from single cells were separated mechanically and a part of the separated colony was genotyped by PCR and subsequent sequencing. Cell colonies with the desired mutations were cultured and used as donor cells for somatic cell nuclear transfer (SCNT).

2.6 | Production of gene-editing SCNT porcine embryos and pigs

For the construction of SCNT embryos, the donor cells and matured oocytes were placed in a drop of micromanipulation solution, incubated and saturated humidity for 10–15 min. The matured oocytes were enucleated by removing the first polar body and the adjacent 10% cytoplasm in micromanipulation buffer media containing TCM199 plus 2% FBS and 5 μg/mL cytochalasin B under an inverted microscope (Olympus IX-71, Japan) equipped with micromanipulation system (Narishige, Japan). The donor cell was injected subsequently into the perivitelline space through the same slot. For fusion and activation, the reconstructed couplets had been recovering for 30 min in a drop of T2 liquid (TCM199 + 2% Fetal Bovine Serum), were transferred in batches into the fusion liquid for 2 min. Finally, couplets were washed in embryo culture media and transferred into chemically assisted activation liquid (PZM-3 10 μg/ml Cycloheximide + 10 μg/mL Cytochalasin B) and incubated for 4 hr, and afterwards fusion results were examined under a stereomicroscope. The somatic cell nuclear transfer reconstructed embryos were transplanted into three surrogate sows. Between 160 and 300 embryos were transferred into each surrogate sow. Cloned piglets were delivered by natural birth after approximate 111 days. Ear tissue DNA was extracted from the clone piglets and the product was used as a PCR template as before.

3 | RESULTS

3.1 | Construction of TALEN and Donor plasmids

The TALEN pair plasmids were successfully constructed, then the activity of TALENs in HEK293T cells was screened with a Gaussia luciferase surrogate reporter assay. The results revealed that Gluc activity was restored in cells co-transfected with pAPN TALEN pair/CMV-PG03-Stop' reporter plasmid/Gluc donor (promoter-less). Compared to cells co-transfected with control TALEN pair/reporter plasmid/Gluc donor (promoter-less), cells transfected with pAPN TALEN had 10-fold (when normalized to internal SEAP activity, Gluc/SEAP ratio) or 11-fold (without normalization) higher Gluc activity (Figure 2 and Supporting Information Table S1). Furthermore,
The Donor plasmid of pAPN was successfully constructed as well. To increase the pAPN site-specific editing efficiency and further accelerate the breeding progress, we constructed a donor for pAPN, which contained both EGFP and puromycin, to select the pAPN site-specific editing cells and breeding more convenient (Figure 3). EGFP and puromycin were flanked by LoxP sites for further marker-free breeding. The left arm is 710 bp and the right arm is 700 bp. The stop codon will be successful to prevent the protein translation and loss function of pAPN.

3.2 | Suppression of the TGEV proliferation in pAPN gene-editing ST cells

The selective medium was added to the cells for 14 days and fresh complete medium was added to the cells. The pAPN gene-editing positive cell colonies were screened for a second time by PCR (Supporting Information Figure S1 and Table S2). Interestingly, 321 nucleotides were missing in the left recombination arm of clone 10 (Supporting Information Figure S1), including 210 nucleotides partial sequence of left arm and LoxP site and so on. Clones 10 and 19 were identified by RT-PCR and qRT-PCR and pAPN mRNA was not detected using different primers which located in different exons (Figure 4a–b and Supporting Information Table S1). Typical cytopathic effects were delayed and the TCID₅₀ assay revealed that the titer of pAPN site-specific edited cell culture supernatants were 100-fold lower in clone 19 than in control ST cells (data not shown). Site-specific editing of pAPN reduced TGEV proliferation in ST cells by 96%–99% at different time periods post-infection (Figure 4c).

3.3 | Production of gene-edited fibroblast cells and pigs

Porcine fetal fibroblast cell line was isolated from 50 to 60 days old fetuses, and homozygous recombined colonies were obtained. We produced transgenic porcine embryos using somatic cell nuclear transfer. The embryos had the ability to develop into blastocysts, i.e., site-specific editing of pAPN was not lethal to the embryos during the early developmental stage. We transferred the transgenic cloned embryos into surrogate sows (160–300 embryos per surrogate sow). Cloned piglets were delivered by natural birth after approximately 111 days. Two surrogates were successfully pregnant and delivered a total of five piglets. Unfortunately, all the piglets were died shortly after birth. Tissue DNA was extracted from the cloned piglets and used for PCR verifying gene-editing

FIGURE 3  Schematic overview of pAPN site-specific editing by TALEN technology. For junction 5' PCR analysis, primer 1 (green) was located upstream of the 5' combination left arm and in the promoter EF1α. For 3' junction PCR analysis, primer 2 (yellow) was located in the SV40 polyA and LoxP sites and downstream of the 3' combination right arm. The primer positions are indicated on the targeted pAPN locus. To assess editing efficiency, the 5' combination left arm was designed as a stop codon (*). For RT-PCR analysis of the pAPN mRNA expression in site-specific edited cells we used primer 3 (red). For qRT-PCR analysis of the pAPN mRNA expression in site-specific edited cells we used primer 4 (black) located in exons 11 and 12.
events. Moreover, all the piglets were positive, the left and right arm amplified fragments size was 1,177 and 1,455 bp, respectively. (Figure 5).

4 | DISCUSSION

TGEV belongs to the coronaviridae family and APN is a host cellular receptor for coronaviridae (Delmas et al., 1992; Li et al., 2018; Nam & Lee, 2010; Wang et al., 2018; Zhu et al., 2018). We speculated that the pathology of TGEV is similar to that of other coronaviruses. Understanding the molecular mechanisms involved in TGEV infections could be beneficial for preventing infections caused by other coronaviruses, such as porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus, severe acute respiratory syndrome coronavirus (SARS-CoV), and Middle East respiratory syndrome coronavirus (MERS-CoV). Similarly, pAPN is a host cellular receptor for porcine deltacoronavirus and K88(Li et al., 2018; Melkebeek et al., 2012; Wang et al., 2018; Xia et al., 2016; Zhu et al., 2018). The production of pAPN site-specific edited pigs could be beneficial for understanding the pathogenesis of porcine deltacoronavirus and K88 for breeding porcine deltacoronavirus and K88-resistant pigs as well. In addition, APN plays a critical role in tumor formation and is a receptor for tumor-homing peptides(Mina-Osorio, 2008; Zhang, Fang, Zhang, Yuan, & Xu, 2011). Investigating the interactions between APN and tumor-homing peptides will lead to a better understanding of cancer biology and more effective APN-based cancer treatments.

In summary, this study showed that TALEN and its donors are effective for the generation of gene-edited pigs. The gene-editing with the homologous recombination is clearly superior to random mutation gene-editing. The production of gene-edited livestock through somatic cell nuclear transfer is also superior to pronuclear injection, because it is easier to determine the sex of the animal and the least genetic variation can be yielded, which will accelerate the livestock breeding progress. In addition, the high off-target rates were often found in gene-editing cells and animals generated by CRISPR-Cas9 technology (Kosicki, Tomberg, & Bradley, 2018; Pattanayak et al., 2013), which could impair animal welfare and cause some unpredictable side effects. In order to protect animal welfare and some unpredictable side-effects in off-target. If the off-target sites on the same chromosome, eliminating the off-target effect is very difficult. If the off-target sites on the different chromosome, eliminating the off-target effect need six generation of breeding in general, which is time-consuming, money-consuming and progress is extremely slow. Here, we strongly recommend TALEN in combination with somatic cell nuclear transfer but not CRISPR-Cas9 in combination with pronuclear injection in livestock gene-editing breeding, especially in single gene-editing in uniparous or twin livestock. In conclusion, pAPN site-specific edited pigs will assist in the development of TGEV-resistant piglets, serve as an important model for studying tumor formation and development, and aid in the development of treatments for infections caused by coronaviruses.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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