Secretion of IFN- γ in goat mammary epithelial cells reduce the mastitis infection risk

Ying Liu

Shandong Agricultural University

Hongyan Zhang

Shandong Agricultural University

Shasha Dong

Taishan Medical University

Boyu Li

Shandong Agricultural University

Weiming Ma

Shandong Agricultural University

Lijiang Ge

Shandong Agricultural University

Zhiyong Hu

Shandong Agricultural University

Feng Su (✉ suf@sdaa.edu.cn)

Shandong Agricultural University <https://orcid.org/0000-0001-8722-9606>

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Abstract

Background: Mastitis results in great economic loss to the dairy goat industry. Many approaches have attempted to decrease the morbidity associated with this disease, and among these, transgenic strategies have been recognized as a crucial development. A previous mammalian study reports that interferon-gamma (IFN- γ) has potential anti-bacterial bioactivity against infection in vitro; however, its capacity in vivo is ambiguous. **Results:** In this study, we first constructed targeting and homologous recombination vectors (containing the IFN- γ gene) and then transferred the vectors into goat mammary gland epithelial cells (GMECs). Enzyme digestion and sequencing analysis indicated that the vectors used in this study were built correctly. Subsequently, monoclonal cells were selected using puromycin and the polymerase chain reaction (PCR) test indicated that IFN- γ was correctly inserted downstream of the casein promoter. Monoclonal cells were then assessed for reducible expression, and reverse transcriptase-PCR (RT-PCR) and Western blot tests confirmed that monoclonal cells could express IFN- γ . Finally, anti-bacterial capacity was evaluated using bacterial counts and flow cytometry analysis. Decreased bacterial counts and cell apoptosis rates in transgenic GMECs demonstrated that the secretion of IFN- γ could inhibit bacterial proliferation and reduce the risk of mammary infection in goats. **Conclusions:** Secretion of IFN- γ could inhibit bacterial proliferation and reduce the risk of mammary infection in goats.

1. Background

Mastitis is a complex disease caused by *Staphylococcus* (*S. aureus*), *Streptococcus* (*S. agalactiae*), and *Escherichia coli* (*E. coli*) infection; it results in great losses to the dairy goat industry. There has been extensive research into this condition, including dietary selection [1, 2], induction of immunity [3], and clinical treatment. However, none of these treatments have fundamentally decreased the incidence of mastitis. Recently, the development of transgenic animal strategies has supplied an effective way to reduce mastitis morbidity[4]. Using this approach, Yu et al. [5] and Liu et al. [6] produced anti-mastitis cattle by transferring the HBD3 gene. Liu et al. also produced transgenic cattle with resistance to mastitis by transfer human lysozyme gene [7]. Jun et al. generated mastitis resistant transgenic dairy goats and evaluated the levels of HBD3 secreted in the milk [8]. All of these animals exhibited an environmentally friendly impact [9]. During these studies, one important step was to evaluate the anti-mastitis capacity of potential genes that could be manipulated during breeding, especially in host cells.

As a remarkable small molecule that participates in immune responses, interferon-gamma (IFN- γ) is widely used for infection resistance and clinical treatment[10]. For goat mastitis, IFN- γ could reduce the level of infection caused by different pathogenic bacteria. In *Staphylococcus* infection, IFN- γ is generated from the capsular polysaccharide simulated T cells and obviously boosts the injurious effects of resistant *staphylococcus* [9]. In *Streptococcal* mammary infection, proliferative *Streptococcus* is inhibited by the highly-expressed IFN- γ that is simulated by the *Streptococcus* [11]. During *E. coli* infection, IFN- γ was first used for control the acute mastitis in bovine during the periparturient period [12]. Application of IFN- γ could elicit functional changes on other lymphocytes and phagocytic cells in the mammary gland which finally reduced the mastitis [13]. To be precise, milk secretion from mammary cells is decreased

and the phagocytic capacity of lymphocytes is increased; together, these reduce mastitis morbidity. Thus, IFN- γ is recognized as a pivotal target site for developing mastitis resistant goats. However, the anti-bacterial capacity of secreted IFN- γ in mammary gland epithelial cells has not been evaluated.

In this study, we transferred the IFN- γ gene into goat mammary epithelial cells using the CRISPR/Cas 9 system and assessed the secretory ability of resultant positive cells. Subsequently, anti-bacterial capacity was evaluated by using bacterial resistance assays.

2. Methods

2.1 Ethics Statement

The lactating dairy goats used in this study were bought from Zhengda Company from China (Taian, China) and accommodated in appropriate livestock housing and fed ad libitum. Following treatment, the Laoshan dairy goats were sacrificed by an injection of sodium barbitol after anesthesia. All procedures involving animals were approved by the Animal Care and Use Committee of Shandong Agricultural University.

2.2 Vector Construction

The gene target LentiCRISPRv2 (Cat: 52961) vector and homologous recombination vector (pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro) were both purchased from Addgene. The sgRNAs were designed from the website <http://crispr.mit.edu/> and then inserted into the LentiCRISPRv2 vector after the vector was digested by BsmB I. The casein promoter (LA: long arm) was amplified into the PMD-18T vector, then the IFN- γ gene, synthesized by the Shanghai Sheng Gong Company (Shanghai, China), was inserted behind the casein promoter by overlap polymerase chain reaction (PCR). The LA-IFN- γ sequence was inserted into the homologous recombination vector after double digestion by the Spe I and EcoR I enzymes. Subsequently, the short arm was amplified and inserted into the recombination vector after digestion by the Kpn I enzyme.

2.3 Preparation of goat mammary epithelial cells (GMECs)

Mammary epithelial tissue was collected from the Laoshan dairy goats. Tissues were washed thrice with PBS, and then minced into several pieces of around 1 mm³. Tissue blocks were placed into 60 mm petri dishes with Dulbecco's Modified Eagle Medium (DMEM)/F12 [containing 10% fetal bovine serum (FBS) and 10 ng/mL epidermal growth factor (EGF)] in a cell incubator at 37°C and 5% CO₂. The medium was refreshed every 2 days.

2.4 Virus package, cell transfection, and single clone cell selection

The recombination vectors and target vectors were packaged separately using human 293T cells. The steps were as follows. Human 293T cells were cultured into 100-mm cell culture dishes, then the target

vector (LentiCRISPRv2, 7.5 µg), homologous recombination vector (7.5 µg), with package vectors psPAX2 (6 µg, Addgene, #12260) and pCMV-VSV-G (6 µg, Addgene, #8454) were co-transfected into human 293T cells to produce lentiviral particles. Cell media were collected after co-infection with 293T cells at 24 h and 48 h and then filtered with a 0.45 µm filter (Millex®-HV). The concentrated virus was obtained after mixing with PEG8000 reagent overnight followed by centrifugation. The virus was then resuspended with fresh DMEM/F12 medium and used to infect the isolated GMECs. The cells were selected using puromycin (1 µg/ml) for 3 weeks and monoclonal cells were exhibited. Then the monoclonal cells were separated and individually transferred into a new dish to proliferate for future assays.

2.5 PCR and RT-PCR assays

The monoclonal cells were proliferated in a 60-mm dish in a cell incubator at 37°C and 5% CO₂. Subsequently, the cells were divided into three aliquots, one of which was frozen, one used for DNA extraction, and the third for RNA extraction and inverse transcription. The primers that were used for insert site detection were designed at both sides of the IFN-γ expression cassette. PCR assay was used to assess IFN-γ insertion after the DNA was extracted using a DNA extraction kit (DNAzol Reagent, Cat: 10503027). Reverse transcriptase-PCR (RT-PCR) primers were used to assess IFN-γ expression in transgenic GMECs. All the primers used in this study are listed in Table 1.

2.6 Inducible expression

The primary cells (non-transgenic GMECs) and monoclonal cells (transgenic GMECs) were separately cultured into 90-mm dishes. When the cells reached 80% confluence, the inducive medium (Opti-MEM with 10 ng/mL EGF, 1% ITS Liquid Media Supplement, 5 ng/mL prolactin (Sigma, cat:L6520), and 1 mg/mL hydrocortisone) was used to replace the previous medium. The supernatants were then collected at 24 h and concentrated for Western blot analysis.

2.7 Western blot analysis

The concentrated proteins were used for Western blot analysis following a standard protocol. Briefly, the proteins were transfer onto a polyvinylidene-fluoride membrane after separation by SDS-PAGE electrophoresis. The membrane was then incubated with IFN-γ antibody (LMAI Bio, Shanghai, China) at a 1:1000 dilution after blocking with blocking buffer for 4 h. Subsequently, the membrane was incubated with HRP goat anti-rabbit IgG (Beyotime Institute of Biotechnology, Shanghai, China) at a 1:2000 dilution for 2 h. The membrane was then exposed after being treated with a chemiluminescence substrate according to the manufacturer's instructions.

2.8 Bacterial infection assay

All bacteria used in this assay were purchased from the Chinese Institute of Veterinary Drug Control (Beijing, China) and preserved in the lab. *E. coli* (ATCC25922), *S. aureus* (ATCC25923), and *S. agalactiae* (ATCC12386) were cultured in 37 °C in 100 mL trypticase soy broth medium until the optical density value (OD 600) is equal or greater than 1. Subsequently, the bacterial were centrifuged at 3000 g for 5 min then washed and resuspended three times in phosphate buffered saline (PBS) to 1 × 10⁷ colony forming units

(CFU)/mL. GMECs were counted and cultured in 12-well dishes, then cultured in DMEM/F12 overnight. After inducible expression of IFN- γ as mentioned above, the cells were then infected with these bacteria separately at a multiplicity of infection (MOI) of 10:1. Cells and their supernatant were separately collected at 2 h and then used for other experiments. Cells were used for flow cytometry analysis and bacterial count evaluation at 6 h. The risk of anti-bacterial infection was evaluated by the bacteria colony-forming unit (CFU) counts in GMECs[14].

2.9 Flow cytometry

The infected cells were then stained using Annexin V PE/7-AAD (Qiagen, Valencia, CA, USA). Anti-bacterial capacity of secretion was evaluated by cell apoptosis and death rates.

2.10 Statistical analysis

Data from the CFU count assay were analyzed using SPSS software (SPSS, Chicago, IL, USA). The CFU data are means $\pm$ SD and were compared using one-way ANOVA followed by the Newman-Keuls test. P values < 0.05 were considered statistically significant.

3. Results

3.1 Inducible secretion of IFN- γ from GMECs was controlled by a transgenic strategy

Transgenic GMECs were processed using the CRISPR/Cas 9 system; the detailed scheme is shown in Fig. 1. From the scheme, one of the target sites was chosen in the 2nd exon of the casein (CSN2) gene, upstream of the signal peptide of the gene. The other one was located upstream of the 8th exon. The homologous arm (long arm: LA) of the recombination vector was designed containing the CSN2 signal peptide and used for the determination of IFN- γ secretion.

3.2 Targeted and homologous combination vector construction

Gene targeted vectors were built based on the Lenti-CRISPR V2 system; the single guide RNAs (sgRNAs) were designed on the 2nd and the 8th exon, separately (Fig. 2A). Accuracy of sgRNA nucleotides were evaluated by sequencing; the sequence, shown in Fig. 2B, shows that the target vectors were built correctly. Subsequently, the digestion efficiency of sgRNAs was assessed by PCR assays after the DNA was cut by the T7E1 enzyme. Shearing efficiency of Sg2-3 and Sg8-3 is higher than for other sgRNAs; this is shown in Fig. 2C. Figure 2D-2G shows the homologous recombination vector construction process. Figure 2D shows the homologous arm amplification. The bands in lane 1 and lane 2 represent the long and the short arm lengths, separately. Subsequently, the IFN- γ was synthesized and inserted downstream of the long arm by overlap PCR, which was about 1358 bp in length (Fig. 2E). Figure 2F shows the long arm inserting test, two bands in lanes 1 and 2 show that the long arm with IFN- γ is completely attached to

the homologous vector. Finally, the short arm was correctly inserted into the recombination vector after sequencing (Fig. 2G).

3.3 Inducible secreted IFN- γ in gene-edited GMECs

Transgenic GMECs were obtained after co-infected target and homologous recombination vectors. Homologous and target vectors were all packaged in 293T cells. Expression of green fluorescent protein (GFP) in 293T cells confirmed that the infection rate of these vectors was efficient for the packaged virus (Fig. 3A). Then the monoclonal GMECs were obtained after selection with puromycin (Fig. 3B). Integrated IFN- γ expression cassettes were then evaluated by PCR tests. Inserted site detection indicated that the expression cassette was correctly integrated into the genome of GMECs (Fig. 3C, 3D). Subsequently, all the monoclonal cells were induced and IFN- γ mRNA proteins were evaluated by RT-PCR and Western blot, respectively. RT-PCR assays in GMECs indicated that the 11th monoclonal cells could express IFN- γ mRNA (Fig. 3E). Finally, Western blot analysis of IFN- γ indicated that the gene-edited GMECs could secrete IFN- γ protein as expected (Fig. 3F).

3.4 Gene-edited GMECs displayed strong bacterial resistance

Bacterial challenge of transgenic GMECs was performed to assess the anti-bacterial capacity of IFN- γ . Transgenic GMECs showed much stronger *Staphylococcus* and *E. coli* resistance activity than the non-transgenic ones ($P < 0.01$), but no obvious differences in anti-*Streptococcus* effects (Fig. 4A, 4B, 4C). GMECs survival rate in different bacteria-treated cells showed that the transgenic GMECs had an obvious increased survival rate over non-transgenic ones (Fig. 4D, 4E).

4. Discussion

Mastitis causes great losses to the dairy goat industry. Many approaches have been investigated to decrease potential mastitis morbidity, with the transgenic strategy being recognized as a crucial development. A previous study reports that IFN- γ has potential bacterial resistance activity against mammalian infection in vitro. However, the anti-bacterial capacity of IFN- γ in vivo is still ambiguous. In this study, we first constructed the gene targeting and homologous recombination vectors and then transferred these vectors into GMECs cells. Enzyme digestion and sequencing analysis indicated that the vectors used in this study were built correctly. Subsequently, the monoclonal cells were selected by puromycin and the PCR test indicated that IFN- γ is correctly inserted downstream of the casein promoter. The monoclonal cells were then used for reducible expression; RT-PCR and Western blot tests confirmed that the monoclonal cells could express IFN- γ . Finally, anti-bacterial ability was evaluated by bacteria counts and flow cytometry analysis. The decreased bacterial counts and cell apoptosis ratio in transgenic GMECs demonstrated that the secretion of IFN- γ could inhibit bacterial proliferation and decrease the risk of mammary infection.

Accurate nucleotide editing technology has been recognized as an effective method for decreasing the morbidity of animal diseases [15]. Regarding bovine mastitis, Liu et al. generated anti-mastitis cows via zinc-finger nickase-mediated insertion of the HBD3 (human-beta-defensin 3) gene [6] and lysostaphin [7]. All transgenic cows exhibited anti-bacterial capacity and mastitis resistance. In his studies, casein (CSN2) promoter was used to drive HBD3/ lysostaphin expression and the signal peptide of CSN2 was used to guide protein secretion [7]. In current study, expression and secretion of IFN- γ was also driven by the CSN2 promoter, as previously reported. The difference between these studies is mainly focused on the cut points. Liu's study used one cut point in the CSN2 promoter while the current study used two main points, one of which was located upstream of the signal peptide and the other one located in exon 8 of the CSN2 gene. The existence of exon 2 and exon 8 in CSN2 may contribute to the stable secretion of IFN- γ .

Target rates of sgRNAs were evaluated by DNA-cleaving rates. The target rate was not as high as previously reported; this may be attributed to species difference [16] as Zhang's laboratory used human cells as targets [17] while this study only used goat cells.

Transgenic GMECs were selected and the expression of IFN- γ was assessed by Western blot analysis. Expression of the GFP protein indicated that the monoclonal cells that were screened had a high purity, as previously reported [9]. Homologous arm detection confirmed that the integration of IFN- γ was correct. Subsequently, inducible expression of IFN- γ was evaluated and only the 11th monoclonal cells presented any expression activity. The main reason for this appearance maybe relevant to the methylation effect of the CSN2 promoter [18].

Anti-mastitis capacity was assessed by bacterial counts and target cell apoptosis rates. Decreased Staphylococcus and E. coli counts in IFN- γ secreted transgenic GMECs indicated that IFN- γ could inhibit Staphylococcus and E. coli proliferation by killing bacteria directly or indirectly, which agrees with reports in previous studies [9]. The survival rates of Staphylococcus and E. coli infected transgenic GMECs also support this finding. In Streptococcus infected transgenic GMECs, secretion of IFN- γ is insufficient to inhibit streptococcal proliferation [19], though the survival rates of transgenic GMECs were obviously higher than non-transgenic ones [11]. The main reason for this difference may be attributed to the potential immune function. On the one hand, inducible expressed IFN- γ could activate GMECs immune reaction to defend against Streptococcal infection which finally decreased the Streptococcus count. However, the GMECs were the main epithelial cells that protected against bacterial invasion rather than immune cells. Immune reaction was unable to activated by Streptococcus because shorten of CD4⁺ receptor in GMECs. Previous study reported B group Streptococcus (Contain Streptococcus agalactiae) could produce IFN- γ in CD4⁺ T cells in vivo or vitro model [20]. A deficiency of CD4⁺ receptor in GMECs is the main reason for shorten of IFN- γ and ineffective inhibition of Streptococcal duplication. On the other hand is the bacterial susceptibility of GMECs. Juliane Günther's study revealed that Streptococcus uberis failed to activate an immune reaction in mammary epithelial cells [21]. Ariffin's assays reported Streptococcus agalactiae was isolated from the dairy goat's milk [22] but there are still no direct evidence confirmed the infection capacity of this bacteria. In addition, the potential existence of T cells in uberis maybe the main factor that simulated the immune reaction in vitro.

In conclusion, this study initially constructed the gene target and homologous recombination vectors and then transferred the IFN- γ into GMECs cells. The transgenic GMECs were then selected and the monoclonal GMECs were used for inducible secretion of IFN- γ . Expression of IFN- γ in transgenic GMECs exhibited a strong anti-bacterial capacity; in particular, it decreased Staphylococcus and E. coli counts. Cell survival rates in transgenic GMECs confirmed that secreted IFN- γ obviously reduced cell apoptosis, which decreased the risk of mastitis. IFN- γ maybe a good prevented treatment that decrease the dairy goat' mastitis, it is also canbe used for gene-editing goat's generation.

5. Declarations

Ethics approval and consent to participate

The lactating dairy goats used in this study were bought from Zhengda Company from China (Taian, China) and accommodated in appropriate livestock housing and fed ad libitum. Following treatment, the Laoshan dairy goats were sacrificed by an injection of sodium barbitol after anesthesia. All procedures involving animals were approved by the Animal Care and Use Committee of Shandong Agricultural University.

Consent for publication

We confirmed all the listed authors have consented to the submission.

Competing interests

None of the authors have any potential financial conflicts of interest related to this manuscript.

Availability of data and materials

Not applicable

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Authors' contributions

YL and FS designed the experiment and drafted the manuscript. HZ, YL, and SD carried out the animal care, samples collection, and performed the experiments. BL and LG performed the data processing and biological information analysis. FS, WM interpretation of data; ZH, and FS conceived the study and writing the manuscript. All authors read and approved the final manuscript.

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Abbreviations

IFN- γ : interferon-gamma; GMECs: goat mammary gland epithelial cells; S. aureus: Staphylococcus. S. agalactiae Streptococcus; E. coli: Escherichia coli

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Table

Table 1. The primers that used in this study

Name	Sequence (5'-3')	Length (bp)	Annealing temperature(°C)	Application
SA-F	AGCAGGTACCGTCTAAGAGGATTC	729	52	Short Arm
SA-R	TGCAGGTACCTAACTCTTCATCTCAC			
LA-I-F	ATCACTAGTTGGGGACTGGGCAAGAGAA	926	60	Long Arm
LA-I-R	TACATATGGGTCCTGTGCAATGGCCAGAGCCACCAGACAGGCA			
A-IFN-F	TGGTGGCTCTGGCCATTGCACAGGACCCATATGTAAAAGA	1358	60	Arm+IFN
A-IFN-R	GAGCGAATTCTTACTGGGATGCTCTTCG			
KY1-F	TGCAATAATATCCTCCCT	1652	56	Integration site detection
KY1-R	CGACAGTTCAGCCATCAC			
KY2-F	AAATCCTGGTTGCTGTCT	1603	54	Integration site detection
KY2-R	GCCTAAGGGTTAATTTATTG			
hx2-F	ACCTTGGCCATATGATAAG	600	55	Digestion efficiency
hx2-R	TCTTGTTGGTCTGTTGCT			
hx8-F	AGAAGAACTTATTGGGA	600	54	Digestion efficiency
hx8-R	AAATCTGTCAACACCATA			

Figures

Figure 1

The entire homologous integration scheme of this research. In the strategy, one target site was chosen in the 2nd exon of the casein (CSN2) gene, upstream of the signal peptide of the gene. The other site was located upstream of the 8th exon. The homologous arm (long arm: LA) of the recombination vector was designed containing the CSN2 signal peptide and used for guiding interferon-gamma (IFN-γ) secretion.

Figure 2

Construction of gene-targeted and homologous integration vectors A: Location of single guide RNAs (sgRNAs) that were used in this study. B: Sequencing of the target vector; the sgRNAs were inserted into the Lenti-CRISPR V2 vector followed by sequencing. C: Digestion efficiency analysis of sgRNAs. D-G: Homologous recombination vector construction process. D: The homologous arm was amplified and assessed by polymerase chain reaction (PCR) assay. The bands in line 1 and lane 2 independently represent the long and the short arm length. E: Interferon-gamma (IFN- γ) was synthesized and inserted downstream of the long arm by overlap PCR, which was about 1358bp in length. F: Long arm inserting assay, the vector was double digested by Spe I and EcoR I enzymes. Two bands in lane 1 and 2 showed that the long arm with IFN- γ was completely inserted into the homologous vector. G: Enzyme identification of the entire combination vector. The short arm was digested from the recombination vector by Kpn I.

Figure 3

Transgenic monoclonal goat mammary gland epithelial cells (GMECs) screening and evaluation A: Lentivirus plasmids were packaged in 293T cells. Homologous and target vectors were all packaged in 293T cells. Expression of green fluorescent protein (GFP) in 293T cells confirmed that the infection rate of homologous vectors was sufficient for the virus concentration. B: The monoclonal GMECs that were obtained after puromycin selection. C-D: Integrated interferon-gamma (IFN- γ) expression cassettes were evaluated by PCR assays. Inserted site detection indicated the expression cassettes were correctly integrated into the genome of GMECs. Middle lane (D) in the figure is the KY2 PCR product, the right lane (D) is KY1 PCR product. E: Detection of IFN- γ mRNA in the monoclonal cells by RT-PCR after inducible expression. F: Western blot analysis of inducible IFN- γ . Analysis of IFN- γ indicated that the gene-edited GMECs could secrete IFN- γ protein as expected.

Figure 4

Anti-bacterial capacity of transgenic monoclonal goat mammary gland epithelial cells (GMECs) that expressed and secreted interferon-gamma (IFN- γ) protein A-C: Bacterial challenge of transgenic GMECs was performed to assess the anti-bacterial capacity of IFN- γ . Transgenic GMECs showed much stronger *Staphylococcus* and *Escherichia coli* resistant activity than the non-transgenic ones ($P < 0.01$), but no obvious differences in anti-*Streptococcus* effects. D-E: Survival rate of GMECs in different bacteria-treated cells at 6h; transgenic GMECs obviously increased the survival rate over non-transgenic ones ($P < 0.05$).

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