Analysis of matrix metalloproteinase-9 promoter region activity and association analysis of promoter region SNPs with lactation traits in dairy goats

Qing Li[1,2], Tianle Chao[1,2], Rong Xuan[1,2], Yunpeng Chu[1,2], Shujun Wang[1,2], Ming Cheng[3], Peipei Li[3], Zhibin Ji[1,2], Jianmin Wang[1,2*]

ABSTRACT

Matrix metalloproteinase-9 (*MMP9*) degrades the extracellular matrix (ECM), participates in mammary gland remodeling, and inhibits mammary epithelial cell apoptosis in goats. To investigate the transcriptional regulatory mechanism of the *MMP9* promoter region, we analyzed the expression pattern of *MMP9* in dairy goats by qRT–PCR and cloned the promoter region by PCR. Deletion analysis indicated that the *MMP9* gene core promoter region was located upstream of the transcription start site in the -715 bp to -926 bp region. We predicted three specificity protein 1 (*Sp1*) binding sites in the *MMP9* core promoter region, and performed targeted mutations on these three sites. The c.1863 G>A mutation in the *MMP9* gene increased the promoter transcriptional activity and may be associated with an additional serum response factor (*SRF*) transcription factor-binding site. Association analysis revealed that c.1863 G>A was significantly associated with milk fat percentage in dairy goats, which was significantly higher in goats with the AG genotype (4.71 ± 0.02%) than in goats with the GG genotype (4.61 ± 0.05%). This study lays a foundation for subsequent analysis of the transcriptional regulatory mechanism of *MMP9* and exploration of its biological functions.

Key words: goat; *MMP9*; promoter; *Sp1*; SNP.

Introduction

Dairy goats are very important economic animals with strong ecological adaptability and unique physiological digestion and lactation characteristics. As an excellent local breed in

[1] Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, College of Animal Science and Veterinary Medicine, Shandong Agricultural University, Tai'an City, Shandong Province, China.
[2] Key Laboratory of Efficient Utilization of Non-Grain Feed Resources (Co-construction by Ministry and Province), Ministry of Agriculture and Rural Affairs，Shandong Agricultural University, Tai'an City, Shandong Province, China.
[3] Qingdao Animal Husbandry and Veterinary Research Institution, Qingdao 266100, Shandong Province, China.
* Corresponding author; E-mail: wangjm@sdau.edu.cn

China, Laoshan dairy goats have many advantages, such as roughage feeding resistance, fast growth and development, high milk production, and stable genetic performance (China National Commission of Animal Genetic Resources, 2011; Chen et al., 2018).

The mammary gland is an important exocrine gland in dairy livestock. Its main function is to produce and secrete milk to nourish offspring (Oftedal, 2002), and its development is a dynamic process. The branching morphogenesis of the mammary gland partly depends on the extracellular matrix (ECM), ECM receptors, and ECM-degrading enzymes (Fata et al., 2003; Loganathan et al., 2020; Buchmann et al., 2021). The *MMP9* gene can degrade collagen and elastin in the ECM under physiological conditions (van Turnhout et al., 2010; Panwar et al., 2018) and participate in mammary gland remodeling (Sternlicht et al., 2006; Takagi et al., 2007).

Our previous study identified miR-143-3p as a miRNA that is differentially expressed in dairy goat mammary tissue during different lactation periods. miR-143-3p inhibits proliferation and promotes apoptosis in goat mammary epithelial cells (Ji et al., 2019), and it regulates matrix metalloproteinase-9 (*MMP9*) by binding to the target site in the *MMP9* gene (Liu, 2020). Other studies have reported that *MMP9* can promote mammary epithelial cell proliferation and inhibit apoptosis in dairy goats (Li et al., 2016). In mice, *MMP* transcriptional activity changes significantly during mammary degeneration (Fata et al., 1999). The mammary epithelium is tightly connected to the stroma via the basement membrane ECM. In dairy cows, degeneration of mammary tissue occurs during the dry phase, followed by tissue remodeling. This change in physiological structure is aimed at obtaining optimal milk yield during the following lactation period (Gifre-Renom et al., 2020). All of these findings indicate that the regulatory function of the *MMP9* gene is essential during mammary gland development.

In this study, we predicted and identified the active region of the *MMP9* promoter and screened possible transcription factor-binding sites (TFBSs) within this promoter. Moreover, we identified single-nucleotide polymorphisms (SNPs) in the *MMP9* promoter, determined the relationships of SNPs in *MMP9* with dairy goat milk yield and milk composition, and further explored the effects of mutations at this locus on the transcriptional activity of *MMP9*. The results provide important insights for elucidating the transcriptional regulatory mechanism of *MMP9* in dairy goats and understanding its role in regulating milk production.

MATERIALS AND METHODS

Ethics statement

The Animal Ethics Committee of Shandong Agricultural University approved this study (SDUAA-2017-40), and efforts were made to reduce the pain of the animals during the procedures.

Animal and sample collection

Twelve 4-year-old Laoshan dairy goats (third parity) were obtained from the Laoshan dairy goat primary farm (Qingdao, Shandong Province, China). Ipsilateral mammary gland was surgically collected under general anesthesia during early lactation (20 days postpartum, n=3), the peak of lactation (90 days postpartum, n=3), the late lactation (240 days postpartum, n=3), and dry period (300 days postpartum, n=3). Heart, lung, ovary, liver, spleen, longissimus muscle tissue, kidney, and mammary gland samples were collected from Laoshan dairy goats during peak lactation (90 days postpartum, n=3) and immediately stored in liquid nitrogen for tissue expression profile construction. The milk composition and milk yield of 254 female Laoshan dairy goats were used for the association analysis, and the milk composition was tested using MilkoScan FT120(Denmark). The dairy goats used for the experiment were all 2-5 litters of multiparous, nonpregnant ewes. Lactation was recorded every 30 days from July to December, and milk production data were recorded once in the morning and once in the evening (6:00; 18:00). Venous blood was collected in a vacuum centrifuge tube with ethylenediaminetetraacetic acid. The goats were healthy and disease-free and had the same feeding conditions.

RNA extraction and quantitative real-time PCR

After the tissues were thoroughly ground in liquid nitrogen, total RNA was extracted by the TRIzol method (Takara, Japan). cDNA was obtained through reverse transcription with the PrimeScript™ cDNA Synthesis Kit (Takara, Japan), and primers were designed using Primer 5.0 (Table 1).

Table 1 Primer sequences designed for amplification, plasmid construction and genotyping in Laoshan dairy goats.

Primer name	Primer sequences (5'-3')	Fragment length/bp	Purpose	TM/℃
GAPDH	F: GCAAGTTCCACGGCACAG	249	qRT–PCR	58.5
	R:GGTTCACGCCCATCACAA			
MMP9	F:CCCATTAGCACGCACGAC	115	qRT–PCR	59
	R:AGCCCACATAGTCCACCTGA			
P1-F	GGGGTACCGCAAAACCCAATCCTTCCCG	419(-299/+120 bp)	Plasmid construction	59.7
P2-F	GGGGTACCAGTCAAGGCAGACTTCAGG	655(-537 /+120 bp)		58.2
P3-F	GGGGTACCAGACGCCGCAATACTCCCAC	831(-715/+120 bp)		61.5
P4-F	GGGGTACCCCAACCTCACTGCCTTTAGC	1047(-926/+120 bp)		59.3
P5-F	GGGGTACCAAGGTCACAGCAGATTAGAGTCC	1741(-1625/+120 bp)		59.7
P6-F	GGGGTACCGAGCTGGGACTGAAAACCAGAACA	2583(-2465/+120 bp)		61.3
P7-F	GGGGTACCCAAAGACCTGAGCCTCCTCTCTGA	3005(-2885/+120 bp)		59.4
R	CCGCTCGAGAGGCTGGTTCGTCGTTCT		Plasmid construction	
Sp1-1	F:CAGCCAGCTATGGGAACCGGGTAAAGTTCAAAAG		Site-directed mutagenesis	76.1
	R:CTTTTGAACTTTACCCGGTTCCCATAGCTGGCTG			
Sp1-2	F: GAATGGTAGGACCCAAAGCTATGGGAAAG		Site-directed mutagenesis	73.8
	R: CTTTCCCATAGCTGGGGGGGTCCTACCATTC			
Sp1-3	F:CTACAATAAGTAGCCCGAAGCCTGGGACATAGCAG		Site-directed mutagenesis	76.4
	R:CTGCTATGTCCCAGGCTTCGGGCTACTTATTGTAG			
MMP9-1863	AlleleFAM: AGTACCTATAAGACAGCTCACAGG		Genotyping	
	AlleleHEX:CAGTACCTATAAGACAGCTCACAGA			
	Common:CAAGGGCTATAGATAATATTCTGAGCCAT			
MUT-1	F:GAGGTACAATTAATCTCCAGTTCCAAGGTCAATT		Site-directed mutagenesis	
	R:GAGATTAATTGTACCTCAAAACAATCCAGGTGA			
MUT-2	F:GCTCACAGaATATGGCTCAGAATATTATCTATAGCCC		Site-directed mutagenesis	
	R:AGCCATATtCTGTGAGCTGTCTTATAGGTACTGAAAC			

The following PCR system was employed (25 μL): cDNA template 2 μl, 10 μM primers, 12.5μL 2×Taq MasterMix, 8.5μl ddH_2O. The PCR procedure included at 95 °C for 30 s and 35 cycles of at 95 °C for 5 s, 59°C for 30 s and 60 °C for 20 s. Finally, the dissolution curve was obtained and analyzed. Three independent repeated experiments were carried out on each sample. The housekeeping gene was used *GAPDH*. The relative gene expression of *MMP9* was calculated by the $2^{-\Delta\Delta Ct}$ method.

Construction of *MMP9* promoter luciferase plasmids

Based on the goat *MMP9* gene sequence in GenBank (accession number: NC_030820.1), a 3005 bp fragment was amplified from goat blood DNA by PCR. The PCR amplification system was as followes (25 μL): 1.0 μL of genomic DNA, 1.0 μL primers (10μmol/L), 12.5μL 2×Easy

Taq PCR Super Mix (+dye), 9.5 μL sterile deionized water (dd H_2O). The PCR reaction conditions were as follows: 94 °C for 5 min; 94 °C for 30 s. 58 °C for 30 s, 72 °C for 2 min, 40 cycles; 72 °C for 10 min. Primers P1–P7 of different lengths were designed using Primer 5.0, and both ends of each primer pair included cutting sites and protective bases for the restriction endonucleases Kpn I and Xho I (Table 1). Then, the amplification product was subcloned into the pGL3-basic. Thus, luciferase reporter gene vectors including different lengths of the dairy goat *MMP9* promoter were obtained.

Cell culture, transient transfection, and luciferase activity assays

293T cells were inoculated into a 24-well plate at 2 x 10^4 cells per well and then cultured in medium without penicillin-streptomycin solution for 24 h. When cells reached 70-80%, the luciferase reporter gene vector was transfected into the cells. Transfection procedures were carried out strictly according to the instructions for Lipofectamine 2000 (Invitrogen, USA). A total of 0.8 μg of the recombinant vector containing a promoter fragment or the pGL3-basic negative control plasmid, along with 0.03 μg of the pRL-TK internal reference plasmid, was transfected into each well. After 4-6 hours of transfection, the medium was changed, and cells were collected after 48 hours for luciferase activity assay.

Bioinformatic analysis of the promoter region fragments

Three bioinformatics software programs were used to analyze TFBSs in the *MMP9* promoter: JASPAR (http://jaspar.binf.ku.dk/), AliBaba2.1 (http://gene-regulation.com/pub/programs/alibaba2/) and TFBIND (http://tfbind.hgc.jp/).

Genotyping of SNPs in the *MMP9* promoter region

SNP in the *MMP9* promoter region was identified by DNA hybrid pool sequencing. Genotyping was performed using competitive allele-specific PCR (KASP) (He et al., 2014). The detection primer sequences (*MMP9*-1863) were listed in Table 1; two upstream primers (Allele FAM and Allele HEX) were specific primers corresponding to the FAM and HEX fluorescence tags, and a single downstream primer was commonly employed. After the reaction, the genotypes were determined based on the two fluorescence signals detected, and different

genotypes were obtained for different fluorescence signals.

Site-directed mutagenesis of the *MMP9* promoter

Using the promoter fragment pGL3-P7 (+120 to -2885 bp) as the template, primers for site-directed mutagenesis were designed as shown in Table 1, defined as pGL3 -MUT-A and pGL3-WT-G, respectively. The PCR amplification products were digested with Dpn I. A recombination reaction was carried out, and the reaction products were used to transform competent cells. Finally, mutant vectors were obtained after plate coating and cloning. The accuracy of the clones was validated by DNA sequencing.

Statistical analysis

Calculation of routine population genetic parameters were used POPGENE (version 1.32), such as observed heterozygosity (Ho), expected heterozygosity (He), the effective number of alleles (Ne), and the Hardy-Weinberg equilibrium (HWE) chi-squared value.

The least squares analysis of GLM was performed in SAS 8.1. The model was as follows:

$Y_{ijk}=\mu+P_i+G_j+e_{ijk}$

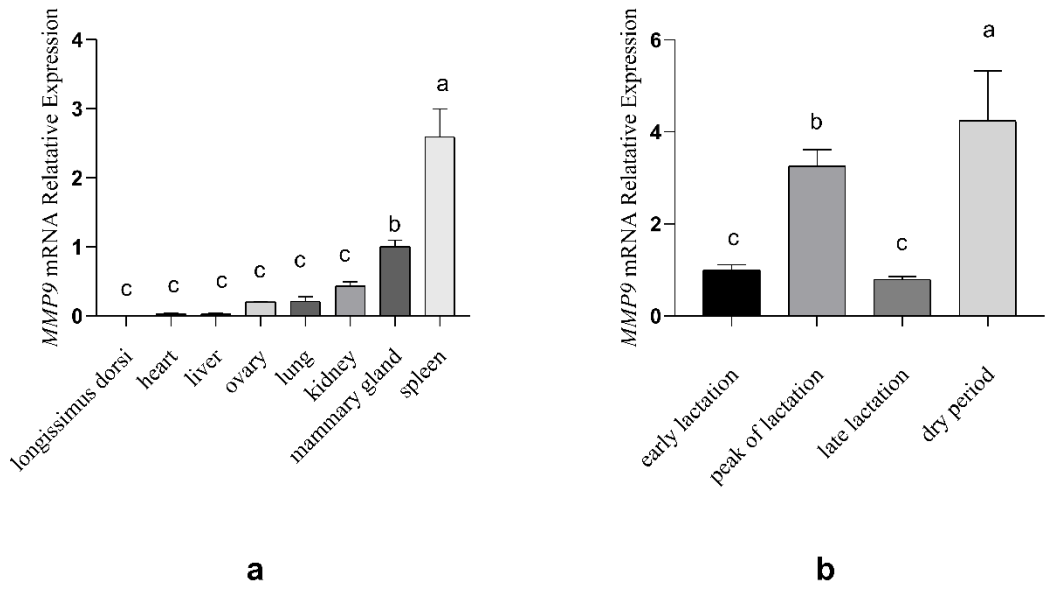
where Y_{ijk} is the phenotypic value of a trait, μ is the group mean, P_i is the fixed effect of parity, G_j is the genotype effect, and e is the random error.

All qRT–PCR and promoter luciferase relative activity results were obtained from three biological replicates. Differential analysis of qRT–PCR and promoter luciferase relative activity using one-way ANOVA, and $P< 0.05$ was considered to be statistically significant.

RESULTS

***MMP9* gene expression pattern analysis**

In this study, qRT–PCR was used to evaluate *MMP9* expression in different tissues and mammary gland tissues of Laoshan dairy goats at different lactation stages. The *MMP9* expression was highest in the spleen and was extremely low in the longissimus muscle (Figure 1A). A comparison of the different developmental stages of mammary tissues in dairy goats revealed that the expression of *MMP9* was highest during the dry period and lowest in late lactation (Figure 1B).



a b

Figure 1. qRT-PCR was used to analyze *MMP9* gene expression in different tissues of Laoshan dairy goats. (A) *MMP9* expression levels in different tissues of Laoshan dairy goats. (B) *MMP9* expression levels in different lactation stages of the mammary gland (mean ± SEM). Different letters represent the significant differences for mean comparisons ($P < 0.05$, n = 3).

Cloning, sequencing, and characterization of the goat *MMP9* promoter

A sequence containing 2885 bp upstream and 120 bp downstream of the TSS was obtained through cloning and sequencing in the dairy goat *MMP9* promoter region (3005 bp). The dual luciferase activity results showed significantly increased activity for the pGL3-*MMP9* fragment compared to the pGL3-basic vector ($P < 0.05$), indicating that the P7 fragment (-2885/+120 bp) functions in transcriptional regulation (Figure 2A).

Identification of the *MMP9* core promoter region

Recombinant pGL3-basic vectors, including P1-P7, were constructed. The *MMP9* gene core promoter region of dairy goat *MMP9*, P4-P7, had the highest activity values ($P < 0.05$), while the luciferase activities of the other recombinant plasmids were not significantly different from the pGL3-basic group ($P > 0.05$) (Figure 2B). These results indicated that the P4-P7 fragment had promoter activity and that the -926 bp/-715 bp region constituted the *MMP9* core promoter.

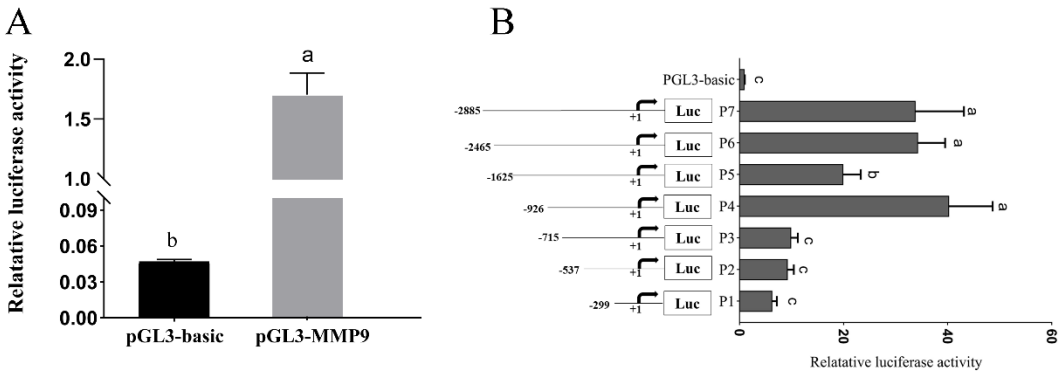


Figure 2. Activity analysis of the *MMP9* promoter. (A). Relative luciferase activity of the full-length *MMP9* promoter. (B). Relative luciferase activity of *MMP9* promoters of different lengths (mean ± SEM). Constructs carrying serial *MMP9* promoter deletions (P1-P7) were used to transiently transfect 293T cells, which were incubated for 48 h for the luciferase assays. Different letters represent the significant differences for mean comparisons ($P < 0.05$, n = 3).

Analysis of the activity of the *MMP9* core promoter region

Using JASPAR, TFBIND, and AliBaba2.1, we analyzed the active region of the *MMP9* promoter (-926 bp/-716 bp) and identified three specificity protein 1 (*Sp1*) TFBSs (Figure 3A). To determine whether the *Sp1* TFBSs exerted a regulatory effect on *MMP9* gene transcription, we introduced targeted mutations of the *Sp1*-1, *Sp1*-2, and *Sp1*-3 sites in the *MMP9* gene promoter region (Figure 3B). The deletion of the *Sp1*-1 and *Sp1*-3 sites resulted in significant downregulation of *MMP9* expression ($P < 0.05$). These findings show that the *Sp1*-1 and *Sp1*-3 loci may have a regulatory effect on *MMP9* transcription, and *Sp1*-3 is more stronger regulated than *Sp1*-1.

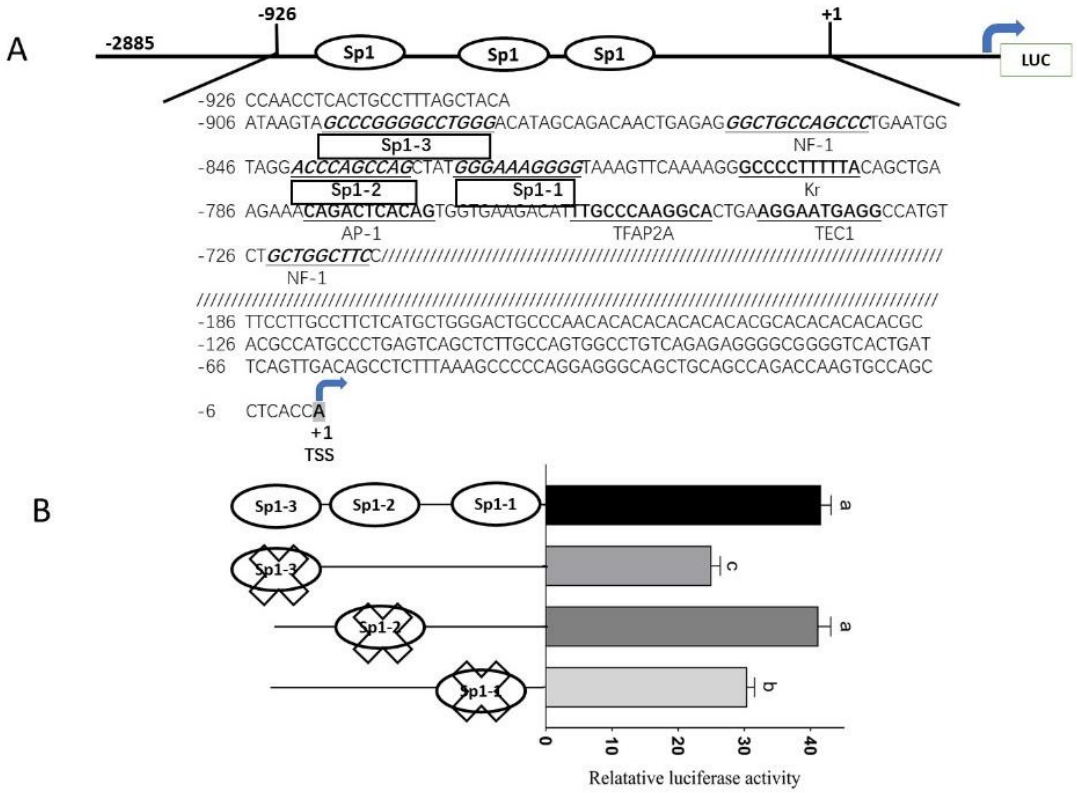


Figure 3. (A). Schematic diagram of the *MMP9* promoter region in dairy goats. Bold italics indicate the predicted TFBS, and the name of the transcription factor is underlined below. (B). The relative dual-luciferase activity of mutations in the *Sp1* TFBS in the promoter region of the *MMP9* gene (mean ± SEM). Different letters represent the significant differences for mean comparisons ($P < 0.05$, n = 3).

MMP9 promoter polymorphism analysis

Through sequencing and analysis, we identified c.-1863 G>A in the promoter region (-2885 bp/+120 bp) (Figure 4A). The genotyping results (Figure 4B) indicated that two genotypes (GG, AG) of the active region of the Laoshan dairy goat *MMP9* gene promoter were present at mutation site c.-1863 G>A. Population genetic analysis of c.-1863 G>A was performed (Table 2). The dominant genotype was GG, with a genotype frequency of 0.60. The dominant allele was G, with a gene frequency of 0.80. The SNP locus did not conform to HWE ($P < 0.05$).

Table 2. Genotypes and allele frequency in the *MMP9* gene promoter.

SNP name	Genotype	Frequency	Allele	Frequency	PIC	Ho	He	Number of effective alleles	χ^2	P-Value
c.-1863G>A	AG	0.40(102)	A	0.20	0.27	0.40	0.32	1.47	16.03	<0.01
	GG	0.60(152)	G	0.80						

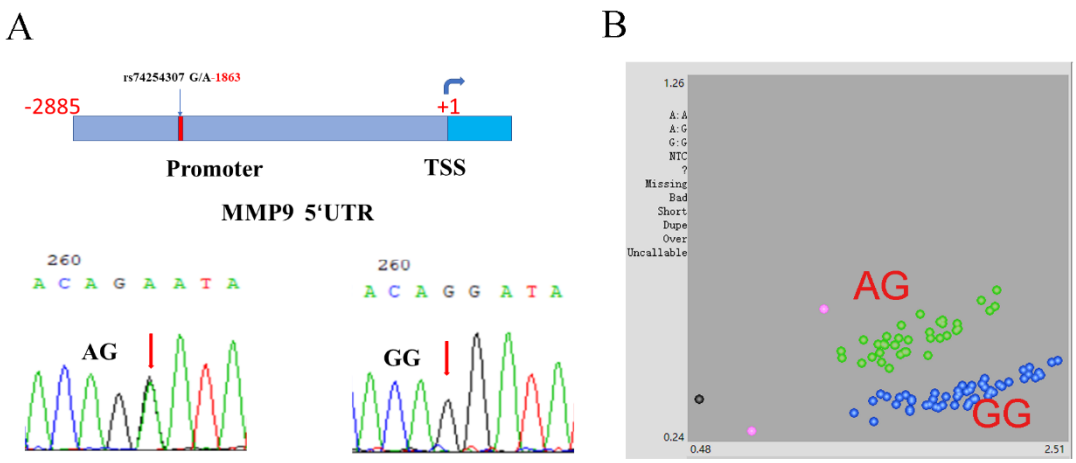


Figure 4. (A) Genetic polymorphisms in the dairy goat *MMP9* promoter region. (B) Sequencing analysis of polymorphisms of the goat *MMP9* promoter.

Association analysis of SNPs in *MMP9* with lactation traits in dairy goats

The analysis of the factorial effects at c. -1863 G>A (Table 3) showed that the effect values of parity at this locus on milk fat percentage, milk protein percentage, total solids content, non-fat solids content, and milk yield reached highly significant levels ($P < 0.01$), and genotype had a significant effect on milk fat ($P < 0.05$). The interaction of parity and genotype also had a significant effect value on milk fat percentage ($P < 0.05$).

The associations of the c.-1863 G>A genotypes in the *MMP9* promoter region with lactation phenotypes were analyzed in 254 Laoshan dairy goats with complete milk yield records (Table 4). The c.-1863 G>A polymorphism had a highly significant effect on milk fat percentage ($P < 0.01$), where the AG genotype (4.71 ± 0.02%) was associated with a significantly higher milk fat rate than the GG genotype (4.61 ± 0.05%). The c.-1863 G>A polymorphism had no significant effect on milk yield or other milk traits ($P > 0.05$).

Table 3. Factorial effects of fecundity and c.1863A>G loci on milk traits in Laoshan dairy goats.

SNP Site	Influence factors	Milk fat percentage (%)	Milk protein percentage (%)	Lactose percentage (%)	True protein percentage (%)	Total solids content (%)	Solid non-fat content (%)	Yield of milk (kg)
c.1863A>G	Genotype	0.02	0.94	0.667	0.811	0.248	0.102	0.184
	Parity	0.001	0.475	0.055	0.075	0.009	0.001	0.002
	Genotype×Parity	0.02	0.055	0.165	0.075	0.101	0.002	0.875

Table 4. Association analysis of a SNPs of *MMP9* promoter region with milk traits of Laoshan dairy goats.

SNP Site	Genotype	Milk fat percentage (%)	Milk protein percentage (%)	Lactose percentage (%)	True protein percentage (%)	Total solids content (%)	Solid non-fat content (%)	Yield of milk (kg)
c.1863G> A	AG	4.71±0.02^{a}	3.72±0.05	3.53±0.05	4.80±0.10	13.30±0.16	8.76±0.06	461.64±8.70
	GG	4.61±0.05^{b}	3.67±0.05	3.48±0.04	4.70±0.07	13.35±0.10	8.79±0.03	475.67±7.40

NOTE: The superscripts of a and b, represent the significance differences in the same column ($P < 0.05$).

c.-1863 G> A polymorphism affects *MMP9* promoter activity

Bioinformatics analysis revealed that the c.-1863 G>A mutation added a new binding site for the *SRF*.

To verify whether the newly added *SRF* TFBS affects the transcriptional activity of the *MMP9* gene, we performed the targeted mutagenesis of c.-1863 G>A. pGL3-MUT-A showed significantly higher dual-luciferase activity than pGL3-WT-G, and the c.-1863 G>A mutation resulted in an approximately 64% increase in the dual-luciferase activity of *MMP9* (Figure 5).

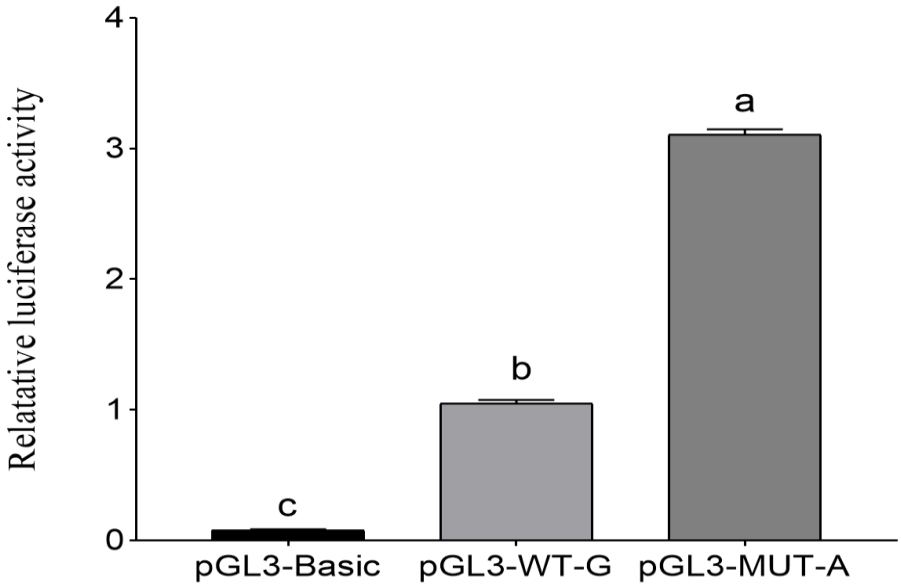


Figure 5. Dual luciferase activity analysis of the *SRF* binding site at c.-1863 G>A (Mean±SEM). The different letters denote significant differences for mean comparisons ($P< 0.05$, n= 3).

DISCUSSION

To elucidate the functions of the *MMP9* gene in dairy goats, *MMP9* gene expression patterns in different tissues and in mammary gland tissues at different lactation stages were analyzed. *MMP9* was expressed in all tested tissues of Laoshan dairy goats. However, *MMP9* expression levels in mammary tissues differed depending on the developmental stage, with a significant increase from early lactation to peak lactation ($P < 0.05$) and a significant decline from peak lactation to late lactation ($P < 0.05$) (Figure 1). This pattern may be related to how *MMP9* promotes synthesis, secretion, and lactation in the mammary gland (Li, et al., 2016). In dairy cows, mammary tissue remodeling occurs during the dry period, and *MMP9* participates in this process (Rabot et al., 2007). In this study, *MMP9* gene expression increased significantly from late lactation to the dry period, which was related to the gene's role in mammary gland development remodeling.

Promoters are DNA sequences upstream of gene coding regions containing many cis-acting elements. (Bai et al., 2015; Yu et al., 2017). *Sp1* is a specific DNA-binding protein found in a wide range of mammalian cells (Briggs et al., 1986). It regulates various biological processes, such as cell proliferation, differentiation, and apoptosis (Vellingiri et al., 2020). To further study the transcriptional regulation of *MMP9*, we confirmed that P3-P6 (-715 to -2465 bp) were

essential segments for the transcription and regulation of *MMP9* (Figure 2). We identified three *Sp1* TFBS in the *MMP9* gene core promoter region and further determined that targeted mutation of the TFBSs at the *Sp1*-1 and *Sp1*-3 sites significantly reduced the transcriptional activity of the *MMP9* gene (Figure 3), suggesting that the *Sp1*-1 and *Sp1*-3 affect the expression of the *MMP9* gene.

The -1954C+/C- polymorphism of *MMP9* was previously reported to be related to egg-laying traits in chickens, and this locus may be related to *MMP9* transcriptional regulation (Zhu and Jiang, 2014). The g.48178429 G>A polymorphism of *MMP9* was previously reported to be related to their resistance to diarrhea in Min piglets (Kou et al., 2020). By analyzing Laoshan dairy goat population genetic data, we found that c.-1863G>A had moderate polymorphism rates (0.25 < PIC < 0.50) (Table 2). The group's genetic richness was high, showing certain heritable variation potential. The SNP locus c.-1863 G>A was not in HWE ($P < 0.05$). Interestingly, we did not observe the AA genotype, and considering that the A allele frequency was 0.2, the theoretical value of the AA genotype frequency was 0.04, which could be related to the small sample size of the dairy goats or the lethality of the AA genotype in Laoshan dairy goat, which lacks further experimental verification. In our study, we identified a SNP locus in the *MMP9* promoter region, c.-1863 G>A, that was significantly associated with milk fat percentage in dairy goats. The AG genotype (4.71±0.02%) had a significantly higher milk fat rate than the GG genotype (4.61 ± 0.05%), as shown in Table 4. This finding suggests that *MMP9* is a possible candidate molecular marker for high milk fat in dairy goats. Furthermore, c.-1863 G>A might be as a potential molecular marker to improve the milk fat rate and produce high-quality dairy goat milk.

SNPs in a promoter region can influence gene regulation by changing the affinity of the sequence for transcription factors (Wray et al., 2003; Savinkova et al., 2013). In humans, c.-1562C>T, a SNP in the *MMP9* gene, can increase *MMP9* expression by preventing nuclear repressors from binding to this region of the promoter (Song et al., 2018).

In this study, we used biological software to analyze the c.-1863 G>A locus and found that c.-1863 G>A mutation created a new TFBS for the *SRF*. Studies have reported that *SRF* can regulate the differentiation, proliferation, and migration of cells (Black and Olson, 1998; Wang

et al., 2001; Taylor and Halene, 2015; Ro 2016). The results show that c.-1863 G>A may lead to an increase in *MMP9* promoter activity by increasing *SRF* transcription factor-binding sites. The role of this *SRF* TFBS in the transcriptional regulation of *MMP9* requires further study.

CONCLUSIONS

In this study, we found that *MMP9* was widely expressed in the tissues of dairy goats. Additionally, we found that the *MMP9* gene was highly expressed in the mammary gland during peak lactation and dry period suggesting that *MMP9* may be involved in mammary epithelial cell development and mammary tissue remodeling. We identified the *MMP9* gene core promoter region and found two *Sp1* TFBSs in the core promoter region that may affect the transcriptional activity of *MMP9*. In addition, we identified c.1863G>A in the active region of the *MMP9* gene promoter. The c.1863G>A mutation provides a new TFBS in the *MMP9 gene*, and c.1863G>A is associated with milk fat percentage in Laoshan dairy goats. This study provides new insights into dairy goat breeding and has significance for the further study of *MMP9* functions.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31672401) and the Shandong Provincial Modern Agriculture Industry Technology System (SDAIT-10).

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