Expression of Stat5a Gene in Bovine Mammary Gland and its Effect on Proliferation of Mammary Epithelial Cells

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ABSTRACT : To understand molecular mechanisms involved in bovine mammary gland growth, expression of stat5a gene was examined in bovine mammary tissues. We found that stat5a gene was highly induced at pregnant 7 and 8 months compared to virgin mammary tissues. To examine function of bovine stat5a in mammary epithelial cell proliferation, stat5a expression vector was transfected into mammary epithelial HC11 cells. Cell proliferation rate in stat5a gene-transfected cells was 26%, 95% and 85% higher at 24 h, 48 h and 72 h after seeding, respectively, compared to control vector-transfected cells. Results demonstrate that bovine stat5a enhances proliferation of mammary epithelial cells. *(Asian-Aust. J. Anim. Sci. 2002, Vol 15, No. 8 : 1198-1203)*

Key Words : Bovine Mammary Tissues, Stat5a, Pregnant, Cell Proliferation

INTRODUCTION

Mammary gland growth during pregnancy involves enormous increases both in branching of ducts and in the number of epithelial cells (Topper and Freeman, 1980). The mammary epithelial cell population influences milk yield considerably. Numbers of mammary epithelial cells are a major determinant of milk yield (Tucker, 1987). Thus, understanding of mechanism that increases epithelial cell numbers will lead to develop new methods to enhance milk yield in the cow. Efforts have been made to understand the role of hormones, growth factors, and unknown factors in the proliferation of mammary epithelial cells (Borellini and Oka, 1987; Imagawa et al., 1990). But many questions on the mechanism that regulates mammary epithelial cell proliferation remain unanswered.

This study was performed to understand molecular events that regulate proliferation of bovine mammary gland. The primary objective was to identify pregnancy-induced clones by differential screening of a cDNA library of bovine mammary gland. One clone was characterized using nucleotide sequencing. The clone was identified as a cDNA encoding the stat5a protein. Stat5 (signal transducer and activator of transcription 5) proteins can be activated by prolactin through the Janus kinase (JAK)-stat pathway and induce gene transcription for mammary gland development and milk production (Watson and Burdon, 1996). Although the action of stat5a has been studied in rodents, its expression and functional role are not studied in bovine mammary tissues. Northern analysis was conducted to examine expression levels of stat5a mRNA at several physiological states in bovine mammary gland. To examine function of bovine stat5a in mammary gland growth, stat5a expression vector was transfected into mammary epithelial HC11 cells, and epithelial cell proliferation rate was examined.

MATERIALS AND METHODS

Bovine mammary tissues

Bovine mammary tissues were obtained by biopsy as described (Knight et al., 1992) at virgin, pregnant (5, 6, 7, and 8 months), lactating (4 months) and at involution (3 and 5 weeks postpartum) states from Holstein cow.

Construction of cDNA library of bovine mammary gland

Total RNA was extracted by the acid/guanidinium thiocyanate/phenol chloroform method (Chomoczynski and Sacchi, 1987). For construction of pregnant-specific cDNA library, poly A RNA was extracted from total RNA using Poly (A) Quick mRNA isolation Kit (Stratagene, USA). The directional cDNA library was constructed in the λTriplEx vector using pooled poly A RNA extracted from mammary gland at pregnant 5, 6, 7 and 8 months according to the manufacturer's protocol (Clontech, USA). Briefly, the first strand cDNA was synthesized using the poly A RNA. Xba 1-(dT)15 primer and AMV reverse transcriptase. The second strand cDNA was synthesized using RNase H. DNA polymerase and DNA ligase. The EcoR I adapted cDNAs were ligated to the λTriplEx vector, and the recombinant vectors were subjected to *in vitro* packaging and transfected into the XLI-Blue cells. The unamplified titers were 3.4×10^6 plaque-forming units per milliliter (pfu/ml). The insert size distribution of the library determined by PCR was 0.3-1.8 kb.

Differential screening, sequencing and northern analysis

Differential screening method was used to isolate pregnancy-induced clones as described earlier (Choi et al., 1996; Kim et al., 2000; Lee et al., 1996, 2001). Briefly, the library was plated with XL1-Blue cells in a low density (about 2,500 pfu/150 mm plate) for the primary screening. Following an 8h incubation at 37°C, the plates were cooled at 4°C for 2 h. Phage DNA from each plate was transferred onto the nylon membranes in duplicate. The differential hybridization was carried out at 68°C overnight with ^{32}P-labeled cDNA probe prepared from mRNAs of virgin tissues and of pregnant tissues, respectively. After comparing signals from the two films, the pregnant-induced positive plaques were identified. The lambda DNA containing the cDNA insert was converted into the pTriplEx clone by in vivo excision.

Partial sequencing of the clone was done by the dideoxy nucleotide chain- termination method using the Sequenase DNA Sequencing Kit (USB, USA) and pTriplEx 5' and pTriplEx 3' primer.

For Northern analysis, twenty micrograms of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, and blotted onto a membrane. The plasmid was digested with EcoR I and Xba I, and the insert was obtained after low melting agarose gel electrophoresis. The insert of cDNA clone was labeled using a Prime-It Random Primer Labeling Kit (Stratagene). The membrane was hybridized with the ^{32}P-labeled insert of the cDNA clone.

Transfection of bovine stat5a expression vector in HC11 cells

To examine function of bovine stat5a in mammary epithelial cell proliferation, expression vector for bovine stat5a cDNA containing full-coding region was constructed by using pBK-CMV expression vector, in which stat5a cDNA expression is driven by cytomegalovirus (CMV) immediate early promoter. Briefly, both pBK-CMV vector and pUC18-stat5a vector were digested by Sac I and Xho I. Digested pBK-CMV vector (4.5 kb) and stat5a insert (2.6 kb) were purified by QIAEX II Gel Extraction kit (QIAGEN, Germany) and ligated using T4 DNA ligase. After transformation, recombinant plasmid was purified. To increase efficiency of stat5a expression in eukaryotic cells, 200 bp region containing bacterial lac promoter and AUG codon was removed from pBK-CMV vector by Nhe I and Sac I digestion. Linear fragments were blunt-ended by fill-in reaction and self-ligated, generating final stat5a expression vector. pBK-CMV vector containing neo^R gene was used as a control. The stat5a expression vector and control vector were transformed to competent XL1-Blue cell. A well-isolated kanamycin resistant-colony was transferred to LB/kanamycin broth. After incubation at 37°C overnight, plasmid was isolated by QIAGEN endo

free kit (QIAGEN). The correct ligation was confirmed by both Not I digestion and sequencing of junction region.

The stat5a expression vector was transfected in HC11 cells. The HC11 cell is a clonal mammary epithelial cell that is derived from spontaneously immortalized COMMA-D epithelial cells, isolated from the mammary gland of midpregnant BALB/c mice (Ball et al., 1988). HC11 cell were grown in RPMI-1640 medium with 10% fetal bovine serum, 5 μg/ml insulin, 10 μg/ml epidermal growth factor (EGF), and 50 μg/ml gentamycin in a 5% CO_2 at 37°C. For transfection of bovine stat5a expression vector, the cells of 3×10^5 were seeded on 6 well dishes and incubated until 80% confluency. After removal medium, the cells were rinsed by 2 ml serum-free medium but containing insulin and EGF. Recombinant DNA was transfected using LIPOFECTAMINE according to the manufacturer's instruction (GIBCO BRL, USA). Briefly, DNA solution (2 μg) and 2, 4 or 8 μl of lipofectamine solution were mixed and the mixtures were incubated at 23°C for 30 min and 0.8 ml serum-free medium were added. The mixtures were overlayed onto the rinsed cells. The cells were incubated for 5 h and further cultured for 24 h after adding medium containing 20% fetal bovine serum (FBS).

Transfected cells were selected by adding the media containing 200 μg/ml Geneticine (G418). Media were changed every 48 h. After 14 days of G418 selection, cells in a colony were obtained by cloning cylinder and transferred onto 24 well dishes. The cells were incubated until confluency, transferred onto 6 well dishes and propagated.

Stat5a gene integration was identified by polymerase chain reaction (PCR). Cells were lysed using 360 μl DNA extraction buffer and 40 μl proteinase K. The genomic DNA was extracted using phenol (25): chloroform (24): isoamylalcohol (1) solution, precipitated by ethanol, and dissolved in TE buffer. PCR amplification was performed with the genomic DNA and CMV 5' and stat5a 3' primers. Control vector integration was also confirmed by polymerase chain reaction (PCR) using CMV 5' and CMV 3' primers. The PCR products were checked by 1.0% agarose gel electrophoresis. Expression of bovine stat5a gene in transfected cells was determined by northern analysis as described above.

MTT assay

Cell proliferation was quantitated by MTT assay as described (Mosmann, 1983). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) is a water-soluble tetrazolium salt, being cleaved to soluble formazan by mitochondrial dehydrogenase enzymes of living cells. Absorbance of formazan dye solution is directly proportional to the number of viable cells (Mosmann, 1983). The cells were seeded at 0.2×10^4 cells per well in 96 well

plates with 100 µl of media. To determine hormonal effects on cell growth, cells were cultured in RPMI1640 media containing 10% FBS with insulin, insulin plus EGF, insulin plus 5 µg/ml of prolactin, and insulin plus 0.1 µM/ml of dexamethasone. The MTT was dissolved in phosphate-buffered saline at 5 mg/ml, filtered through a Millipore filter (pore size, 0.22 µm), and added to the culture medium at a dilution of 1:10. The formazan crystals formed after reduction of the tetrazolium ring were solubilized in acidic isopropanol (0.04-0.1 N HCl in absolute propanol). The optical densities of the samples were read at 570 nm by using an enzyme-linked immunosorbent assay (ELISA)-type microplate spectrophotometer. Data were analyzed by the General Linear Model procedure of SAS (SAS, 1987).

RESULTS AND DISCUSSION

Differential screening and mRNA levels of stat5a gene in bovine mammary tissues

To understand molecular events involved in bovine mammary gland development, pregnancy-specific cDNA library of mammary gland was constructed in the λTriplEx vector, and pregnant-induced clones were identified from a cDNA library using differential screening. After the primary screening of a total 40,000 pfu of the cDNA library, 109 positive phage candidates were isolated. One clone was selected for nucleotide sequencing. Partial sequencing revealed that the clone was a stat5a cDNA (Schroder et al., 1998; Seyfert et al., 2000).

Although the action of stat5a has been studied in rodents, its expression and functional role are not well studied in bovine mammary tissues. In the present study, the expression of stat5a gene was examined by a northern analysis using total RNA extracted from mammary gland at several physiological states of Holstein cow. Northern analysis revealed that stat5a gene (approximately 3.9 kb) was highly expressed at late pregnant (7 and 8 months) bovine mammary tissues (figure 1). Expression of stat5a gene in mammary tissues was not detected at virgin, lactation, and involution 3 and 5 weeks. Similar results were reported in rat mammary gland: the mRNA levels of stat5a increased during pregnancy, were highest in late pregnancy, and then decreased during lactation (Kazansky et al., 1995). A marked increase of stat5a protein expression was also observed during late pregnancy in mouse mammary tissues (Philp et al., 1996).

Effect of bovine stat5a gene transfection on mammary epithelial cell proliferation

We have found that expression of stat5a gene is highly induced at late pregnant stage at which proliferation of mammary epithelial cells is extensively occurred. Functional role of stat5a has not been studied in bovine
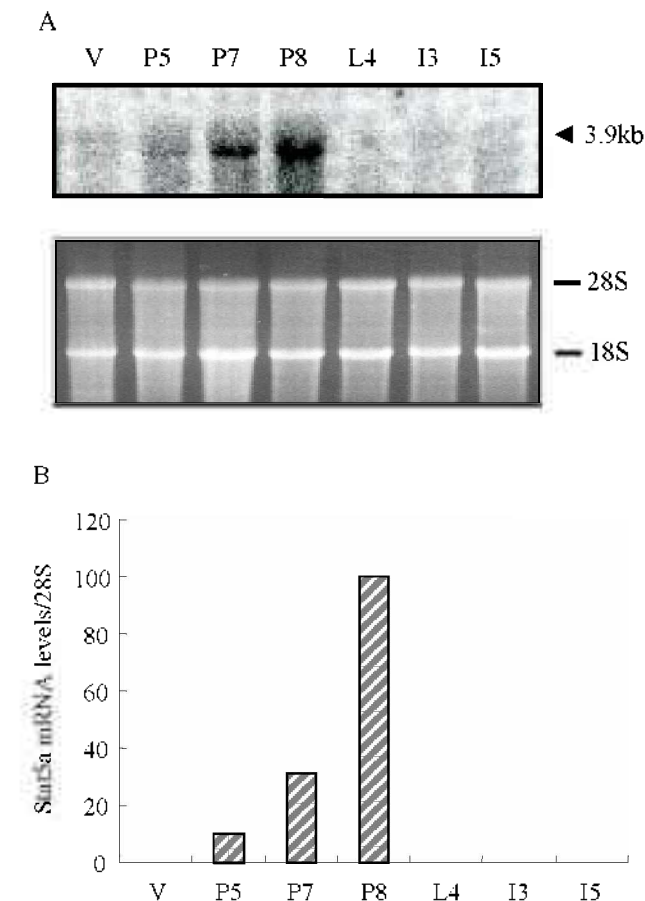
A

B

Figure 1. Northern analysis of the stat5a gene in bovine mammary gland at several physiological states. A. Twenty micrograms of total RNA isolated from mammary gland of Holstein cow at virgin (V), pregnant 5 months (P5), pregnant 7 months (P7), pregnant 8 months (P8), lactating 4 months (L4), involution 3 weeks (I3), and involution 5 weeks (I5) were separated on a 1% formaldehyde/agarose gel. Total RNA on the gel was transferred onto the membrane by capillary reaction. The blot was hybridized with the ^{32}P-labeled cDNA probe. That amounts of RNA were present in each lane was checked by the intensities of 28S and 18S bands as shown, and the efficiency of RNA transfer was monitored by ethidium bromide staining. B. The stat5a mRNA levels were normalized with corresponding 28S RNA band. The mRNA levels at pregnant 8 months were expressed as 100.

mammary gland development. To examine functional role of bovine stat5a in mammary epithelial cell proliferation, expression vector for bovine stat5a cDNA was constructed, and transfected in mouse mammary epithelial HC11 cells. After about two weeks of G418 selection, each of four resistant colonies for control vector and stat5a expression vector was isolated, and two control vector-transfected and three stat5a-transfected colonies were analyzed. Integration

of stat5a recombinant DNA was confirmed with PCR. Expected 0.7 kb fragments were amplified in stat5a-transfected cells (figure 2). Expression of bovine stat5a mRNA was detected in the transfected cells (figure 3).

The cell proliferation rate was quantitated by MTT assay. Stat5a transfection increased mammary epithelial cell proliferation under all hormonal treatments (figure 4). Overall mean values of cell proliferation at 24 h. 48 h and 72 h after seeding were 26%. 95% and 85% higher in stat5a-transfected cells than in control vector-transfected cells.
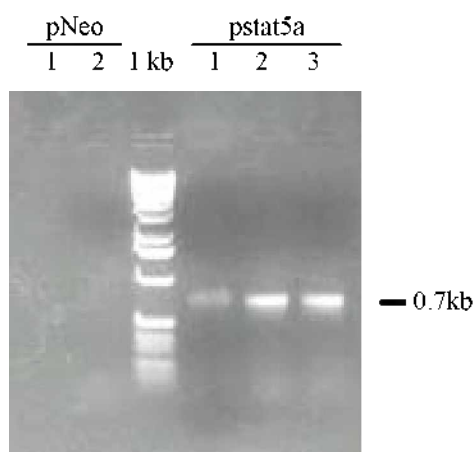
Mammary gland growth and differentiation requires the

Figure 2. PCR identification of stat5a cDNA integration into HC11 cells. Integration of stat5a gene was confirmed by PCR using genomic DNA isolated from pNeo (colony # 1 and 2)- and pstat5a (colony # 1, 2, and 3) vector-transfected HC11 cells and CMV5'- and stat5a 3'- primer. 1 kb. 1 kb ladder.
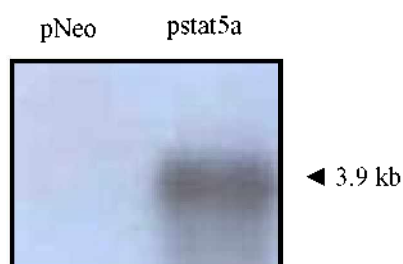

Figure 3. Northern analysis of stat5a gene expression in transfected mammary epithelial HC11 cells. The twenty micrograms of total RNA were isolated from pNeo- and pstat5a-transfected cells. The mRNA levels of bovine stat5a gene were analyzed by northern method.

coordinate action of growth factors and hormones including epidermal growth factor (EGF), insulin, prolactin and glucocorticoid (GR). Prolactin, growth hormone, EGF, and many cytokines use stat proteins to regulate the transcription of specific genes through Janus kinase (JAK)-stat pathway (Schindler and Darnell, 1995). Prolactin binds to its receptor and causes the prolactin receptor to dimerize. Receptor-associated tyrosine kinase JAK2 phosphorylates the prolactin receptor and stat5a and stat5b. Activated stat5a and stat5b are transported into the nucleus. bind to γ-interferon activation sites (GAS) (TTCNNNGAA). and induce transcription of target genes that promote proliferation, differentiation and lactogenesis of mammary gland (Hennighausen et al., 1997). Glucocorticoids affect milk protein gene expression via association of the GR with stat5. It has been demonstrated that there is a functional coupling between stat-dependent and nuclear hormone receptor-dependent gene transcription (Cella et al., 1998). To examine whether EGF, prolactin and dexamethasone are effective on the proliferation of stat5a-transfected HC11 cells. the cell proliferation rate was quantitated by MTT assay under the addition of these factors. Cell proliferation effects of stat5a transfection were more pronounced by EGF. prolactin and dexamethasone treatments (75%. 73% and 71% increase in average compared to control cells. respectively) than by insulin treatment (58% increase in average compared to control cells). Experiments of cell proliferation with other two stat5a- transfected independent colonies showed similar results. Cell proliferation rate determined by cell number counting using hemocytometer showed similar results as MTT assay (data not shown).

In cell growth experiments, bovine stat5a transfection increased proliferation of mammary epithelial cells. This is the first report demonstrating that bovine stat5a increases mammary epithelial cell growth. Our results demonstrate that bovine stat5a is responsible for mammary gland development. It has been demonstrated that stat5a is a survival factor for the mammary epithelial cells in transgenic mouse model (Humphreys and Hennighausen, 1999). Stat5a knockout mice showed impaired lobuloalveolar outgrowth during pregnancy and defective lactation, indicating that stat5a is required for both mammopoiesis and lactogenesis (Liu et al., 1997). In other study, stat5a-deficient mice exhibited defective mammary gland development (Akira, 1999). Stat5a and stat5b proteins are able to act independently as homodimers or in combination as heterodimers. Stat5a homodimers were more abundant than the stat5b homodimers in HC11 cells treated with lactogenic hormones (Cella et al., 1998). Our results suggest that bovine stat5a are also able to act as homodimers on the mammary cell proliferation.

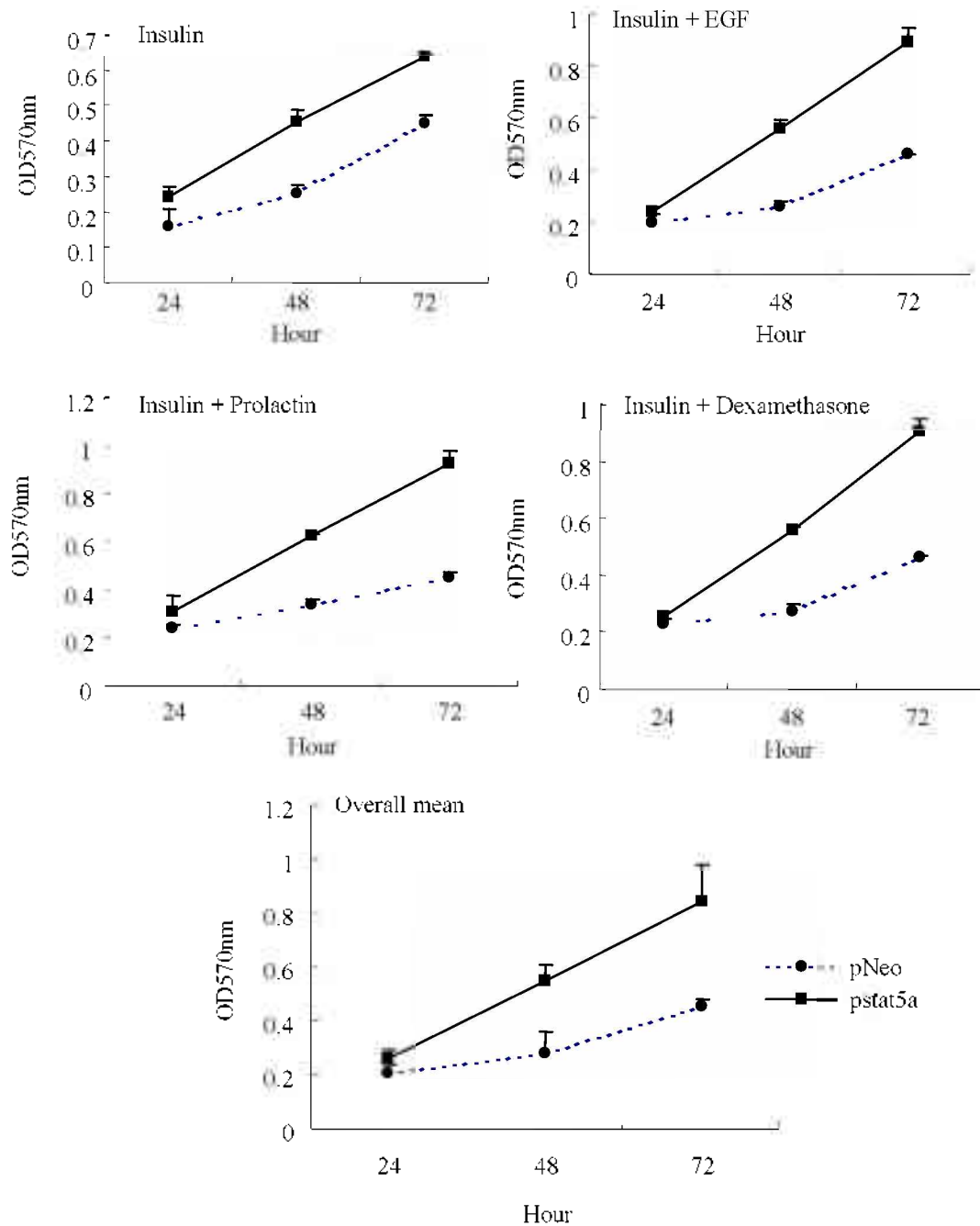We observed that cell proliferation effects of stat5a

Figure 4. Proliferation of mammary epithelial HC11 cells expressing bovine stat5a gene under various hormonal treatments. The pNeo- and pstat5a-transfected HC11 cells were cultured in RPMI1640 media containing 10% FBS with insulin, insulin plus EGF, insulin plus prolactin, and insulin plus dexamethasone. Cell proliferation was quantitated by MTT assay as described in Materials and Methods at 24 h, 48 h and 72 h after seeding. The optical densities (OD570nm) are directly proportional to the number of viable cells. The values are the averages + the standard deviations (n=5). Overall mean are the averages of all hormonal treatments. All data except insulin plus prolactin and insulin plus dexamethasone treatments at 24 h (no differences: p>0.05) showed statistical differences (p<0.01) between stat5a-transfected cells and control cells at each time.

transfection were more effective by prolactin and dexamethasone than by insulin treatment. These results suggest that bovine stat5a is involved in prolactin/JAK signaling pathway for proliferation of mammary epithelial cells and that glucocorticoids affect growth-related gene expression via association of the glucocorticoid with bovine stat5a.

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