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BRIEF COMMUNICATION: DNA methylation events in the α S1-casein-encoding gene associated with involution and re-initiation of lactation in dairy cows

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Introduction

During lactation, prolactin activates STAT5 (Signal Transducer and Activator of Transcription5), which binds to conserved DNA sequence motifs in promoters of milk protein genes to stimulate their expression (Liu et al. 1997). Mammary gland involution induced by termination of milking is characterised by a rapid decrease in milk protein gene expression (Singh et al. 2008). Whereas milk production can be fully restored after seven days of non-milking (Dalley & Davis 2006), mammary lactation is less likely to be restored with longer non-milking periods (Capuco et al. 1997). The major bovine milk protein α S1-casein is down-regulated by mammary gland involution (Singh et al. 2008), and is associated with an increase in methylation of three CpG dinucleotides immediately upstream of a doublet STAT5 binding site at the distal α S1-casein promoter (~10 kb) (Vanselow et al. 2006; Singh et al. 2009). The aim of this study was to determine if methylation of a CpG dinucleotide within the distal doublet STAT5 binding site is reversible with resumption of milking following extended non-milking.

Materials and methods

Twenty five non-pregnant primiparous Friesian dairy cows at mid-lactation (97 ± 2 days in milk) were divided into five groups of five cows per group. Mammary alveolar tissue was obtained at slaughter from lactating cows six hours post-milking (Control), cows with non-milking intervals of either seven or

28 days, and cows where milking was resumed for seven days following these dry periods. A liver sample (Positive control) was also collected from a lactating cow. The pasture-grazed cows had a mean milk production of 14.0 ± 2.0 L/d (Alfa Laval Cow Management System, Ruakura Dairy, AgResearch, Hamilton) and a somatic cell count less than 150,000 cells/mL (Flow Cytometry, LIC) for a seven day period prior to the non-milking intervals. All procedures were approved by the Ruakura Animal Ethics Committee.

Total RNA was extracted from alveolar tissue and α S1-casein mRNA levels determined by real-time reverse transcription polymerase chain reaction as described by Singh et al. (2005). Differences between means were analysed by ANOVA in GenStat (Payne et al. 2009). The means for each group were backtransformed and expressed as the fold change \pm standard error of mean relative to the lactating mean. Genomic DNA was isolated from mammary alveolar tissue and bisulfite treated using an EZ DNA methylation-gold™ kit (Zymo Research, Orange, CA, USA). PCR amplification products were prepared for quantitative methylation analysis of the CpG dinucleotide located within the STAT5 binding site (~10 kb) in the α S1-casein promoter (Fig. 1) and analysed using Sequenom MassARRAY quantitative methylation analysis (Ehrich et al. 2005). The resultant spectra's methylation ratios were generated by the EpiTyper software (v1.0.6; Sequenom). Data are expressed as mean percentage methylation for each treatment group.

Figure 1 Genomic map around the distal α S1-casein promoter (modified from Vanselow et al. 2006). STAT5 binding sites (lines with carets) between -10453 and -8534 in the α S1-casein promoter, showing the functional doublet STAT5 binding site. Arrows show the position of CpG dinucleotides including the site that was analysed in this study (*).

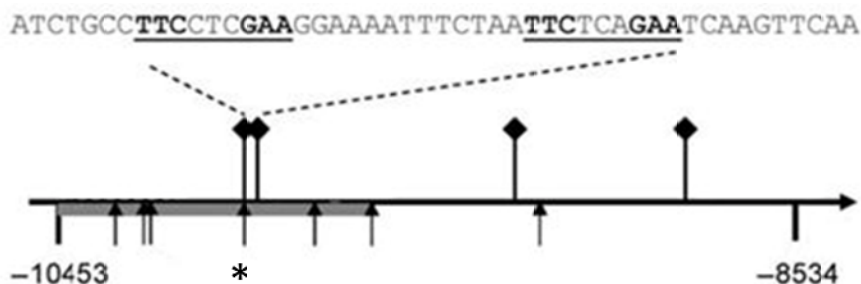
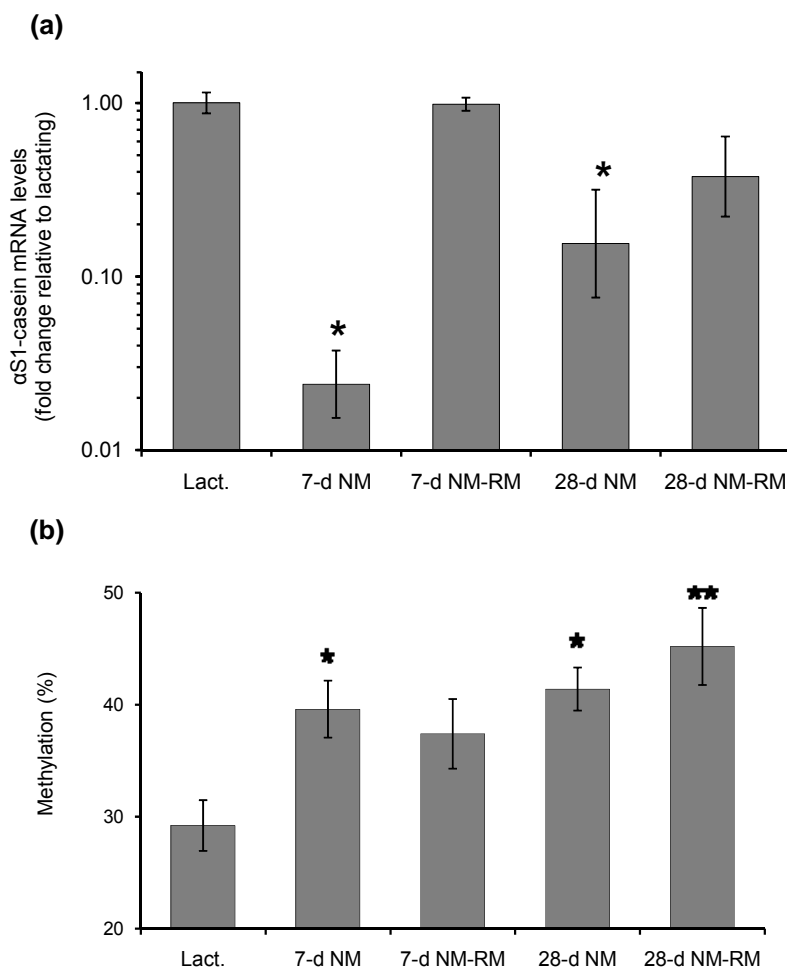


Figure 2 Average fold change in α S1-casein mRNA level relative to lactating (Lact.) (a) and average percentage of chromosomes methylated at the CpG dinucleotide within the STAT5 binding site of the distal α S1-casein promoter in mammary alveolar tissue (b) with seven or 28 days of non-milking (7-, 28-d NM) and with seven day re-milking following seven or 28 days non-milking (7-d, 28-d NM-RM). Error bars indicate the standard error of the mean with significance relative to the lactating cows (* $P < 0.05$, ** $P < 0.01$; (a) ANOVA, (b) *t*-test).



Results and discussion

The α S1-casein mRNA level in mammary alveolar tissue decreased 42- and 6.5-fold following seven and 28 days of non-milking ($P < 0.05$; Fig. 2a), respectively, relative to lactating cows. This agrees with the decrease in milk protein gene expression previously observed with induced involution (Singh et al. 2008). In contrast to the seven day non-milked groups, there was a large degree of variability in levels of α S1-casein mRNA in the 28 day non-milked group, with some cows still demonstrating a high level of α S1-casein mRNA following extended non-milking. This suggests that involution of the bovine mammary gland occurs at different rates between cows supporting earlier findings that demonstrated variability in milk protein gene expression between cows with extended periods of non-milking (Molenaar et al. 2004). Methylation of the CpG dinucleotide within the STAT5 binding site of the distal α S1-casein promoter increased ($P < 0.05$) from 29% in alveolar tissue of lactating cows to 40% and

41% with seven and 28 day non-milking, respectively (Fig. 2b). This negative association between methylation and α S1-casein gene expression has been previously described for other CpG dinucleotides within this promoter (Vanselow et al. 2006; Singh et al. 2009). Although the percentage of methylated chromosomes following seven and 28 day non-milking were increased compared to lactation, the values were still low compared with liver from a lactating cow where 72% of the chromosomes were methylated at this CpG site (S Pryor, Unpublished data). As involution progresses it would be expected that the level of methylation in the alveolar tissue would approach that of liver, which does not express α S1-casein (Johnson et al. 1983).

A milk yield recovery of 91% was observed with seven days of re-milking following seven days of non-milking, compared with 29% recovery after 28 days of non-milking (Singh et al. 2012). Moreover, re-milking resulted in the α S1-casein mRNA levels returning to that of lactating cows after the seven day non-milking period; however, after

28 days of non-milking the levels remained two fold lower than that of lactating cows (Fig. 2a). These results suggest that involution is more readily reversible after seven than 28 days of non-milking, supporting an earlier study where 25% of mammary epithelial cells retained secretory activity after seven days of non-milking, in contrast to 25 days of non-milking where no secretory activity was noted (Capuco et al. 1997).

Despite the increase in α S1-casein mRNA levels with re-milking compared to non-milking (Fig. 2a), the percentage of chromosomes methylated at the CpG dinucleotide within the STAT5 binding site were not reversible (Fig.2b). That is, the methylation levels remained higher than lactating samples and similar to non-milked groups following re-milking (Fig. 2b). As DNA methylation increased with non-milking, a decrease in methylation may have been expected with the recovery of α S1-casein mRNA level. Although controversy exists over the concept of active CpG demethylation (Ooi & Bestor 2008), a dynamic role for methylation/demethylation has been reported for gene promoters in human cells (Metivier et al. 2008). In the present study the level of methylation obtained with seven and 28 days non-milking may not have been sufficient to observe reversibility in methylation levels with re-milking. Moreover, the interaction of DNA methylation with potentially more dynamic histone modification may play a role in regulating α S1-casein gene transcription. Future research will focus on the involvement of histone modifications with the onset of involution, in addition to the methylation of other CpG sites located around the distal promoter region of α S1-casein.

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