Negative regulation of α_{S1} -casein (*CSN1S1*) improves β -casein content and reduces allergy potential in goat milk

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ABSTRACT

Compared with other milk proteins, α_{S1} -casein (encoded by *CSN1S1*) is associated with higher rates of allergies in humans. Although some studies have revealed that *CSN1S1* affects a variety of cellular physiological processes such as immune response and proliferation in various cells, whether *CSN1S1* regulates other milk proteins in ruminants is not known. In this study, we observed a negative Pearson correlation between the contents of α_{S1} -casein and β -casein in goat milk. Thus, we used isolated primary goat mammary epithelial cells along with adenoviral infection or small interference RNA to alter abundance of *CSN1S1* and examine its role in milk protein synthesis regulation. Overexpressing *CSN1S1* through adenoviral transfection decreased β -casein mRNA (*CSN2*) and protein abundance, whereas interference of *CSN1S1* resulted in a significant increase in β -casein abundance. *CSN1S1* reduced phosphorylation level of Janus kinase 2 (JAK2) and signal transducer and activator of transcription 5a (STAT5a). The transcription factor STAT5a activates *CSN2* transcription by binding with its promoter region and then promotes β -casein synthesis. Furthermore, *CSN1S1* inhibited *CSN2* promoter activity and β -casein synthesis by modulating the JAK2/STAT5a signaling pathway. No changes in abundance were detected for α_{S2} -casein (*CSN1S2*), κ -casein (*CSN3*) and major whey proteins (*LALBA*, *BLG*). Overall, our results underscored a mechanism whereby *CSN1S1* inhibits β -casein synthesis via inhibition of STAT5a. The data suggested that knocking down *CSN1S1* not only reduced the content of α_{S1} -casein, but also increased the abundance of major milk proteins including β -casein. Thus, the present study provides a theoretical basis for manipulating α_{S1} -

casein mRNA to improve quality of ruminant milk for human consumption.

Key words: α_{S1} -casein, β -casein, STAT5a, goat mammary epithelial cells

INTRODUCTION

Milk protein consists mainly of 4 caseins (α_{S1} -casein, α_{S2} -casein, β -casein, and κ -casein) and whey proteins, all of which provide essential amino acids for humans. Despite those benefits, the prevalence of milk protein allergy worldwide can be as high as 2 to 7.5% (Høst, 2002; Fiocchi et al., 2010). Approximately 65% of patients with milk protein allergy are susceptible to α_{S1} -casein (Wal, 2001; Schulmeister et al., 2009). This casein iso-type contains antigen epitopes that can be recognized by human IgE causing allergic rhinitis, diarrhea, and asthma symptoms, among others (Martorell-Aragonés et al., 2015; Hochwallner et al., 2016). Clinical studies have shown that the antigen epitopes of α_{S1} -casein in cow milk and goat milk are similar (Vila et al., 2001; Lisson et al., 2014). Compared with cows, goat milk contains a very low content of α_{S1} -casein, which is the main reason it is not prone to elicit allergic reactions (Ceballos et al., 2009; Ballabio et al., 2011).

To solve the problem of milk allergy caused by α_{S1} -casein in ruminant milk, it is necessary to understand functions of the α_{S1} -casein gene (*CSN1S1*). Compared with other milk proteins, α_{S1} -casein is most likely to induce specific T-cell reactions in human peripheral blood mononuclear cells (Archila et al., 2017). α_{S1} -Casein and its derived bioactive peptides can promote the release of cytokines, regulate the immune response and inhibit proliferation of leukemia cells (Ledesma-Martinez et al., 2019). Although previous studies have shown *CSN1S1* plays crucial roles in the immune response and proliferation in various cell types, whether *CSN1S1* can regulate the synthesis of other milk proteins in ruminants remains unclear.

The synthesis of milk proteins is regulated by various signaling pathways among which the Janus kinase 2

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(**JAK2**) signal transducer and activator of transcription 5a (**STAT5a**) and mammalian target of rapamycin (**mTOR**) signaling play key roles (Bionaz and Loor, 2011; Castro et al., 2016). Our hypothesis was that *CSN1S1* gene regulates the synthesis of other milk proteins in goat mammary epithelial cells (**GMEC**). Thus, the objective was to determine regulatory mechanisms whereby *CSN1S1* on the synthesis of other milk proteins in GMEC could alter abundance of other milk proteins associated with milk allergies in humans.

MATERIALS AND METHODS

Ethics Statement

All experimental procedures were carried out in accordance with the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Northwest A&F University, Yang Ling, China (protocol number 15–516).

α_{S1} -Casein and β -Casein Abundance in Goat Milk

Milk samples from Xinong Saanen dairy goats ($n = 40$) were collected at peak lactation (60 d after parturition). Content of α_{S1} -casein and β -casein in goat milk were determined by ELISA assay of sandwich technique (mlbio, Shanghai, China). Total protein content in goat milk was determined via milk component analyzer (Foss FT 120, Hillerød, Denmark).

Animals, Mammary Tissue Collection, and Cell Culture

Mammary tissue samples were collected from 5 healthy Xinong Saanen dairy goats (average of about 57 kg) during early, peak, mid, and end lactation (15, 60, 120, and 270 d after parturition, respectively), and the dry period (60 d before parturition; Yao et al., 2016; Chen et al., 2016). A total of 25 mammary tissue samples were frozen in liquid nitrogen immediately after washing with diethylpyrocarbonate-treated PBS (Zhang et al., 2018a).

Cell culture methods were according to previous studies from our group (Shi et al., 2014; Zhu et al., 2015). Briefly, the GMEC were obtained from mammary gland biopsies of 3 Xinong Saanen dairy goats at peak of lactation. Mammary tissue was trimmed of visible fat and connective tissue and washed with D-Hank's solution. Tissue sections were then minced into about 1-mm³ cubes, plated in 60-mm culture dishes, and cultured in 5% CO₂ at 37°C until epithelial cells separated from the tissue block. Culture medium was changed 2 d after initial incubations. Cells were subsequently digested

from the tissue block with 0.25% trypsin-EDTA solution. The GMEC from each goat were purified using a differential adhesion method to remove fibroblasts, which adhered to culture dishes faster than GMEC (i.e., 30–40 min of culture). The suspended GMEC and culture medium were removed and transferred to a new culture dish. This process was performed for 5 passages to purify the GMEC used in subsequent experiments. The GMEC from each goat were cultured at 37°C in a humidified atmosphere with 5% CO₂. The culture medium contained basal DMEM/F12 medium (Hyclone, Logan, UT), 1 μ g/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), 5 μ g/mL insulin, 100 U/mL penicillin, and 100 mg/mL streptomycin (Harbin Pharmaceutical Group, Harbin, China), 5 mM sodium acetate (Sigma-Aldrich), 10 ng/mL epidermal growth factor (**EGF**, Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (Gibco, Gaithersburg, MD). Lactogenic medium (Kadegowda et al., 2009a) consisted of the culture medium together with 1 mg/mL BSA and 2 μ g/mL prolactin (Sigma-Aldrich).

Cloning of Goat *CSN1S1*, *STAT5a*, and *CSN2*

Primers were designed based on the goat *CSN1S1* (GenBank no. XM_018049127.1), *STAT5a* (GenBank no. XM_018065112.1), and *CSN2* promoter (GenBank no. NC_030813.1); primer sequences for amplification are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2020-18595>). PCR reactions for *CSN1S1* and *STAT5a* were performed using samples from the 5 Xinong Saanen dairy goats after cDNA synthesis. The sequence of the *CSN2* promoter was amplified from goat genomic DNA, which was extracted from blood samples of the same 5 goats using a genomic DNA extraction kit (Tiangen, Beijing, China). The DNA fragments were cloned into pMD-19T plasmid vectors (Takara, Shiga, Japan) for sequencing and the positive plasmids were conserved.

Vector Construction

Generation and proliferation of the adenovirus were performed as previously described by our group (Shi et al., 2017). Briefly, the coding sequence of goat *CSN1S1* was subcloned into the pAdTrack-CMV shuttle vector between the *XhoI* and *HindIII* (Takara) restriction enzyme sites to generate pAdTrack-CMV-*CSN1S1* vector. Subsequently, this vector was linearized by *PmeI* (New England BioLabs, Ipswich, MA) and inserted into an adenoviral vector pAdEasy-1 to generate adenoviral plasmids in BJ5183 bacterial cells. The adenoviral plasmid linearized by *PacI* (New England BioLabs) was transfected into HEK293A cells to generate the

adenovirus Ad-CSN1S1. After 4 generations of packaging, high titer Ad-CSN1S1 was obtained (Supplemental Figure S1, <https://doi.org/10.3168/jds.2020-18595>). The recombinant adenovirus with green fluorescent protein (**Ad-GFP**) was used as a negative control.

Primers containing *Hind*III and *Not*I restriction enzyme sites were designed (Supplemental Table S1, <https://doi.org/10.3168/jds.2020-18595>) using pMD-19T-STAT5a plasmid as template for PCR. *STAT5a* fragments containing enzyme sites and pcDNA3.1 plasmid, after cutting with *Hind*III and *Not*I enzymes (Takara), were linked to construct pcDNA3.1-STAT5a vector via T4 DNA Ligase (Takara). Similarly, we constructed the pGL3-Basic-CSN2 vector with the *CSN2* promoter fragment and a pGL3-Basic plasmid.

Preparation of Inhibitors

The specific JAK2 inhibitor AG490 (CAS 133550–30–8, Sigma-Aldrich) and STAT5 inhibitor STAT5-IN-1 (CAS 285986–31–4, MedChemExpress, Monmouth Junction, NJ) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to produce 50 mM stock solutions. Before applications in cell treatments, stock solutions were diluted to working concentrations.

RNA Interference

To knock down expression of *CSN1S1*, 3 small interference RNA (**siRNA**, siCSN1S1-47, siCSN1S1-195, siCSN1S1-507) targeting *CSN1S1* were designed using online software (<http://sirna.wi.mit.edu/home.php>), and synthesized by GenePharma (Shanghai, China). The 3 siRNA and negative control siRNA (**siNC**) sequences are listed in Supplemental Table S2 (<https://doi.org/10.3168/jds.2020-18595>). For *CSN1S1* mRNA interference, cells cultured in 6-well plates at a density of 5×10^5 cells per well were transfected with 100 nM siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA). Transfected GMEC were harvested at 48 h for RNA extraction and subsequent assays. For JAK2 inhibition assays, cells were treated with DMSO or AG490 (30 μ M) followed by siNC or siCSN1S1 transfection, respectively. After 48 h of treatment in lactogenic medium, cells were collected for protein extraction and Western blot analysis.

DNA Transfection

For Western blot analysis, GMEC were transfected with pcDNA3.1-NC or pcDNA3.1-STAT5a plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were subsequently infected with Ad-

GFP or Ad-CSN1S1 (multiplicity of infection of 200), respectively. After 48 h of treatment, cells were collected for protein extraction and Western blot analysis. For promoter luciferase assays, cells were seeded in 48-well plates at a density of 5×10^4 cells per well and pGL3-Basic-CSN2 vector was transfected along with Renilla vector (pRL-TK) as a control at a ratio of 50:1 using X-treme GENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). For STAT5a overexpression assays, cells were co-transfected with both pGL3-Basic-CSN2 vector and pcDNA3.1-STAT5a (or pcDNA3.1-NC) at a ratio of 1:1, then cells were subsequently infected with Ad-GFP or Ad-CSN1S1 (multiplicity of infection of 200) for 48 h, respectively. For STAT5 inhibition assays, cells were incubated with DMSO or STAT5-IN-1 (200 μ M) followed by both pGL3-Basic-CSN2 and siCSN1S1 (or siNC, 100 nM) co-transfection for 48 h, respectively.

RNA Extraction and Quantitative Real-Time PCR

The GMEC from each of the 3 goats were seeded in 12-well plates at a density of 2×10^5 cells per well until 80% confluence before applying treatments. Total RNA from each sample was extracted using RNAiso Plus (9109, Takara). The RNA quality was detected with a NanoDrop2000 spectrophotometer (Thermo Scientific), and the optical density 260/280 ratio was 1.9–2.1:1. The RNA integrity was examined by agarose gel electrophoresis analysis of 28S and 18S rRNA, and 28S/18S ratio was ~2:1. First-strand cDNA from each sample was synthesized using 1 μ g of purified total RNA by the PrimeScript RT kit with a gDNA Eraser (RR047A, Perfect Real Time, Takara). Quantitative real-time PCR (**qRT-PCR**) was performed according to manufacturer's instructions using SYBR green (RR820A, TB Green II, Perfect Real Time; Takara). Ubiquitously expressed transcript (*UXT*) and ribosomal protein S9 (*RPS9*) were used as internal control genes (Kadegowda et al., 2009b, Zhao et al., 2014). All qRT-PCR reactions were performed in a Bio-Rad CFX96 sequence detector (Bio-Rad, Hercules, CA), and data were normalized to internal controls. Data were analyzed using the relative quantification ($2^{-\Delta\Delta C_t}$) method. Primers for qRT-PCR are shown in Table 1.

Western Blot

The GMEC from each of the 3 goats were grown in 6-well plates at a density of 5×10^5 cells per well until 80% confluence before applying treatments. Cellular protein was harvested after cells were lysed with ice-cold RIPA buffer (R0010, Solarbio, Beijing,

China) containing protease and phosphatase inhibitor cocktail tablets (04693132001, Roche, Mannheim, Germany). Protein concentration was measured using BCA protein assay kit (23227, Thermo Fisher, Rockford, IL). The protein was separated with SDS-PAGE and transferred onto a PVDF membrane (Roche, Mannheim, Germany) by a Bio-Rad Trans-Blot SD semidry transfer cell, and membranes blocked for 2 h using 5% skim milk (232100, BD, Franklin Lakes, NJ). Membranes were incubated overnight at 4°C with primary antibodies. After membranes were washed with TBST 3 times, incubated with secondary antibodies. Secondary antibody horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (CW0103, CW Biotech; Beijing, China; 1:5,000) was used for primary antibodies α_{S1} -casein (SAB1401093, Sigma-Aldrich; 1:1,000), α_{S2} -casein (bs-10034R, Bioss, Beijing, China; 1:500), β -casein (orb2053, Biorbyt, Cambridgeshire, UK; 1:500), κ -casein (GTX60145, GeneTex, Irvine, CA; 1:500), JAK2 (3230, Cell Signaling Technology, Danvers, MA; 1:1,000), p-JAK2 Tyr1007/1008 (ab32101, Abcam, Cambridge, UK; 1:1,000), mTOR(2972, Cell Signaling Technology; 1:1,000) and p-mTOR Ser2448 (2971; Cell Signaling Technology; 1:1,000). Secondary antibody HRP-conjugated goat anti-mouse IgG (CW0102, CW Biotech; 1:5,000) was used for primary antibodies STAT5a (610191, BD; 1:1000), p-STAT5a Tyr694 (611964, BD; 1:500), and β -actin (CW0096, CW Biotech; 1:2000). Signals were measured using an enhanced chemiluminescent (ECL) Western blot system (1705061, Bio-Rad). The intensity of indicated bands was quantified by densitometry using ImageJ software (<http://imagej.nih.gov/ij/>). Relative protein abundance of α_{S1} -casein, α_{S2} -casein, β -casein, κ -casein

and STAT5a was normalized to β -actin. Relative protein abundance of p-JAK2 Tyr1007/1008 and p-mTOR Ser2448 was normalized to total kinase. Relative protein abundance of p-STAT5a Tyr694 was normalized to β -actin or total kinase.

Luciferase Assays

After 48 h incubation, cells were washed twice with PBS and lysed with Promega passive lysis buffer to measure luciferase activity. Reporter activity was measured using the Dual-Luciferase Reporter assay kit (Promega, Madison, WI) on a Fluoroskan Ascent apparatus (Thermo Scientific). The relative luciferase activity was calculated as the ratio of firefly compared with renilla luciferase activity.

Statistical Analysis

All experiments were conducted with each goat as a biological replicate ($n = 3$). Statistical analyses were performed with SPSS 20.0 (SPSS, Chicago, IL). The association between α_{S1} -casein and β -casein content in goat milk was analyzed by Pearson correlation analysis after testing for normal distribution with the Shapiro-Wilk test. The data were analyzed by Student's *t*-test (2 tailed) when only 2 groups were compared. For multiple comparisons, data were analyzed by one-way ANOVA (ANOVA) using the general linear model procedure, and mean separation was performed via Tukey test. Values are presented as means $\pm$ standard error of means. *P*-values < 0.05 were considered statistically significant (* $P < 0.05$, ** $P < 0.01$).

Table 1. Primers used for amplification of gene coding sequence, promoter or 3' untranslated region (<https://www.ncbi.nlm.nih.gov>)

NCBI accession ID	Gene	Primer sequence (5'-3') ¹	Size (bp)
XM_018049127.1	<i>CSN1S1</i>	F, TCCACTAGGCACACAATACACTGA R, GCCAATGGGATTAGGGATGTC	61
NM_001285585.1	<i>CSN1S2</i>	F, CTGGTTATGGTTGGACTGGAAAA R, AACATGCTGGTTGTATGAAGTAAAGTG	76
XM_005681721.2	<i>CSN2</i>	F, CCCAGGCACAGTCTCTAGTCT R, GGCTCAACTGGATATTTAGGGA	196
NM_001285587.1	<i>CSN3</i>	F, AGGTGCAATGATGAAGAGTTTTTC R, CCCAAAAATGGCAGGGTTAA	66
NM_001285635.1	<i>LALBA</i>	F, TCTCTGCTCCTGGTAGGCAT R, GGCAAACTGACACCTCCGTA	113
XM_018054687.1	<i>BLG</i>	F, CATCGTCACCCAGACCATGA R, CAAGTCCCCGCCACCTTC	54
NM_001037471	<i>UXT</i>	F, CAGCTGGCCAAATACCTTCAA R, GTGTCTGGGACCACTGTGTCAA	125
XM_005709411.1	<i>RPS9</i>	F, CCTCGACCAAGAGCTGAAG R, CCTCCAGACCTCACGTTTGTTTC	64

¹F = forward; R = reverse.

Figure 1. α_{S1} -Casein and β -casein content in goat milk and mammary gland tissue. (A) Content of α_{S1} -casein and β -casein in goat milk ($n = 40$) was determined by ELISA. (B) Total protein content in goat milk was determined with a milk component analyzer. The percentage of casein in total protein is expressed as the ratio of casein content to total protein content in goat milk. (C) α_{S1} -Casein gene (*CSN1S1*) and β -casein gene (*CSN2*) mRNA expression profiles in goat mammary tissue at different stages of lactation. Mammary tissue samples were collected from Xinong Saanen goats ($n = 5$) during early lactation, peak lactation, mid lactation, end of lactation, and dry period. Data were normalized to the end of lactation. Values are presented as means $\pm$ SEM. Different letters represent significant differences ($P < 0.05$) in gene expression.

RESULTS

Association Analysis Between α_{S1} -Casein and β -Casein in Dairy Goats

Results showed that the content of α_{S1} -casein and β -casein in goat milk averaged 0.50 mg/mL and 11.38 mg/mL, respectively (Figure 1A). The percentage of α_{S1} -casein and β -casein in total protein of goat milk averaged 1.55 and 36.73%, respectively (Figure 1B). Pearson correlation analysis revealed a negative correlation of moderate intensity between the concentration of α_{S1} -casein and β -casein in goat milk ($r = -0.508$, $P = 0.001$, $n = 40$).

Compared with end of lactation and the dry period, abundance of mRNA for *CSN1S1* and *CSN2* was higher in early-, peak-, and mid-lactation periods. Abundance of *CSN1S1* was ~1.9-fold higher in peak lactation compared with mid lactation ($P = 0.004$). On the contrary, *CSN2* abundance in mid lactation was ~2.3-fold higher than peak lactation ($P = 0.001$, Figure 1C).

Overexpression of *CSN1S1* Suppresses Expression of β -Casein

To investigate whether *CSN1S1* could regulate the expression of other milk proteins, GMEC were infected with Ad-*CSN1S1* or Ad-GFP for 48 h (Figure 2A). Compared with Ad-GFP control, *CSN1S1* mRNA abun-

dance was upregulated over 500-fold in the Ad-*CSN1S1* group (Figure 2B, $P = 0.0005$). The mRNA abundance of *CSN2* was decreased after *CSN1S1* overexpression ($P = 0.008$). Compared with Ad-GFP control, no significant effect on the abundance of *CSN1S2* and *CSN3* in cells infected with Ad-*CSN1S1* was observed, similarly with whey protein genes *LALBA* and *BLG* (Figure 2C). α_{S1} -Casein expression was also ~1.7-fold higher in the Ad-*CSN1S1* group compared with Ad-GFP group ($P = 0.028$). *CSN1S1* overexpression markedly down-regulated β -casein expression ($P = 0.035$), but had no significant effect on the expression level of α_{S2} -casein and κ -casein (Figure 2D).

Interference of *CSN1S1* Increases β -Casein Synthesis

Compared with siNC control, siRNA for siC-*SN1S1*-47, siC*SN1S1*-195, siC*SN1S1*-507 effectively knocked down endogenous expression of *CSN1S1* by an average of 43%, 50% and 64%, respectively ($P < 0.01$, Figure 3A). For subsequent experiments, we selected siC*SN1S1*-507 for interference of *CSN1S1*. The qRT-PCR results indicated that incubation with siC*SN1S1* increased abundance of *CSN2* ($P = 0.004$) and had no effect on the mRNA abundance of *CSN1S2*, *CSN3*, *LALBA* and *BLG* (Figure 3B). Relative to the siNC control, incubation with siC*SN1S1* clearly reduced α_{S1} -casein expression ($P = 0.033$). Compared with the siNC

control, β -casein expression was enhanced by *CSN1S1* knockdown ($P = 0.023$). Consistent with results of *CSN1S1* overexpression, knockdown of *CSN1S1* had no significant effect on α_{S2} -casein and κ -casein (Figure 3C).

CSN1S1 Downregulates JAK2/STAT5a Signaling Pathway

Compared with the Ad-GFP control, overexpression of *CSN1S1* by adenovirus vector significantly reduced the phosphorylation level of JAK2 and STAT5 ($P = 0.041$ and 0.040 , respectively), but did not affect phos-

phorylation of mTOR (Figure 4A). On the other hand, *CSN1S1* knockdown by siCSN1S1-507 interference also enhanced p-JAK2 at Tyr1007/1008 and p-STAT5a at Tyr694 ($P = 0.025$ and 0.043 , respectively). p-mTOR at Ser2448 did not differ between the siNC and siCSN1S1 group (Figure 4B).

CSN1S1 Represses β -Casein Synthesis via JAK2/STAT5a Signaling Pathway

Overexpression of STAT5a increased both total STAT5a and p-STAT5a at Tyr694, with enhanced β -casein abundance ($P < 0.05$). Furthermore, when

Figure 2. Overexpression of *CSN1S1* suppressed β -casein expression in primary goat mammary epithelial cells (GMEC). (A) Fluorescent images of adenovirus with green fluorescent protein (Ad-GFP) or adenovirus Ad-CSN1S1 [multiplicity of infection (MOI) of 200] infection of GMEC for 48 h. The image on the left panel is in bright field, and the image on the right panel is green fluorescence in the same field. Scale bar is 500 μ m. (B) *CSN1S1* mRNA expression level in GMEC infected with Ad-GFP or Ad-CSN1S1 (MOI of 200) for 48 h. (C) mRNA expression levels of caseins and main whey proteins in GMEC infected with Ad-GFP or Ad-CSN1S1 (MOI of 200) for 48 h. (D) Protein abundance of caseins in GMEC infected with Ad-GFP or Ad-CSN1S1 (MOI of 200) for 48 h. Relative protein abundance was normalized to β -actin. Values are presented as means $\pm$ SEM from 3 individual cultures ($n = 3$ goats). * $P < 0.05$ and ** $P < 0.01$ compared with control (Ad-GFP).

Figure 3. Interference of *CSN1S1* increases β -casein expression primary goat mammary epithelial cells (GMEC). (A) Interference efficiency detection of small interfering (si)CSN1S1-47, siCSN1S1-195 and siCSN1S1-507 (100 nM) targeting *CSN1S1*. (B) mRNA expression levels of caseins and main whey proteins in GMEC transfected with negative control siRNA (siNC) or siCSN1S1-507 (100 nM) for 48 h. (C) Protein abundance of caseins in GMEC transfected with siNC or siCSN1S1-507 (100 nM) for 48 h. Relative protein abundance was normalized to β -actin. Values are presented as means $\pm$ SEM from 3 individual cultures (n = 3 goats). * $P < 0.05$ and ** $P < 0.01$ compared with control (siNC).

both overexpressing *CSN1S1* and *STAT5a* in GMEC, the repression of β -casein caused by *CSN1S1* was alleviated (Figure 5A).

In DMSO-treated cells, knockdown of *CSN1S1* elevated the intracellular β -casein abundance ($P < 0.05$). However, this effect of *CSN1S1* was completely abrogated in cells deprived of JAK2/STAT5a signaling as a result of AG490 treatment (Figure 5B).

CSN1S1 Reduces Transcription of β -Casein Gene by Modulating STAT5a

Compared with the pGL3-Basic group, after 48 h transfection of pGL3-Basic-CSN2 into GMEC we observed a significant increase of promoter activity ($P = 0.008$, Figure 6A) indicating strong activation of *CSN2*. Compared with the Ad-GFP group, overexpression of

Figure 4. *CSN1S1* downregulates Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5a (STAT5a) signaling pathway in primary goat mammary epithelial cells (GMEC). (A) Phosphorylation (p) rates of JAK2 at Tyr1007/1008, STAT5a at Tyr694, and mammalian target of rapamycin (mTOR) at Ser2448 in GMEC infected with adenovirus with green fluorescent protein (Ad-GFP) or adenovirus Ad-CSN1S1 (multiplicity of infection of 200) for 48 h. (B) Phosphorylation rates of JAK2 at Tyr1007/1008, STAT5a at Tyr694, and mTOR at Ser2448 in GMEC transfected with negative control small interfering RNA (siNC) or siCSN1S1-507 (100 nM) for 48 h. Relative protein abundance was normalized to total kinase. Values are presented as means $\pm$ SEM from 3 individual cultures (n = 3goats). * $P < 0.05$ compared with control (Ad-GFP or siNC).

STAT5a markedly increased *CSN2* promoter activity ($P < 0.01$), whereas Ad-*CSN1S1* decreased *CSN2* promoter activity ($P < 0.05$). By co-transfecting Ad-*CSN1S1* and pcDNA3.1-*STAT5a*, *CSN1S1* inhibited the enhancement of *CSN2* promoter activity induced by *STAT5a* (Figure 6B).

Knockdown of *CSN1S1* markedly enhanced *CSN2* promoter activity ($P = 0.025$), whereas *STAT5-IN-1*

could reduce *CSN2* promoter activity regardless of absence or presence of *CSN1S1* in GMEC (Figure 6C).

DISCUSSION

α_{S1} -Casein and α_{S2} -casein function as molecular chaperones and complex together to stabilize the α_S -casein structure (Treweek et al., 2011). Different

Figure 5. *CSN1S1* represses β -casein expression via Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5a (STAT5a) signaling pathway in primary goat mammary epithelial cells (GMEC). (A) GMEC were transfected with pcDNA3.1-NC or pcDNA3.1-*STAT5a* followed by infection with adenovirus with green fluorescent protein (Ad-GFP) or adenovirus Ad-*CSN1S1* (multiplicity of infection of 200), respectively. After 48 h of treatment, protein abundances of phosphorylated (p)-*STAT5a*, *STAT5a*, α_{S1} -CN, and β -CN were measured. (B) GMEC were treated with dimethyl sulfoxide (DMSO) or JAK2 inhibitor (AG490; 30 μ M) followed by negative control small interfering RNA (siNC) or siCSN1S1-507 (100 nM) transfection, respectively. At 48 h treatment, protein abundances of p-*STAT5a*, *STAT5a*, α_{S1} -CN, and β -CN were measured. Relative protein abundance was normalized to β -actin or total kinase. Values are presented as means $\pm$ SEM from 3 individual cultures ($n = 3$ goats). The different letters represent significant differences ($P < 0.05$) in protein abundance.

Figure 6. *CSN1S1* reduces the transcription of β -CN gene by modulating signal transducer and activator of transcription 5a (STAT5a). (A) Relative luciferase activity of primary goat mammary epithelial cells (GMEC) transfected with pGL3-Basic or pGL3-Basic-CSN2. (B) GMEC were co-transfected with both pGL3-Basic-CSN2 and pcDNA3.1-STAT5a (or pcDNA3.1-NC, plasmid ratio is 1:1) followed by infection with adenovirus with green fluorescent protein (Ad-GFP) or adenovirus Ad-CSN1S1 (multiplicity of infection of 200), respectively. After 48 h treatment, *CSN2* promoter activity was measured. (C) GMEC were incubated with dimethyl sulfoxide (DMSO) or STAT5 inhibitor (STAT5-IN-1; 200 μ M) followed by both pGL3-CSN2 and siCSN1S1-507 [or negative control small interfering RNA (siNC), 100 nM] co-transfection, respectively. After 48 h treatment, *CSN2* promoter activity was measured. Values are presented as means $\pm$ SEM from 3 individual cultures ($n = 3$ goats). * $P < 0.05$ and ** $P < 0.01$ compared with control (pGL3-Basic, Ad-GFP, or siNC).

β -casein variants were associated with phosphorylation status of α_{S1} -casein (Fang et al., 2017). The ChIP-seq for H3K4me2 at the casein gene cluster regions and flanking regions showed interactions among casein gene promoters and distal regulatory elements in lactating mammary gland (Rijnkels et al., 2013). The present study found a negative correlation of moderate intensity between the contents of α_{S1} -casein and β -casein in goat milk, underscoring the need for more detailed and controlled studies such as could be conducted in vitro (e.g., using GMEC; Shi et al., 2014; Zhu et al., 2015).

Compared with wild-type control cows with the same genetic background, β -casein and κ -casein transgenic cows had lower concentrations of α -casein and α -lactalbumin in milk harvested at early, mid, peak, and late lactation (Laible et al., 2016). There is negative correlation between transcription levels of *CSN1S1* and *BLG* in lactating goat mammary gland, underscoring a significant interaction between these proteins (Morammazi et al., 2016). In the present study, we confirmed the negative regulatory effect of *CSN1S1* gene on β -casein, but *CSN1S1* did not significantly affect the expression of α_{S2} -casein, κ -casein and whey proteins (α -lactalbumin and β -lactoglobulin). The regulatory relationship among various milk proteins is complex and not exactly same in different species or populations.

Previous studies provided strong evidence for the central role of JAK2/STAT5 and mTOR signaling pathway in regulating milk protein synthesis in ruminants (Laplanche and Sabatini, 2012; Qian and Zhao,

2016). Transcription factor E74-like factor 5 (**ELF5**) is essential for expression of milk-related genes, and its expression is under control of STAT5 and appears to encompass and enhance STAT5 activity (Bionaz and Looor, 2011). Suppressor of cytokine signaling 3 (**SOCS3**) is a cytokine-induced negative feedback-loop regulator of cytokine signaling. In bovine mammary epithelial cells (**BMEC**), the inhibition of STAT5a activation correlated with reduced SOCS3 expression, which indicated that *SOCS3* might be one of the targets of STAT5a activation (Huang et al., 2013).

Ghrelin peptides and methionyl-methionine dipeptides increase *CSN2* expression and β -casein synthesis in BMEC by activating JAK2/STAT5 and mTOR signaling (Li et al., 2016; Wang et al., 2018). In fact, both of these intracellular signaling pathways are known to participate in the control of bovine milk protein synthesis (β -casein and κ -casein) as it relates to lysine and leucine supply (Lin et al., 2018; Zhao et al., 2019) or the supply of glucose and amino acids (Zhang et al., 2018b). Thus, based on those data, we sought to determine if α_{S1} -casein (*CSN1S1*) could regulate β -casein expression via JAK2/STAT5 or mTOR signaling in GMEC. Overexpressing *CSN1S1* via adenovirus vector downregulated both p-JAK2 and p-STAT5a in GMEC, but mTOR phosphorylation was not affected. We found similar results by knocking down *CSN1S1* in GMEC. Although β -casein can be regulated by both JAK2/STAT5 and mTOR signaling pathways, it seems that *CSN1S1* inhibits β -casein expression via JAK2/STAT5a but not mTOR.

Prolactin- and glucocorticoid-mediated induction of β -casein gene expression in HC11 mouse mammary epithelial cells involves 2 principal regulatory regions, a proximal promoter and a distal enhancer located in the mouse approximately 6 kb upstream of the transcription start site (Kabotyanski et al., 2009). In the present study, overexpression of total STAT5a alleviated the inhibitory effect of *CSN1S1* on β -casein by enhancing STAT5a signaling, and inhibition of JAK2/STAT5 activity by AG490 rescued the effect of interfering *CSN1S1* on β -casein expression.

As a key transcription factor regulating milk protein synthesis, STAT5 recognizes its specific binding motifs and binds with the promoter region of milk protein genes (Shin et al., 2019). For instance, STAT5a binds the promoter region of *CSN2* and activates transcription both in HC11 mammary epithelial cells and immortalized GMEC (Kung et al., 2015, Ghosh and Radhakrishnan, 2019). Consistent with these results, in the present study *CSN2* promoter activity could also be activated by STAT5a in GMEC. We further revealed that *CSN1S1* mediated the regulation of *CSN2* promoter activity via STAT5a, which is consistent with the negative effect of *CSN1S1* on β -casein expression.

CONCLUSIONS

This study provided evidence that α_{S1} -casein (*CSN1S1*) affects synthesis of other milk protein in dairy goat mammary cells. The *CSN1S1* gene suppressed β -casein synthesis by affecting the binding of STAT5a to the promoter region of *CSN2* in GMEC, which explained in part the negative correlation between expression of α_{S1} -casein and β -casein in goat milk. Understanding the regulatory role of *CSN1S1* on β -casein synthesis may contribute to manipulating *CSN1S1* gene to improve quality of ruminant milk for human consumption.

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