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Full Length Article

Role of IGFBP5 on Growth of Goat Mammary Epithelial Cells (MECs) during Different Lactation Stages *via* the PI3K/Akt Signaling Pathway

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Abstract

The mammary glands of dairy goats undergo physiological degeneration after peak lactation, resulting in a decline in milk yield. This phenomenon is closely related to mammary epithelial cells (MECs) apoptosis. The present study examined the expression levels of the insulin-like growth factor-binding protein-5 (IGFBP5) gene in the mammary tissues of dairy goats at various lactation stages and found that this gene was particularly highly expressed in the early and late lactation stages. In addition, the expression of IGFBP5 was assessed in different tissues from dairy goats in late lactation. IGFBP5 was expressed in various tissues, especially in the mammary glands. Overexpression of the IGFBP5 gene *in vitro* in cultured MECs from dairy goats inhibited the proliferation of the MECs and caused an increased in cell apoptosis. Western blot analysis of the proteins involved in the phosphoinositide-3-kinase (PI3K)/Akt signaling pathway revealed that overexpression of the IGFBP5 gene affected the PI3K/Akt signaling pathway, reduced the expression levels of PI3K and Akt, altered the balance between B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) and enhanced the expression level of the apoptosis-related protein caspase 3. The above results reveal that IGFBP5 is an essential regulatory factor in the growth of MECs. IGFBP5 inhibits the proliferation of MECs and promotes the apoptosis of MECs through the PI3K/Akt signaling pathway.

Keywords: Dairy goat; Mammary epithelial cells; IGFBP5; PI3K/Akt; Apoptosis

Introduction

Milk yield is closely related to the quantity and physiological activity of mammary epithelial cells (MECs). Among mammals in general, the mammary gland undergoes gradual degeneration during the late lactation period (Akhtar *et al*. 2016) and the state of balance between the proliferation and apoptosis of MECs has an important impact on changes in milk yield (Knight 2000; Capuco *et al*. 2003). There are many factors that influence the growth of MECs, including various hormones and cytokines, which are involved in regulating and controlling various biological processes such as gene transcription and key signaling pathways (McCormick *et al*. 2014). IGFBP5 is a major member of the insulin-like growth factor-binding protein family, able to bind to insulin-like growth factors and the extracellular matrix (ECM) as well as mucopolysaccharides (Mohan and Baylink 2002). Studies have shown that changes in the expression changes of IGFBP5 are associated with mammary gland development. For instance, during mammary gland degeneration in rodents, the expression of the IGFBP5 gene increased in MECs (Tonner *et al*. 1997). Analysis of the expression profile of IGFBP5 protein in *Bos indicus* and *Bubalus bubalis* at various lactation stages has revealed that IGFBP5 protein is highly expressed in the late stage of lactation. Additionally, compared with normal mammals, animals with a history of short lactation length (short-lactating animals) express increased higher levels of IGFBP5 protein (Mohapatra *et al*. 2014).

IGFBP5, with its unique structure, is an important regulator of activity of IGFs in the mammary gland. This is because the N-terminal domain of IGFBP5 contains special sites that bind to IGF-I (Ravid *et al*. 2008). Additionally, although the C-terminal domain of IGFBP5 does not bind directly to IGF-I, it potentially affects the IGF-I binding affinity of the protein. Studies have shown that the IGFBP5 gene participates in the regulation of MECs apoptotic processes and may influence cell survival (Dupont *et al*. 2002; Allan *et al*. 2004). Subcutaneous injection of mice with a recombinant IGFBP5 vector late in pregnancy led to mammary gland injuries, as evidenced by a decrease in mammary fat pad infiltration (Allan *et al*. 2002). Additionally, a study in IGFBP5 transgenic mice revealed that the DNA content was significantly reduced in the mammary glands of transgenic mice on the tenth day of gestation. Moreover, the number of MECs and the level of

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milk yield were decreased by approximately 50% within the first 10 d of lactation (Allan *et al*. 2004). However, the above studies merely explored the effect of IGFBP5 on MECs growth; little has been reported concerning further details or clear regulatory mechanisms. Recently, the PI3K/Akt signaling pathway has become a research hotspot. PI3K/Akt signal transduction plays as a crucial role in cell metabolism, survival, proliferation and migration (Kawiak and Lojkowska 2016). Activated PI3K and Akt activate or inhibit a series of downstream substrates such as Bcl-2-associated agonist of cell death (BAD), caspase 3 and Bcl-2 through phosphorylation, thereby regulating cell proliferation, differentiation, apoptosis and migration (Manning and Cantley 2007). For example, adolescent mammary gland development is affected by hormones and growth factors that activate the PI3K/Akt signaling pathway to induce epithelial cell proliferation and stimulate terminal end bud (TEB) formation and ductal branching (Meng *et al*. 2017).

The objectives of this study were to search for any changes in the expression of IGFBP5 gene in the mammary gland tissues of dairy goats at three different lactation stages, to explore the effect of the IGFBP5 gene on the growth of MECs, and to reveal the molecular mechanism by which IGFBP5 promotes the apoptosis of MECs through the PI3K/Akt signaling pathway. This study provides a new theoretical reference for the mechanism of MECs apoptosis in late lactation.

Materials and Methods

Animals and ethical statement

The five 4-year-old, third-parity Laoshan dairy goats used in the present study were obtained from the Aote goat breeding farm (a Laoshan dairy goat stock farm) in the Shandong Province, China. The goats were healthy and free of disease and were kept under the identical feeding and housing conditions. Mammary gland tissues were collected surgically under general anesthesia from goats at the early lactation stage (20 d postpartum) and peak lactation stage (90 d postpartum). The goats were sacrificed in the late lactation stage (210 d postpartum). Goat heart, liver, spleen, lung, colon, muscle, brain and mammary tissues were collected, frozen rapidly in liquid nitrogen and cryopreserved. All animal experiments were carried out under the guidance of the Shandong Agricultural University Animal Care and Use Committee (SDAUA-2017-40), and best efforts were made to reduce animal suffering during the operation.

Cell culture and transfection

Mammary tissue specimens were collected from Laoshan dairy goats, and the MECs were isolated and cultured using the tissue-block method. The MECs culture medium was prepared according to the following formula: 100 mg/mL streptomycin, 10 ng/mL epidermal growth factor (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin, 12% fetal bovine serum (Gibco, Grand Island, NY, USA), and 88% Dulbecco's modified Eagle's medium (DMEM)/F12, 5 mg/L insulin (Sigma, St. Louis, MO, USA). A culture environment was provided for cells at 37°C and 5% CO_2 concentration in a temperature-controlled incubator. The *in vitro*-purified dairy goat MECs were plated in 24-well plates. The cells were subjected to transfections when grown to approximately 65% confluence. Transfection with the overexpression vector was performed using Lipofectamine™ 2000 Transfection Reagent (Invitrogen). The IGFBP5 interference vector was synthesized by RiboBio (Guangzhou, China). Transfection with the interference vector was performed using a small interfering RNA kit.

RNA sample preparation and gene expression profile analysis

In this study, the tissues or cells were disrupted using a Bionoon-48 homogenizer (BIONOON, Shanghai, China), and RNA was extracted by adding TRIzol reagent (Transgen, Biotech, Beijing, China). The quality and purity of RNA were examined using agarose gel electrophoresis (5% gel) and a NanoDrop ND-2000 ultramicrospectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) was applied to perform a reverse transcription reaction. The PCR system (20 μL) was set up according to the instructions provided in the TB Green Premix Ex Taq kit (Takara) as follows: 10 μL of TB Green, 0.4 μL of forward primer, 0.4 μL of reverse primer, 2 μL of DNA template and 7.2 μL of sterile water (primers of IGFBP5: forward 5′-GGGTTTGCCTGAACGA-3′, reverse 5′-TCTCCTCTGCCATCTCG-3′, product size: 102 bp; primers of GAPDH: forward 5′-AGATAGCCGTAACTTCTGTG-3′, reverse 5′-GGGTGGAATCATACTGGA-3′, product size: 198 bp). PCR amplification was carried out using the LightCycler 96 PCR system (Roche, Basel, Switzerland). The PCR conditions included a pre-denaturation step at 95°C for 30 s, followed by 35 cycles of denaturation at 95°C for 5 sec, and extension at 60°C for 20 s. The melting curves were then analyzed. Each sample was subjected to three independent amplifications. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the internal reference. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Detection and analysis of protein expression

RIPA lysis buffer (Beyotime, Shanghai, China) was applied to lyse cells; the liquid lysate was then centrifuged and total protein was obtained from the supernatant. The Enhanced BCA Protein Assay Kit (Tiangen Biotech, Beijing, China) was used to measure the protein concentration. A separation gel with a concentration of 10% and an upper gel with a

concentration of 5% were prepared and then the target proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The electrophoresis time was 150 min. Next, the proteins in the gel were transferred to polyvinylidene fluoride (PVDF) membranes by electrophoresis. Subsequently, the PVDF membranes were blocked for 1 h using a blocking solution (5% fat-free milk in 1× Tris-buffered saline/TWEEN 20 (TBST)) and then were incubated with specific antibodies overnight. The antibodies used in this experiment were as follows: rabbit anti-goat IGFBP5 polyclonal antibody (Abcam, Shanghai, China), rabbit anti-goat PI3K polyclonal antibody (CST, Shanghai, China), rabbit anti-goat Akt polyclonal antibody (Abcam), rabbit anti-goat Bcl-2 monoclonal antibody (Abcam), and rabbit anti-goat Bcl-2-associated X (Bax) polyclonal antibody (Abcam), rabbit anti-goat casepase3 polyclonal antibody (Abcam), mouse anti-goat β-actin monoclonal antibody (Abcam), rabbit anti-goat GAPDH monoclonal antibody (CST), goat anti-mouse IgG HRP conjugated antibody (CWBIO, Beijing, China), and goat anti-rabbit IgG HRP conjugated antibody (CWBIO). After being washed three times with 1× TBST, the protein-loaded PVDF membranes were incubated with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. The proteins were then visualized using a BeyoECL Plus kit (Beyotime) on an Azure Biosystem C300 apparatus (Azure Biosystem, Dublin, CA, USA). The protein image was processed and quantified by ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA). The relative levels of the proteins of interest were calculated based on GAPDH or β-actin as an internal control protein.

Cell viability evaluation

MECs were recovered and cultured in 96-well plates. The number of cells seeded into each well was approximately 3,000. Then, the cells were cultured overnight at a temperature of 37°C under an atmosphere of 5% CO_2. A Cell Counting Kit-8 (CCK8; Sigma-Aldrich, Beijing, China) was used to examine cell viability. Specifically, cells were collected after 0, 24, 48 and 72 h of cell culture, and 10 ul of CCK8 reagent was added to each well. Then the 96-well plate was incubated at 37°C for 40 min. After incubation, the absorbance of the samples was measured at 450 nm using a SpectraMax iD3 microplate reader (Molecular Devices, San Jose, CA, USA). An overexpression group, an interference group, a blank control group and a negative control group were set up for the experiment. Each group contained 5 replicate wells, and every experiment was repeated three times independently.

Apoptotic assay

After 48 h of cell transfection, the MECs were resuspended in PBS buffer, and the number of cells was counted. The cells were mixed with binding buffer at a concentration of 1×10^6 cells/mL. According to the instructions provided in the Dead Cell Apoptosis Kit with Annexin V-FITC and PI (Invitrogen, Carlsbad, CA, USA), the cells were mixed thoroughly with 5 μL of Annexin V-fluorescein isothiocyanate (FITC) and 5 μL of propidium iodide (PI) staining solution and incubated at 4°C for 10 min in the dark. The cells were examined immediately after the incubation period using a flow cytometry sorter (BD, Franklin Lake, NJ, USA). FlowJo 7.6.1 software (Ashland, OR, USA) was applied to analyze and visualize the original flow cytometry data.

Data statistics and processing

Data collation and statistical analysis were performed using R software (Version 3.5.1). Statistical significance was analyzed by repeated-measures ANOVA and Tukey's honestly significant difference (HSD) test. When the *P* value was less than 0.05, the difference between different groups was considered significant. Unless otherwise stated, all the data shown in the present study were obtained from at least three biological replicates. The data are expressed as the mean ± standard error of the mean (SEM).

Results

The expression profile of the IGFBP5 gene in the mammary tissue of dairy goats at various lactation stages

The qRT-PCR was employed to examine the expression level of the IGFBP5 gene in the mammary tissue of dairy goats during the early (20 d), peak (90 d) and late (210 d) stages of lactation. The expression level of the IGFBP5 gene was significantly higher at the early and late lactation stages than at the peak lactation stage (Fig. 1A). Compared with the peak lactation stage, the expression level of the IGFBP5 gene was increased by 6.78- and 5.92-fold at the early and late lactation stages, respectively ($P < 0.01$). Additionally, examination of the IGFBP5 gene expression levels in multiple tissues of dairy goats revealed that the IGFBP5 gene was widely expressed in many tissues (Fig. 1B). Among the multiple tissues of the dairy goat, the highest expression of IGFBP5 was observed in ovarian tissue, followed by kidney and mammary tissues. In contrast, IGFBP5 gene expression was significantly reduced in the colon, heart, spleen, muscle, brain, liver and adipose tissues ($P < 0.05$).

MECs proliferation was regulated by IGFBP5

To discover and clarify the effect of the IGFBP5 gene on the growth activity of MECs in dairy goats, we constructed IGFBP5 overexpression and interference vectors and then transfected them into the *in vitro*-cultured dairy goat MECs. In the protein expression assay (Fig. 2), the group transfected with the IGFBP5 overexpression vector had a significantly increased level of IGFBP5 ($P < 0.01$) compared with the control group, while in the group transfected with the interference vector, the expression level markedly decreased

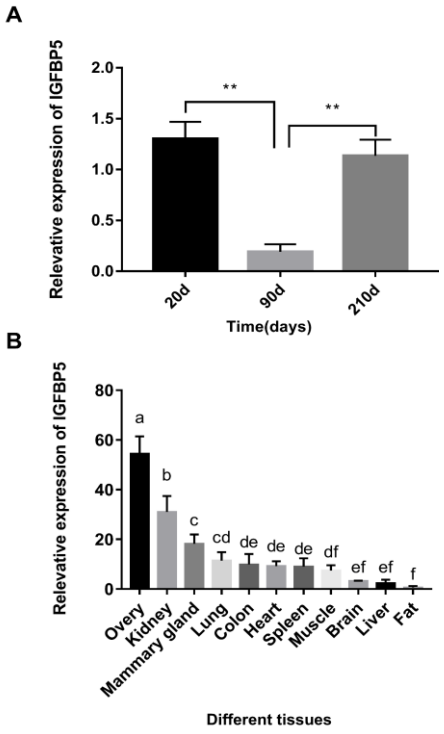

Fig. 1: Analysis of the expression profile of the IGFBP5 gene in various tissues of dairy goats

(**A**) The results of real-time fluorescence-based quantitative PCR analysis of IGFBP5 gene expression at different lactation stages. The abbreviation "20 d" refers to 20 days postpartum (early lactation stage), "90 d" refers to 90 days postpartum (peak lactation stage), and "210 d" refers to 210 days postpartum (late lactation stage). (**B**) Analysis of the expression abundance of the IGFBP5 gene in various tissues of dairy goats at the late stage of lactation. ** indicates highly significant differences ($P < 0.01$). Different lowercase letters above the columns indicate that the differences between the groups reached the significance level ($P < 0.05$)

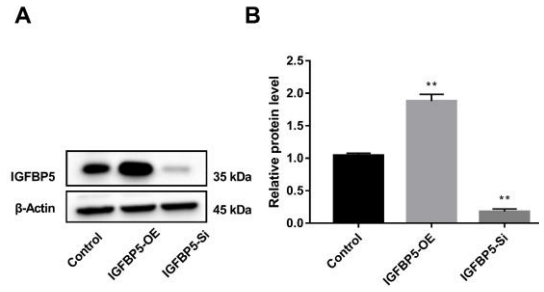

Fig. 2: Western blot analysis of the expression levels of the IGFBP5 gene *in vitro* in cultured MECs after overexpression or inhibition of the IGFBP5 gene

(**A**) Western blot results. (**B**) Gray value analysis of Western blot bands using ImageJ. Control refers to the blank control group, IGFBP5-OE refers to the IGFBP5 overexpression group, and IGFBP5-Si refers to the IGFPB5 interference group. ** indicates highly significant differences ($P < 0.01$)

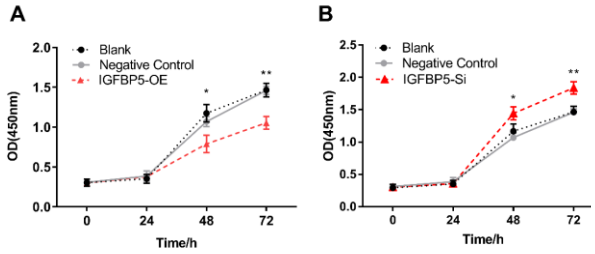

Fig. 3: Examination of the effect of IGFBP5 on the proliferative activity of *in vitro*-cultured MECs by the CCK8 assay

(**A**) Effect of IGFBP5 overexpression on the proliferative activity of MECs cultured *in vitro*. (**B**) Effect of the inhibition of IGFBP5 expression on the proliferative activity of MECs cultured *in vitro*. Blank refers to the blank control group, IGFBP5-OE refers to the IGFBP5 overexpression group, and negative control refers to the negative control group for the IGFBP5 overexpression vector. The cells were examined at 0, 24, 48 and 72 h after transfection using the SpectraMax iD3 microplate reader. * indicates significant differences ($P < 0.05$) and ** indicates highly significant differences ($P < 0.01$)

($P < 0.01$) after 48 h. Additionally, in order to examined the proliferative capacity of the cells, a CCK8 test kit was specifically used for detection at four different time points—0, 24, 48 and 72 h after cell transfection. The proliferation of the group overexpressing the IGFBP5 gene began to slow down at 24 h after transfection (Fig. 3A). Compared to negative group, the overexpression group exhibited significantly reduced viability at 48 h ($P < 0.05$). As shown in Fig. 3B, the group with interfered IGFBP5 gene expression exhibited proliferative activity similar to that of the negative control group until the 24-h time point. However, at 48 h, the proliferative activity was significantly higher in the IGFBP5 interference group than in the negative control group ($P < 0.05$). The above data indicate that the IGFBP5 gene exerts a certain inhibitory effect on the proliferation of MECs.

The IGFBP5 gene promotes the apoptosis of MECs

After 48 h of transfection, the cells were harvested into a sterile centrifuge tube with a capacity of 1.5 mL and the fluorescent dyes Annexin V-FITC and PI were used to stain the cells. The effect of the IGFBP5 gene on apoptosis was examined by flow cytometry and the results are shown in Fig. 4A-D. Based on comparison with the results of the control group, the number of apoptotic cells (Q2+Q3) was significantly increased in the group overexpressing the IGFBP5 gene ($P < 0.01$) but was markedly reduced in the interference group ($P < 0.05$). Flow cytometric analysis also found that the number of early apoptotic cells was increased by 8.08%, while the number of late apoptotic cells was increased by 4.9%, in the group overexpressing the IGFBP5 gene. By contrast, the percentages of early and late apoptotic cells were reduced in the group with interfered IGFBP5 gene expression compared with those in the control group (3.24% *vs*. 6.32% and 5.01% *vs*. 11.4%, respectively). Additionally, Western blot analysis was also applied to examine the expression of the apoptosis-related factor caspase 3 in this study; experimental results are shown in Fig. 4E-F. Based on comparison with the results of the control group, the expression level of caspase 3 protein was significantly increased in the IGFBP5 overexpression group ($P < 0.01$). The opposite results were found in the group with interfered IGFBP5 gene expression. The above results demonstrate that the IGFBP5 gene is related to the apoptosis of MECs. Overexpression of the IGFBP5 gene promoted the early

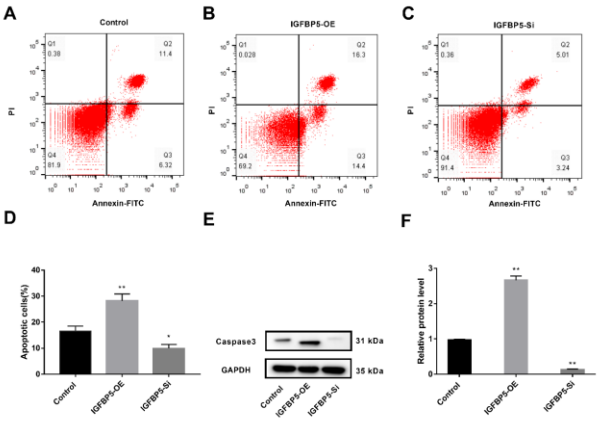

Fig. 4: Flow cytometric analysis of the effect of IGFBP5 on the apoptosis of *in vitro*-cultured MECs

(**A-D**) Effects of the *in vitro* overexpression and inhibition of IGFBP5 expression on apoptosis. (**E-F**) Western blot analysis of caspase 3 expression after *in vitro* overexpression and inhibition of IGFBP5 expression. The data are expressed as the mean ± SEM. Control refers to the blank control group, IGFBP5-OE refers to the IGFBP5 overexpression group, and IGFBP5-Si refers to the IGFPB5 interference group. * indicates significant differences ($P < 0.05$), and ** indicates highly significant differences ($P < 0.01$)

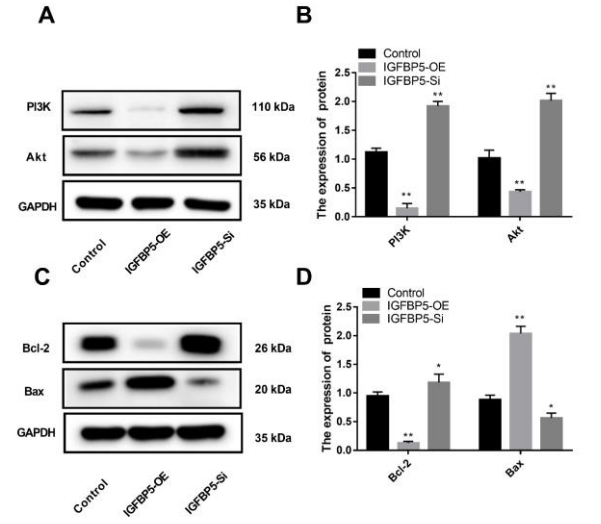

Fig. 5: Effects of *in vitro* overexpression and inhibition of IGFBP5 expression on the relative expression of the proteins in the PI3K/Akt signaling pathway

(**A-B**) Western blot analysis of the expression of PI3K and Akt proteins. (**C-D**) Western blot analysis of the expression of Bcl-2 and Bax protein. All the data are expressed as the mean ± SEM. Control refers to the blank control group, IGFBP5-OE refers to the IGFBP5 overexpression group, and IGFBP5-Si refers to the IGFPB5 interference group. * indicates significant differences ($P < 0.05$), and ** indicates highly significant differences ($P < 0.01$)

apoptosis of MECs.

Regulatory effect of the IGFBP5 gene on the components of the PI3K/Akt signaling pathway

To elucidate whether the effect of the IGFBP5 gene on MECs growth is related to the PI3K/Akt signaling pathway, we applied Western blotting to examine the changes in the expression levels of PI3K and Akt proteins in the MECs of the dairy goats from the normal control, IGFBP5 overexpression and interference groups. The experimental results showed that the expression of PI3K and Akt proteins was markedly reduced in MECs after overexpression of the IGFBP5 gene for 48 h ($P < 0.01$). By contrast, the interference group expressed significantly increased levels of PI3K ($P < 0.01$) and Akt proteins ($P < 0.05$) (Fig. 5A–B). Additionally, we examined the changes in the expression of the apoptotic factor Bax and the antiapoptotic factor Bcl-2 in MECs after overexpression and inhibition of the IGFBP5 gene. In the group overexpressing the IGFBP5 gene, the Bcl-2 expression level was significantly decreased, while the Bax expression level was considerably elevated. Interference with IGFBP5 gene expression yielded completely opposite results ($P < 0.05$) (Fig. 5C–D). Thus, from the above experiments, regulation of IGFBP5 gene expression altered the expression of PI3K and Akt, which are two key proteins in the PI3K/Akt signal transduction pathway. Moreover, the expression levels of apoptosis-related factors also changed significantly. Therefore, IGFBP5 could promote the apoptosis of MECs through the PI3K/Akt signaling pathway.

Discussion

The mammary glands of dairy goats undergo structural and physiological functional changes at different stages of lactation, often accompanied by changes in various physiological activities of the MECs, which may include gene replication, transcription, translation, material transport, energy metabolism and other activities (Tong and Hotamisligil 2007; Ji *et al*. 2013). As an important gene that can influence the growth of mammary gland epithelial cells, IGFBP5 is a very highly conserved member of IGF-binding protein family (James *et al*. 1993). The expression pattern of this protein in breast tissues is closely related to mammary gland development, milk secretion and breast cancer diseases (Liu *et al*. 2012). The results from our study indicated that the IGFBP5 gene was highly expressed in the mammary tissue of dairy goats at the early and late stages of lactation. In contrast, IGFBP5 gene expression was low in the peak lactation period. This expression pattern has also been found in previous lactation-related studies conducted in mice (Affolter *et al*. 2003) and cattle (Plath-Gabler *et al*. 2001). Interestingly, most studies have focused on the high expression of IGFBP5 in the mammary gland in late lactation, ignoring the role of IGFBP5 in mammary gland development and lactation during early lactation. On the one hand, the reasons for the high expression level of IGFBP5 during early lactation may be related to the higher expression of IGFBP5 in puberty and pregnancy (Allar and Wood 2004); on the other hand, it may be that IGFBP5 expression level is affected by related hormones, such as prolactin (PRL), which inhibits the production of IGFBP5 during lactation (Colitti and Farinacci 2009). In addition, IGFBP5 can be produced either by local cells in the mammary gland

or from the liver to the mammary gland region (Phillips *et al*. 1993). To further understand the expression pattern of the IGFBP5 gene, we examined the expression status of IGFBP5 in various tissues of dairy goats at the late lactation stage (Fig. 1B). The IGFBP5 gene was particularly highly expressed in mammary tissue. A study has reported that a high level of IGFBP5 expression damages the mouse mammary gland and promotes the apoptosis of mouse MECs (Phillips *et al*. 1993). Therefore, we speculated that the IGFBP5 gene might have a similar effect on the growth of dairy goat MECs. In the present study, the IGFBP5 gene was overexpressed or inhibited in *in vitro*-cultured dairy goat MECs. The results showed that IGFBP5 overexpression inhibited the proliferation of MECs and promoted the apoptosis of MECs in dairy goats. Combined with the expression characteristics of IGFBP5 in different lactation stages, the potential function of IGFBP5 in mammary gland-related physiological activities was further revealed.

IGF-I is a well-known survival-promoting antiapoptic factor. The PI3K/Akt pathway is related to IGF-I-induced cell survival (Kim and Park 2018). IGFBP5 binds to IGF-I, thereby affecting cell proliferation and apoptosis, and several studies have confirmed this conclusion. For example, intact IGFBP5 promotes chondrocyte proliferation through binding to IGF-I. The C-terminal domain of IGFBP5 binds firmly to the cell membrane, improving the presentation of IGF-I to its receptor (Kiepe *et al*. 2005). Additionally, IGFBP5 is a potential tumor suppressor that inhibits the signal transduction and functional output of IGF-I and blocks the proliferation and migration of cancer cells (Ding *et al*. 2016). Therefore, we speculated whether IGFBP5 participates in the regulation of the PI3K/Akt signaling pathway by affecting IGF-I. However, IGFBP5 was also reported to exert its regulatory effects independently. For example, a study has shown that when exerting its effect independently, IGFBP5 acts as a survival factor to promote the proliferation of muscle cells (Cobb *et al*. 2004). IGFBP5 not only exerts its effect through different modes of action but also plays distinct roles in various cell types and environments. As mentioned earlier, IGFBP5 promotes apoptosis in nerve cells and mouse MECs. However, in an inflammatory environment, recombinant human IGFBP5 stimulated the proliferation and migration of mesenchymal stem cells and induced the differentiation of mesenchymal stem cells toward bone/dentine (Schmidt *et al*. 2014). Therefore, we speculate that the diversity of IGFBP5 function is related to cell type, environment, and the dependence status on its ligands (such as IGF-I).

The activated PI3K/Akt signaling pathway participates in the regulation of proliferation of various cells, including muscle cells (Fang *et al*. 2016), fibroblasts (Jung *et al*. 2007), cancer cells (Vara *et al*. 2004), stem cells (Ling *et al*. 2013) and MECs (Zhu and Nelson 2013). Additionally, mammary gland development is under the potential regulation of the PI3K/Akt signaling pathway (Wickenden and Watson 2010; Schmidt *et al*. 2014). Many functions of PI3K in the regulation of cellular physiological activity are related to its ability to activate the serine/threonine kinase Akt. Therefore, Akt, located downstream of the signaling pathway can be regulated by PI3K. Activated Akt can affect the morphology of MECs and the branches of mammary ducts (Engelman 2009). IGF-I inhibited the expression of connective tissue growth factor through the PI3K-Akt signaling pathway, thereby promoting the proliferation of MECs in dairy cows (Zhou *et al*. 2008). Recently, a study reported that lauric acid stimulated the development of the mammary gland in adolescent mice by activating the PI3K/Akt signaling pathway (Meng *et al*. 2017). The present study showed that overexpression of the IGFBP5 gene in the MECs of dairy goats inhibited the PI3K/Akt signaling pathway, resulting in the downregulation of PI3K and Akt expression levels. Interference with IGFBP5 gene expression yielded completely opposite results. Bcl-2 and Bax are significant factors involved in apoptotic regulation, and they are part of the PI3K/Akt signal transduction pathway. Their interaction and balance affect cell survival. Therefore, changes in Bcl-2 and Bax were also detected after upregulation or interference of IGFBP5 in MECs of dairy goats. When the IGFBP5 gene was overexpressed in MECs, the expression of Bcl-2 was inhibited, while the expression of Bax was increased. The above experimental data demonstrated that IGFBP5 is a key factor in the regulation of MECs survival in dairy goats. Changes in the expression of IGFBP5 can induce changes in apoptosis-related proteins and then cause a transition of cell fate. IGFBP5 could inhibit the proliferation and promote the apoptosis of dairy goat MECs by participating in the regulation of the PI3K/Akt signaling pathway. However, the process of apoptosis is complex and changeable. This study did not verify whether IGFBP5 relies on IGF-I to participate in the regulation of the PI3K/Akt pathway or exerts a regulatory effect independently. In summary, our results demonstrate that IGFBP5 is an important regulatory factor capable of affecting mammary epithelial growth. IGFBP5 can promote the apoptosis of MECs through the PI3K/Akt signaling pathway.

Conclusion

By constructing IGFBP5 overexpression and interference vectors and employing *in vitro*-cultured MECs as the experimental model, the present study demonstrated that the IGFBP5 gene exhibits distinct differential expression patterns in various tissues of dairy goats at different lactation stages. The IGFBP5 gene is highly expressed in the early and late lactation periods. Upregulation of the IGFBP5 gene can prevent cell growth and accelerate apoptosis of MECs. The changes in the PI3K/Akt signaling pathway in MECs were also verified, indicating that the IGFBP5 gene can affect the survival of mammary cells via this pathway. The present study explored the molecular mechanisms with regard to the effects of the IGFBP5 gene on the growth of dairy goat MECs, providing a theoretical basis to understand the role of

IGFBP5 in mammary gland development and lactation in dairy goats.

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