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Research Paper

Effects study of SCGB2A2 on cell proliferation and milk components biosynthesis in ovine mammary epithelial cells

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ABSTRACT

The Small-Tailed Han (STH) sheep milk has significantly higher fat and protein than DairyMeade (DM) sheep milk. Further study found that the expression of whey protein secretoglobin family 2 A member 2 (SCGB2A2) in STH sheep milk was significantly higher than in DM sheep milk. Thus we surmise that the SCGB2A2 may be involved in the regulation of the mammary biological function. Here, the expression of SCGB2A2 at different lactation periods was detected first. Then the cell proliferation and milk component synthesis effects on ovine mammary epithelial cells (OMECs) were analyzed. And the CUT&Tag analysis was used to identify SCGB2A2 target binding sites at the genome. The transcriptome of *SCGB2A2* overexpression and knockdown OMECs was analyzed. Two co-analyses were conducted to further screen out SCGB2A2 potential target genes. Finally, the potential interacting protein was verified by CO-IP and UHLP-MS analysis. Results showed that the SCGB2A2 was expressed highest at the colostrum stage and lowest at the dry milk stage. Immunohistochemical analysis showed that it was mainly expressed in mammary epithelial cells. CCK8 and cell cycle analysis showed that SCGB2A2 promotes the OMECs proliferation. Milk components synthesis detection found that the SCGB2A2 was positively correlated with the CSN2, lactose, and triglyceride (TG). CUT&Tag and transcriptome co-analyses found that 20 genes were consistently detected, including *FBP2*, *IFIT3* and so on. CO-IP analysis demonstrated that mTOR interacted with SCGB2A2. Taken together, we demonstrated that SCGB2A2 plays a positive role in OMECs proliferation and biosynthesis of milk components. Some SCGB2A2 direct regulated genes are involved in the cell proliferation. The regulation of SCGB2A2 for milk components biosynthesis mainly interacts with mTOR. However, the specific regulatory mechanism is needed to further study.

1. Introduction

Except for casein and lactoglobulin, there are various types of whey proteins in milk. These whey proteins play roles in anti-inflammatory, antibacterial, anti-oxidation, cell proliferation regulation, milk component synthesis regulation and other biological functions [1,2]. In addition, the characteristic analysis of whey protein composition is also one of the methods to distinguish different breeds of milk [3]. On one hand, the study of whey proteins supplies a basic understanding of the milk's biological function. On the other hand, it also is a feasible way to explore the regulatory mechanism of dairy livestock lactation traits [4]. Previously, we found that concentrations of protein and fat were significantly higher in Small-tailed Han (STH) sheep milk than DairyMeade (DM) [5]. Further whey proteome analysis discovered that the abundance of

Abbreviations: CCK8, Cell Counting Kit-8; CSN2, casein beta; CO-IP, Co-Immunoprecipitation; CUT&Tag, Cleavage Under Targets and Tagmentation; DEGs, differentially expressed genes; EGF, epidermal growth factor; FBS, fetal bovine serum; FPKM, Fragments Per Kilobase per Million; Gln, glutamine; GO, Gene Ontology; GSH, glutathione; HCT, hydrocortisone; ITS, insulin-transferrin-selenite; KEGG, Kyoto Encyclopedia of Genes and Genomes; MS, Mass Spectrometry; OD, optical density; OMECs, ovine mammary epithelial cells; PRL, prolactin; qRT-PCR, quantitative real-time PCR; SCGB2A2, secretoglobin family 2 A member 2..

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secretoglobin family 2 A member 2 (SCGB2A2) was significantly higher in STH milk than DM [6]. Thus, we suppose that the SCGB2A2 may be involved in the biosynthesis regulation of the milk components in sheep.

SCGB2A2, also called MGB1, belongs to the hemoglobin secretory family. Series tumor-related research found that the expression of SCGB2A2 is abnormal during the progression of breast cancer, cervical cancer, endometrial cancer, ovarian cancer, cutaneous melanoma and salivary gland cancer [7–13]. Studies related to breast cancer have shown that the expression of SCGB2A2 in the tumor tissue is extremely higher than normal [14,15]. A circulating tumor cells study demonstrated that the transcript level of *SCGB2A2* is related to the survival period [16]. SCGB2A2 is involved in multiple biological processes, including cellular signal transduction, immune response, chemotaxis and potential hormone transporting [17]. However, the mechanism of action is still unclear. In addition, the regulatory role of SCGB2A2 during lactation is needed to clarify.

The protein, fat and lactose are the main nutritional components in milk. Among discovered regulatory pathways, the mammalian target of rapamycin (mTOR) is considered to be playing the central role. It integrates various signals from the environment and regulates specific lactating processes, such as mammary epithelial cell proliferation, milk protein and fat synthesis. The mTORC1 is a major regulatory factor in the mTOR pathway and regulates cell development and milk synthesis under the action of growth stimulation, nutrition and energy [18]. And the phosphatidylinositol 3-kinase (PI3K), Akt, p38/ERK/MAPK, nuclear factor kappa B and c-Jun N-terminal kinase signaling pathways were all demonstrated to interact with the mTOR pathway [19]. Considering that SCGB2A2 can activate the p38, JNK and ERK in the MAPK signaling pathway [20], we further surmise that it may participate in the regulation of the development of mammary and lactation.

In order to clarify the regulatory effect of the SCGB2A2 in ovine lactation, we analyzed the expression at different lactation periods first. Then the *SCGB2A2* over-expressed (OE) and knock-down (KD) ovine mammary epithelial cell lines (OMECs) were established to evaluate its effects on cell proliferation and biosynthesis of milk protein, fat and lactose. Finally, the potential regulatory mechanism was explored through transcription, CUT&Tag and Co-IP analyzes. The results clarified the biological effects of SCGB2A2 on mammary epithelial cells in dairy sheep and its possible regulatory mechanisms, which can provide theoretical assistance for the breeding of dairy sheep.

2. Materials and methods

2.1. Ethics statement

This study received approval from the Inner Mongolia University Research Ethics Committee of China (approval number: 2021002). All experiments were performed according to Chinese laws and institutional guidelines. Informed consents were obtained from Monterra Husbandry Technology Development Co., Ltd. and Inner Mongolia Fengdongzhiying Husbandry Technology Co. Ltd. (Inner Mongolia, China) for the use of experimental sheep.

2.2. Dairy sheep mammary tissue sampling of different lactation periods

The dairy sheep in this study were obtained from Monterra Husbandry Technology Development Co., Ltd. and Inner Mongolia Fengdongzhiying Husbandry Technology Co. Ltd. (Inner Mongolia, China). Healthy DM sheep of second parity at pregnancy (D120 ~ 135 after pregnancy), early lactation (D0 ~ 3 after parturition), peak lactation (D75 ~ 90 after parturition) and dry milk (D240 ~ 280 after parturition) periods were selected, and three of each period. After being anesthetized with lidocaine hydrochloride, about 1 cm^3 of mammary tissues were obtained. The mammary tissues were washed with saline and frozen with liquid nitrogen or immobilized with 4% paraformaldehyde to further use.

2.3. Cell culture and establishment of SCGB2A2 OE and KD OMECs

The used OMECs in this study were obtained from the preservation of our laboratory, which cultured from the mid-lactated mammary tissue of DM sheep. The OMECs were cultured with DMEM-based medium containing 15% FBS, 1 μg/mL HCT, 10 ng/mL EGF, 200 nM L-Gln, 5 μg/mL ITS and 5 μg/mL PRL. All cell culture reagents were purchased from Yeasen (Shanghai, China). All of them were cultured at 37 °C under 95% air and 5% CO_2.

The establishments of *SCGB2A2* OE, not treated (NT), KD and non-specific control (NS) cell lines were all through lentiviral vector. The vectors were plenti-Ef1α-SCGB2A2, lenti-Ef1α-empty and pLKO.1-shRNA. The interference targets of *SCGB2A2* were displayed in the Fig. S1.

The lentivirus packaging was processed with a three vector system. Briefly, the plenti-Ef1α-SCGB2A2\plenti-Ef1α-empty\pLKO.1-shRNA \pLKO.1-nsRNA-puro, psPAX2 and pMD2.G vectors were co-transfected into HEK-293 T cells with HieffTrans® Polyethylenimine Linear (PEI) MW40000 transfection regent (YEASEN, China) and cultured with DMEM based medium containing 10% FBS at 37 °C under 95% air and 5% CO_2. The supernatant were collected after 48 h of culture and filtered with a 0.45 μm filter. The HEK-293 T cells supernatant and OMECs medium (2:1, *V*/V) and 10 μg/mL polybrene (YEASEN, China) were added when OMECs 70% ~ 80% adhering. After 2 days of culture, 0.6 μg/mL puro (YEASEN, China) was added to screen positive cells.

2.4. Cell proliferation analysis

The SCGB2A2 OE, KD, NT and NS OMECs proliferation analysis after 2 days of culture was followed as Wang et al. [21]. Briefly, the cell cycle of OMECs was assessed through the Cell Cycle Analysis Kit (YEASEN, China) according to the manufacturer's recommendations and followed as Chen et al. [22]. Then the Cell Counting Kit (CCK-8) (YEASEN, China) was used to determine the OMECs proliferation according to the manufacturer's recommendations.

2.5. RNA extraction and quantitative real time PCR (qRT-PCR)

The total RNAs of mammary tissue and OMECs were extracted using Total RNA Extraction reagent (YEASEN, China) following the manufacturer's procedure. The Hifair® III 1st Strand cDNA Synthesis SuperMix reagent kit (YEASEN, China) was used for cDNA synthesis. Hieff® qPCR SYBR® Green Master Mix (YEASEN, China) was used for qRT-PCR. The procedures were followed as the manufacturer's instruction book and followed as Han et al. [23]. The *GAPDH* was used as the reference gene. The primers were followed as: *SCGB2A2*-F: 5′ - CTGTACTGCTATGCAGGGTCTT - 3′, *SCGB2A2*-R: 5′ - GGCCAGAGTTTCATTTGACTGC - 3′, 189 bp. *GAPDH*-F: 5′ - TCCGTTGTGGATCTGACCTG - 3′, *GAPDH*-R: 5′ - CCCTGTTGCTGTAGCCGAAT - 3′, 250 bp. The qPCR procedure is 95 °C for 30 s, followed by 95 °C for 10 s and 60 °C for 30 s and the cycle was set as 40. Then the melting curve was obtained from the default program of the instrument. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method with 3 repetitions.

2.6. Western blot analysis

The RIPA lysis solution and protease inhibitor (Coolaber, China) were used for the mammary tissue and OMECs protein extraction and followed as Zhang et al. [24]. The proteins (30 μg of each sample) were separated on a 12% SDS-polyacrylamide gel by electrophoresis and then transferred onto a poly-vinylidene difluoride (PVDF) membrane (Pall, USA). After being blocked with a 5% skimmed milk solution for 1 h at room temperature, the membrane was incubated overnight at 4 °C with a primary antibody against sheep anti-rabbit SCGB2A2 (1:1000) (EIAab, China) and then incubated with an HRP-conjugated secondary antibody

goat-anti-rabbit IgG (1:10000) (Proteintech, China). After washing with PBS 3 times, the protein bands in the PVDF membrane were visualized with a Super ECL Plus detection kit (YEASEN, China) using the Chemiluminescent Imaging and Analysis System (SAGECREATION, China), and the α-Tubulin (1:1000) (Proteintech, China) was used as an internal control. The densitometry analysis was performed using Sage Capture software (SAGECREATION, China).

2.7. Mammary tissue immunohitochemistry (IHC) analysis

Expressions of SCGB2A2 at pregnancy, early lactation, peak lactation and dry milk periods were analyzed via immunohitochemistry. Briefly, 5-μm sections were treated with the Citrate Antigen Retrieval Solution (Coolaber, China) for 30 min. Then incubated with goat serum (Zsbio, China) for 15 min. Sections were incubated with sheep anti-rabbit SCGB2A2 (1:800, EIAab, China) for 60 min and stains were visualized using Mach 2 Rabbit HRP polymer (Proteintech, China). Five photomicrographs per tissue section (Zeiss microscope) per sample were taken to analyze the expression of SCGB2A2. Positive cells were labeled as brown.

2.8. Triglyceride (TG), lactose and CSN2 assay of cells and supernatants

The OE, NT, KD and NS OMECs were cultured at 6-well plates. When cells were fully confluent, the fresh medium was added, and concentrations of TG, lactose and CSN2 of cells and supernatants were measured after 2 days of culture using the TG Assay Kit, Lactose Assay Kit (Grace Biotechnology, China) and CSN2 Assay Kit (Abebio, China) and followed as Feng et al. [25].

For the cell's assay, the lysis was added and the ultrasonication was processed to obtain cell lysates. After centrifuged with 12,000 RPM at 4 °C for 10 min, the supernatant was collected for further analysis.

For the TG concentration assay, 20 μL supernatants were mixed with chromogenic agents and incubated for 30 min at 25 °C. The absorbance was measured at 510 nm. TG (μg/mL) = 500 × ($A_{sample} - A_{blank}$) ÷ ($A_{control} - A_{blank}$).

For the lactose concentration assay, 10 μL supernatants plus agent 1 and agent 2 were mixed and incubated for 20 min at 25 °C. Then agent4 and agent5 were added and mixed and incubated for 30 min at 37 °C. The absorbance was measured at 510 nm. Lactose (mg/mL) = 0.95 × ($A_{sample} - A_{blank}$) ÷ ($A_{control} - A_{blank}$).

For the CSN2 concentration assay, 100 μL supernatants were added into a 96-well plate and incubated for 2 h at 37 °C. Then 100 μL biotin-conjugate of each well was added and incubated for 1 h at 37 °C. And then 100 μL strepatavidin-HRP of each well was added and incubated for 1 h at 37 °C. And 100 μL substrate solution of each well was added and incubated for 20 min at 37 °C. The absorbance was measured at 450 nm. Data was calculated through "Stand curve".

2.9. OMECs transcriptome analysis

The transcriptome analysis of OMECs was performed by OE. Co. Ltd. (Shanghai, China) and followed as Wang et al. [21]. Briefly, full confluent OE, NT, KD and NS OMECs were collected by Trizol (Yeasen, China) lysing ($n = 3$). These samples were snap-frozen in liquid nitrogen and stored for the RNA libraries generation. The library was generated on the Illumina Novaseq6000 platform. Raw data (raw reads) of fastq format were firstly processed through fastp software and the clean data were calculated. The clean reads were mapped to the sheep reference genome (Oar_v4.0, GCA_000298735.2) using Hisat2 v2.0.5. The read counts of each gene were obtained by Feature Counts v1.5.0-p3 and FPKM of each gene was calculated.

The differentially expressed genes (DEGs) analysis was performed using the DESeq2 R package (1.20.0). Genes with an adjusted $P \leq 0.05$ were assigned as DEGs. The Gene Ontology (GO) enrichment analysis of DEGs was implemented by the clusterProfiler R package. GO terms with corrected $P \leq 0.05$ were considered significantly enriched by differential expression. The enrichment analyses of Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/) were performed by using the cluster Profiler R package to test the statistical enrichment of DEGs.

2.10. SCGB2A2 CUT&tag analysis

The early lactation mammary tissue was used for SCGB2A2 CUT&Tag analysis and performed by OE. Co. Ltd. (Shanghai, China). The library construction was performed using the Hyperactive™ In-Situ ChIP Library Prep Kit (Vazyme Biotech, China) according to the manufacturer's instructions [26]. Briefly, prepared concanavalin A-coated magnetic beads (ConA beads) were added and incubated at room temperature first. Then non-ionic detergent digitonin was used to permeate the cell membrane. And the primary SCGB2A2 antibody, secondary antibody and the hyperactive pA-Tn5 transposase were co-incubated with cells that were bounded by ConA beads in order. The cut DNA fragments can be ligated with P5 and P7 adaptors by Tn5 transposase and the libraries were amplified by PCR with the P5 and P7 primers. The purified PCR products were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Finally, these libraries were sequenced on the Illumina NovaSeq6000 platform and 150 bp paired-end reads were generated for the following analysis.

The clean reads were obtained through raw sequence data quality trimmed by FATSP software [27]. The clean reads were aligned to the sheep reference genome (Oar_v4.0, GCA_000298735.2) using Bowtie2. The putative binding sites detection was performed by the SEACR software based on the "stringent" parameter.

2.11. SCGB2A2 protein pulldown and mass spectrometry (MS) analysis

The early lactation mammary tissue was used for SCGB2A2 protein pulldown and mass spectrometry analysis and performed by OE. Co. Ltd. (Shanghai, China). Briefly, the washed tissue was treated with the immunoprecipitation (IP) lysis/wash buffer (YEASEN, China) and phenyl methane sulfonyl fluoride (PMSF) (YEASEN, China) and lysis on ice for 20 min. The supernatant was obtained through centrifugation at 12000 RPM for 15 min. Then the SCGB2A2 and IgG IP antibody complex was added and incubated at 4 °C to form an IP complex. The Protein A/G Magnetic Beads (YEASEN, China) were used to target protein enrichment. And the SDS-PAGE Loading Buffer (YEASEN, China) was used to dissociate SCGB2A2 IP proteins for Western blot or MS analyses.

For the MS analysis, SCGB2A2 IP proteins were enzymolyzed with trypsin and desalinized with the Nano-HPLC Buffer (Thermo, USA). Then the Nano HPLC liquid phase system (UltiMate 3000 RSLCnano, Thermo, USA) was used for separation. The Q-Exactive Plus mass spectrometer (Thermo, USA) was used for MS. The molecular ion scanning range is 300–1500 *m/z*. The 20 most intense peaks in MS2 were fragmented with higher-energy collisional dissociation with an NCE of 28. The MS1 resolution is 70,000 with an AGC target of 3e6 and a maximum injection time of 100 ms. The MS2 resolution is 17,500 with an AGC target of 1e5 and a maximum injection time of 50 ms.

The Q Exactive raw data was searched by the the Proteome-Discoverer (v.2.5). The database is uniprot-*Ovis aries* (Sheep) UP000002356_9940. The database search was performed with trypsin digestion specificity.

2.12. Statistical analysis

The data were indicated as the means ± S.E. ($n = 3$). The normal distribution analysis was performed using the Shapiro-Wilk test, and the significance was calculated using one-way ANOVA test in GraphPad Prism statistical software (Version 8.3.0). The significant difference is defined as $P < 0.05$.

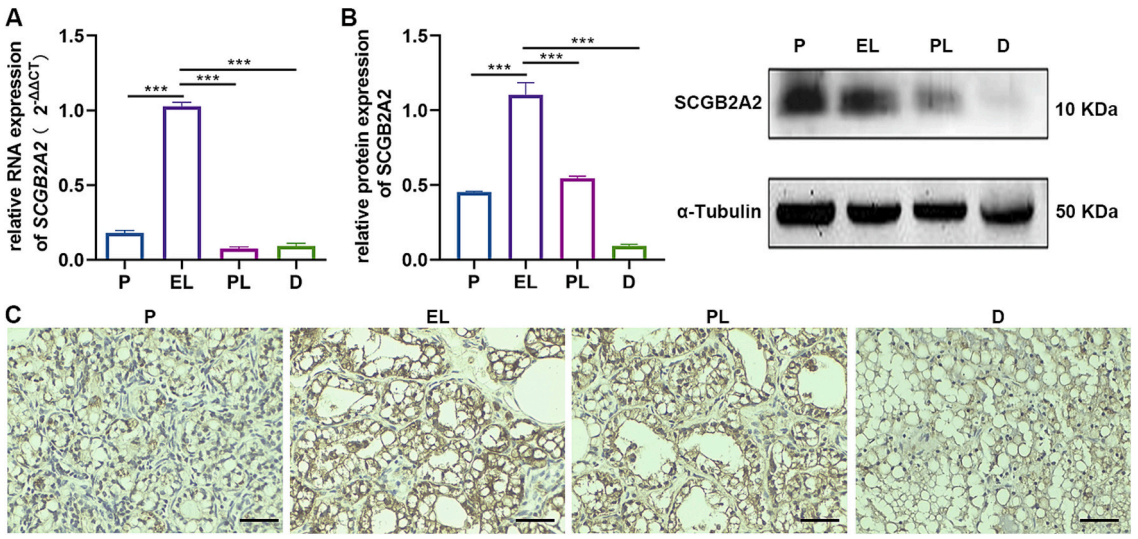

Fig. 1. The expression pattern of SCGB2A2 at different lactation periods in dairy sheep mammary. A. qRT-PCR result. B. Western blot result. C. Immunohistochemistry result. Positive cells were labeled as brown. ($N = 3$. P: pregnancy; EL: early lactation; PL: peak lactation; D: dry milk period. ***: $P < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

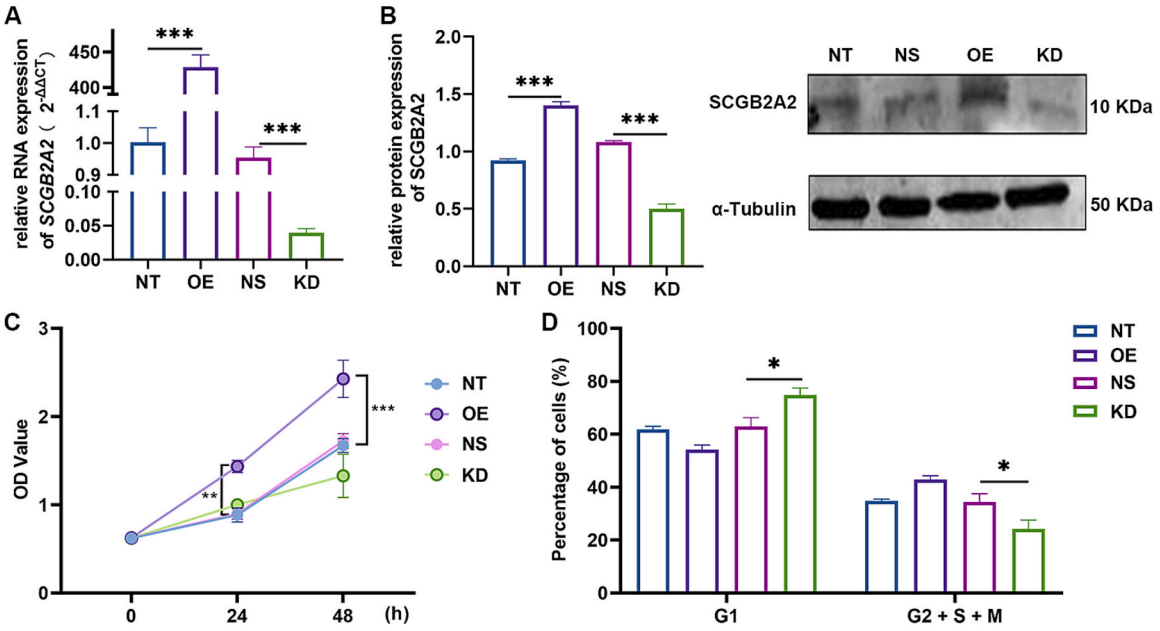

Fig. 2. The effect on cell proliferation in OMECs. A. qRT-PCR result. B. Western blot result. C. CCK-8 result. D. Cell cycle result. (N = 3. NT: Not Treated OMECs; OE: *SCGB2A2* Overexpressed OMECs; NS: Non-specific control RNA transfected OMECs; KD: *SCGB2A2* Knockdown OMECs. *: $P < 0.05$. ***: $P < 0.001$).

3. Results

3.1. The expression pattern of SCGB2A2 at different lactation periods in dairy sheep mammary

In order to clarify the expression pattern of SCGB2A2 at different lactation periods in dairy sheep mammary, the pregnancy, early lactation, peak lactation and dry milk periods mammary tissues were collected. The qRT-PCR and Western blot analysis showed that the expression of SCGB2A2 at early lactation was extremely significantly higher than at other periods ($P < 0.001$) (Fig. 1A, B). The IHC analysis result showed that it is mainly expressed at the mammary epithelial cells (Fig. 1C). It suggests that the SCGB2A2 may be involved in the development of mammary glands and the biosynthesis of milk components.

3.2. SCGB2 A2 promotes the proliferation of OMECs

In order to analyze the effect of SCGB2 A2 on the proliferation of OMECs, we established the *SCGB2 A2* OE and KD OMECs. As shown in Fig. S1B, the shRNA2 presented a better *SCGB2 A2* KD effect. And we selected it to establish the *SCGB2A2* KD OMECs.

Compared with NT OMECs, the expression of SCGB2A2 of OE OMECs at RNA and protein levels was extremely significantly higher ($P < 0.001$) (Fig. 2A, B). And the expression of SCGB2A2 of KD OMECs at the RNA and protein levels was extremely significantly lower than NS OMECs ($P < 0.001$) (Fig. 2A, B).

The CCK8 analysis result showed that the proliferation capacity of OE OMECs was significantly higher than others ($P < 0.01$ at 24 h and $P < 0.001$ at 48 h) (Fig. 2C). The cell cycle analysis result showed that the G1 phase of KD OMECs was significantly higher than NS ($P < 0.05$),

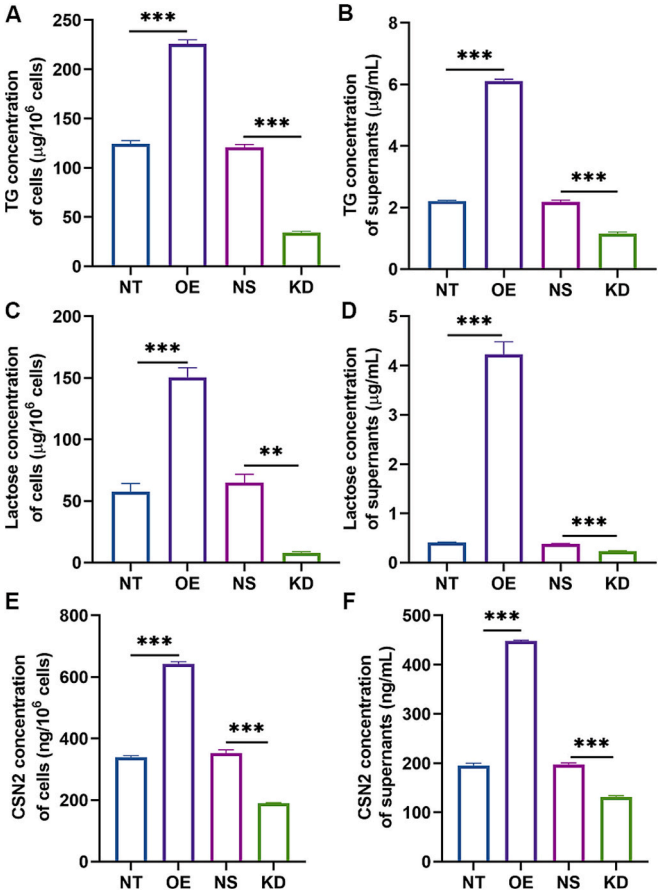

Fig. 3. Effect of SCGB2A2 for the biosynthesis of milk components in OMECs. A. TG analysis result of cells. B. TG analysis result of cell supernatant. C. Lactose analysis result of cells. D. Lactose analysis result of cell supernatant. E. CSN2 analysis result of cells. F. CSN2 analysis result of cell supernatant. (N = 3. NT: Not Treated OMECs; OE: *SCGB2A2* Overexpressed OMECs; NS: Non-specific control RNA transfected OMECs; KD: *SCGB2A2* Knockdown OMECs. ***: $P < 0.001$).

while the G2 + S + M phases were significantly lower ($P < 0.05$) (Fig. 2D). It suggests that the SCGB2A2 presents a positive regulatory effect on the proliferation of OMECs.

3.3. *SCGB2A2 promotes the biosynthesis of milk components*

In order to study the effect of SCGB2A2 on the biosynthesis capacity of milk components, we analyzed concentrations of TG, lactose and CSN2 in cells and supernatants after 2 days of culturing. As shown in Fig. 3, all three milk components were positively regulated by SCGB2A2 in OMECs. Compared with the NT OMECs, concentrations of TG, lactose and CSN2 in cells were all extremely significantly higher in OE OMECs, and these concentrations in KD OMECs were significantly lower than NS OMECs ($P < 0.01$) (Fig. 3A, C and E). And concentrations of TG, lactose and CSN2 in supernatants presented similar trends ($P < 0.001$) (Fig. 3B, D and F). It suggests that the SCGB2A2 promotes the biosynthesis of milk components in OMECs.

3.4. *Analysis of the SCGB2A2 regulatory mechanisms in OMECs*

In order to discover the SCGB2A2 regulatory mechanisms in OMECs, the transcriptome analysis was performed first. In the OE VS NT comparison, 1367 DEGs were screened, of which 704 were up-regulated and 663 were down-regulated (Fig. 4A, Table S1). In the KD VS NS comparison, 4627 DEGs were screened, of which 2248 were up-regulated and 2379 were down-regulated (Fig. 4B, Table S2). And 430 DEGs were consistently detected across two comparisons (Fig. 4C). The GO analysis showed that OE VS NT and KD VS NS DEGs were annotated to 703 and 838 terms, respectively (Table S3, S4). The KEGG result showed that DEGs of OE VS NT comparison were enriched to 309 pathways, including the MAPK signaling pathway, cell cycle and protein export (Table S5, Fig. 4D). DEGs of KD VS NS comparison were enriched to 333 pathways, including MAPK and PI3K-AKT signaling pathways (Table S6, Fig. 4F).

To investigate the SCGB2A2 DNA binding sites, the CUT & Tag assay was conducted utilizing an anti-flag antibody to isolate the DNA sequences that interact with SCGB2A2. Subsequently, these DNA sequences were subjected to analysis through DNA sequencing. The results indicated that SCGE2A2 has the capability to bind to the promoter-transcription start sites of 1286 genes (Table S7). According to the transcriptome and CUT & Tag co-analyses results, 20 genes were consistently detected, including *FBP2*, *IFIT3* and so on (Fig. 4F).

To identify potential co-interacted proteins, the Co-IP combined HPLC-MS analysis was performed. A total of 1474 co-interacted proteins were detected (Table S8). Novelty, the mTOR was screened. The Co-IP assay confirmed that there was an interaction between SCGB2A2 and mTOR (Fig. 4G). It suggests that the SCGB2A2 plays a role in OMECs through interaction with the mTOR.

4. Discussion

The SCGB2A2 is found to be abnormally expressed in multiple cancers. Here, we demonstrated that it plays a role in the regulation of OMECs proliferation and biosynthesis of milk components. Partial biological effects of SCGB2A2 are performed through binding with the mTOR.

Series studies have found that the SCGB2A2 promotes cancer cells malignant features, such as proliferation. Especially in most mammary carcinomas, it is overexpressed. In the breast cancer cells, the overexpressed SCGB2A2 promotes cells proliferation [20,28,29]. And the loss of SCGB2A2 decreases cell proliferation and invasion capacities [20,30]. In the ovarian normal and cancer cells, the concerted overexpression of SCGB2A2 and LIPB leads to a significant increase in the proliferation [31]. In this study, we found it presents higher expression in the early lactation and pregnancy periods, which are accompanied by the mammary development. The cell experiment also showed that the SCGB2A2 promotes the proliferation of OMECs. These findings suggest that the SCGB2A2 is positively regulating the proliferation in both cancer and normal cells.

SCGB2A2 is a transcription factor. A previous study showed that the loss of SCGB2A2 suppresses expressions of Cyclin D1, A2 and p-p65 in the breast cancer cells [28]. In this study, we also found that the expression of CCNA2 was regulated by SCGB2A2. It demonstrates that the regulation of SCGB2A2 for cell proliferation genes is conserved among species.

In addition, we found that expressions of cell proliferation-related genes *ADRA2C*, *TM7SF2*, *ARHGEF37*, *KCNN1*, *KANK2* and *PAWR* are affected by SCGB2A2. ADRA2C is a G protein-coupled receptor subtype. Except to play a crucial role in the regulation of neurotransmitter release [32], ADRA2C also inhibits the apoptosis of glioma cells [33]. Here, we demonstrated that SCGB2A2 positively regulates the expression of ADRA2C in OMECs and may decrease its apoptosis. The TM7SF2 is previously to be involved in the process of cholesterol biosynthesis [34]. However, another study showed that the disruption of TM7SF2 does not affect the biosynthesis of cholesterol in mice [35]. However, the liver cell proliferation is impaired in TM7SF2 knock-out mice [36]. In cervical cancer cells, the TM7SF2 regulates cell proliferation and apoptosis via the C-Raf/ERK pathway [37]. In this study, we found that the expression of *TM7SF2* is upregulated in the *SCGB2A2* OE OMECs (Table S1), which suggests that it may play a role in the OMECs proliferation., The overexpression of ARHGEF37 in hepatocellular carcinoma promotes the

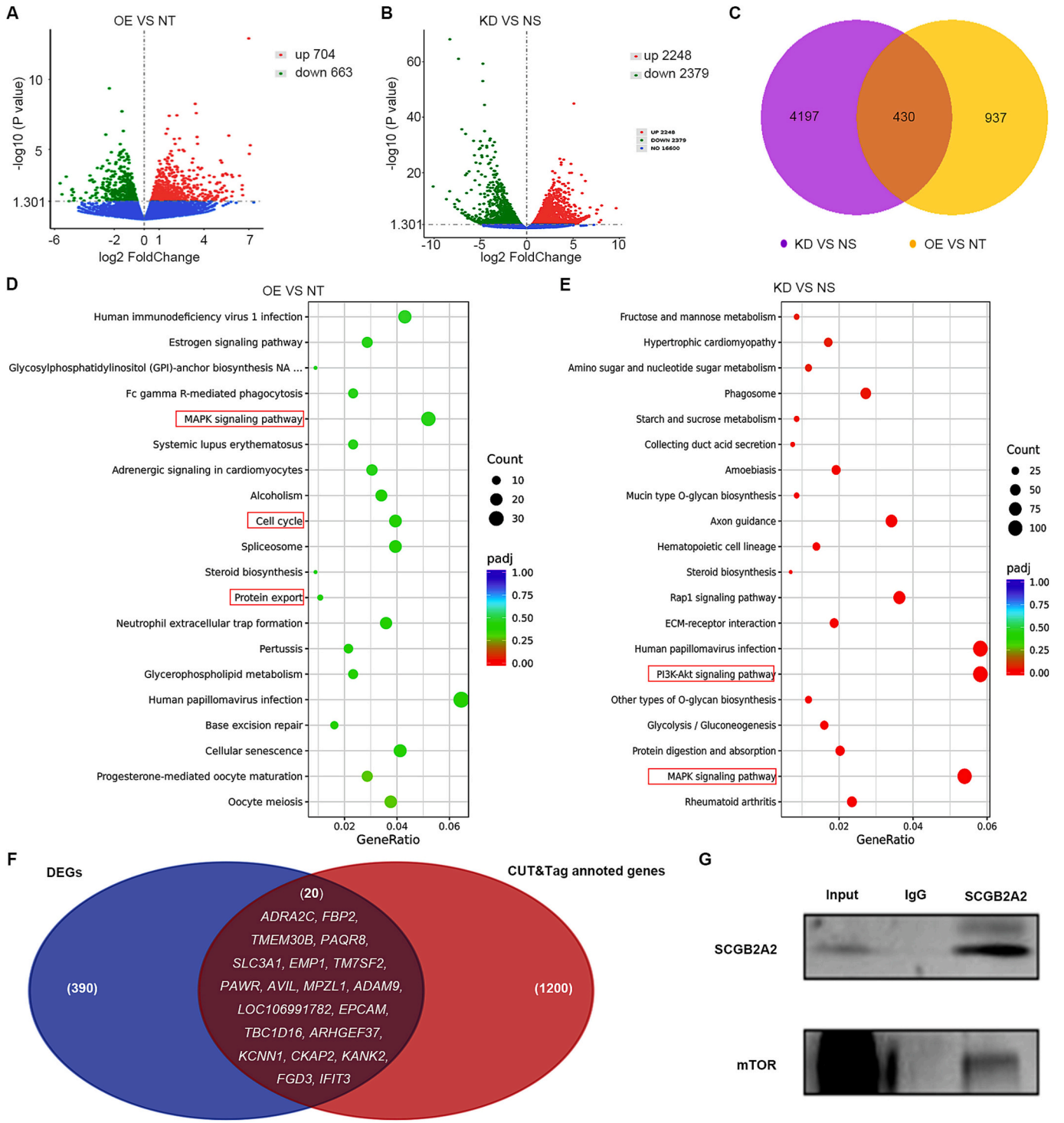

Fig. 4. Potential regulatory mechanism of SCGB2A2 in OMECs. A: Volcano plot of OE vs NT OMECs comparison DEGs. B: Volcano plot KD VS NS OMECs comparison DEGs. C: Venn diagram of DEGs. D: KEGG result of OE vs NT OMECs comparison DEGs. E: KEGG result of KD vs NS OMECs comparison DEGs. F: Venn diagram of transcriptome DEGs and CUT&Tag annotated genes. G: Co-immunoprecitation (co-IP) showed the interaction of SCGBA2A and mTOR in OMECs.

extravasation and metastasis [38]. Further study found that ARHGEF37 assists DNM2 during clathrin-mediated endocytosis [39]. Here, we surmise that ARHGEF37 participates in cell proliferation under the regulation of SCGB2A2. KCNN1 belongs to the KCNN family that widely exists in cells cytoplasmic membranes and intima. Recent studies showed that KCNN1 also co-locates with cytoskeleton actin filaments [40]. In breast cancer cells, KCNN1 is found to promote cell proliferation and metastasis via ERLIN2-mediated stabilization and K63-dependent Cyclin B1 ubiquitination [41]. Here, we found its expression is upregulated in SCGB2A2 OE OMECs and may participate the OMECs proliferation. KANK2 is a steroid receptor coactivator (SRC)-interacting protein [42]. It interacts with SRC co-activators and helps with transactivation [43]. A previous study showed that KANK2 suppressed the caspase-independent apoptosis through the prevention of mitochondria AIF releasing [44]. Another research demonstrated that it interacted with HSP70 to regulate cell apoptosis [45]. Here, we found that the

expression of KANK2 is upregulated in the *SCGB2A2* OE OMECs (Table S1), which suggests that it may suppress the apoptosis of OMECs. The PAWR is associated with cell apoptosis and autophagy [46–48]. It suppresses the expression of BCL2. In this study, we found that it is suppressed by SCGB2A2. Taken together, we surmise the cell proliferation effect of SCGB2A2 is not only the regulation of cell cycle but also the inhibition of cell apoptosis.

Here, we found that SCGB2A2 can enhance the biosynthesis capacities of milk fat, lactose and CSN2 in OMECs. However, most of SCGB2A2 regulated DEPs have no related function. The prolactin promotes milk production by activating Jak2/Stat5, mTOR, PI3K, MAPK, and other molecular signaling pathways in mammary epithelial cells. A study showed that SCGB2A2 can activate the MAPK (p38, JNK and ERK) and NFκB signaling pathways [20]. This study demonstrated that mTOR co-interacted with SCGB2A2. Among the numerous regulations governing the synthesis of cellular milk components, mTOR plays a central role. The mTOR is an atypical serine/threonine kinase and highly conserved through evolution. In multiple cells, it is involved in cell proliferation and metabolism in response to a variety of signals [49]. Its upstream regulators include AKT, PI3K, SREBP1, PPARγ, SCD and FASN [50]. And mTORC1 regulates milk fat and protein synthesis via RPL8, FASN, SREBP1 and SCD [18]. The SREBP1 and PPARγ pathways occupy main pathways in milk fat synthesis. In milk protein synthesis, mTOR signaling has also been shown to regulate the expression of various downstream effectors, including 4EBP1 and S6K1. The phosphorylation of S6K and 4EBP1 by mTOR activates the translation machinery and enhances the synthesis of milk proteins [18]. However, most of the common milk regulation-related mTOR pathway genes were not identified in this study. It suggests that it may affect these genes post-transcriptional regulation, such as translation and phosphorylation.

Interestingly, IFIT3 was identified as SCGB2A2 regulated gene in this study. It is one of the most important members in both the IFIT family and interferon-stimulated genes family [51]. It integrates into many key signaling pathways, including the JAK-STAT, IFN and Toll-like receptor (TLR)-mediated recognition pathways [52]. The expression of IFIT3 is upregulated during many virus infection procession [53]. Further studies revealed that it can inhibit the replication of some viruses [54]. In our experience, the STH sheep has better disease resistance than DM sheep. Considering that the expression of SCGB2A2 in STH milk is higher than DM milk, we surmise that it may be the upregulation of IFIT3.

5. Conclusion

In this study, we demonstrated that SCGB2A2 plays a positive role in OMECs proliferation and biosynthesis of milk components. The omics sequencing analysis revealed that some SCGB2A2 direct regulated genes are involved in cell proliferation, such as *ADRA2C*, *TM7SF2*, *ARHGEF37*, *KCNN1*, *KANK2* and *PAWR*. The regulation of SCGB2A2 for milk components biosynthesis mainly interacts with mTOR. However, the specific regulatory mechanism is needed to further study.

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CRediT authorship contribution statement

Sijia Ma: Writing – original draft, Methodology, Investigation. **Hui Bai:** Methodology, Investigation, Funding acquisition, Data curation. **Lidong Han:** Methodology. **Yijing Zhu:** Methodology. **Anping Xie:** Methodology. **Ruilin Fang:** Methodology. **Xvhui Hu:** Methodology. **Liguo Zhang:** Supervision, Conceptualization. **Chao Bian:** Writing – review & editing, Project administration, Investigation, Conceptualization. **Xiaohu Su:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Ethics approval and consent to participate

This study received approval from Inner Mongolia University Research Ethics Committee (approval number: 2021002). All experiments were performed according to Chinese laws and institutional guidelines. Informed consents were obtained from Monterra Husbandry Technology Development Co., Ltd. and Inner Mongolia Fengdongzhiying Husbandry Technology Co. Ltd. (Inner Mongolia, China) for the use of experimental sheep.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

The original contributions presented in the study are included in the article, and further inquiries can be directed at the corresponding author. The transcriptome data that support the findings of this study have been deposited into CNSA with accession number CNP0008772.

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