Enhancing the antibacterial activities of sow milk via site-specific knock-in of a lactoferrin gene in pigs using CRISPR/Cas9 technology

Xiaosong Han1, Yang Gao1, Guanglei Li1, Youcai Xiong1, Changzhi Zhao1, Jinxue Ruan1, Yunlong Ma1, Xinyun Li1,2, Changchun Li1,2, Shuhong Zhao1,2 and Shengsong Xie1,2*

Abstract

Colostrum quality is a vital factor in mortality and growth performance for piglets. Lactoferrin is an immuno-active milk protein that contributes to the formation of a protective layer above intestinal mucosa, possesses the antibacterial and antiviral activities that are favorable for piglet development. However, there is a notable reduction in lactoferrin in sow milk during lactation after the first few days, which causes many piglets to fail to ingest enough colostrum thereby leading to an increase in piglet mortality. In this study, we successfully constructed genome-edited Large-White pigs with marker-free site-specific knock-in of lactoferrin gene in the 3′-end of Casein alpha-s1 via CRISPR/Cas9 mediated homologous recombination. Thus, the lactoferrin protein can be expressed in the mammary gland in the control of Casein alpha-s1 promoter. As expected, the lactoferrin protein in genetically modified pigs sustained high expression in both colostrum and milk when compared with wild-type pigs. Moreover, the bacterial plate assay indicated that the milk from genetically modified pigs showed bacteriostatic effects when compared with control pigs. Taken together, our study demonstrated that the milk from genetically modified pigs had antibacterial activity which may reduce the costs of veterinary drug and improve the surviving rate of piglets, which is promising for pig breeding.

Keywords: Pig, Lactoferrin, Colostrum, CRISPR/Cas9, Homologous recombination

Dear Editor,

Due to the lack of a fully developed immune system, newborn piglets are susceptible to pathogenic bacteria, thus causing billions of dollars in annual global losses. Sow milk, especially colostrum, containing abundant immune active compounds, plays a key role in piglet thermoregulation, acquisition of passive immunity, and intestinal development [1]. Colostrum quality is a vital factor in mortality and growth performance for piglets. Lactoferrin (LF) is an immune-active milk protein that contributes to the formation of a protective layer above intestinal mucosa, possesses antibacterial and antiviral properties [2], therefore, piglets suffer less from intestinal inflammation or diarrhea. However, the secretion volume for porcine lactoferrin (pLF) decreases in sow milk along with the lactation. Previous studies have shown that artificial supplementation of the piglet diet with LF can enhance piglet growth [3]. We assume that overexpression of the LF gene in sow milk might help to improve piglet survival rate. To demonstrate this hypothesis, we established gene-edited pigs with the targeted insertion of LF using CRISPR/Cas9-mediated knock-in system.
Overexpressing LF using transgenesis methods have been reported in many species, such as dairy cattle, goat, and mouse. In 2015, Cui et al. reported the production of transgenic pigs overexpressing human LF which brought LF content to reach 6.5 g/L [4]. While, most of the researches used random insertion of the LF gene, which arose safety concerns for the animals. In recent years, many kinds of genetically modified animals using CRISPR/Cas9 technology have been generated to improve animal traits [5]. Inspired by these studies, we attempted to generate genetically modified pigs that could produce LF at a high level throughout the entire lactation period. To minimize the effect of the inserted gene, we intended to insert the pLF gene before the stop codon site of the Casein alpha-s1 gene (CSN1S1) linked by a self-cleavage peptide, P2A sequence (Fig. 1a). CSN1S1 is expressed specifically in the mammary gland and sustains high expression during lactation [6]. Thus, the promoter of CSN1S1 was employed to drive the expression of pLF, which may minimize the effect in other tissues.

Fig. 1 Construction of CRISPR plasmids and activity assessment. a Schematic representation of the design of the CSN1S1-targeting vector. Exons of CSN1S1 and homologous arms are shown as gray boxes, and the purple triangle in Exon 17 represents the sgRNA targeting site. The pLF gene flanked by P2A and polyA in green is located in the middle of the targeting vector. Three pairs of primers were designed for genotyping. P1/P2 and P3/P4 are designed for genotyping the 5′ and 3′ junctions in pLF-KI colonies, respectively. P5/P6 are designed for detecting the donor vector region within the genetically modified pigs. LF, lactoferrin; HR, Homologous Recombination; sgRNA, single-guide RNA. b Assessment of the activity of 18- and 20-nt sgRNAs targeted to the same genomic loci via the T7EN1 cleavage assay in vitro. The upper sequences indicate the 18- and 20-nt sgRNAs, respectively, with the protospacer adjacent motif (PAM) sequence underlined. NC, negative control; M, DL2000 Marker. c Verification of activity of different length sgRNAs by Sanger sequencing. Characters in purple indicate the sgRNA sequences; The PAM sequences are underlined; Δ, deletion; +, insertion; WT, wild-type; No., Numbers of Sanger sequencing results.
Construction of CRISPR plasmids and assessment of its activity
To verify this strategy, we constructed a homologous recombination donor vector targeting the CSN1S1 locus in the pig genome. The single-guide RNA (sgRNA) closest to the stop codon was selected for construction of sgRNA-expressing vector. Previous studies showed that the length of the sgRNA may affect genome editing activity [7, 8]. To optimize the efficiency of the sgRNA, one 18-nt sgRNA and another 20-nt sgRNA were synthesized and assembled. A T7 endonuclease I (T7ENI) cleavage assay was used to evaluate the targeting efficiency for the two sgRNAs (Fig. 1b). Interestingly, when the length of sgRNA was decreased to 18-nt, the cleavage efficiency was more than 2-fold than that of a 20-nt sgRNA. Then, Sanger sequencing was used to identify the mutation (Fig. 1c). Therefore, we chose the 18-nt sgRNA for subsequent experiments. To achieve pLF knock-in at the 3′-end of the CSN1S1 locus, a homologous recombination donor vector was constructed (Fig. 1a). We chose the modified PCI vector with a deleted promoter as the backbone. The left homologous arm was approximately 731 bp and the right homologous arm was approximately 1129 bp. We used P2A as the linker between the pLF coding sequence (CDS) and the left homologous arm.

Generation of site-specific lactoferrin knock-in pigs using CRISPR/Cas9 technology
Porcine fetal fibroblast (PFF) cells isolated from 35-day-old embryos of Large-White pigs were electroporated with circular plasmids of CSN1S1-sgRNA, Cas9, and donor vector. We used G418 selection (400 μg/mL for 10 days) to identify the positive cell colonies, and their genotype was identified using PCR and Sanger sequencing. Among the 109 surviving cell colonies, twelve of them (12/109, 11%) were identified as positive for pLF insertion (Fig. 2a and Additional file 1: Figure S3A). Next, the verified colonies were used as donor cells for nuclear transfer. A total of 1750 cloned embryos were successfully reconstructed in vitro, and 39% of which were developed into blastocysts (Additional file 1: Figure S1). Subsequently, these blastocysts were transferred into 7 surrogate mothers (Additional file 1: Table S2). Fortunately, five surrogates were confirmed pregnant through ultrasound detection 35 days post embryo transfer. Finally, all of these surrogates developed to term and gave birth to 25 genetically modified piglets (Fig. 2b). Genomic DNA was extracted from the ear tissues of these cloned piglets 3 days after birth, and their genotype was identified by PCR (Fig. 2c and Additional file 1: Figure S3B), the results showed that all of the piglets were heterozygous.

![Fig. 2](https://example.com/image)

**Fig. 2** Generation of site-specific lactoferrin knock-in pigs using CRISPR/Cas9 technology. a Detection of site-specific insertion of selected clones using PCR genotyping. Lanes 1–12 represent 12 different clones, lanes 2, 3, 7, 9, 10 represent clone 25#, 27#, 68#, 75# and 81#, respectively; lane N represents the negative control wild-type cell line. b A genetically modified piglet at day 3 postpartum. c Detection of site-specific insertion of donor DNA in the genetically modified pigs using a PCR genotyping assay. Lanes 1–7 represent 7 individuals, and lane WT represents one negative control wild-type pig. WT, wild-type. d Nucleotide sequence analysis of junctions between endogenous and exogenous DNA corresponding to homologous recombination (HR) events. P5/P6 primers were used to amplify the specific region for the left- and right-hand junctions. Primary nucleotide sequence data corresponding to transition regions between the homologous arms of the targeting vector and the internal transgene DNA. LF, lactoferrin
The sequencing results further confirmed the desired precise knock-in location in the genetically modified piglets (Fig. 2d). Furthermore, to detect whether there were off-target effects in the cloned piglets, potential off-targets (OTS) were predicted by CRISPR-offinder [9]. A total of 20 putative OTS with less than 4 mismatches were considered and were examined via T7EN1 cleavage assay, some of the OTS were furtherly verified by Sanger sequencing (Additional file 1: Figure S2). No mutations were found at the potential off-target sites in all of the genetically modified piglets. These results indicated that seamless site-specific modification for the pLF gene in pig had been successfully achieved. Furthermore, this results in modified pigs with minimal safety concerns for further pig breeding in agriculture.

**Detection of the antibacterial activity of milk from pLF-KI pigs in vitro**

To test whether pLF gene expression was up-regulated in the mammary gland tissue during the lactation period, three sexually mature genetically modified sows were artificially inseminated at 7-8 months old. One of the sows was pregnant and gave birth to 7 piglets. Three

| Components | pLF-KI | WT |
|------------|--------|----|
| Fat        | 6.7%   | 5.9%|
| Protein    | 18.1%  | 17.2%|
| Lactose    | 2.3%   | 2.8%|
| Solids     | 20.8%  | 20.1%|

**Fig. 3** Detection of the antibacterial activity of milk from pLF-KI pigs in vitro. **A** Raw components of genetically modified colostrum compared with wild-type colostrum. **B** Detection of the expression of LF in colostrum stage and milk stage in pLF-KI pigs by qRT-PCR assay. **C** Western blot analysis for the expression and accumulation of LF protein between pLF-KI pigs and wild-type pigs. KD, Kilodalton. **D** Bacterial plate assay for *Escherichia coli* (*E. coli*) survival in response to colostrum treatment. Depletion zones developed on a lawn of *E. coli* 12 h after sample application: (1) 10 μL milk from genetically modified pig, (2) 10 μL milk from wild-type pig, (3) 10 μL sterile water and (4) 10 μL antibiotic. **E** Plate dilution colony counting assay to test the antibacterial activity of colostrum. pLF-KI, porcine lactoferrin knock-in; WT, wild-type; Anti, Antibiotic; CFU, colony-forming unit; Blank: sterile water; the cycles in orange indicate the transparent zones. **P** < 0.01, ***P** < 0.001, ****P** < 0.0001; **P** values were determined by two-sided Student’s t test.
of them were demonstrated as pLF-KI pigs. Firstly, we analyzed the quality of colostrum collected from genetically modified and wild-type pigs. The results showed that there was no significant difference in the percentage of fat, protein, lactose, and solids in the colostrum of pLF-KI and wild-type pigs (Fig. 3a). Mammary gland tissue was collected on day 1 (colostrum stage) and day 12 (milk stage) after parturition. To determine the relative transcriptional expression levels of the pLF gene in pLF-KI pigs, qRT-PCR assays were performed. The results showed that the LF gene expression was significantly higher during the pLF-KI sow lactation period (Fig. 3b).

In addition, Western blot assays of mammary tissue also showed that LF protein accumulated to high abundance in both the colostrum and milk of pLF-KI pigs compared to that of wild-type pigs (Fig. 3c). Additionally, we performed antibacterial experiments to determine the function of the colostrum from the genetically modified pig. Filter paper containing colostrum was plated on the agar plates containing Escherichia coli (E. coli) and the antibacterial activity was estimated by the presence of transparent zones appearing around the filter paper after a 24 h incubation at 37 °C (Fig. 3d), we found that the pLF-KI milk produced a transparent zone, while no transparent zone was found for the wild-type milk. Subsequently, the antibacterial activity was also verified by plate dilution colony counting (Fig. 3e). These results indicated that the milk from genetically modified pigs had bacteriostatic effects when compared to the wild-type control.

Conclusion
In summary, we successfully generated seamlessly-engineered pigs by CRISPR/Cas9-mediated homologous recombination, and we knocked in the pLF gene at the 3′-end of endogenous CSN1S1 locus in the pig genome for the first time. Thus, the pLF gene can be over-expressed specifically in the mammary gland during the lactation period under the control of the CSN1S1 promoter. Also, this study demonstrated that the milk from genetically modified pig possessed antibacterial activity without significant changes to its nutritional composition, which may reduce the costs associated with veterinary drug treatment and improve the mortality rate of piglets. Our work demonstrates a promising strategy for pig breeding and trait improvement efforts and provides hope that this strategy can be adopted in other species in the future.

Supplementary information
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