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# Spleen tyrosine kinase regulates mammary epithelial cell proliferation in mammary glands of dairy cows

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## ABSTRACT

Spleen tyrosine kinase (SYK) is a nonreceptor tyrosine kinase that has been considered a hematopoietic cell-specific signal transducer involved in cell proliferation and differentiation. However, the role of SYK in normal mammary gland is still poorly understood. Here we show that SYK is expressed in mammary glands of dairy cows. Expression of SYK was higher in dry period mammary tissues than in lactating mammary tissues. Knockdown and overexpression of SYK affected dairy cow mammary epithelial cell proliferation as well as the expression of signal molecules involved in proliferation, including protein kinase B (PKB, also known as AKT1), p42/44 mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription 5 (STAT5). Dual-luciferase reporter assay showed that SYK increased the transcriptional activity of the *AKT1* promoter, and *cis*-elements within the *AKT1* promoter region from −439 to −84 bp mediated this regulation. These results suggest that SYK affects mammary epithelial cell proliferation by activating *AKT1* at the transcriptional level in mammary glands of dairy cows, which is important for the mammary remodeling process in dry cows as well as for increasing persistency of lactation in lactating cows.
**Key words:** mammary gland, SYK, proliferation, AKT1

## INTRODUCTION

Bovine milk is an agricultural product with tremendous economic importance worldwide because it is the primary source of nutrition for growth and development of the calf (Nissen et al., 2013), as well as an important part of human nutrition (Haug et al., 2007). Bovine milk is synthesized in the mammary epithelial cells of dairy cows (Ollier et al., 2007). Many physiological factors, such as nutrition and pregnancy status, and management factors, such as milking frequency and efficiency, influence milk yield and composition (Stefanon et al., 2002; Nørgaard et al., 2008a; Dessauge et al., 2011). At the cell level, studies on mammary cell loss and replacement during lactation indicate that the number of mammary secretory cells and the secretory activity per cell determine milk yield (Capuco et al., 2003). The number of mammary epithelial cells is greatest during early lactation and declines with advancing lactation (Capuco et al., 2001). Thus, it is important to maintain high cell number by either increasing mammary cell survival and proliferation or reducing apoptosis during lactation.

Following cessation of milking or weaning of the young, a dry period occurs. The dry period is necessary to facilitate cell turnover in the bovine mammary gland and to optimize milk production in the next lactation cycle (Capuco et al., 1997). A typical dry period in a dairy cow involves active involution after cessation of milk removal followed by a period of redevelopment before the next lactation cycle (Hurley, 1989). During the first days of active involution, extensive histological changes affect the capability of synthesis and secretion of milk components and a loss of integrity of the mammary epithelial cells are observed (Seeth et al., 2015). Thus, apoptosis is high early in the dry period and then cell proliferation increases (Nørgaard et al., 2008b).

The molecular mechanisms affecting mammary gland lactation cycle and milk quality are numerous, and they include gene expression, micro-RNA, and epigenetic regulation (Singh et al., 2012; Wang et al., 2012). Spleen tyrosine kinase (**SYK**) is a nonreceptor tyrosine kinase that is most highly expressed in hematopoietic cells and has the ability to regulate development and proliferation (Uckun et al., 2010). Upon receptor stimulation, a phospho-ITAM/(SYK-$(SH2)_2$ interaction induces an allosteric activation of SYK by changing the conformation of SYK from a closed inactive structure to an open active form (Kulathu et al., 2009). Expres-

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sion of SYK has been detected in normal mouse and human mammary tissues (Flück et al., 1995; Ruschel and Ullrich, 2004). The expression of SYK in the mammary gland promotes research into its potential role in lactation cycle and milk production. Studies in an IL-2-dependent natural killer cell line have shown that SYK is a critical effector essential for IL-2-mediated survival signaling upstream of protein kinase B (PKB, also known as **AKT1**; Jiang et al., 2003). In a murine hematopoietic cell line, expression of cytoplasmic TEL-SYK fusion protein leads to the constitutive activation of phosphoinositide 3-kinase (**PI3K**)/AKT, mitogen-activated protein kinase (**MAPK**) and signal transducer and activator of transcription 5 (**STAT5**), which are closely involved in cell proliferation (Kanie et al., 2004). However, a study directly comparing the expression of SYK in lactation cycle or in lactating cows with different milk quality has not been reported. Whether SYK affected lactation cycle and milk quality by inducing mammary epithelial cell proliferation has not been well established.

As SYK has the ability to induce cell proliferation, we hypothesized SYK may participate in mammary epithelial cell turnover in lactation cycle of dairy cows. Additionally, SYK may have a relationship with milk quality in lactating cows. To meet these objectives, we compared SYK expression in mammary glands of dry cows versus lactating cows and detected the effect of SYK on mammary epithelial cell proliferation. The expression and activity of proteins known to be involved in pathways regulating cell proliferation were examined by Western blot when SYK was knocked down or overexpressed. To evaluate if SYK was related to milk quality in lactating cows, we compared SYK expression in mammary glands of lactating cows with high-quality milk (milk protein >3% and milk fat >3.5%) versus low-quality milk (milk protein <3% and milk fat <3.5%).

## MATERIALS AND METHODS

### Animals and Tissue Collection

In our study, all animal experimental protocols were approved by Northeast Agricultural University (Harbin, China). Nine multiparous Holstein cows with the similar genetic background were used to obtain mammary tissues: 6 were in the lactating period and 3 were in the dry period. All animals were clinically healthy. All 6 lactating cows were in the third parity (calving at 52 to 54 mo of age) and at 90 DIM. Lactating cows were milked at 0800 and 1530 h daily. Three dry cows were pregnant and dried off at 310 DIM. Animals were housed in tiestalls and fed a ration based on grass silage and concentrate (Supplementary Table S1; http://dx.doi.org/10.3168/jds.2015-10118). The feeding regimen was ad libitum throughout lactation and restricted during the dry period according to their requirements. Animals had free access to fresh water. Lactating cows were allocated to high-quality milk group (milk protein >3% and milk fat >3.5%, n = 3) and low-quality milk group (milk protein <3% and milk fat <3.5%, n = 3) according to their milk protein and fat contents (Table 1). Milk yield of lactating cows with high-quality milk was 33.9 ± 2.1 kg/d. Milk yield of lactating cows with low-quality milk was 33.7 ± 0.5 kg/d. Somatic cell count was <50,000 cells/mL for all cows.

**Table 1**. Milk components of lactating Holstein dairy cows

| Milk component | Dairy cows with high-quality milk | Dairy cows with low-quality milk |
|---|---|---|
| Milk protein (%) | 3.27 ± 0.04 | 2.89 ± 0.02 |
| Milk fat (%) | 4.17 ± 0.01 | 3.20 ± 0.06 |
| Lactose (%) | 4.84 ± 0.03 | 4.52 ± 0.09 |
| DM (%) | 11.93 ± 0.01 | 10.89 ± 0.03 |

The 6 lactating cows were slaughtered at 90 DIM and the 3 dry cows were slaughtered at 30 d after dry off. The lactating cows were milked 1 h before slaughter. Immediately after exsanguination, several pieces of mammary parenchyma tissue were aseptically removed from the midregion of the mammary glands. Tissue samples were trimmed of visible connective and adipose tissues. Small pieces of mammary tissue were frozen in liquid nitrogen and stored at −80°C for RNA and protein extraction or were fixed for immunofluorescence study. For cell culture, fresh tissue was placed in sterilized tubes containing ice-cold Hanks' balanced salt solution (HBSS, Life Technologies, Carlsbad, CA) and immediately transported to the laboratory.

### Reagents and Antibodies

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum (FBS), TRIzol reagent, M-MLV, and Lipofectamine 2000 transfection reagent were purchased from Life Technologies. The RIPA buffer (P0013C) and mammalian genomic DNA extraction kit were purchased from Beyotime Biotechnology (Shanghai, China). The BCA protein assay kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). KeyFluor488 Click-iT 5-ethynyl-2'-deoxyuridine (**EdU**) Imaging Kit was purchased from KeyGEN BioTECH (Nanjing, China). The SYK, cyclin D1, STAT5, phospho-STAT5, and AKT1 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phospho-AKT1 antibody was purchased from Abcam (Cambridge, MA). The p42/44