

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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

A thesis presented in partial fulfilment of the requirements
for the degree of

Doctor of Philosophy

in

Animal Science

at Massey University

Palmerston North

New Zealand



Regan James Murney

2015

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.

Acknowledgements

I would like to thank my supervisors Dr Jean Margerison, Dr Kuljeet Singh, Dr Kerst Stelwagen and Dr Thomas Wheeler for all their support and advice during all stages of this project.

I would also like to thank all the members of what was the AgResearch Lactation Biology Team, past and present: Adrian, Chris, Eric, Gagan, Jo, Juliane, Kara, Kim and Shona, for all their support and assistance through this journey. In addition I would also like to thank the wider residents of the Dairy Science Building who have contributed to making the building an interesting and exciting place to work.

I would like to thank the farm staff and the AgResearch Tokanui research farm for all their hard work preparing cows and assistance during the animal trial.

Finally I would also like to thank AgResearch for giving me the opportunity to attempt this endeavour.

This study was funded as part of the MSI programme [CX10X0702] entitled "Future Proofing of the NZ Dairy Cow".

Publications arising from this thesis

Murney, R., K. Stelwagen, T. T. Wheeler, J. K. Margerison, and K. Singh. 2015. The effects of milking frequency on insulin-like growth factor I signaling within the mammary gland of dairy cows. *Journal of Dairy Science*. 98:5422–5428. DOI: 10.3168/jds.2015-9425

Murney, R., K. Stelwagen, T. T. Wheeler, J. K. Margerison, and K. Singh. 2015. Activation of signal transducer and activator of transcription 5 (STAT5) is linked to β 1-integrin protein abundance in unilaterally milked bovine mammary glands *Journal of Dairy Science*. 98:3133-3142. DOI: <http://dx.doi.org/10.3168/jds.2014-9003>

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. *Journal of Dairy Science*. 98:305-311. DOI: <http://dx.doi.org/10.3168/jds.2014-8745>

Murney R, Stelwagen K, Wheeler TT, Margerison JK, Singh K 2013. Effects of milking frequency on integrin signaling in mammary glands of dairy cows. *Journal of Dairy Science* 96 e-suppl. 1: 151.

Murney R, Stelwagen K, Singh K 2012. BRIEF COMMUNICATION: The effect of milking frequency in early lactation on milk yield and milk protein gene expression in the bovine mammary gland. *Proceedings of the New Zealand Society of Animal Production* 72: 67-69.

Murney, R., K. Stelwagen, T. T. Wheeler, J. K. Margerison and K. Singh. 2012. Effects of milking frequency on integrin signalling in mammary glands of dairy cows. *Queenstown Molecular Biology Conference, Queenstown, New Zealand.*

Murney, R., K. Stelwagen and K. Singh. 2011. The effect of short-term milking frequency during early lactation on milk yield and mammary IGF signalling in dairy cows. 8th International Symposium on Milk Genomics and Human Health, Melbourne, Australia.

Table of contents

Abstract	i
Acknowledgements	iii
Publications arising from this thesis	iv
Table of contents	vi
List of figures	xi
List of tables	xiii
Abbreviations	xiv
Chapter I: Review of literature	1
1.1 Introduction	2
1.2 Bovine mammary gland	3
1.2.1 Functional anatomy	3
1.2.2 Mammogenesis	4
1.2.3 Lactogenesis	5
1.2.4 Galactopoiesis	5
1.2.5 Cessation of milking and mammary gland involution	6
1.2.6 Heterogeneity of mammary alveoli tissue	7
1.3 Milking frequency	7
1.3.1 Effects of milking frequency on milk yield and composition	7
1.3.2 Long term effects of short term changes in milking frequency during early lactation	10
1.3.3 Unilateral milking frequency experiments	12

1.4 Prolactin and its role in the response of the mammary gland to changes in milking frequency .13	
1.4.1 Prolactin signalling pathway in mammary epithelial cells.....13	
1.4.2 Effect of prolactin on the mammary gland14	
1.4.3 Other pathways which may interact with prolactin signalling pathway15	
1.4.4 The effects of milking frequency and role of prolactin17	
1.5 Insulin-like growth factor I and its role in the mammary gland response to milking frequency18	
1.5.1 Insulin-like growth factor I axis18	
1.5.2 Insulin-like growth factor I signalling in mammary epithelial cells19	
1.5.3 Insulin-like growth factor I effects on mammary function21	
1.5.4 Insulin-like growth factor I and milking frequency22	
1.6 Other factors that control milk production within the mammary gland in response to milking frequency23	
1.6.1 Growth hormone.....23	
1.6.2 Feedback inhibition of lactation.....24	
1.6.3 Mammary epithelial cell tight junction permeability.....24	
1.7 Objectives of this study25	
Chapter II: The effects of milking frequency in early lactation on milk yield, mammary cell turnover and secretory activity in grazing dairy cows.....27	
2.1 Abstract28	

2.2 Introduction.....	29
2.3 Materials and methods.....	31
2.3.1 Animals and treatments.....	31
2.3.2 Half udder milk yield and composition data.....	32
2.3.3 Cell proliferation.....	32
2.3.4 RNA isolation and reverse transcription.....	33
2.3.5 Statistical analysis.....	36
2.4 Results.....	36
2.5 Discussion.....	40
2.6 Conclusions.....	45
2.7 Acknowledgements.....	45
Chapter III: STAT5 activation is linked to β1-integrin protein abundance in unilaterally milked bovine mammary glands.....	46
3.1 Abstract.....	47
3.2 Introduction.....	48
3.3 Materials and methods.....	50
3.3.1 Animals and treatments.....	50
3.3.2 RNA isolation and reverse transcription.....	51
3.3.3 Protein extraction and western blotting.....	52
3.3.4 Histological analysis.....	53
3.3.5 Statistical analysis.....	54

3.4 Results	56
3.4.1 Changes in prolactin signalling pathway in response to milking frequency.....	56
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	61
3.4.3 Effects of milking frequency on mammary alveoli phenotype and correlation with STAT5 activation and β 1-integrin.....	61
3.5 Discussion	65
3.6 Conclusions	67
3.7 Acknowledgements	68
Chapter IV: The effects of milking frequency on insulin-like growth factor I signalling within the mammary gland of dairy cows	69
4.1 Abstract	70
4.2 Introduction	71
4.3 Materials and methods	74
4.3.1 Animals and treatments.....	74
4.3.2 RNA isolation and reverse transcription	75
4.3.3 Protein extraction and western blotting	78
4.3.4 Statistical analysis	79
4.4 Results	79
4.4.1 The effects of milking frequency on genes of the IGF-I axis	79

4.4.2 The effects of milking frequency on the activation of PI3K/Akt and ERK1/2.....	80
4.5 Discussion.....	83
4.6 Conclusions	84
4.7 Acknowledgements	85
Chapter V: General discussion and conclusions	86
Conclusions	91
Chapter VI: References	93

List of figures

Figure 1.1 Structure of alveoli and ductal system of the bovine mammary gland	4
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	16
Figure 1.3 Schematic diagram of the insulin-like growth factor I (IGF-I) signalling pathways in mammary epithelial cells	20
Figure 2.1 Mean milk yield (MY) of udder halves for cows unilaterally milked for fourteen day in early lactation.....	39
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)	41
Figure 2.3 Difference in mRNA abundance for milk protein genes α S1-casein (<i>CSN1S1</i>), β -casein (<i>CSN2</i>), α -lactalbumin (<i>LALBA</i>), β -lactoglobulin (<i>LGB</i>), and lactoferrin (<i>LTF</i>) 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)	42
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.....	58-59

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, STAT5-P protein abundance and β 1-integrin protein abundance, STAT5-P protein abundance and phosphorylated STAT3 (STAT3-P) protein abundance, STAT5-P protein abundance and proportion of alveoli exhibiting the “lactating” phenotype, β 1-integrin protein abundance and proportion of alveoli exhibiting the “lactating” phenotype, and STAT3-P protein abundance and proportion of alveoli exhibiting the “lactating” phenotype in mammary tissue extracted from udder halves of dairy cows which had been subjected to differential milking for either four times a day (4x) or once a day (1x) for fourteen days in early lactation60

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..... 63-64

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 lactation82

List of tables

Table 1.1 Studies demonstrating the effect of milking frequency during different stages of lactation on milk yield in dairy cows.....	9
Table 1.2 Studies demonstrating the effect of short-term changes of milking frequency in early lactation on milk yield in dairy cows.....	11
Table 2.1 Primers used for real time PCR.....	35
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 frequency	38
Table 3.1 Primers used for real time PCR.....	55
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.....	57
Table 4.1 Primers used for real time PCR.....	77
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.....	80

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