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

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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 ($4.71 \pm 0.02\%$) than in goats with the GG genotype ($4.61 \pm 0.05\%$). 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

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

31 The mammary gland is an important exocrine gland in dairy livestock. Its main function is to
32 produce and secrete milk to nourish offspring (Oftedal, 2002), and its development is a dynamic
33 process. The branching morphogenesis of the mammary gland partly depends on the
34 extracellular matrix (ECM), ECM receptors, and ECM-degrading enzymes (Fata et al., 2003;
35 Loganathan et al., 2020; Buchmann et al., 2021). The *MMP9* gene can degrade collagen and
36 elastin in the ECM under physiological conditions (van Turnhout et al., 2010; Panwar et al.,
37 2018) and participate in mammary gland remodeling (Sternlicht et al., 2006; Takagi et al., 2007).

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

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

57 MATERIALS AND METHODS

58 Ethics statement

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

62

63 Animal and sample collection

64 Twelve 4-year-old Laoshan dairy goats (third parity) were obtained from the Laoshan dairy
65 goat primary farm (Qingdao, Shandong Province, China). Ipsilateral mammary gland was
66 surgically collected under general anesthesia during early lactation (20 days postpartum, n=3),
67 the peak of lactation (90 days postpartum, n=3), the late lactation (240 days postpartum, n=3),
68 and dry period (300 days postpartum, n=3). Heart, lung, ovary, liver, spleen, longissimus muscle
69 tissue, kidney, and mammary gland samples were collected from Laoshan dairy goats during
70 peak lactation (90 days postpartum, n=3) and immediately stored in liquid nitrogen for tissue
71 expression profile construction. The milk composition and milk yield of 254 female Laoshan
72 dairy goats were used for the association analysis, and the milk composition was tested using
73 MilkoScan FT120(Denmark). The dairy goats used for the experiment were all 2-5 litters of
74 multiparous, nonpregnant ewes. Lactation was recorded every 30 days from July to December,
75 and milk production data were recorded once in the morning and once in the evening (6:00;
76 18:00). Venous blood was collected in a vacuum centrifuge tube with
77 ethylenediaminetetraacetic acid. The goats were healthy and disease-free and had the same
78 feeding conditions.

79

80 RNA extraction and quantitative real-time PCR

81 After the tissues were thoroughly ground in liquid nitrogen, total RNA was extracted by the
82 TRIZOL method (Takara, Japan). cDNA was obtained through reverse transcription with the
83 PrimeScript™ cDNA Synthesis Kit (Takara, Japan), and primers were designed using Primer
84 5.0 (Table 1).

85

86

87

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

Primer name	Primer sequences (5'-3')	Fragment length/bp	Purpose	TM/°C
<i>GAPDH</i>	F: GCAAGTTCCACGGCACAG R:GGTTCACGCCATCACAA	249	qRT-PCR	58.5
<i>MMP9</i>	F:CCCATTAGCACGCACGAC R:AGCCCACATAGTCCACCTGA	115	qRT-PCR	59
P1-F	GGGGTACCGCAAACCCAATCCTTCCCG	419(-299/+120 bp)	Plasmid construction	59.7
P2-F	GGGGTACCGTCAAGGCAGACTTCAGG	655(-537 /+120 bp)		58.2
P3-F	GGGGTACCGACGCCGCAATACTCCAC	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	CCGCTCGAGAGGCTGGTTCGTCTTCT		Plasmid construction	
Sp1-1	F:CAGCCAGCTATGGGAACCGGTAAAGTTCAAAAAG R:CTTTTGAACTTTACCCGGTTCATAGCTGGCTG		Site-directed mutagenesis	76.1
Sp1-2	F: GAATGGTAGGACCCAAAGCTATGGGAAAAG R: CTTTCCCATAGCTGGGGGGTCTACCATTC		Site-directed mutagenesis	73.8
Sp1-3	F:CTACAATAAGTAGCCCGAAGCCTGGGACATAGCAG R:CTGCTATGTCCAGGCTTCGGGCTACTTATTGTAG		Site-directed mutagenesis	76.4
<i>MMP9</i> -1863	AlleleFAM: AGTACCTATAAGACAGCTCACAGG AlleleHEX:CAGTACCTATAAGACAGCTCACAGA Common:CAAGGGCTATAGATAATATTCTGAGCCAT		Genotyping	
MUT-1	F:GAGGTACAATTAATCTCCAGTTCGAAGGTCAATT R:GAGATTAATTGTACCTCAAAACAATCCAGGTGA		Site-directed mutagenesis	
MUT-2	F:GCTCACAGaATATGGCTCAGAATATTATCTATAGCCC R:AGCCATATCTGTGAGCTGTCTTATAGGTACTGAAAC		Site-directed mutagenesis	

90
 91 The following PCR system was employed (25 μ L): cDNA template 2 μ L, 10 μ M primers,
 92 12.5 μ L 2 \times Taq MasterMix, 8.5 μ L ddH₂O. The PCR procedure included at 95 °C for 30 s and 35
 93 cycles of at 95 °C for 5 s, 59°C for 30 s and 60 °C for 20 s. Finally, the dissolution curve was
 94 obtained and analyzed. Three independent repeated experiments were carried out on each
 95 sample. The housekeeping gene was used *GAPDH*. The relative gene expression of *MMP9* was
 96 calculated by the 2^{- $\Delta\Delta$ Ct} method.

98 Construction of *MMP9* promoter luciferase plasmids

99 Based on the goat *MMP9* gene sequence in GenBank (accession number: NC_030820.1), a
 100 3005 bp fragment was amplified from goat blood DNA by PCR. The PCR amplification system
 101 was as follows (25 μ L): 1.0 μ L of genomic DNA, 1.0 μ L primers (10 μ mol/L), 12.5 μ L 2 \times Easy

102 Taq PCR Super Mix (+dye), 9.5 μ L sterile deionized water (dd H₂O). The PCR reaction
103 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
104 cycles; 72 °C for 10 min. Primers P1–P7 of different lengths were designed using Primer 5.0,
105 and both ends of each primer pair included cutting sites and protective bases for the restriction
106 endonucleases Kpn I and Xho I (Table 1). Then, the amplification product was subcloned into
107 the pGL3-basic. Thus, luciferase reporter gene vectors including different lengths of the dairy
108 goat *MMP9* promoter were obtained.

109

110 Cell culture, transient transfection, and luciferase activity assays

111 293T cells were inoculated into a 24-well plate at 2×10^4 cells per well and then cultured in
112 medium without penicillin-streptomycin solution for 24 h. When cells reached 70-80%, the
113 luciferase reporter gene vector was transfected into the cells. Transfection procedures were
114 carried out strictly according to the instructions for Lipofectamine 2000 (Invitrogen, USA). A
115 total of 0.8 μ g of the recombinant vector containing a promoter fragment or the pGL3-basic
116 negative control plasmid, along with 0.03 μ g of the pRL-TK internal reference plasmid, was
117 transfected into each well. After 4-6 hours of transfection, the medium was changed, and cells
118 were collected after 48 hours for luciferase activity assay.

119

120 Bioinformatic analysis of the promoter region fragments

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

124

125

126 Genotyping of SNPs in the *MMP9* promoter region

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

133 genotypes were obtained for different fluorescence signals.

134

135 **Site-directed mutagenesis of the *MMP9* promoter**

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

142

143 **Statistical analysis**

144 Calculation of routine population genetic parameters were used POPGENE (version 1.32),
145 such as observed heterozygosity (H_o), expected heterozygosity (H_e), the effective number of
146 alleles (N_e), and the Hardy-Weinberg equilibrium (HWE) chi-squared value.

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

$$148 Y_{ijk} = \mu + P_i + G_j + e_{ijk}$$

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

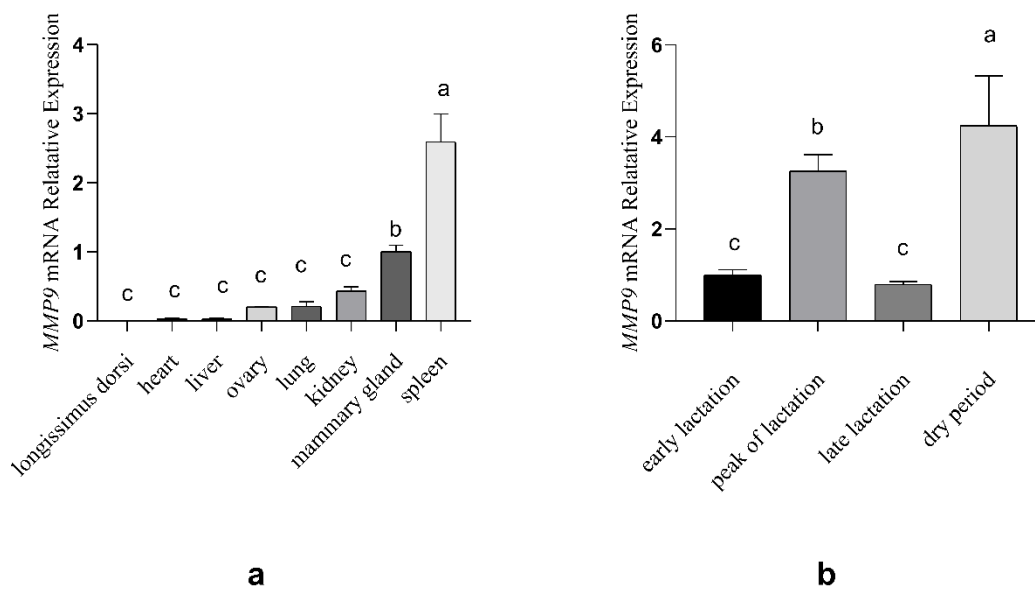
151 All qRT-PCR and promoter luciferase relative activity results were obtained from three
152 biological replicates. Differential analysis of qRT-PCR and promoter luciferase relative activity
153 using one-way ANOVA, and $P < 0.05$ was considered to be statistically significant.

154

155 **RESULTS**

156 ***MMP9* gene expression pattern analysis**

157 In this study, qRT-PCR was used to evaluate *MMP9* expression in different tissues and
158 mammary gland tissues of Laoshan dairy goats at different lactation stages. The *MMP9*
159 expression was highest in the spleen and was extremely low in the longissimus muscle (Figure
160 1A). A comparison of the different developmental stages of mammary tissues in dairy goats
161 revealed that the expression of *MMP9* was highest during the dry period and lowest in late
162 lactation (Figure 1B).



163

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

168

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

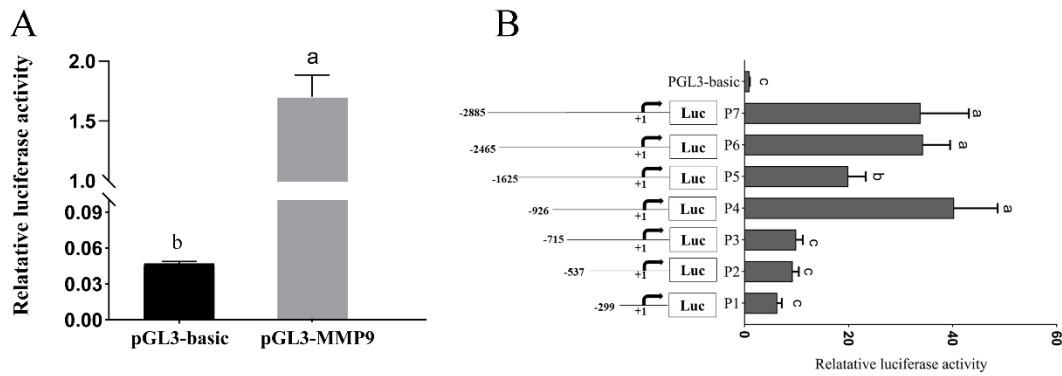
170 A sequence containing 2885 bp upstream and 120 bp downstream of the TSS was obtained
 171 through cloning and sequencing in the dairy goat *MMP9* promoter region (3005 bp). The dual
 172 luciferase activity results showed significantly increased activity for the pGL3-*MMP9* fragment
 173 compared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

175

176 Identification of the *MMP9* core promoter region

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

182

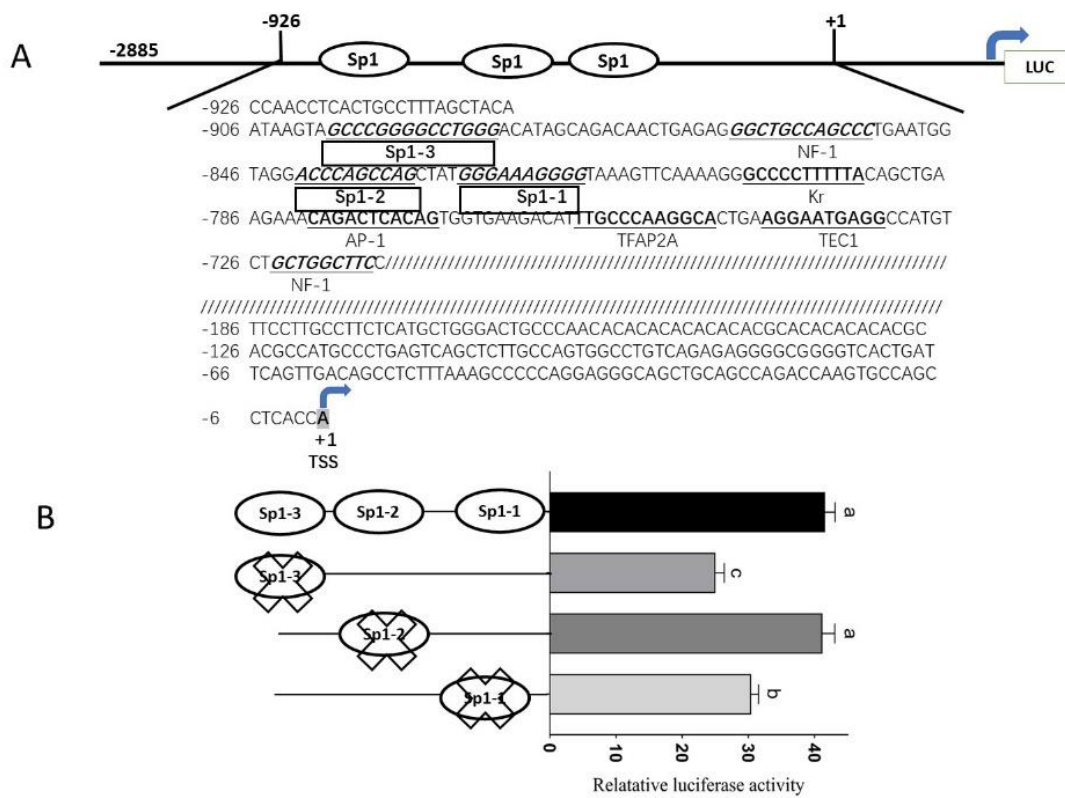


183 **Figure 2.** Activity analysis of the *MMP9* promoter. (A). Relative luciferase activity of the full-
 184 length *MMP9* promoter. (B). Relative luciferase activity of *MMP9* promoters of different
 185 lengths (mean \pm SEM). Constructs carrying serial *MMP9* promoter deletions (P1-P7) were used
 186 to transiently transfect 293T cells, which were incubated for 48 h for the luciferase assays.
 187 Different letters represent the significant differences for mean comparisons ($P < 0.05$, $n = 3$).
 188

189

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

191 Using JASPAR, TFBIND, and AliBaba2.1, we analyzed the active region of the *MMP9*
 192 promoter (-926 bp/-716 bp) and identified three specificity protein 1 (*Sp1*) TFBSs (Figure 3A).
 193 To determine whether the *Sp1* TFBSs exerted a regulatory effect on *MMP9* gene transcription,
 194 we introduced targeted mutations of the *Sp1*-1, *Sp1*-2, and *Sp1*-3 sites in the *MMP9* gene
 195 promoter region (Figure 3B). The deletion of the *Sp1*-1 and *Sp1*-3 sites resulted in significant
 196 downregulation of *MMP9* expression ($P < 0.05$). These findings show that the *Sp1*-1 and *Sp1*-
 197 3 loci may have a regulatory effect on *MMP9* transcription, and *Sp1*-3 is more stronger
 198 regulated than *Sp1*-1.



199

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

205

206 ***MMP9* promoter polymorphism analysis**

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

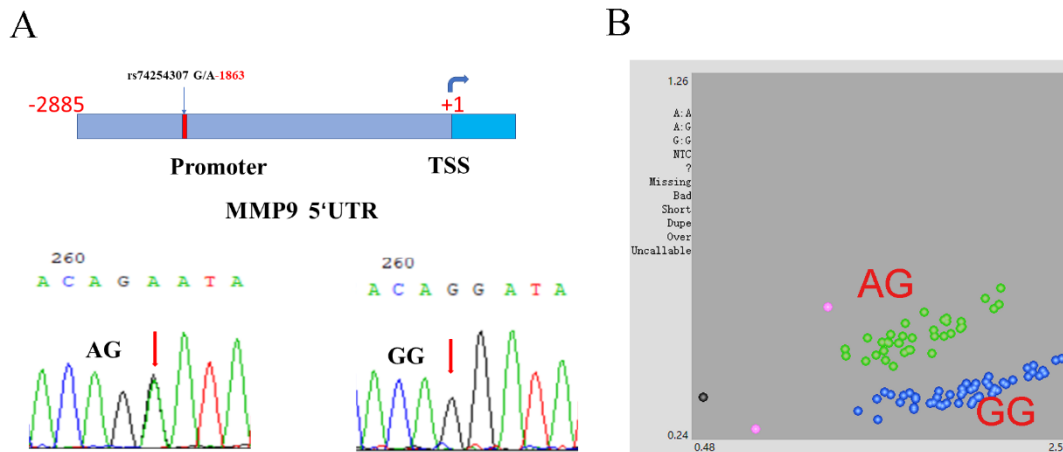
213

214

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						

215



216

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

219

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

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

226 The associations of the c.-1863 G>A genotypes in the *MMP9* promoter region with lactation
 227 phenotypes were analyzed in 254 Laoshan dairy goats with complete milk yield records (Table
 228 4). The c.-1863 G>A polymorphism had a highly significant effect on milk fat percentage ($P <$
 229 0.01), where the AG genotype ($4.71 \pm 0.02\%$) was associated with a significantly higher milk
 230 fat rate than the GG genotype ($4.61 \pm 0.05\%$). The c.-1863 G>A polymorphism had no
 231 significant effect on milk yield or other milk traits ($P > 0.05$).

232

233 **Table 3.** Factorial effects of fecundity and c.1863A>G loci on milk traits in Laoshan dairy
 234 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

235
 236 **Table 4.** Association analysis of **a** SNPs of *MMP9* promoter region with **milk** traits of Laoshan
 237 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.1863 G> 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

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

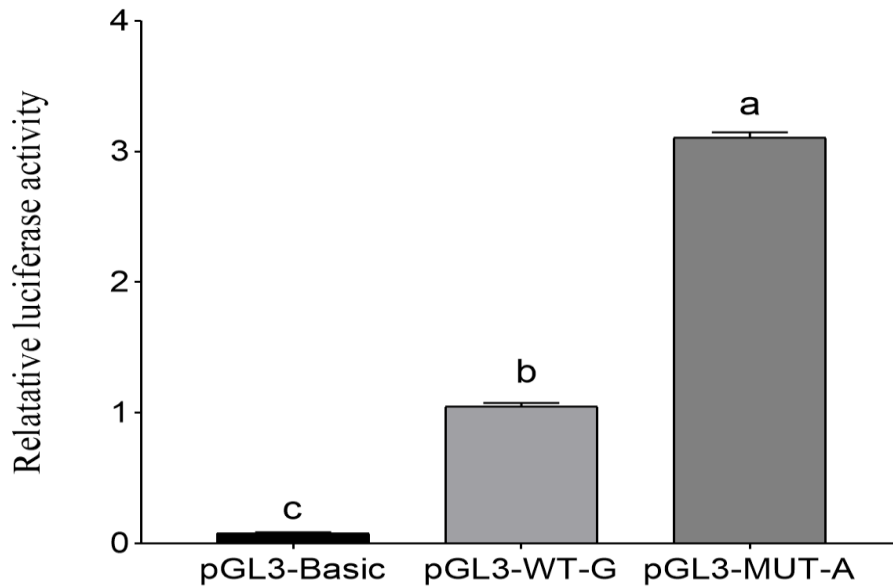
239

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

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

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

247



248
 249 **Figure 5.** Dual luciferase activity analysis of the *SRF* binding site at c.-1863 G>A (Mean±SEM).
 250 The different letters denote significant differences for mean comparisons ($P < 0.05$, $n = 3$).
 251

252 DISCUSSION

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

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

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

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

291 SNPs in a promoter region can influence gene regulation by changing the affinity of the
292 sequence for transcription factors (Wray et al., 2003; Savinkova et al., 2013). In humans, c.-
293 1562C>T, a SNP in the *MMP9* gene, can increase *MMP9* expression by preventing nuclear
294 repressors from binding to this region of the promoter (Song et al., 2018).

295 In this study, we used biological software to analyze the c.-1863 G>A locus and found that c.-
296 1863 G>A mutation created a new TFBS for the *SRF*. Studies have reported that *SRF* can
297 regulate the differentiation, proliferation, and migration of cells (Black and Olson, 1998; Wang

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

301

302 CONCLUSIONS

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

313

314 ACKNOWLEDGEMENTS

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317

318 REFERENCES

- 319 1. Bai, Y., Zhang, J. B., Xue, Y., Peng, Y. L., Chen, G., and Fang, M. Y. 2015. Differential
320 expression of *CYB5A* in Chinese and European pig breeds due to genetic variations in the
321 promoter region. *Anim Genet.*, 46:16–22.
- 322 2. Black, B. L., and Olson, E. N. 1998. Transcriptional control of muscle development by
323 myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol.*, 14: 167–196.
- 324 3. Briggs, M. R., Kadonaga, J. T., Bell, S. P., and Tjian, R. 1986. Purification and biochemical
325 characterization of the promoter-specific transcription factor, *Sp1*. *Science*, 234: 47–52.
- 326 4. Buchmann, B., Engelbrecht, L. K., Fernandez, P., Hutterer, F. P., Raich, M. K., Scheel, C.
327 H., and Bausch, A. R. 2021. Mechanical plasticity of collagen directs branch elongation in

- 328 human mammary gland organoids. *Nat Commun.*, 12: 2759.
- 329 5. China National Commission of Animal Genetic Resources. 2011. *Animal Genetic*
330 *Resources in China: Sheep and Goats (In Chinese)*. Beijing: *China Agriculture Press.*, 423-425.
- 331 6. Chen, D., Zhao, X., Li, X., Wang, J., and Wang, C. 2018. Milk compositional changes of
332 Laoshan goat milk from partum up to 261 days postpartum. *Anim Sci J.*, 89: 1355–1363.
- 333 7. Fata, J. E., Leco, K. J., Moorehead, R. A., Martin, D. C., and Khokha, R. 1999. Timp-1 is
334 important for epithelial proliferation and branching morphogenesis during mouse mammary
335 development. *Dev Biol.*, 211: 238–254.
- 336 8. Fata, J. E., Werb, Z., and Bissell, M. J. 2003. Regulation of mammary gland branching
337 morphogenesis by the extracellular matrix and its remodeling enzymes. *Breast Cancer Res.*, 6:
338 1–11.
- 339 9. Gifre-Renom, L., Carratalá, J. V., Parés, S., Sánchez-García, L., Ferrer-Miralles, N.,
340 Villaverde, A., Bach, A., Garcia-Fruitós, E., and Arís, A. 2020. Potential of MMP-9 based
341 nanoparticles at optimizing the cow dry period: pulling apart the effects of MMP-9 and
342 nanoparticles. *Sci Rep.*, 10: 11299.
- 343 10. Ji, Z., He, R., Chao, T., Xuan, R., Liu, S., Wang, G., and Wang, J. 2019. chi-miR-143-
344 3p promotes apoptosis of mammary gland epithelial cells from dairy goats by
345 targeting *Ndfip1*. *DNA Cell Biol.*, 38: 1188–1196.
- 346 11. He, C., Holme, J., and Anthony, J. 2014. SNP genotyping: the KASP assay. *Methods*
347 *Mol Biol.* 1145:75-86.
- 348 12. Kou, M., Guo, D., Liu, L., Gao, X., Xing, G., Zha, A., Shao, B., Sun, Y., Yang, X.,
349 Wang, X., Di, S., Cai, J., and Niu, B. 2020. Expression pattern and association analysis of
350 porcine matrix metalloproteinase 9 (*MMP9*) with diarrhea and performance traits in piglets. *Res*
351 *Ver Sci.*, 129:53-58.
- 352 13. Li, H., Zheng, H., Li, L., Shen, X., Zang, W., and Sun, Y. 2016. The effects of matrix
353 metalloproteinase-9 on dairy goat mastitis and cell survival of goat mammary epithelial
354 cells. *PloS one*, 11: e0160989.
- 355 14. Liu, S. 2020. Role of miR-143 on dairy goat mammary gland epithelial cells by
356 targeting *MMP9* and *LOC108635657*. Master's thesis, Shandong Agricultural University, Tai'an,

357 China.

358 15. Loganathan, R., Little, C. D., and Rongish, B. J. 2020. Extracellular matrix dynamics
359 in tubulogenesis. *Cell Signal.*, 72: 109619.

360 16. Oftedal O. T. 2002. The mammary gland and its origin during synapsid evolution. *J*
361 *Mammary Gland Biol Neoplasia.*, 7: 225–252.

362 17. Panwar, P., Butler, G. S., Jamroz, A., Azizi, P., Overall, C. M., and Brömme, D. 2018.
363 Aging-associated modifications of collagen affect its degradation by matrix
364 metalloproteinases. *Matrix Biol.*, 65: 30–44.

365 18. Rabot, A., Sinowatz, F., Berisha, B., Meyer, H. H., and Schams, D. 2007. Expression
366 and localization of extracellular matrix-degrading proteinases and their inhibitors in the bovine
367 mammary gland during development, function, and involution. *J Dairy Sci.*, 90: 740–748.

368 19. Ro S. 2016. Multi-phenotypic Role of Serum Response Factor in the Gastrointestinal
369 System. *J Neurogastroenterol Motil.*, 22: 193–200.

370 20. Savinkova, L., Drachkova, I., Arshinova, T., Ponomarenko, P., Ponomarenko, M., and
371 Kolchanov, N. 2013. An experimental verification of the predicted effects of promoter TATA-
372 box polymorphisms associated with human diseases on interactions between the TATA boxes
373 and TATA-binding protein. *PloS one*, 8: e54626.

374 21. Song, C., Tan, P., Zhang, Z., Wu, W., Dong, Y., Zhao, L., Liu, H., Guan, H., and Li, F.
375 2018. REV-ERB agonism suppresses osteoclastogenesis and prevents ovariectomy-induced
376 bone loss partially via *FABP4* upregulation. *FASEB J.*, 32: 3215–3228.

377 22. Sternlicht, M. D., Kouros-Mehr, H., Lu, P., and Werb, Z. 2006. Hormonal and local
378 control of mammary branching morphogenesis. *Differentiation*, 74: 365–381.

379 23. Takagi, M., Yamamoto, D., Ohtani, M., and Miyamoto, A. 2007. Quantitative analysis
380 of messenger RNA expression of matrix metalloproteinases (*MMP-2* and *MMP-9*), tissue
381 inhibitor-2 of matrix metalloproteinases (*TIMP-2*), and steroidogenic enzymes in bovine
382 placentomes during gestation and postpartum. *Mol Reprod Dev.* 74:801–807.

383 24. Taylor, A., and Halene, S. 2015. The regulatory role of serum response factor pathway
384 in neutrophil inflammatory response. *Curr Opin Hematol.*, 22: 67–73.

385 25. van Turnhout, M. C., Schipper, H., Engel, B., Buist, W., Kranenbarg, S., and van

- 386 Leeuwen, J. L. 2010. Postnatal development of collagen structure in ovine articular
387 cartilage. *BMC Dev Biol.*, 10: 62.
- 388 26. Vellingiri, B., Iyer, M., Devi Subramaniam, M., Jayaramayya, K., Siama, Z.,
389 Giridharan, B., Narayanasamy, A., Abdal Dayem, A., and Cho, S. G. 2020. Understanding the
390 Role of the Transcription Factor *Spl* in Ovarian Cancer: from Theory to Practice. *Int J Mol*
391 *Sci.*, 21: 1153.
- 392 27. Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg,
393 P. A., and Olson, E. N. 2001. Activation of cardiac gene expression by myocardin, a
394 transcriptional cofactor for serum response factor. *Cell*, 105: 851–862.
- 395 28. Wray, G. A., Hahn, M. W., Abouheif, E., Balhoff, J. P., Pizer, M., Rockman, M. V., and
396 Romano, L. A. 2003. The evolution of transcriptional regulation in eukaryotes. *Mol Biol*
397 *Evol.*, 20: 1377–1419.
- 398 29. Yu, S., Liao, J., Tang, M., Wang, Y., Wei, X., Mao, L., Zeng, C., and Wang, G. 2017.
399 A functional single nucleotide polymorphism in the tyrosinase gene promoter affects skin color
400 and transcription activity in the black-boned chicken. *Poult Sci.*, 96: 4061–4067.
- 401 30. Zhu, G., and Jiang, Y. 2014. Polymorphism, genetic effect and association with egg
402 production traits of chicken matrix metalloproteinases 9 promoter. *Asian-Australas J Anim*
403 *Sci.*, 27: 1526–1531.