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**Intra-mammary molecular mechanisms
involved in the response to changes in
milking frequency**

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Abstract

In dairy cows, short-term changes of milking frequency (MF) in early lactation have been shown to produce an immediate and a long-term effect on milk yield (MY). The effect is controlled locally within the mammary gland by as yet unknown factors. To investigate the intra-mammary molecular mechanisms that are involved in the MY response to MF, a unilateral milking frequency (UMF) experiment was conducted with udder halves of 17 multiparous pasture-fed dairy cows milked either four times a day (4x) or once a day (1x) for fourteen days from 5 ± 2 days in milk (DIM). Mean udder-half MY during the treatment period was higher from the 4x compared to 1x-udder halves and once returned to twice a day milking, continued to be higher until 200 DIM. Mammary biopsies were obtained on day fourteen of treatment from both udder halves of ten cows. Proliferation of mammary cells was higher in 4x-udder halves compared to 1x, whereas no difference in apoptosis levels was detected. Abundance of major milk protein gene mRNA was higher in tissue samples from 4x-udder halves compared with 1x. The effects of changes of MF in early lactation on the prolactin (PRL) and insulin-like growth factor I (IGF-I) pathways to determine their role in the MY response to MF. The activation of signal transducer and activator of transcription (STAT) 5 was measured as an indication of PRL signalling, which was higher following 4x-milked mammary tissue samples compared to 1x-milked, and correlated highly with milk protein gene mRNA abundance. Activation of STAT5 also correlated with the protein abundance of the extracellular matrix (ECM) interacting protein β 1-integrin, which suggest a

link between PRL/STAT5 and ECM/ β 1-integrin signalling. The mRNA abundance of IGF binding protein (IGFBP)3 and IGFBP5 were lower in 4x-milked mammary tissue samples relative to 1x-milked. Both IGFBP3 and IGFBP5 are thought to inhibit IGF-I, so the decrease in their mRNA abundance may serve to stimulate the IGF-I signal in the 4x-milked mammary gland. However, two cellular pathways downstream of IGF-I (phosphoinositide 3-kinase (PI3K)/Akt and extracellular-signal-regulated kinase (ERK)1/2) were not positively affected by 4x milking. The activation of PI3K/Akt pathway was lower in 4x-milked mammary tissue samples relative to 1x-milked, and the activation of the ERK1/2 was unaffected by MF. Overall, the results obtained in this thesis have increased the understanding of the changes in intra-mammary molecular mechanisms in response to differing MF.

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Abbreviations

1x	once a day milking
2x	twice a day milking
3x	three times a day milking
4x	four times a day milking
B2M	β -2 microglobulin
BAX	Bcl-2-associated X protein
BCL-XL	B-cell lymphoma-extra-large
BSA	bovine serum albumin
C_t	threshold cross-over value
CIS	cytokine inducible SH2 protein
CP	crude protein
CSN1S1	α_{S1} -casein
CSN2	β -casein
DIM	days in milk
DM	dry matter
ECM	extracellular matrix
ERK	extracellular-signal-regulated kinase
FAK	focal adhesion kinase
GRB2	growth factor receptor-bound protein 2
GH	growth hormone

HRP	horseradish peroxidase
IGF1	insulin-like growth factor I gene
IGF1R	insulin-like growth factor type I receptor gene
IGFBP	IGF binding protein
IGF-I	insulin-like growth factor I
IGFIR	insulin-like growth factor type I receptor
IRS	insulin receptor substrate
JAK	janus kinase
LALBA	α -lactalbumin
LGB	β -lactoglobulin
LTF	lactoferrin
LIF	leukaemia inhibitory factor
Lu	lumen
ME	metabolisable energy
MEC	mammary epithelial cell
MF	milking frequency
MY	milk yield
NS	not significant
PI3K	phosphoinositide 3-kinase
PRL	prolactin
PRLR	prolactin receptor

Rac1	Ras-related C3 botulinum toxin substrate 1
RIN	RNA integrity number
SCC	somatic cell count
SED	standard error of the difference
SEM	standard error of the mean
SHC	src/collagen homology protein
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
TBS	tris buffered saline
TBST	tris buffered saline containing 1% tween 20
UBB	ubiquitin B
UMF	unilateral milking frequency

Chapter I

Review of literature

1.1 Introduction

Adjusting the frequency with which dairy cows are milked, both upwards and downwards, can be an effective management practice that farmers can use to alter milk production, nutrient requirements, animal management and operating costs as on-farm conditions change. Increasing milking frequency (MF) above the most commonly employed practice of twice a day (2x) to three- (3x) and four times a day (4x) can increase milk yield (MY) by as much as 10 to 20 % (Erdman and Varner, 1995, Stelwagen, 2001). However, there are increased costs associated with greater MF (Erdman and Varner, 1995). In low-cost and low-input dairy systems, such as those employed in New Zealand and Ireland, the use of once a day (1x) milking can provide an effective management tool to reduce farm inputs such as feed requirements and labour (Davis et al., 1999). Decreasing MF from 2x to 1x can decrease MY by approximately 20 % (Stelwagen, 2001), but the associated decreasing fixed and variable cost of milk production and labour requirements associated with milking can be beneficial to the farm business overall. Furthermore in these pasture-based systems, 1x milking can also be utilised to decrease the amount of nutrients required for milk production in response to shortfalls in feed supply, that typically occur in pastoral milk production systems when adverse weather conditions, such as drought, affect grass growth rates and supplement feed costs.

Although there has been considerable research into the effects of MF on milk production, there remains a lack of understanding of the mechanisms that mediate the response of the mammary gland to changes in MF. Therefore, the objectives of this review are to present the current literature available on MF and to describe the potential molecular mechanisms involved in the response by the mammary gland to MF, with specific attention being given to the effects of MF during early lactation.

1.2 Bovine mammary gland

1.2.1 Functional anatomy

The mammary gland of the dairy cow is located in the inguinal region and consists of four simple functional glands, referred to in practice as quarters, which make up the complex mammary gland that is collectively known as an udder. Each of the quarters are separated by connective tissue and functions separately with their own milk synthesising tissue consisting of alveoli from which milk is drained via an intricate system of ducts into the mammary cisterns (Figure 1.1). Milk is discharged from the cisterns via a single teat which is made up of a smaller teat cistern and a single streak canal.

The bovine mammary gland consists of ductal and secretory alveolar mammary epithelial cells (MEC) referred to as parenchyma that contains a heterogeneous matrix of cells (stroma), which includes myoepithelial cells, adipocytes and fibroblasts. Milk synthesis occurs in the alveoli, which are considered the basic functional units of the lactating mammary gland. Each alveolus is made up of a single layer of polarised secretory MEC surrounded by myoepithelial cells and blood vessels. Milk is produced by the MEC and transported into the alveolar lumen where it accumulates. Milking and suckling stimulates systemic oxytocin release from the pituitary which induces contraction of the myoepithelial cells. The milk stored in the alveolar lumen is forced out into the gland's ductal system, flowing down the ducts into the cistern and then out through the teat. Milk synthesis is controlled by the availability of nutrients, hormonal stimulation and the amount of milk accumulated in the alveolar lumen.

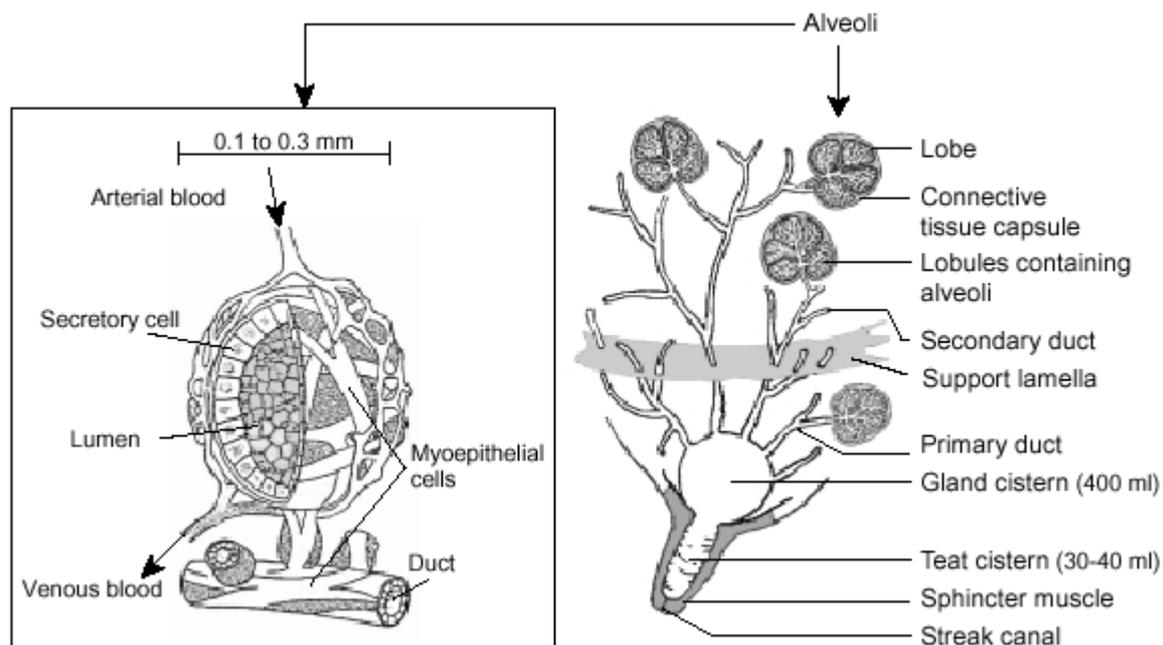


Figure 1.1 Structure of alveoli and ductal system of the bovine mammary gland (Adapted from <http://babcock.wisc.edu/node/202>).

1.2.2 Mammogenesis

Mammary growth (mammogenesis) is a major determinant of bovine MY capacity, as the number of alveolar MEC directly influences MY (Capuco et al., 2003). The bovine mammary gland experiences relatively little development from birth to puberty, with a growth rate which is consistent with body growth rate (isometric growth) until two to three months of age. However, recent studies have shown that faster growth rates in young dairy calves up to eight weeks of age, can increase subsequent milk yield (Khan et al., 2011, Bach, 2012, Soberon et al., 2012, Margerison et al., 2013), potentially through greater mammary development. The next phase coincides with the onset of ovarian activity preceding puberty (Sinha and Tucker, 1969) and from this point the mammary gland begins a phase of allometric growth, developing at a faster rate than the rest of the body, which is stimulated by the interaction of oestrogen, prolactin (PRL) and growth hormone (GH; Kleinberg, 1997, Tucker, 2000). At approximately nine months of age, following several oestrous cycles, the mammary gland returns to an

isometric growth status until conception (Sinha and Tucker, 1969). Once pregnant, the mammary gland enters a second phase of allometric growth, which continues throughout pregnancy until parturition. During pregnancy, the growth of the mammary gland is mediated by the presence of progesterone, oestrogen, PRL and GH, which collectively stimulate the proliferation and differentiation of the MEC (Tucker, 2000). After three to four months of gestation, mammary ducts elongate further, and alveoli form and begin to replace the stromal cells (adipocytes) in the supra-mammary fat pad.

1.2.3 Lactogenesis

The induction of milk synthesis, referred to as lactogenesis, is defined as a two-stage process (McManaman and Neville, 2003). The initiation phase of lactogenesis occurs during pregnancy once sufficient differentiation of the alveolar cells has occurred. This is characterised by the induction of milk protein gene expression with a limited secretion of some milk components (Linzell and Peaker, 1971, Hartmann, 1973). The second phase of lactogenesis in dairy cows commences shortly before parturition and is characterised by the copious secretion of all milk components (Linzell and Peaker, 1971, Hartmann, 1973), accompanied by a decrease in the levels of circulating progesterone (McManaman and Neville, 2003). In the early post-partum period the secretion product of mammary glands is called colostrum, which contains high concentrations of immunoglobulins and other proteins for the immune protection of the newborn calf (Linzell and Peaker, 1971).

1.2.4 Galactopoiesis

Once lactation has been established, mammary glands enter the maintenance phase of lactation, referred to as galactopoiesis. Post parturition, MY steadily increases, reaching a peak at approximately 60 days in milk (DIM) followed by which MY gradually declines until the cessation of

milking when the cow is 'dried-off' in preparation for calving and the initiation of next lactation. Prior to peak lactation the increase in MY is predominantly mediated by increasing MEC secretory activity rather than cell numbers as MEC already present differentiate into secretory MEC (Capuco et al., 2001). Whereas, the decline in MY post-peak is predominantly due to a steady decrease in MEC number (Capuco et al., 2001). In dairy cows which are usually mated and are pregnant while lactating, there is an increase in the MEC number during late lactation in preparation for the next lactation (Capuco et al., 1997). However, the increase in MEC number does not influence MY which continues its decline (Capuco et al., 1997).

1.2.5 Cessation of milking and mammary gland involution

Once milking has ceased in late lactation, mammary gland involution is initiated. However, the magnitude of mammary gland regression in dairy cows is far less pronounced than that documented in rodent species (Holst et al., 1987, Lund et al., 1996, Capuco and Akers, 1999, Stein et al., 2007). In dairy cows, following cessation of milking there is no actual loss of parenchyma tissue (Capuco et al., 1997), but there is evidence of an increased rate of cell turnover, with MEC apoptosis and proliferation occurring simultaneously, during involution of the bovine mammary gland (Capuco et al., 1997, Wilde et al., 1997). The morphology of the mammary tissue is affected by the cessation of milking, but to a far lesser degree than has been observed in rodent species (Holst et al., 1987, Capuco et al., 1997), which most likely reflects a change in the secretory state of the MEC (Capuco and Akers, 1999). The most notable changes are a reduction in luminal size and an increase in the area of intra-alveolar space, while the alveolar structures remain predominantly intact (Holst et al., 1987, Capuco et al., 1997).

1.2.6 Heterogeneity of mammary alveoli tissue

Interestingly, during lactation not all the alveoli in bovine mammary glands actively secrete milk components. Instead there is a heterogeneous mix of actively and quiescent alveoli. The actively secreting alveoli have MEC expressing genes associated with milk synthesis, while quiescent alveoli have MEC that are expressing immune related genes such as lactoferrin (Molenaar et al., 1992). It is possible that the proportion of active and quiescent alveoli subpopulations may be altered, which has been postulated as a possible mode of action of changes in MF (Vetharanim et al., 2003). Increasing MF is purported to stimulate alveoli to switch from quiescent into a secreting state, therefore increasing the overall activity of the gland (Vetharanim et al., 2003).

1.3 Milking frequency

1.3.1 Effects of milking frequency on milk yield and composition

The effects of MF on milk production in dairy cows are well established (Table 1.1). The response to a change in MF are immediate (Hillerton et al., 1990) and have been documented to occur at most stages of lactation (reviewed in Erdman and Varner 1995), provided that concurrent changes in nutrition has been provided to support any increases in MY (Phillips et al., 1980). The effect of MF on MY can be variable, but by comparing multiple studies it has been proposed that the effects of MF of 1x is - 20 %, 3x is + 17 % and 4x + 20 %, compared to 2x (Stelwagen, 2001). However, in the majority of experiments, the examination of MF has been completed with housed cows fed total (TMR) or partial mixed rations (PMR). There are a few studies that have attempted to measure the effects of increased MF on dairy cows in pasture based systems during early lactation (McNamara et al., 2008, Phyn et al., 2011). These studies found that 3x milking for three weeks resulted in a modest increase (Phyn et al., 2011) and no effect on MY (McNamara et al., 2008). Therefore, there remains doubt about whether

increasing MF has any positive effect in pasture based farming systems where feed supply is typically restricted.

The effect of MF on milk composition was summarised in a review by Erdman and Varner (1995). Increasing MF reduces the percentage of fat and protein in milk, whereas the milk fat and protein yield was greater due to the increase in MY. Equally, researchers have measured an increase in fat and protein percentage in milk when cows are milked 1x, although this was also due to the lower MY, the net result was a lower yield of fat and protein (Carruthers et al., 1993b, Stelwagen et al., 1994). Finally, several studies have also detected no effect on milk composition in response to changes in MF (Amos et al., 1985, DePeters et al., 1985). The reasons behind these discrepancies have not been fully explored but may be the result of variability in milk sampling methods, the nutritional status of the animals, an effect of the stage of lactation and/or breed of the cows utilised in the various studies.

Table 1.1 Studies demonstrating the effect of milking frequency during different stages of lactation on milk yield in dairy cows.

Reference	Daily milking frequencies ¹	Stage of lactation	Diet ²	Period (weeks)	Milk yield response ³
Whole udder					
DePeters et al. (1985)	3x vs 2x	Whole	TMR		+17%
Amos et al. (1985)	3x vs 2x	Whole	TMR		+18%
Campos et al. (1994)	3x vs 2x	Whole	TMR		+17%
Claesson et al. (1959)	1x vs 2x	Whole	B		-50%
Carruthers et al. (1991)	1x vs 2x	Late	P+S	2	-11%
Holmes et al. (1992)	1x vs 2x	Whole	P		-35%
Unilateral milking					
Carruthers et al. (1993b)	1x vs 2x	Early to mid	P	2	-16%
Hillerton et al. (1990)	4x vs 2x	Mid to late	S+S	4	+10.4%
Knight et al. (1992)	4x vs 2x	Mid	S+S	2	+14%
Stelwagen and Knight (1997)	1x vs 2x	Early	P+S	3	-38%
		Late	P+S	3	-28%

¹ 1x = once a day milking; 2x = twice a day milking; 3x = three times a day milking; 4x = four times a day milking.

² TMR = total mixed ration; B = barn-fed; P+S = pasture with supplementation; P = pasture only; S+S = silage with supplementation;

³ Milk yield change in response to milking frequency compared to control (2x). NS = not significant.

1.3.2 Long term effects of short term changes in milking frequency during early lactation

The short-term application of increased MF has been demonstrated to have longer-term effect on MY, that continues beyond the period that the increased MF was applied. This is referred to as a 'carry over' effect (Table 1.2) and is additional to the immediate effects of MF on MY. This carry-over effect was first reported by Bar-Peled et al. (1995), who compared 3x and six times a day (6x) during the first six weeks of lactation. In this study Bar-Peled et al. (1995) found that cows milked 6x have a greater MY during the six-week MF treatment period and that this greater MY continued for up to twelve weeks following the cessation of the 6x MF when all cows had a MF of 3x. Subsequent experiments by Dahl et al. (2004) compared 3x with 6x MF and concluded that three weeks of greater MF was sufficient to stimulate the carry-over effect on MY. Moreover, Hale et al. (2003) compared 2x with 4x MF for the first three weeks of lactation, followed by a 2x MF, and found a carry-over with cows having a greater MY in response to greater MF. A more recent study reported a 4x milking for two weeks within the first three weeks of lactation was sufficient to produce a measurably greater MY during the carry-over period when all cows had a MF of 2x (Wall and McFadden, 2007b).

Studies that reduced the MF, from 2x to 1x, for three to six weeks during early lactation were shown to elicit a negative effect on MY and that the six week period of reduced MF resulted in a negative carry-over effect on MY (Rémond et al., 1999). This was in contrast with studies that increased the MF, demonstrating that a period of at least six weeks is required to achieve a significant reduction in MY during the post-treatment carry over period (Rémond et al., 1999).

Table 1.2 Studies demonstrating the effect of short-term changes of milking frequency in early lactation on milk yield in dairy cows

Reference	Milking Frequency ¹	Period (DIM) ²	Diet ³	Treatment Milk yield ⁴	Carry over Milk yield ⁴
Whole udder					
Bar-Peled et al. (1995)	6x & 3x	1 to 42	TMR	+21 %	+14 %
Dahl et al. (2004)	6x & 3x	1 to 21	TMR	14 kg/d	3.7 kg/d
Hale et al. (2003)	4x & 2x	1 to 21	TMR	+26 %	+11 %
	4x & 2x	4 to 21	TMR	+14 %	+ 9 %
McNamara et al. (2008)	3x & 2x	1 to 28	S+S	NS	NS
	1x & 2x	1 to 28	S+S	-25 %	-11 %
Phyn et al. (2011)	3x & 2x	1 to 21	P+S	+7 %	NS
	3x & 2x	1 to 42	P+S	+8 %	NS
	1x & 2x	1 to 21	P+S	-15 %	NS
	1x & 2x	1 to 42	P+S	-19 %	NS
Rémond et al. (1999)	1x & 2x	1 to 21	S+S	-22 %	NS
	1x & 2x	1 to 42	S+S	-34 %	-16 %
Unilateral milking					
Wall & McFadden (2007a)	4x & 2x	1 to 21	TMR	3.5 kg/d	1.8 kg/d
Wall & McFadden (2007b)	4x & 2x	1 to 14	TMR	3.7 kg/d	1.2 kg/d
	4x & 2x	7 to 21	TMR	2.9 kg/d	1.5 kg/d

¹ 1x = once a day milking; 2x = twice a day milking; 3x = three times a day milking; 4x = four times a day milking; 6x = six times a day milking.

² DIM = days in milk.

³ TMR = total mixed ration; S+S = silage with supplementation; P+S = pasture with supplementation; P = pasture only;;

⁴ Milk yield change in response to milking frequency treatment compared to control (2x or 3x). NS = not significant.

1.3.3 Unilateral milking frequency experiments

Unilateral MF (UMF) experiments, in which udder halves are milked independently at differing MF, have demonstrated that the effects on MY are predominantly controlled locally within the mammary gland (Table 1.1 and Table 1.2). Hillerton et al (1990), showed that 4x of the udder half increased MY by 10 %, while the 2x udder half was unaffected. These findings confirmed previous work that showed this relationship using goats (Henderson et al., 1983, Wilde et al., 1987) and suggested the presence of a local intra-mammary regulator of MY, modulated by MF. A further study demonstrated that lower MF showed that the 1x udder halves produced 38 % less MY compared with their opposing udder half milked 2x (Stelwagen and Knight, 1997). Furthermore, Wall and McFadden (2007a) showed that the carry-over effect was controlled by intra-mammary factors. In this study, udder halves milked 4x for fourteen days during early lactation, produced 1.5 kg/d more MY once returned to 2x milking, compared with their opposing 2x udder half (Wall and McFadden, 2007b). All taken together, these results confirm the value of within-animal (UMF) experiments for studying the effects of MF on MY. While the responses to MF are remarkably similar between whole animal and half-udder experiments, the half-udder studies have the advantage of minimising variation from factors such as the genetic merit of the animal, as well as nutrition and environmentally related factors.

1.4 Prolactin and its role in the response of the mammary gland to changes in milking frequency

1.4.1 Prolactin signalling pathway in mammary epithelial cells

Prolactin (PRL) is a cytokine secreted by the pituitary gland in response to many physiological stimuli, such as eating, mating, photoperiod, ovulation and suckling of young and machine milking. It has a multitude of functions within the body and has approximately 300 known actions on various tissues. This review will however be specifically limited to the relevant role of PRL in mammary gland development and function.

Predominantly PRL functions as an endocrine hormone produced by the pituitary gland, which circulates in the bloodstream. There is some evidence from rodent models that PRL is also expressed in other tissues other than the pituitary gland, including the mammary gland (Kurtz et al., 1993, Iwasaka et al., 2000). However, the role and importance of locally synthesised PRL in bovine mammary glands is unclear.

In mammary glands, multiple isoforms of PRL receptor (PRLR) are expressed by the MEC (Freeman et al., 2000). Rodents and humans have three to four isoforms of PRLR (Bole-Feysot et al., 1998), whereas the bovine has the long and short isoforms (Schuler et al., 1997). All of the PRLR isoforms have identical extracellular domains and differ in the length of the intracellular domains (Freeman et al., 2000). The long isoform is thought to be the PRLR that conducts the PRL signal into the cell, whereas the short isoforms are unable to conduct a signal internally, due to their truncated intracellular domains, and are thought to antagonise the PRL signal by sequestering free PRL (Freeman et al., 2000).

A schematic diagram of the PRL signalling pathway within MEC is shown in Figure 1.2. The PRL protein has two binding sites for its receptor, PRLR. A PRLR molecule must be bound to PRL at site 1 to allow exposure of site 2 allowing for the binding of a second PRLR molecule (Freeman et al., 2000). Dimerisation of the two PRLR molecules is then induced. The intracellular portion of the receptor complex recruits Janus Kinase 2 (JAK2) which facilitates the phosphorylation of signal transducer and activator of transcription 5 (STAT5). When phosphorylated, STAT5 forms a homodimer and is transported to the nucleus where it activates genes involved in multiple cellular processes including milk synthesis. In addition STAT5 also up-regulates the expression of suppressor of cytokine signalling (SOCS) and cytokine inducible SH2 proteins which then act as feedback inhibitors of STAT5 activation dampening the response to the PRL signal (Pezet et al, 1999).

1.4.2 Effect of prolactin on the mammary gland

The effects of PRL on mammary gland development and function have been studied widely (reviewed in Tucker, 2000). In laboratory animals, PRL is essential for the induction and maintenance of lactation (Topper and Freeman, 1980). However, in cows PRL role in lactation is less clear (Knight, 2001). Administration of bromocriptine, a dopamine agonist which inhibits milking induced PRL secretion, to cows at parturition has been shown to delay but did not prevent the initiation of lactation (Schams et al., 1972). Although once lactation has been established bromocriptine has little or no effect on MY in dairy cows (Karg et al., 1972). Although, it is important to note that in these experiments using bromocriptine the PRL concentrations were reduced and PRL was still detectable, albeit at low levels. More recently, studies have investigated the effects of quinagolide, another PRL-release suppressor, on lactating dairy cows in early lactation (Lacasse et al., 2011, Boutinaud et al., 2012), demonstrating that a nine week quinagolide treatment lowered MY (Lacasse et al., 2011, Boutinaud et al., 2012),

decreased the expression of milk protein genes and potentially lowered MEC number by decreasing levels of proliferation and increasing the rate of apoptosis within the mammary gland (Boutinaud et al., 2012). These results suggest that under the right conditions levels of PRL may in fact influence MEC activity and/or MEC number. However, the interactions of PRL with the MEC in the bovine mammary gland does not seem to be as straight forward as it is in laboratory animals. One possibility is that in the bovine mammary gland the response to PRL is highly regulated by intracellular factors that mediate the MEC responsiveness to PRL. Therefore, further investigation into the response to PRL in mammary glands at the cellular level is required.

1.4.3 Other pathways which may interact with prolactin signalling pathway

1.4.3.1 Extracellular matrix/integrin signalling

A schematic diagram of the integrin signalling pathway and how it may interact with the PRL pathway within MEC is shown in Figure 1.2. Signalling from the extracellular matrix (ECM) via integrins is necessary for appropriate MEC differentiation and milk synthesis (Sympson et al., 1994, Roskelley et al., 1995). Integrins are receptors for cell adhesion to ECM proteins and also play an important role in certain cell-cell adhesions. In addition to mediating cell adhesion, integrins make transmembrane connections to the cytoskeleton and activate many intracellular signalling pathways including the phosphoinositide 3-kinase (PI3K)/Akt and extracellular-signal-regulated kinase (ERK)1/2 pathways. Integrins are heterodimers made up of two subunits (α and β), of which there are multiple forms.

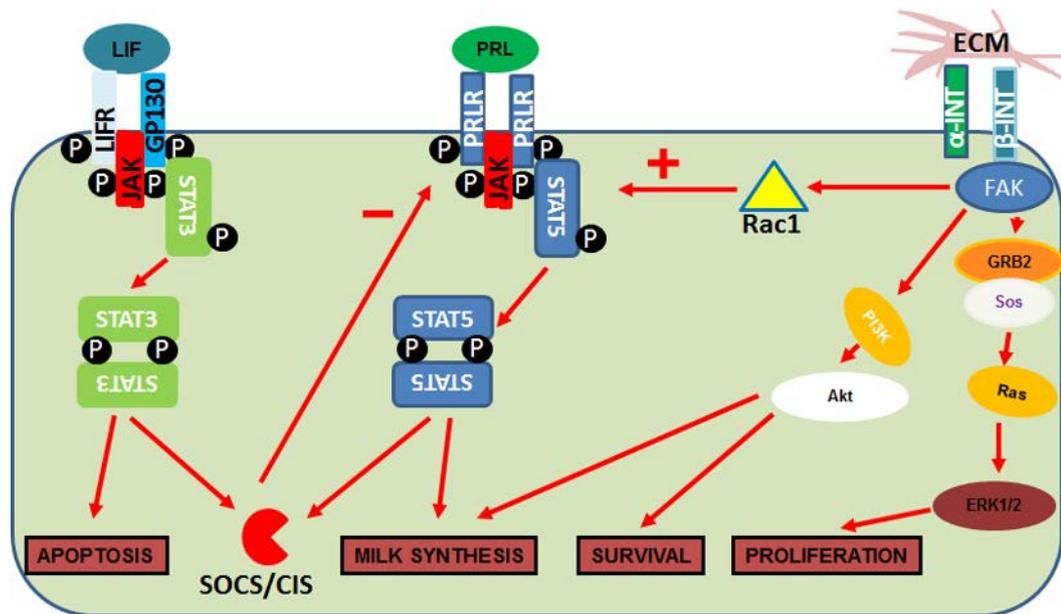


Figure 1.2 Schematic diagram of the prolactin (PRL), leukaemia inhibitory factor (LIF) and extracellular matrix (ECM)/integrin (INT) signalling pathways in mammary epithelial cells. PRLR = PRL receptor; LIFR = LIF receptor; JAK = janus kinase; STAT = signal transducer and activator of transcription; SOCS = suppressor of cytokine signalling; CIS = cytokine inducible SH2 protein; FAK = focal adhesion kinase; Rac1 = Ras-related C3 botulinum toxin substrate 1; PI3K = phosphoinositide 3-kinase; GRB2 = growth factor receptor-bound protein 2; ERK = extracellular-signal-regulated kinase.

There are different $\alpha\beta$ integrin combinations that make various non-redundant integrin complexes capable of recognising different ligands. It has been demonstrated in mouse knockout models that $\beta 1$ -integrin is essential for mammary gland development and sustained STAT5 activation (Naylor et al., 2005, Akhtar and Streuli, 2006). In mouse mammary glands the interaction between ECM/integrin signalling and PRL signalling appears to be mediated by the protein Ras-related C3 botulinum toxin substrate 1 (Rac1; Akhtar and Streuli, 2006). The interaction between the PRL/STAT5 and ECM/integrin pathways in mammary epithelial cells has not been well characterised but it is currently thought that interactions between $\beta 1$ -integrin and its ECM ligands activate Rac1, which in turn interacts with STAT5 by an

as yet unknown mechanism (Akhtar and Streuli, 2006). This Rac1-STAT5 interaction either enhances STAT5 activation or protects already activated STAT5 from degradation. Culminating in an overall increase in the abundance of activated STAT5. In normal mammary glands, the levels of β 1-integrin protein decrease during involution (McMahon et al., 2004, Singh et al., 2008) which suggests the abundance of this protein is modulated during physiological changes in mammary tissue function. Furthermore, this decrease coincides with a decrease in STAT5 activation (Singh et al., 2009).

1.4.3.2 STAT3 signalling

The ability of MEC to respond to PRL can be perturbed by the presence of antagonistic factors. In mouse mammary glands, STAT3 activation is pro-apoptotic and can be induced by the leukaemia inhibitory factor (LIF) (Kritikou et al., 2003). The activation of STAT3 has been shown to occur during involution in both mouse and bovine mammary glands (Kritikou et al., 2003, Singh et al., 2009) and coincides with the inactivation of STAT5. There is evidence from *in vitro* models that suggests that the activation of STAT3 can inhibit the activation of STAT5, possibly through the up-regulation of SOCS proteins, but whether this occurs *in vivo* is less clear (Clarkson et al., 2006, Granillo et al., 2007). A schematic diagram of the LIF/STAT3 signalling pathway and how it may interact with the PRL pathway within MEC is shown in Figure 1.2.

1.4.4 *The effects of milking frequency and role of prolactin*

Studies indicate that the ability of mammary glands to respond to PRL may be modulated by changes in the level of MF. McKinnon et al (1988), using a goat UMF experimental design, found that 3x-milked udder halves had almost twice the PRL binding capacity of the 2x-milked halves. The authors attributed this increase in PRL binding potential to an increase in the number of PRLR within the 3x-milked mammary glands, although they did not

measure this directly (McKinnon et al., 1988). A further study in dairy cattle measured the expression of PRLR using real time RT-PCR in udders milked unilaterally 1x and 3x daily (Bernier-Dodier et al., 2010) and found that the expression of both the long and short isoform of PRLR were up-regulated in the 3x-milked udder halves (Bernier-Dodier et al., 2010). A study by Lacasse et al (2011) examined the effects of the PRL release inhibitor quinagolide in lactating dairy cows milked unilaterally 1x and 2x. A small, but significant decrease in MY was detected in the 2x-milked udder halves in response to quinagolide, while the 1x udder halves were unaffected (Lacasse et al., 2011). The 1x-milked udder halves produced a lower MY so it is unclear whether the differential response to quinagolide treatment is due to a MF interaction or due to the lower MY (Lacasse et al., 2011). Overall, the results from these studies suggest that changes in MF may affect, at a cellular level, how mammary glands respond to the levels of PRL. Therefore, MF is an appropriate model for examining the underlying mechanisms that mediate how MEC respond to systemic PRL.

1.5 Insulin-like growth factor I and its role in the mammary gland response to milking frequency

1.5.1 Insulin-like growth factor I axis

Insulin-like growth factor I (IGF-I) has been a major focus of research in the area of mammary gland development and function (reviewed in Dembinski and Shiu, 1987). The majority of systemic IGF-I is produced in the liver and is controlled by the levels of pituitary GH. Most (approximately 95%) of the circulating IGF-I is bound to one of six IGF binding proteins (IGFBP1-6) (Ballard et al., 1989, Flint et al., 2008), which protects IGF-I from degradation (free IGF-I has a short half-life), but also shields IGF-I from its target receptors (Zapf, 1995). Once the IGFBP-bound IGF-I has entered target tissues, such as the mammary gland, release can be mediated by the presence of proteases that specifically break down IGFBPs (Flint et al.,

2008). Another possible mechanism that can mediate release of IGF-I is interactions with other extracellular components that are present in the ECM and on the cell surface, which interact with the IGFBPs and modulate the binding affinity of the IGFBPs to IGF-I (Flint et al., 2008). These mechanisms, coupled with free IGF-I short half-life, ensure that the effects of IGF-I are targeted precisely to the specific area where it is required.

1.5.2 Insulin-like growth factor I signalling in mammary epithelial cells

A schematic diagram of the IGF-I signalling pathway within MEC is shown in Figure 1.3.

Free IGF-I acts on MEC by binding IGF type I receptor (IGFIR), which is a transmembrane receptor that has intrinsic tyrosine kinase activity (Ullrich et al., 1986, Adams et al., 2000). Binding of IGF-I to IGFIR causes the activation of the tyrosine kinase domain of the receptor, leading to auto-phosphorylation as well as tyrosine phosphorylation of multiple cytoplasmic substrates, such as insulin receptor substrate (IRS) 1/2/3/4 and src/collagen homology (SHC) protein which serve as signalling nodes for distinct intracellular pathways (Myers et al., 1992, Sasaoka et al., 1994). The IRS and SHC signalling proteins activate two major intracellular pathways, the PI3K/Akt and ERK1/2 pathways (Peruzzi et al., 1999).

1.5.2.1 PI3K/Akt pathway

The serine/threonine kinase Akt is an important intracellular node in cell signalling which is activated by IGF-I as well as other growth factors, cytokines and the ECM. Phosphorylated IRS activates PI3K which in turn activates phosphoinositide-dependent kinases, which then phosphorylates and activates Akt. The activation of Akt is known to protect against apoptosis (Kennedy et al., 1997) and overexpression of activated Akt within the

mammary glands of transgenic mice can delay the onset of involution (Hutchinson et al., 2001).

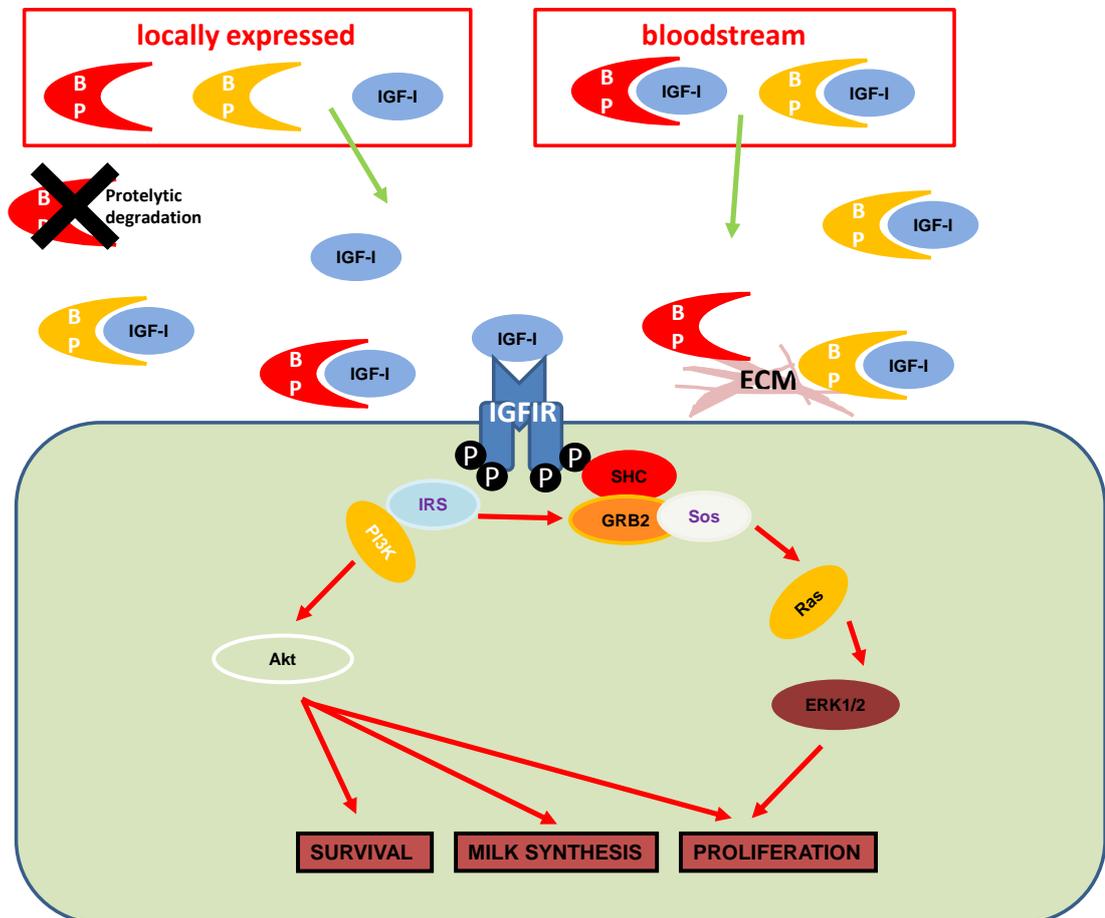


Figure 1.3 Schematic diagram of the insulin-like growth factor I (IGF-I) signalling pathways in mammary epithelial cells. BP = IGF binding proteins; ECM = extracellular matrix; IGFIR = IGF type I receptor; IRS = insulin receptor substrate; PI3K = phosphoinositide 3-kinase; SHC = src/collagen homology; GRB2 = growth factor receptor-bound protein 2; ERK = extracellular-signal-regulated kinase.

1.5.2.2 ERK1/2 pathway

The ERK1/2 pathway is also stimulated by IGF-I along with other mitogenic stimuli. Activation of ERK1/2 involves recruitment to both IRS-1 and SHC of the guanine nucleotide-exchange factor Sos. This leads to activation of the small G-protein Ras, which in turn activates ERK1/2. The activation of the ERK1/2 pathway is involved in a diverse range of cellular processes which control such functions as the cell cycle progression, cell proliferation, cell division and cell differentiation (O'Connor, 2003). The activation of ERK1/2 is essential for appropriate development of mammary glands controlling MEC proliferation (Whyte et al., 2009).

1.5.3 *Insulin-like growth factor I effects on mammary function*

In rodent mammary glands, locally synthesised IGFBP3 and IGFBP5 are thought to be important for controlling IGF-I function (Flint et al., 2008). The up-regulation of IGFBP5 has been demonstrated in rodent mammary glands at the onset of involution and is thought to inhibit the cell survival signal of IGF-I (Tonner et al., 1997, Flint et al., 2005). Insulin-like growth factor I is expressed in other tissues including the bovine mammary gland (Glimm et al., 1992) and it is thought this locally produced IGF-I has a greater effect on mammary gland function than systemic IGF-I (Kleinberg, 1997, Akers et al., 2000, Plath-Gabler et al., 2001). Insulin-like growth factor-I is a potent mammary mitogen that has been shown to have proliferative properties in the bovine MEC *in vitro* (McGrath et al., 1991), and to protect against apoptosis in mouse mammary glands during involution (Neuenschwander et al., 1996). Experiments investigating IGF-I's ability to stimulate milk synthesis in goats have shown when IGF-I is injected via the jugular it has no effect on MY, even though the treatment elevated systemic levels of IGF-I two-fold (Davis et al., 1989). However, introduction of IGF-I directly into one side of the mammary gland, by close-arterial infusion, can significantly increase MY of the treated gland but not the opposing side (Prosser et al., 1990). Interestingly, this effect is lost if the IGF-I treatment is preceded by a

treatment of increased MF, suggesting some link between the effect of IGF-I and the effect of MF (Prosser and Davis, 1992).

1.5.4 Insulin-like growth factor I and milking frequency

Changes in the several components of the IGF-I signalling pathway within the mammary gland in response to MF have been reported (Bernier-Dodier et al., 2010, Littlejohn et al., 2010, Wall and McFadden, 2010, Boutinaud et al., 2013). Bernier-Dodier et al., (2010) measured an increase in the abundance of IGF-I (*IGF1*) and IGFIR (*IGF1R*) mRNA in mammary tissue that was subjected to 3x milking during mid-lactation compared to 1x, whereas *IGFBP5* mRNA was not affected. In contrast, microarray analysis detected an increase of *IGFBP5* mRNA abundance in 1x-milked udder halves compared with 2x (Littlejohn et al., 2010, Boutinaud et al., 2013). Additionally, a study comparing 2x and 4x during early lactation, showed that MF had no effect on *IGF1* and *IGF1R* mRNA abundance, but lower amounts of *IGFBP3* mRNA were observed in 4x-milked mammary tissues compared to 2x (Wall and McFadden, 2010). Overall, these findings only serve to illustrate the differing, complex and dynamic nature of IGF-I signalling in bovine mammary gland, which is not fully understood. These results suggest that MF can modulate factors that affect the response of the mammary gland to IGF-I and that MF is an appropriate model to investigate the underlying mechanisms that mediate the response of MEC to IGF-I stimulus.

1.6 Other factors that control milk production within the mammary gland in response to milking frequency

1.6.1 Growth hormone

The galactopoietic effect of GH in dairy cows is well documented (Tucker, 2000) and recombinant GH, has been available to farmers, mainly in the United States, for many years to increase the MY of dairy cattle. The mechanisms underlying the galactopoietic actions of GH on the mammary gland are not fully understood. It may act directly on mammary tissue since GH receptor mRNA has been detected in mammary tissues (Glimm et al., 1990), and/or indirectly by increasing the systemic level of IGF-I, which then acts on the mammary gland (Davis et al., 1987). There is also evidence that GH effects nutrient partitioning from other bodily functions towards the mammary gland, therefore making more nutrients available for milk synthesis (Etherton and Bauman 1998).

Knight et al (1992) compared the effects of GH on MY in cows with and without increasing the MF. Cows were either treated or untreated with GH and then milked unilaterally 2x compared with 4x. The GH alone increased MY by 13%, while 4x had a positive effect on MY increasing it by 10 %, and in combination (GH + 4x milking) increased MY by 24 %. The effect of the combined treatment was considered additive and no synergism was detected (Knight et al., 1992). Furthermore the mode of action of the treatments were different, i.e. the response to MF was more immediate, increasing MY significantly following three days of greater MF, while the effects of GH were not apparent until seven days of greater MF (Knight et al., 1992).

1.6.2 Feedback inhibition of lactation

Increased MF results in reduction of milk accumulation in the alveolar lumen. One mechanism proposed to account for this a decrease in negative feedback mechanisms (Linzell and Peaker, 1971). It has been suggested that a component of milk may act as a negative feedback signal accumulating between milking sessions and at sufficient amounts to inhibit milk secretion (Linzell and Peaker, 1971). Increased MF decreases the action of this signal by decreasing the interval between milking and therefore the accumulation of the factor. Studies in goats have shown that a fraction of whey protein injected into the teat canal has an immediate, but short-lived negative effect on MY over one to two days, whereas a similar volume of an isosmotic solution has no effect (Wilde et al., 1988). The authors labelled the causative agent, feedback inhibitor of lactation (FIL) but it remains uncharacterised and little more beyond these initial experiments has been reported. The accumulation of factors such as breakdown products of casein proteins in the alveolar lumen (Shamay et al., 2003), and serotonin (Matsuda et al., 2004, Collier et al., 2012) have been shown to inhibit milk secretion in dairy cows, but it remains to be seen whether these factors are related to FIL.

1.6.3 Mammary epithelial cell tight junction permeability

Milk accumulation can affect the integrity of tight junctions between the MEC, which become 'leaky' allowing the interchange of solutes between blood and milk (Stelwagen et al., 1997). In dairy cattle, the change in permeability of tight junctions is detectable approximately eighteen hours following milking when the gland could be reaching its milk storage capacity, which coincides approximately with a decrease in milk secretion rate (Stelwagen et al., 1997). Tight junction permeability has been shown to be involved in the response to lower MF (Stelwagen, 2001, Bernier-Dodier et al., 2010) and may account for the dramatic decrease in MY of cows milked 1x compared to 2x, but its role in increased MF is less clear. However, Sorensen et al. (2001) demonstrated

that long-term 3x milking maintained MEC integrity, which usually declines as lactation progresses, suggesting an increased rate of milk removal may reduce tight junction permeability.

1.7 Objectives of this study

The objective of this study was to use a bovine unilateral MF model to investigate the intra-mammary molecular mechanisms underlying the MY response to short-term changes in MF during early lactation. The effects of MF during early lactation on MY are two-fold:

- 1) The immediate MY response to changes in MF observed during the MF treatment,
- 2) The long-term carry-over effect on MY in response to short-term changes in MF during early lactation.

The immediate and long-term carry-over effects of MF on MY is controlled by intra-mammary changes rather than changes in systemic factors. Therefore a UMF model comparing a 4x and 1x MF was applied during early lactation to investigate the following hypotheses.

- 1: Does alteration of MF modify the mammary glands secretory activity by adjusting the proportion of actively secreting and quiescent alveoli.
- 2: Does the alteration of MF during early lactation change the levels of proliferation and/or apoptosis of MEC, which effects the number of secretory MEC and therefore long-term MY.

The global effects of MF on mammary glands are controlled at the cellular level by alteration in the MEC ability to respond to the systemic hormones PRL and IGF-I.

This study attempts to increase the understanding of the molecular mechanisms that control the activation of secretory MEC and MEC number in response to changes in MF of dairy cows.

Chapter II

The effects of milking frequency in early lactation on milk yield, mammary cell turnover and secretory activity in grazing dairy cows

The material presented in this chapter has been published in the Journal of Dairy Science (Murney, R., K. Stelwagen, T. T. Wheeler, J. K. Margerison, and K. Singh. 2015. The effects of milking frequency in early lactation on milk yield, mammary cell turnover and secretory activity in grazing dairy cows. J. Dairy Sci. 98:305-311)

2.1 Abstract

In dairy cows, short-term changes of milking frequency in early lactation have been shown to produce an immediate and a long-term effect on milk yield in stall-fed cows. The effect is controlled locally within mammary glands and could be a function of either secretory mammary epithelial cell number or activity. To resolve this and determine its applicability in other feed management systems, a unilateral milking frequency experiment was conducted with udder halves of 17 multiparous, pasture-fed dairy cows milked either four times (4x) or once a day (1x) for fourteen days from 5 ± 2 days in milk (DIM). Mean half-udder milk yield during the treatment period was higher from the 4x compared with 1x udder halves and continued to be higher until 200 DIM once returned to twice a day milking. Mammary biopsies were obtained on day fourteen of treatment from both udder halves of 10 cows. Proliferation of mammary cells was higher in 4x udder halves compared with 1x, whereas no difference in apoptosis levels was detected. Abundance of α S1-casein, β -casein, α -lactalbumin, and β -lactoglobulin mRNA was higher in tissue samples from 4x udder halves compared with 1x, whereas lactoferrin mRNA abundance was lower in 4x udder halves. In summary, change in milking frequency during early lactation affects proliferation of mammary cells as well as expression of the major milk protein genes, which both contribute to the observed changes in milk yield during and after unilateral milking frequency treatment.

Key words: milking frequency; grazing dairy cow; early lactation; milk yield; cell proliferation

2.2 Introduction

Dairy cows are commonly milked twice a day (2x). Increasing milking frequency (MF) to three (3x) or four (4x) times a day has a dramatic effect on milk yield (MY; Amos et al., 1985; Hale et al., 2003). This response is immediate (Hillerton et al., 1990) and has been observed at most stages of lactation (reviewed in Erdman and Varner, 1995), provided sufficient nutrition has been supplied to the animals to accommodate the increased production (Phillips et al., 1980). In addition, short-term increased MF can have a long-term carry-over effect on MY even after the cows have been returned to a normal milking regimen (Bar-Peled et al., 1995; Hale et al., 2003; Wall and McFadden, 2007a). This effect is most notable in early lactation, where two to three weeks of increased MF can positively influence MY for the remainder of lactation (Hale et al., 2003; Wall and McFadden, 2007a), but has also been demonstrated in midlactation following eight weeks of 3x versus once daily (1x) milking (Bernier-Dodier et al., 2010). In most cases, experiments examining increased MF have been conducted in confinement settings, where cows are housed indoors and fed a controlled diet. A few studies have attempted to measure the effects of increased MF during early lactation in grazing dairy cows (McNamara et al., 2008; Phyn et al., 2011). However, in these studies, a treatment of 3x milking for three weeks yielded a modest increase in MY, but with no long-term effect post-treatment (Phyn et al., 2011) or no effect on MY at all (McNamara et al., 2008).

Similarly, decreasing MF below 2x to 1x has a negative effect on MY (Carruthers et al., 1993a; Rémond et al., 1999; Phyn et al., 2011). As well as having an effect on MY during the treatment, if applied in early lactation, a temporary reduction in MF can also illicit a long-term negative carryover effect on MY (Rémond et al., 1999) or the treatment negatively affects levels of milk solids rather than MY (Phyn et al., 2011).

Studies using unilateral MF (UMF) models, where udder halves are milked independently at differing MF, have demonstrated that the effects on MY are predominantly controlled locally within the treated mammary gland (Hillerton et al., 1990; Stelwagen and Knight, 1997; Wall and McFadden, 2007a). This has been observed for both the immediate effects of increased (Hillerton et al., 1990; Wall and McFadden, 2007a) and decreased (Stelwagen and Knight, 1997) MF, as well as the carry-over effect associated with temporary increased MF in early lactation (Wall and McFadden, 2007a) and midlactation (Bernier-Dodier et al., 2010). Furthermore, the MY response to UMF is equivalent to what has been seen in whole animal MF experiments, which suggest the underlying mechanisms are the same (Wall and McFadden, 2007a). The advantage of utilising a UMF model is that it is within animal and therefore the effects of genetics, nutrition, and environmental factors are largely negated.

The mechanisms underlying the MY response to MF are not well understood, but are most likely a function of secretory mammary epithelial cell (MEC) number or activity. To date, studies reporting the effects of MF during early lactation on cell turnover (a factor of the rate of proliferation and apoptosis) have been unclear. Hale et al. (2003), comparing 4x to 2x MF, were unable to detect any significant difference in proliferation. However, a significant increase in apoptosis in samples from 4x-milked glands was observed after three days of treatment, but this difference was not detected at a later time point (Hale et al., 2003). Conversely, Nørgaard et al. (2005) saw no difference in proliferation or apoptosis following a seven day 4x MF compared with 2x. Grala et al. (2011) reported an increase in apoptosis-related genes in mammary samples from cows milked 1x for three and six weeks compared with 2x. However, no proliferation markers were measured to ascertain the effects of 1x milking on combined cell turnover. Bernier-Dodier et al. (2010) detected an increase in both proliferation and apoptosis when 1x was compared with 3x by UMF in midlactation dairy cows, suggesting the decreased MF initiated tissue remodelling. In the current

study, a UMF model was used to compare udder halves of grazing dairy cows milked 4x versus 1x for fourteen days to investigate how short-term MF changes in early lactation can affect MY both during and after treatment, along with cell turnover and cellular activity in the mammary glands.

2.3 Materials and methods

2.3.1 Animals and treatments

All animal manipulations were conducted in compliance with the rules and guidelines of the Ruakura Animal Ethics Committee. Seventeen multiparous Holstein-Friesian and Holstein-Friesian x Jersey cows were freely grazed on pasture and given access to 2 kg/d of commercial supplement (Topcow dairy, Ingham Feeds & Nutrition, Te Aroha, New Zealand; 12.9 MJ/kg of ME, 12% CP based on DM). Initially the cows were milked 2x and then, from 5 ± 2 to 19 ± 2 days in milk (DIM), randomly assigned udder halves of all cows were unilaterally milked (1x in one udder half at 1100 h, and 4x in the other half at 0500, 1100, 1700, and 2300 h). Milk samples were collected from the rear quarters of all cows and SCC was measured. Cows with low SCC in both rear quarters and high overall MY were selected for biopsy. On the final day of treatment, 3 to 5 h following the 1100-h milking, mammary tissue was obtained by biopsy from both rear quarters of 10 cows, as previously described by Farr et al. (1996). A portion of the mammary tissue was fixed overnight in 4% paraformaldehyde and processed into wax as described previously (Singh et al., 2005). The remainder was snap-frozen in liquid nitrogen for subsequent molecular analysis. The cows were then returned to 2x milking for the remainder of lactation.

2.3.2 Half udder milk yield and composition data

Half-udder MY data were measured using a custom-built 2-cup milk claw installed on a commercial rotary milking parlour (Tokanui Research Farm, Waikato, New Zealand), with the yields calculated using an in-line iNTELSKAN Plus milk meter (Milfos International Ltd., Hamilton, New Zealand). Milk yield data were collected daily during the treatment period and monthly following treatment until 200 ± 2 DIM. During the post-treatment period, milk samples were collected and analysed by infrared spectrometry for fat, protein and lactose, and by flow cytometry for SCC (Fossomatic equipment, LIC Herd Testing Station, Hamilton, New Zealand).

2.3.3 Cell proliferation.

Immunohistochemical localisation of the Ki-67 cell proliferation antigen was optimised from the method previously described by Capuco et al. (2001). Slides were deparaffinised, rehydrated, and antigen retrieval was carried out as described previously (McMahon et al., 2004). Slides were then quenched with Dual Endogenous Block (Dako Cytomation California Inc., Carpinteria, CA), washed 3 times in Tris-buffered saline solution (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6) containing 0.05% Tween 20 (TBST), blocked with Biotin Blocking System (Dako), washed in TBST, and then blocked in 2.5% bovine serum albumin (Life Technologies, Grand Island, NY) in TBST. Slides were then incubated either with 1:100 anti-Ki-67 rabbit monoclonal antibody (Abcam, Cambridge, UK) diluted in antibody diluent (Stressmarq, Victoria, Canada) or diluent alone as a negative control overnight at 4°C. Slides were washed three times in TBST and incubated with a 1:50 biotinylated anti-rabbit secondary antibody (Biogenex, Fremont, CA) in antibody diluent (Stressmarq) for 30 min. After three washes in TBST the slides were then incubated with 1:50 horseradish peroxidase (HRP)-conjugated streptavidin (Biogenex) diluted in antibody diluent (Stressmarq) for 30 min. Slides were then washed three times in TBST and to visualise the Ki-67 positive nuclei the slides were incubated with 3,3'-diaminobenzidine substrate (Roche,

Basel, Switzerland) for 2 min. Slides were rinsed in deionised water and counterstained with haematoxylin, dehydrated, washed with xylene and then mounted with DePeX mounting medium (VWR International Ltd., Poole, UK). The proportion of Ki-67-positive cells was determined by comparing the number of Ki-67-positive cells to the total number of cells per field at 100x magnification using a binocular microscope (Olympus Optical Co. Ltd., London, UK). Images were captured of four fields per cow per treatment using a Prog14 digital camera (Jenoptik GmbH, Jena, Germany). Cells were counted manually using Image-Pro Plus Version 7.0 (Media Cybernetics Inc., Bethesda, MD) software and a total of 6,000 or more cells were counted across the four fields for each section.

2.3.4 RNA isolation and reverse transcription

Total RNA was isolated from 100 mg of mammary tissue using Trizol reagent (Life Technologies) according to the manufacturer's instructions and quantified using a Nanodrop 1000 (Nanodrop, Wilmington, DE). The RNA integrity was measured on an Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA) with an RNA integrity number (RIN) >5 considered sufficient for real time reverse transcription PCR analysis. Total RNA (1 µg) was purified using columns (Qiagen, Valencia, CA) and used as a template to produce cDNA using the SuperScript III reverse transcription kit (Life Technologies) according to the manufacturer's instructions. The amount of cDNA equivalent to 5 ng of starting total RNA was used as template for each real time PCR reaction. Primers for pro-apoptotic Bcl-2-associated X protein (*BAX*), antiapoptotic B-cell lymphoma-extra-large (*BCL-XL*), α_{s1} -casein (*CSN1S1*), α -lactalbumin (*LALBA*), and β -lactoglobulin (*LGB*) mRNA were designed using Primer3 (Rozen and Skaletsky, 2000) and are listed in Table 2.1. Primers for β -casein (*CSN2*), lactoferrin (*LTF*), and ubiquitin B were from Singh et al. (2008) and primers for β -2 microglobulin were from Wall and McFadden (2010). Abundance of mRNA of milk protein genes *CSN1S1*, *CSN2*, *LALBA*, *LGB*, and *LTF* was normalised to the geometric mean of the

mRNA abundance of two housekeeper genes (ubiquitin B and β 2-microglobulin). The housekeeper genes were selected due to their expression remaining unchanged relative to each other as previously described by Bionaz and Looor (2007). To measure relative apoptosis levels, the ratio of *BAX* and *BCL-XL* mRNA abundance was compared between treatments. The PCR products were verified by sequencing (Waikato DNA Sequencing Facility, Hamilton, New Zealand)

Table 2.1 Primers used for real time PCR

Target ¹	GeneBank no.	Forward	Reverse	Reference
<i>BAX</i>	NM_173894	cgagtgccgctgaaatggt	gcagccgctctcgaaggaagt	-
<i>BCL-XL</i>	NM_001077486	gcgtagacaaggagatgcag	gttcacaaaagtgtcccag	-
<i>CSN1S1</i>	NM_181029	taccctgagcctttcagaca	cataactgtggagtcctca	-
<i>CSN2</i>	NM_181008	gtgaggaacagcagcaaca	aggyaagggcattcttgt	Singh et al., 2008
<i>LALBA</i>	NM_174378	tggtctgtaccacgttca	gcttatgggccaaccagta	-
<i>LGB</i>	NM_173929	cattgtcacccaagacatga	cactcacccgttctccattt	-
<i>LTF</i>	NM_180998	ggccttggccttgyaatgtatc	attagccacagctcctcctggag	Singh et al., 2008
<i>UBB</i>	NM_174133	ggcaagaccatcacccctggaa	gccacccctcagacgaagga	Singh et al., 2008
<i>B2M</i>	NM_173893	aaggatggctcgccttcgt	ttcaaatctgatgtgtctg	Wall & McFadden, 2010

¹ *BAX* = BCL2-associated X protein; *BCL-XL*= B-cell lymphoma-extra-large; *CSN1S1* = α S1-casein; *CSN2* = β -casein; *LALBA* = α -lactalbumin; *LGB* = β -lactoglobulin; *LTF* = lactoferrin; *UBB* = ubiquitin B; *B2M* = β -2 microglobulin.

2.3.5 Statistical analysis

Differences between half-udder MY, assessed as means of two subsequent days, milk composition, and \log_{10} -transformed SCC were analysed by ANOVA (Minitab 16.2.2, 2010, State College, PA) by treatment, DIM, and their interaction, with cow as a random effect. Differences between the proportion of Ki-67-positive cells, *BAX-to-BCL-XL* ratio, and \log_{10} -transformed normalised mRNA abundance were analysed by ANOVA (Minitab 16.2.2) by treatment with cow as a random effect. The MY, proportion of Ki-67-positive cells, and *BAX-to-BCL-XL* ratios were expressed as means \pm SEM. The differences in MY, milk composition, and \log_{10} -transformed SCC were shown as means with standard error of the difference. The differences in mRNA abundance were back-transformed and expressed as the 4x mean fold change \pm standard error of the difference. Differences between means were analysed by paired t-test and considered significant at $P < 0.05$.

2.4 Results

Pretreatment MY (3 to 4 DIM) was similar for 4x and 1x udder halves (Table 2.2). By day two of treatment (i.e., 6 DIM), the MY of 4x-milked udder halves was higher compared with 1x and remained elevated during the UMF treatment period. This resulted in an 80% higher MY from the 4x udder halves (Figure 2.1A, Table 2.2). Once both udder halves had been returned to 2x, the 4x udder halves continued to produce more milk than the 1x udder halves (Figure 2.1B, Table 2.2). The increase in 4x MY was detectable until 200 DIM and corresponded to an overall increase in MY of 12% across the post-treatment period (55–200 DIM). Percentage of fat was higher in milk samples collected from 4x udder halves compared with 1x, whereas lactose and protein percentage were not significantly different between 4x and 1x (Table 2.2). Total daily yields of fat, protein, and lactose were 16, 10, and 12% higher, respectively, from 4x udder halves compared with 1x (Table 2.2). No difference in SCC was detected between the 4x and 1x udder halves (Table 2.2).

The relative number of proliferating cells within the 4x- and 1x-milked mammary tissue samples was measured by Ki-67 localisation. The number of Ki-67-positive cells in tissue samples from 4x udder halves was 32% higher ($P < 0.05$) than in 1x (Figure 2.2A). Representative images of the Ki-67 assay are shown for 4x (Figure 2.2C) and 1x (Figure 2.2D) mammary tissue samples. No significant difference in *BAX*-to-*BCL-XL* ratio was detected between treatments (Figure 2.2B).

Abundance of mRNA was higher for *CSN1S1*, *CSN2*, *LALBA*, and *LGB* in tissue samples from 4x-milked udder halves compared with 1x (Figure 2.3). In contrast, mRNA abundance for *LTF*, which is elevated in milk during drying off, was lower in tissue samples from 4x-milked udder halves compared with 1x (Figure 2.3).

Table 2.2 Half-udder milk yield and milk composition for four times (4x) and once (1x) daily milking frequencies measured pre, during and post allocation of differing milking frequency¹

	Milking frequency			SED	P value
	4x	1x	4x/1x		
Pre-treatment, 3 to 4 DIM					
Milk yield, kg/d	8.5	8.4	0.1	0.3	NS
During treatment, 5 to 19 DIM					
Milk yield, kg/d	13.8	7.7	6.1	0.2	< 0.001
Post-treatment, 55 to 200 DIM					
Milk yield, kg/d	10.0	8.9	1.1	0.2	< 0.001
Fat, %	5.20	5.00	0.20	0.08	< 0.05
Protein, %	3.44	3.47	-0.03	0.02	NS
Lactose, %	4.93	4.90	0.03	0.02	NS
Fat, g/d	500	430	70	10	< 0.001
Protein, g/d	330	300	30	10	< 0.001
Lactose, g/d	480	430	50	10	< 0.001
SCC, Log ₁₀ cells/ml	1.81	1.77	0.04	0.06	NS

¹ Cows were milked twice daily during pretreatment (3 to 4 days in milk (DIM)) and then udder halves were milked either four times a day (4x) or once a day (1x) during treatment (5 to 19 DIM). Following the treatment period, cows were returned to twice daily milking for the remainder of lactation (post-treatment, 55 to 200 DIM). Half-udder MY were collected for all three periods, whereas milk composition and SCC data were collected during the post-treatment period only. Data (4x and 1x) are expressed as treatment means and the difference between the means (4x-1x) and standard error of the difference (SED) are indicated.

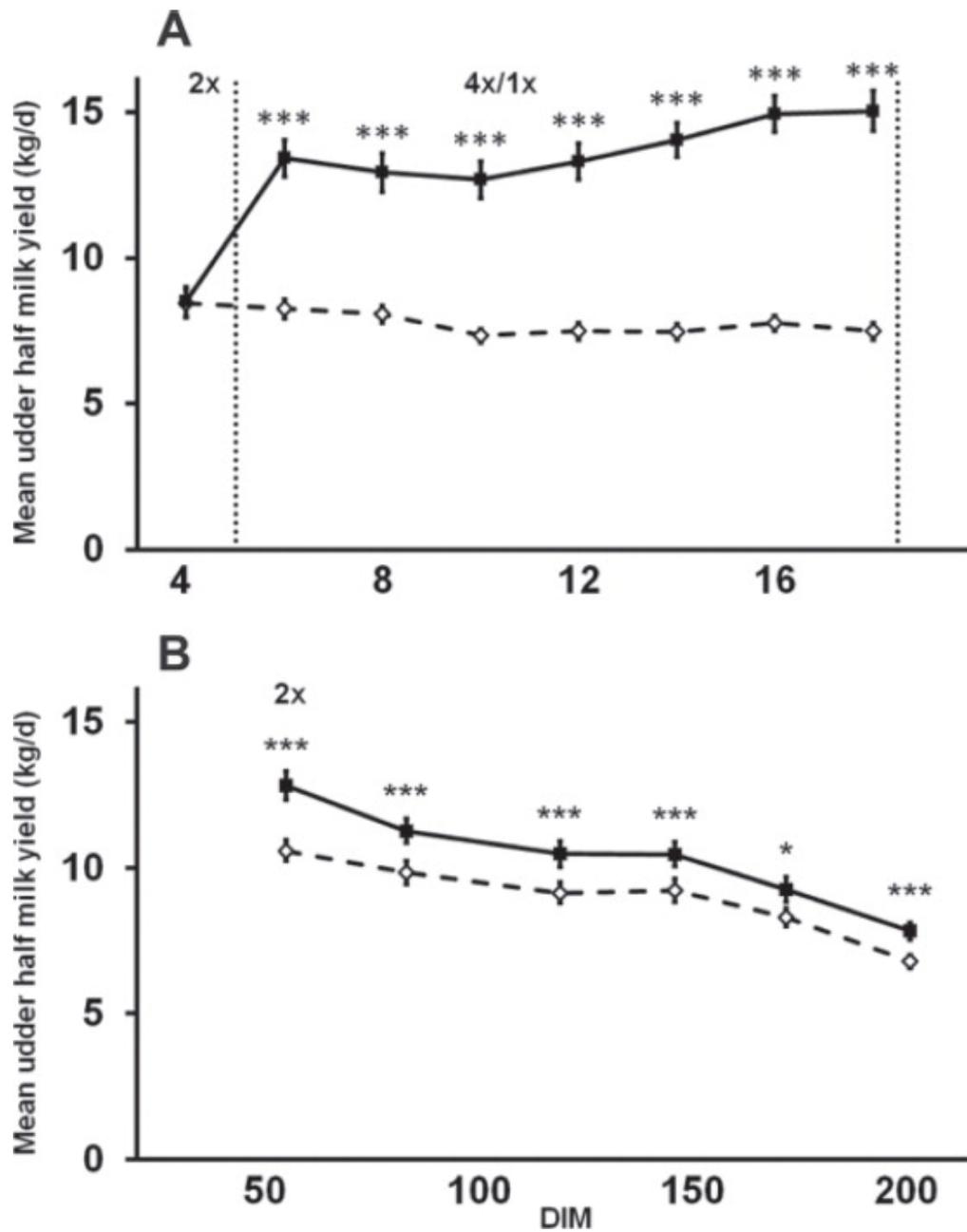


Figure 2.1 Mean milk yield (MY) of udder halves for cows unilaterally milked for fourteen days in early lactation. (A) Cows were milked twice daily (2x; 3 to 4 days in milk (DIM)) and then udder halves were milked either four times a day (4x, solid line) or once a day (1x, dashed line; 5 to 19 DIM). (B) Following the treatment period, cows were returned to 2x milking for the remainder of lactation (55 to 200 DIM). Data are expressed as means \pm SEM; * $P < 0.05$, *** $P < 0.001$ are for comparisons between 4x and 1x MY at each time point.

2.5 Discussion

Ultimately, milk production in the mammary gland is determined by the number of secretory MEC and the activity of those cells. Secretory MEC number is controlled by the balance between proliferation and apoptosis. Both of these latter processes produce effects gradually over the course of lactation, whereas secretory activity is regulated more acutely. The udder halves milked 4x responded rapidly, increasing their MY to 60% more than both pretreatment and 1x udder halves by day two of treatment. Due to the swiftness of response, this acute increase in MY can only be attributed to increased MEC secretory activity in response to the change in MF. This higher secretory activity was maintained for the treatment period and was associated with an increase in expression of the genes encoding the major milk proteins in the 4x-milked mammary tissue samples compared with the 1x on day fourteen.

Stelwagen and Knight (1997) reported a small transient compensatory increase of the MY from 2x-milked udder halves when the opposing halves were milked 1x. A similar effect may have contributed to the increase in MY of the 4x udder halves reported in our study. However, its contribution could only be minor, as the increase of 4x-milked udder halves was substantially greater than the decrease detected in 1x-milked halves. This effect was attributed to nutrient availability, and would not be expected to persist beyond the UMF treatment period.

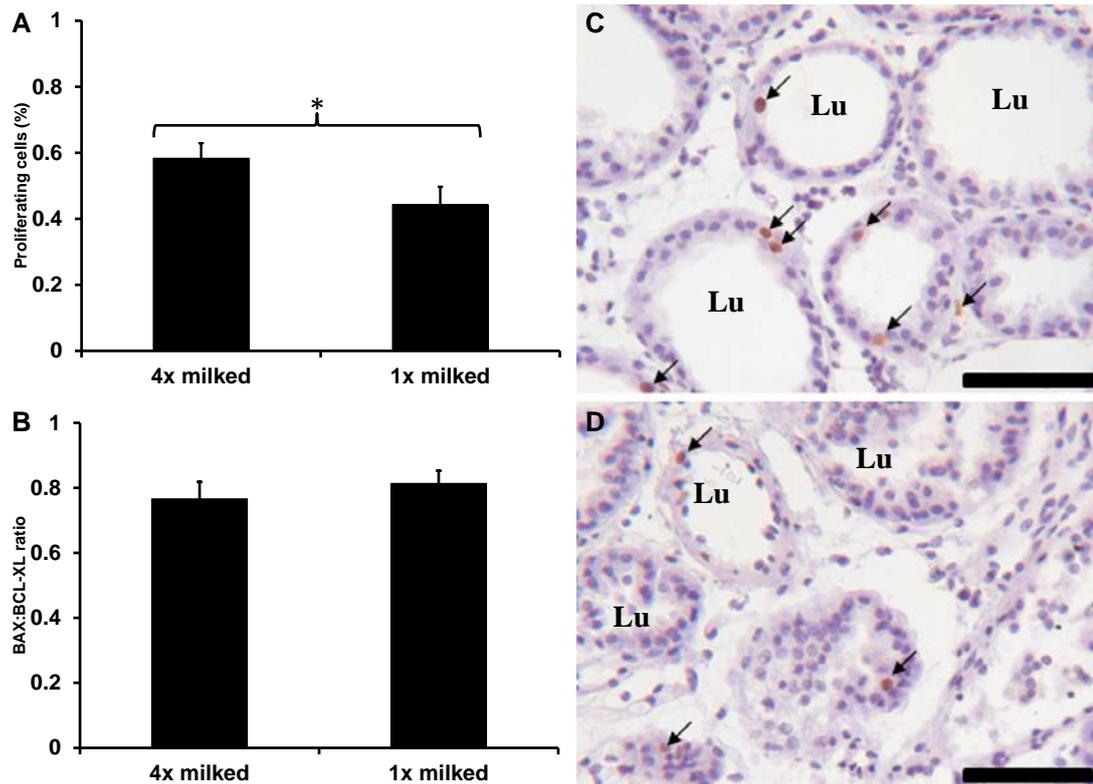


Figure 2.2 Changes in proliferation and apoptosis in mammary tissue of dairy cows unilaterally milked either four times a day (4x) or once a day (1x) for fourteen days in early lactation (5 to 19 days in milk). (A) Percentage of cells stained positively for the proliferation marker, Ki-67, on sections of formaldehyde-fixed, paraffin-embedded mammary tissue biopsied from unilaterally milked udder halves. (B) Ratio of pro-apoptotic Bcl-2-associated X (*BAX*) and anti-apoptotic B-cell lymphoma-extra-large (*BCL-XL*) mRNA measured by real time reverse transcription-PCR in mammary tissue biopsied from unilaterally milked udder halves. Bars indicate SEM; * $P < 0.05$. (C and D) Representative Ki-67 staining in formaldehyde-fixed, paraffin-embedded mammary tissue biopsied from unilaterally milked udder halves either milked 4x (C) or 1x (D). Arrows indicate positively stained cells, alveolar lumen (Lu), bar represents 50 μm .

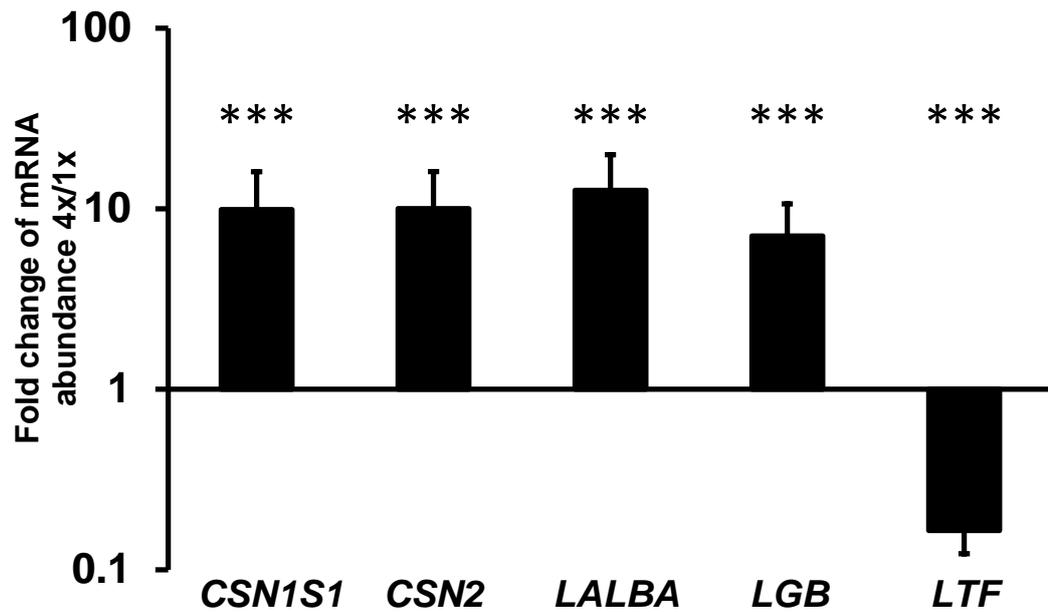


Figure 2.3 Difference in mRNA abundance for milk protein genes α_{S1} -casein (*CSN1S1*), β -casein (*CSN2*), α -lactalbumin (*LALBA*), β -lactoglobulin (*LGB*), and lactoferrin (*LTF*) expressed in mammary tissue from udder halves of dairy cows which had been subjected to unilateral milking four times a day (4x) or once a day (1x). Abundance of mRNA was normalised to the geometric mean of mRNA abundance of 2 house keeper genes (ubiquitin B and β 2-microglobulin) and expressed as a fold change in 4x compared to 1x. Data are expressed as mean fold change with bars indicating standard error of the difference; ***P < 0.001.

Once both udder halves were returned to 2x, the 4x-milked udder halves continued to produce more milk than 1x udder halves. This carryover effect was detected at all of the time points measured until the end of the trial (i.e., 200 DIM). This observation can be attributed to changes in secretory cell number in response to MF. Proliferation and apoptosis were therefore measured on day fourteen of the treatment. Proliferation was significantly higher in the 4x-milked mammary tissue samples compared with 1x, whereas no difference in apoptosis-related gene expression was noted. This indicates that the carryover effect on MY is due to increased proliferation, resulting in greater numbers of secretory cells in the 4x compared with 1x treatment. Some previous studies investigating the effects of increased MF in early lactation did not detect a significant change in proliferation or apoptosis (Hale et al., 2003, Nørgaard et al., 2005, Wall and McFadden, 2010). Grala et al. (2011) did detect an increase in apoptotic markers in 1x-milked mammary tissue samples compared with 2x, but effects on proliferation were not reported; therefore, the overall effect on cell turnover was not ascertained. Whereas, when 3x and 1x MF were compared in midlactation, an increase in both proliferation and apoptosis was detected in the 1x-milked udder halves following four weeks of treatment (Bernier-Dodier et al., 2010). The discrepancy between reports remains unclear, but it may be due to the differences in the parameters of the experiments (i.e., stage of lactation, duration of treatment, and time of sample collection in relation to previous milking). Furthermore, the results reported in the present study are the first description of a carryover effect on MY in response to MF in pasture-fed dairy cows. Unlike previous experiments in pasture-based systems (McNamara et al., 2008, Phyn et al., 2011), the present study used a unilateral milking model, which by decreasing the confounding effects of nutrition and genetics increases the likelihood of detecting small changes in MY and cell turnover in response to MF. In addition, the difference between the treatments (4x vs. 1x) was more extreme than in previous studies.

In the current study it has not been ascertained how changes in MF modulate the secretory activity and number of MEC, but previous studies have investigated possible signalling pathways that could be involved. Expression of prolactin receptors has been demonstrated to be increased with higher MF in mid lactation in bovine mammary glands (Bernier-Dodier et al., 2010). Whereas McKinnon et al. (1988) detected an increase in the prolactin-binding capacity of goat mammary glands when subjected to increased MF. This would facilitate an increased sensitivity to prolactin, and thereby increase MEC activity and cell number (reviewed in Knight, 2001). Changes in the expression of IGF-I and IGF binding proteins within the mammary gland may increase the bioavailability of IGF-I, potentially increasing MEC activity and stimulating proliferation (reviewed in Cohick, 1998). Bernier-Dodier et al. (2010) detected an increase in IGF-I mRNA in response to increased MF in mid lactation. Whereas in early lactation Wall and McFadden (2010) identified a decrease in IGF binding protein 3 mRNA in response to milk removal associated with increased MF. Evidence suggests that, in 1x-milked mammary glands at 18 h post-milking, the mammary gland reaches a level of milk capacity that begins to detrimentally affect the rate of milk synthesis (Stelwagen, 2001). A loss of mammary cell tight junction integrity occurs at this time, which interrupts the barrier between the alveolar lumen and the extracellular space, allowing the passage of milk constituents out of and blood constituents into the lumen, which could be the signal for secretory activity to cease (Stelwagen, 2001). Previous studies in sheep and beef cattle mammary glands have shown that *LTF* is expressed in alveoli that were not expressing *LALBA* and may be indicative of when the mammary alveoli enter a non-secretory state (Molenaar et al., 1992). Therefore, the significant increase in *LTF* expression in 1x-milked mammary tissue samples relative to 4x-milked may indicate a switch of secretory cells into a more non-secretory quiescent state compared with 4x, as postulated by Vetharanim et al. (2003).

A net increase in fat, protein, and lactose yield was measured in the post-treatment period in the 4x udder halves compared with 1x, which was consistent with previous studies (Bar-Peled et al., 1995, Wall and McFadden, 2007a). In comparison, Phyn et al. (2011), when measuring the effects of a three week treatment of 1x compared with 2x, did not detect any difference in MY; however, a carryover decrease in milk solids was detected following 1x treatment.

2.6 Conclusions

During early lactation in pasture-fed cows, a fourteen day UMF treatment of 4x versus 1x had an effect on MY, mammary cell proliferation, and major milk protein gene expression. Following the UMF treatment, a long-term carryover effect on MY was also observed. The results demonstrate that, even in pasture-fed systems, in which nutrient availability can be limiting, bovine mammary glands can still positively respond to MF stimulus. The increase of MEC proliferation, with 4x compared with 1x MF, may explain how this carryover effect on MY is established.

2.7 Acknowledgements

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Chapter III

STAT5 activation is linked to β 1-integrin protein abundance in unilaterally milked bovine mammary glands

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3.1 Abstract

Prolactin (PRL) is important in the regulation of milk synthesis in mammary epithelial cells (MEC). In cattle, the circulating levels of PRL are not limiting, suggesting the possible involvement of other factors that may control the response to PRL at the cellular level. The effects of milking frequency (MF) on milk synthesis are controlled locally within mammary glands and involve PRL signalling. To investigate this relationship between MF and PRL signalling further, udder halves of 17 dairy cows were milked either four times a day (4x) or once a day (1x) for fourteen days in early lactation. Mammary biopsies were obtained between 3 and 5 h following milking from both udder halves of 10 cows and changes in PRL and associated pathways were measured. The abundance of *STAT5A* mRNA was higher after 4x milking, whereas the mRNA abundance of the *PRL receptor* and *STAT3* were lower relative to 1x milking. In 4x mammary tissues, the protein levels of STAT5, activated STAT5 and β 1-integrin were higher whereas the protein levels of the long isoform of PRL receptor and activated STAT3 were lower than 1x milking. The activation of STAT5 correlated strongly with major milk protein gene mRNA abundance ($r = 0.86 - 0.94$) and β 1-integrin protein levels ($r = 0.91$). These results confirm that major milk protein gene expression is associated with STAT5 activation and also suggests that the STAT5 and β 1-integrin signalling pathways are linked. Modulation of β 1-integrin abundance in response to changes in MF may be a mechanism that controls the MEC ability to respond to PRL and therefore its secretory activity.

Key Words: milking frequency, mammary, milk synthesis, cell signalling

3.2 Introduction

In lactating mammary glands, prolactin (PRL) is important for the regulation of milk synthesis in mammary epithelial cells (MEC; Flint and Knight, 1997). The PRL signal is transmitted into the cell through its receptor (PRLR), which facilitates the activation of two closely related signalling proteins, signal transducer and activator of transcription (STAT)5A and –B. When activated, the STAT5 proteins form homodimers and are transported to the nucleus where they activate expression of genes involved in milk synthesis. This relationship between PRL signalling and milk synthesis has been well established in rodent models (reviewed in Flint and Knight, 1997). Administration of bromocriptine, which blocks PRL release from the pituitary gland, inhibits further milk secretion in rodents (Shaar and Clemens, 1972, Knight et al., 1986) however in cattle the relationship is unclear. During lactogenesis, in the period leading up to parturition, there is a surge in circulating PRL (Ingalls et al., 1973). Inhibition of this surge with bromocriptine can decrease subsequent milk yield (MY; Akers et al., 1981). However, once lactation has been established, blocking PRL-release with bromocriptine has little effect on MY (Smith et al., 1974). Nevertheless, recent studies have shown that another PRL-release blocking agent, quinagolide, applied over a longer period in mid-lactation does decrease MY (Lacasse et al., 2011), suggesting that PRL may have a galactopoietic effect under certain conditions.

Unilateral MF (UMF) models, where udder halves are milked independently at differing MF, have demonstrated that the effects on MY and MEC activity are predominantly controlled locally within the treated mammary gland (Stelwagen and Knight, 1997, Wall and McFadden, 2007a, Murney et al., 2015a). Dairy cows are commonly milked twice a day (2x), but increasing MF to four times a day (4x) can increase MY up to 20% compared to 2x (Stelwagen, 2001). This response is immediate (Hillerton et al., 1990, Murney et al., 2015a) and has been observed at most stages of lactation

(reviewed in Erdman and Varner, 1995). Similarly, decreasing MF from 2x to once a day (1x) has a negative impact on MY (Carruthers et al., 1993b, Rémond et al., 1999).

The mechanisms underlying the MY response to MF are not well understood, but are most likely a function of secretory MEC number and/or activity. In mammary glands during lactation, MEC number is controlled by the rates of proliferation and apoptosis (Capuco et al., 2001), which may be modulated by MF (Boutinaud et al., 2013, Murney et al., 2015a). However, changes in cell number are thought to be incremental and occur over the course of days to weeks (Capuco et al., 2001), and are unlikely to account for the 60% shift in MY in response to differential MF that occurs within two days of commencement of treatment (Murney et al., 2015a). Therefore, at least part of the response to MF may be through the modulation of the secretory activity of MEC. An increase in the mRNA abundance of both the long and short isoforms of the PRLR (Bernier-Dodier et al., 2010) occurs in response to increased MF. In addition, quinagolide treatment had more of an effect on the MY of udder-halves milked 2x compared to 1x (Lacasse et al., 2011). These results suggest a possible link between MF and PRL signalling, that is manifested through alteration of the sensitivity of MEC to circulating PRL.

Signalling from the extracellular matrix (ECM) via integrins is necessary for appropriate MEC differentiation and milk synthesis (Sympson et al., 1994, Roskelley et al., 1995). It has been demonstrated in mouse knockout models that β 1-integrin is essential for mammary gland development and sustained STAT5 activation (Naylor et al., 2005, Akhtar and Streuli, 2006). In normal mammary glands of both in mice (McMahon et al., 2004) and cows (Singh et al., 2008) the level of β 1-integrin protein decreases during involution, which suggests that the abundance of this protein is modulated during physiological changes in mammary tissue function. Furthermore in cow mammary glands,

this decrease coincides with a decrease in STAT5 activation (Singh et al., 2009).

The ability of MEC to respond to PRL can be perturbed by the presence of antagonistic factors. In mammary glands, STAT3 activation is pro-apoptotic and can be induced by the Leukaemia Inhibitory Factor (LIF) pathway (Kritikou et al., 2003). Activation of STAT3 has been shown to occur during involution in both mouse and bovine mammary glands (Kritikou et al., 2003, Singh et al., 2009) and coincides with the inactivation of STAT5. There is evidence from *in vitro* models suggesting that the activation of STAT3 can inhibit activation of STAT5 and vice versa (Clarkson et al., 2006, Granillo et al., 2007), but whether this occurs *in vivo* is unclear.

In this study, a UMF model was used to investigate PRL signalling in mammary glands of dairy cows. We hypothesised that the MY response to increased MF is a result of an increase in MEC sensitivity to circulating PRL. If so, this could be manifested through changes in the abundance of constituents of the PRL signalling pathway and/or cross-talk from other stimuli, such as ECM interactions via β 1-integrin or STAT3 activation. We therefore investigated changes in these signalling proteins, and their encoding mRNA, in response to altered MF.

3.3 Materials and methods

3.3.1 Animals and treatments

All animal manipulations were conducted in compliance with the rules and guidelines of the Ruakura Animal Ethics Committee. Animal management and treatments have been described previously (Murney et al., 2015a). Briefly, the udder halves of 17 cows were randomly assigned a MF either 4x

or 1x (4x in one udder half at 0500, 1100, 1700, and 2300 h, and 1x in the other udder half at 1100 h) for fourteen day from 5 ± 2 days in milk (DIM). The MY of 4x and 1x udder halves were 8.5 ± 0.5 kg/d and 8.4 ± 0.5 kg/d respectively before commencement of the treatment and by the end of the fourteen day period were 15.0 ± 0.7 kg/d and 7.5 ± 0.3 kg/d for 4x and 1x treatments, respectively. Biopsies of mammary gland tissue were taken from both rear quarters of 10 cows 3 to 5 h following the 1100 h milking as previously described (Farr et al., 1996). Briefly, this involved excision of approximately 0.5 g of alveolar tissue from a site distal from the cistern and major ducts using a modified purpose-built drill bit. A portion of the mammary tissue was fixed overnight in 4 % paraformaldehyde and processed into wax as described previously (Singh et al., 2005). The remainder was snap frozen in liquid nitrogen for subsequent molecular analysis of mRNA and protein.

3.3.2 RNA isolation and reverse transcription

Total RNA was isolated from 100 mg of mammary tissue using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and quantified using a Nanodrop 1000 spectrophotometer (Nanodrop, Wilmington, DE). The RNA integrity was measured on an Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA) with a RIN > 5 considered sufficient for real time RT-PCR analysis. For cDNA synthesis, 1 μ g of total RNA was purified using RNAeasy columns (Qiagen, Valencia, CA), as per the supplied RNA clean-up protocol (Qiagen) and converted using the SuperScript III reverse transcription kit (Life Technologies) according to the manufacturer's instructions. The amount of cDNA equivalent to 5 ng of starting total RNA was used as template for each real-time PCR reaction. Primer pairs for genes of interest and housekeeper genes are listed in Table 3.1. Primers for *PRLR* short and long isoforms, *STAT5A* and *STAT3* were designed using Primer3 (Untergasser et al., 2012). Primers have previously been reported for β 1-integrin (*ITGB1*) and focal adhesion kinase (*FAK*; Singh et al., 2005), α_{S1} -casein (*CSN1S1*), α -lactalbumin (*LALBA*), and β -

lactoglobulin (*LGB*) (Murney et al., 2015a), β -casein (*CSN2*) and ubiquitin B (*UBB*; Singh et al., 2008) and β 2 microglobulin (*B2M*; Wall and McFadden, 2010). Real-time PCR was carried out on a Corbett Rotorgene 6000 (Qiagen) with SYBR ExTaq Mix (Takara Bio Inc., Shiga, Japan) with a 3-min denaturation, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec. Relative mRNA abundance for genes of interest were calculated in relation to the geometric mean of the mRNA abundance of two housekeeper genes (*UBB* and *B2M*) as previously described (Smith et al., 2010), with these modifications; threshold cross-over values (C_t) were replaced with take-off values and amplification efficiencies were calculated using the comparative quantification analysis tool of Rotorgene 6000 series software 1.7 (Qiagen). The housekeeper genes were selected due to their expression remaining unchanged relative to each other as previously described by Bionaz and Loor (2007). PCR products were verified by sequencing (Waikato DNA Sequencing Facility, Hamilton, New Zealand).

3.3.3 Protein extraction and western blotting

Total protein was isolated from previously snap-frozen mammary biopsy samples by homogenisation in a buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl, 10 mM KCl, and protease inhibitors as described previously (McMahon et al., 2004). Protein concentration was determined using the Bradford method (Bradford, 1976) and 20 μ g of total protein was separated on 10% BisTris NUPAGE gels (Life Technologies) and transferred onto nitrocellulose membranes (Pall Corporation, East Hills, NY, USA). Membranes were blocked in Tris-buffered saline (TBS) solution (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6) containing 0.1% Tween 20 (TBST), 0.1% bovine serum albumin (BSA) and 4 % nonfat milk for 2 h. After three washes in TBST/BSA, membranes were incubated for 2 h in TBST/BSA with antibodies targeting; PRLR (long; 1:1000, sc-30225, Santa Cruz Biotechnology, Santa Cruz, CA), PRLR (short; 1:2000, C61-K5, kindly provided by A.J. Nixon, AgResearch Ltd, Hamilton, New Zealand), STAT5A/B (STAT5, 1:10000, sc-

835, Santa Cruz Biotechnology), Tyr 694 phosphorylated STAT5A/B (STAT5-P, 1:30000; Wheeler et al., 2001), STAT3 (1:10000, sc-482, Santa Cruz Biotechnology), Tyr 705 phosphorylated STAT3 (STAT3-P, 1:10000, sc-7993-R, Santa Cruz Biotechnology), β 1-integrin (1:1000, sc-8978, Santa Cruz Biotechnology) and FAK (1:1000, sc-557, Santa Cruz Biotechnology). Following three further washes in TBST/BSA, membranes were incubated for 1 h in TBST/BSA with 1:10000 goat anti rabbit secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich Co Ltd, Gillingham, United Kingdom), except for STAT5-P which was incubated in TBST/BSA with 1:15000 sheep anti mouse secondary antibody conjugated to horseradish peroxidase (Silenus Labs Pty Ltd, Boronia, Australia). Finally membranes were washed in TBST/BSA three times and then a further four washes in TBS. To visualise the immunoreactive bands, membranes were incubated for 1 min in ECL Western blotting detection reagents (Amersham, GE Healthcare, Buckinghamshire, United Kingdom) and then exposed to X-Omat AR film (Carestream Health, Inc., Rochester, NY) for 2 to 30 min, depending on the protein of interest. Films were scanned using a densitometer (GS 800, BioRad, Auckland, New Zealand) and the densities of immunoreactive bands were determined using Quantity One software (BioRad).

3.3.4 Histological analysis

Sections were cut from paraffin embedded formaldehyde fixed mammary tissue samples and then stained with haematoxylin and eosin as previously described in Singh et al. (2005). One section per mammary tissue sample (n=10 per treatment) was assayed. Sections were viewed at 100x magnification using a binocular microscope (Olympus Optical Co. Ltd, London, United Kingdom). All alveoli of each section were categorised into either the “lactating” phenotype or the “involuting” phenotype using descriptions from Holst et al. (1987) as a guide. Alveoli were considered to have the “lactating” phenotype when cells had a regular cuboidal appearance

and the alveolar lumen occupied most of the tissue area with little interalveolar space. Alveoli were considered to have the “involuting” phenotype when cells were irregular and often contained several large vacuoles, the lumen appeared collapsed and the interalveolar space was more obvious. The “lactating” phenotype proportion of alveoli for each section was calculated by dividing the number of “lactating” phenotype alveoli by total number of alveoli counted. Total number of alveoli counted per section was > 250.

3.3.5 Statistical analysis

Differences between \log_{10} -transformed relative mRNA abundance, \log_{10} -transformed protein densities and “lactating” phenotype proportion were analysed by ANOVA (Minitab® 16.2.2, 2010, State College, PA) by treatment (MF), with cow as a random effect. \log_{10} -transformed relative mRNA abundance was expressed as treatment mean and fold change, which was calculated by back-transformation of the difference between the treatment means (4x-1x). \log_{10} -transformed protein densities was expressed as a ratio of treatment mean (4x:1x) \pm standard error of the difference. “Lactating” phenotype proportion was expressed as treatment mean \pm SEM. Differences between means were analysed by paired t-test and considered significant at $P < 0.05$. Correlations between \log_{10} -transformed relative mRNA abundance of milk protein genes, \log_{10} -transformed protein densities of STAT5-P, STAT3-P and β 1-integrin, and \log_{10} -transformed “lactating” phenotype proportion were performed with Minitab.

Table 3.1 Primers used for real time PCR.

Target ¹	GeneBank no.	Forward (5`-3`)	Reverse (5`-3`)	Product length (bp)	Reference
<i>PRLR</i> (short)	NM_174155	tgattcccagtgaggacac	tagacacaaggcgagaaggc	199	-
<i>PRLR</i> (long)	NM_001039726	tgacttcccagtgaggacac	cttcggacttgcctcttc	187	-
<i>STAT5A</i>	NM_001012673	cgcagctccagaacacgltac	accagtcgcagttcctcaaa	186	-
<i>STAT3</i>	NM_001012671	gtgcattgacaagaactccg	aatcaggaggcatcaaat	195	-
<i>ITGB1</i>	NM_174368	cagatgagggtgaaccagcga	atgcaggaagtgtaccag	288	Singh et al. (2005)
<i>FAK</i>	NM_001075250	ctggggccatgagcgaagta	cttggtggtyggcaagttcata	264	Singh et al. (2005)
<i>CSN1S1</i>	NM_181029	taccctgagctttcagaca	cataactgtggagtcctca	200	Murney et al. (2015a)
<i>CSN2</i>	NM_181008	gtgaggaacacgacgcaaa	aggyaagggcatttctgt	232	Singh et al. (2008)
<i>LALBA</i>	NM_174378	tgggtctgtaccacgttca	gctttatgggccaaccagta	251	Murney et al. (2015a)
<i>LGB</i>	NM_173929	cattgtcaccacagaccatga	cactcaccgftctccatt	194	Murney et al. (2015a)
<i>UBB</i>	NM_174133	ggcaagaaccatcacccctggaa	gccacccctcagacgaagga	201	Singh et al. (2008)
<i>B2M</i>	NM_173893	aagatggtctcgtctgt	tcaaatctgatgtgtctg	381	Wall and McFadden 2010

¹ *PRLR* (short) = prolactin receptor short isoform; *PRLR* (long) = prolactin receptor long isoform; *STAT5A* = signal transducer and activator of transcription 5A; *STAT3* = signal transducer and activator of transcription 3; *ITGB1* = β 1-integrin; *FAK* = focal adhesion kinase; *CSN1S1* = α _{s1}-casein; *CSN2* = β -casein; *LALBA* = α -lactalbumin; *LGB* = β -lactoglobulin; *UBB* = ubiquitin B; *B2M* = β -2 microglobulin.

3.4 Results

3.4.1 Changes in prolactin signalling pathway in response to milking frequency

Relative mRNA and protein levels of PRLR and STAT5 were measured in the two treatment groups to determine the effects of MF. The relative mRNA abundance of both the long and short isoforms of *PRLR* and the protein abundance of the long isoform PRLR were lower in 4x mammary tissue samples compared with 1x samples (Table 3.2 and Figure 3.1). The relative abundance of *STAT5A* mRNA and STAT5 protein abundance were higher in 4x mammary tissue samples compared with 1x samples (Table 3.2 and Figure 3.1). Activation of the pathway was evaluated by measuring the phosphorylation of tyrosine 694 within the STAT5 protein. The level of STAT5-P was 40 times higher in 4x mammary tissue samples compared with 1x samples (Figure 3.1). Within all mammary tissue samples the levels of STAT5-P correlated strongly with the mRNA abundance of *CSN1S1* (Figure 3.2A). Similar correlations were observed with the mRNA abundance of *CSN2*, *LALBA* and *LGB* ($r = 0.95$, 0.96 , and 0.91 respectively, data not shown).

Table 3.2 Relative mRNA abundance for genes of interest in mammary tissue collected from dairy cow udder halves unilaterally milked four times a day (4x) or once a day (1x) in early lactation.

Target ¹	4x ²	1x ²	fold change ³	P value ⁴
<i>PRLR</i> (short)	-1.487	-1.391	0.79	0.002
<i>PRLR</i> (long)	-0.876	-0.681	0.69	0.001
<i>STAT5A</i>	-2.137	-2.263	1.33	0.05
<i>STAT3</i>	-1.598	-1.351	0.57	0.001
<i>ITGB1</i>	-1.204	-1.142	0.87	NS
<i>FAK</i>	-1.901	-1.842	0.87	NS

¹ *PRLR* (short) = prolactin receptor short isoform; *PRLR* (long) = prolactin receptor long isoform; *STAT5A* = signal transducer and activator of transcription 5A; *STAT3* = signal transducer and activator of transcription 3; *ITGB1* = β 1-integrin; *FAK* = focal adhesion kinase.

² Mean \log_{10} -transformed relative mRNA abundance derived from take-off value as described in the methods.

³ Fold change in abundance derived by back-transformation of the difference of the transformed values between treatments (4x-1x).

⁴ Probability of the difference between treatments occurring by chance. Difference was considered not significant (NS) at $P > 0.05$.

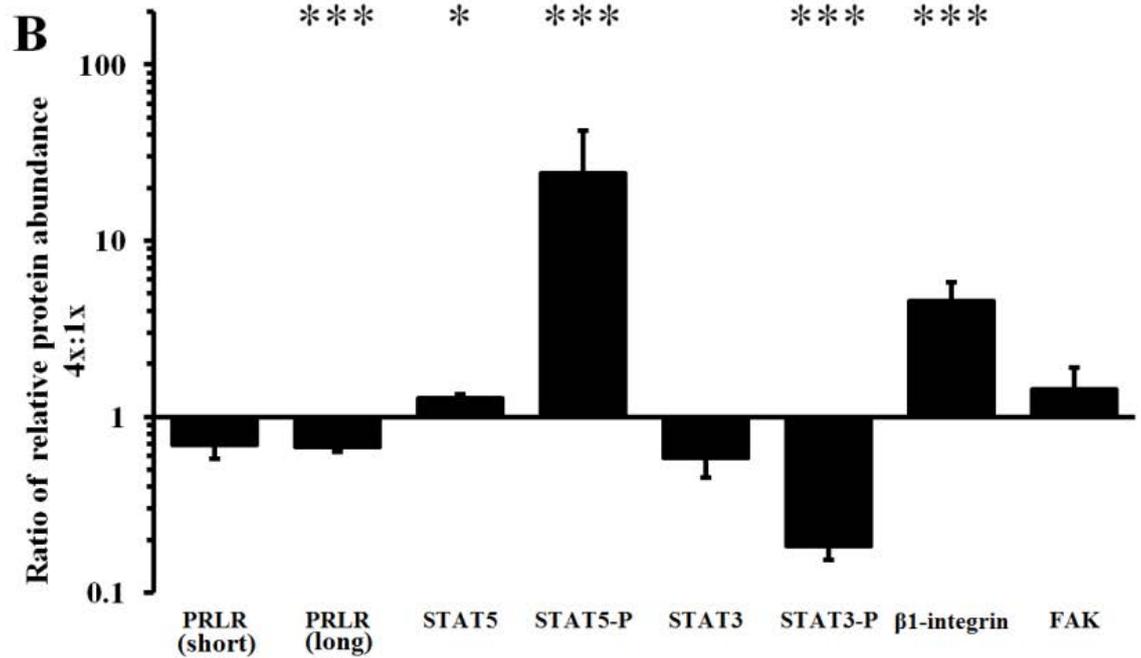
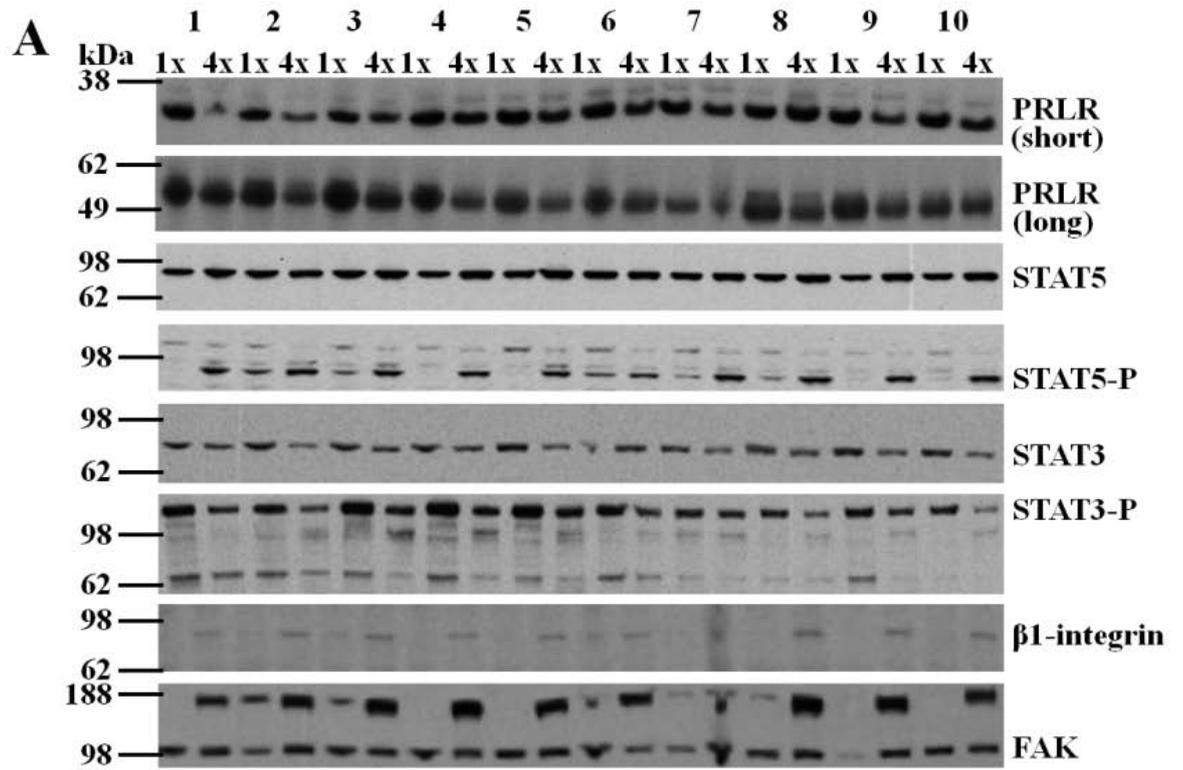


Figure 3.1 Western blot analysis of key proteins in the prolactin and integrin signalling pathways in mammary tissue extracted from udder halves of dairy cows which had been unilaterally milked either four times a day (4x) or once a day (1x) for fourteen days in early lactation. Panel A, independent western blots for prolactin receptor short and long isoforms (PRLR (short), PRLR (long)), signal transducer and activator of transcription 5 A/B (STAT5), phosphorylated STAT5A/B (STAT5-P), STAT3, phosphorylated STAT3 (STAT3-P), β 1-integrin, and focal adhesion kinase (FAK) in total protein samples extracted from both the 4x and 1x milked udder halves of 10 dairy cows. Panel B, Difference of the mean protein levels as determined by densitometry of PRLR (short), PRLR (long), STAT5, STAT5-P, STAT3, STAT3-P, β 1-integrin, and FAK, expressed as the ratio of abundance in 4x compared to the 1x \pm standard error of the difference, * $P \leq 0.05$ and *** $P \leq 0.001$.

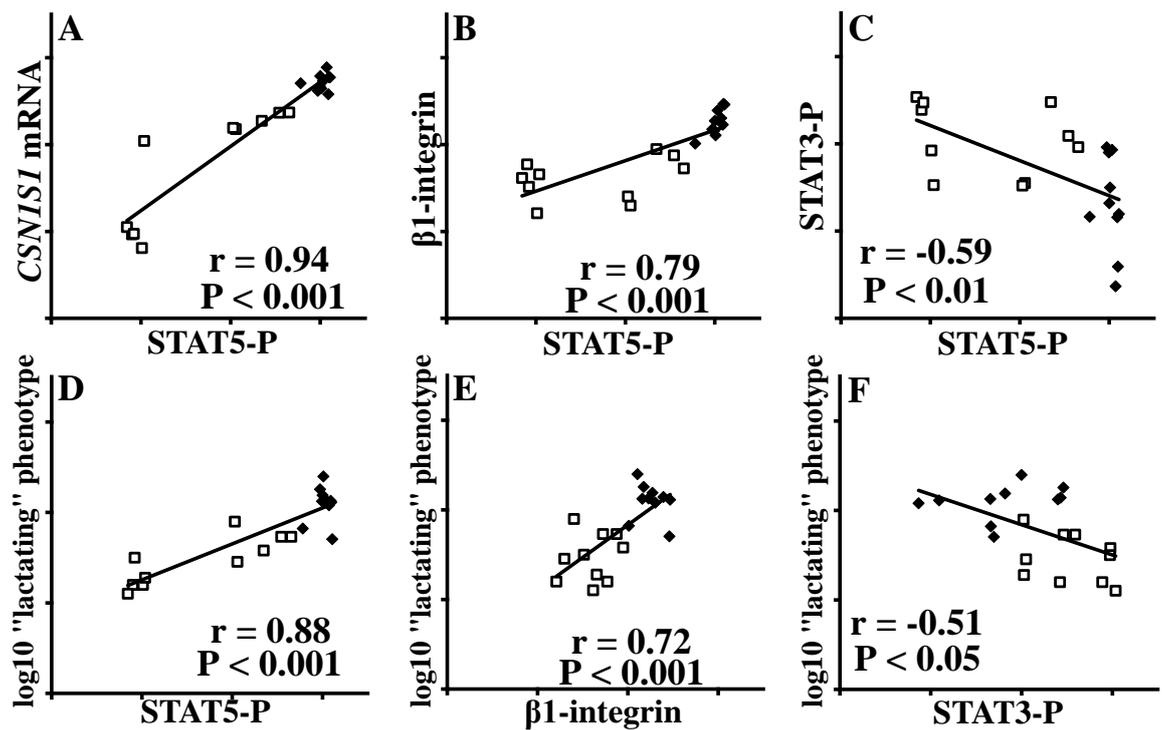


Figure 3.2 Correlations between phosphorylated signal transducer and activator of transcription 5 A/B (STAT5-P) protein abundance and α_{S1} -casein (CSN1S1) mRNA abundance (panel A), STAT5-P protein abundance and β 1-integrin protein abundance (panel B), STAT5-P protein abundance and phosphorylated STAT3 (STAT3-P) protein abundance (panel C), STAT5-P protein abundance and proportion of alveoli exhibiting the “lactating” phenotype (panel D), β 1-integrin protein abundance and proportion of alveoli exhibiting the “lactating” phenotype (panel E), and STAT3-P protein abundance and proportion of alveoli exhibiting the “lactating” phenotype (panel F) in mammary tissue extracted from udder halves of dairy cows which had been subjected to differential milking for either four times a day (4x, black diamonds) or once a day (1x, white squares) for fourteen days in early lactation. All data is \log_{10} -transformed with regression fits and Pearson correlation (r) for all data points (4x and 1x combined).

3.4.2 Changes in STAT3 activation and extracellular matrix/ β 1-integrin pathway constituents in response to milking frequency and correlation with STAT5 activation

To investigate whether cross-talk between pathways may play a role in STAT5 activation, the abundance and activation of STAT3, and the abundance of β 1-integrin and FAK were measured. The level of *STAT3* mRNA was lower in 4x mammary tissue samples compared with the 1x samples (Table 3.2). At the protein level, STAT3 was not significantly different between the two treatments (Figure 3.1), whereas the levels of phosphorylated STAT3 was 5 times lower in the 4x mammary tissue samples compared with 1x samples (Figure 3.1). The levels of β 1-integrin protein was five times higher in 4x mammary tissue samples compared with 1x samples (Figure 3.1), whereas the relative abundance of *ITGB1* and *FAK* mRNA (Table 3.2) and the levels of FAK protein were not significantly different between the two treatments (Figure 3.1). Within all mammary tissue samples the levels of STAT5-P protein correlated highly with the levels of β 1-integrin protein (Figure 3.2B), whereas a negative correlation was detected between the levels of STAT5-P and STAT3-P proteins (Figure 3.2C).

3.4.3 Effects of milking frequency on mammary alveoli phenotype and correlation with STAT5 activation and β 1-integrin

Generally, the mammary tissue from both treatment groups was heterogeneous with areas of alveoli either displaying a “lactating” phenotype (a representative image shown in Figure 3.3A) or an “involuting” phenotype (a representative image shown in Figure 3.3B). Areas of the mammary tissue sections consisting of predominantly “lactating” phenotype typically had larger ordered alveoli with clearly defined lumen and less interalveolar space. The alveoli consisted of cuboidal MEC that were regularly spaced (Figure 3.3A). In contrast, alveoli typical in areas of the mammary tissue sections consisting of predominantly “involuting” phenotype were smaller and

irregularly shaped, with collapsed lumen. These alveoli consisted of disordered MEC, with numerous cells containing unstained cytoplasmic vacuoles (Figure 3.3B). A four-fold higher proportion of “lactating” phenotype alveoli was detected in the 4x mammary tissue samples compared to 1x samples (Figure 3.3C). The proportion of “lactating” phenotype alveoli correlated highly with the levels STAT5-P protein (Figure 3.2D) and β 1-integrin protein (Figure 3.2E), and correlated negatively with the levels of STAT3-P protein (Figure 3.2C).

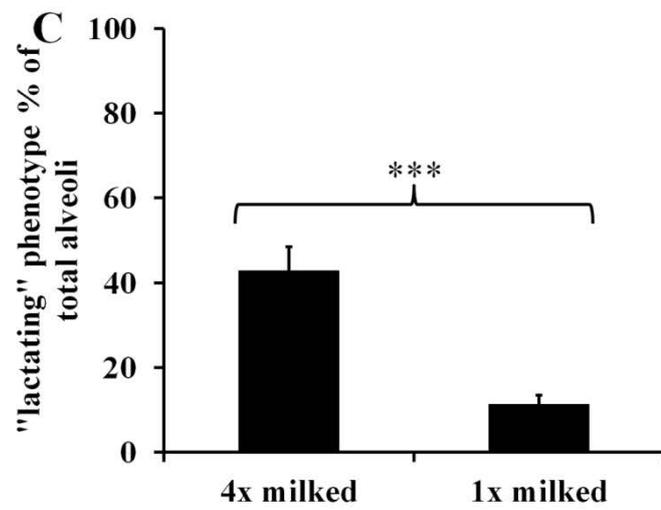
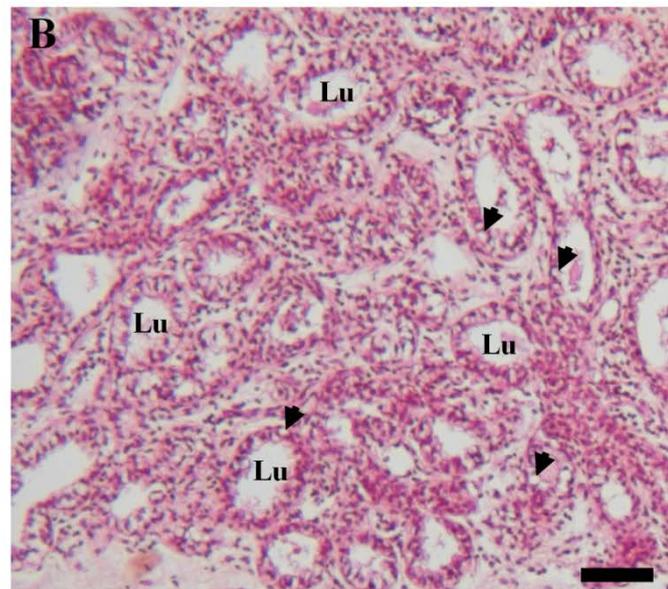
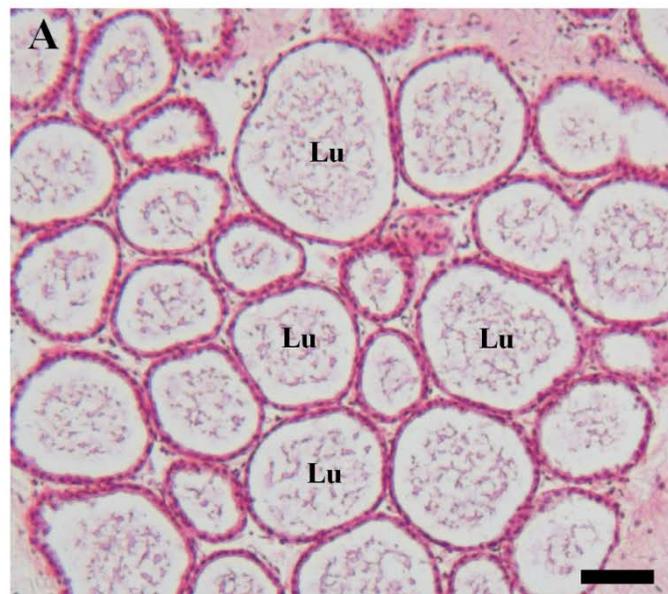


Figure 3.3 Phenotypic analysis of mammary tissue extracted from udder halves which had been unilaterally milked either four times a day (4x) or once a day (1x) for fourteen days in early lactation. Panel A, representative image of mammary parenchyma from the 4x milked udder half of cow 10, exhibiting the “lactating” phenotype. Panel B, representative image of mammary parenchyma from the 1x milked udder half of cow 10, exhibiting the “involuting” phenotype. Sections were stained with haematoxylin and eosin. Several alveolar lumen (Lu) are evident in both images; black arrows indicate a number of the cytoplasmic vacuoles within the mammary epithelial cells in alveoli exhibiting the “involuting” phenotype. Panel C, mean % of alveoli exhibiting the “lactating” phenotype in 4x and 1x mammary tissue sections with SEM, *** $P \leq 0.001$.

3.5 Discussion

Previous studies have demonstrated that the effects of MF on milk production are controlled locally within the mammary gland (Hillerton et al., 1990, Stelwagen and Knight, 1997). In accordance with this, the increase of STAT5 activation in the 4x mammary tissue reported in the present study is likely due to local intra-mammary signals. Furthermore, the activation of STAT5 is highly correlated with major milk protein gene mRNA abundance confirming the importance of this transcription activator in the control of milk synthesis in bovine mammary glands. In MEC, activation of STAT5 is mediated by PRL, which signals through PRLR (Hynes et al., 1997). Previous studies in mid-lactation dairy cows have demonstrated *PRLR* mRNA abundance increased following increased MF (Bernier-Dodier et al., 2010). In contrast, we report a decrease of both *PRLR* mRNA and protein in the 4x tissue samples compared to 1x. The discrepancy between reports remains unclear, but it may be due to the differences in the parameters of the experiments, i.e. stage of lactation, duration of treatment and time of sample collection in relation to previous milking. Due to the lack of association between the levels of STAT5 activation and PRLR protein, our results suggest the abundance of PRLR is not a limiting factor in the ability of MEC to respond to PRL stimulus.

In mice, the β 1-integrin cell surface receptor influences the PRL sensitivity of MEC (Naylor et al., 2005, Akhtar and Streuli, 2006). In agreement with this, the elevated levels of β 1-integrin protein in the 4x tissue samples and correlation with STAT5 activation suggest that modulation of β 1-integrin levels might also mediate the responsiveness of the MEC to PRL in bovine mammary glands. One possible mechanism for a causative link between these two signalling proteins is via Ras-related C3 botulinum toxin substrate 1 (Rac1; Akhtar and Streuli, 2006). In the present study, Rac1 was unable to be detected thus its role linking STAT5 and integrin signalling in the bovine mammary gland remains unverified. The regulation of β 1-integrin is post-

transcriptional since the levels of *ITGB1* mRNA were unaffected by MF. It is unknown how protein levels of β 1-integrin are maintained in MEC or how MF modulates their abundance and this question requires further study.

During involution, the activation of STAT5 diminishes and at the same time activation of STAT3 increases (Kritikou et al., 2003, Singh et al., 2009). It is thought that STAT3 can act as an antagonist to STAT5, preventing further activation when the gland shuts down milk production in response to infection or cessation of milking (Granillo et al., 2007). A negative relationship between STAT5 and STAT3 activation was also demonstrated in the data presented here. Whether activation of STAT3 in the 1x tissue samples is inhibiting STAT5 activation or vice versa in the 4x tissue samples is unclear. Activation of STAT3 occurs as part of the immune response in mammary glands and is increased during mastitis (M. Prewitz & K. Singh, unpublished data) and involution (Singh et al., 2009). Therefore, the increase in STAT3 activation detected within the 1x tissue samples may indicate that an immune response has been initiated in response to decreased MF. In accordance with this an increase in lactoferrin mRNA abundance in these 1x milked samples has previously been reported (Murney et al., 2015a), which is also up-regulated during mastitis (Harmon et al., 1975) and involution (Schanbacher et al., 1993). It is therefore possible that STAT5 and STAT3 activation never coincide in the same MEC and occur independent of each other, being activated only in different MEC states, i.e. actively secreting MEC for STAT5 and inflammatory response activated MEC for STAT3.

Previous studies have shown that normally lactating bovine mammary glands are heterogeneous (Molenaar et al., 1992). The secretory MEC can be divided into two distinct sub-populations, either actively secreting MEC that are expressing genes associated with milk synthesis, or quiescent MEC which instead are expressing immune related genes, such as lactoferrin (Molenaar et al., 1992). The results presented in this study demonstrate that

the proportions of the two sub-populations of MEC can be modulated by MF. These results suggest that MEC switch between active secretory and quiescence, increasing or decreasing the proportions in response to MF stimuli, which has been postulated as a possible mode of action of MF (Vetharaniem et al., 2003). Furthermore, the results demonstrate a link between the overall activity of the mammary tissue, i.e. milk protein gene expression and STAT5 activation, and the proportion of phenotypically “lactating” alveoli suggesting that this switching of quiescent MEC to actively secreting MEC is indeed the mechanism that controls the increase in milk synthesis in bovine mammary glands, rather than a uniform increase in activity across all MEC. How the switching of MEC from quiescent to actively secretory is controlled is not fully understood, but the correlation between β 1-integrin the secretory activity of the mammary tissues may indicate a role for ECM signalling through β 1-integrin and the switching of MEC from quiescent to actively secretory.

3.6 Conclusions

The data presented here suggests that β 1-integrin protein abundance is closely linked to STAT5 activation in bovine mammary glands subjected to UMF. We propose that it is β 1-integrin that permits the sustained STAT5 activation and abundance of β 1-integrin protein is controlled as a mechanism to alter the MEC sensitivity to PRL rather than constituents of the PRL signalling pathway. It appears that secretory activity is controlled at the alveolar level and the proportion of phenotypically “lactating” alveoli is increased in the 4x mammary tissue samples compared to 1x mammary tissue samples.

3.7 Acknowledgements

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Chapter IV

The effects of milking frequency on insulin-like growth factor I signalling within the mammary gland of dairy cows

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4.1 Abstract

In dairy cows, short-term changes of milking frequency (MF) in early lactation have been shown to produce both an immediate as well as a long-term effect on milk yield. The effect of MF on milk yield is controlled locally within mammary glands and could be a function of changes in either secretory mammary epithelial cell (MEC) number or their activity. Insulin-like growth factor I (IGF-I) signalling is one candidate factor that could mediate these effects, as it can be controlled locally within mammary glands and can effect both MEC number and activity by activating the phosphoinositide 3-kinase (PI3K)/Akt and extracellular-signal-regulated kinase (ERK)1/2 pathways. To investigate the relationship between MF and IGF-I signalling, udder halves of 17 dairy cows were milked either four times a day (4x) or once a day (1x) for fourteen days in early lactation. On day fourteen between 3 and 5 h following milking, mammary biopsies were obtained from 10 cows from both udder halves and changes in the expression of genes associated with IGF-I signalling and the activation of the PI3K/Akt and ERK1/2 pathways were measured. The mRNA abundance of IGF type I receptor, IGF binding protein (IGFBP)-3 and IGFBP-5 were lower following 4x milking relative to 1x milking. However, the mRNA abundance of IGF-I was not effected by MF. Both IGFBP3 and IGFBP5 are thought to inhibit IGF-I, so the decrease in their mRNA abundance may serve to stimulate the IGF-I signal in the 4x-milked mammary gland. The activation of PI3K/Akt pathway was lower in response to 4x milking relative to 1x, and the activation of the ERK1/2 was unaffected by MF, suggesting they do not mediate the effects of MF.

Key Words: milking frequency, mammary, milk synthesis, cell signalling.

4.2 Introduction

In early lactation, a short-term changes of milking frequency (MF) have an immediate effect on milk yield (MY), as well as a long-term MY effect after normal milking is resumed (Bar-Peled et al., 1995, Rémond et al., 1999). The positive long term carry-over effect on MY of increased MF can be achieved with a two to three week treatment of four times a day (4x) milking compared to twice a day (2x) milking alone (Hale et al., 2003, Wall and McFadden, 2007b). Whereas, the negative carry-over effect on MY in response to decreased MF has also been demonstrated following once a day (1x) milking, however in this case, a 1x treatment of at least six weeks is required (Rémond et al., 1999).

Unilateral MF (UMF) experiments, in which udder halves are milked at different frequencies independently of one another, have demonstrated that the effects of MF on MY are predominantly controlled by intra-mammary factors (Stelwagen and Knight, 1997, Wall and McFadden, 2007b, Murney et al., 2015a). Milk yield is ultimately affected by a combination of the number of secretory mammary epithelial cells (MEC) and their level of activity, therefore factors and signalling pathways that modulate these parameters are the most plausible candidate mechanisms driving the response to changes in MF. Comparison of 4x milking with 1x milking by UMF in early lactation demonstrated an increase in both proliferation and the mRNA abundance of the major milk protein genes in response to the higher MF (Murney et al., 2015a). Furthermore, analysis of mammary tissue from these treatments showed that signalling proteins involved in prolactin and extracellular matrix signalling pathways seem to be affected by MF (Murney et al., 2015b). However, these effects do not provide a full mechanistic description of how the MF effects are mediated in the mammary gland. Thus, further candidate pathways and factors should be investigated.

Insulin-like growth factor-I (IGF-I) is a candidate factor that may be involved in the effects of MF. This endocrine hormone is synthesised in the liver in response to growth hormone and circulates in the bloodstream at a relatively high concentration. In addition, IGF-I can be locally produced within several different tissues including mammary glands, thereby acting as an autocrine hormone (Glimm et al., 1992). This locally produced IGF-I is thought to be as important to mammary gland development as systemic IGF-I (Akers et al., 2000).

The majority of IGF-I found in circulation is bound with high affinity to one or more of the six known IGF binding proteins (IGFBP 1-6), which modulate the bio-availability of IGF-I to target tissues (Flint et al., 2008). In mammary glands, locally synthesised IGFBP3 and IGFBP5 are thought to be important for controlling IGF-I function (Flint et al., 2008). The upregulation of IGFBP5 has been demonstrated in rodent mammary glands at the onset of involution and is thought to inhibit the cell survival signal of IGF-I (Tonner et al., 1997, Flint et al., 2005). The effects of IGF-I on cellular function can also be moderated by changes in the levels of the IGF Type I receptor (IGFIR), which is the primary signalling receptor for IGF-I (Baumrucker and Erond, 2000).

Insulin-like growth factor-I is a potent mammary mitogen and has been shown to have both proliferative properties in bovine MEC in vitro (McGrath et al., 1991), and to protect against apoptosis in mouse mammary glands during involution (Neuenschwander et al., 1996). Furthermore in goats, IGF-I has been shown to increase milk secretion when administered via close arterial injection (Prosser et al., 1990). Interestingly, this effect is masked by a preceding treatment of increased MF, suggesting some link between the effect of IGF-I and the effect of MF (Prosser and Davis, 1992).

Previous studies have reported changes in components of the IGF-I signalling pathway within mammary glands in response to MF (Bernier-Dodier et al., 2010, Littlejohn et al., 2010, Wall and McFadden, 2010, Boutinaud et al., 2013). The abundance of IGF-I (*IGF1*) and IGFIR (*IGF1R*) mRNA increased in mammary tissue that was subjected to three times a day (3x) milking in mid-lactation compared to 1x, whereas *IGFBP5* mRNA was not affected (Bernier-Dodier et al., 2010). In contrast, microarray analysis of genes modulated in response to 1x milking revealed an increase of *IGFBP5* mRNA abundance in 1x-milked udder halves compared to 2x (Littlejohn et al., 2010, Boutinaud et al., 2013). In another study comparing 4x and 2x milking in early lactation, no MF effect was observed on *IGF1* and *IGF1R* mRNA abundance, but lower amounts of *IGFBP3* mRNA were observed in 4x-milked mammary tissues compared to 2x (Wall and McFadden, 2010). This response of *IGFBP3* mRNA to MF was only detected when the tissue was collected following a 4x milking, whereas samples taken when both udder halves were milked showed no difference in *IGFBP3* mRNA abundance (Wall and McFadden, 2010). Overall, the differing findings of these studies only serve to illustrate the complex and dynamic nature of IGF-I signalling in bovine mammary glands, which is not fully understood.

There are two intracellular pathways that are thought to be stimulated by IGF-I, which are the phosphoinositide 3-kinase (PI3K)/Akt and extracellular-signal-regulated kinase (ERK)1/2 pathways (Peruzzi et al., 1999). The serine/threonine kinase Akt is an important intracellular node in cell signalling which is activated by IGF-I as well as other growth factors, cytokines and the extracellular matrix. Activation of Akt is known to protect against apoptosis (Kennedy et al., 1997) and overexpression of activated Akt within the mammary glands of transgenic mice can delay the onset of involution (Hutchinson et al., 2001). The ERK1/2 pathway is also stimulated by IGF-I as well as other mitogenic stimuli. Activation of the ERK1/2 pathway is involved in a diverse range of cellular processes which control such functions as the cell cycle progression, cell proliferation, cell division and cell differentiation.

The activation of ERK1/2 is essential for appropriate development of mammary glands controlling MEC proliferation (Whyte et al., 2009). These signalling intermediates thus represent likely downstream candidate mediators of the MF response in mammary glands.

We hypothesised that MF modulates the IGF-I signalling pathway within mammary glands by altering the expression of locally produced IGF-I, IGFbps and IGFIR. This would in turn lead to activation of the PI3K/Akt and/or ERK1/2 pathways. This results in modulation of the rate of MEC turnover by altering levels of apoptosis and proliferation. To test this hypothesis we measured a number of key components of the IGF-I signalling pathway in a 4x/1x UMF model.

4.3 Materials and methods

4.3.1 Animals and treatments

All animal manipulations were conducted in compliance with the rules and guidelines of the Ruakura Animal Ethics Committee. Animal management and treatments have been described in detail previously (Murney et al., 2015a). Briefly, half udders from 17 Holstein-Friesian and Holstein-Friesian x Jersey dairy cows in the first week of lactation (5 ± 2 DIM) were randomly assigned a MF treatment either 4x or 1x (4x in one udder half at 0500, 1100, 1700, and 2300 h, and 1x in the other udder half at 1100 h) for fourteen days. The pre-treatment MY of the udder halves were 8.5 ± 0.5 kg/d and 8.4 ± 0.5 kg/d for 4x and 1x respectively. By day fourteen of treatment the 4x and 1x udder half MY were 15.0 ± 0.7 kg/d and 7.5 ± 0.3 kg/d respectively. Biopsies of mammary gland tissue were taken from both rear quarters of 10 cows at between 3 and 5 h following the 1100 h milking on day fourteen as previously described (Farr et al., 1996). Briefly, this involved excision of approximately 0.5 g of alveolar tissue from a site distal from the cistern and

major ducts using a modified purpose-built drill rotating stainless steel cannula. The tissue samples were snap frozen in liquid nitrogen for subsequent molecular analysis of mRNA and protein.

4.3.2 RNA isolation and reverse transcription

The total RNA was isolated from 100 mg of mammary tissue using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and quantified using a Nanodrop 1000 spectrophotometer (Nanodrop, Wilmington, DE). The RNA integrity was measured on an Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA) with a RIN > 5 considered sufficient for real time RT-PCR analysis. For cDNA synthesis, 1 µg of total RNA was purified using RNAeasy columns (Qiagen, Valencia, CA), as per the supplied RNA clean-up protocol (Qiagen) and converted using the SuperScript III reverse transcription kit (Life Technologies) according to the manufacturer's instructions. The amount of cDNA equivalent to 5 ng of starting total RNA was used as template for each real-time PCR reaction. Primer pairs for genes of interest and housekeeper genes are listed in Table 1. Primers have previously been reported for IGF-I (*IGF1*) and *IGFBP5* (Wall et al., 2005), IGF type I receptor (*IGF1R*) and β 2 microglobulin (*B2M*) (Wall and McFadden, 2010), *IGFBP3* (Zhou, 2007), and ubiquitin B (*UBB*) (Singh et al., 2008). Real-time PCR was carried out on a Corbett Rotorgene 6000 (Qiagen) with SYBR ExTaq Mix (Takara Bio Inc., Shiga, Japan) with a 3 min denaturation, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The mRNA abundance for genes of interest were calculated relative to the geometric mean of the mRNA abundance of two housekeeper genes (*UBB* and *B2M*) as previously described (Smith et al., 2010), with these modifications; threshold cross-over values (Ct) were replaced with take-off values and amplification efficiencies were calculated using the comparative quantification analysis tool of Rotogene 6000 series software 1.7 (Qiagen). The housekeeper genes were selected due to their expression remaining unchanged relative to each other as previously

described by Bionaz and Looor (2007). The PCR products were verified by sequencing (Waikato DNA Sequencing Facility, Hamilton, New Zealand).

Table 4.1 Primers used for real time PCR.

Target ¹	Gene no.	Bank	Forward (5`-3`)	Reverse (5`-3`)	Product length (bp)	Reference
<i>IGF1</i>	X15726		ctcctcgcacatcttctatct	actcatccacgattcctgtct	201	Wall et al. (2005)
<i>IGF1R</i>	BM482617		tgagagtgtgtatgcctctgt	ggtctcgyggtcctcctt	177	Wall and McFadden (2010)
<i>IGFBP3</i>	AF305199.1		cagagcagacagacacccagaa	tgccccfactatccacaca	230	Zhou (2007)
<i>IGFBP5</i>	BF0762255		tccgagatggcagaggag	ggtcacagttggcaggt	289	Wall et al. (2005)
<i>UBB</i>	NIM_174133		ggcaagaccatcacccttggaa	gccaccctcagacgaagga	201	Singh et al. (2008)
<i>B2M</i>	NIM_173893		aaggatggtctcgtctcgt	ttcaatctgattggtgtctg	381	Wall and McFadden (2010)

¹ *IGF1* = insulin-like growth factor I; *IGF1R* = IGF type I receptor; *IGFBP* = IGF binding protein; *UBB* = ubiquitin B; *B2M* = β -2 microglobulin.

4.3.3 Protein extraction and western blotting

Total protein was isolated from snap-frozen mammary biopsy samples by homogenisation in a buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and protease inhibitors as described previously (McMahon et al., 2004). Protein concentration was determined using the Bradford method (Bradford, 1976) and 20 µg of total protein was separated on 10% BisTris NUPAGE gels (Life Technologies) and transferred onto nitrocellulose membranes (Pall Corporation, East Hills, NY, USA). Membranes were blocked in Tris-buffered saline (TBS) solution (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6) containing 0.1% Tween 20 (TBST), 0.1% bovine serum albumin (BSA) and 4 % nonfat milk for 2 h. After three washes in TBST/BSA, membranes were incubated for 2 h in TBST/BSA with antibodies targeting; ERK1/2 (1:10000, sc-94, Santa Cruz Biotechnology, Santa Cruz, CA), Thr 202/Thr 204 phosphorylated ERK1/2 (ERK-P, 1:500, sc-16982-R, Santa Cruz Biotechnology), Akt1/2/3 (1:1000, sc-8312, Santa Cruz Biotechnology), and Ser 473 phosphorylated Akt1/2/3 (Akt-P, 1:5000, sc-7985-R, Santa Cruz Biotechnology). Following three further washes in TBST/BSA, membranes were incubated for 1 h in TBST/BSA with a 1:10000 dilution of goat anti rabbit secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich Co Ltd, Gillingham, United Kingdom). Finally membranes were washed in TBST/BSA three times and then washed a further four times in TBS. To visualise the immunoreactive bands, membranes were incubated for 1 min in ECL Western blotting detection reagents (Amersham, GE Healthcare, Buckinghamshire, United Kingdom) and then exposed to X-Omat AR film (Carestream Health, Inc., Rochester, NY) for 2 to 30 min, depending on the protein of interest. Films were scanned using a densitometer (GS 800, BioRad, Auckland, New Zealand) and the densities of immunoreactive bands were determined using Quantity One software (BioRad).

4.3.4 Statistical analysis

Differences between \log_{10} -transformed relative mRNA abundance and \log_{10} -transformed protein densities were analysed by ANOVA (Minitab® 16.2.2, 2010, State College, PA) by treatment (MF), with cow as a random effect. \log_{10} -transformed relative mRNA abundance was expressed as treatment mean. The fold change with treatment was calculated by back-transformation of the difference between the treatment mean (4x-1x). \log_{10} -transformed protein densities were expressed as a ratio of treatment mean (4x:1x) \pm standard error of the difference. Differences between means were analysed by paired t-test and considered significant at $P < 0.05$.

4.4 Results

4.4.1 The effects of milking frequency on genes of the IGF-I axis

The relative abundance of *IGF1*, *IGF1R*, *IGFBP3* and *IGFBP5* mRNA were measured in mammary tissue extracted from udder halves either milked 4x or 1x for fourteen days in early lactation. There was no significant difference between 4x and 1x-milked mammary tissues for *IGF1* mRNA (Table 2). On the other hand, a > two-fold decrease in the relative abundance of *IGFBP3* and *IGFBP5* mRNA was detected in 4x-milked mammary tissues compared to 1x-milked mammary tissues (Table 2). In addition, a significant decrease in the relative abundance of *IGF1R* mRNA was also detected in 4x-milked mammary tissues compared to 1x-milked mammary tissues (Table 2). However, the magnitude of the change of *IGF1R* mRNA in response to MF was modest, at less than two-fold change between the treatments. These results show that in mammary glands, MF does alter the expression of some components of the IGF-I signalling pathway.

Table 4.2 Relative mRNA abundance for genes of interest in mammary tissue collected from dairy cow udder halves unilaterally milked four times a day (4x) or once a day (1x) in early lactation.

Target ¹	4x ²	1x ²	SED ³	Change ⁴	P value
<i>IGF1</i>	-2.642	-2.433	0.091	0.62	NS
<i>IGF1R</i>	-2.547	-2.422	0.053	0.75	< 0.05
<i>IGFBP3</i>	-1.615	-1.171	0.052	0.36	< 0.001
<i>IGFBP5</i>	-1.499	-1.084	0.052	0.39	< 0.001

¹ *IGF1* = insulin-like growth factor 1; *IGF1R* = IGF type I receptor; *IGFBP* = IGF binding protein

² Mean log₁₀-transformed relative mRNA abundance derived from take-off value as described in the methods.

³ Standard error of the difference (SED) between the treatment means.

⁴ Fold change in abundance derived by back-transformation of the difference of the transformed values between treatments (4x-1x).

4.4.2 The effects of milking frequency on the activation of PI3K/Akt and ERK1/2

Insulin-like growth factor-I is known to stimulate both the PI3K/Akt and ERK1/2 pathways resulting in the phosphorylation of the Akt and ERK1/2 proteins. The abundance of Akt and ERK1/2, and the abundance of the phosphorylated forms of these proteins were therefore measured in mammary tissue extracted from udder halves either milked 4x or 1x for fourteen days in early lactation. The abundance of Akt protein was similar between both treatments, whereas the abundance of the phosphorylated form of Akt (Akt-P) in 4x-milked mammary tissues was approximately one third of the abundance of Akt-P in the 1x-milked mammary tissues (Figure 1). This difference was significant, indicating that increased MF decreased Akt signalling.

The ERK antibody detected two protein bands, ERK1 (~44 kDa band) and ERK2 (~42kDa band) in the mammary tissue samples. The total protein abundance of ERK1 in 4x-milked mammary tissues was approximately one third of the abundance of ERK1 in the 1x-milked mammary tissues (Figure 1), whereas there was no difference between treatments for the protein abundance of ERK2 (Figure 1). However, there was no significant difference between the treatments in the abundance of the phosphorylated forms of either ERK1 (ERK1-P) and ERK2 (ERK2-P; Figure 1). This indicates that MF does not affect the overall activation of the ERK1/2 signalling pathway, but that it nevertheless affects ERK1 protein abundance.

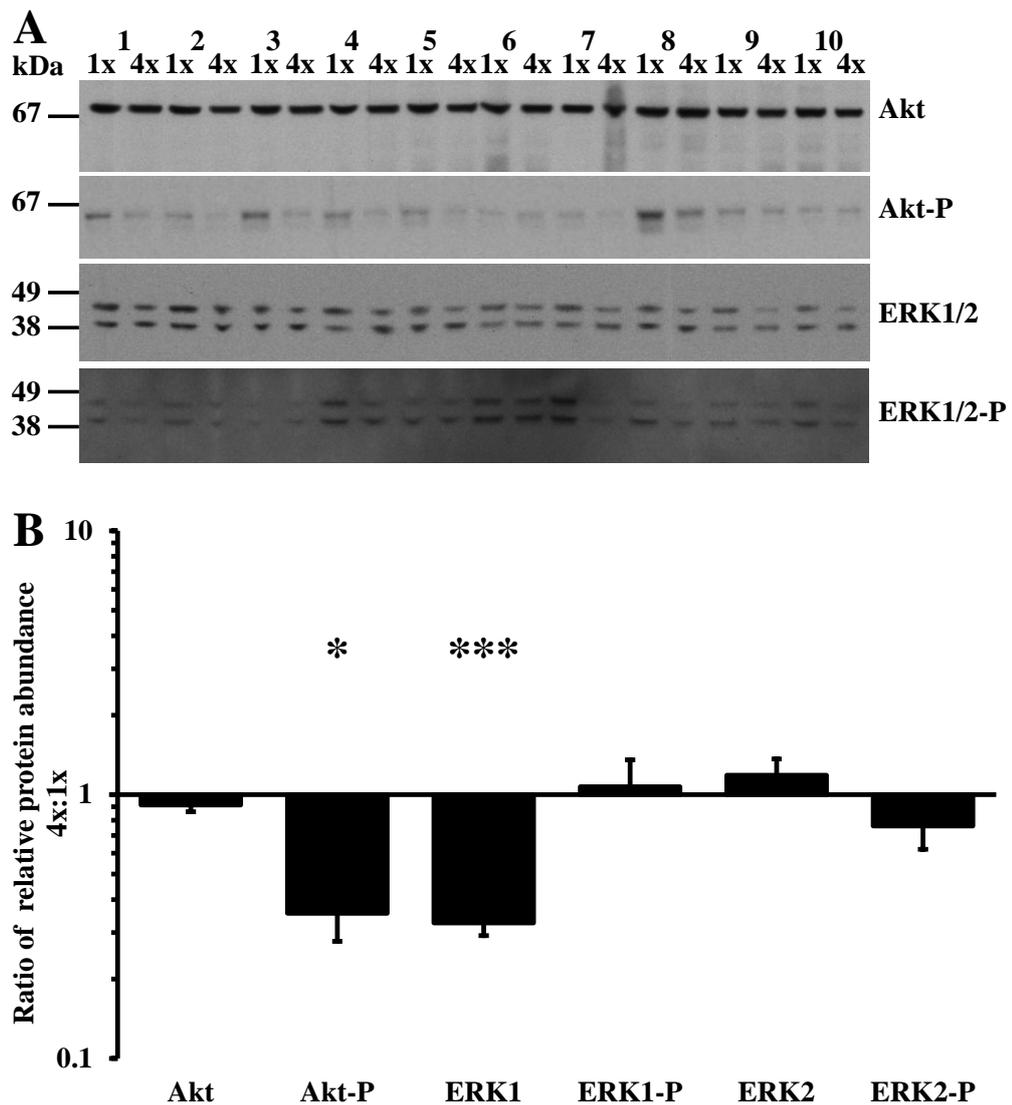


Figure 4.1 Western blot analysis of key proteins in the phosphoinositide 3-kinase/Akt and extracellular-signal-regulated kinase (ERK) signalling pathways in mammary tissue extracted from udder halves of dairy cows which had been unilaterally milked four times a day (4x) or once a day (1x) for fourteen days in early lactation. Panel A, independent western blots for Akt, phosphorylated Akt (Akt-P), ERK1/2 and phosphorylated ERK1/2 (ERK1/2-P) in total protein samples extracted from both the 4x and 1x-milked udder halves of 10 dairy cows. Panel B, Difference of the mean protein levels as determined by densitometry of Akt, Akt-P, ERK1 (~44kDa band), ERK2 (~42 kDa band), ERK1-P and ERK2-P expressed as a ratio in the 4x compared to the 1x \pm standard error of the difference, * $P \leq 0.05$ and *** $P \leq 0.001$.

4.5 Discussion

Expression of components of the IGF-I axis were measured to determine whether alteration of MF in early lactation can affect the IGF-I signalling in bovine mammary glands. The results revealed that MF can affect the IGF-I signalling pathway in mammary glands by decreasing *IGFBP3* and *IGFBP5* mRNA expression with increased MF, which should conceivably increase the bio-availability IGF-I in the 4x-milked mammary tissues.

Previous studies have shown there appears to be a window of opportunity in early lactation (the first three weeks postpartum) where short-term treatments of increased MF can elicit a long-term effect on MY (Hale et al., 2003, Wall and McFadden, 2007b, Murney et al., 2015a). Interestingly, Plath-Gabler et al. (2001) reported that this stage of lactation is when the expression of *IGFBP5* mRNA in the mammary gland is at its peak. The data from this study revealed in early lactation, 4x-milked mammary tissues had a lower abundance of *IGFBP3* and *IGFBP5* mRNA compared to 1x-milked mammary tissues. It is plausible that the drop in the IGFBPs expression could increase the bio-availability of IGF-I in the 4x-milked mammary tissues and this could be one factor contributing to the higher level of proliferation previously reported in the 4x-milked mammary tissues (Murney et al., 2015a). The idea of down regulation of IGFBPs in the mammary gland, in response to increased MF, increasing the bio-availability of IGF-I may also explain the results obtained by Prosser and Davis (1992). In the study of Prosser and Davis (1992) it was demonstrated that the lactogenic effects of close arterial infusion of IGF-I into the mammary gland in goats is not apparent if an increased MF treatment is applied to the mammary gland on the day preceding infusion. It is conceivable that the MF treatment suppressed the expression of IGFBPs and therefore increased the IGF-I bio-availability to a level close to saturation. Under these conditions, subsequent introduction of exogenous IGF-I would have little effect on milk production, as was observed (Prosser and Davis, 1992).

An increase in expression of IGFBP5 in rodent mammary glands during involution coincides with an increase in MEC apoptosis (Tonner et al., 1997). It is thought that the increase in IGFBP5 sequesters any free IGF-I and therefore inhibits the IGF-I activation of the anti-apoptotic PI3K/Akt pathway (Tonner et al., 2002). The data from this study suggest that in bovine mammary glands during early lactation, there seems to be no link between the expression of IGFBP5 and the activation of PI3K/Akt pathway. Instead the activation of Akt was independent of the abundance of IGFBP5 mRNA with Akt activation being highest in the 1x-milked mammary tissues. One possible explanation is that another as yet unidentified signal activates the PI3K/Akt pathway in response to 1x milking. Furthermore, the activation of the PI3K/Akt pathway in 1x-milked mammary tissues may explain the why no increase in apoptosis was observed in these tissues in response to 1x milking (Murney et al., 2015a).

4.6 Conclusions

The data presented suggests that the IGF-axis can be modulated by changes in MF in bovine mammary glands in early lactation. This modulation is primarily mediated by the levels of *IGFBP3* and *IGFBP5* mRNA expression which decreased in response to increased MF. This may indicate an increased bio-availability of IGF-I in the 4x-milked mammary tissues which could account for the increased proliferation and secretory activity measured in the 4x-milked mammary tissues (Murney et al., 2015a). However, it is unclear what pathway is mediating the IGF-I signal within MEC, as both ERK1/2 and PI3K/Akt pathways were not stimulated by increased MF.

The PI3K/Akt pathway activation was greater in the mammary tissues from udder halves 1x-milked, which suggests IGF-I may not be the major factor stimulating this pathway in bovine mammary glands in early lactation. However, as activation of Akt is known to be anti-apoptotic, the stimulation of

Akt activity during 1x milking may account for the lack of apoptosis that was detected in the mammary tissues following this treatment.

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Chapter V

General discussion and conclusions

Overall this study aimed to investigate the effects of short-term alteration of milking frequency (MF) during early lactation on milk yield (MY) from dairy cows and investigate the underlying intra-mammary mechanisms that potentially play a role in mediating the effect of MF.

The effect of short-term changes in MF during early lactation are two-fold, an immediate MY response that lasts as long as the treatment is applied and a long-term carry-over which continues beyond the treatment period. Both an immediate MY response and a long-term carry-over MY response were observed in this study. The effects of applying a fourteen day period during which udder halves were milked four times a day (4x), while the opposing udder half was milked once a day (1x) in a unilateral MF (UMF) experiment completed during early lactation (Chapter II; Murney et al., 2015a).

Milk yield is a function of mammary epithelial cell (MEC) number and MEC secretory activity, therefore the greater MY observed in response to 4x milking must be modulated by an increase in one or both of these factors. The initial MY response to 4x milking occurred rapidly, i.e. within two days (Chapter II; Murney et al., 2015a), suggesting the immediate MY response to 4x milking was predominantly a function of an increase in MEC activity rather than increased number of MEC, because changes in MEC number would have taken place more slowly and as such too slowly to accommodate such a large gain in MY over a two day period, therefore according to this study MEC number could only have conceivably make a relatively minor contribution to the observed MY response during the period when UMF was applied.

Secretory activity of the mammary tissue samples taken by biopsy at the end of the fourteen day period of the UMF treatment was measured by milk protein mRNA abundance, activation of signal transducer and activator of

transcription (STAT)5 and histological analysis (Chapter II and III; Murney et al., 2015a&b). Milk protein mRNA abundance, activation of STAT5 determined that the activity of mammary tissue samples from the 4x-milked were significantly greater than the 1x-milked mammary tissue samples (Chapter II and III; Murney et al., 2015a&b). Whereas from the histological analysis it was observed that phenotypic changes in the alveoli were correlated to the activation of STAT5 (Chapter III, Murney et al., 2015b) and was consistent with the hypothesis that MF modulated the proportion of alveoli in secretory and quiescent subpopulation (Vetharanim et al., 2003).

The long-term carry-over MY response to increased MF during early lactation is most likely a consequence of increases in MEC number by either an increase in the proliferation of MEC and/or a decrease in MEC apoptosis. Changes in the amount proliferation and apoptosis of MEC in the mammary tissue samples collected by biopsy at the end of the fourteen day UMF treatment were investigated (Chapter II; Murney et al., 2015a). The proliferation of MEC in the mammary tissue samples collected from udder halves milked 4x daily was observed to be significantly greater, whereas, the levels of apoptosis were observed not to be different between the 4x and 1x MF treatments. Therefore, there could be an overall increase in the number of MEC in the mammary tissue collected from udder halves milked 4x. Although the increase in MEC proliferation measured in this study was relatively small, should this difference in proliferation be applied across the whole treatment period it would be expected that the greater proliferation could account for the observed increase in MY from the udder halves milked 4x during the post MF treatment period (Chapter II; Murney et al., 2015a).

Another objective of this study was to investigate the effects of 4x and 1x MF on prolactin (PRL) and insulin-like growth factor I (IGF-I) signalling pathways and to determine whether these pathways are involved in the change in MEC secretory activity and MEC number in response to differing MF (Chapter III

and IV; Murney et al., 2015b&c). In 4x-milked mammary tissue samples, the activation of STAT5, which is the predominant transcription activator stimulated by the PRL pathway, was significantly greater than the level of activation in response to 1x-milked mammary tissue samples (Chapter III; Murney et al., 2015b). The UMF model utilised in this experiment was used to have 4x and 1x MF treatments being applied concurrently to the same cows, thus both treatments would have been exposed to the same levels of systemic PRL. Therefore, any difference in the activation state of the PRL pathway between the 4x and 1x must be due to intra-mammary differences as a consequence of UMF treatments. Molecular analysis of the mammary tissue samples revealed that the protein abundance of β 1-integrin was highly correlative with the activation level of STAT5 activation, suggesting a link between STAT5 activation and β 1-integrin. β 1-integrin is essential for proper mammary gland development and function in mice (Naylor et al., 2005) and a lack of the extracellular matrix (ECM) signal via β 1-integrin has been shown to inhibit activation of STAT5 in mice mammary glands (Akhtar and Streuli, 2006). Therefore, the interaction of β 1-integrin with ECM appears to be necessary for activation of STAT5 in mice mammary glands and the results presented here suggest that it may also be the case for bovine mammary glands. How the protein abundance of β 1-integrin is controlled remains unknown, but it is most likely due to post-translational degradation, because the β 1-integrin mRNA abundance was lower in tissue samples collected from udder halves milked 4x daily compared to those milked 1x daily (Chapter III; Murney et al., 2015

Within the MEC it appears that activation of the PRL signalling pathway requires a signal from the ECM via β 1-integrin. It is plausible that this ECM signal may be like a switch and is required to convert quiescent MEC into actively secreting MEC. One possible mechanism may occur when localised accumulation of milk, which would occur more predominantly in 1x-milked mammary glands, could modulate the composition of the ECM that the MEC are surrounded, either through changing the expression of the ECM

components or the expression of ECM remodelling proteases. Remodelling of the ECM may then decrease the availability of β 1-integrin binding motifs which would decrease the amount of β 1-integrin which is bound to the ECM. Unbound membrane proteins are more likely to be recycled through the cells protein recycling proteases and therefore an overall decrease in β 1-integrin protein abundance would be observed. Without the β 1-integrin/ECM signal the MEC would not be able to be activated by PRL and therefore would be in a quiescent state. Once the localised milk accumulation was rectified the ECM could be returned to a milk synthesis conducive conformation with a higher level of β 1-integrin binding motifs available. A higher level of β 1-integrin binding would increase the overall β 1-integrin protein abundance and the β 1-integrin/ECM signal. Which would then allow for the activation of the STAT5 signalling pathway by PRL. This type of mechanism would be controlled at the alveolar level and could be rapidly switched as local conditions change. The mechanics of how this signal is controlled are unknown and it remains a topic for future study.

The mRNA abundance of IGF-I binding proteins (IGFBP)3 and IGFBP5 were lower in the mammary tissue samples collected from 4x compared with 1x-milked udder halves (Chapter IV; Murney et al., 2015c). Expression of IGFBPs has been shown to block the effects of IGF-I in rodent mammary glands (Tonner et al., 1997, Flint et al., 2005), therefore, a decrease in their mRNA abundance may indicate a greater bio-availability of IGF-I in the bovine mammary (Chapter IV; Murney et al., 2015c). However, these changes in the mRNA abundance of IGF axis components did not affect the activation of the phosphoinositide 3-kinase (PI3K)/Akt and the extracellular-signal-regulated kinase (ERK)1/2 pathways, which are thought to be the major effector pathways downstream from IGF-I. The activation of Akt was lower in the mammary tissue samples from 4x milked udder halves compared to 1x milked halves and there was no difference in the activation state of ERK1/2 (Chapter IV; Murney et al., 2015c). These results suggest that either the expression of IGFBP3 and IGFBP5 in bovine mammary

glands during early lactation has no effect the IGF-I signalling pathway which contrasts what has been seen in rodent models (Flint et al., 2005), or the IGF-I signal is routed through alternative pathways in bovine mammary glands during early lactation, therefore bypassing PI3K/Akt and ERK1/2. Should this be the case then the alternate pathway maybe an important focus for future research.

The greater activation of Akt observed in mammary tissue samples collected from 1x-milked udder halves may have been stimulated by another factor, because Akt is a central node with multiple signals feeding into it. Activation of Akt is known to be a survival signal and therefore this greater activation found in mammary tissue samples from 1x-milked udder halves may be the reason for which MEC apoptosis levels were not affected (Chapter II; Murney et al., 2015a).

Finally, the levels of activation for Akt and ERK1/2 detected in the mammary tissue samples from this study were not positively affected by 4x milking (Chapter IV; Murney et al., 2015c). Therefore, this suggests that these pathways are not involved in the cellular response by the mammary gland to greater MF.

Conclusions

Milking of udder halves 4x and 1x daily elicited both immediate and a long-term carry-over MY responses, which were associated with MEC secretory activity and cell proliferation. Secretory activity seemed to be controlled at the alveolar level, with the proportion of actively lactating and quiescent alveoli, being modulated by changes in MF. Activation of STAT5 in MEC was involved in the MY response and this was linked to the protein abundance of β 1-integrin, suggesting ECM signalling via β 1-integrin is necessary for MEC

to be secretory active. The mRNA abundance of IGFBP3 and IGFBP5 were affected by MF, however these changes did not correspond to a modulation of the activation state of Akt and ERK1/2. This suggests that the PI3K/Akt and ERK1/2 signalling pathways are not involved in the MY response by the mammary gland to MF, however the PI3K/Akt pathway may be involved by exerting a pro-survival signal in mammary tissue samples collected from 1x-milked glands and in the inhibiting greater rates of apoptosis.

As a whole the results obtained in this thesis have increased the understanding of the changes in intra-mammary molecular mechanisms in response to differing MF. It is possible that these molecular mechanisms can be modulated by other means and therefore may represent a novel process for increasing MY in dairy cows without having to alter MF or increase the MY of cows milked 1x.

Chapter VI

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