

Controlled synchronization of prolactin/STAT5 and AKT1/mTOR in bovine mammary epithelial cells

Baosheng Wang¹ · Linlin Shi¹ · Jingjing Men¹ · Qingzhang Li² · Xiaoming Hou² · Chunmei Wang² · Feng Zhao¹

Received: 14 November 2019 / Accepted: 27 January 2020 / Published online: 20 February 2020 / Editor: Tetsuji Okamoto
© The Society for In Vitro Biology 2020

Abstract

The prolactin/STAT5 and AKT1/mTOR pathways play a key role in milk protein transcription and translation, respectively. However, the correlation between them in bovine mammary epithelial cells remains unclear. Here, mRNA and protein expression levels of AKT1, STAT5, and mTOR and the phosphorylation of these proteins were determined. Cell proliferation and viability were examined using the CASY-TT assay. Purified bovine mammary epithelial cells were cultured in differentiation media for different periods. The basic differentiation medium contained a lactogenic hormone cocktail of insulin (5 µg/mL), hydrocortisone (1 µg/mL), and prolactin (5 µg/mL). The cells cultured in this medium grew slowly and expressed higher levels of p-STAT5, p-AKT1, and p-mTOR (activated form) than those of control cells. Although the phosphorylation ratio was not increased, transcription and translation of these proteins were upregulated by the addition of insulin-like growth factor-1 or growth hormone, which further increased β -casein mRNA expression. Furthermore, the three proteins were upregulated or downregulated synchronously, even after RNA interference silencing of either *Stat5* or *Akt1*. These findings indicate that a few hormones and other factors play lactogenic and galactogenic roles by promoting two key lactogenic signaling associated with milk protein expression. We provide evidence of prolactin/STAT5 and AKT1/mTOR synchronization, establishing a direct correlation between transcription regulation and translation regulation of milk protein in bovine mammary epithelial cells.

Keywords Bovine mammary epithelial cell · AKT1 · mTOR · STAT5 · Milk protein synthesis

Introduction

Classic lactation studies have shown that a lactogenic phenotype can be induced in mammary epithelial cells (MECs) by a combination of glucocorticoids (GCs), insulin (INS), and prolactin (PRL) and assessed based on milk protein production (Johnson et al. 2010). This lactogenic combination has been considered necessary for milk protein production (whether murine or ruminant) *in vitro*, because it induces milk protein mRNA transcription. However, only β -casein-encoding gene (*CSN2*) expression has been well studied because few stable mammary epithelial cell lines are available, in addition to murine HC11 and bovine MAC-T cells that produce only β -

casein (Huynh et al. 1991; Choi et al. 2004; Zhou et al. 2008; Sornapudi et al. 2018; Qian and Zhao 2014). Moreover, as mentioned by Tucker (2000) and Hovey et al. (2002), there are major differences in the hormonal regulation of mammogenesis and lactation in rodents, cattle (ruminants), and humans (Tucker 2000; Hovey et al. 2002). This highlights the need for bovine-specific research models.

Several specific signaling pathways involved in milk protein synthesis are worth studying; stimulation by a single hormone or growth factor alone generally activates multiple signaling pathways, sometimes synchronously (Wartmann et al. 1996). However, analyses of single signaling have indicated that information on interactions among them is missing (Qian and Zhao 2014).

Milk protein expression can be initiated by activated prolactin/STAT5 signaling, and the maximum expression occurs in fully differentiated alveoli *in vivo* (Riley et al. 2010). Among them, *CSN2* expression is regulated by active STAT5 induced by prolactin bound with its receptor (prolactin/STAT5 signaling) (Gouilleux et al. 1994; Faraci-Orf et al. 2006). In recent years, interactions between STAT5 and a 63-ku serine-phosphorylated protein have been identified; this unknown

✉ Feng Zhao
erjinzhi@126.com

¹ Department of Food Science, Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin 150030, China

² College of Life Science, Northeast Agricultural University, Harbin 150030, China

protein was later confirmed to be AKT (Dinerstein-Cali et al. 2000; Chen et al. 2010). More recently, AKT1/mTOR signaling has been shown to play an important role in milk protein synthesis by controlling the translation of a few main milk proteins in cattle. Morrison and Cutler (2009) found that prolactin complexes cultured with HC11 mouse cells for 3–5 days promoted cell differentiation and protein synthesis. In addition, Burgos et al. (2010) stimulated mTOR signaling with different nutrients and a combination of dexamethasone (a type of GC), insulin, and prolactin, which is abbreviated as DIP, and determined changes in milk protein synthesis rate and mTOR signaling. They revealed that milk protein synthesis may be enhanced by hormones other than DIP (Burgos and Cant 2010). Similar results have been found following exogenous growth hormone (GH) or insulin-like growth factor 1 (IGF-1) treatment. DIP increased growth hormone receptor (GHR) mRNA expression, and GH might have acted directly on transfected GHR-overexpressing MAC-T cells (in contrast to native MAC-T, which shows low expression of GHR) to stimulate the transcription of major milk protein genes (Sakamoto et al. 2005; Zhou et al. 2008). IGF-1 mainly participates by binding with IGF-1R. The PI3K/AKT pathway is activated by IGF-1/IGF-1R, and IGF-1 stimulated global protein synthesis in MAC-T cells through changes in the phosphorylation and association state of components of the mTORC1 signaling pathway (Burgos et al. 2010; Kfir and Barash 2019).

Although a few lactogenic stimulants can activate specific signaling, their effects are often temporary, as shown in numerous experiments *in vitro*. One cause for this may be an imbalance in positive and negative regulatory factors and dysregulation of mRNA transcription (prolactin/STAT5) and protein translation (AKT1/mTOR). Sufficient expression and stable activity of key signal proteins are needed to induce and maintain lactation, particularly in dairy animal mammary glands (Qian and Zhao 2014).

In the present study, we aimed to detect the expression and activity of STAT5, AKT1, and mTOR through positive hormone stimulation or negative gene knockdown to evaluate the correlation between prolactin/STAT5 and AKT1/mTOR signaling in milk protein production in bovine mammary epithelial cells (BMECs). A comprehensive understanding of the synchronization of milk protein expression signaling may help improve mammary epithelial cell models for studies on lactation function.

Materials and Methods

Cell preparation and treatments Primary BMECs were obtained from parenchymal tissues of lactation Holstein dairy cows. The cells were purified according to a method by Zhao et al. (2015). In brief, cells were placed in culture flasks and digested using trypsin to purify mammary epithelial cells.

Purified cells plated in six-well plates at 1×10^5 cells/well with growth medium consisting of DMEM/F12 (Gibco, Grand Island, NY), 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (0.1 mg/mL) until 80% confluent. We performed lactogenic differentiation of MECs as described earlier. In brief, 80% confluent native and logarithmic growing cells were treated with differentiation medium consisting of DMEM/F12, penicillin/streptomycin (100 U/mL and 0.1 mg/mL, respectively), a HIP combination including 1 μ g/mL hydrocortisone (Sigma-Aldrich), 5 μ g/mL insulin (bovine, Sigma-Aldrich, St. Louis, MO), and 5 μ g/mL prolactin (ovine, Sigma-Aldrich), and either bovine GH (ProSpec, St. Louis, MO; 0 or 100 ng/mL) or IGF-1 (Sigma-Aldrich, MO; 0 or 100 ng/mL) for various periods (0 h, 0.25 h, 1 h, 24 h).

To obtain cell growth curves in growth medium and differentiation medium, the cells grown in each medium were collected every 12 h for 6 d and counted. Based on these curves, the start times for RNA interference and differentiation induction were established.

Immunocytochemistry Purified cells were cultured on glass coverslips in six-well plate and incubated with 10% FBS growth medium for 48 h until 80% confluence. After two PBS washes, cells were covered with 1 mL of methanol pre-cooled for 10 min at 4°C. Cells were then immersed in 5% BSA in PBS for 1 h at 37°C followed by incubation with rabbit anti-cytokeratin 18 polyclonal antibody (1:50; Bioss, Beijing, China, bs-2043R-AF488), mouse anti-prolactin receptor monoclonal antibody (1:100; Novus, Littleton, CO, NB300-561) in PBS for 1 h at 37°C. PBS was used instead of primary antibody as the control. Lastly, cells were washed three times in PBS for 5 min each. Nuclei were stained by DAPI. Images were acquired and analyzed with a Leica TCS SP2 Confocal Laser Scanning Microscope.

Small interfering RNA-mediated gene knockdown Small interfering RNAs (siRNAs) against *Akt1* or *Stat5* and negative control RNA oligonucleotides were chemically synthesized by GenePharma Co., Ltd. (Shanghai, China). Gene knockdown was achieved by transient transfection with *Akt1* or *Stat5* siRNA or a negative control using Lipofectamine 2000 (LF2000, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. To investigate the effects of *Akt1* and *Stat5* knockdown on milk protein synthesis, the medium was changed to DMEM/F12 with different lactogenic hormone combinations after a 24-h transfection period. After 24 h of treatment, the cells were collected for further analysis.

Cell viability and proliferation Cell viability and proliferation were assessed in parallel cultures grown as described above. After transfection treatment, the cells were differentiated for

24 h with HIP alone, HIP + 100 ng/mL GH, or HIP + 100 ng/mL IGF-1 and assessed using the CASY TT Analyser System (Schärfe System GmbH, Reutlingen, Germany) according to instrument manual.

Quantitative real-time PCR The total RNA from BMECs was extracted using TriZol reagent (Invitrogen) according to the manufacturer’s protocol. RNA samples were identified by agarose gel electrophoresis. Then, the total RNA (1 µg) was transcribed into cDNA using PrimeScript™ reverse transcriptase (TaKaRa, Dalian, China) according to the manufacturer’s protocol. Quantitative real-time polymerase chain reaction (q-PCR) was carried out using the TB Green™ Premix Ex Taq™ II Kit. *ACTB* (*β-actin* gene) was used as the reference gene. The primers sequences are shown in Table 1. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression.

Western blotting The total protein was extracted from cells subjected to various treatments. Cell pellets were homogenized with RIPA buffer to which protease inhibitors had been added as previously described (Woodward et al. 1998). Protein content was determined using a bicinchoninic acid (BCA) protein assay (Bio-Rad Laboratories, Inc., Berkeley, CA). Proteins (20 µg) were separated on 10% SDS-PAGE gel and transferred onto nitrocellulose (NC) membranes. After blocking with 5% non-fat milk in TBS/T (0.1% Tween-20), the blots were probed with primary antibodies for mTOR (Cell Signaling Technology, Danvers, MA), phospho-mTOR (p-mTOR, Cell Signaling Technology, Danvers, MA), and *β-actin* (Santa Cruz Biotechnology, Santa Cruz, CA). This was followed by a second incubation with the secondary antibodies, goat anti-rabbit IgG with HRP and goat anti-mouse IgG with HRP (ZSGB-BIO, Beijing, China). Gray-scale scans of western blots were detected using Super ECL Plus and analyzed using ImageJ.

Statistical analysis All experiments were conducted in triplicates. The results are reported as mean ± standard error (SE). Data were normalized by log transformation and were tested

using the one-way analysis of variance (ANOVA) with SPSS 19.0 software.

Results

Identification and characteristic of purified BMECs Purified bovine mammary cells were visualized under a confocal laser scanning microscope and exhibited a normal epithelial cell shape. More than 90% of cells were strong positive staining for luminal epithelial marker cytokeratin 18 and moderate positive staining for prolactin receptor (PRLR) (Fig. 1). It confirmed that purified cells are BMECs with responsiveness to prolactin.

By comparing the growth of BMECs in differentiation medium (with HIP) with that of BMECs in growth medium, we found that the number of cells in serum-free differentiation medium did not change until cell death over a longer culture time. Proliferation was most apparent in growth medium containing 10% FBS. In fact, proliferation of MECs *in vitro* was hardly affected by stimulation with the combination of lactogenic hormones, because regulated withdrawal from the cell cycle is required for cells of any kind to differentiate, which showed sporadic and slow growth during lactation because the majority of MECs (normal and differentiated) were arrested in the G0/G1 stage during lactogenic differentiation (Sornapudi et al. 2018). Subsequent treatments were administered for 24 h, during which the cell number was stable, and the results were not influenced by cell proliferation (Fig. 1).

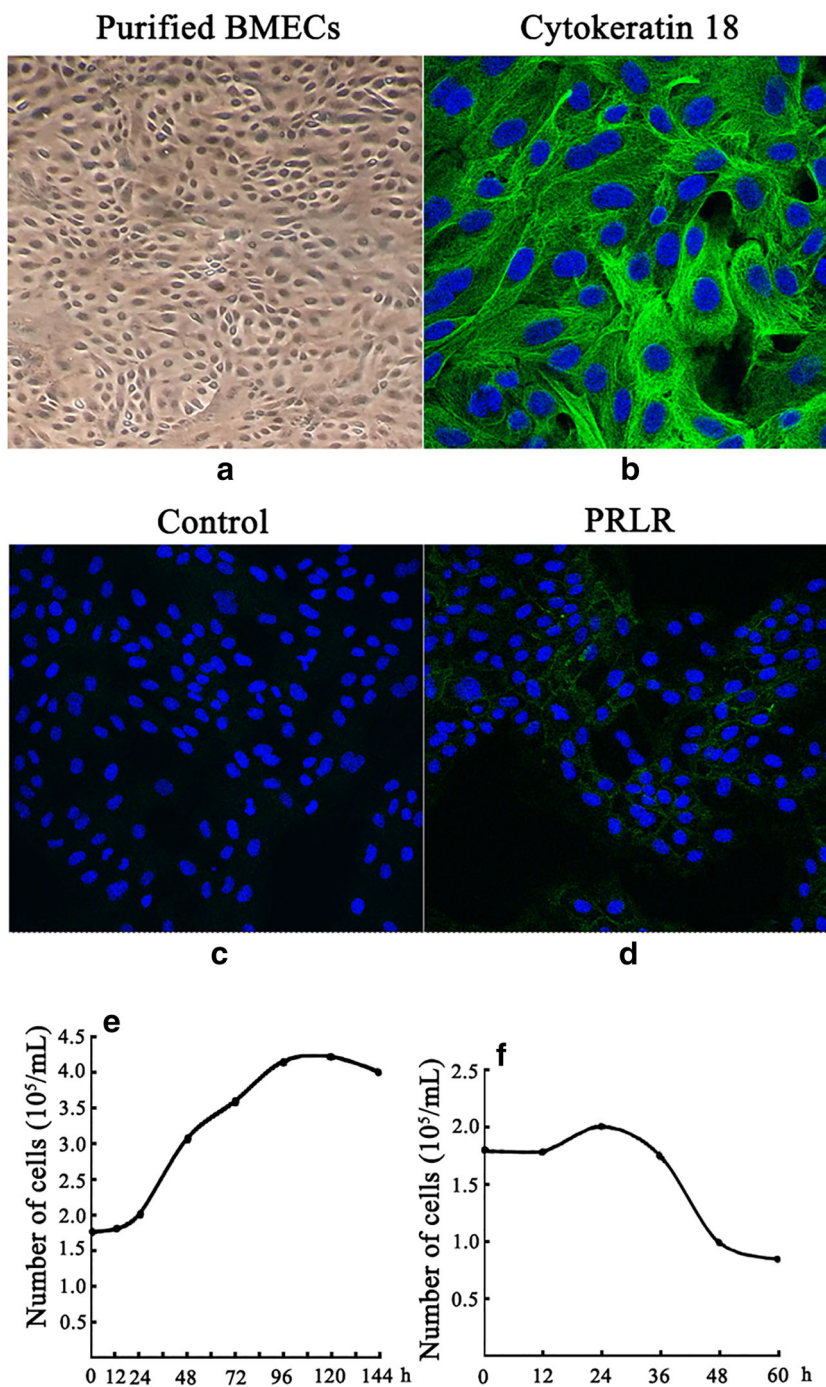
Effects of lactogenic stimulation on lactogenic signaling in BMECs To investigate whether lactogenic signaling is regulated in this study, mRNA and protein expression of a few vital signal molecules in BMECs cultured for various times was measured by q-PCR and western blotting, respectively. The q-PCR results revealed that each lactogenic stimulant induced a significant increase in mRNA expression of all three signal molecules within 24 h. As shown in Fig. 2, there were small differences in mRNA and protein levels of STAT5 with HIP alone, but with a sharp rise in the total p-STAT5 level due to a significant increase of phosphorylation ratio (Fig. 2). A large increase was apparent within the first hour, with a small increase observed from 1 to 24 h. The addition of IGF-1 induced a significant increase in *Stat5* mRNA levels and an extremely significant increase in *Akt1* mRNA levels compared with those in cells cultured with HIP alone. There was no significant difference in mTOR mRNA abundance between the control culture and cultures with IGF-1. The addition of GH showed a positive effect on *Stat5*, *Akt1*, and mTOR mRNA expression. There was a significant increase in *AKT1* mRNA levels, a moderate increase in *Stat5* mRNA levels, and a marginal increase in mTOR mRNA levels between cells cultured with GH or HIP alone.

Table 1. Primers for q-PCR

Gene	Primer (5'—3')	Amplicon size (bp)
<i>ACTB</i>	F: AAGGACCTCTACGCCAACACG	249
	R: TTTGCGGTGGACGATGGAG	
<i>Akt1</i>	F: TGGAGAACCTCATGCTGGACA	206
	R: GCCGCACATCATCTCGTACAT	
mTOR	F: ATGCTGTCCCTGGTCCTTATG	178
	R: GGTCAGAGAGTGGCCTTCAA	
<i>Stat5</i>	F: CCCTTCCCGTGGTTGT	265
	R: ATGCCGTTGTAGTCCTC	
<i>CSN2</i>	F: CAAGCAGCGAGGAATCAA	249
	R: GAAAGGGACAGCACGGAC	

The q-PCR analysis was conducted using the $2^{-\Delta\Delta CT}$ method

Figure 1. Characteristics of purified bovine mammary epithelial cells. (a) Purified bovine mammary epithelial cells ($\times 400$). (b) Cytokeratin 18 staining of BMECs ($\times 800$). (c) Primary antibody negative control ($\times 400$). (d) Prolactin receptor staining of BMECs ($\times 400$). (e) The growth curve of BMECs in growth medium. (f) The growth curve of BMECs in differentiation medium.



The protein expression profiles were similar to the mRNA expression profiles (Fig. 2). In brief, the addition of IGF-1 marginally increased total STAT5 protein level, but did not significantly affect the total p-STAT5 levels compared with that in cells treated with HIP alone. The larger increase in AKT1, mTOR, and total phosphorylated protein of them implies that IGF-1 preferentially regulates AKT1/mTOR over prolactin/STAT5. However, adding GH had a stronger positive effect on BMECs than IGF-1. When the total protein or total

phosphorylated protein of AKT1, mTOR, and STAT5 was increased significantly, GH displayed an enhanced induction of both AKT1/mTOR and prolactin/STAT5 pathways; however, both the addition of GH and IGF-1 had no additional effects on their phosphorylated ratio compared with HIP alone.

As shown in Fig. 2 (k to m), the highest phosphorylated ratios of AKT1 and mTOR appeared at 0 h followed by the lowest level significantly at 0.25 h, because AKT1/mTOR signaling was also vital to cell growth and retained a higher

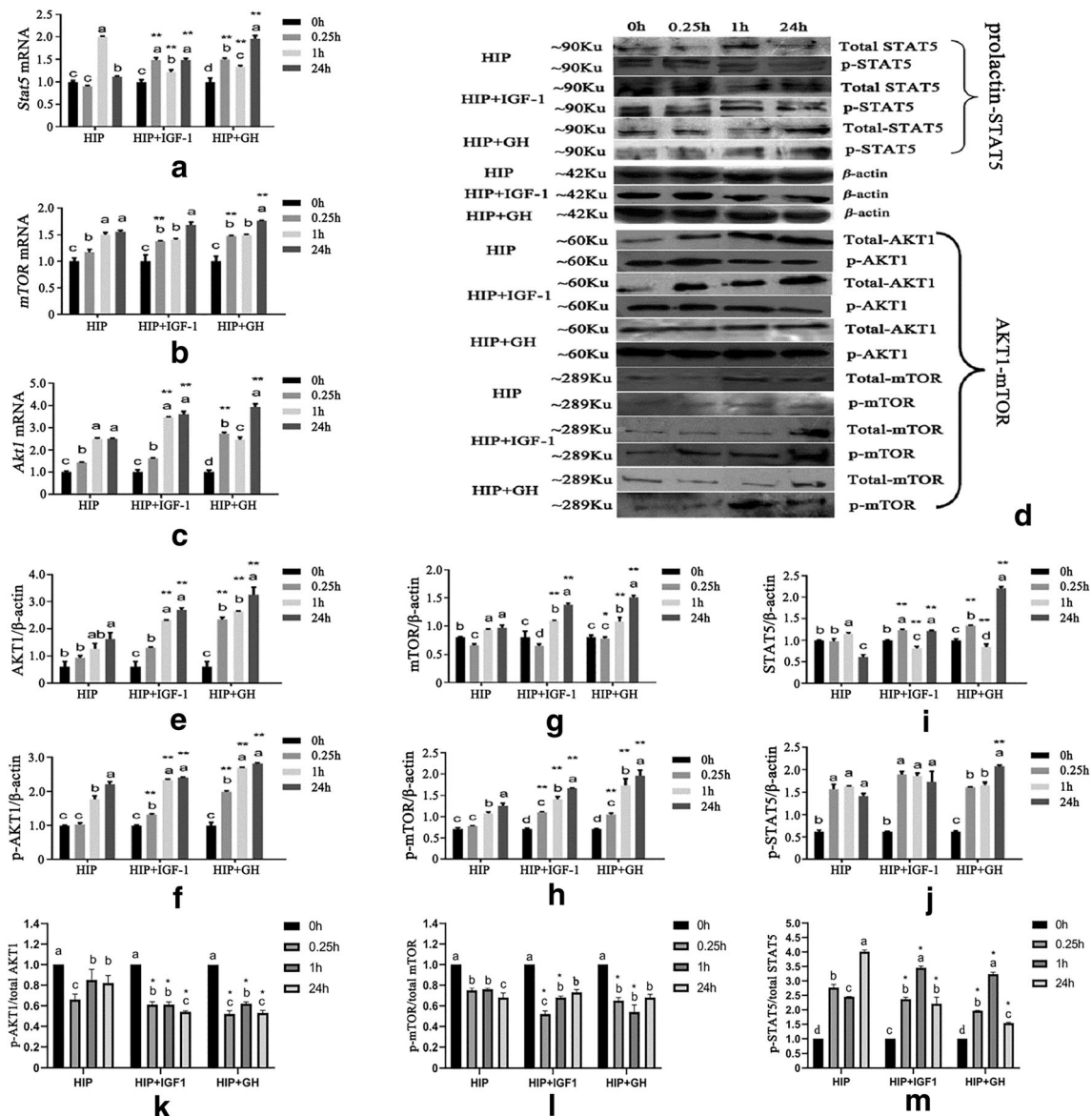


Figure 2. (a–m) Induced lactogenic signaling in BMECs cultured for various periods. $n = 3$, data from the same group with different letters are significantly different, $P < 0.05$; asterisks indicate significant differences between different groups (vs. HIP), * $P < 0.05$, ** $P < 0.01$.

activity with 10% FBS; therefore, they would be downregulated when serum deprivation until the effects of the new stimulations appeared. However, the phosphorylated ratio of STAT5 was low at the present of serum during cell growth and was easily increased by HIP treatment (Wartmann et al. 1996; Finucane et al. 2008).

Taken together, these results suggest that IGF-1, GH, and HIP are needed for induction, and that the maximum lactogenic effect usually required a combination of lactogenic hormones or factors.

Effect of Akt1 and Stat5 gene knockdown on cell viability and proliferation AKT1 and STAT5 are key members of the AKT1/mTOR and prolactin/STAT5 signaling pathways, respectively. In this assay, we assessed their roles on the viability

and proliferation of BMECs. The data revealed that *Akt1* or *Stat5* gene knockdown significantly decreased the viability and number of BMECs at 48 h post-transfection with the mimic compared with that of the control groups (Fig. 3). These results confirm that AKT1 and STAT5 are also vital positive regulators of cell growth and survival in BMECs during lactation differentiation. The addition of IGF-1 or GH reversed this decrease in cell viability and proliferation compared with that by HIP alone. As described above, both of them might induce higher *Akt1* and *Stat5* gene expression.

Effect of gene knockdown on lactogenic signaling in BMECs According to the q-PCR analysis of all treatment groups, *Akt1*, *mTOR*, *Stat5*, and *CSN2* mRNA expression in the interference groups decreased significantly in comparison with that in the

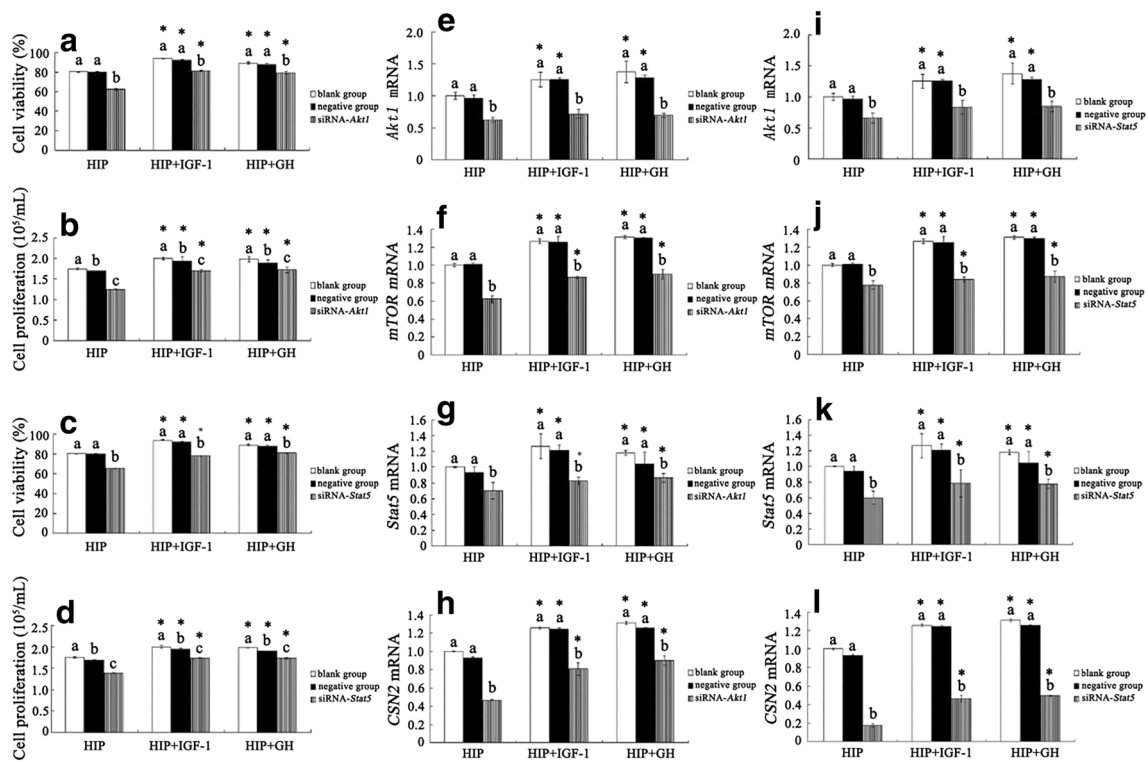


Figure 3. Effect of *Akt1* and *Stat5* gene knockdown by RNA interference on cell viability, proliferation, and lactogenic signaling molecule transcription. (a, c) Cell viability after gene interference. (b, d) The number of viable cells after gene interference. (e–h) *Akt1* gene

knockdown by RNAi. (i–l) *Stat5* gene knockdown by RNAi. $n = 3$; data from the same group with different letters are significantly different, $P < 0.05$; asterisk indicates a significant difference between different groups (vs. HIP), $P < 0.05$.

control. Namely, the synthesis of milk protein mRNA was reduced following the inhibition of lactogenic signaling by the knockdown of key genes. Notably, key signal molecule gene expression in another pathway was inhibited when either *Akt1* or *Stat5* was silenced by RNA interference.

The mRNA expression in BMECs treated with HIP+IGF-1 or HIP+GH was compared with that in the groups treated with HIP alone, and there were significant differences in the corresponding mRNA levels in the blank control, negative control, and interference groups, but no difference in the mRNA levels between the blank and negative controls with the same treatment (Fig. 3).

As shown in Fig. 3, the abundance of silenced *Akt1* and *Stat5* mRNA following 24-h treatment with HIP alone decreased by 30–40%, demonstrating RNA interference efficiency. Although other undisturbed genes showed a smaller decrease (approximately 20%), it is possible that synchronization of reduced mRNA expression related to two vital lactogenic signaling genes resulted in a significant decrease of 60–80% in β -casein transcription by a superposition effect. On the contrary, the addition of IGF-1 or GH resulted in higher mRNA expression with similar RNA interference efficiency in almost all treatment groups compared with that in the HIP control, confirming their galactogenic action resisting impaired differentiation and reduced milk protein expression.

The protein expression profiles resembled that of their transcripts, but there was a smaller decrease in protein levels than in mRNA levels in the interference groups (Fig. 4). This may be because of the time delay from gene transcription to protein translation and a difference in the optimum interference times to affect mRNA and protein expression. Total phosphorylated protein levels decreased synchronously with total protein levels; however, phosphorylated ratio changed unsynchronously and irregularly, it was possible that there was the compensatory increase due to a rapid protein reduction (Fig. 4). Independent of gene knockdown, the application of exogenous IGF-1 or GH resulted in the increase of all three target phosphorylated proteins (Figs. 2 and 4).

In summary, these data indicate that single-gene knockdown by siRNA negatively affected the expression of two undisturbed genes other than the target gene, resulting in the down-regulation of their expression and total phosphorylated protein.

Discussion

Lactogenic stimulation can synchronously enhance lactogenic signaling in BMECs The microarray results indicated that upregulation of genes implicated in milk synthesis is concomitant with the inhibition of those related to cell proliferation in

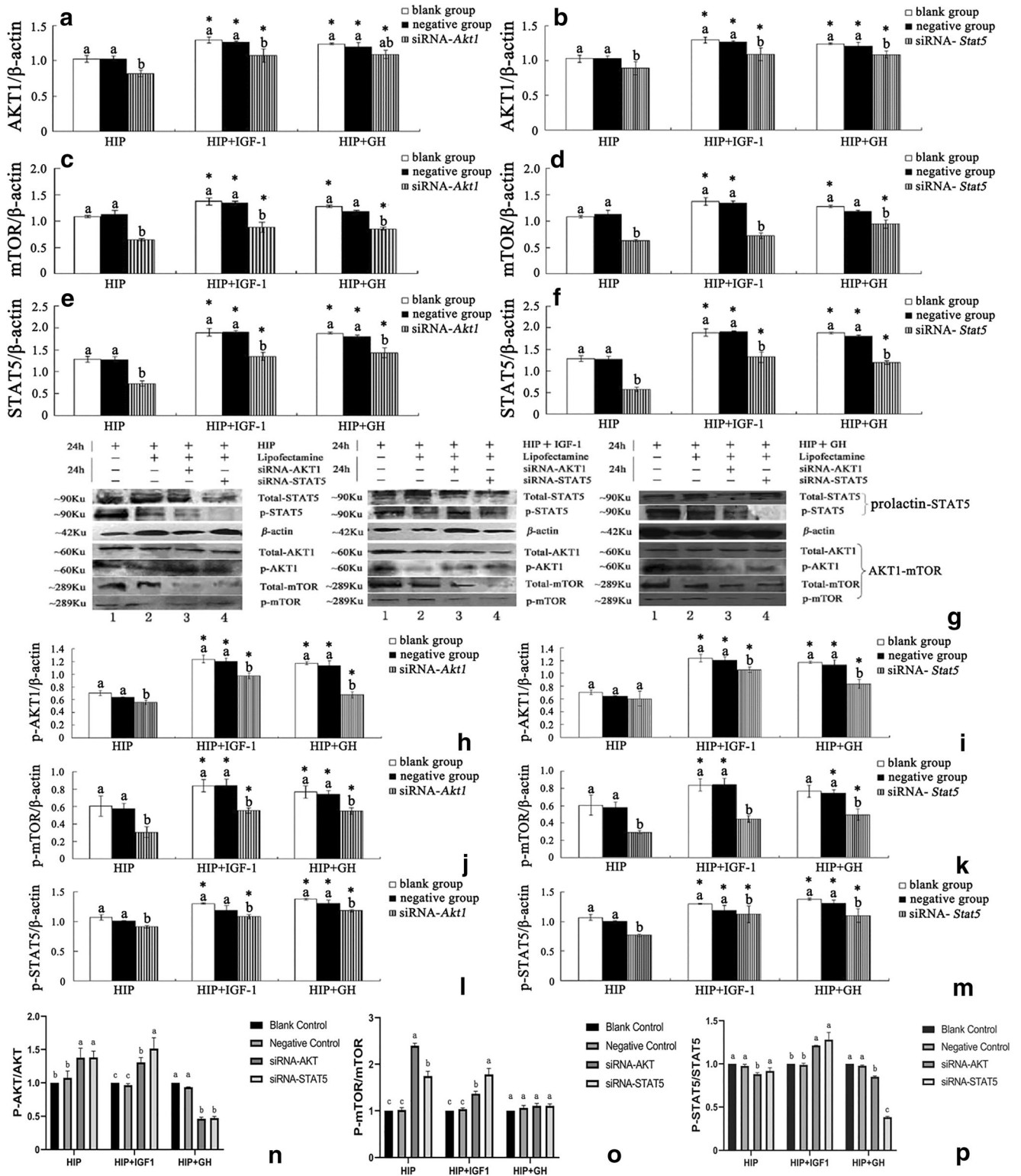


Figure 4. (a–p) Effect of *Akt1* and *Stat5* gene knockdown by RNA interference on lactogenic signaling molecule translation and activity. $n=3$; data from the same group with different letters are significantly

different, $P < 0.05$; asterisk indicates significant differences between different groups (vs. HIP), $P < 0.05$.

bovine mammary glands at the onset of lactation (Finucane et al. 2008). In fact, basal and lactogen-induced *CSN2* promoter activities were found to be elevated in MAPK inhibitor-

treated cells, and HC11 cells transformed by potent activators of the MAP kinase pathway were found to no longer synthesize β -casein in response to lactogenic hormones (Wartmann

et al. 1996). This may be due to impairment of prolactin-induced *Stat5* DNA-binding activity in the transformed HC11 cells. This led to our hypothesis that slow proliferation or growth arrest is a prerequisite for functional differentiation of BMECs. Here, we showed that differentiation medium with HIP alone had no significant mitogenic effect (Fig. 1), which was the first step in regulating lactation function in our experiment.

In addition to inhibited proliferation, mammary gland differentiation requires the coordinated action of hormones and growth factors that contribute to morphological development and milk protein production in the lactating glands (Wartmann et al. 1996). A lactogenic phenotype is generally induced in BMECs by a HIP or DIP combination and has typically been assessed by milk protein production (Shao et al. 2013). Nevertheless, GH and IGF-1 are believed to have a positive regulatory effect on mammary development and lactation (Lee et al. 2013; Sciascia et al. 2013; Qian and Zhao 2014). Some *in vitro* studies have shown that their roles are associated with prolactin/STAT5 and PI3K/AKT1/mTOR (Zhou et al. 2008; Burgos and Cant 2010; Johnson et al. 2010; Qian and Zhao 2014; ; Akers 2017).

It has been elaborated how lactogenic hormone combinations can positively regulate the transcription of milk protein genes. However, there has been no clear conclusion on the translation of milk proteins, especially in cattle. This is partly due to inexplicable results in studies; for example, the change in mRNA and protein expression being inconsistent. One of the reasons is inappropriate detection time, which is suitable for the transcription of mRNA from DNA and transient, but not enough for translation, which is a delayed second major step in protein expression (Qian and Zhao 2014; Akers 2017). Therefore, we set a time gradient in the experiments to screen suitable time points for transcription, translation, and long-term stable protein phosphorylation (Fig. 2). According to our data, both gene and protein expression changed significantly within several hours of exposure to the lactogenic compounds, and higher levels were maintained until 24 h post-treatment in most experiment groups. Therefore, we used 24 h as the detection time.

Upregulation or activation of prolactin/STAT5 signaling, which is essential for milk protein mRNA transcription, is insufficient to sustain stable high-level milk protein translation (Qian and Zhao 2014; Akers 2017). Differential rates of expression of the major milk proteins in mammary secretory epithelial cells support this view. The rate of secretion of α s2-casein in bovine milk is approximately 25% that of β -casein, yet the expression of their respective mRNA transcripts (*csn1s2* and *csn2*) in mammary glands is not different. This is because the translational efficiency of *csn2* is five times that of *csn1s2* (Kim et al. 2015). In fact, the effects of nutritional regulation and management measures on bovine milk production are likely to occur at the post-transcriptional

and translational levels rather than increasing the abundance of transcripts (Burgos and Cant 2010; Kim et al. 2015; Akers 2017; Li et al. 2017). Optimal ratios of essential amino acids can stimulate β -casein synthesis via the activation of mTOR signaling in MAC-T cells and bovine mammary tissue explants (Li et al. 2017).

Synergistic enhancement of both transcription and translation signaling induced by a combination of hormones and factors was observed in this study. Our research showed that whether HIP was used individually or in combination with GH or IGF-1, all treatments promoted the synchronous upregulation of *CSN2* expression. Furthermore, it has been reported that lactogenic hormones significantly affected the negative regulators of these two pathways (Qian and Zhao 2014; Akers 2017).

Synchronous changes in lactogenic signaling by gene knock-down in BMECs As a transcription factor, STAT5 is directly related to the expression of transcription promoter genes of a few major milk proteins, especially β -casein. Furthermore, regulation of STAT5 expression involving multiple levels—transcription, translation, and phosphorylation—is essential to evaluate lactation ability in an *in vitro* cell model (Radler et al. 2017). However, the dominant role of STAT5 in milk protein expression has been challenged recently (Qian and Zhao 2014; Akers 2017). Many unexpected results may be temporary positive effects rather than stable long-term effects on milk proteins that reflect inadequate translation and failed secretion *in vitro*, even if STAT5 is increased and activated (Qian and Zhao 2014; Akers 2017).

A competitor of prolactin/STAT5 has emerged, AKT1/mTOR, which is closely associated with protein synthesis. Because milk protein production is a complex process and has distinct and specific inhibitors/negative regulators, the lack of positive lactogenic hormones and growth factors and unrestrained negative regulators may hinder milk protein gene expression. Both AKT1/mTOR and prolactin/STAT5 with sustained activation can promote milk protein production. Our data show that additional GH and IGF-1 can positively and synergistically regulate AKT1/mTOR and prolactin/STAT5 by mediating AKT1, mTOR, and STAT5 expression resulting in the increase of total phosphorylated proteins, however, phosphorylation ratio unchanged. Therefore, what is the negative regulatory effect of single gene knockdown? Are there synergies? With such questions, we conducted RNA interference of the *Akt1*, *mTOR*, and *Stat5* genes, and the results showed that transcription and translation of the three genes were always synchronous (Figs. 3 and 4).

This finding is consistent with the latest research on crosstalk between the JAK2/STAT5 and PI3K/AKT1 pathways to mediate the proliferation of alveolar progenitors and survival of their functionally differentiated descendants in the mice mammary glands (Radler et al. 2017). STAT5 induces

the transcription of the *Akt1* gene from a novel promoter and controls the expression of regulatory and catalytic subunits of PI3K (p85 and p110), as well as directly regulates PI3K kinase activity by binding to the p85 subunit SH2 domain of PI3K to significantly augment signaling via the prosurvival PI3K/AKT pathway (Abell and Watson 2005; Sakamoto et al. 2007; Creamer et al. 2010; Schmidt et al. 2014; Singh et al. 2016; Radler et al. 2017). Therefore, ATK1 is an essential mediator for the biological function of STAT5 as a survival factor (Chen et al. 2010; Radler et al. 2017). The interference of just one signaling mediator in regulatory signaling networks can have severe consequences that manifest in the proliferation, survival, and differentiation of BMECs. There may be a crosstalk between prolactin/STAT5 and AKT1/mTOR in BMECs similar to that in mice (Chen et al. 2010; Radler et al. 2017).

Conclusion

In conclusion, GH and IGF-1 play lactogenic and galactogenic roles by promoting two key lactogenic signaling during lactation differentiation induced by HIP. The prolactin/STAT5 and AKT1/mTOR pathways can be controlled synchronously for milk protein expression, which connects transcription regulation to translation regulation of β -casein, in BMECs.

Acknowledgments We would like to thank Editage (www.editage.cn) for English language editing.

Author contributions Conceptualization, F.Z.; methodology, F.Z., B.W. and L.S.; validation, B.W. and J.M.; formal analysis, L.S. and B.W.; data curation, J.M. and X.H.; writing-original draft preparation, F.Z. and L.S.; writing-review and editing, Q.L. and C.W.; supervision, X.H. and Q.L.; project administration, F.Z. and Q.L.; funding acquisition, F.Z. and X.H.

Funding information This research was funded by the National Natural Science Foundation of China, grant numbers 31571338 (to F.Z.) and 31671285 (to X.H.).

References

Abell K, Watson CJ (2005) The Jak/Stat pathway: a novel way to regulate PI3K activity. *Cell Cycle* 4:897–900

Akers RM (2017) A 100-year review: mammary development and lactation. *J Dairy Sci* 100:10332–10352

Burgos SA, Cant JP (2010) IGF-1 stimulates protein synthesis by enhanced signaling through mTORC1 in bovine mammary epithelial cells. *Domest Anim Endocrinol* 38:211–221

Burgos SA, Dai M, Cant JP (2010) Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. *J Dairy Sci* 93:153–161

Chen CC, Boxer RB, Stairs DB, Portocarrero CP, Horton RH, Alvarez JV, Birnbaum MJ, Chodosh LA (2010) Akt is required for Stat5 activation and mammary differentiation. *Breast Cancer Res* 12:R72

Choi KM, Barash I, Rhoads RE (2004) Insulin and prolactin synergistically stimulate beta-casein messenger ribonucleic acid translation by cytoplasmic polyadenylation. *Mol Endocrinol* 18:1670–1686

Creamer BA, Sakamoto K, Schmidt JW, Triplett AA, Moriggl R, Wagner KU (2010) Stat5 promotes survival of mammary epithelial cells through transcriptional activation of a distinct promoter in Akt1. *Mol Cell Biol* 30:2957–2970

Dinerstein-Cali H, Ferrag F, Kayser C, Kelly PA, Postel-Vinay M (2000) Growth hormone (GH) induces the formation of protein complexes involving Stat5, Erk2, Shc and serine phosphorylated proteins. *Mol Cell Endocrinol* 166:89–99

Faraci-Orf E, McFadden C, Vogel WF (2006) DDR1 signaling is essential to sustain Stat5 function during lactogenesis. *J Cell Biochem* 97:109–121

Finucane KA, McFadden TB, Bond JP, Kennelly JJ, Zhao FQ (2008) Onset of lactation in the bovine mammary gland: gene expression profiling indicates a strong inhibition of gene expression in cell proliferation. *Funct Integr Genomics* 8:251–264

Gouilleux F, Wakao H, Mundt M, Groner B (1994) Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO J* 13:4361–4369

Hovey RC, Trott JF, Vonderhaar BK (2002) Establishing a framework for the functional mammary gland: from endocrinology to morphology. *J Mammary Gland Biol Neoplasia* 7:17–38

Huynh HT, Robitaille G, Turner JD (1991) Establishment of bovine mammary epithelial cells (MAC-T): an *in vitro* model for bovine lactation. *Exp Cell Res* 197:191–199

Johnson TL, Fujimoto BA, Jimenez-Flores R, Peterson DG (2010) Growth hormone alters lipid composition and increases the abundance of casein and lactalbumin mRNA in the MAC-T cell line. *J Dairy Res* 77:199–204

Kfir SH, Barash I (2019) Calorie restriction and rapamycin administration induce stem cell self-renewal and consequent development and production in the mammary gland. *Exp Cell Res* 382:111477

Kim JJ, Yu J, Bag J, Bakovic M, Cant JP (2015) Translation attenuation via 3' terminal codon usage in bovine *csn1s2* is responsible for the difference in alphas2- and beta-casein profile in milk. *RNA Biol* 12:354–367

Lee HY, Heo YT, Lee SE, Hwang KC, Lee HG, Choi SH, Kim NH (2013) Short communication: retinoic acid plus prolactin to synergistically increase specific casein gene expression in MAC-T cells. *J Dairy Sci* 96:3835–3839

Li SS, Looor JJ, Liu HY, Liu L, Hosseini A, Zhao WS, Liu JX (2017) Optimal ratios of essential amino acids stimulate beta-casein synthesis via activation of the mammalian target of rapamycin signaling pathway in MAC-T cells and bovine mammary tissue explants. *J Dairy Sci* 100:6676–6688

Morrison B, Cutler ML (2009) Mouse mammary epithelial cells form mammospheres during lactogenic differentiation. *J Vis Exp* 32:1265

Qian X, Zhao FQ (2014) Current major advances in the regulation of milk protein gene expression. *Crit Rev Eukaryot Gene Expr* 24:357–378

Radler PD, Wehde BL, Wagner KU (2017) Crosstalk between STAT5 activation and PI3K/AKT functions in normal and transformed mammary epithelial cells. *Mol Cell Endocrinol* 451:31–39

Riley LG, Gardiner-Garden M, Thomson PC, Wynn PC, Williamson P, Raadsma HW, Sheehy PA (2010) The influence of extracellular matrix and prolactin on global gene expression profiles of primary bovine mammary epithelial cells *in vitro*. *Anim Genet* 41:55–63

Sakamoto K, Creamer BA, Triplett AA, Wagner KU (2007) The Janus kinase 2 is required for expression and nuclear accumulation of cyclin D1 in proliferating mammary epithelial cells. *Mol Endocrinol* 21:1877–1892

Sakamoto K, Komatsu T, Kobayashi T, Rose MT, Aso H, Hagino A, Obara Y (2005) Growth hormone acts on the synthesis and secretion

- of alpha-casein in bovine mammary epithelial cells. *J Dairy Res* 72: 264–270
- Schmidt JW, Wehde BL, Sakamoto K, Triplett AA, Anderson SM, Tschlis PN, Leone G, Wagner KU (2014) Stat5 regulates the phosphatidylinositol 3-kinase/Akt1 pathway during mammary gland development and tumorigenesis. *Mol Cell Biol* 34:1363–1377
- Sciascia Q, Pacheco D, McCoard SA (2013) Increased milk protein synthesis in response to exogenous growth hormone is associated with changes in mechanistic (mammalian) target of rapamycin (mTOR)C1-dependent and independent cell signaling. *J Dairy Sci* 96:2327–2338
- Shao Y, Wall EH, McFadden TB, Misra Y, Qian X, Blauwiel R, Kerr D, Zhao FQ (2013) Lactogenic hormones stimulate expression of lipogenic genes but not glucose transporters in bovine mammary gland. *Domest Anim Endocrinol* 44:57–69
- Singh K, Vetharanim I, Dobson JM, Prewitz M, Oden K, Murney R, Swanson KM, McDonald R, Henderson HV, Stelwagen K (2016) Cell survival signaling in the bovine mammary gland during the transition from lactation to involution. *J Dairy Sci* 99:7523–7543
- Sornapudi TR, Nayak R, Guthikonda PK, Pasupulati AK, Kethavath S, Uppada V, Mondal S, Yellaboina S, Kurukuti S (2018) Comprehensive profiling of transcriptional networks specific for lactogenic differentiation of HC11 mammary epithelial stem-like cells. *Sci Rep* 8:11777
- Tucker HA (2000) Hormones, mammary growth, and lactation: a 41-year perspective. *J Dairy Sci* 83:874–884
- Wartmann M, Cella N, Hofer P, Groner B, Liu X, Hennighausen L, Hynes NE (1996) Lactogenic hormone activation of Stat5 and transcription of the beta-casein gene in mammary epithelial cells is independent of p42 ERK2 mitogen-activated protein kinase activity. *J Biol Chem* 271:31863–31868
- Woodward TL, Sia MA, Blaschuk OW, Turner JD, Laird DW (1998) Deficient epithelial fibroblast heterocellular gap junction communication can be overcome by co-culture with an intermediate cell type but not by E-cadherin transgene expression. *J Cell Sci* 111(Pt 23): 3529–3539
- Zhao F, Liu C, Hao YM, Qu B, Cui YJ, Zhang N, Gao XJ, Li QZ (2015) Up-regulation of integrin $\alpha\beta 4$ expression by mitogens involved in dairy cow mammary development. *In Vitro Cell Dev Biol Anim* 51(3): 287–299
- Zhou Y, Akers RM, Jiang H (2008) Growth hormone can induce expression of four major milk protein genes in transfected MAC-T cells. *J Dairy Sci* 91:100–108