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Epigenetics: a possible role in acute and transgenerational regulation of dairy cow milk production

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A potential role for epigenetic mechanisms in the regulation of mammary function in the dairy cow is emerging. Epigenetics is the study of heritable changes in genome function that occur because of chemical changes rather than DNA sequence changes. DNA methylation is an epigenetic event that results in the silencing of gene expression and may be passed on to the next generation. However, recent studies investigating different physiological states and changes in milk protein gene expression suggest that DNA methylation may also play an acute, regulatory, role in gene transcription. This overview will highlight the role of DNA methylation in the silencing of milk protein gene expression during mastitis and mammary involution. Moreover, environmental factors such as nutrition may induce epigenetic modifications of gene expression. The current research investigating the possibility of in utero*, hence cross-generational, epigenetic modifications in dairy cows will also be discussed. Understanding how the mammary gland responds to environmental cues provides a potential to enhance milk production not only of the dairy cow but also of her daughter.*

Keywords: epigenetics, DNA methylation, transgeneration, milk production, dairy cows

Implications

Epigenetic regulation of gene expression is emerging as a hitherto unknown level of biological control. We will discuss how epigenetics may be part of the enormously complex regulatory pathways underpinning milk production. Not only may epigenetic regulation be directly involved in milk production of the mother ('acute epigenetics'), but it may also indirectly affect milk production in its offspring through transgenerational epigenetics, by effects on foetal development *in utero*. Gaining a clearer understanding of this level of regulatory control may lead to new insights in optimizing milk production not only in today's cows but also in future generations.

Introduction

Milk production in dairy cows is influenced by numerous factors, including the environment (e.g. nutrition, photoperiod and heat stress), hormones, local factors within the mammary gland (e.g. autocrine, paracrine and homeostatic feedback systems), diseases (e.g. mastitis) and management practises (e.g. milking frequency). At the cellular level, these factors influence both the number and the activity of the mammary epithelial cells that synthesize milk. Multiple cell signalling pathways have been identified that play a role in regulating milk synthesis (Suchyta *et al.*, 2003; Connor *et al.*, 2008; Finucane *et al.*, 2008; Singh *et al.*, 2008). At the initiation of lactation, the rapid increase to peak milk yield following parturition is primarily because of an increase in mammary epithelial activity in response to milking. The gradual decline in milk production following peak lactation is anomalous to gradual involution and is predominantly a result of mammary epithelial loss via apoptosis (Wilde *et al.*, 1997; Capuco *et al.*, 2001). Microarray studies on rodent (Master *et al.*, 2002; Clarkson *et al.*, 2004; Stein *et al.*, 2004) and bovine (Suchyta *et al.*, 2003; Singh *et al.*, 2008) mammary tissues have identified multiple cell signalling pathways that may orchestrate the switch from a lactating to a non-lactating phenotype. Moreover, the cell-to-cell communications via tight junction proteins (Cooper *et al.*, 2004) and the cell–extra cellular matrix communication (Gilmore *et al.*, 2000; McMahon *et al.*, 2004; Singh *et al.*, 2005) are also disrupted during involution.

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It is becoming increasingly evident that epigenetic mechanisms play a role in the molecular regulation of milk production in dairy cows. Epigenetics refers to changes in genome function that occur because of chemical changes in DNA and its surrounding chromatin rather than DNA sequence changes. These changes can remain stable through rounds of cell division and even from mother to offspring (i.e. transgenerational; Riggs *et al.*, 1996). Epigenetic mechanisms modulate chromatin structure and may either repress or enhance gene expression. These mechanisms include DNA methylation (Jones and Takai, 2001; Jaenisch and Bird, 2003); histone modifications, such as acetylation, ubiquitination, methylation and phosphorylation (Strahl and Allis, 2000); and microRNAs (Bartel, 2009). DNA methylation is the most extensively studied epigenetic regulators (Jaenisch and Bird, 2003). A series of complex changes in methylation patterns are essential for development and differentiation, and influence housekeeping, X-chromosome-linked, imprinted and tissue-specific genes. DNA methylation occurs predominantly at the 5′-position of cytosine in cytosine–phosphate–guanine (CpG) dinucleotides in motifs known as CpG islands. These CpG islands are associated with protein-coding genes with the majority being located in the promoter regions (Bird, 2002). It is proposed that DNA is methylated by atleast two *de novo* methyltranferases – Dnmt3a and Dnmt3b. The Dnmt1, through its actions on hemi-methylated DNA, ensures complete methylation, and hence is responsible for maintaining the methylation pattern. Demethylation may be passive or can also occur actively, although the process of demethylation mechanisms is less clear (Reik and Walter, 2001). Methylation may also occur at isolated CpG dinucleotides in close proximity to functional transcription factor binding sites (Vanselow *et al.*, 2006). Furthermore, a small fraction of methylation also occurs in non-CpG cytosines (Ramsahoye *et al.*, 2000). Two mechanisms have been proposed to explain the mechanisms whereby DNA methylation represses gene transcription. First, methylated CpGs may prevent the binding of the transcriptional activators (Bird and Wolffe, 1999). Second, transcriptional repressors with methyl-CpG-binding domains may associate with methylated CpGs and block transcription by modifying the surrounding chromatin or prevent interaction by activators. The methyl-CpG-binding proteins are thought to play a role in this interaction between DNA methylation and chromatin remodelling and modification (Klose and Bird, 2006).

Epigenetic marks are generally maintained for the life of an organism; however, the acute nature of DNA methylation for regulating gene expression has been described (Vanselow *et al.*, 2006; Kangaspeska *et al.*, 2008; Métivier *et al.*, 2008) and will be discussed below with respect to milk protein gene expression (see section 'Acute DNA methylation regulation of casein expression in mammary epithelial cells'). In mammals, there is limited evidence that these marks can be passed on to the next generation. The direct evidence in rodents and indirect evidence in humans and livestock will be discussed below (see section 'Transgenerational epigenetic inheritance studies in mammals'). Unravelling the epigenetic mechanisms that regulate milk production may explain how environmental factors influence lifetime lactation performance of the dairy cow, as well as the lactation performances of her offspring. Studies investigating epigenetic mechanisms regulating milk protein gene expression in rodent mammary gland development and functional differentiation (Rijnkels *et al.*, 2010), and bovine mammary gland regression, have recently been reviewed (Singh *et al.*, 2010a). This current review will focus on DNA methylation and will provide an update of, first, the evidence that DNA methylation may play an acute role in regulating the decline in milk protein gene expression during bovine mammary involution and disease, and, second, the possibility of transgenerational epigenetic inheritance in dairy cows.

Acute DNA methylation regulation of casein expression in mammary epithelial cells

Epigenetic modifications and chromatin conformation during normal mammary gland development has recently been reviewed (Rijnkels *et al.*, 2010). Studies indicate that lactogenic hormones can induce an open chromatin conformation at regulatory regions, which correlates with milk protein gene expression. Focussing on DNA methylation, in this review, methylation levels are associated with the expression of several casein genes. Studies in rodents demonstrate that β- and γ-casein genes are hypomethylated in the lactating mammary gland. In contrast, in the liver, these genes are hypermethylated, and thus not expressed (Johnson *et al.*, 1983). The κ-casein gene is also hypomethylated in lactating mammary glands but hypermethylated in non-mammary and non-lactating tissue (Thompson and Nakhasi, 1985). Hypomethylation during lactation has also been described for the bovine αS1-casein gene (Platenburg *et al.*, 1996). Methylation and expression of this gene has been studied in mammary tissue of cows at different physiological states and during disease.

DNA methylation of bovine αS1-casein gene during mastitis and involution

Vanselow *et al.* (2006) have described the association of DNA methylation and chromatin structure around a signal transducer and activator of transcription (STAT)5-binding lactation enhancer, which occurs at approximately −10 kb of the αS1-casein-encoding gene. In the bovine lactating mammary gland, this region of DNA is hypomethylated. During *Escherichia coli* infection of the mammary gland, this region becomes methylated at three CpG dinucleotides and is associated with chromatin condensation. These changes accompany the shutdown of αS1-casein synthesis, with the mRNA levels dropping to 50% and protein levels to 2.5% of that in the non-mastitic control glands (Vanselow *et al.*, 2006). Similar results have been demonstrated with a *Streptococcus uberis* infection of the mammary gland. The αS1-casein mRNA declined by 2.3-fold in mastitic tissue compared with non-mastitic controls (Swanson *et al.*, 2009), and there was an increase in methylation levels of the three CpG sites in the mastitic tissue (ranging from 28% to 68%) compared with non-mastitic controls (ranging from 10% to

25%; Singh *et al*., 2010b). Preliminary results demonstrated that DNA compaction in this region of the αS1-casein gene was increased from 31% to 45% during infection (Molenaar *et al*., 2010).

Furthermore, in healthy mammary tissue, there is an increase in DNA methylation at these three CpG dinucleotides at the functional STAT5-binding site of the αS1-casein promoter, following an 8-day non-milking period when milking was ceased at mid-lactation (Singh *et al*., 2009a). Interestingly, there was a decline in methylation following an 18 h non-milking period compared with lactation. This is in the same time frame in which multiple physiological events start to occur in response to the cessation of milk removal in dairy cows (Stelwagen, 2001). These events occur before the decline in prolactin–STAT5 signalling (Singh *et al*., 2009b) and milk protein gene expression that occurs at 24 to 36 h post milking (Singh *et al*., 2008). The average percentage compaction of 15% appeared relatively constant for the first 24 h, but then increased by an average of 1.9% per day to 30% by day 8 of non-milking (Molenaar *et al*., 2010). Although DNA methylation may not initiate the decline in αS1-casein mRNA during involution, it plays a role in silencing αS1-casein expression in both involution and infection of the udder.

Variability in DNA methylation of the bovine αS1-casein gene and rate of involution between cows

There is wide variation in the rate of DNA compaction in mammary glands from different cows, with most exhibiting a relatively moderate to high rate of compaction and a few showing very little compaction. Comparison of the casein mRNA expression and DNA compaction revealed a close but inverse relationship (Molenaar *et al*., 2010). The variability in DNA methylation and association with gene expression are supported by a subsequent study demonstrating an increase in DNA methylation in mammary tissue in mid-lactation dairy heifers that have undergone non-milking periods of up to 28 days (Swanson *et al*., 2011). In this study, the between-cow variation in the rate of involution was also observed both by quantitative real time-PCR and by histology, with two out of five cows showing high casein expression and histological characteristics of lactation, whereas the remaining three had low to no casein expression and histological characteristics of involution (data not shown). The causes and mechanisms of the variation in the rate of involution are currently under investigation.

DNA methylation changes of the bovine αS1-casein gene during reversible and irreversible involution

Studies of involution of the rodent mammary gland demonstrate that this process occurs during two distinct stages, that is, reversible and irreversible involution. The regression of the mammary gland results in irreversibility of involution during the second stage; however, this process is reversible within the first 48 h (Jaggi *et al*., 1996; Li *et al*., 1997; McMahon *et al*., 2004). The reversible nature of the initial stages of involution in the rodent mammary gland has been demonstrated following re-suckling of engorged and involuting rat mammary glands (McMahon *et al*., 2004). However, the extent to which mammary gland involution is reversible varies widely among species. In contrast to the rodent mammary gland, involution in cows does not occur to the same extent (Capuco *et al*., 2001). During the extended process of involution, which occurs in the bovine mammary gland, many alveolar structures are retained (Holst *et al*., 1987) and Capuco *et al*. (1997) suggest that during involution of the bovine mammary gland senescent and damaged cells are being replaced, rather than extensive remodelling of the mammary epithelial cell compartment. Thus, in the cow, mammary involution may potentially be reversible following extended non-milking periods (Noble and Hurley, 1999).

Indeed, cessation of milk removal for up to 7 days from mid-lactation cows demonstrated that lactation could be fully restored following re-initiation of milking twice daily for 7 days (Dalley and Davis, 2006). Numerous other studies have demonstrated re-initiation of lactation following short-term non-milking periods (Wheelock *et al*., 1965; Stelwagen *et al*., 2008). Extended non-milking periods of 14 days demonstrated recovery of milk yield to pre-trial levels following 16 days of re-milking (Hamann and Reichmuth, 1990). However, cessation of milking was in one udder half, whereas the contralateral udder half was continuously milked. Therefore, the presence of lactogenic signals and hormones such as prolactin may have delayed the onset of involution in the unmilked glands (Akers and Keys, 1985; Feng *et al*., 1995; Noble and Hurley, 1999). Previous studies from our laboratory have shown a significant down-regulation in bovine milk protein gene expression following 24 to 36 h post milking. By 8 days post milking, apoptotic factors increased and mammary epithelial cells undergoing apoptosis were identified, suggesting that the mammary gland had entered the process of involution (Singh *et al*., 2008). However, the complete recovery of milk yield following cessation of milking for up to 7 days suggests that the process of involution is fully reversible at this stage (Dalley and Davis, 2006). Following which, previous studies have suggested that the process of involution becomes increasingly irreversible (Noble and Hurley, 1999).

Research from our laboratory support and further extend the current studies on lactation re-initiation in the dairy cow. Extended non-milking periods in mid-lactation heifers for either 7, 14 or 28 days resulted in large variation in the recovery of milk yield and composition for the different treatment groups (Swanson *et al*., 2011). Greater than 92% recovery of milk yield was observed by 5 days after resumption of milking following a 7-day non-milking period. This recovery decreased to 48% for the 14-day non-milking period and to less than 20% for the 28-day non-milking period. Results suggest that STAT5/STAT3 and insulin-like growth factor-I signalling pathways play a central role in the reversible and irreversible phases of bovine mammary gland involution (Singh *et al*., 2011). Results from this and previous reports suggest that alveoli may enter the process of involution at varying rates and the observed flexibility in bovine mammary function may be because of this heterogeneity.

Heterogeneity within the bovine mammary gland

The retention of functional alveoli has been demonstrated using histological techniques where small populations of involuting alveoli are present in lactating tissue (Molenaar *et al*., 1992and 1996). Cessation of milk removal in the bovine mammary gland induces morphological, physiological and structural changes, which result in the initiation of involution. However, the retention of alveolar structures ensures that unlike rodents, lactation can still be re-initiated in the bovine mammary gland after extended non-milking periods. Therefore, the shutdown of milk production may be partially due to other regulatory mechanisms, such as epigenetics. This is currently being addressed by investigating the re-initiation of milking following extended periods of non-milking in the trial described above (Swanson *et al*., 2011). Methylation levels of CpG dinucleotides at the STAT5-binding site −10 kb in the αS1-casein promoter were increased following both 7 and 28 days non-milking, compared with lactating cows (Swanson *et al*., 2011). Furthermore, the full recovery of lactation following re-initiation of milking after the 7 days non-milking was associated with demethylation of DNA, returning to levels similar to those in lactating control cows. However, the DNA methylation levels remained high following the re-initiation of milking after 28 days non-milking, which corresponded with the low partial recovery of milk yields. These results suggest that DNA methylation at a functional STAT5-binding site of the αS1-casein-encoding gene may play a role in regulating both reversible and irreversible involution. Future studies are focussed on addressing the heterogeneity within a gland to understand the relationship of DNA methylation events with gene expression. An understanding of how chromatin remodelling mechanisms may be manipulated for the regulation of milk production is necessary to potentially devise novel approaches and/or technologies to enhance the lactation performance of dairy cows.

Transgenerational epigenetic inheritance studies in mammals

The epigenetic state of the mammalian genome undergoes dynamic reprogramming events in the germ cells and in the early embryo (Dean *et al*., 2003; Jirtle and Skinner, 2007). There is increasing evidence that prenatal and early postnatal environmental factors can modify the epigenome to develop stable alterations in the phenotype (Jirtle and Skinner, 2007). Barker *et al*. (1993) proposed the 'foetal origin hypothesis', whereby the environment *in utero* may lead to permanent effects in subsequent generations. Evidence supporting this is from human epidemiological studies, suggesting foetal undernutrition, is associated with foetal growth and programming of several adult diseases (Barker, 1995). Although it is possible that permanent influences on the foetus may be because of the direct influences of the environment on the foetus, rather than mediated via maternal epigenetic mechanisms, transgenerational inheritance in humans has been suggested by epidemiological studies of the Dutch Famine of 1944, referred to as the Dutch Famine Birth Cohort Study (Lumey, 1992). This investigation showed that women who were pregnant during the extreme famine conditions gave birth to children who were smaller than average and who were also more susceptible to health problems (e.g. diabetes, obesity, cardiovascular disease, microalbuminuria, etc). The children of these children were also smaller. Although these early findings suggest that nutrition may have an influence for at least two subsequent generations, the effect of severe *in utero* maternal malnutrition on offspring size was not confirmed in a subsequent analysis, perhaps because of sampling variability or study design (Lumey *et al*., 1995). Morgan and Whitelaw (2008) have reviewed several studies in humans, suggesting that environmental factors may play a role in transgenerational inheritance effects; however, there is no direct molecular evidence and it is difficult to interpret whether transgenerational effects are due to social factors or epigenetic mechanisms.

In contrast to human studies, in mice, there is direct evidence of transgenerational epigenetic inheritance. This is from studies investigating nutritional supplementation of methyl donors and genes with metastable epialleles (Morgan *et al*., 1999), although, even here, there are contradicting results, such that the evidence for transgenerational inheritance of these marks is not robust. The A^{vy} allele is a dominant mutation of the *agouti* (A) locus, caused by the insertion of an intracisternal A-particle (IAP) retrotransposon upstream of the agouti coding exons. A cryptic promoter in the proximal end of the A^{vy} IAP promotes constitutive ectopic expression of the *agouti* gene, resulting in a yellow coat colour (Duhl *et al*., 1994). However, DNA methylation at this promoter results in its silencing and the agouti colour is expressed (Morgan *et al*., 1999). Furthermore, the dam's nutrition when pregnant can influence coat colour of the offspring, for example, supplementing the diet with methyl donors shifts the offspring's coat colour from yellow to brown (Wolff *et al*., 1998; Waterland and Jirtle, 2003). Although, there is evidence that transgenerational inheritance of these DNA methylation marks occur (Cropley *et al*., 2006), there is also a report suggesting that the increased DNA methylation levels at the A^{vy} locus were not due to transgenerational inheritance (Waterland *et al*., 2007). Another possible direct example of transgenerational epigenetic inheritance reported in mice describes the axin-fused, $Axin^{Fu}$, allele. This allele contains a retrotransposon within an intron of the gene, which may result in a kinked tail Vasicek *et al*., 1997; (Zeng *et al*., 1997). Both the A^{vy} and $Axin^{Fu}$ alleles demonstrate phenotypic variability (Morgan *et al*., 1999).

Genomic imprinting is a non-Mendelian epigenetic process that is inherited through the germline. The epigenetic mechanisms involve DNA methylation and histone modifications for gene expression in a parent-of-origin-specific manner, that is, the paternally and maternally inherited alleles are expressed to different degrees. Lactation is a maternal trait, hence it is possibly imprinted and in agreement with the parental conflict hypothesis. In livestock, reviewed by Ruvinsky (1999), studies in multiple pig breeds,

reciprocal crosses between different sheep breeds, callipyge mutant allele studies in heterozygous sheep and reciprocal crosses between horse and donkey provide evidence of genomic imprinting. In cattle, it has been shown that manipulation of pre-implantation embryos *in vitro* (i.e. *in vitro* fertilization, nuclear transfer and embryo transfer) can result in a condition known as large calf syndrome, leading to developmental abnormalities. This may possibly be because of shifts in a balance between paternal and maternal contributions (Moore and Reik, 1996). Cloning may lead to epigenetic errors resulting from incomplete reprogramming of the donor cell's nucleus. Nevertheless, milk produced by cloned cows was similar to that of cows in commercial herds managed under similar conditions (Laible *et al.*, 2007).

We are currently investigating whether transgenerational epigenetic inheritance, with regard to milk production, occurs in dairy cows. Epigenetic regulation is not specifically accounted for in current animal genetic models. The current animal genetic models (Van Tassell *et al.*, 1997) assume that 30% of phenotypic variance in milk yield is due to Mendelian genetic (additive) effects, whereas 15% is associated with the cow's permanent environment (PE) effect. PE effects refer to those factors that are responsible for a consistent deviation from the cow's expected yield following adjustment for genetic gain, management, herd effects and herd by sire interactions. The PE effect can account for as much as 40% of variance in milk production (Bormann *et al.*, 2003) and likely include epigenetic effects. When subdivided into the within- and across-lactation components, PE effects for milk production ranged from 22% to 26% and 6% to 16%, respectively, for phenotypic variation, suggesting that a significant portion of PE within an individual cow is carried forward across lactations (Bormann *et al.*, 2003).

Diverse environmental factors and management practices influence dairy cow milk production. Nutrition of the animal is a key environmental factor affecting milk production, either directly, during lactation, or indirectly, through mammary development. Sejrsen *et al.* (1982) showed that a high plane of nutrition during the first 9 months of a heifer's life may lead to changes in the mammary tissue, resulting in a lower subsequent milk yield. Furthermore, nutrition during gestation may influence not only subsequent lactation (Park *et al.*, 1989) but also performance during the second lactation (Ford and Park, 2001). In this study, heifers were subjected to energy restriction during mid-gestation, followed by re-feeding during late gestation. It was hypothesized that epigenetic mechanisms may play a role in these carryover effects on lactation (Park, 2005). To support this, there was a decrease in 5′-methyldeoxycytidine levels in late gestation mammary tissue from heifers subjected to the stair-step compensatory nutrition compared with controls, which was associated with increased casein gene expression (Choi *et al.*, 1998).

Adverse environmental effects, relating to suboptimal nutritional and health status, are often associated with a reduction in milk yield and the depletion of body energy reserves. Such adverse maternal conditions may in turn have an impact on the phenotype of the offspring of the affected cows, which may be, in part, due to epigenetic modifications *in utero*. Body condition score (BCS) is often used as an indicator of a cow's energy or nutritional status. Previous New Zealand studies suggest a significant effect of BCS on subsequent reproductive performance of female progeny (Roche *et al.*, 2006; Pryce and Harris, 2006). In a recent Irish study, using their national database, intrauterine conditions were quantified by a maternal variance component to determine the potential relationship between dam milk production and daughter milk yield in the Irish Holstein population (Berry *et al.*, 2008). Maternal milk production effects on daughter milk yields were small and negatively related. This association increased with gestation trimester. Further, there was no PE effect of dam on daughter performance. High-producing cows tend to be in greater negative energy balance. Thus, greater metabolic stress during pregnancy may have subsequent negative effects on the daughters' lactation performance.

In contrast, our preliminary results suggest a positive association of dam's milking performance and nutritional status with her daughter's first lactation performance. The dam's nutritional status was indicated by BCS measured either during late first trimester or early second trimester of the dam's second pregnancy (i.e. during their first lactation). A total of 11 593 unique dam–daughter pairs with data on the first lactation and BCS of the dam and the daughter's first lactation milk yield were analysed. These dam–daughter pairs were physically distributed throughout New Zealand, belonging to 1373 different herds, representing three different genetic groups (Holstein–Friesian, Jersey and Kiwi-cross). The dam data were obtained over the period between 2002 and 2006 and the daughter's performance was measured between 2005 and 2009. After correcting for the environmental effects (year, herd) and the additive genetic effect on milk yield (via the daughters estimated breeding value for that trait), the dam's maternal environment was found to influence their daughter's subsequent milk production in the Jersey breed, but not in the Holstein–Friesian or Kiwi-cross. The contradictory results between this and the Irish studies (Berry *et al.*, 2008) are likely a reflection of the different management systems between the two countries. Furthermore, Berry *et al.* (2008) kept only animals with greater than 81% Holstein–Friesian lineage with 100% Holstein–Friesian sires. There is currently no direct evidence for transgenerational epigenetic inheritance in dairy cows. The limited indirect evidence suggests that the lactation performance of the dam has a strong influence on the daughter's lactation performance in the Jersey breed. Further analyses of larger datasets are required to more accurately account for the different genetic effects using an animal model. It is a logistical challenge to design transgenerational cow trials in order to distinguish between genetic and epigenetic mechanisms because of the large generation intervals of cattle. However, this type of a study may result in novel approaches to enhancing the lifetime lactation performance of the dam and also that of its offspring. As some of the phenotypic variation

could be due to epigenetic factors, continued research into the epigenetic regulation of milk production may have significant economic and environmental benefits.

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