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A novel fluorescence reporter system for the characterization of dairy goat mammary epithelial cells

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ABSTRACT

Goat mammary epithelial cells (GMECs) are a useful model to understand the physiological function of mammary glands and to assess the efficiency of mammary-specific vectors. The aim of this study was to develop an effective and convenient way to evaluate the secretory capacity of GMECs in primary culture. In this study, we developed a reporter system using fluorescent proteins driven by the CSN2 (Capra hircus beta-casein) gene promoter to detect the secretory capacity of GMECs. Additionally, we evaluated the efficiency of the reporter system by determining the expression of cytoskeletal proteins and beta-casein protein. The results suggest that this reporter system provides an easy, convenient and effective method to detect the function of milk synthesis in GMECs. Primary cultures of GMECs were homogeneous and retained the function of milk synthesis, prompting their usefulness as a model for further studies.

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1. Introduction

The mammary gland is the milk production organ of mammals, and reproductive hormones regulate it. Milk is produced during lactation, and it is generally considered the best source of nutrition for the offspring because its composition is optimized for healthy growth and development [1,2]. Previous reports have showed that goat milk provides 94% of the recommended adult daily dietary allowances of essential amino acids, 83% of calcium, 78% of riboflavin, as well as other minerals and vitamins to a lesser degree, while cow milk provides up to 81%, 74% and 89%, respectively [3,4]. The amounts of essential fatty acids, crucial monounsaturated and medium chain fatty acids are higher in goat milk than in cow milk [4]. In addition, goat milk is more easily digested owing to the smaller size of its fat globules and different casein types [5]. Hence, goat milk is considered superior to cow milk and is easily absorbed by the human digestive tract. The importance of goat milk in overall milk production has been rising steadily. Therefore it is important to understand the mechanisms of milk production and the response of the mammary gland to pathogenic infections [3,6].

Mammary epithelial cells are responsible for the synthesis and secretion of milk proteins [6]. They can reasonably be used as a model to understand the physiological function of the mammary gland [7]. Additionally, mammary epithelial cells can also be used as a model for transgenic screening systems. By identifying superior transgenes prior to transgenic animal production, the probability of the foreign gene being silenced in the offspring can be significantly decreased [8,9]. Therefore, an *in vitro* model that preserves species-specific mammary gland functions is of great importance in the investigation of the development, differentiation, and involution of the mammary gland.

There are many reports on culture of mammary epithelial cells, while the culture conditions did not consistent with each other and the characteristics of mammary gland specific functions are usually determined by the expression of the beta-casein gene at RNA and protein levels. To develop an effective and convenient way to evaluate the secretory capacity of goat mammary epithelial cells (GMECs), firstly, we isolated GMECs, and the cells were assigned to one of four group to study the effect of serum, ITS (Insulin–Transferrin–Selenium Solution, Gibco, USA) and insulin (ProSpec, Israel) on cell proliferation, cell cycle synchronization, apoptosis and transfection efficiency. Secondly, a reporter vector containing

Abbreviations: GMECs, goat mammary epithelial cells; GEFCs, goat ear skin-derived fibroblast cells; CSN2, Capra hircus beta-casein.

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enhanced green fluorescence protein (EGFP), driven by the CSN2 (Capra hircus β-casein) promoter, was constructed. The vector was introduced into GMECs and induced expression for 48 h, and the secretory capacity of GMECs was represented by the expression of EGFP. Finally, to evaluate the efficiency of the reporter system, we measured the expression of cytoskeletal proteins and the beta-casein protein. To our knowledge, this is the first report of a fluorescent protein reporter system under the CSN2 promoter, designed to evaluate the secretory capacity of GMECs in primary cell culture. In summary, the presented system provides an easy, convenient, and effective way to detect the function of milk synthesis in GMECs. Primary culture of GMECs preserved the mammary gland specific function, indicating that it could be used as a model for further studies.

2. Materials and methods

2.1. Isolation and culture of GMECs

Goat mammary gland tissue was obtained from a lactating Saanen dairy goat for isolation of GMECs. We followed essentially protocols [10] for isolation and purification of GMECs with minor modifications. Fresh tissues were placed in sterilized tubes containing ice-cold DPBS (Gibco, USA) containing 500 U/ml penicillin (Gibco) and 500 μg/ml streptomycin (Gibco) and immediately transported to the laboratory. Tissues were washed with DPBS solution for three times. Then they were cut into 0.5–1 mm^3 cubes and washed with DPBS until clean. These smaller pieces of tissues were transferred with sterile tips into empty plastic cell culture flasks (Corning, USA). Culture flasks were incubated at 37 °C and 5% CO_2. After 4 h, 3 mL DMEM-F12 (Gibco) containing 20% FBS (Gibco) were added to every flanks ensuring that the tissue would not be floated and separated from the bottom of the culture flanks. The medium was replaced with fresh medium every 3 d. Selective trypsinization steps were used to enrich the GMECs and removed the fibroblast cells from the primary culture. The purified mammary epithelial cells were isolated after an additional 3 to 4 passages.

2.2. Experimental design

Purified GMECs were cultured in DMEM-F12 containing 10% FBS, and cells from the fifth passage were assigned to one of four experimental groups (Table 1). After an additional five passages, cells from the tenth passage were used to assess the following cellular characteristics. (1) Cell proliferation was detected using a Cell Counting Kit-8 (CCK8, Beyotime, China) assay, with optical density (OD) values measured at 450 nm by a Multiskan Go Microplate Reader (Thermo Fisher Scientific, USA). (2) The cell cycle was visualized by staining using DNA staining solution with propidium iodide (PI) and RNase A (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and analyzed using a flow cytometer (FACS Calibur, Becton, Dickinson and Company). (3) Cell apoptosis was assessed using an Alexa Fluor®488 annexin V/dead cell apoptosis kit (Invitrogen, Carlsbad, CA, USA) and analyzed by flow cytometry. (4) Transient transfection efficiency was measured by transfecting cells with a pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA) using Lipofectamine™ LTX and PLUS reagents (Invitrogen) according to the manufacturer's protocol. After 24 h of culture, transfection efficiency was evaluated using a flow cytometer.

2.3. Construction of a fluorescence protein reporter vector driven by the CSN2 promoter

The fragment of EGFP was amplified from the vector of pEGFP-N1. The primers F1/R1 used for the synthesis of EGFP, were capped with *Xho*I sites in the 5′ terminal end to facilitate the subcloning of pBC1. The synthesized fragment was inserted into the pMD®19-T vector (Takara, China), followed by sequencing. The EGFP fragment was isolated from pMD-EGFP and inserted into the pBC1 [8] vector by *Xho*I site, followed by enzymatic digestion, PCR amplification and partial DNA sequencing. The plasmid obtained was named pBC1-EGFP. A schematic representation of this vector was shown in Fig. 3A and primers used for the plasmid construction were listed in Table 2.

2.4. Cell culture and transfection

Goat mammary epithelial cells and goat ear skin-derived fibroblast cells (GEFCs) were transfected with pBC1-EGFP or pEGFP-N1 plasmid by the Lipofectamine™ LTX and PLUS reagents according to the manufacturer's protocol. After transfection 6 h, the GMECs were cultured in the growth medium supplemented with 5 μg/ml prolactin (ProSpec, Israel). After incubation for 48 h, the confluent cells were harvested for RT-PCR analysis.

2.5. RNA extraction and RT-PCR assay

Total RNA from GMECs and GEFCs were prepared using PureLink® RNA Mini Kit (Invitrogen) according to manufacturer's protocol, cDNA was synthesized using RT reagent Kits with gDNA Eraser (Takara, China) by reverse transcription PCR. Primers for EGFP gene and *Gapdh* were listed in Table 2.

2.6. Immunofluorescence

Cytoskeletal proteins (Cytokeratin 18, Vimentin) in GMECs and GEFCs were examined by immunocytochemical analysis. Cells were seeded in glass bottom dishes and fixed with ice-cold methanol for 10 min at room temperature and permeabilized (0.25% Triton X-100) for 10 min. Then they were blocked with Immunol Staining Blocking Buffer (Beyotime) for 60 min at room temperature on a rocking platform. The primary antibodies of mouse anti-cytokeratin 18 (1:200, Abcam, USA) or mouse anti-vimentin (1:200, Abcam) was added to cells and incubated at 4 °C for overnight. The cells were washed with DPBS 3 × 10 min. The secondary antibody, FITC-conjugated Donkey anti-mouse antibody (1:500) or 594-conjugated Donkey anti-mouse antibody (1:500) was added to cells and incubated in the dark for 2 h at room temperature. The samples were co-stained with DAPI (10 μg/ml in PBS) for 10 min and then examined with a confocal laser-scanning microscope (Zeiss LSM 710 META, Germany).

2.7. Western blotting analysis of CSN2 protein

Proteins were isolated from GMECs and goat mammary gland tissues using RIPA Lysis Buffer (Beyotime). Western blotting was conducted as previous report [8]. A primary rabbit anti-CSN2 antibody (1:500, Biorbyt, UK) and HRP-conjugated GAPDH (1:500, Abcam) (loading control) were used in the western blot analysis.

2.8. Statistical analysis

The data was expressed as mean ± standard error (SEM). Statistically significant differences were performed using the SPSS software (version 18.0). Differences of $p < 0.05$ were considered to be significant.

Table 1
Experimental treatment groups.

Group	5% FBS	10% FBS	1% ITS	Insulin (5 μg/ml)	Hydrocortisone (5 μg/ml)	EGF (10 ng/μl)
5ITS	+		+		+	+
5INS	+			+	+	+
10ITS		+	+		+	+
10INS		+		+	+	+

Note: 5: 5% FBS; 10: 10% FBS; ITS: insulin–transferrin–selenium solution; INS: insulin.

3. Results

3.1. Morphology of GMECs

A primary culture of GMECs was established (Fig. 1A–D). The freshly isolated cells developed as a mixed population of epithelial cells and fibroblast cells (Fig. 1E). For additional passages with selective enzymatic digestion caused the fibroblast cells to disappear and a homogenous population of GMECs to remain (Fig. 1F). When

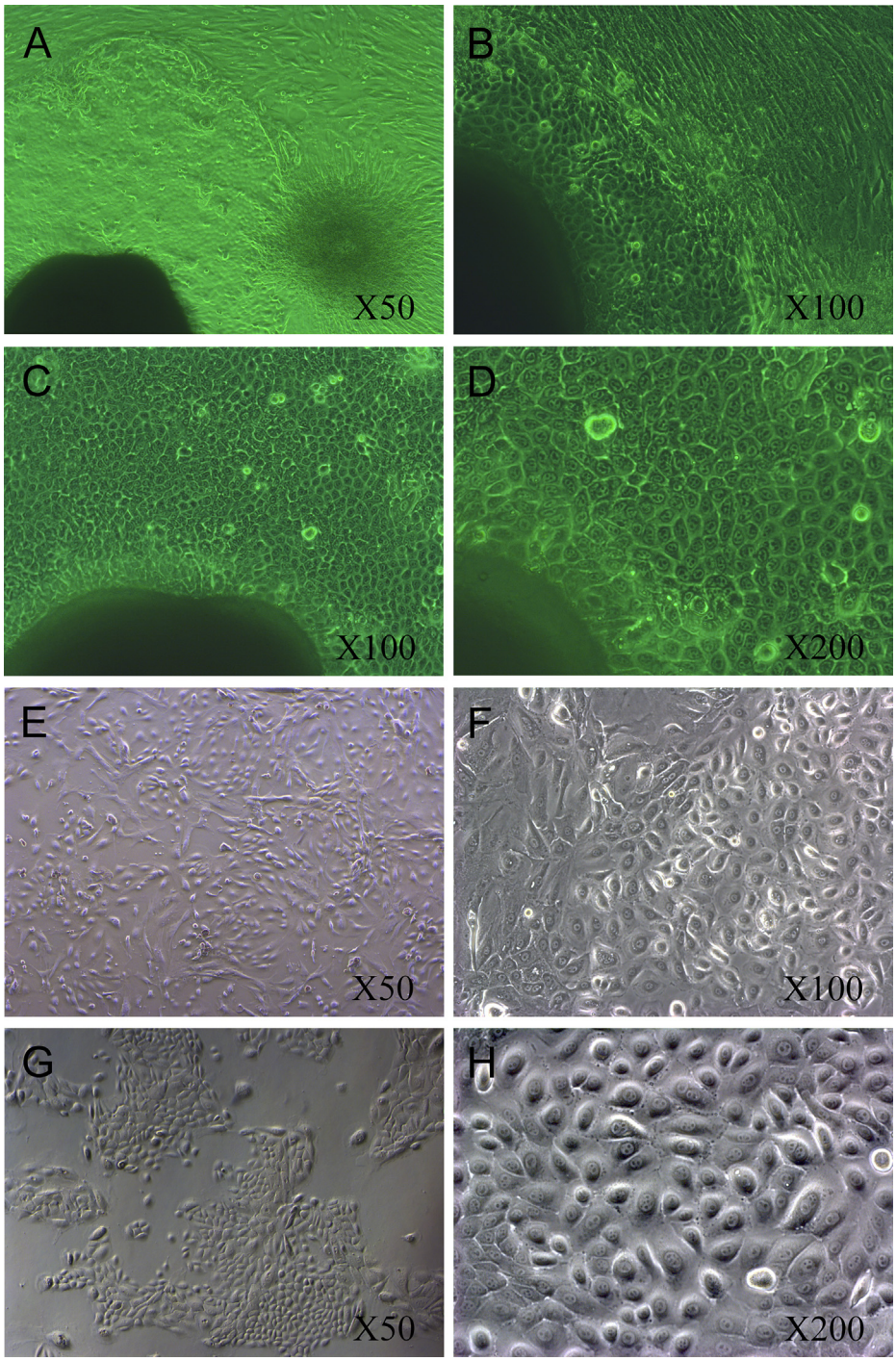

Fig. 1. Morphology of goat mammary epithelial cells. (A–D) Primary culture of goat mammary epithelial cells. (E) The mixture of goat mammary epithelial cells and fibroblast cells (×50). (F) Purified goat mammary epithelium cells (×100). (G) Goat mammary epithelial cells formed islands (×50). (H) Cobble-stone morphology of goat mammary epithelial cells (×200).

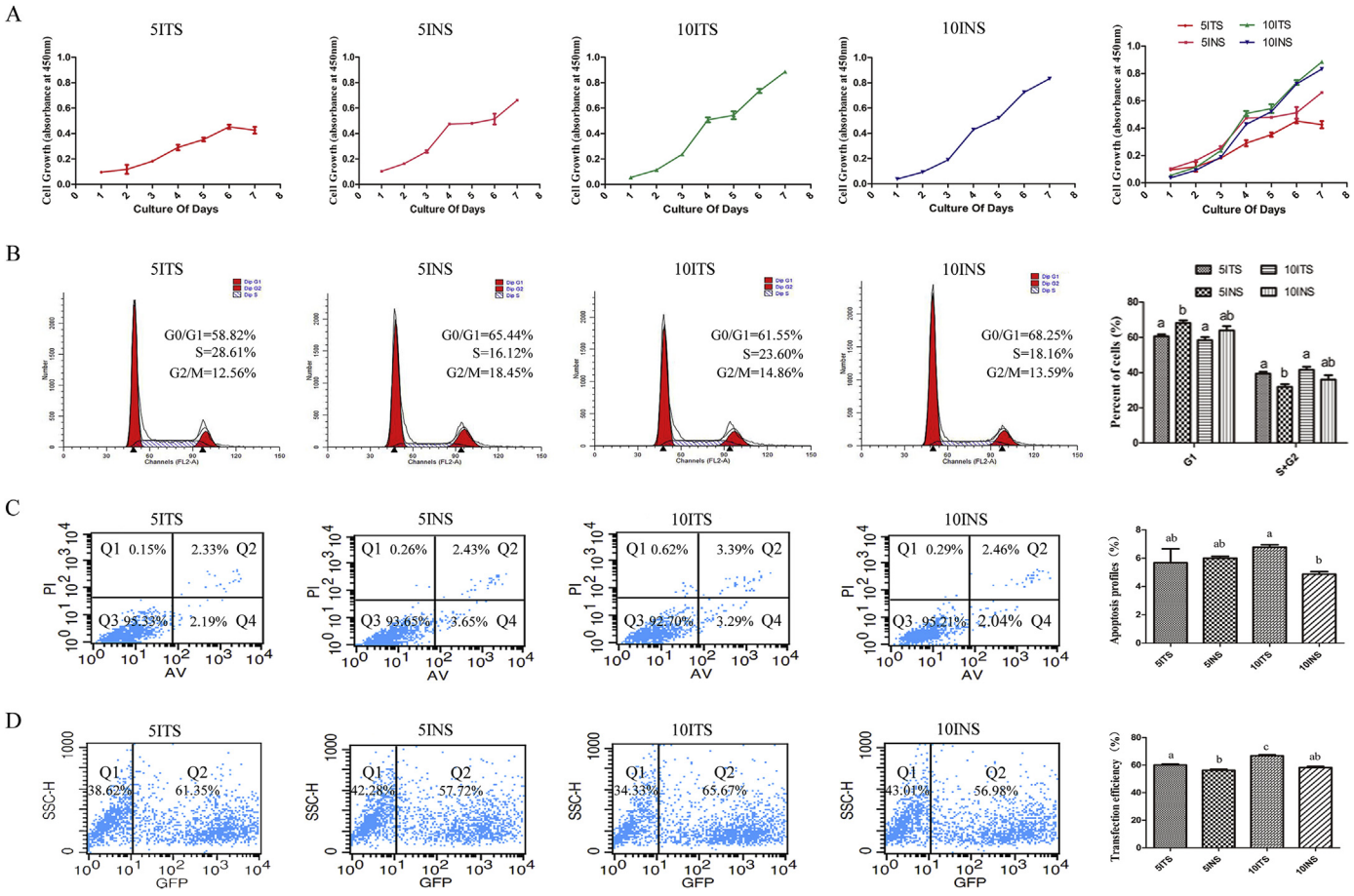

Fig. 2. The characterization of goat mammary epithelial cells (GMECs) under different culture conditions. Cells from the fifth passage were assigned to one of four groups. After an additional five passages, cells from the tenth passage were used to assess the following cellular characteristics. (A) The growth curves of GMECs. (B) The cell cycle proportions of GMECs. (C) The cell apoptosis rate of GMECs. (D) The transfection efficiency of GMECs. All results are the average of three independent experiments in duplicates. $^{a-c}$Means without a common superscript differed ($p < 0.05$). Note, 5: 5% FBS; 10: 10% FBS; ITS: insulin–transferrin–selenium solution; INS: insulin.

grown at low density on a plastic substratum, the cells formed islands (Fig. 1G), and when grown to confluency, they displayed a monolayer and cobblestone, epithelial-like morphology (Fig. 1H).

3.2. Characterization of GMECs under different culture conditions

3.2.1. Growth curves of GMECs under different culture conditions

The four groups of cells experienced a rapid increase in cell proliferation 48 h after passaging. The 10ITS and 10INS groups had similar growth curves, with a more rapid increase than groups 5INS and 5ITS on days 5–7. Group 5ITS reached its maximal growth rate on days 6–7, producing a sinusoidal growth curve, whereas the other groups continued to exhibit an increase in cell proliferation on days 6–7 (Fig. 2A).

3.2.2. Cell cycle synchronization of GMECs under different culture conditions

Previous studies showed that the rate of cell growth is governed by the concurrent cell cycle stage [11]. To study the growth of GMECs under different culture conditions, cell cycle progression was analyzed by flow cytometry (Fig. 2B). We observed that the population of the 5ITS group in S-phase was larger than the one of the 5INS group ($p < 0.05$). The 10ITS group was larger than 10INS ($p > 0.05$), the 5ITS group was smaller than 10ITS ($p > 0.05$), and the 5INS group was smaller than 10INS group. The population size of cells in G0/G1 phase was also measured and was opposite to the S-phase, while there were no changes in the percentage of cells in G2/M phase.

3.2.3. Apoptosis of GMECs under different culture conditions

Annexin V and PI for flow cytometry were used to determine the apoptosis rate of GMECs after different treatments (Fig. 2C). Apoptosis rates of GMECs were lower in the 5ITS group than in 5INS ($5.67 \pm 0.99\%$ vs. $6.00 \pm 0.14\%$, $p > 0.05$), and higher in the 10ITS group than in 10INS ($6.77 \pm 0.18\%$ vs. $4.86 \pm 0.19\%$, $p < 0.05$). Apoptosis rates were lower in 5ITS compared with 10ITS ($5.67 \pm 0.99\%$ vs. $6.77 \pm 0.18\%$, $p > 0.05$), and higher in 5INS compared with 10INS ($6.00 \pm 0.14\%$ vs. $4.86 \pm 0.19\%$, $p > 0.05$).

3.2.4. Transfection efficiency of GMECs under different culture conditions

The transfection efficiency in the 5ITS group was higher than in 5INS ($60.11 \pm 0.65\%$ vs. $56.33 \pm 0.80\%$, $p < 0.01$) (Fig. 2D). The efficiency in the 10ITS group was significantly higher than in 10INS ($66.81 \pm 0.66\%$ vs. $58.38 \pm 0.81\%$, $p < 0.01$). Compared with 5ITS, the efficiency in the 10ITS group was increased ($66.81 \pm 0.66\%$ vs. $60.11 \pm 0.65\%$, $p < 0.01$). The efficiency was lower in the 5INS group compared with the 10INS group ($56.33 \pm 0.80\%$ vs. $60.11 \pm 0.65\%$, $p > 0.05$).

3.3. Construction and assessment of the plasmid by restriction digestion

The EGFP fragment was amplified from the pEGFP-N1 plasmid, and the cloned sequence was 100% homologous with the template. The mammary gland specific vector of pBC1-EGFP was confirmed by PCR,

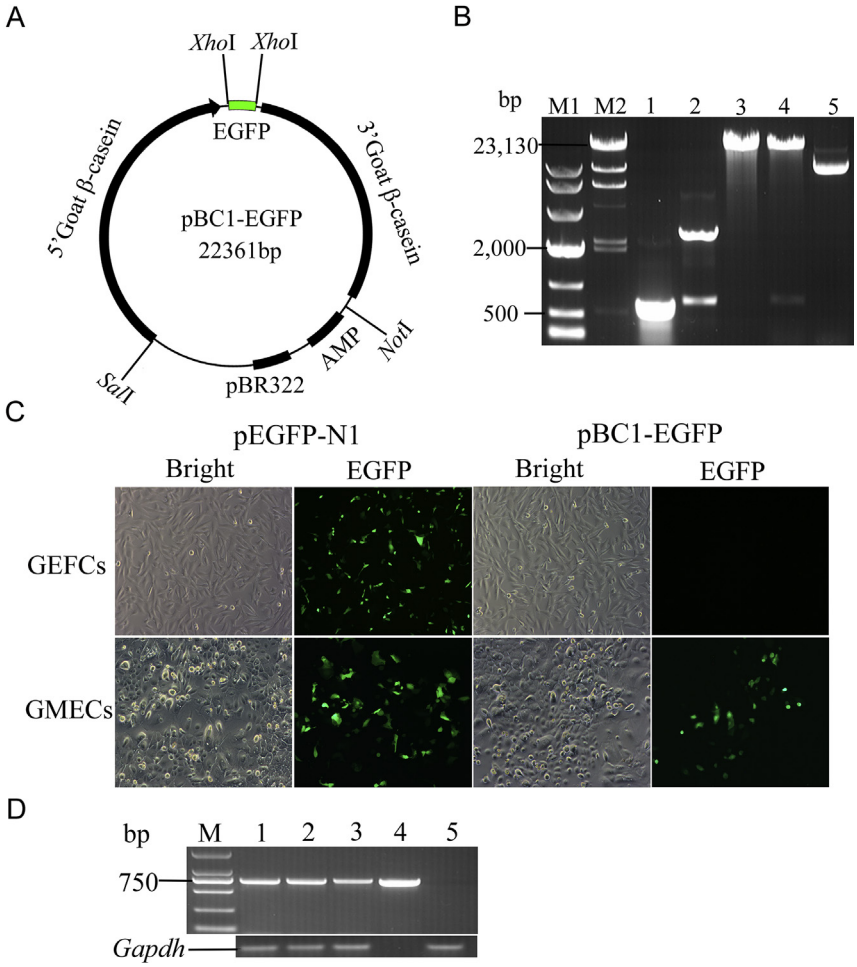

Fig. 3. Assessment and expression of a report vector pBC1-EGFP. (A) DNA construct for EGFP mammary gland-specific vector using a goat β-casein (CSN2) promoter. (B) Restriction enzymatic digestion of pBC1-EGFP. Lane 1: EGFP fragment (729 bp); Lane 2 to 4: pMD-EGFP, pBC1 and pBC1-EGFP digested by *Xho*I; Lane 5: pBC1-EGFP plasmid, M1: DL 10,000 DNA Marker, M2: λ-*Hind*III digest DNA Marker. (C) Fluorescence of EGFP was detected 48 h post transfection (×100). (D) Detected the expression of EGFP in GMECs and GEFCs by RT-PCR, Lane 1 to 3: transgenic GMECs, Lane 4: pBC1-EGFP plasmid as template, Lane 5: transgenic GEFCs, M: DL 2000 DNA Marker, *Gapdh* was used as a reference gene.

restriction enzyme digestion and partial sequencing. As predicted, *Xho*I cleaved pBC1-EGFP into 21 kb and 729 bp fragments (Fig. 3B).

3.4. Expression of the fluorescence protein reporter vector driven by the CSN2 promoter

GMECs and GEFCs were transiently transfected with pBC1-EGFP plasmid, and the expression of EGFP was analyzed by RT-PCR. Inspection of cultures under UV light 48 h after transfection revealed that the plasmid was expressed in GMECs but not in GEFCs (Fig. 3C). RT-PCR further confirmed that the pBC1-EGFP plasmid could be induced only in GMECs (Fig. 3D).

3.5. Immunofluorescence characterization of GMECs

Although the purified cells appeared to have epithelial morphology, and the reporter vector could be induced expression in GMECs, we further evaluated the homogeneity of the cells by staining with anti-cytokeratin 18 antibody to detect the expression of cytoskeleton 18 which is specific for epithelial cells, and with anti-vimentin antibody to detect vimentin which is usually expressed in various non-epithelial cells [12,13]. Almost all of the epithelial cells revealed a strong positive fluorescence signal for cytokeratin 18 (Fig. 4A). No positive staining was detected in fibroblast cells. In contrast, only a few GMECs were stained lightly

Table 2
Primers for the plasmid construction and assessment.

Gene name and DNA fragment	Primer sequences	Size of PCR product (bp)	Annealing temperature, °C
EGFP	F1: CTCGAGGTCGCCACCATGGTGAGCAAGGG R1: CTCGAGTTACTTGTACAGCTCGTCCATGCC	729	64
Gapdh	F2: CGACTTCAACAGCGACACTCAC R2: CCCTGTTGCTGTAGCCGAATTC	119	60

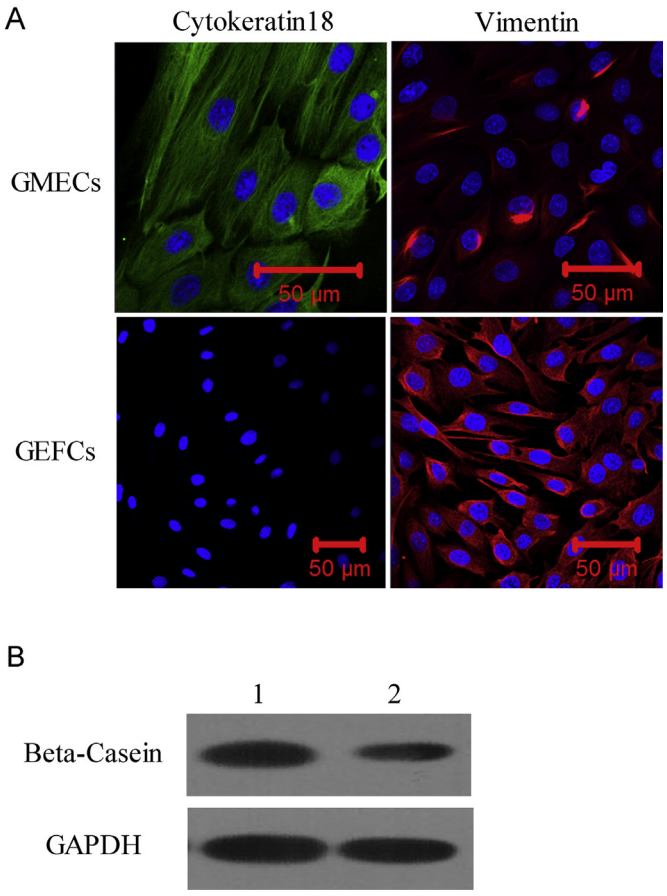

Fig. 4. The expression of cytoskeletal proteins and beta-casein protein. (A) Fluorescent image of GMECs and GEFCs stained for cytokeratin 18 and vimentin, bar: 50 μm. (B) Western-blotting was performed to detect the expression of beta-casein protein. GAPDH served as an internal control. Lane 1: goat mammary gland tissues; Lane 2: goat mammary epithelial cells.

by anti-vimentin antibody. Almost all of the fibroblast cells were stained strongly by the anti-vimentin antibody (Fig. 4A).

3.6. Expression of CSN2 protein

To confirm the lactation function in GMECs, we also evaluated the beta-casein protein synthesis in GMECs by western blotting. In the western blot analysis, lysates of both mammary gland tissues and GMECs reacted positively with an anti-CSN2 antibody. GAPDH was used as a loading control (Fig. 4B).

4. Discussion

Goat mammary epithelial cells have been widely used as a model to study the physiological function of mammary glands. For the current study, we obtained GMECs from a lactating dairy goat by tissue culture. To optimize culture conditions, the cells were designated to one of four groups to determine the effects of serum, ITS and insulin on cell proliferation, cell cycle synchronization, apoptosis and transfection efficiency. Groups 10ITS and 10INS had similar growth curves, and their populations grew more rapidly than the others. The amount of cells in the 5ITS group that were in S-phase was higher than in the other groups. It is generally believed that cells in the S-phase of the cell cycle possess higher proliferation capacities than those in other cell cycle stages [11]. The apoptosis rate and transfection efficiency of the 10ITS group was the highest of the four groups. Taking into consideration the proliferation, cell cycle synchronization, apoptosis and transfection efficiency, the 10ITS group was found to be the best suited for cell culture and was chosen for further experiments.

GMEC models for the study of milk synthesis are not only required to maintain morphological features, but also to preserve mammary gland specific functions [14]. The research of lactation function in GMECs is mainly on milk protein (the main component is casein) gene expression and secretory capacity. The expression of beta-casein can be used to represent the capacity of milk protein synthesis [15]. A mammary-specific expression vector, pBC1, harboring the CSN2 promoter, was used as the vector backbone. CSN2 promoter targets expression of the gene of interest almost exclusively to the lactating mammary gland [16,17,18]. In this study, we utilized a CSN2 promoter driven reporter as a strategy to detect the expression of beta-casein protein in GMECs. RT-PCR and EGFP fluorescence showed that reporter vector only be induced expression in GMECs.

To confirm the efficiency of the reporter system, we further studied the homogeneity of cells by immunofluorescence and the secretory capacity by western blot analysis. The epithelial cells were strongly stained by cytokeratin-18 but only lightly stained by vimentin. The positive staining to cytokeratin-18 was a powerful proof of the cells' epithelial nature. The weak staining by vimentin was possibly associated with the culture conditions [10,19]. Western blot analysis showed that both the mammary gland tissues and mammary epithelial cells positively reacted with the beta-casein antibody. These findings indicate that the GMECs were able to produce beta-casein protein and to maintain their secretory capacity.

In conclusion, the new reporter system was an easy, convenient, and efficiency method to detect the secretory capacity of GMECs in primary culture. GMECs retained their mammary gland specific function, which can be used for the study of milk synthesis in the goat mammary glands. Therefore, this work lays a foundation for both the evaluation of lactation ability of GMECs in primary culture and study of the physiological function of mammary glands.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Transparency document

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