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Expression of ESR1, PRLR, GHR, and IGF1R in mammary glands of Hu sheep with four teats

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Abstract: Supernumerary teats are commonly found in sheep. To investigate the expression levels of hormone receptors in supernumerary teats of Hu sheep, mammary tissue samples were collected from two groups of Hu sheep, four- and two-teat sheep, during adolescent (A), pregnancy (P), lactation (L), and non-pregnancy (N). Using hematoxylin and eosin staining, we found that mammary alveoli were denser in the four-teat sheep than in the two-teat sheep during P and L. Immunohistochemistry and Western blotting were used to detect the expression patterns and relative protein expression levels of the estrogen receptor 1 (ESR1), prolactin receptor (PRLR), growth hormone receptor (GHR), and insulin-like growth factor 1 receptor (IGF1R) in Hu sheep mammary glands during the four periods. All four receptors were mainly expressed in mammary epithelial cells and adipose cells. Furthermore, the expression levels of PRLR and GHR in the four-teat sheep were significantly higher than those in the two-teat sheep during P and L. Our data suggest that four-teat sheep have more developed mammary gland tissue compared with two-teat sheep.

Keywords: supernumerary teats; mammary tissue; immunohistochemistry; hormone receptor

Hu sheep, a unique breed from China, is a famous multiple-birth breed in the world. However, the ewes that give birth to a large litter may not produce enough milk for their lambs. Supernumerary teats (SNTs) are commonly seen in cattle (Brka et al. 2002; Pausch et al. 2016), humans (Kotrulja 2015), sheep (Peng et al. 2017), and goat (Kashyap et al. 2014). In Hu sheep, four-teat ewes have lacteal output comparable to two-teat ewes. Therefore, if four teats of Hu sheep are at a suitable distance and of the same size, lambs have a better chance to be fed. What means that up to four lambs can suck the milk, but the amount would be the same as in two-teat ewes.

Mammary glands are composed of stromal tissue and essential tissue, and stromal tissue includes adipose tissue, connective tissue, and the circulatory system (Silberstein 2015). Lobes divided into many lobules by connective tissue are the structural unit of the mammary essential part, and lobules are composed of alveoli and mammary ducts (Lawrence et al. 2002). The vascular and lymphatic systems in the mammary mesenchyme play a key role in supplying nutrients to mammary alveoli.

Estrogen (E2), growth hormone (GH), and prolactin (PRL) induce the development and differentiation of mammary glands. Experiments showed that E2 and its receptor are regulatory factors of the mammary gland development (Mueller et al. 2002). The calf mammary gland, which failed to develop after ovariectomy, recovered after an injection of E2 (Wallace 1953). Ovariectomy induced a rapid decrease in the level of serum E2 and the expression of the estrogen receptor 1 (*ESR1*) in mammary epithelial cells (MECs) of dairy goat (Dessauge et al. 2009). PRL is a key hormone promoting the mammary growth and lactation. PRL and E2 work together to

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promote the synthesis of α_2-lactoglobulin (Goodman et al. 1980), and GH, which can bind to and activate PRL, also promotes the mammary development (Ilkbahar et al. 1999). Studies in ruminants have shown that treatment with GH during adolescence can promote the late mammary gland development. Furthermore, treatment with GH during late pregnancy can promote the mammary gland development and lactation, as well as proliferation of mid-lactation mammary parenchyma, but not the breast growth during early lactation (Sejrsen et al. 1999). Resection of the pituitary gland greatly reduced development of mammary ducts in rats, which was restored after treatment with IGF1, showing that IGF1 mediates the PRL action and promotes the mammary gland development similar to GH, what induces the production of IGF1 in the liver and increases the *IGF1* mRNA expression in breast tissue (Walden et al. 1998).

Thus, the processes of mammary gland development and lactation are regulated by many bioactive factors, including E2, PRL, GH, and IGF1. The aim of this study was to investigate whether four-teat sheep have more developed mammary gland tissue than two-teat sheep.

MATERIAL AND METHODS

Ethics statement. All animals in this study were maintained according to the No. 5 proclamation of the Ministry of Agriculture, P.R. China. Animal protocols were approved by the Review Committee for the Use of Animal Subjects of Northwest A&F University. The sheep were euthanized following intravenous injection of a barbiturate (30 mg/kg).

Mammary tissue collection. All Hu sheep were from the YuanSheng sheep farm, Yongchang County, Jinchang City, Gansu Province, China. The mammary tissues of four-teat sheep from the lobules that correspond to two-teat (normal) sheep were collected during the following four periods: adolescent (A), pregnancy (P), lactation (L), and non-pregnancy (N). The sheep of group A were all adolescent and about 200 days old. The sheep of group P were all in the mid-trimester of pregnancy (80 days of pregnancy). The sheep of group L were all in the mid-trimester of lactation (30 days of lactation). The sheep of group N were all non-pregnant and at 30 days after stopping lactation. Each group had three ewes for four-teat sheep and two-teat sheep, respectively.

Hematoxylin and eosin staining. Mammary gland tissue samples were fixed in Bouin's solution at 4°C for 48 h and then embedded in paraffin. Paraffin blocks were cut into 5-μm sections. The paraffin sections were dewaxed with dimethylbenzene and ethyl alcohol, then stained with hematoxylin for 2 min, washed with 5% acetic acid, followed by differentiation in 0.3% acid alcohol for 30 s, washed again with distilled water for 5 min, and stained with eosin for 1 min. After staining, the sections were destained with dimethylbenzene and ethyl alcohol, mounted on a microscope slide, and sealed with neutral gum.

Immunohistochemistry. Paraffin sections were dewaxed in xylene and rehydrated in decreasing concentrations of alcohol. After citrate buffer antigen retrieval, the sections were blocked with normal goat serum (CWBio, China) and incubated overnight with primary antibody. The primary antibodies used included rabbit anti-PRLR (BA3818, 1 : 100), rabbit anti-ESR1 (BM4463, 1 : 30), rabbit anti-GHR (BA1619, 1 : 100), and rabbit anti-IGF1R (BA0498, 1 : 100) antibodies, which were purchased from Wuhan Boster Biological Technology, Ltd., China. Then, the sections were washed three times with phosphate-buffered saline (PBS; pH 7.4), and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 30 min at 37°C. After the sections had been washed three times with PBS, the colour reaction was developed with the substrate diaminobenzidine, and then slides were washed under running water for 5 min before being counterstained with haematoxylin (Carnaxide). After restaining the nucleus with haematoxylin and ammonia, the slides were dehydrated with increasing concentrations of ethanol and sealed with xylene for 5 min. The sample treated with only secondary antibody without primary antibody was used as Control.

Western blotting. Samples of mammary gland tissue from each group were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium dodecyl sulfate (SDS)) with phenylmethylsulfonyl fluoride (0.1 mg/ml), then homogenized, and put on ice for 1 h. The lysates were centrifuged at 4°C for 5 min at 12 000 rpm, and the supernatants containing soluble proteins were collected. Protein concentrations in the supernatants were measured using the BCA protein assay kit (Solarbio, China). The supernatants were then mixed with loading buffer and boiled for 5 min. The samples were run on 8% SDS-polyacrylamide gel and transferred to a

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polyvinylidene difluoride membrane. The membranes were blocked in 5% skim milk at room temperature for 2 h and incubated with a primary antibody overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody at room temperature for 2 h. After three washes with Tris-buffered saline Tween-20 (TBST) for 5 min each time, the proteins were detected using enhanced chemiluminescence (Advansta, USA). The primary antibodies were the same as reported above (dilution 1 : 200 for all). Goat anti-β-actin (BM0626, 1 : 200) was used as internal reference, quantification was performed using the Quantity One program (Bio-Rad, USA) in this study.

Statistical analysis. Statistical analysis was performed using the SPSS 19.0 software package. Data are presented as the mean ± standard error of the mean (SE) of three independent experiments. Differences between the groups were considered significant at $P < 0.05$.

RESULTS

Mammary gland structure of four-teat Hu sheep. To examine the differentiation patterns of mammary glands of Hu sheep and the difference between the two groups, hematoxylin and eosin (HE) staining was used. As shown in Figure 1, adipose tissue and connective tissue were stained during A (Figure 1A, 1a). In the mammary glands of the four-teat sheep, more gland catheters were seen compared with those of the two-teat sheep. During P (Figure 1B, 1b), mammary alveoli rapidly developed close to each other. Note that more mammary gland alveoli were stained in the four-teat sheep than in the two-teat sheep and more alveoli replaced adipose tissue and connective tissue in both groups when compared with the pattern were observed during A. During L, the mammary glands of both groups were filled with alveoli for secreting milk (Figure 1C, 1c). During N, because milk secretion by the mammary glands decreased, alveoli became smaller, and some debris of apoptotic cells was seen. In addition, the amounts of adipose tissue and connective tissue continued to grow (Figure 1D, 1d).

Expression patterns of ESR1 in mammary glands during A, P, L, and N. Immunohistochemistry (IHC) was used to examine the ESR1 expression patterns in mammary glands of the four-teat and two-teat sheep during the four periods (Figure 2). ESR1 was detected in adipose tissue, connective tissue, and alveolar buds during A (Figure 2A, 2a). During P, ESR1 expression was mainly detected in MECs as the mammary gland entered the period of rapid growth and differentiation (Figure 2B, 2b). ESR1 was also detected in MECs during L (Figure 2C, 2c) and N (Figure 2D, 2d). Western blotting (WB) results showed there was no significant difference on the protein level of ESR1 between four-teat sheep and two-teat sheep (Figure 2E).

Expression patterns of PRLR in mammary glands during A, P, L, and N. Because PRL plays a key role in mammary gland development and lactation, changes were detected in the prolactin receptor (PRLR) expression. Similar to the ESR1 expression patterns, the PRLR antibody stained adipose tissue, connective tissue, and alveolar buds during A (Figure 3A, 3a). During P and L, PRLR expression was mainly found in MECs, which rapidly increased during these two periods (Figure 3B, 3b, 3C, 3c). During N (Figure 3D, 3d), PRLR was expressed in MECs and connective tissue similar to ESR1. Moreover, the protein expression levels of PRLR in the four-teat sheep were higher than those in the two-teat sheep (Figure 3E) during P ($P < 0.05$), L ($P < 0.01$), and N ($P < 0.05$).

Expression patterns of GHR in mammary glands during A, P, L, and N. In order to investigate the changes in growth hormone receptor (GHR) expression in mammary glands of the four-teat and two-teat sheep, we examined its patterns of expression during the four periods. During A, the antibody to GHR stained adipose tissue, connective tissue, and alveolar buds (Figure 4A, 4a). During P and L, GHR was detected in MECs of mammary alveoli (Figure 4B, 4b, 4C, 4c). Then, during N (Figure 4D, 4d), mammary alveoli and connective tissue were obviously stained. Using WB, we found that the four-teat sheep had a higher GHR expression level compared with those in the control (Figure 4E) during P ($P < 0.01$) and L ($P < 0.05$).

Expression patterns of IGF1R in mammary glands during A, P, L, and N. As mentioned above, IGF1 and GH may work together to promote the growth of mammary glands. Therefore, IHC was used to detect the expression pattern of its receptor in the mammary gland. Similar to the GHR expression pattern, insulin-like growth factor 1 receptor (IGF1R) was detected in adipose tissue, connective tissue, and alveolar buds during A (Figure 5A, 5a) and was seen in MECs of mammary alveoli during P and L (Figure 5B, 5b, 5C, 5c). During L, the ex-

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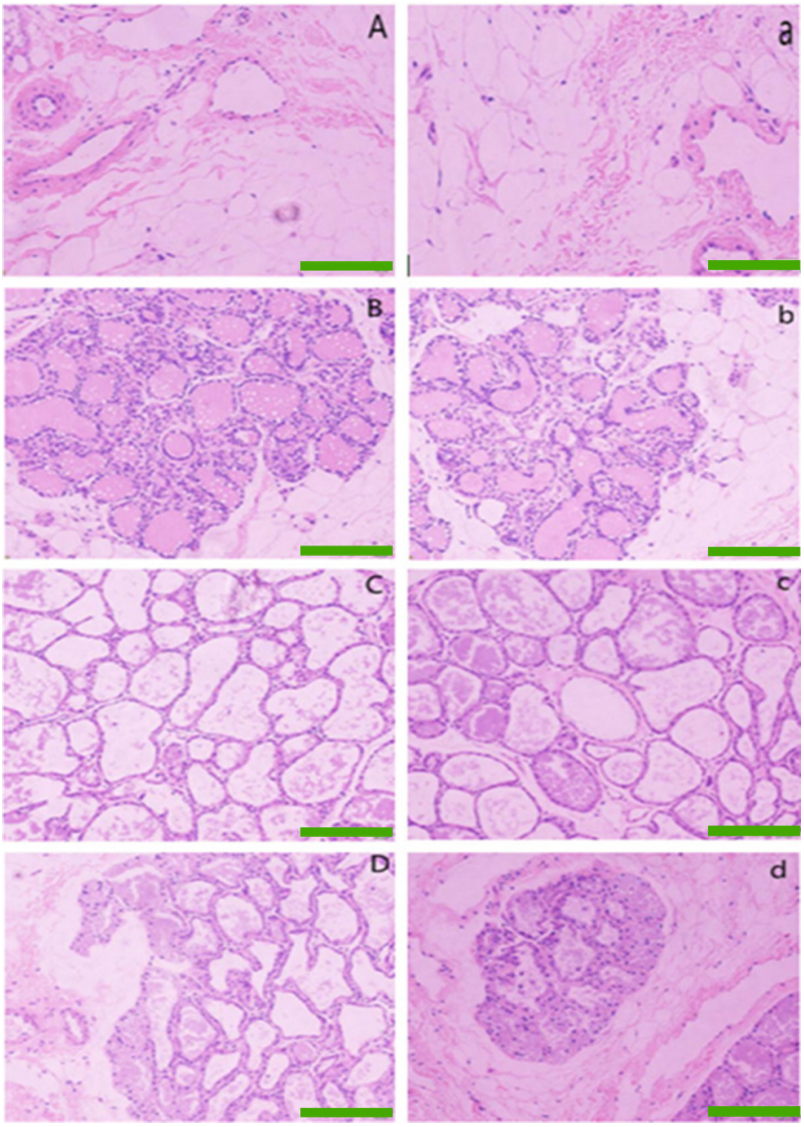

Figure 1. HE-stained sections of mammary glands of four-teat sheep (**A**–**D**) and two-teat sheep (**a**–**d**) during four periods: adolescent (**A**, **a**), pregnancy (**B**, **b**), lactation (**C**, **c**), and non-pregnancy (**D**, **d**). Green scale bars are 80 μm

pression level of IGF1R in the four-teat sheep was significantly higher than that in the two-teat sheep (Figure 5C, 5c). During N, the antibody for IGF1R mainly stained alveoli in the essential mammary part. Unlike GHR, staining could hardly be seen in connective tissue (Figure 5D, 5d). In addition, we noticed that the IGF1R expression in the four-teat sheep was significantly higher than that in the two-teat sheep during L ($P < 0.01$) and N ($P < 0.05$) (Figure 5E).

DISCUSSION

The postnatal mammary gland development is divided into several stages, including virginity, pregnancy, lactation, and involution, during which the mammary gland exhibits significant structural and functional changes. The mammary gland alveolus is the basic unit of lactation, and the number of alveoli is proportional to the lactation ability of the mammary gland (Brisken and Rajaram 2006). The developmental period of the gland and its nutritional status are closely related to the number of lobules and alveoli (Knight and Peaker 1978). In this study, four-teat sheep have a better potential for lactation based on the observations of the microstructure of mammary glands in the two groups of sheep.

Previous studies have shown that the mammary gland development and lactation were mainly regulated by the endocrine system, including *ESR1*, PR, GH, PRL, insulin, and the adrenal cortical hormone, and their receptor (Ilkbahar et al. 1999; Kelly et al. 2002). Our current study revealed the ESR1, PRLR,

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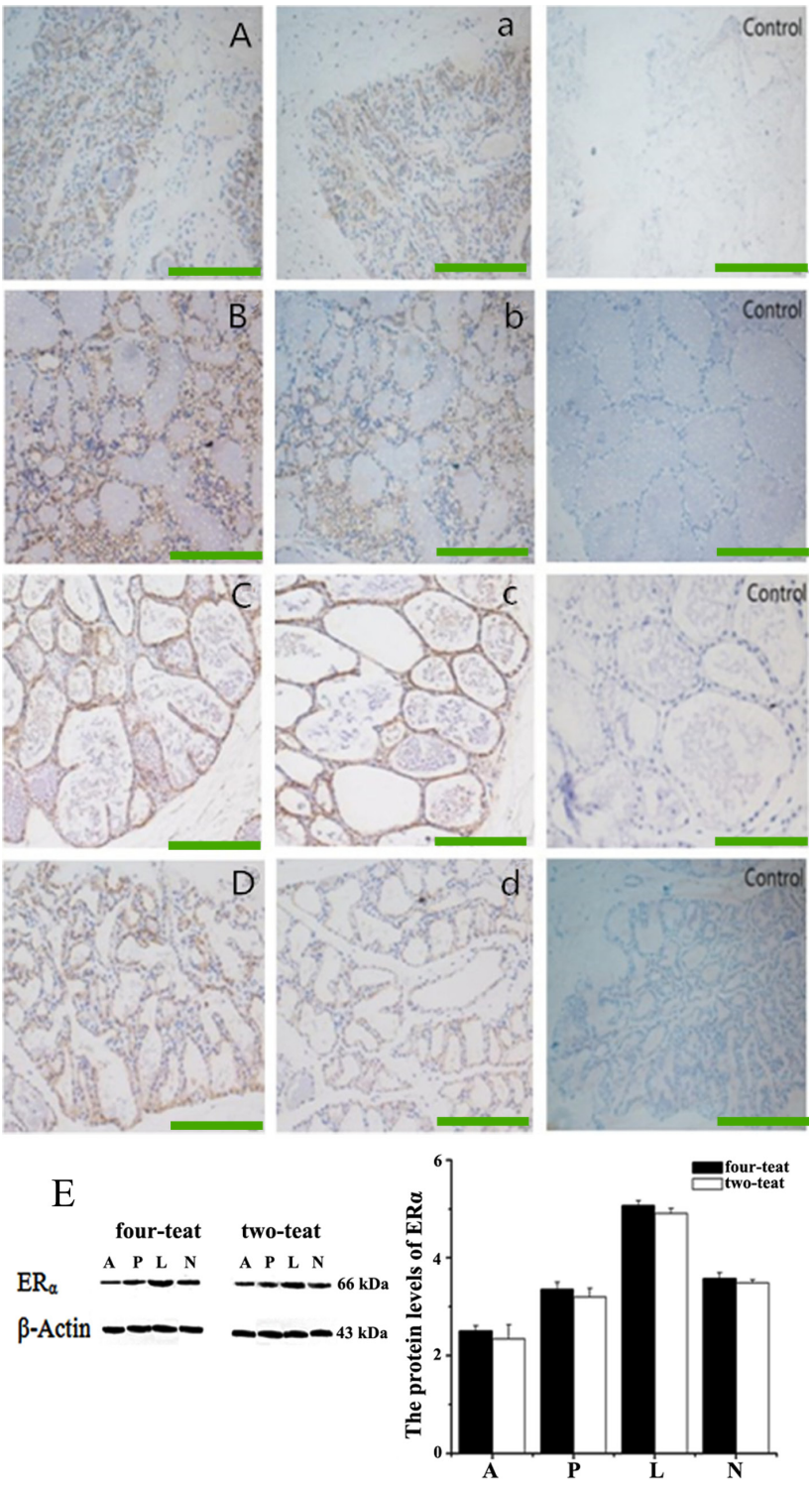

Figure 2. Expression patterns of ESR1 in mammary gland tissue. Immunohistochemistry for the ESR1 protein was performed in mammary gland sections during adolescent (**A**, **a**), pregnancy (**B**, **b**), lactation (**C**, **c**), and nonpregnancy (**D**, **d**) periods for four-teat sheep (**A**–**D**) and two-teat sheep (**a**–**d**); the sample treated with only secondary antibody without primary antibody was used as Control. Green scale bars are 80 μm. (**E**) Protein expression levels of ESR1 in breast tissue during different stages; Western blotting was used to measure proteins levels of *ESR1* in mammary glands during adolescent (A), pregnancy (P), lactation (L), and non-pregnancy (N) periods; β-actin was used as a loading control. All experiments were performed in duplicate and repeated three times. Values are presented as means ± SE

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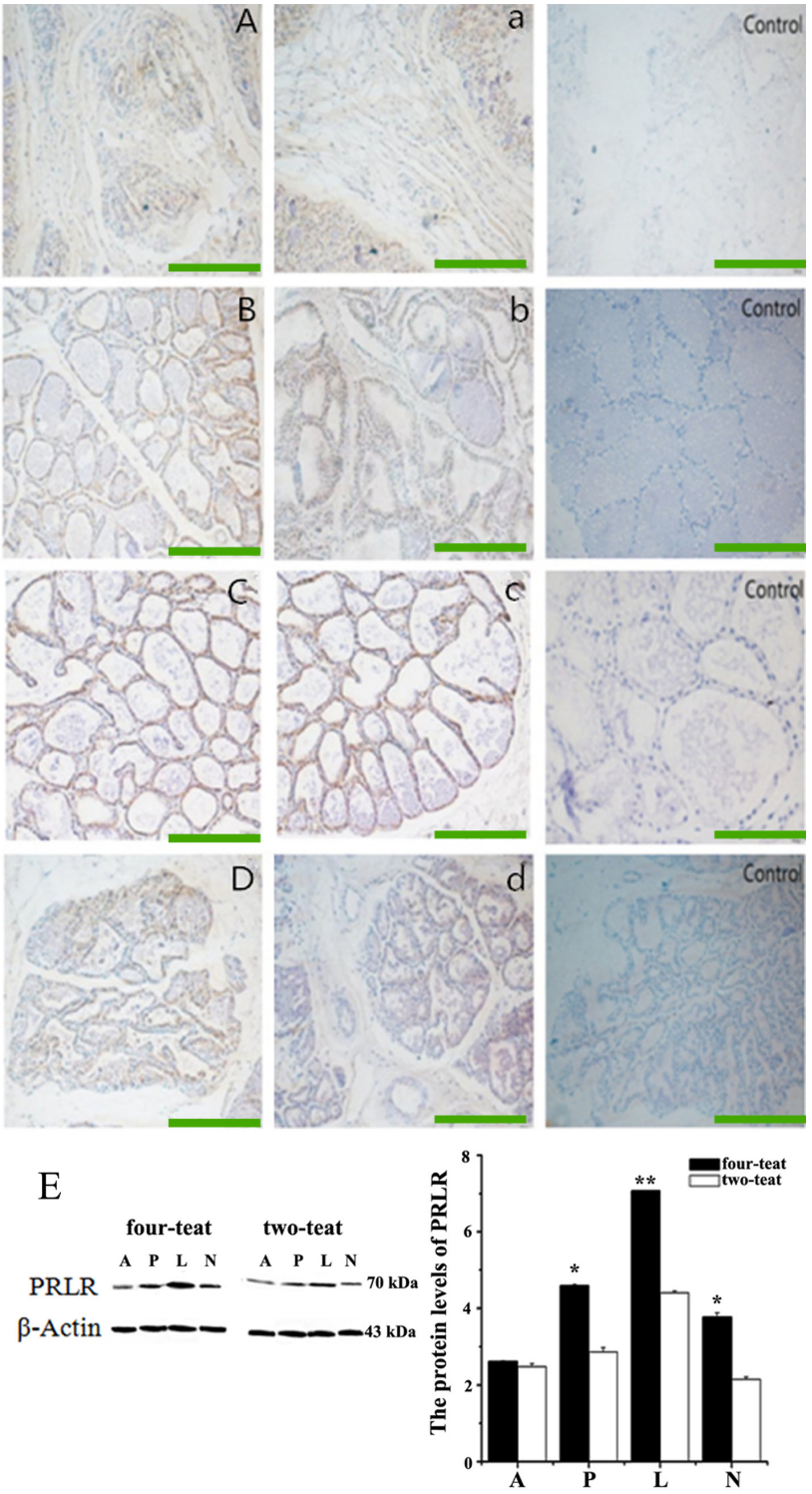

Figure 3. Expression patterns of PRLR in mammary gland tissue. Immunohistochemistry for the PRLR protein was performed in mammary gland sections during adolescent (**A**, **a**), pregnancy (**B**, **b**), lactation (**C**, **c**), and non-pregnancy (**D**, **d**) periods for four-teat sheep (**A**–**D**) and two-teat-sheep (**a**–**d**); the sample treated with only secondary antibody without primary antibody was used as Control. Green scale bars are 80 μm. (**E**) Protein expression levels of PRLR in breast tissue during different stages; Western blotting was used to measure proteins levels of PRLR in mammary glands during adolescent (A), pregnancy (P), lactation (L), and non-pregnancy (N) periods; β-actin was used as a loading control. All experiments were performed in duplicate and repeated three times; values are presented as means ± SE; $^{*}P < 0.05$, $^{**}P < 0.01$

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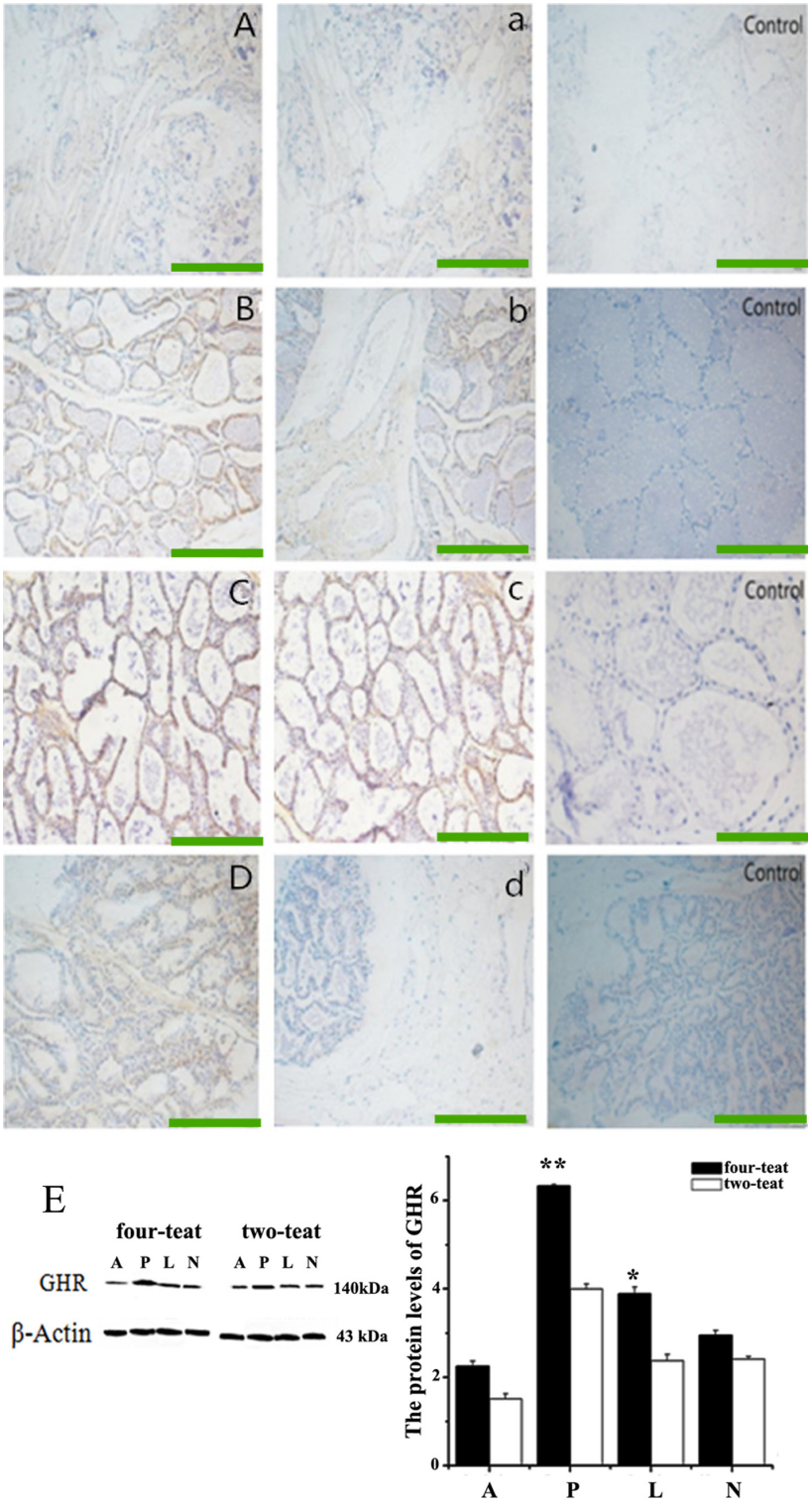

Figure 4. Expression patterns of GHR in mammary gland tissue. Immunohistochemistry for the GHR protein was performed in mammary gland sections during adolescent (A, a), pregnancy (B, b), lactation (C, c), and non-pregnancy (D, d) periods for four-teat sheep (A–D) and two-teat-sheep (a–d); the sample treated with only secondary antibody without primary antibody was used as Control. Green scale bars are 80 μm. (E) Protein expression levels of GHR in breast tissue during different stages; Western blotting was used to measure proteins levels of GHR in mammary glands during adolescent (A), pregnancy (P), lactation (L), and nonpregnancy (N) periods; β-actin was used as a loading control. All experiments were performed in duplicate and repeated three times; values are presented as means ± SE; $^{*}P < 0.05$, $^{**}P < 0.01$

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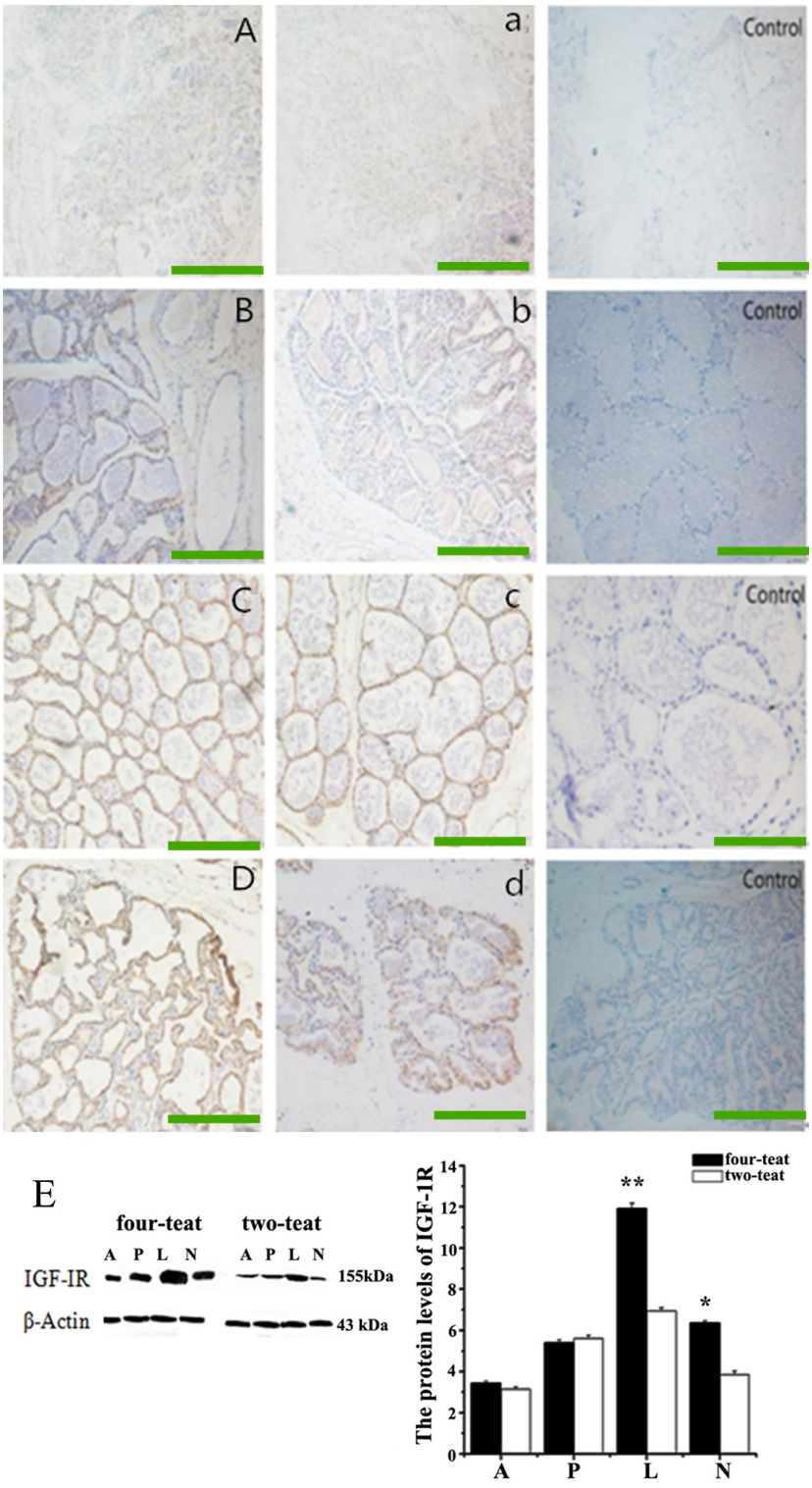

Figure 5. Expression patterns of IGF1R in mammary gland tissue. Immunohistochemistry for the IGF1R protein was performed in mammary gland sections during adolescent (A, a), pregnancy (B, b), lactation (C, c), and non-pregnancy (D, d) periods for four-teat sheep (A–D) and two-teat-sheep (a–d); the sample treated with only secondary antibody without primary antibody was used as Control. Green scale bars are 80 μm. (E) Protein expression levels of IGF1R in breast tissue during different stages; Western blotting was used to measure proteins levels of IGF1R in mammary glands during adolescent(A), pregnancy (P), lactation (L), and non-pregnancy (N) periods; β-actin was used as a loading control. All experiments were performed in duplicate and repeated three times; values are presented as means ± SE; $^{*}P < 0.05$, $^{**}P < 0.01$

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GHR, and IGF1R expression patterns and changes in the expression levels of the four proteins in two groups of sheep during A, P, L, and N periods. IHC showed that the ESR1, PRLR, GHR, and IGF1R proteins were mainly expressed in mammary epithelial cells and adipose cells. Moreover, we used WB to examine the expression levels of the four receptors during the four periods. *ESR1* expression increases to promote the mammary gland development (Feng et al. 2007) when essential mammary tissue begins to expand during pregnancy. However, expression of ESR1 decreased during L and N, which contradicts to the previous research data (Connor et al. 2005) obtained for dairy goats. This discrepancy is most likely due to the species difference.

PRLR has long and short isoforms in mammals. The former mediates the PRL-induced mammary development, milk protein synthesis, and differentiation of mammary epithelial cells, while the latter mediates the PRL regulation of mammary development (Binart et al. 2003; Saunier et al. 2003). Previous research of the *PRLR* mRNA expression during different physiological stages in rats showed that PRLR was expressed in adipose tissue, stromal cells, and mammary epithelial cells of the rat mammary gland. Therefore, the expression of PRLR increased when the number of mammary epithelial cells rose during the process of mammary development. In addition, four-teat sheep's mammary glands showed higher expression of PRLR compared to that in the two-teat sheep during P and L, which can promote better development of the mammary gland in four-teat sheep.

GHR is a single membrane-spanning receptor belonging to class 1 of the cytokine receptor superfamily, which binds GH to initiate biological actions (Loesch 2006). GH interaction with GHR on stromal cells induces the IGF1 release, which subsequently interacts with IGF1R on epithelial cells to mediate their survival and proliferation (Buckels 2013). ESR1 can also induce the expression of IGF1, which may then act on adjacent mammary epithelial cells (Marshman and Streuli 2002). High expression of GHR during pregnancy can promote the proliferation of mammary ducts.

Hyperthelia was a highly heritable trait, and inbreeding would increase the probability of its happening (Oppong and Gumedze 1981). SNTs in Hu sheep show normal lacteal output, while excision of a sheep SNT results in a significant decrease of lactation yield, with no difference between four-teat and two-teat sheep (Palacios and Abecia 2014). A study on Holstein and Brown Swiss cattle has shown that the lactation yield is unrelated to the teat number (Kaygisiz 2010). These results contradict to our conjecture, and thus, the estimation of the milk yield is considered our next step.

CONCLUSION

ESR1, PRLR, GHR, and IGF1R were mainly expressed in mammary epithelial cells and adipose cells in Hu sheep. Furthermore, four-teat Hu sheep have more developed mammary gland tissue compared with two-teat.

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