The Effects of Matrix Metalloproteinase-9 on Dairy Goat Mastitis and Cell Survival of Goat Mammary Epithelial Cells

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Abstract

Matrix metalloproteinase-9 (MMP-9) is a zinc-dependent enzyme, and plays a crucial role in extracellular matrix degeneration, inflammation and tissue remodeling. However, the relationship between MMP-9 and somatic cell count (SCC) in goat milk and the role of MMP-9 in the regulation of mastitis are still unknown. In this study, we found MMP-9 was predominantly expressed in the spleen, intestine and mammary gland. The SCC in goat milk was positively correlated with MMP-9 expression, and staphylococcus aureus could markedly increase MMP-9 expression in goat mammary epithelial cells (GMEC) in dosage and time dependent manner. We also demonstrated that SB-3CT, an inhibitor of MMP-9, promoted apoptosis and inhibited proliferation in GMEC. Thus, MMP-9 may emerge as an easily measurable and sensitive parameter that reflects the number of somatic cells present in milk and a regulatory factor of apoptosis in GMEC.

Introduction

Matrix metalloproteinase-9 (MMP-9) is a multifunctional protein that is synthesized and secreted widely in various cells, such as capillary endothelial cell, neutrophils and macrophages, and plays important roles in degradation of extracellular matrix, cell migration, cell apoptosis and inflammation [1-4]. The somatic cells in goat milk consist of mammary epithelial cells and leukocytes including neutrophils, lymphocytes and macrophages [5]. The proportion of leukocytes is about 90% and only 10% of somatic cells are mammary epithelial cells in milk [6, 7]. When breast tissue infected by pathogenic microorganisms, a large number of white blood cells is recruited to the milk, which markedly increases milk somatic cells count (SCC) and activates the immune response [8, 9]. The SCC in milk is an important indicator to measure the quality of milk [10, 11]. The milk SCC should be maintained at low levels because acute increase in milk SCC will patently affect the nutrient content of the milk [8, 12]. Previous research showed that age, parity, lactation stage and bacterial infection could impact the SCC in milk [13, 14]. Mastitis, caused by bacterial infection, has a profound impact on milk yield and leads to great financial losses. Staphylococcus aureus is one of the most important mastitis-causing pathogen in dairy goats [9, 15]. Meanwhile, studies have suggested that somatic cells in
milk secreted by cows which suffer from mastitis, present a high MMP-9 protein activity [13, 14]. MMP-9 plays important roles in recruiting neutrophils and inducing cell apoptosis in bovine mammary epithelial cell with mastitis [10, 16]. The plasmin and MMP-9 increase rapidly in mastitis tissue corresponding to the rising SCC [10, 11]. However, the role of MMP-9 in regulating milk SCC in dairy goats is not well known.

In this study, we investigated the relationship between MMP-9 expression and SCC in the goat milk, and the effect of *Staphylococcus aureus* on MMP-9 expression in goat mammary gland epithelial cells (GMEC). Moreover, we also explored the effect of MMP-9 on GMEC apoptosis.

**Materials and Methods**

**Animals and reagents**

All dairy goats were maintained according to the No. 5 proclamation of the Ministry of Agriculture, P.R. China. Sample collection was approved by the Institutional Animal Care and Use Ethics Committee of Northwest A&F University and performed in accordance with the ‘Guidelines for Experimental Animals’ of the Ministry of Science and Technology (Beijing, China). Three-year-old Xinong Saanen dairy goats at peak lactation (n = 6) slaughtered by captive bolt stunning followed by exsanguination [17]. Mammary gland, subcutaneous adipose, skeletal muscle, heart, liver, spleen, lung, kidney, rumen, marrow, and small intestine tissues were collected. All tissue samples were obtained under sterile conditions and washed with diethylpyrocarbonate (DEPC) treated water, then immediately frozen in liquid nitrogen. The *Staphylococcus aureus* strains were isolated from local Xinong Saanen goats and kept in our laboratory. SB-3CT, an inhibitor of MMP-9, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Milk somatic cell count analysis**

Milk samples were collected from 24 Xinong saanen dairy goats at peak lactation in August, and all dairy goats were healthy without symptoms of mastitis with milk production of 1.8–2.3 kg/d. The 24 dairy goats were divided into two groups (group A, two-year-old and first-born does; group B, four-year-old and third-born does), and each group had twelve dairy goats. The SCC and composition of milk samples were detected by Shaanxi Provincial Animal Husbandry and Veterinary Research Center. Meanwhile, the milk samples were used for collecting somatic cells, RNA extraction and quantitative real time PCR (RT-qPCR). For somatic cell collection, briefly, the milk samples were diluted by phosphate buffered saline (PBS), and centrifuged to remove the supernatant and milk fat. Then the somatic cells were washed with PBS and centrifugation for four times. Finally, the depositing somatic cells were diluted by cell lysis buffer with 1% β-mercaptoethanol stored at -80°C until RNA extraction.

**Cells culture and treatments**

The GMEC were isolated from Xinong Saanen goats at peak lactation, and the details of the cell culture were described previously [17–19]. The GMEC at nearly 80–90% confluence were plated at 5 × 10^5–1 × 10^6 cells/well in 6-well plates or 1 × 10^4 cells/well in 96-well plates in complete DMEM/F-12 medium and incubated overnight at 37°C with 5% CO₂. When GMEC grow to approximately 80% confluence, they were treated with (a) 0, 10^5, 10^6, 10^5 cfu/ml *Staphylococcus aureus*, respectively; (b) 0, 5 or 10 μM SB-3CT (dissolved in dimethyl sulfoxide), respectively. After 2, 4, 6 or 24 h incubation, the GMEC with different treatments were used for RNA extraction, cell proliferation assay, cell apoptosis assay and Hoechst33342/PI staining analysis.
Total RNA extraction and RT-qPCR

The GMEC were lysed in 1 mL of Trizol Reagent (Invitrogen) and proteins were removed using 200 μL of chloroform. Total RNA was precipitated with an equal volume of isopropanol, and the RNA pellet was washed with 75% ethanol for two times. cDNA was synthesized using a transcript first-strand cDNA synthesis kit (TaKaRa, Japan). RT-qPCR primers were shown in Table 1, and the accepted rang of primer efficiencies is 95–105%. Three genes were used as internal control genes including glyceraldehyde phosphate dehydrogenase (GAPDH), ubiquitously expressed transcript (UXT), and mitochondrial ribosomal protein L39 (MRPL39). All treatments of RT-qPCR were replicated six times in a Bio-Rad master cycler using the SYBR Green PCR Master Mix (Takara, Japan) according to the manufacturer’s protocol. The RT-qPCR data were analyzed using the $2^{-ΔΔCt}$ method.

Cell cycle assay

Cell cycle analysis was performed using Cell Cycle Testing Kit (Multisciences, China). The GMEC were grown in 6-well plates (1 × 10^6 cells/well). The GMEC were treated with indicated concentration of SB-3CT for 24 h, and subsequently the cells were harvested and centrifuged at 800 g/min for 5 min. The supernatant was discarded, and the cells were washed once by cold PBS. GMEC were resuspended using 1 mL of reagent A and 10 μL of reagent B, subsequently blended by vortexing for 10 s and incubated for 30 min, and then analysis was done using a flow cytometry (FACS Canto™ II, BD BioSciences, USA).

Cell proliferation assay

Cell proliferation assay was performed using cell counting kit-8 (CCK-8) assay and EdU incorporation assay. For CCK-8 assay, the GMEC were plated in 96-well plates at the density of

| Gene   | Primer Sequence (5‘→3’)       |
|--------|-------------------------------|
| MMP-9  | Forward: AGCCCACATAGTCCACCTGA |
|        | Reverse: CCCATTAGCACGCACGAC    |
| TNF-α  | Forward: GTCCGGCAGGTGTGATCTCA |
|        | Reverse: CAGGAAGCGTGGTCAAGTAG |
| IL-6   | Forward: GGCTCCCATGATGTGGAGTT |
|        | Reverse: GCCCAGTGGACAGTTCCTCG |
| IL-8   | Forward: GGGAAAATGGTTGACAGAAGT |
|        | Reverse: CTAGGGCCATACAGCCTCCAC |
| Bcl-2  | Forward: ATGTGTGTGGAGAAGCTCAA |
|        | Reverse: CTCTGTGTGCTGATTTGT    |
| Bcl-xl | Forward: GTGTTGTGTGGAGAAGCTCAA |
|        | Reverse: CTCTGTGTGCTGATTTGT    |
| Bax    | Forward: CTTCCCCGAGAGGTCTTTTT |
|        | Reverse: TGATGGTGCTCTATCCTACG  |
| Bad    | Forward: GGAGTCGGGCACACGCTCCAC |
|        | Reverse: ACTCGTGCTCATCTCCCTCG  |
| GAPDH  | Forward: GCAAGTTACACAGGCAGCA   |
|        | Reverse: GGTTACGCCCATACCAA     |
| UXT    | Forward: TGGCCGCTGAGATTTGT     |
|        | Reverse: GGTTGCTGCTGAGCTCTTG   |
| MRPL39 | Forward: AGGTCTTCTTGTGGGATCC   |
|        | Reverse: TTGGTCAGAGCCCAAGAAGT  |

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1 × 10^4 cells/well with 100 μL DMEM/F-12 medium. The GMEC were treated with indicated concentration of SB-3CT for 24 h, and subsequently each well was incubated with 10 μL of CCK-8 reagent (Vazyme, China) and 90 μL refreshed DMEM/F-12 medium at 37°C for 2 h. Then the absorbance was detected at 450 nm using a microplate reader (Molecular Devices, USA). Cell proliferation was also assessed by Cell-Light EdU DNA cell proliferation kit (RiboBio, Guang zhou, China), according to the manufacturer’s instructions.

**Hoechst 33342/PI dual staining assays**

Cell apoptosis was detected with Hoechst 33342 and PI double staining analysis (Solarbio, China). In brief, the GMEC were washed with PBS and subsequently stained with Hoechst 33342 (100 μg/mL) for 15 min at room temperature. The cells were washed with PBS twice, and then the PI (100 μg/mL) was added and incubated for 10 min at room temperature. The presence of fluorescence was observed under a fluorescence microscope. Hoechst 33342^-/PI^- cells (viable cells) were in light blue; Hoechst 33342^-/PI^+ cells (early apoptotic cells) were with blue fragmentations; Hoechst 33342^+/PI^- cells (late apoptotic cells) were with red fragmentations; PI^+ cells (necrotic cells) were with debris signals.

**Cell apoptosis measurement by Annexin V-FITC/PI staining assay**

After incubations, the GMEC were washed with PBS three times, harvested by trypsinization, washed with PBS again and resuspended in 500 μL 1 × binding buffer. Five microliters of FITC-conjugated Annexin V (Vazyme, China) and five microliters of PI (Vazyme, China) were added to 100 μL of the sample solution, and incubated for 15 min in the dark. Then each sample solution was mixed with 400 μL PBS. The cells were immediately analyzed using a flow cytometry.

**Gelatin zymography**

The GMEC were treated with indicated concentration of SB-3CT for 24 h, and cell culture medium was collected and performed as previously described [20].

**Western blot analysis**

The GMEC were collected, pelleted and lysed. The total protein was prepared and protein concentration was measured by Bradford assay. Then proteins were separated by the SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transfered to nitrocellulose membranes, blocked with milk powder solution for 1.5 h at room temperature and overnight incubation with the primary antibody. Then the members was washed with PBS-tween and incubated for 1.5 h with horseradish peroxidase-conjugated secondary antibody. The protein bands were detected using Super signal West Femto Signal Fire agent of Thermo.

**Statistical analysis**

All treatments were replicated six times and the data were analyzed by SPSS 19.0 software (Chicago, USA) using one-way analysis of variance (ANOVA) and Tukey’s test. Values are means ± SEM using the following significant values: *P < 0.05, **P < 0.01, and ***P < 0.001.
Results

The relationship between SCC and MMP-9 expression

The goat MMP-9 was widely expressed in different tissues, with the highest messenger RNA (mRNA) levels observed in spleen, followed by intestine and mammary gland, and the lowest levels were detected in heart (Fig 1A).

The SCC and composition of goat milk were shown in Table 2 (group A) and Table 3 (group B). We found the SCC in group B (four-year-old and third-born does) was higher than group A (two-year-old and first-born does) which means that the SCC increased with age and parity in goat milk. We also found that the SCC was positively correlated with the expression of MMP-9 in dairy goats with the same age and parity (Fig 1B and 1C). The correlation and regression relationship

![Diagram](https://example.com/diagram1.png)

**Fig 1.** The relationship between somatic cell count (SCC) and matrix metalloproteinase-9 (MMP-9) expression. (A) Relative mRNA levels of MMP-9 in goat various tissues. (B) The relative expression levels of MMP-9 in somatic cell of goat milk. Group A, two-year-old and first-born dos; group B, four-year-old and third-born dos. Each group had twelve dairy goats. (C) The SCC in goat milk. (D) The correlation and regression relationship analysis between MMP-9 mRNA expression and SCC were presented using the regression equations. The mRNA expression of MMP-9 as the independent variable and the SCC (lg) as the dependent variable.

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analysis between MMP-9 mRNA expression and SCC were presented using the regression equations $y = 0.6185 \ln(x) + 4.9009$ (group A) and $y = 0.5943 \ln(x) + 4.9095$ (group B), and the relevant coefficients were 0.9949 ($P < 0.05$) and 0.9953 ($P < 0.05$), respectively (Fig 1D).

The effects of *Staphylococcus aureus* on the MMP-9 mRNA expression

The GMEC were treated with different concentrations of *Staphylococcus aureus* ($10^3$, $10^5$ and $10^7$ cfu/mL) for 2, 4, 6 or 24 h, respectively. When the cells were infected with $10^5$ cfu/mL *Staphylococcus aureus*, the MMP-9 mRNA expression increased to 1.03 fold ($P > 0.05$) at 2 h, 1.13 fold ($P > 0.05$) at 4 h, 1.37 fold ($P > 0.05$) at 6 h, and 6.35 fold ($P < 0.05$) at 24 h (Fig 2A). The mRNA expression levels of MMP-9 were increased to 1.1, 1.56, 2.65 and 14.3 fold ($P < 0.05$) in GMEC treated with $10^5$ cfu/mL *Staphylococcus aureus* at 2, 4, 6 and 24 h, respectively (Fig 2A). Treating with a concentration of $10^7$ cfu/mL, the MMP-9 mRNA expression increased to 1.35, 2.48, 9.19 and 19.7 fold ($P < 0.05$) at 2, 4, 6 and 24 h, respectively (Fig 2A). Thus, these observations suggested that *staphylococcus aureus* could increase MMP-9 mRNA expression in goat mammary epithelial cells.

We also found the mRNA expression of tumour necrosis factor (TNF-α), interleukin (IL)-6 and IL-8 increased with the increased treatment concentration of *Staphylococcus aureus* in GMEC (Fig 2B). However, the SB-3CT, an inhibitor of MMP-9, promoted the mRNA expression of TNF-α and inhibited the expression of IL-6 and IL-8 (Fig 2C). It is worth mentioning

### Table 2. Somatic cell count and composition of goat milk (group A, n = 12).

| Number of goat | Protein (%) | Fat (%) | Fat percentage (%) | Total solid (%) | SCC (10^3 cells/mL) | Urea (mg/dL) |
|----------------|-------------|---------|--------------------|----------------|---------------------|-------------|
| A1             | 3.68        | 3.08    | 4.21               | 11.98          | 2694                | 102.8       |
| A2             | 3.18        | 2.75    | 4.54               | 11.43          | 2688                | 94.3        |
| A3             | 2.25        | 2.66    | 4.59               | 10.37          | 1037                | 89.4        |
| A4             | 4.83        | 3.89    | 4.57               | 14.14          | 619                 | 108.7       |
| A5             | 2.5         | 2.51    | 4.73               | 10.61          | 458                 | 84.1        |
| A6             | 2.37        | 2.35    | 4.64               | 10.22          | 179                 | 88.9        |
| A7             | 1.28        | 2.81    | 4.87               | 9.74           | 156                 | 94.6        |
| A8             | 2.79        | 3.09    | 4.51               | 11.4           | 142                 | 92.3        |
| A9             | 8.2         | 2.9     | 4.86               | 16.74          | 100                 | 125.4       |
| A10            | 2.29        | 2.62    | 4.8                | 10.53          | 66                  | 97.4        |
| A11            | 2.26        | 3.27    | 4.85               | 11.22          | 35                  | 93.3        |
| A12            | 3.16        | 2.8     | 4.73               | 11.52          | 32                  | 99.8        |

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### Table 3. Somatic cell count and composition of goat milk (group B, n = 12).

| Number of goat | Protein (%) | Fat (%) | Fat percentage (%) | Total solid (%) | SCC (10^3 cells/mL) | Urea (mg/dL) |
|----------------|-------------|---------|--------------------|----------------|---------------------|-------------|
| B1             | 2.17        | 3.45    | 3.89               | 10.64          | 7915                | 89.3        |
| B2             | 3.62        | 5.4     | 3.16               | 13.35          | 3637                | 137.1       |
| B3             | 3.39        | 3.1     | 4.52               | 12.02          | 3620                | 113.4       |
| B4             | 2.43        | 2.96    | 4.82               | 11.01          | 704                 | 89.9        |
| B5             | 3.13        | 3.01    | 4.6                | 11.7           | 339                 | 92.1        |
| B6             | 2.62        | 2.89    | 4.68               | 11.05          | 157                 | 97.8        |
| B7             | 3.25        | 2.94    | 4.7                | 11.82          | 110                 | 90.9        |
| B8             | 5           | 3.04    | 4.6                | 13.61          | 90                  | 106.5       |
| B9             | 2.12        | 2.96    | 4.79               | 10.75          | 89                  | 91.2        |
| B10            | 2           | 2.98    | 4.83               | 10.59          | 68                  | 101.5       |
| B11            | 3.67        | 3.08    | 4.92               | 12.57          | 40                  | 99          |
| B12            | 2.01        | 2.48    | 4.54               | 9.96           | 23                  | 80.1        |

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that SB-3CT did not decrease the mRNA expression of MMP-9 (S1 Fig), but gelatin zymography results showed that SB-3CT (at indicated concentration) in GMEC inhibited the activity of MMP-9 in dosage dependent manner (S1 Fig).

The effects of MMP-9 on GMEC survival

To assess the effect of MMP-9 on cell survival, we treated GMEC with SB-3CT. FITC-AnnexinV/PI and Hoechst 33342/PI dual staining assays showed that Staphylococcus aureus and SB-3CT markedly induced GMEC apoptosis (Fig 3A and 3B). To make sure the role of MMP-9 on

Fig 2. The effects of Staphylococcus aureus on the matrix metalloproteinase-9 (MMP-9) expression. The GMEC were treated with 0, 10^3, 10^5, 10^7 cfu/ml Staphylococcus aureus for 2, 4, 6 or 24 h, respectively, then the mRNA expression levels of MMP-9 (A) and inflammation related genes (B) were analyzed by quantitative real time PCR (RT-qPCR). (C) The GMEC were treated with indicated concentration of SB-3CT for 24 h, and the mRNA expression levels of inflammation related genes were analyzed by RT-qPCR. SB-3CT, an inhibitor of MMP-9. Values are means ± SEM for six individuals, using the following significant values: *P < 0.05, **P < 0.01, and ***P < 0.001.

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Fig 3. The effects of matrix metalloproteinase-9 (MMP-9) on goat mammary epithelial cells (GMEC) apoptosis. The GMEC were treated with indicated concentration of SB-3CT or 10^7 cfu/ml Staphylococcus aureus for 24 h. Cell apoptosis was determined by Hoechst33342/PI dual staining assays (A) and Annexin V-FITC/PI binding followed by flow cytometry (B). For Hoechst33342/PI dual staining assays, Hoechst 33342-/PI- cells (viable cells) were in light blue; Hoechst 33342+/PI- cells (early apoptotic cells) were with blue fragmentations; Hoechst 33342+/PI- cells (late apoptotic cells) were with red fragmentations; PI+ cells (necrotic cells) were with debris signals. SB-3CT, an inhibitor of MMP-9. Values are means ± SEM for three individuals, using the following significant values: *P < 0.05, **P < 0.01, and ***P < 0.001.

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survival, we performed cell cycle and CCK-8 cell viability assays to evaluate the effects of SB-3CT on cell survival. Cell cycle analysis revealed SB-3CT significantly increased the number of GMEC in G0/G1 phase, and decreased the proportion of cells in S and G2/M phases (Fig 4A and 4B). We found cell proliferation was inhibited with the increased treatment concentration of SB-3CT, and thus confirmed the growth inhibitory effect of SB-3CT in GMEC (Fig 4C).

Besides the CCK-8 assay, cell proliferation was also analyzed with EdU incorporation assay. We found that SB-3CT inhibited cell proliferation in a dosage dependent manner (Fig 4D).

Previous studies have shown that Bcl-2 promotes cell survival in mammary epithelial cells and breast cancer cells [21, 22]. To know whether Bcl-2 is implicated in the proapoptosis actions of SB-3CT in mammary epithelial cells, we treated GMEC with SB-3CT and detected the expression of Bcl-2. In GMEC, SB-3CT decreased Bcl-2 expression in mRNA and protein levels (Fig 5A and 5C). Then we wanted to know whether the other Bcl-2 family members were regulated by MMP-9: pro-survival gene Bcl-xl and proapoptotic genes Bax and Bad. We found SB-3CT significantly increased Bax and Bad expression and suppressed Bcl-xl expression in GMEC (Fig 5A and 5C). We also found that SB-3CT decreased both Bcl-2/Bax and Bcl-xl/Bax ratios in GMEC (Fig 5B and 5D).

Thus, these observations suggested that MMP-9 promoted cell survival in GMEC.

Discussion

In this investigation, we propose that the SCC in goat milk was positively correlated with MMP-9 mRNA expression, and staphylococcus aureus could markedly increase MMP-9
mRNA expression in GMEC in dosage and time dependent manner. We also demonstrated that inhibition of MMP-9 activity promoted apoptosis and inhibited proliferation in GMEC.

Goats with subclinical mastitis or in the late lactation, the milk plasmin and plasminogen activator levels were higher [23, 24]. The physiological changes caused by plasmin which can activate MMP-9 involving some normal and pathological process including cell migration and tissue remodeling [23, 24]. It had been reported that the increased milk SCC induced markedly increasing of milk gelatinases and plasmin, which revealed that there is a certain relationship between the activity of MMP-9 and milk SCC [10, 16]. Consistent with this study, we found the expression of MMP-9 increased in the milk somatic cells of goats, and the expression of MMP-9 and SCC were positively correlated in dairy goats with the same age and parity. On average, the SCC in goat free of intramammary infection is less than 5 × 10^6 cells/mL. Interestingly, the SCC of goat numbered B1 in group B reached about 7.9 × 10^6 cells/mL, but this goat was healthy without symptoms of mastitis. Probably because milk samples were collected in August. A number of factors have an impact on SCC such as seasonal variations. Generally, somatic cells are at highest level during the summer and decrease in the winter [8, 9].

The pathogenic microorganism infection was the main cause of mastitis, and *staphylococcus aureus* was an important pathogen [25, 26]. MMP-9 in inflammatory process could change vascular permeability and blood brain barrier, and transfer white blood cells to the inflammation areas [27, 28]. Therefore, we speculate that the reason for MMP-9 highly expressing in inflammation cells is that the organism has to recruit immune cells to inflammation areas which induces MMP-9 to degrade extracellular matrix. We found *staphylococcus aureus* could markedly increase MMP-9 expression in dosage and time dependent manner in goat mammary epithelial cells.
Bovine mammary epithelial cell infected with *Staphylococcus aureus* showed a dose dependent increase of IL-8 secretion, and the expression of TNF-α also increased markedly [29]. MMPs had been found to promote the release of TNF-α, activate pro-IL-1β and cleave the activated form of IL-1β [30, 31]. Inhibition of MMP-9 in mice skin epithelial cells of burn injuries could accelerate wound healing [30]. The mature of TNF-α was dependent on matrix metalloproteinase-like enzyme by cleaving TNF-α precursor [31]. In this study, we found *Staphylococcus aureus* increased the mRNA expression of inflammation related genes (*TNF-α, IL-6* and *IL-8*) with the increase of treatment concentration in GMEC. We demonstrated that SB-3CT, an inhibitor of MMP-9, increased the mRNA expression of TNF-α and inhibited the expression of IL-6 and IL-8.

We also demonstrated that SB-3CT induced apoptosis and inhibited proliferation in GMEC. MMP-9 overexpression in MCF-7 human mammary carcinoma cells induced migration and invasion [32, 33]. SB-3CT in MCF-7 cells could induce cell apoptosis and inhibit cell viability by regulating the expression of Bcl-2 [34]. MMP-9 activity had also been shown to be important for the angiogenic and metastatic phenotypes of human tumors. MMP-9 upregulation in three tumor models, the K14 HPV16 skin cancer, the RIP1 TAG2 insulinoma and DU145 prostate tumor resulted in an increase in tumor growth and angiogenesis by activating the gene expression of vascular endothelial growth factor (VEGF) and intercellular adhesion molecule 1 (ICAM-1) [35–37]. By contrast, MMP-9 had been shown to promote chondrocyte apoptosis, leading to abnormal vascularization and ossification [38]. One possible reason is that MMP-9 degrades ECM and controls angiogenesis, and lead to apoptosis. MMP-9-deficient mice revealed granular precursor migration was delayed, and cell apoptosis was reduced [39]. The possibility is that transforming growth factor β (TGF-β), which modulates programmed cell death, can be activated by MMP-9. TGF-β could induce apoptosis in cerebellar granule cell, and inhibit granular precursor expansion during postnatal cerebellar development [40].

In conclusion, the SCC in goat milk was positively correlated with MMP-9 expression, and *staphylococcus aureus* can markedly increase MMP-9 expression in GMEC in dosage and time dependent manner. We also demonstrated that SB-3CT, an inhibitor of MMP-9, promoted apoptosis and inhibited proliferation in GMEC. Therefore, MMP-9 may emerge as an easily measurable and sensitive parameter that reflects the number of somatic cells present in milk and a regulatory factor of apoptosis in GMEC.

**Supporting Information**

**S1 Fig. The effect of SB-3CT on the expression and activity of MMP-9 in goat mammary gland epithelial cells (GMEC).** The GMEC were treated with 0 μM, 5 μM or 10 μM SB-3CT for 24 h, and subsequently the mRNA expression and protein activity of MMP-9 were detected by quantitative real time PCR (RT-qPCR) and Gelatin Zymography, respectively. SB-3CT, an inhibitor of MMP-9. Values are means ± SEM for six individuals, using the following significant values: *P* < 0.05.

(TIF)

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**Author Contributions**

Conceived and designed the experiments: HZ.
Performed the experiments: HL LL XS WZ.

Analyzed the data: HL LL XS.

Contributed reagents/materials/analysis tools: HZ XS HL LL YS.

Wrote the paper: HL LL YS HZ.

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