Mechanistic target of rapamycin (mTOR) activation during ruminant mammary development and function

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This thesis is dedicated to my daughter

Caitlyn Grace Sciascia

“You lift me when all else fails”

Whāia te iti kahurangi - Ki te tūohu koe, me he maunga teitei

Pursue excellence – should you stumble, let it be to a lofty mountain

ABSTRACT

This thesis examines the abundance of total and activated mechanistic target of rapamycin (mTOR) pathway components in the developing and functional ruminant mammary gland. mTOR pathway activation is stimulated by a wide range of intra- and extracellular signals, such as amino acids (AA) and hormones, making the mTOR pathway a potential candidate for the development of intervention strategies designed to increase ruminant lactation potential.

Tissues from two trials shown to improve lactation potential; dam-fetal nutrition and exogenous growth hormone (GH) administration during lactation, were used to measure changes in total and activated mTOR pathway protein abundance. Results show mammary glands of d 140 fetal lambs carried by maintenance fed dams and dairy cows administered exogenous GH, had increased abundance of total and activated mTOR and mitogen activated protein kinase (MAPK) pathway proteins. Increased abundance was associated with changes in biochemical indices. In the GH study MAPK pathway activation was stimulated by IGF-1 signaling whilst mTOR pathway activation was proposed to be mediated by AA signalling. Data from the GH study shows, L-arginine a known activator of the mTOR pathway, was the only AA reduced in both plasma and the lactating gland. Upstream factors were not identified for the phenotype observed in the dam-fetal nutrition study, but similar mechanisms were proposed.

To elucidate the potential regulation of mTOR pathway activation by L-arginine and examine the effect on milk production, *in vitro* bovine cell culture models were evaluated.

Results show that none of the models evaluated produced a lactating phenotype – a pre-requisite to accurately study the lactating gland *in vitro*.

Finally, this thesis shows L-arginine administration from d 100 to d 140 of pregnancy, in twin bearing ewes had no effect on mTOR protein abundance or activation. However, administration from d 100 to parturition improved maternal gland health.

In summary, this thesis associates improved lactation potential with increased total and activated mTOR pathway protein abundance, and the administration of L-arginine during late gestation with improved gland health. These findings provide fundamental knowledge that may lead to the development of novel technologies to increase ruminant gland performance and health.

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To my fellow Ph.D. students "***He waka eke noa***"

Last but not least to my whanau.

I could not have made it this far without your love, dedication and emotional support. ***Aroha nui***

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

4EBP1	Eukaryotic initiation factor 4E (eIF4E)-binding protein 1
4E-SE	Eukaryotic initiation factor 4E (eIF4E)-sensitivity element
AA	Amino acids
AAT	Amino acid transporter
BCAA	Branched-chain amino acids
DNA	Deoxyribonucleic acid
EAA	Essential amino acid
eIF4E	Eukaryotic initiation factor 4E
eIF4G	Eukaryotic initiation factor 4G
FAA	Free amino acid
GH	Growth hormone
IGF	Insulin-like growth factor
IGF1	Insulin-like factor 1
IGF1r	Insulin-like factor 1 receptor
IGFBP3	Insulin-like factor 1 binding protein 3
IGFBP5	Insulin-like factor 1 binding protein 5
NEAA	Non-essential amino acid
MAPK	Mitogen activated protein kinase
MKNK1	Mitogen activated protein kinase (MAPK) interacting serine/threonine kinase 1
mLST8	Mammalian lethal with SEC13 protein 8
mRNA	Messenger ribonucleic acid

mSin1	Mammalian stress-activated protein kinase (SAPK)-interacting protein 1
mTOR	Mechanistic target of rapamycin
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
PI3K	Phosphoinositide-3-kinase
Pl	Placental lactogen
PRAS40	Proline-Rich Akt Substrate, 40 KDa
Prl	Prolactin
Raptor	Regulatory associated protein of mechanistic target of rapamycin (mTOR), complex 1.
Ras	Rat sarcoma (Protein family)
Rictor	Regulatory associated protein of mechanistic target of rapamycin (mTOR), complex 1 (RPTOR) independent companion of mTOR, complex 2
RPS6	Ribosomal protein S6
RPS6KA1	Ribosomal protein S6 kinase A1
rRNA	Ribosomal ribonucleic acid
S6K1 / S6K2	Ribosomal protein S6 kinase 1 / ribosomal protein S6 kinase 2
TSC1/TSC2	Tuberous sclerosis 1 / tuberous sclerosis 2

GENERAL INTRODUCTION

Ruminant milk production is a cornerstone of the New Zealand economy, earning in 2010 close to $15.6-billion per annum in foreign currency. In addition, the dairy industry employs locally, almost seventy-five thousand people.

The nutritional importance of milk has seen global demand rise, creating an opportunity for New Zealand dairy farmers to sell more milk, but simultaneously placing pressure on farmers to make constant productivity increases. Productivity increases that are being met through the application of novel environmental, genetic and nutritional intervention strategies. Environmental (soil maintenance, grazing intensity, water management, the introduction of new pasture species / cultivars) and genetic (breed selection, cross-breeding) advances have traditionally focused on the whole animal, whilst nutritional strategies have focused on provision of an adequate nutrient supply to the mammary gland, in an effort to increase milk production.

The ruminant mammary gland goes through very distinct developmental stages, *fetal, pre-pubertal, post-pubertal, pregnancy and lactation* with each stage susceptible to nutrient manipulation that can negatively or positively influence lactation potential. Traditionally, nutrition studies in the dairy industry have focused on plane of nutrition (high, low, and stair-step), however, results have been inconsistent, with factors such as species, age of intervention and diet composition producing conflicting results. Current research shows supplementation with specific nutrients, such as amino acids (AA), at crucial stages of

development (pregnancy, gestation, lactation), may play an important role in helping the mammary gland reach full production potential.

Amino acid uptake studies using arterio-venous differences across the lactating ruminant mammary gland show valine, leucine, arginine, lysine, and threonine are extracted in excess of milk protein outputs indicating possible roles in maintaining lactation. Arginine supplementation studies with late-pregnant Holstein cows increased milk yield, whilst the arginine-free diets retards mammary gland growth in rats. Glutamine is proposed to be limiting for milk protein synthesis due to three unique factors: uptake by the mammary gland is close to 99% of the arterial supply, high levels of glutamate are synthesised from glutamine and both glutamine, and glutamate are the most abundant AAs found in milk proteins. Arginine and glutamine belong to the arginine-family of AA (arginine, leucine and glutamine), long known to be important regulators of protein synthesis in multiple organisms. Studies show the arginine-family of AA enhance lactation potential, via possible direct or indirect stimulation of the nutrient-sensing mechanistic target of rapamycin (mTOR) pathway and reciprocal control of amino acid transporter (AAT) activity.

The mTOR pathway integrates multiple environmental signals and metabolic pathways to regulate protein synthesis, cellular proliferation and development via phosphorylation of downstream targets involved in mRNA translation and nucleocytoplasmic export, cell size, rRNA and gene transcription. The mTOR pathway may be a rate-limiting step in the cellular development and function of the ruminant mammary gland, and the ability to enhance

mTOR signalling via AA may provide a novel intervention to enhance the production of milk in ruminants.

The main objectives of this thesis were to understand the mTOR pathways role in the development and function of the ruminant mammary gland in response to external stimuli including nutrients and hormones, and to identify the potential role AAs play in stimulating mammary gland development and function through mTOR pathway signalling. The second objective was to develop a chronic *in vitro* model of bovine lactation to study the molecular mechanisms employed by AAs to regulate mTOR pathway signalling and the reciprocal control of AAT function.

Knowledge gained from this research could potentially contribute to the development of future nutritional intervention strategies designed to enhance milk protein yield, with potential economic benefits to the dairy industry.

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

In this chapter, a summary of current literature regarding the regulation of ruminant mammary gland development and milk production will be presented. In particular, focus will be given to the role of mechanistic target of rapamycin (mTOR) signalling pathway, amino acids (AA) used as regulators of mTOR signalling and reciprocal control of AA transporters (AAT) due to the nature of the research conducted in subsequent chapters.

1.2 Milk production in New Zealand

1.2.1 Economic significance

The production of ruminant milk has become a cornerstone of the New Zealand economy. In 2010, the New Zealand dairy industry accounted for almost one-third of cross-border trade, earned $15.6-billion dollars in total revenue and employed locally nearly seventy-five thousand people (http://www.mpi.govt.nz).

As price and global demand for milk products from ruminants continues to rise, New Zealand dairy farmers are under increasing pressure to make constant productivity increases, through the application of novel production strategies. These production strategies can be broadly classed into three groups, environmental (1, 2), genetic (3) and nutritional (4).

1.2.2 Production strategies

1.2.2.1 Environmental

Environmental strategies encompass on-farm activities which directly support livestock productivity gains. These include factors such as soil maintenance, grazing intensity, effluent and water management, which have become increasingly important as stocking rates and conversions to dairy production continue to rise (1). Efficient management of these factors is now possible through integrated systems developed by on-farm dairy partners such as DairyNZ (http://www.dairynz.co.nz/).

1.2.2.2 Feed

New Zealand dairy livestock are predominately grass/forage fed with hay and silage cut in summer, stored and then used as supplement feed over winter and in dry summers. This provides a low input system that can be easily targeted for productivity gains, through the introduction of new pasture species and cultivars (5). Research has been on-going to establish forage species with greater persistence (5), yield, ability to grow in a variety of soil environments (6), and disease/pest resistance.

1.2.2.3 Genetics

Breed selection and cross-breeding were the two earliest forms of genetic management, with traits such as reproductive performance, superior milking and growth rate used as indicators and selection criteria (7). Technological advancements have taken genetic management down to the molecular level, where individual animals or whole herds can be screened for genes encoding desirable traits (7, 8). Research has also begun to focus on

unravelling the complex signalling pathways, interactions and activation factors involved in the growth and development of livestock or economically important tissues, in particular, their function in the ruminant mammary gland (9, 10).

1.3 Nutritional and health significance of ruminant milk

1.3.1 Humans

Humans are the only species to consume milk sourced from other species throughout all stages of development. Ruminants provide over 90% of the milk consumed by humans mainly due to their ease of domestication, scale of production and because milk sourced from ruminants provides many of the essential factors necessary to support healthy growth and development throughout the human lifecycle (11, 12).

Milk is also viewed as a functional food i.e. possessing the ability to positively impact the maintenance of human health and diseases prevention. Milk fat components (fatty acids) have been extensively studied and shown to possess anti -bacterial, -viral and -cancer properties (13-15), whilst milk proteins are an essential source of bioactive proteins and peptides that can regulate immune function and blood pressure (16).

1.3.2 Ruminant neonates

At birth, the digestive functions of the ruminant neonate resemble that of a monogastric animal, and as such, the ruminant neonate cannot partake of an adult ruminant diet. To survive, ruminant neonates need to quickly access milk to maintain body temperature and

survive (17). During the first few weeks of life milk supplied by the mother is the major nutrient source. Variations in composition and yield of maternally supplied milk have a direct effect upon neonate survival and future production performance (18). Milk protein provides essential amino acids (EAA) and peptides required for organ growth and development, and the maintenance of cellular functions, while milk fats and lactose are important sources of energy (19).

In ruminants there is no exchange of immune factors *in utero* thus an important function of milk, especially the colostrum stage, is acquisition of passive immunity from the mother to the ruminant neonate (20). Colostrum has a high immunoglobulin content and proteins which have anti- bacterial and –microbial functions. In addition, colostrum contains hormones, growth factors, cytokines, enzymes, polyamines and nucleotides, which aid development of the ruminant neonate and exert protective functions.

1.4 Mammary gland development and function – Physiology

1.4.1 Fetal

Through the very early stages of mammary development the nascent gland goes through many distinct, well defined morphological changes, that rely on signalling between the epithelium and the mesenchyme to lay down the framework for future specialised secretory cells to proliferate (21, 22). Hand in hand with these changes, first signs of the essential ancillary structures (i.e. blood vessels, nerves, lymphatics, myoepithelial cells and connective tissue) appear (23). Adipose tissue begins to develop at the same time (23).

This forms the mammary fat pad which is essential for mammary epithelium proliferation and differentiation. It also provides the necessary space, support and local control for duct elongation and, ultimately, lobulo-alveolar proliferation (23).

1.4.2 Neonatal

During the neonatal developmental stage the gland continues the ordered extension of the duct system within the fat pad whilst increasing growth of the adipose and connective tissue. Growth of the fat pad occurs, primarily as a result of hypertrophy (increasing cell size) rather than hyperplasia (increasing cell number). Although the level of mammary growth during this developmental stage is considered low in comparison to some stages, research shows that it is a key point in regulating future mammary output (24, 25).

1.4.3 Pregnancy

During pregnancy, the majority of mammary growth occurs, driven by the simultaneous elevation of blood concentrations of estrogen and progesterone, though nutrition has been shown to play a role (26). This is a continuous, exponential process from conception to parturition, with the greatest increase in parenchymal mass occurring in late pregnancy (27-29). Differentiation of mammary epithelial cells completes and the components of milk protein begin to be synthesised. Towards the end of pregnancy (~final month), alveoli secretory activities begins, increasing udder size due to the accumulation of the secretory material in preparation for the onset of lactation (30).

1.4.4 Lactation

Growth of the mammary gland continues in early lactation until the peak of lactation, and only occurs because the exponential growth during pregnancy cannot be abruptly stopped (31). This period of growth accounts for less than 10 percent of total mammary development, and is the time when the rate of secretory cells loss exceeds the rate of cell growth and as a result, the gland contains more secretory cells at the beginning than at the end of lactation (32).

Pregnancy also marks the end of lactogenesis, a co-ordinated differentiation process where mammary cells switch from a non-secretory to secretory state (33). Copious secretion of all milk products begins approximately 0 to 4 days before parturition and extends through to a few days postpartum. The galactopoiesis stage takes over and continues the process of milk secretion initiated by lactogenesis. Lactogenesis is hormonally regulated and stimulation involves prolactin, growth hormone, adrenocorticotropin (via stimulation of secretion of glucocorticoids), and oestrogens (9). It is negatively regulated by progesterone (34).

Crucial elements of lactation are peak milk yield, which is the maximum daily yield reached during lactation, and lactation persistency, which is the average rate of milk yield decrease after the lactation peak. Increasing peak milk yield and lactation persistency depends on the number of secretory cells, their production capacity and their continual survival (33). Many studies influencing peak milk yield and lactation persistency have been conducted, and have identified genetics (35), stem cell number (33), hormonal status and

administration (9), udder morphology, seasonal changes, management, animal health (e.g. mastitis), stress and nutrition (36) as factors that can influence this process.

1.5 Models to study mammary gland development and function

Mammary gland development and function is a complex program of cell proliferation, differentiation, and morphogenesis that is regulated by the nutritional environment and the interactions of multiple hormones (23, 37). Two well established models used to study mammary gland development and function in ruminants are hormonal and nutritional intervention. Whilst other treatment models are employed by researchers, the focus of the proceeding section will be on those utilised in the experiments outlined in this thesis.

1.5.1 Growth hormone

Growth hormone (GH), along with Prolactin (Prl) and placental lactogen (Pl) are structurally related members of the somatotrophin family secreted by the anterior pituitary gland. In ruminants, Prl and Pl, in co-ordination with GH are crucial for the initiation of lactogenesis, but only play limited roles in the maintenance of lactation. Instead, GH is considered to be the primary galactopoietic hormone, with Prl a minor partner, in contrast to other species where the opposite appears true. To date, of the somatotrophins, only GH has an identified role during embryonic and pre-pubertal mammogenesis (38).

Early studies using pituitary extracts, and later, purified and recombinant GH showed exogenous administration dramatically increased milk yield and lactation persistency in lactating dairy cows (39). Subsequent studies in sheep and goats confirmed the galactopoietic effect of exogenously administered GH in ruminants, and today GH is one of the most established treatment models used by researchers to study mechanisms involved in the regulation milk yield and composition by the lactating ruminant mammary gland (40). Whilst the galactopoietic effect of GH has been well investigated; the cell signalling mechanisms that mediate its action on the mammary gland are not fully understood (39).

In vitro models utilising bovine mammary epithelial cells suggest GH may signal directly to mammary epithelial cells via the growth hormone receptor, through pathways linked to protein synthesis and cellular proliferation (41). Mammary explant studies show GH alone increases cell survival by suppressing expression of the pro-apoptosis gene insulin-like growth factor-binding protein 5 (42). Evidence from *in vivo* studies postulate that GH can also act indirectly on the mammary gland via stimulation of liver insulin-like growth factor 1 (IGF1) secretion (37). IGF1 belongs to the IGF family of mitogens, which play a central role in regulating cell proliferation, differentiation, and apoptosis (43). Both GH and IGF1 increase epithelial cell proliferation and/or secretory activity in the lactating mammary gland (37) indicating the involvement of overlapping signalling pathways regulating cell turnover and renewal, RNA transcription and protein synthesis. A common signalling pathway shared by GH (44) and IGF1 (45) is the mechanistic target of rapamycin (mTOR). The mTOR pathway integrates mitogenic signalling to control factors involved in protein synthesis, cellular differentiation and proliferation.

1.5.2 Nutrition

1.5.2.1 Plane of nutrition

Ruminant mammary gland development studies have traditionally focused on plane of nutrition (low, high and stair-step) and the subsequent effect on lactation performance (46, 47). A low plane of ewe nutrition from d 21 – d 140 of pregnancy increases first-lactation milk production of their offspring (36). A low plane of nutrition from d 98 - parturition negatively impacts ewe milk yield and composition (48). A high plane of ewe nutrition from d 70 – d 140 of pregnancy increases milk yield, in early lactation (49). Prepubertal heifers on an *ad libitum* plane of nutrition have reduced parenchyma and hyperplasia, but increased fat pad (4). A high plane of nutrition reduces subsequent milk yield in prepubertal Holstein heifers (50).

During the neonatal stage of ruminant growth, increasing the plane of nutrition hastens puberty, decreases both the percentage of proliferating mammary epithelial cells and the mass of fat-free mammary parenchyma per unit of carcass; leading to reduced mammary parenchymal mass at puberty (51). Compensatory growth regimes (stair-step nutrition) started in the pre-pubertal stage and ending just prior to parturition, are regularly employed to increase lactation performance (52-55). The stair-step nutrition regimen is a growth plane with a combination of alternating dietary energy restriction and re-alimentation phases. It has been designed to reduce energy levels when dams do not require it and to increase energy input during phases of high requirement (54). Studies show that mean daily milk yield across multiple lactation cycles is significantly increased

and growth efficiencies are improved (less feed consumption) (52, 54), and that the response is associated with elevated levels of plasma GH (46).

1.5.2.2 Amino Acids

The original concept behind the use of amino acids (AA)s to enhance milk production was the "limiting amino acid" hypothesis (56). It was proposed that as the mammary gland approaches peak lactation a specific AA (first-limiting) or set of AA (co-limiting) becomes limiting as a direct substrate for milk protein synthesis or incorporation into the Krebs cycle for the production of other AAs, thus preventing milk production from increasing further. Studies based on the limiting or co-limiting AA hypothesis have included AAs such as glutamine, lysine and methionine (56). Glutamine was identified because ruminants have low plasma glutamine levels compared to monogastrics and uptake of glutamine by the mammary gland is effectively 100% of the arterial supply (57). Lysine is taken up in excess of requirements for milk protein synthesis and is limiting in corn based diets used widely in the US dairy industry (58). Methionine has been the focus of a large number of studies because it is an EAA that cannot be adequately supplied by rumen microbial protein synthesis (59). Attempts to increase milk protein yield by increasing the supply of glutamine, lysine or methionine, alone or in combination have given inconsistent results (60, 61). This suggests that glutamine, lysine and/or methionine are not limiting AA, or there is another concept being overlooked.

One thing the aforementioned studies have in common is that they focused on AAs as a nutritional component and their potential limitation as a substrate. It has now been shown

that AA play a much wider role than just as an intracellular substrate. AAs are involved in the regulation of tissue development and function by blocking protein degradation (62), activating metabolic pathways (63) and/or acting as efflux agents for the influx of critical AAs (64). Lactation research has now begun to focus on arginine as a potential signalling molecule to enhance mammary gland development and function (65).

Arginine is a nutritionally essential amino acid for embryonic development in mammals (65). In addition to being a building block for proteins, arginine is the precursor for synthesis of many biologically active molecules, such as polyamines and nitric oxide (NO) (66, 67). Polyamine (putrescine, spermidine and spermine) synthesis supports a large number of cellular processes, such as DNA stabilization (68), cell growth, proliferation, differentiation (69) and membrane stability (70). Nitric oxide production stimulates cell proliferation and migration, cellular remodelling, angiogenesis, and dilation of blood vessels to increase blood flow (65)

In ruminants, arginine is critical for embryonic growth and development. *In vivo* studies show arginine stimulates the proliferation and migration of ovine trophectoderm cells by enhancing the production of polyamines and NO (71). *In vitro* studies of ovine intrauterine growth restriction (IUGR) in multiple bearing ewes show parenteral administration of arginine increases the percentage of lambs born alive and enhances the birth weights of quadruplets (72) . Studies show L-arginine enhances fetal development and lactation potential, via possible stimulation of the nutrient-sensing mechanistic target of rapamycin (mTOR) pathway (64, 73). mTOR signalling increases the survival and proliferation of stem

cells and secretory cells (74), regulates mRNA translation, autophagy, ribosome biogenesis and gene transcription (75-77).

1.6 The mechanistic target of rapamycin (mTOR) pathway

1.6.1 A brief history of mTOR

The mechanistic target of rapamycin (mTOR) pathway could be described as the most studied yet least understood molecular checkpoint in molecular biology. The core protein of the mTOR pathway; mTOR, was identified during a screen for resistance to the immunosuppressant drug rapamycin (78). Not long after the mammalian ortholog was cloned work began to unravel the vast array of processes, partners, regulators and functions mTOR interacts with (79). The mTOR protein is a highly conserved serine/threonine kinase that is currently known to exist in two different complexes (**Figure 1.1**), the rapamycin sensitive mTORC1 and rapamycin insensitive mTORC2 (80). The mTORC1 complex is generally regarded as a regulator of ribosome biogenesis, mRNA translation, autophagy, mitochondrial metabolism, cell growth and size, and is primarily composed of the factors mLST8, raptor, mTOR (79). The mTORC2 complex has been implicated in the control of cytoskeletal dynamics, actin association and cell survival, and is primarily composed of mSin1, mLST8, rictor and mTOR (81, 82). While the identified functions of each mTOR complex is "generally" true, research shows there is significant crosstalk and co-regulation of cellular functions between each complex that makes studying and manipulating this pathway a significant challenge (83). Of the two complexes, mTORC1 is the best characterised and plays the primary role in cellular development and protein synthesis, two processes being investigated in the experimental chapters of this thesis. As such, the remainder of this review section will focus on mTORC1, upstream control and downstream regulation of cellular development and protein synthesis.

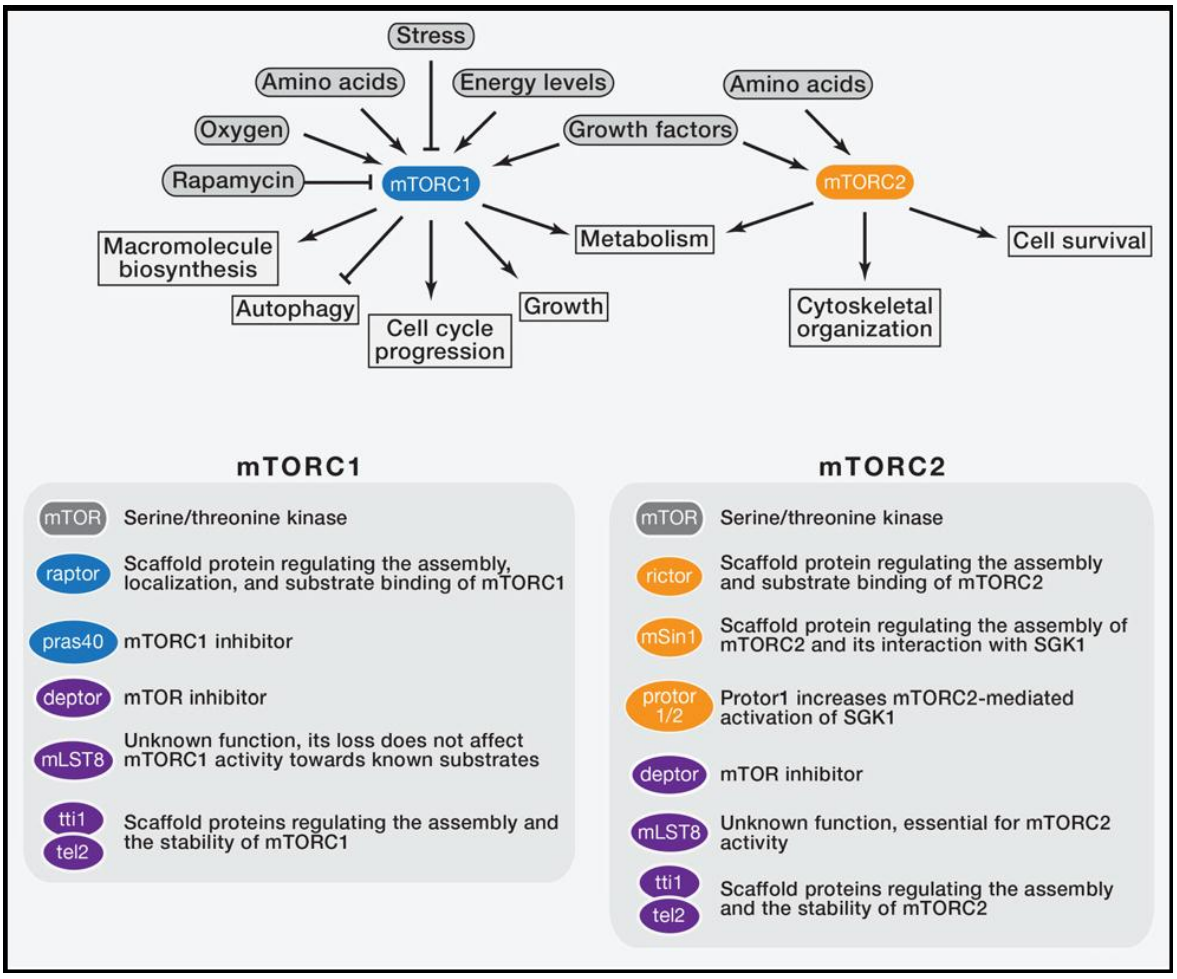


Figure 1.1. The mTOR protein is a highly conserved serine/threonine kinase that is currently known to exist in two different complexes, the rapamycin sensitive mTORC1 and rapamycin insensitive mTORC2. mTORC1 responds to amino acids, stress, oxygen, energy, and growth factors to promote ribosome biogenesis, mRNA translation, autophagy, mitochondrial metabolism, cell growth and size. mTORC2 responds to growth factors, amino acids to regulate cytoskeletal dynamics, actin association and cell survival. mTORC2 is insensitive to acute rapamycin treatment but chronic exposure to the drug can disrupt its structure. The bottom panel describes the known functions of the proteins that form each mTOR complex (Adapted from Laplante and Sabatini., 2012)

1.6.2 Upstream of mTORC1

The mTORC1 pathway acts as the nexus point for at least five major upstream signalling cascades - growth factors, stress, energy status, oxygen, and AAs, to regulate downstream processes such as protein synthesis, autophagy and ribosome biogenesis (78). Growth hormone (a growth factor) is one of the most established treatment models used by researchers to study the regulation of milk yield and composition in ruminants, whilst AA are a potential nutritional intervention to improve milk yield, and both are known to stimulate mTOR signaling, thus the remainder of this review section will focus on these two factors.

1.6.2.1 Amino acids

In vitro and *in vivo* studies show activation of mTOR signalling can be stimulated by AA sufficiency and/or specific AA accumulation (84, 85). Inactive mTORC1 relocates from the cytoplasm to the lysosome surface in response to AA sufficiency or specific AA accumulation within the lysosomal lumen (86). Once in the lysosome, mTORC1 is activated via phosphorylation at mTOR (Ser2448) (87, 88). In addition to AA sufficiency and accumulation of specific AA mammalian studies show that leucyl-tRNA synthetase (LeuRS) acts as a direct sensor for the AA leucine and is involved in the activation of mTORC1 (89). In response to leucine, LeuRS translocates to the lysosomal membrane where it participates in the stimulation of factors that control mTORC1 activation (89). Leucine belongs to the arginine-family of amino acids (arginine, leucine and glutamine). Studies show they enhance lactation potential, via possible stimulation of the nutrient-sensing mechanistic target of rapamycin (mTOR) pathway (64, 73).

1.6.2.2 Growth factors (Growth hormone and IGF1)

While GH and IGF utilise different cell surface receptors to regulate cell growth and proliferation they both converge on the PI3K and Ras (**Figure 1.2**) pathways to mediate their cellular functions (44, 90, 91). The core proteins of these pathways: protein kinase B (Akt/PKB), extracellular-signal-regulated kinase 1/2 (ERK1/2), and ribosomal S6 kinase (RSK1), directly phosphorylate the TSC1/TSC2 complex to inactivate it and thus activate mTORC1 (79). The Akt protein can regulate mTORC1 independently of TSC1/2 by phosphorylating PRAS40, an mTORC1 inhibitor (78).

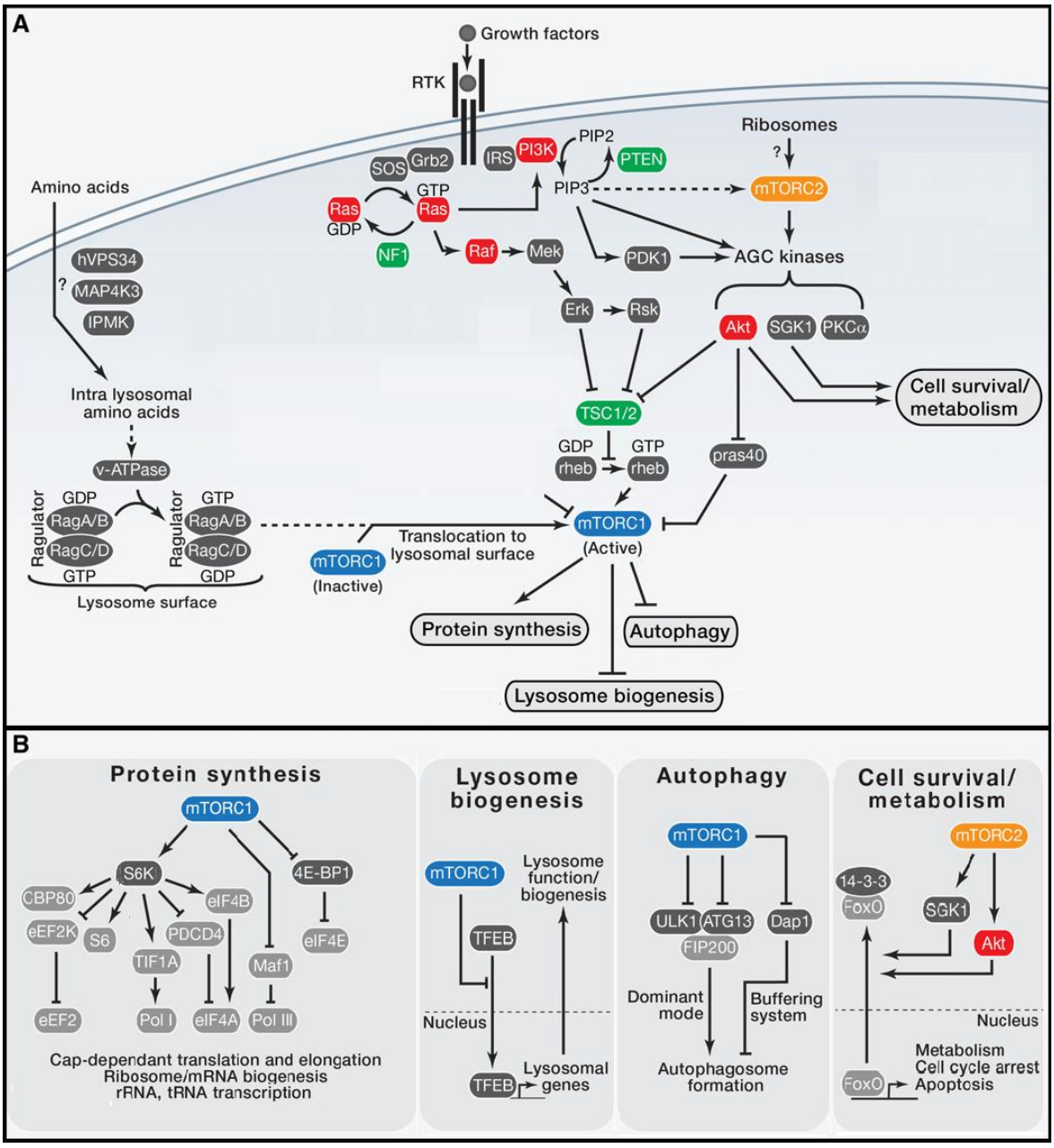


Figure 1.2. (*A*) Critical mTORC1 (and mTORC2) signalling factors involved in cellular development and protein synthesis. Inputs regulating mTORC1 (growth factors, amino acids), with a focus on those downstream, processes applicable to the experimental chapters of this thesis (protein synthesis, lysosome biogenesis, autophagy and cell survival/metabolism). (*B*) The key processes and protein factors, regulating and regulated by, mTORC1 (and mTORC2). (Adapted from Laplante and Sabatini., 2012)

1.6.3 Downstream of mTORC1

For the purposes of the research outlined in this thesis this review will focus on those factors involved in protein synthesis, which is by far the best-characterised process controlled by mTORC1 (**Figure 1.2**). mTORC1 directly phosphorylates eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and members of the S6 kinase (S6K) family, well established promoters of protein synthesis. The phosphorylation of 4E-BP1 prevents its binding to eIF4E, enabling eIF4E to participate in the formation of the eIF4F complex that is required for the initiation of cap-dependent translation. The activation of the S6K family leads, through a variety of effectors, to an increase in mRNA transcription, as well as translational initiation and elongation (**Figure 1.2**). mTORC1 also regulates the protein synthetic machinery in other ways: (1) it activates the regulatory element tripartite motif-containing protein-24 (TIF-1A), which promotes its interaction with RNA Polymerase I (Pol I) and the expression of ribosomal RNA (rRNA) (92); (2) mTORC1 phosphorylates and inhibits Maf1, a Pol III repressor, and so induces 5S rRNA and transfer RNA (tRNA) transcription (93) and (3) mTORC1 regulates the transcription and export of eIF4E-sensitivity element (4E-SE) containing mRNAs known to control cell growth and differentiation (94).

1.6.4 Role in mammary development and function

Identification and understanding of mTOR signalling within the ruminant mammary gland is currently very poor. This seems an extraordinary oversight since the mammary gland is a protein synthetic machine and the mTOR pathway is known to regulate protein synthesis in a wide range of tissues in other species (78). What data is available suggests the mTOR pathway functions in a manner identical to that of other tissues (**see Sections 1.6.2 – 3**) to regulate protein synthesis and cellular differentiation (**Figure 1.3**). Bovine microarray studies show mTOR and insulin play central roles in milk protein synthesis, during lactogenesis and lactation (95). *In vitro* and *in vivo* studies show AA, GH (via IGF1) and the lactogenic hormones hydrocortisone, insulin an prolactin can increase protein synthesis through mTOR activation and subsequent phosphorylation of the downstream effectors 4E-BP1, eEF2 S6K1 and RPS6 (9, 84, 96). To date, no studies have been published regarding the expression or potential role mTOR signalling may play in fetal mammary development, and thus, presents a unique research opportunity.

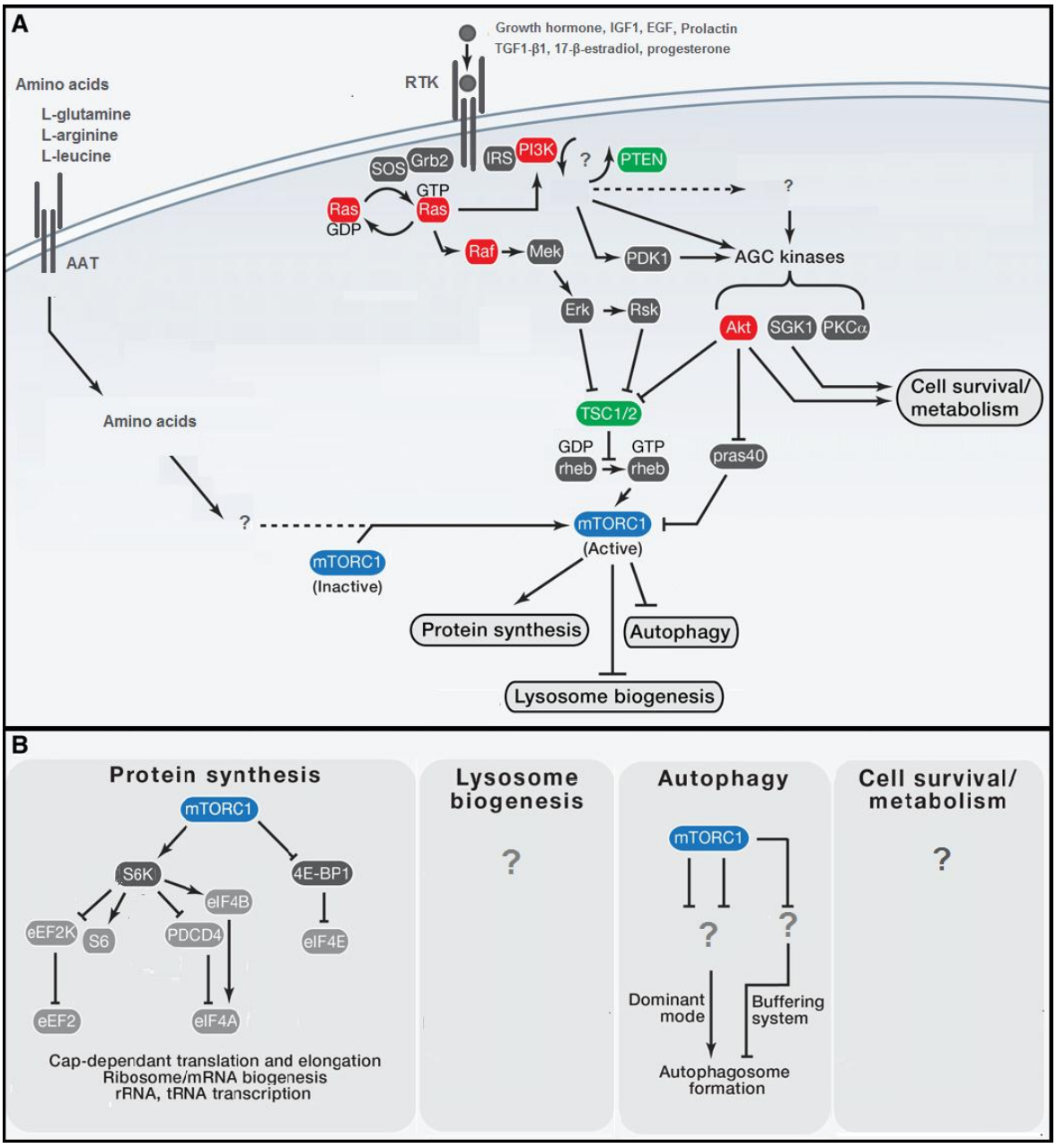


Figure 1.3. (*A*) mTORC1 signalling factors that have been identified in ruminant mammary epithelial cells. (*B*) Key processes and protein factors identified in ruminant mammary epithelial cells, regulating and regulated by, mTORC1. (?) – highlight comparisons between Figure 1.2 and 1.3 where these factors have been identified in other cell / tissue types, but yet to be identified in ruminant mammary epithelial cells. (Adapted from Laplante and Sabatini., 2012)

1.7 Rationale for the study

Lactation research in ruminants has progressed to the stage where a greater understanding of the molecular pathways regulating the development and function of the mammary gland is needed. While treatments such as exogenous GH administration provide a well-established experimental model to study the molecular pathways involved in regulating increased milk protein synthesis by the lactating mammary gland, they are banned from commercial use in New Zealand. Nutrition, in particular the use of specific AAs, are a more commercially and socially acceptable solution, than GH, to increasing ruminant lactation performance. A pathway stimulated by AAs, mitogens and hormones is the mechanistic target of rapamycin (mTOR), which controls downstream proteins involved in cellular proliferation, differentiation and protein synthesis.

The mTOR pathway may be a rate-limiting step in the cellular development and lactation potential of the ruminant mammary gland, and as such the research outlined in this study had the following core objectives:

1. To determine if improved first lactation performance observed in lambs born to ewes fed maintenance nutrition levels was linked to changes in fetal mammary gland biochemical makeup and the abundance of total and phosphorylated mTOR and MAPK signalling proteins (Chapter 2).
2. To determine if increased milk protein synthesis observed in dairy cows treated with GH is associated with changes in mTOR and MAPK signalling and downstream factors regulating nucleocytoplasmic export and translation of mRNA (Chapter 3).
3. To determine if increased milk protein synthesis observed in dairy cows treated with GH is associated with changes in plasma and mammary gland intracellular amino acid concentrations and their reciprocal plasma membrane transporters (Chapter 4).
4. To determine if L-arginine supplementation during mid-late pregnancy influences maternal mammary gland development, mTOR abundance and activation, and lactation performance in the early post-partum period (Chapter 5)
5. Develop an *in vitro* model of bovine lactation to study interactions between mTOR pathway signalling and stimulation by GH and potential AA identified in Chapter 4 (Chapters 6, 7)

CHAPTER 2: NUTRITIONAL PLANE OF TWIN-BEARING EWES ALTERS FETAL MAMMARY BIOCHEMICAL COMPOSITION AND MTOR / MAPK PATHWAY SIGNALLING.

The material presented in this Chapter will be submitted to the Journal of Animal Science.

2.1 Abstract

Identifying the molecular pathways that regulate fetal mammary development is important for understanding the potential link between fetal programming of mammary gland development and future lactation potential. While there are a few published studies regarding biochemical changes in the developing mammary gland there is currently no data available on the involvement of molecular pathways. In this study, we used an ovine model of restricted maternal nutrition to study potential changes in fetal mammary biochemical indices, and mTOR / MAPK signalling at d 100 and d 140 of gestation. We show that mammary glands of fetuses carried by dams on a maintenance plane of nutrition were heavier at d 100 than controls; however by d 140 they were lighter due to atrophy, with increased protein synthetic capacity. The abundance of fetal mammary MAPK and mTOR pathway proteins was unaffected by dam nutrition at d 100 of gestation, yet at d 140, mammary glands of fetuses carried by dams on a maintenance plane of nutrition had increased abundance of MAPK pathway signalling proteins eIF4E, eIF4E^{Ser209}, and mTOR pathway signalling proteins 4EBP1, 4EBP1^{Ser65}, RPS6, and RPS6$^{Ser235/236}$. Increased abundance of MAPK and mTOR pathway proteins and their regulation of ribosomal RNA synthesis, ribosome biogenesis and the availability of factors required to initiate protein translation and cellular differentiation, may explain improved lactation performance in another cohort from the same trial. Increased abundance of 4EBP1^{Ser65} is regulated by mTOR and mTOR^{Ser2448}, whose abundance was unchanged in the mammary glands of d 140 fetuses carried by dams on a maintenance plane of nutrition. Utilising mammary samples separated into parenchyma and fat pad, from an independent trial, we show mTOR^{Ser2448} was primarily localized to the fat pad, potentially explaining why no difference was

measured in the mammary glands of d 140 fetuses carried by dams on a maintenance plane of nutrition. We conclude that these mechanisms are activated in response to restricted maternal nutrition to support fetal mammary gland development and improve subsequent lactation performance of offspring.

2.2 Introduction

Lactation is an essential part of the reproductive cycle, with sufficient production of colostrum and milk essential for early life survival in ruminants (97). In line with this, the ruminant mammary gland undergoes specific developmental changes related to reproductive development: fetal, pubertal, pregnancy and lactation (98). A well-established model system used to study mammary gland development at each stage of the reproductive cycle is nutritional restriction [fetal: (99); pubertal: (100); pregnancy: lactation: (101).

Restricted nutrition during lactation negatively impacts milk yield in dairy cows (102), sheep (101) and goats (103), by reducing mammary epithelial cell (MEC) number and activity (102, 103). In contrast, restricted nutrition during pre-pubertal development improves mammary gland development with no loss in growth performance (100). Altered development of the mammary gland *in utero* due to restricted maternal nutrition and its effects on subsequent milk production and milk composition have been identified (99). The molecular mechanisms regulating fetal mammary development in ruminants are still poorly understood, with studies suggesting involvement of the IGF receptor and IGF

signalling proteins (36). A pathway known to be stimulated by the IGF signalling cascade using *in vitro* models of the lactating ruminant mammary gland is the mechanistic target of rapamycin (mTOR) pathway (90, 104, 105).

At the centre of the mTOR pathway is the evolutionarily conserved serine threonine kinase mTOR, which exists in two known complexes, mTORC1 and mTORC2 (106). In response to nutrients, hormones, mitogens and energy both mTOR complexes regulate a wide array of cellular processes including hypertrophy, hyperplasia and differentiation (107). In the ruminant fetus, mTORC1 is important for survival and development of the ovine conceptus (71), growth and differentiation of ovine (108) and bovine *longissimus dorsi* muscle (109). A role for mTORC2 in ruminant fetal development is yet to be defined, however murine studies implicate a role in embryo survival (110). Signalling through mTORC1 is mediated through a large number of downstream effector proteins including ribosomal protein S6 (RPS6: (71)) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1: (111)). While the role of RPS6 is yet to be fully elucidated, 4E-BP1 exerts control over cellular processes by sequestering and releasing eIF4E (112). Phosphorylation of 4E-BP1 by mTOR, at Ser65, releases eIF4E. Once released the activity of eIF4E is regulated by Mitogen Activated Protein Kinase Interacting Serine/Threonine Kinase 1 / 2 (MKNK1 / 2), through signalling regulated by the Mitogen Activated Protein Kinase (MAPK) pathway (113). In the nucleus this signalling cascade leads to down regulation of eIF4E-mediated export of growth promoting mRNAs, while in the cytoplasm this leads to increased initiation of protein synthesis (114). To date, no studies have investigated the potential role mTOR / MAPK signalling plays in fetal ovine mammary gland development.

Results from a previous study show ewe lambs born to dams on a maintenance plane of nutrition had greater milk yields at d 7 and d 28, greater lactose percentage at d 14, d 21, and d 28, greater accumulated lactose and CP yields and tended to have greater accumulated milk yields compared with offspring of ewes fed *ad libitum* (99). The observed change in milk yield and composition from 2 year old ewes born to dams on a maintenance plane of nutrition were associated with increased fetal mammary size at d 100 of gestation (99). This study aims to identify the molecular mechanisms regulating fetal mammary gland development in another cohort from that trial. The objectives of this study were to measure (1) the biochemical composition and abundance of mTOR and MAPK pathway signalling proteins in the developing ovine fetal mammary gland at d 100 and d 140 of gestation and (2) investigate the potential effect of maternal nutrition on objective (1).

2.3 Materials and methods

2.3.1 Ethics

All procedures involving animals were carried out in compliance with the guidelines of the Massey University Animal Ethics Committee (Palmerston North, New Zealand) or the University of Auckland Animal Ethics Committee (Auckland, New Zealand) in accordance with the Animal Welfare Act 1999.

2.3.2 Animals, treatments and tissue processing

The trial design and sample collection methodology has been described in detail elsewhere (115). Briefly, 900 mixed aged Romney dams were randomly allocated to *ad libitum* (A) or maintenance (M) nutritional regimens under New Zealand pastoral grazing conditions, from d 21 until d 140 of pregnancy. The M-feeding regimen ensured that throughout pregnancy, total dam BW gain was similar to that of the expected increase in conceptus mass. The A-feeding regimen provided *ad libitum* grazing conditions throughout pregnancy. At d 100 and d 140 of pregnancy, a sub-group of twin bearing dams were euthanized and whole fetal mammary glands (fibre, skin, fat and ducts) collected. Fetal mammary glands were weighed, cut in half to separate the two hemispheres, with one half being used for histology and the other stored at -80^{o}C. For the purposes of this study the -80^{o}C stored half was pulverized in a MM301 mixer mill (Retsch, Düsseldorf, Germany) for at least 2 minutes. The frozen powder was aliquoted into 10 ml cryovials and stored at -80^{o}C for subsequent molecular analysis. In total, 23 fetal mammary glands were harvested at d 100 (*ad libitum* group, n = 16; maintenance group, n = 7) and 20 fetal mammary glands at d 140 (*ad libitum* group, n = 13; maintenance group, n = 7). Fetal mammary glands from

this trial were collected whole, so additional fetal mammary glands (separated into parenchyma and fat pat) were harvested from an unrelated trial to study potential tissue specific differences in biochemical indices and mTOR pathway protein abundance. The trial design and sample collection methodology has been described in detail in Appendix A. Briefly, multiparous twin-bearing Romney ewes were housed indoors and fed once daily (between 0800 and 0900 h) a lucerne-based pellet diet (University B mix, Camtech Nutrition, Cambridge NZ) formulated to meet 100% of the NRC-recommended maintenance requirements for twin-bearing pregnant ewes. At d 140 of pregnancy, a sub-group of twin bearing dams was euthanized and fetal mammary glands collected (control group, n = 14). Mammary glands were weighed, separated into fat pad and parenchyma sections, snap frozen in liquid nitrogen and stored at -80^{o}C for subsequent molecular analysis. The parenchymal section refers to a 1 cm^2 section encompassing the developing duct and teat, whilst the fat pad was a 1 cm^2 section underlying the developing duct and teat.

2.3.3 Mammary gland weight and biochemical indices

Mammary gland weight at d 100 was previously published by van der Linden et al (2009. Day 140 mammary gland weight was analysed using the MIXED procedure (SAS Institute. Inc., Cary, NC) with a linear model that included the fixed effect of dam nutrition, ewe size and ewe size by dam nutrition interaction. Total RNA, DNA and protein were extracted, purified and quantified as previously published (105). Differences in the biochemical indices of cell size (protein:DNA ratio), protein synthetic efficiency (protein:RNA ratio), protein synthetic capacity (RNA:DNA ratio), DNA, RNA, protein concentration, and total

parenchymal DNA, RNA and protein content between treatment groups were determined using the MIXED procedure in SAS (SAS Institute) with a linear model that included the fixed effect of dam nutrition. Data that conformed to normal distribution (Kolmogorov–Smirnov: $P \leq 0.05$) with no transformation were reported as least squared means ± SE, data that required log transformation were reported as least squared means (95% CI). Differences between means were considered significant at $P \leq 0.05$.

2.3.4 Immunoblotting

SDS-PAGE: Total protein from d 140 fetal mammary parenchyma and fat pad was extracted and quantified as previously published (105). Total protein samples were separated via reducing SDS-PAGE. Protein were separated on 3-8% Tris-acetate gels (Invitrogen) for the detection of targets mTOR, mTOR^{Ser2448}, or 4-12% Bis-tris gradient gels (Invitrogen) for the detection of eIF4E, eIF4E^{Ser209}, 4EBP1, 4EBP1^{Ser65}, RPS6, and RPS6$^{Ser235/236}$, according to manufacturer's instructions. Due to the limited number of wells available only 11 of the 14 parenchyma and fat pad total protein samples were analysed.

Western Blots: Proteins were transferred using an iBlot® Gel Transfer Device to a polyvinylidene difluoride membrane (PVDF; Invitrogen). The PVDF membrane was blocked according the antibody manufacturer's instructions Cell Signaling Technologies (Boston, MA, USA), then incubated with primary antibodies [mTOR, mTOR^{Ser2448}, eIF4E, eIF4E^{Ser209}, 4EBP1, 4EBP1^{Ser65}, RPS6, RPS6$^{Ser235/236}$ (Cat No: 9742, 9741, 9452, 9451, 2211, 4857, 2672, 2971 respectively)] followed by a horseradish peroxidase-conjugated secondary antibody (Cat No: 7074). All antibodies were purchased from Cell Signaling Technologies. Proteins

were then visualized using SuperSignal West Pico enhanced chemiluminescence reagents (BioRad, Auckland, NZ) and Kodak Biomax XAR film (Rochester, NY, USA). At least three repeat films were scanned using an HP photosmart B110 (Hewlett Packard, Auckland, NZ) set at 300 dpi. Where films were used for densitometric measurement (Figure 2.1) the peak area of individual bands was determined using ImageJ software (National Institutes of Health, Bethesda, MD). The peak area of individual bands within each film was calculated per unit of DNA. The calculated values were then expressed as a percentage of the total peak area from all bands on the film to reduce variation between repeat films (abundance of total and phosphorylated protein). Differences between treatment groups were analysed using the MIXED procedure in SAS (SAS Institute) and reported as normalised peak area (per unit of DNA). All data conformed to Kolmogorov-Smirnov normality ($P \leq 0.05$) and differences between means were considered significant at $P \leq 0.05$. Where films were used to assess the presence or absence of mTOR / MAPK pathway proteins (Figure 2.2), no densitometric measurements were made. Films were over-exposed to ensure samples with low target protein could also be detected.

2.4 Results

2.4.1 Effect of dam nutrition on fetal mammary gland weight and biochemical indices (Table 2.1)

The mammary gland weight of d 100 twin fetuses carried by M-fed dams was heavier than those from A-fed dams. There was a trend (P = 0.12) for decreased cell number and increased cell size (P = 0.13) in the mammary glands of fetuses carried by M-fed dams, compared to A-fed at d 100. Dam nutrition at d 100 had no effect upon fetal mammary total RNA, protein, DNA, RNA concentration, protein and RNA to DNA, protein to RNA. At d 140 of gestation, fetal mammary gland weight tended to be lighter (P = 0.07) in fetuses carried by M-fed dams compared to A-fed. Total DNA and protein was lower, DNA concentration tended (P = 0.10) to be lower and potential growth rate (RNA:DNA) was increased in mammary glands from d 140 fetuses carried by M-fed compared to A-fed ewes. Dam nutrition at d 140 had no effect upon fetal mammary gland RNA content, RNA and protein concentration, and protein to DNA, protein to RNA.

Table 2.1. Maintenance (M) levels of ewe nutrition, from day 21 of pregnancy, increased the mammary gland weight of fetuses harvested at day 100, compared to fetuses from *ad libitum* (A) fed ewes‡. This was associated with trends for lower cell number and higher cell size, indicating size difference was due to cellular hypertrophy not hyperplasia. At day 140, fetal mammary glands harvested from M-fed ewes were significantly lighter than those harvested from fetuses carried by A-fed ewes. This was associated with decreased DNA concentration and total per gland, and increased protein synthetic potential, indicating that whilst fetal mammary glands from A-fed ewes were heavier due to increased hyperplasia, M-fed glands were developmentally active. Each biochemical indices value (excluding mammary gland weight) represents the average of three independent extractions. *$P \leq 0.05$; †$P \leq 0.15$. ‡Data previously published (99).

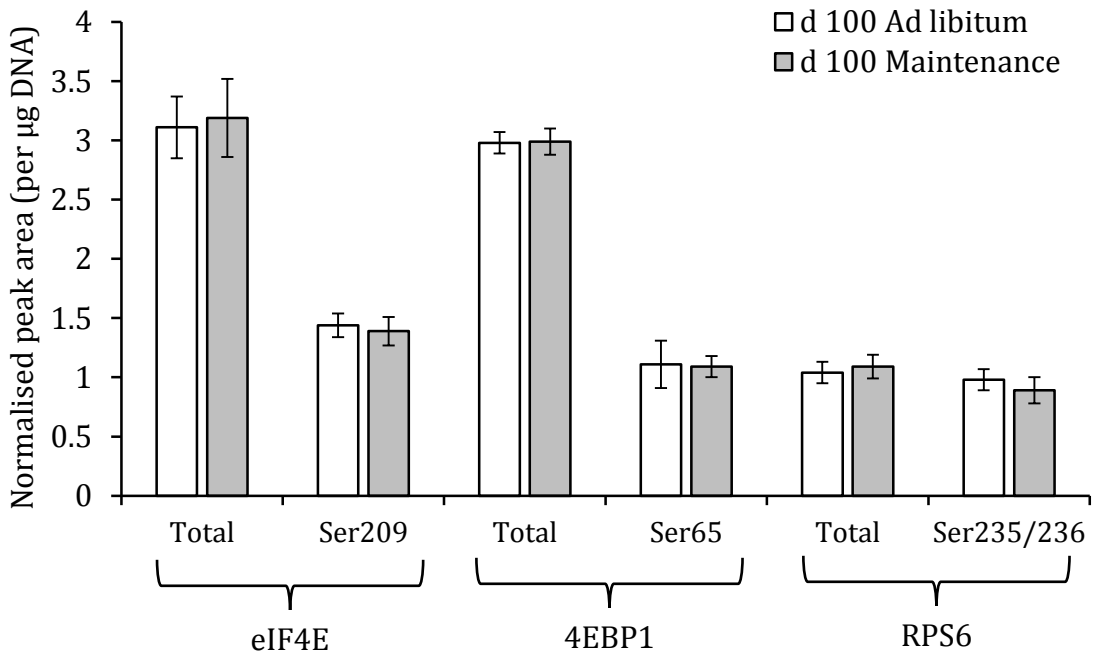
	Day 100			Day 140		
	A (*n=16*)	M (*n=7*)	*P* value	A (*n=11*)	M (*n=6*)	*P* value
Fetal mammary gland weight (g)	3.98 ± 0.12	4.52 ± 0.20	0.03*‡	13.90 ± 0.98	10.37 ± 1.31	0.07†
Total mammary gland content (mg)						
DNA	6.91 ± 0.37	5.8 ± 0.55	0.12†	8.01 (6.91-9.30)	4.5 (3.68-5.50)	0.04*
RNA	3.81 ± 0.19	3.57 ± 0.28	0.48	8.65 ± 0.94	7.54 ± 1.28	0.49
Protein	100.9 ± 6.73	100.56 ± 10.18	0.98	196 (177-217)	145 (126-166)	0.09†
Total mammary gland concentration (mg/g tissue)						
DNA	1.68 ± 0.09	1.45 ± 0.13	0.16	0.61 (0.55-0.69)	0.44 (0.38-0.51)	0.10†
RNA	0.94 ± 0.04	0.85 ± 0.06	0.26	0.64 ± 0.04	0.73 ± 0.06	0.23
Protein	24.57 ± 1.21	23.3 ± 1.83	0.58	15.6 ± 1.28	14.36 ± 1.73	0.58
Mammary gland ratio						
RNA:DNA	0.56 ± 0.04	0.64 ± 0.06	0.31	1.01 ± 0.15	1.75 ± 0.20	0.02*
Protein:DNA	14.76 ± 1.64	19.41 ± 2.49	0.13†	26.4 ± 3.78	35.09 ± 5.12	0.19
Protein:RNA	27.43 ± 2.00	28.29 ± 3.01	0.81	24.32 (22.04-26.85)	19.41 (16.98-22.10)	0.19

2.4.2 Effect of dam nutrition on the abundance of mTOR and MAPK signalling proteins in the fetal mammary gland

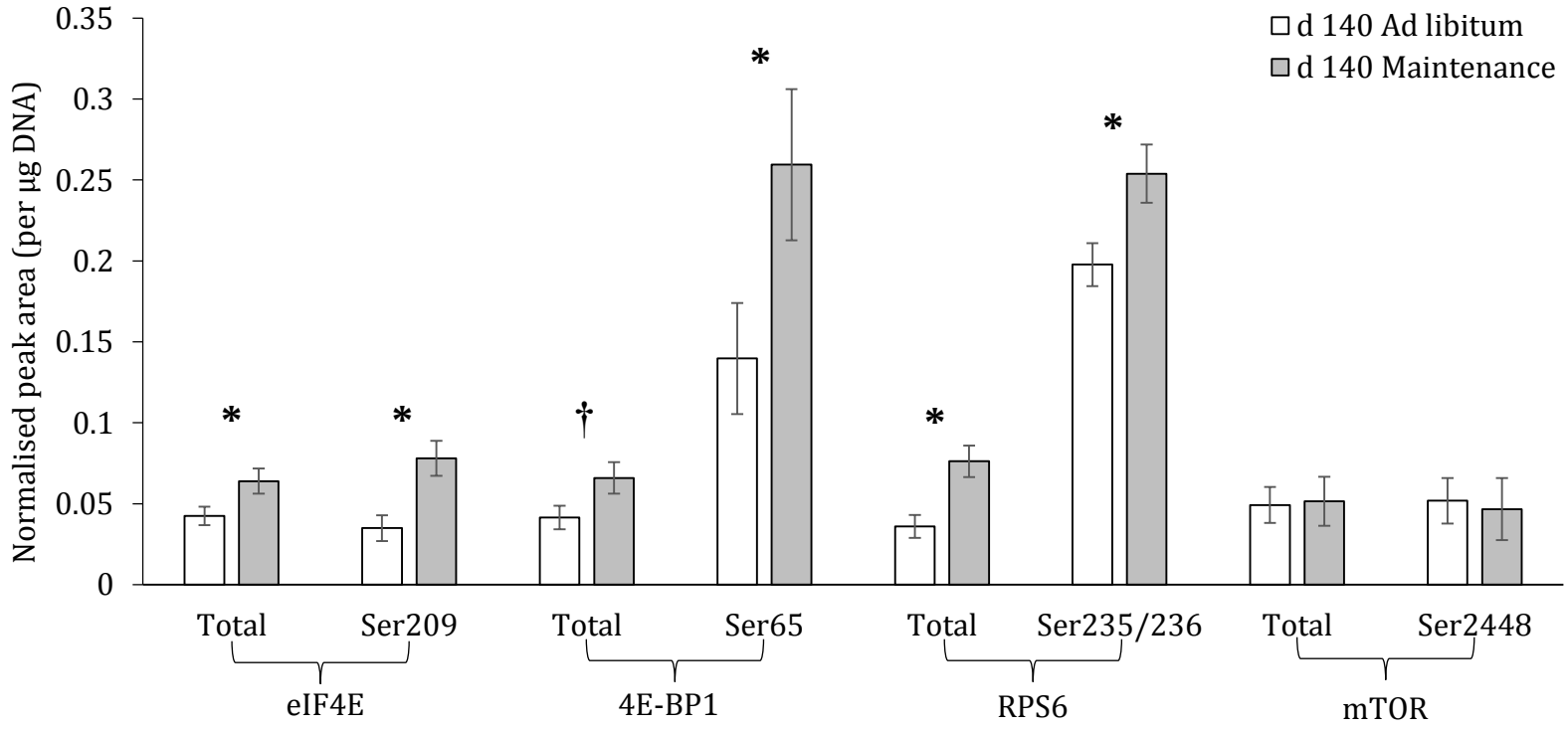
Dam nutrition had no effect on the abundance of total eIF4E, eIF4E^{Ser209}, 4EBP1, 4EBP1^{Ser65}, RPS6 and RPS6$^{Ser235/236}$ in the mammary glands of d 100 fetuses (Figure 2.1*A*). Mammary glands from d 140 fetuses carried by M-fed ewes had increased abundance of total eIF4E, eIF4E^{Ser209}, 4EBP1, 4EBP1^{Ser65}, RPS6$^{Ser235/236}$, and a trend for increased RPS6 compared to mammary glands of fetuses carried by A-fed ewes (Figure 2.1*B*). Dam nutrition had no effect on the abundance of total mTOR and mTOR^{Ser2448} in the mammary glands of d 140 fetuses (Figure 2.1*B*).

Figure 2.1. (*A*) **Level of ewe nutrition, from d 21 until d 100 of pregnancy, had no effect on the abundance of fetal mammary gland eIF4E, eIF4E^{Ser209}, RPS6, RPS6$^{Ser235/236}$, 4E-BP1 and 4E-BP1^{Ser65}.** (*B*) **At d140, mammary glands from fetuses carried by M-fed ewes had increased abundance of eIF4E, eIF4E^{Ser209}, RPS6, RPS6$^{Ser235/236}$, 4E-BP1 and 4E-BP1^{Ser65}. No significant difference in the abundance of mTOR and mTOR^{Ser2448} was observed. *$P \leq 0.05$; †$P \leq 0.15$.**

(*A*)



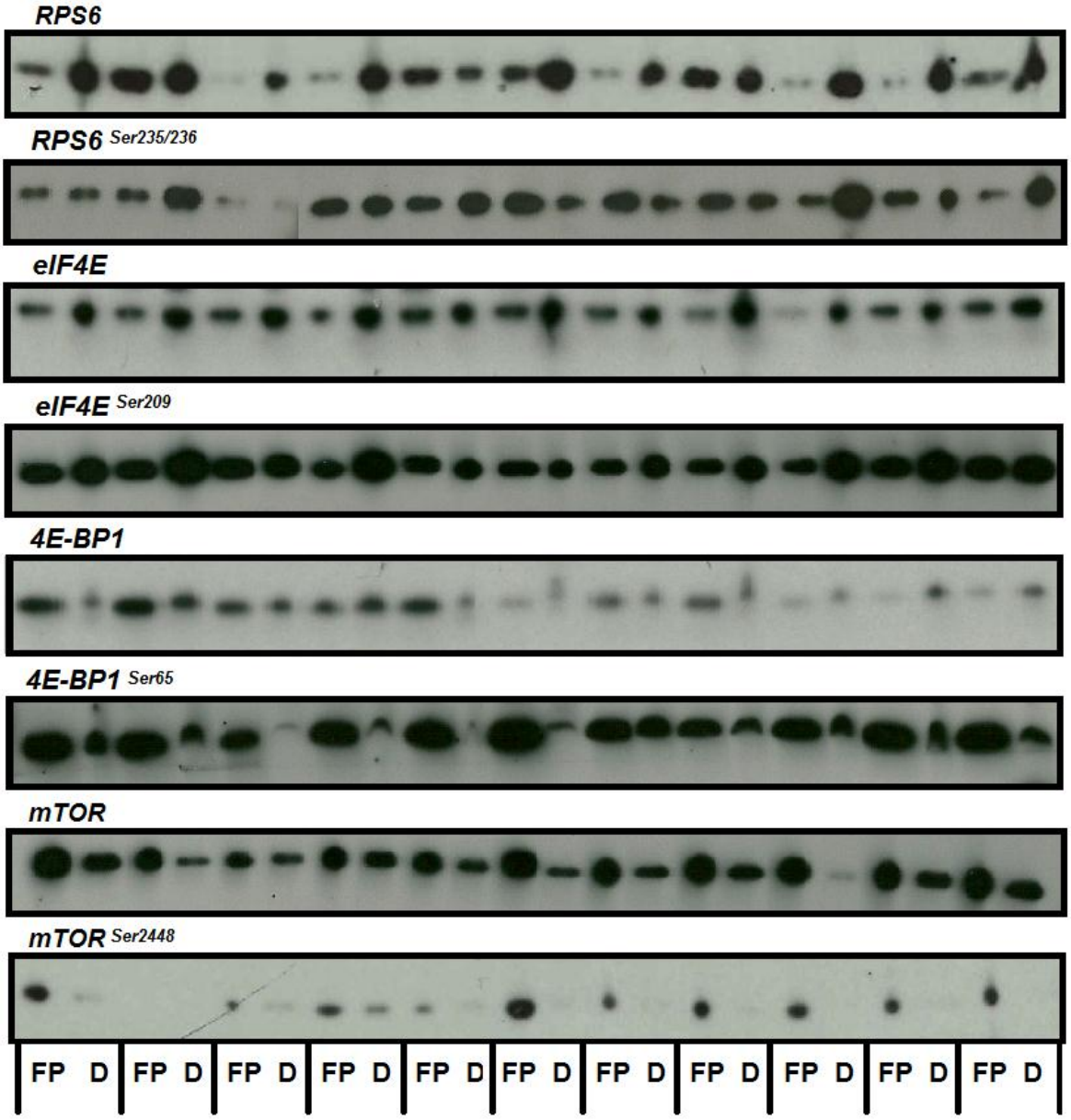
(*B*)


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Normalised peak area (per µg DNA)
d 140 Ad libitum
d 140 Maintenance
*
*
†
*
*
*
Total
Ser209
Total
Ser65
Total
Ser235/236
Total
Ser2448
eIF4E
4E-BP1
RPS6
mTOR

2.4.3 Localisation of mTOR protein in the fetal mammary gland at d 140 of gestation

mTOR was detected in all 11 of the d 140 fetal mammary fat pad and parenchyma protein extracts analysed, whilst mTOR^{Ser2448} was detected in 10 of the 11 fat pad and 4 of the 11 parenchymal protein extracts (Figure 2.2). mTOR signalling targets RPS6, RPS6$^{Ser235/236}$, 4E-BP1, 4E-BP1^{Ser65} and MAPK signalling target eIF4E, eIF4E^{Ser209} were detected in all 11 of the d 140 fetal mammary fat pad and parenchyma protein extracts (Figure 2.2).

Figure 2.2. At d 140, mTOR^{Ser2448} was mostly observed in the fetal mammary fat pad (FP), mTOR, eIF4E, eIF4E^{Ser209}, 4EBP1, 4EBP1^{Ser65}, RPS6 and RPS6$^{Ser235/236}$ were detected in both the fat pad (FP) and parenchyma (D).



2.5 Discussion

The objectives of this study were to measure (1) the biochemical composition and abundance of mTOR and MAPK pathway signalling proteins in the developing ovine fetal mammary gland at d100 and d140 of gestation and (2) investigate the potential effect of maternal nutrition on objective one. The main findings of this study are that significant changes in biochemical indices of protein synthetic capacity and hyperplasia at d 140 are linked to activation of mTOR and MAPK pathway signalling and improved first-lactation performance in the mammary glands of fetuses carried by dams exposed to maintenance levels of nutrition.

2.5.1 Maternal nutrition alters d 100 and d140 fetal mammary gland weight through changes in cell number and size

Nutrition is a well-established model for studying the effects of the maternal environment on fetal development in sheep (116). The effect of maternal nutrition on fetal growth is broadly divided into three developmental time points, the first third of gestation (d 0 – 45) when mammary gland structure, oocyte quality, number of blastocysts and placentomes are established, the second third (d 45 – 90) when there is increased proliferative growth of the placenta and the last third (d 90 – 147) when rapid fetal growth occurs (117). The last third of gestation is generally regarded as the critical time point for nutritional intervention because during this developmental stage the nutritional requirements of the pregnant dam increase markedly, while requirements of the fetus prior to the last third are considered to be very low (117). Results published in another study from the same trial, using the same tissues, showed mammary glands at d 100 were heavier from fetuses carried by M-fed

dams (99). We show that at d 100, fetal mammary glands from fetuses carried by M-fed dams were potentially heavier than those from A-fed due to increased hypertrophy, or mammary glands from fetuses carried by A-fed dams were lighter due to decreased hypertrophy. The current study highlights that the phenotype is reversed at d 140 where mammary glands from fetuses carried by M-fed dams are lighter than those from A-fed dams. The aforementioned phenotype change could be due to decreased mammary gland hyperplasia in d 140 fetuses carried by M-fed dams compared to A-fed, or mammary glands of fetuses carried by A-fed dams have increased hyperplasia.

In this study, the timeframe for treatment was d 21 - d 140 of pregnancy, with changes in fetal mammary weight and biochemical composition observed between d 100 and d 140. The observed decrease in d140 mammary gland weight and hyperplasia in fetuses carried by M-fed dams is consistent with the theory that restricted maternal nutrition has the greatest impact on fetal organ size during the last trimester of pregnancy (116). The results from this study also agree with Rattray *et al* (1975) who show that total DNA, RNA, protein and protein to DNA ratios were lower in the brain, heart, kidney and muscle of singletons and twins carried by dams fed a low plane of nutrition compared to singletons and twins carried by dams on a high plane of nutrition (118). Results published in two previous studies from the same trial show dam nutrition had no effect on fetal mammary gland duct area, lumen area, secretory cell area, number of ducts or estimated cell size at d 100 (99) and d140 of gestation [41]. These observations indicate that the reduction in fetal mammary gland weight from d 100 to d 140 of gestation was most likely due to reduced mammary fat pad hyperplasia. The fat pad is indispensable for development of the

parenchyma at all stages of development and reduced fat pad size is positively correlated with improved lactation performance (119), and may provide an insight to why 2 year old ewes born to M-fed dams were reported to have improved first-lactation performance in another study from this trial (99).

2.5.2 Increased abundance of total and activated MAPK and mTOR signalling proteins is associated with increased protein synthetic capacity

Development of the fetal mammary gland depends on complex interactions between the mammary mesenchyme and epithelium, interactions which are mediated by locally and systemically produced hormones and mitogens (120). A family of mitogens known to regulate fetal ovine growth (116) and fetal mammary growth in murine models are the insulin-like growth factors (IGF) (121). The IGF family are mitogens that play a central role in regulating cell proliferation, differentiation, and apoptosis (122). In ruminants, mesenchyme-epithelium signalling in the mammary gland is mediated through local mesenchyme production of IGF-I and IGF-II (120). These mitogens interact with the epithelium IGF-1 receptor to regulate functional differentiation and prepare the epithelium for future milk production (120). A pathway known to mediate IGF signalling that regulates downstream factors involved in cell growth, differentiation and development is the MAPK. In this study, the abundance of MAPK signalling target eIF4E^{Ser209} was increased in the mammary glands of fetuses carried by M-fed ewes at d 140 of gestation, indicating members of the IGF family could be using the MAPK pathway to increase phosphorylation of eIF4E at Ser209. The cellular function of eIF4E^{Ser209} is still under investigation, but two models have been proposed. Topisirovic (114), suggests eIF4E^{Ser209} aids in eIF4E-mediated

transport of growth-promoting mRNAs from the nucleus, while Zuberek (2003) proposes that eIF4E^{Ser209} increases the initiation of protein translation. As the maternal nutritional environment of M-fed ewes did not increase d 140 fetal mammary hypertrophy and total protein content, the model proposed by Zuberek is unlikely, based on data reported in the current study. We propose that the mammary glands of d 140 fetuses carried by M-fed dams utilise IGF-MAPK signalling to increase the abundance of eIF4E^{Ser209} to mediate the nucleocytoplasmic transport of growth-promoting mRNAs (94). Insulin-like growth factor-MAPK signalling is most likely via localised mesenchyme-interactions as studies show restricted maternal nutrition decreases fetal plasma levels of IGF-I (116).

The most abundant RNA species in mammalian cells is ribosomal RNA (rRNA), accounting for >80% of the total cellular RNA content (123). Ribosomes are factories used by cells to translate mRNA into protein and are indicative of cells that are more "developmentally active (124)." Mammalian ribosomes are comprised of two subunits, the 40S (small) and 60S (large). The 40S subunit consists of a single RNA molecule (18S rRNA), and 33 protein partners; the larger 60S subunit is comprised of three RNA molecules (5S, 5.8S, 28S rRNA), and 47 partner proteins. RPS6 is a protein with diverse roles in ribosome biogenesis and function. Ribosomal protein S6 can act as a functional unit within the small-subunit processome, a large ribonucleoprotein involved in the biogenesis of 18S rRNA (125), mediate small-large subunit joining to form functional ribosome assemblies (126) and when activated by phosphorylation of residue Ser235/236 enhance translation of 5′-terminal oligopyrimidine tract (5′TOP) mRNAs (127), (128). In this study, the observed increase in the ratio of RNA to DNA, a measure of protein synthetic capacity, is associated

with increased abundance of RPS6, RPS6$^{Ser235/236}$. This suggests mammary glands of d 140 fetuses carried by M-fed dams have increased levels of ribosome formation, per cell, mediated in a potentially RPS6-dependent manner. Interestingly, phosphorylation of RPS6 at Ser235/236 can be regulated by MAPK signalling and suggests a link between eIF4E^{Ser209} 4E-SE mediated mRNA export and RPS6 mediated translation.

The rate-limiting step in mRNA translation is formation of the initiation complex eIF4F. eIF4F is a multiprotein / peptide complex whose core is composed of an mRNA-cap binding protein (eIF4E), a large scaffolding subunit (eIF4G), and an RNA helicase (eIF4A). Formation of the eIF4F complex is regulated at two levels. Firstly, in its hypophosphorylated form, 4E-BP1 can inhibit cap-dependent translation by sequestering eIF4E in an inactive complex. Hyperphosphorylation of 4E-BP1, terminating at Ser65, by mTOR results in the release of eIF4E, allowing association with eIF4G and assembly of an active eIF4F complex (112). Secondly, mammalian cells undergoing oncogenic transformation (increased cellular hyperplasia and hypertrophy) can increase mRNA translation by increasing the abundance of eIF4E, preventing hypophosphorylated 4E-BP1 from shutting down eIF4F formation (129). In this study, the abundance of mTOR signalling targets 4EBP1, 4EBP1^{Ser65} and MAPK signalling target eIF4E, was increased in mammary glands of d 140 fetuses carried by M-fed dams. This result is consistent with the observed increase in the ratio of RNA to DNA, an indicator of protein synthetic capacity. The cytoplasmic role of 4E-BP1 and eIF4E is the regulation of mRNA translation, but in the nucleus, where >70% of 4E-BP1 resides, they regulate the export of eIF4E-sensitivity element (4E-SE) containing mRNAs (112). Like 5'-TOP mRNAs, 4E-SE play roles in cell

growth and differentiation. We propose that mammary glands from fetuses carried by M-fed dams increase the abundance of 4E-BP1 and eIF4E to support eIF4E^{Ser209} mediated export of 4E-SE containing mRNAs. The phosphorylation of 4E-BP1 at Ser65 would aid RPS6$^{Ser235/236}$ mediated translation of 5'TOP mRNAs and eIF4E^{Ser209} 4E-SE-containing mRNAs by increasing the available pool of eIF4E, supporting cell growth and differentiation (94, 128).

Ribosomal RNA biogenesis (123) and co-regulation of 4E-SE / 5'TOP mRNA nucleocytoplasmic export and global mRNA translation (130) is mediated by the mTOR pathway. The mTOR pathway responds to four major signals; nutrients, growth factors, energy and stress to control multiple downstream functions (78). Activation of mTOR signalling regulates mRNA translation by two distinct mechanisms that are cytoplasmic and nuclear in origin. Phosphorylation of 4EBP1 by mTOR, at Ser65, promotes mRNA translation, while in the nucleus this negatively regulates the export of 4E-SE containing mRNAs (78). In this study, the abundance of d 140 fetal mammary gland mTOR, mTOR^{Ser2448} was not affected by the maternal nutrition environment. This result was inconsistent with observed changes in the activation of downstream signalling components of the mTOR pathway. Using d 140 fetal mammary tissue collected from an independent trial, and separated into fat pad and parenchyma, we were able to show that mTOR^{Ser2448} was primarily detected in the fat pad. While, mTOR, eIF4E, eIF4E^{Ser209}, 4EBP1, 4EBP1^{Ser65}, RPS6, and RPS6$^{Ser235/236}$ were detected in both tissue compartments. These results indicate that mTOR^{Ser2448} abundance is higher in the fat pad than the parenchyma at d 140 of gestation, and may explain why no difference in mTOR^{Ser2448} abundance could be detected

in protein extracts from fetal mammary glands not separated into parenchyma and fat pad. We do not believe the parenchyma samples collected from the second, independent trial, had no mTOR^{Ser2448}, as several developmental animal models show this to be lethal (131, 132).

In conclusion, this study proposes restricted maternal nutrition between d 21 – d 140 of pregnancy enhances fetal mammary development and first-lactation performance by decreasing fat pad hyperplasia. This is associated with increased abundance of MAPK and mTOR pathway signalling proteins that regulate the export of growth promoting 4E-SE and 5'TOP mRNA and protein synthetic capacity in response to potential paracrine IGF-1 / IGF-2 signalling. The abundance of mTOR^{Ser2448} was higher in the fat pad than the parenchyma at d 140 suggesting the main function of mTOR signalling resides in the fat pad. The findings in this study are associated with improved first-lactation performance and provide new knowledge about the mechanisms regulating this phenotype. However, the regulation of these pathways and the identification of the cell or groups of cells these changes are occurring in is not clear and deserves further investigation.

CHAPTER 3: INCREASED MILK PROTEIN SYNTHESIS IN RESPONSE TO EXOGENOUS GROWTH HORMONE IS ASSOCIATED WITH CHANGES IN MECHANISTIC (MAMMALIAN) TARGET OF RAPAMYCIN (MTOR)C1-DEPENDENT AND INDEPENDENT CELL SIGNALING.

The material presented in this Chapter has been published as a paper:

Sciascia Q, Pacheco D, McCoard SA. (2013). Increased milk protein synthesis in response to exogenous growth hormone is associated with changes in mechanistic (mammalian) target of rapamycin (mTOR)C1-dependent and independent cell signaling. J Dairy Sci. 96(4):2327-38.

Chapter Emendations

Animal production data as published in (9). This has been included to show the milk production phenotype in response to treatment with exogenous growth hormone (GH).

Mean daily milk and protein production for all cows before treatment (mean ± SE) was 8.5 ± 1.17 L/cow and 0.47 ± 0.055 kg/cow, respectively. There were significant differences between GH and control groups in milk and protein yields by d 4 of GH treatment. Six days after treatment, GH had increased milk yield by 42% compared with controls, with corresponding increases in protein, fat, and lactose yields. Somatic cell analyses showed that there were no significant differences between treatments and there were no significant differences within each day (data not shown). The serum concentrations of IGF-1 on d 4 and 6 of GH administration were significantly ($P < 0.001$) higher for the GH group compared with controls (GH: 928.0 ± 26.6 and 582.0 ± 64.2 ng/mL; control: 196.0 ± 26.6 and 74.0 ± 64.2 ng/mL, respectively), indicating that the dosing and timing protocol for GH treatment was successful.

3.1 Abstract

The objective of this study was to determine if increased milk protein synthesis observed in lactating dairy cows treated with growth hormone (GH) was associated with mechanistic (or mammalian) target of rapamycin complex 1 (mTORC1) regulation of downstream factors controlling nucleocytoplasmic export and translation of mRNA. To address this objective, biochemical indices of mammary growth and secretory activity and the abundance and phosphorylation status of mTORC1 pathway factors were measured in mammary tissues harvested from non-pregnant lactating dairy cows 6 d after treatment with a slow-release formulation of GH or saline (n = 4/group). Treatment with GH increased mammary parenchymal weight and total protein content and tended to increase ribosome number and cell size, whereas protein synthetic efficiency, capacity, and cell number were unchanged. Cellular abundance of the mTORC1 components mTOR and (phosphorylated) mTOR^{Ser2448} increased, as did complex eukaryotic initiation factor 4E:eukaryotic initiation factor 4E binding protein 1 (eIF4E:4EBP1), whereas no change was observed for mTORC1-downstream targets 4EBP1, 4EBP1^{Ser65}, p70/p85^{S6K} and p70^{S6K}Thre389/p85^{S6K}Thre412. Changes in activation were not observed for any of the targets measured. These results indicate that GH treatment influences signalling to mTORC1 but not downstream targets involved in the nucleocytoplasmic export and translation of mRNA. Increased eIF4E:4EBP1 complex formation indicates involvement of the mitogen-activated protein kinase (MAPK) pathway. Abundance of MAPK pathway components eIF4E, eIF4E^{Ser209}, eIF4E:eIF4G complex, MAP kinase-interacting serine/threonine-protein kinase 1 (MKNK1), MKNK1$^{Thr197/202}$, and ribosomal protein S6 kinase, 90kDa, polypeptide 1 (RPS6KA1) increased significantly in response to GH, whereas

relative activation of the proteins was unchanged. Expression of IGFBP3 and IGFBP5 increased, that of IGF1R decreased, and that of IGF1 remained unchanged in response to GH. PatSearch analysis of the milk caseins αS1-casein, αS2-casein, and β-casein, MAPK signalling target RPS6KA1, and proliferation gene IGFBP3 mRNA indicated that all contained putative eIF4E-sensitivity elements. In response to GH, these genes were all up-regulated, suggesting that increased abundance of eIF4E and eIF4E^{Ser209} plays a role in mediating their nucleocytoplasmic export. We propose that, in response to GH, the IGF1-IGF1R-MAPK signalling cascade regulates eIF4E-mediated nucleocytoplasmic export and translation of mRNA, whereas mTOR controls cell renewal, cell turnover, and rRNA transcription through an alternative signalling cascade.

3.2 Introduction

Cellular regulation of protein synthesis is a tightly coordinated system that utilizes common factors located in the cytoplasm and nucleus (133). Cytoplasmic factors are widely recognized as regulators of mRNA translation, whereas their nuclear counterparts control the export of specific mRNAs linked to cell survival, proliferation, and growth (94). In the ruminant mammary gland, coordination of these factors relies upon a complex endocrine, mitogen, and nutritional signalling cascade that has yet to be fully elucidated (134). Identifying and understanding the molecular mechanisms that underpin this coordination may provide fundamental knowledge that leads to the development of novel technologies to increase dairy cow lactation performance. A well-established treatment model used to study increased milk protein synthesis by the ruminant mammary gland is growth hormone (GH). The galactopoietic effect of GH has been well investigated; however, cell signalling mechanisms that mediate its action on the mammary gland are not fully understood (37, 135).

Studies utilising in vitro models suggest GH may signal directly to mammary epithelial cells via the growth hormone receptor, through pathways linked to protein synthesis and cellular proliferation (41). Mammary explant studies show GH alone increases cell survival by suppressing expression of the pro-apoptosis gene insulin-like growth factor-binding protein 5 (IGFBP5) (42). Evidence from in vivo studies postulate that GH can also act indirectly on the mammary gland via stimulation of liver insulin-like growth factor 1 (IGF1) secretion or by increasing blood flow and nutrient availability to the gland (37, 136). These physiological stimuli increase epithelial cell proliferation and/or secretory activity in the

lactating mammary gland (37) indicating the involvement of signalling pathways regulating cell turnover and renewal, RNA transcription and protein synthesis in mediating the effects of GH on milk production. We have previously reported that treatment with GH increases plasma IGF1 concentrations, increases ribosomal protein S6 (RPS6) abundance and phosphorylation, and increases eukaryotic initiation factor 4E (eIF4E) abundance in the mammary gland of lactating dairy cows (9). Furthermore, Burgos and Cant (2010) using the bovine mammary epithelial cell line MAC-T, showed that IGF1 increases formation of the eIF4E:eukaryotic initiation factor 4G (eIF4G) complex, $p70^{S6K}$ phosphorylation and reduces eIF4E:eukaryotic initiation factor 4E binding protein 1 (4EBP1) complex formation. Interestingly, RPS6 phosphorylation and eIF4E:4EBP1 complex formation can be influenced by the mechanistic target of rapamycin (mTOR) pathway (137, 138).

The mTOR protein is an evolutionarily conserved serine threonine kinase that exists in two known complexes, mTORC1 and mTORC2. The mTORC1 complex responds to four major signals: nutrients, growth factors, energy and stress to control protein synthesis, cell size, cellular proliferation and gene expression, while mTORC2 responds to nutrients and growth factors to regulate cytoskeleton formation (139). Activation of mTORC1 signalling elevates protein synthesis by two distinct mechanisms that are cytoplasmic and nuclear in origin. Cytoplasmic mTORC1 phosphorylation of $4EBP1^{Ser65}$ uncouples it from eIF4E allowing the formation of the 5'-m7G cap-dependent eIF4E:eIF4G protein synthesis initiation complex (137). Once protein synthesis has begun mTORC1 regulates the speed of translation by phosphorylating $p70^{S6K}$, which in turn phosphorylates its downstream target RPS6 (140). Nuclear signalling by mTORC1 involves the phosphorylation of MAF1 to

increase ribosomal RNA (rRNA) transcription and $p85^{S6K}$ to increase messenger RNA (mRNA) transport (93).

The aim of this study was to determine if increased milk protein synthesis observed in dairy cows treated with GH is associated with changes in mTORC1 signalling and downstream factors regulating nucleocytoplasmic export and translation of mRNA.

3.3 Materials and methods

3.3.1 Animals and treatments

All procedures involving animals were carried out in compliance with the guidelines of the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand), in accordance with the 1999 Animal Welfare Act of New Zealand. The trial design and sample collection methodology has been described in detail elsewhere (9). Briefly, eight non-pregnant, lactating second parity Jersey cows (178-200 days postpartum) were housed in separate indoor stalls and fed a diet formulated to exceed requirements for metabolisable energy, protein and essential amino acids. They were fed and milked twice daily. A single subcutaneous injection of either a slow-release formulation of commercially available GH (Lactotropin®) 500 mg or saline (n=4/group) was administered. Six days following injection, cows were milked, euthanized, and mammary parenchymal tissue (alveolar tissue free of large ducts and blood vessels) collected and snap frozen in liquid nitrogen (-80^{o}C) within 5 min, for subsequent analyses.

3.3.2 Biochemical indices

Mammary tissue from each cow was homogenised in TRIzol® reagent (Invitrogen, Auckland, NZ) and total RNA, DNA and protein were extracted according to the manufacturer's protocol. Nucleic acids were quantified using a NanoDrop Spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, DE) and protein was quantified using the Bradford assay (141). DNA, RNA and protein were then used to determine biochemical indices measures of cell size (protein:DNA ratio), protein synthetic efficiency (protein:RNA ratio), protein synthetic capacity (RNA:DNA ratio), DNA, RNA,

protein concentration, and total parenchymal DNA, RNA and protein content. Differences between treatment groups were determined using the T-TEST procedure in SAS (SAS Inst. Inc., Cary, NC). Differences between means were considered significant at $P \leq 0.05$.

3.3.3 Quantitative real-time PCR

***RNA Extraction and cDNA Synthesis*.** RNeasy minikits, with on-column DNase I treatment (Qiagen, San Diego, CA, USA) to remove any residual DNA contamination, were used to purify total RNA extracted for biochemical indices. Purified RNA was quantified using a NanoDrop Spectrophotometer (ND-1000; Nanodrop Technologies), and RNA quality assessed by running 1 μg on a 1% non-denaturing agarose gel, stained with SYBR® Safe (Invitrogen). Total RNA (500 ng) was reverse transcribed to make cDNA using the SuperScript® VILO™ cDNA Synthesis Kit and the manufacturers modified protocol of 120 min at 42^{o}C (Invitrogen).

Primer Design. Primers were designed against mRNA sequences from both *Bos taurus* and *Ovis aries* using publicly available data at NCBI (www.ncbi.nlm.nih.gov) and CSIRO (www.livestockgenomics.csiro.au/sheep/), using the Roche Universal ProbeLibrary assay design centre (www.roche-applied-science.com/sis/rtpcr). IGF1 primers had been previously published (142). Primers were made by Integrated DNA Technologies (IDT, Antwerp, Belgium) and purified using desalting to remove short truncated products and small organic contaminants. Primer sequences are presented in Table 3.1.

Table 3.1. Name, symbol, gi number, primer sequence and amplicon size of genes analysed by qPCR.

Gene Name	Symbol	gi number	Hybridisation	Primers (5′ to 3′)	Amplicon Size (bp)
Reference Genes					
Mitogen-activated protein kinase 1	MAPK1	31343545	Forward	TCGCAGGAAGACCTGAATTG	165
			Reverse	TCCTCTTGTGAGGGTTGAACG	
SHC – both variants (Src homology 2 domain containing) transforming protein 1	SHC1	255759964	Forward	CAGTCCATCTCGTTTGCATC	260
		255759963	Reverse	GGCTCTTCCTCCTCCTCATC	
Target Genes					
Insulin-like growth factor 1	IGF1	148747337	Forward	TCGCATCTCTTCTATCTGGCCCTGT	240
			Reverse	GCAGTACATCTCCAGCCTCCTCAGA	
Insulin-like growth factor 1 receptor	IGF1R	347446670	Forward	CTGTATGCCTCTGTGAACCC	197
			Reverse	TCGTTCACCGTCTTAATGGC	
Insulin-like growth factor binding protein 3	IGFBP3	27807006	Forward	AGGAAATGGCAGTGAGTCG	172
			Reverse	CTCGTAGTCAACCTTGTAGCG	
Insulin-like growth factor binding protein 5	IGFBP5	157427781	Forward	GACCCAGTCCAAGTTCGTG	200
			Reverse	TCTTGTAGAACCCTTTGCGG	
Ribosomal protein S6 kinase, 90kDa, polypeptide 1	RPS6KA1	139948214	Forward	CTTCTACTTTGACACTGAGTTCACG	108
			Reverse	TGGCCACGAAACTGAAGC	
18S Ribosomal RNA	18S	307691242	Forward	AAACGGCTACCACATCCAAG	250
			Reverse	CGCTCCCAAGATCCAACTAC	

qPCR Assay. Quantitative real time PCR was performed using the SensiFAST™ SYBR No-ROX Kit (Bioline, London, UK) in a Rotor-Gene™ 6000 (Qiagen), with the template and all reagents at half of the manufacturers recommended volume. The PCR efficiency and quantification cycle (Cq) values were obtained for each sample using LinRegPCR (143). Two reference genes, MAPK1 and SHC1 were identified using NormFinder (http://www.mdl.dk/publicationsnormfinder.htm; (144)). Reactions were performed in triplicate with all data entered into REST 2009 (http://www.gene-quantification.de/rest-2009.html; (145)) and the fold change in expression ratios between the means of two treatment groups determined. All qPCR data was reported as per the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE; (146)) guidelines.

3.3.4 Protein precipitation and immunoblotting

Protein Extraction. Tissue was homogenised in cold protein extraction buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8, 1 mM EGTA, pH 8, 1% Triton X-100, 0.5 mM sodium vanadate, 1 mM benzamidine hydrochloride, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonylfluoride, 1 µg/ml pepstatin, 1 µg/ml antipain, 1 µg/ml leupeptin), centrifuged at 7,000 rpm for 10 min at 4^{o}C. The supernatant was collected and protein concentration determined using the Bradford assay (141).

Protein precipitation. For eIF4E complex analysis protein supernatants were treated with 7-methyl GTP sepharose beads following the manufacturer's protocol (GE Healthcare, Auckland, NZ). Immunoprecipitation of total mTOR was performed with the primary antibody (Cat No: 2672; Cell Signaling Technologies, Boston, MA, USA) using the protein G-Dynabead® magnetic separation kit and DynaMag™-2 magnetic separator according to the manufacturer's protocol (Invitrogen).

SDS-PAGE and Western Blotting. All protein samples were separated via reducing SDS-PAGE. Protein targets mTOR, mTOR^{Ser2448}, eIF4G, p70^{S6K}/p85^{S6K}, and p70^{S6K} Thre389/p85^{S6K} Thre412 (Cat No: 2672, 2971, 2469, 9202, 9205 respectively; Cell Signaling Technologies) were separated on 3-8% Tris-acetate gels (Invitrogen) whilst eIF4E, eIF4E^{Ser209}, 4EBP1, 4EBP1^{Ser65}, RPS6KA1, MKNK1 and MKNK1$^{Thr197/202}$ (Cat No: 9742, 9741, 9452, 9451, 9333, 2195, 2111 respectively; Cell Signaling Technologies) were separated on 4-12% Bis-tris gradient gel (Invitrogen), according to manufacturer's instructions. Proteins were then transferred using an iBlot® Gel Transfer Device to a

polyvinylidene difluoride membrane (Invitrogen) and blocked according to the antibody manufacturer's instructions. The membrane was then incubated with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody, according to the manufacturer's instructions. Proteins were then visualized using SuperSignal West Pico enhanced chemiluminescence reagents (BioRad, Auckland, NZ) and Kodak Biomax XAR film (Rochester, NY, USA). At least three repeat films were scanned using an HP photosmart B110 (Hewlett Packard, Auckland, NZ) set at 300 dpi, and the signal intensity of individual bands determined by densitometric measurement using ImageJ software (National Institutes of Health, Bethesda, MD). Within each film individual band intensities were calculated per unit of DNA to express relative abundance per cell. The calculated values were then expressed as a ratio of the total signal from all bands on the film to reduce band intensity variation between repeat films. To determine activation the intensities of phosphorylated signals were normalized to total protein intensities. Differences between treatment groups were analysed using the TTEST procedure in SAS (SAS Inst. Inc., Cary, NC). Differences between means were considered significant at $P \leq 0.05$.

3.3.5 Analysis of mRNA 3'UTR for putative 4E-SE

The publicly available Ensembl (www.ensembl.org) bovine database was used to obtain the 3'UTR (UnTranslated Region) sequences of alpha-S1-casein (gi:31341348), alpha-S2-casein (gi: 31341749), beta-casein (gi:31341343), kappa-casein (gi:27881411), alpha-lactalbumin (gi:31342165), beta-lactoglobulin (gi:81294269), IGFBP3 (gi:27807006), IGFBP5 (gi:157427781), RPS6KA1 (gi:139948214) and G1/S-specific cyclin-D1 (gi:114053226). The PatSearch algorithm (http://itbtools.ba.itb.cnr.it/patsearch) was used to identify putative 4E-SE (eIF4E-sensitivity element) corresponding to the conserved sequence element UX2UX2A within the 3'UTR.

3.4 Results

3.4.1 Effect of GH treatment on mammary gland weight and biochemical indices

Total parenchymal RNA (33%) and protein (34%) tended to be increased, while DNA concentration tended to decrease (20%) in GH-treated compared to control cows (Table 3.2). Cell size tended to be increased by 30% in GH-treated compared to control cows, while there was no change in total parenchymal DNA content (cell number). Mammary gland weight was increased such that GH-treated cows had a 33% heavier mammary gland compared to control cows. These results indicate greater total ribosome number (protein synthetic capacity), which supports increased mammary gland protein content leading to increased cell size, mammary gland weight and decreased DNA concentration.

Table 3.2. Growth hormone (GH) treatment increased mammary gland weight, total parenchymal RNA and protein content and cell size (Protein:DNA), whilst a decrease in DNA concentration was observed. Each control and GH value (excluding mammary gland weight) represents the average of three independent extractions and their pooled SEM. *P* ≤ 0.05; *P* ≤ 0.15.

Measurement	GH n = 4	Control n = 4	SEM	P-value
Mammary gland weight, g	9336	7000	472	0.030
Total parenchymal content, mg				
DNA	17.8	16.5	1.9	0.678
RNA	35.3	26.6	2.8	0.104
Protein	806	601	52	0.058
Parenchymal concentration, mg/g				
DNA	1.9	2.4	0.1	0.093
RNA	3.8	3.9	0.4	0.909
Protein	86.8	85.6	6.8	0.910
Parenchymal ratio				
RNA:DNA	2.13	1.63	0.29	0.318
Protein:DNA	47.1	36.1	4.0	0.142
Protein:RNA	24.0	22.8	2.5	0.762

3.4.2 Effect of GH treatment on the mTORC1 signalling pathway

The mTOR pathway has been identified in mammalian cells to regulate ribosomal RNA transcription (123) and mRNA nucleocytoplasmic export and translation (147). Treatment with GH increased the abundance of total and $mTOR^{Ser2448}$ (Figure 3.1) and the abundance of complexed 4EBP1:eIF4E compared to control cows (Figure 3.2). No change in the abundance of total 4EBP1, $4EBP1^{Ser65}$ total $p70/p85^{S6K}$ and $p70^{S6K}Thre389/p85^{S6K}Thre412$ (Figure 3.1) was observed between treatment groups. No change in relative activation was observed for any of the mTOR pathway proteins measured between treatment groups (Figure 3.3).

Figure 3.1. Growth hormone (GH) treatment increased the abundance of total and Ser2448 phosphorylated mTOR compared to controls. No treatment effect was observed for 4EBP1, 4EBP1^{Ser65}, p70S6K, p70^{S6K}Thre389, p85S6K or p85^{S6K}Thre412. The figure shows values for each animal measured, Growth hormone (GH) treatment increased the abundance of total and Ser2448 phosphorylated mTOR compared to controls. No treatment effect was observed for 4EBP1, 4EBP1^{Ser65}, p70^{S6K}, p70^{S6K}Thre389, p85^{S6K} or p85^{S6K}Thre412. The figure shows values for each animal measured, n=4/treatment (•), the mean of those values (represented by the bars) ± SE (represented by the lines). *P ≤ 0.05, †P ≤ 0.15.

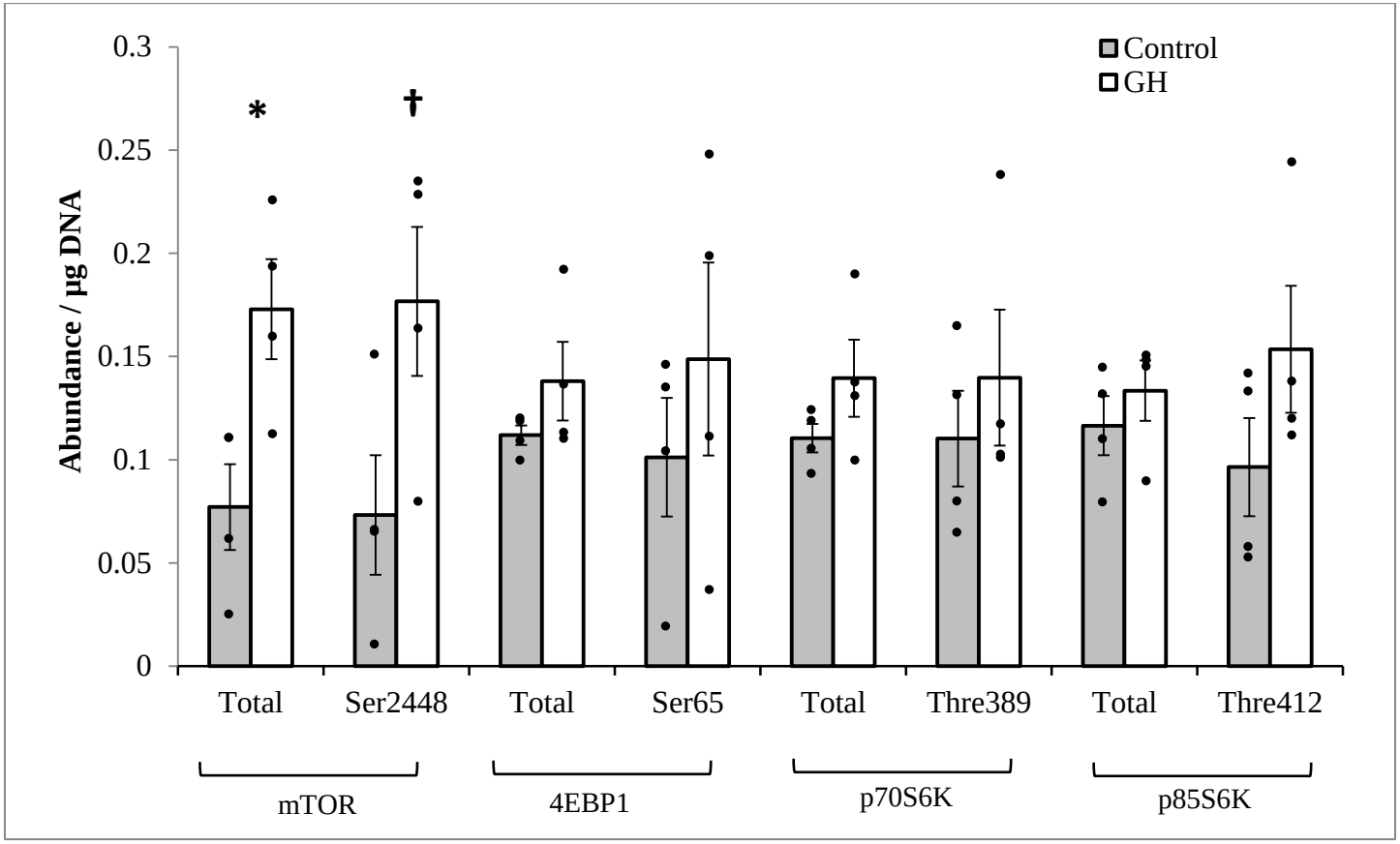
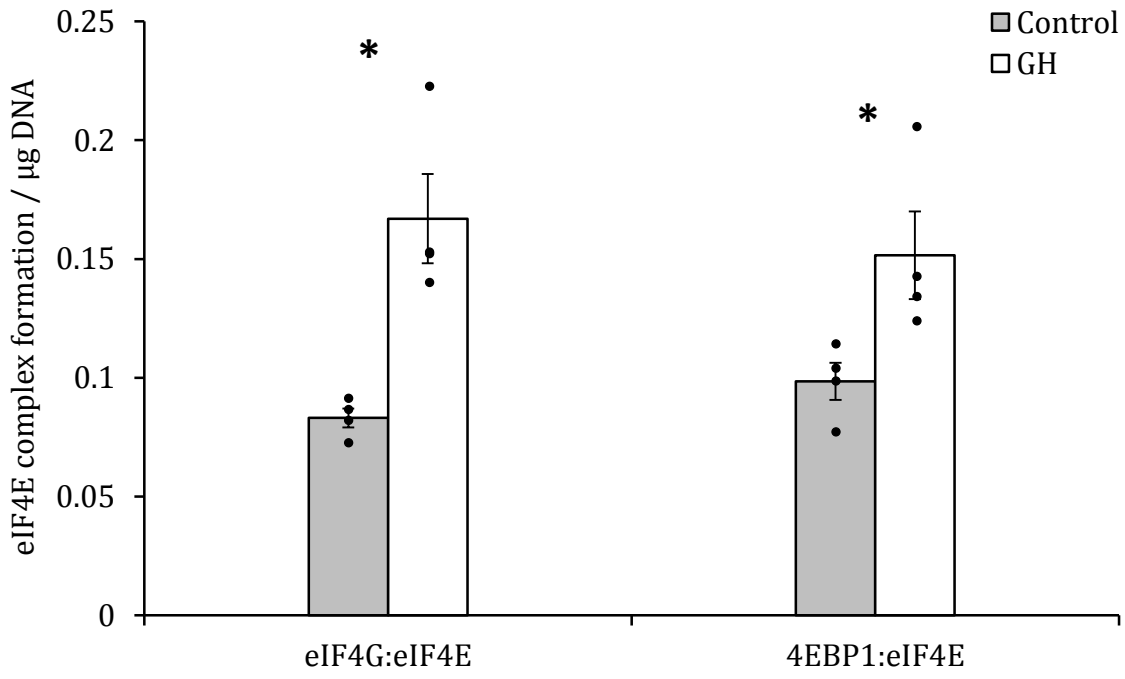


Figure 3.2. Growth hormone (GH) treatment increased the association of total 4EBP1 and eIF4G with total eIF4E. The figure shows values for each animal measured, n=4/treatment (*), the mean of those values (represented by the bars) ± SE (represented by the lines), *$P \leq 0.05$.



3.4.3 Effect of GH treatment on the MAPK signalling pathway

The MAPK pathways regulate proliferation and differentiation, in part, by controlling the protein translation machinery (148). They are activated by mitogenic stimuli such as growth factors and cytokines, such as IGF1. The abundance of MAPK pathway proteins total eIF4E, eIF4E^{Ser209}, total MKNK1 and MKNK1$^{Thre197/202}$, total RPS6KA1 (Figure 3.4) and eIF4E:eIF4G complex formation (Figure 3.2) was increased in GH-treated compared to control cows. No change in relative activation was observed for any of the MAPK pathway proteins measured between treatment groups (Figure 3.3).

Figure 3.3. Growth hormone (GH) treatment had no effect on the relative activation of mTOR or MAPK signalling. The figure shows values for each animal measured, n=4/treatment (*), the mean of those values (represented by the bars) ± SE (represented by the lines).

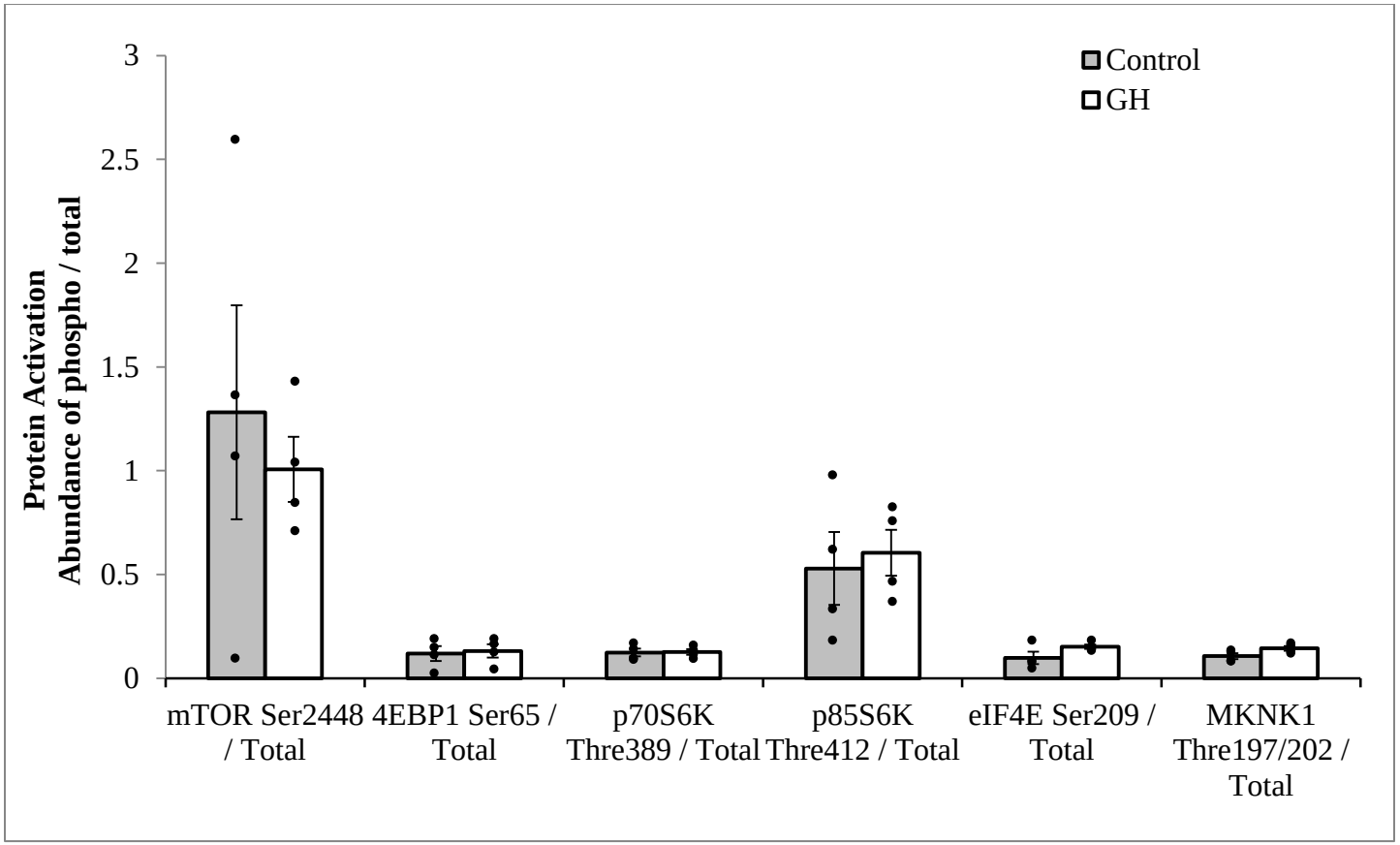
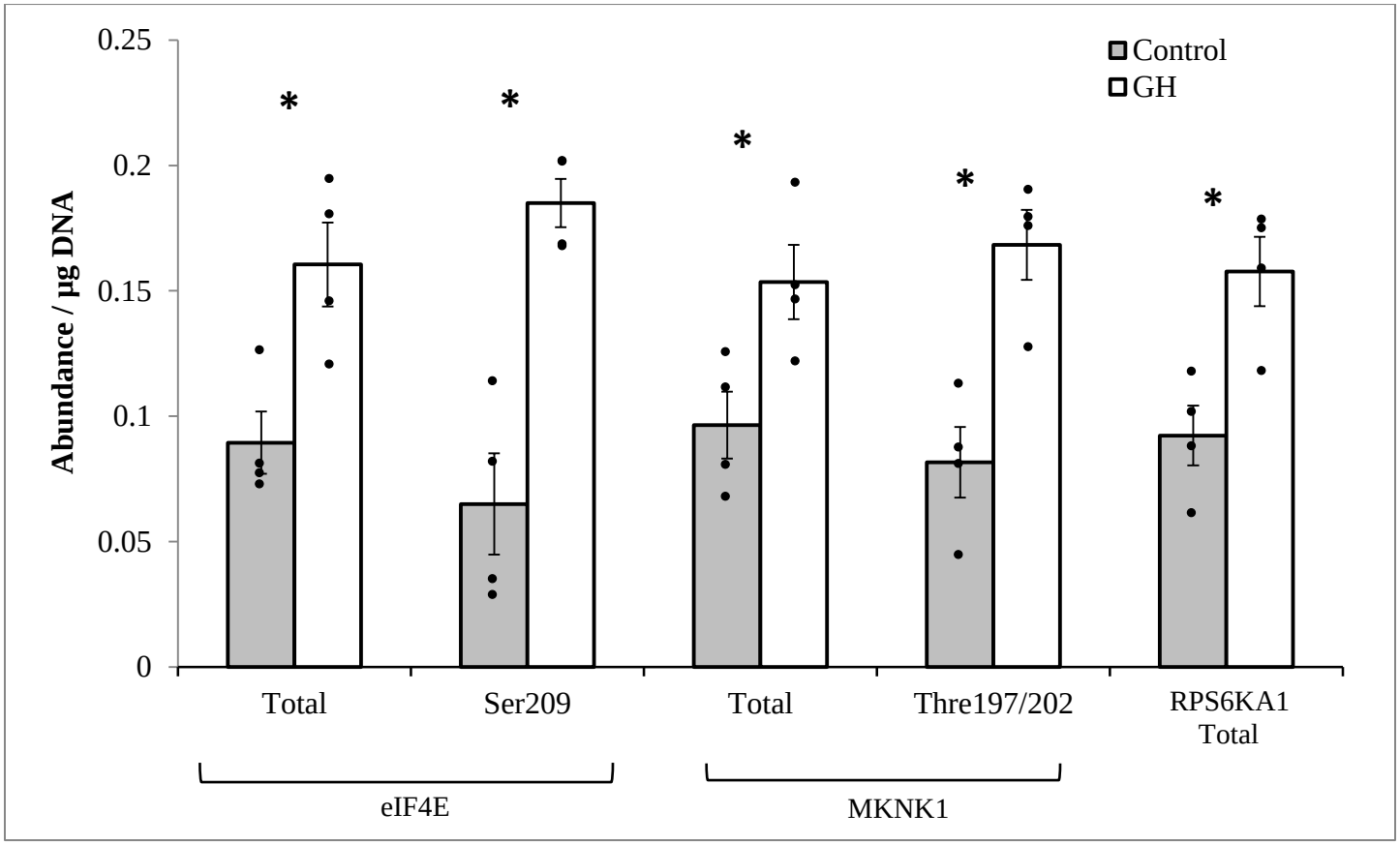


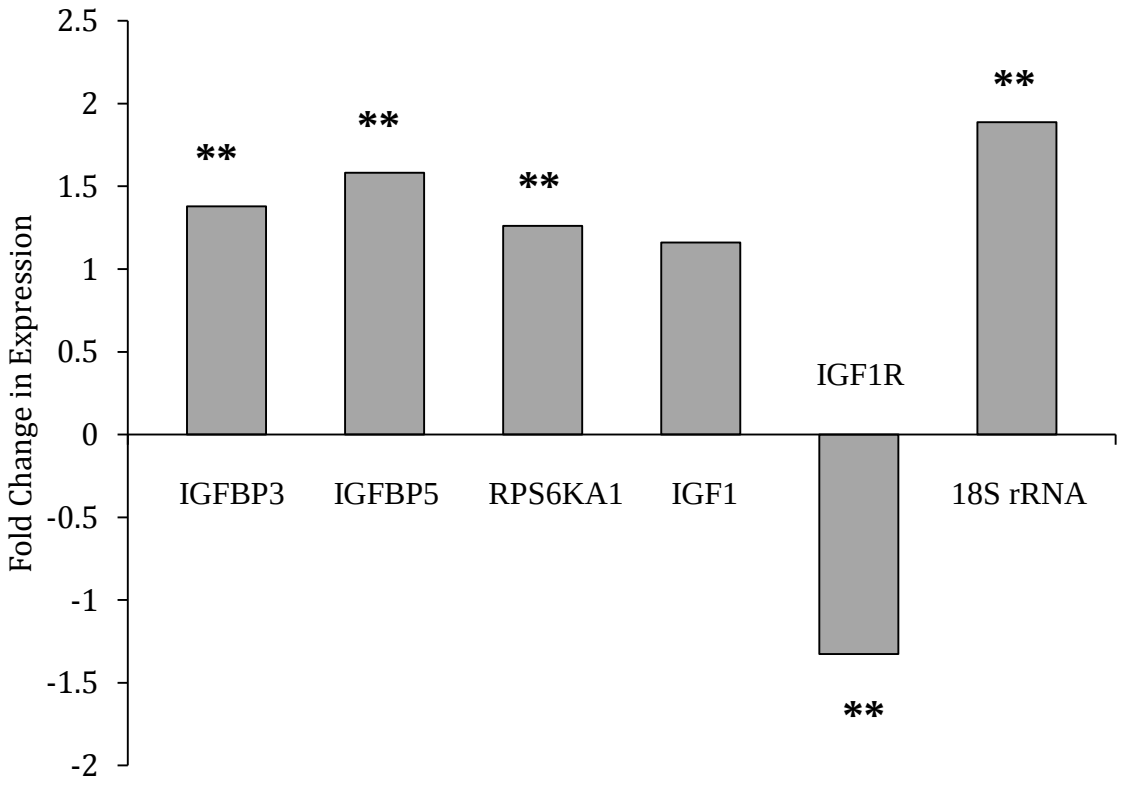
Figure 3.4. Growth hormone (GH) treatment increased the abundance of MAPK signalling proteins eIF4E, eIF4E^{Ser209}, MKNK1, MKNK1$^{Thre197/202}$ and RPS6KA1. The figure shows values for each animal measured, n=4/treatment (*), the mean of those values (represented by the bars) ± SE (represented by the lines), *$P \leq 0.05$.



3.4.4 Effect of GH treatment on mammary gland gene expression

The mRNA abundance of IGFBP3, IGFBP5, 18S rRNA, and RPS6KA1 were increased by 1.4, 1.6, 1.8, and 1.3-fold, respectively, of GH animals compared to controls. IGF1R decreased by 1.3-fold and IGF1 remained unchanged in the mammary glands of cows treated with GH compared with controls (Figure 3.5).

Figure 3.5. Growth hormone (GH) treatment increased the abundance of MAPK signalling proteins eIF4E, eIF4ESer209, MKNK1, MKNK1Thre197/202 and RPS6KA1. The figure shows values for each animal measured, n=4/treatment (·), the mean of those values (represented by the bars) ± SE (represented by the lines), *P ≤ 0.05.



3.4.5 Effect of GH treatment on eIF4E-mediated nucleocytoplasmic export of mRNA

Growth promoting IGFBP3 and RPS6KA1, and milk proteins alpha-S1-casein, alpha-S2-casein and beta-casein mRNAs were shown to contain putative 4E-SE, linking their increased expression in mammary glands of GH cows to the increased abundance of eIF4E and eIF4E^{Ser209}. Pro-apoptosis marker IGFBP5 and milk protein kappa-casein and alpha-lactalbumin mRNAs were elevated in mammary glands of GH compared with control cows, yet contained no 4E-SE, indicating their export is driven by an eIF4E-independent mechanism.

3.5 Discussion

This study provides new knowledge of potential molecular mechanisms regulating milk protein synthesis in the mammary gland of lactating dairy cows treated with GH. We postulate that in response to GH, the IGF1-IGF1R-MAPK signalling cascade regulates eIF4E-mediated nucleocytoplasmic export and translation of mRNA; whereas an as yet unidentified signalling cascade mediated by mTOR controls cell renewal, turnover and rRNA transcription.

3.5.1 GH treatment increases mammary gland protein synthetic capacity

In mammalian cells the most abundant RNA species is rRNA, accounting for >80% of the total cellular RNA content (149). RNA is made up of three subspecies, 5S, 18S and 28S which are used by cells to manufacture ribosomes, which are factories used to translate mRNA into protein. In this study, we demonstrate that GH treatment potentially increased the protein synthetic capacity of the lactating mammary gland by increasing 18S rRNA mRNA abundance, total parenchymal RNA, and by association total parenchymal rRNA. We also observed no effect on parenchymal cell number, consistent with previous cow and goat lactation studies which show GH does not exert its effect on the mammary gland by increasing secretory cell number, but by increasing cellular hypertrophy (37, 150).

3.5.2 Elevated total and activated mTOR abundance is associated with increased mammary gland protein synthetic capacity

The observed increase in total parenchymal RNA was associated with an increase in total parenchymal protein, indicating that increased protein synthesis occurs in response to GH

treatment. The regulation of protein synthesis is a highly coordinated and intricately linked three-step process involving the expression and nucleocytoplasmic export of specific mRNAs and their translation into protein by ribosomes. Ribosomal RNA transcription (149) and mRNA nucleocytoplasmic export and translation (147) in mammalian cells are regulated by mTOR signalling. In this study the abundance of total and mTOR^{Ser2448} was increased in response to GH compared to controls, indicating that the mTOR pathway plays a potential role in mediating the effect of GH in the lactating mammary gland.

The nucleocytoplasmic export of specific mRNAs and their translation into protein is regulated by the mTOR pathway and is mediated by several downstream factors common to each process, 4EBP1, p70^{S6K} and p85^{S6K} (112, 147). The 4EBP1 protein has an estimated nuclear to cytoplasmic ratio of 30:70, where its principal role in both sub-cellular compartments is to bind eIF4E (112). Phosphorylation of 4EBP1 by mTOR, at Ser65, releases eIF4E. In the nucleus this leads to down regulation of eIF4E-mediated mRNA export, while in the cytoplasm this leads to increased initiation of protein synthesis. In response to GH treatment, 4EBP1:eIF4E complex formation was increased, but no change in the abundance of total and activated 4EBP1 was observed. This result agrees with the findings of Toerien and Cant (2007) who reported increased 4EBP1:eIF4E complex in lactating dairy cows compared with non-lactating and concluded that this was due to the gland not operating at maximum capacity. Based on observations from the present study it is more likely that in response to increasing levels of protein synthesis, secretory cells sequester more eIF4E into the nucleus via its interaction with 4EBP1 to support elevated nucleocytoplasmic export of specific mRNAs.

Regulation of protein synthesis by mTOR signalling also occurs via Thre389/412 phosphorylation of p70/p85^{S6K}, enhancing the transcription of specific mRNAs and phosphorylation of RPS6 at Ser235/236 (151). We have previously shown that GH treatment increased RPS6$^{Ser235/236}$ phosphorylation (9), a well-established change associated with increased cell size (151). In this study cell size was increased however the abundance of total and p70/p85^{S6K} Thre389/412 was unaffected. These results indicate that the regulation of RPS6$^{Ser235/236}$ phosphorylation and potentially cell size occurred via an mTOR-independent mechanism. The phosphorylation of RPS6$^{Ser235/236}$ can also be regulated by RPS6KA1, a downstream target of the MAPK signalling cascade (152). We observed that in response to GH treatment the abundance of RPS6KA1 increased; suggesting MAPK signalling also controls RPS6$^{Ser235/236}$ phosphorylation.

The relative abundance of phosphorylated mTOR^{Ser2448}, 4EBP1^{Ser65} or p70/p85^{S6K} Thre389/412 to total abundance (activation) did not change in GH compared to control cows, contrasting other ruminant lactation studies that showed elevated protein synthesis was associated with increased activation of signalling components upstream and downstream of the mTOR pathway (9, 153, 154). We postulate, based on the results from this study that in response to GH treatment, the protein synthetic capacity of the mammary gland is increased via mTOR regulation of factors that control rRNA transcription, consistent with the model of chronic increased protein synthesis proposed by Wang and Proud (2006).

3.5.3 Changes in MAPK signalling are associated with increased nucleocytoplasmic mRNA export and translation

Located at the 5'-end of an mRNA molecule is the m7G cap, a structure required for its cytoplasmic translation into protein (94). In the nucleus, a subset of mRNAs utilise the 5'-m7G cap and an extra stem-loop pair in the 3-UTR known as the 4E-SE, to mediate their export to the cytoplasm (94). Each process is mediated by the protein eIF4E, which is bound to the 5'-m7G cap. Cytoplasmic eIF4E initiates mRNA translation by binding eIF4G and recruiting the multiprotein eIF4F complex to the 5'-m7G cap (155). We observed that formation of the eIF4E:eIF4G complex is increased in response to GH, suggesting that the observed increase in total parenchymal protein could be a function of elevated mRNA translation. Nuclear eIF4E binds both the 5'-m7G cap and 4E-SE to mediate the export specific mRNAs involved in cellular growth and proliferation (94). Results from this study using a PatSearch analysis of genes known to be up-regulated in response to GH showed growth promoting IGFBP3 and RPS6KA1, and milk proteins alpha-S1-casein, alpha-S2-casein and beta-casein mRNAs which contain putative 4E-SE. Therefore, nuclear eIF4E may play a role in mediating the export of mRNAs that encode proteins used for cell survival and milk protein synthesis in response to GH. This is the first study to show that milk protein mRNAs contain 4E-SE, and that their presence is associated with increased expression in the lactating mammary gland of the GH supplemented cow.

The abundance of total and eIF4E^{Ser209}, and the eIF4E:eIF4G complex increased in response to GH. These results suggest that rather than utilising the mTOR signalling pathway to phosphorylate 4EBP1^{Ser65} and relieve the repression of protein synthesis, secretory cells in

the mammary gland increase eIF4E production. This method of elevating protein synthesis has been well characterized in mammalian cells undergoing oncogenic transformation (129). There is also evidence that mammalian cells may increase eIF4E production in response to increased expression of mRNA containing 4E-SE to mediate their export from the nucleus (94). The role of eIF4E^{Ser209} is still the subject of investigation, but its elevation in response to GH fits with the two mechanisms proposed in the literature. Firstly, Zuberek (2003) (156) using intein-mediated protein ligation generated pure eIF4E, selectively phosphorylated at Ser 209. Results from this study showed eIF4E^{Ser209} has less affinity for the 5'-m7G. These authors propose that the reduced interaction allows eIF4E to utilise a "skipping mechanism." In this model eIF4E recruits an eIF4F complex to the 5'-m7G. Once translation has begun, eIF4E can dissociate from the complex and "skip" to the 5'-m7G of another mRNA molecule, recruiting another eIF4F complex, leading to increased protein translation initiation. Secondly, Topisirovic (2004) (114) reported that nuclear eIF4E^{Ser209} has less affinity for 5'-m7G, and propose this feature aids in eIF4E-mediated transport of mRNA from the nucleus of NIH3T3 cells. Interestingly, eIF4E:eIF4G complex formation and elevation of eIF4E^{Ser209} phosphorylation are controlled by MKNK1, a kinase regulated by the MAPK pathway (148). We observed that the abundance of total and MKNK1$^{Thre197/202}$ was increased in response to GH, providing further support to our hypothesis that elevated eIF4E^{Ser209} and eIF4E:eIF4G formation in response to GH treatment occurs via MAPK signalling.

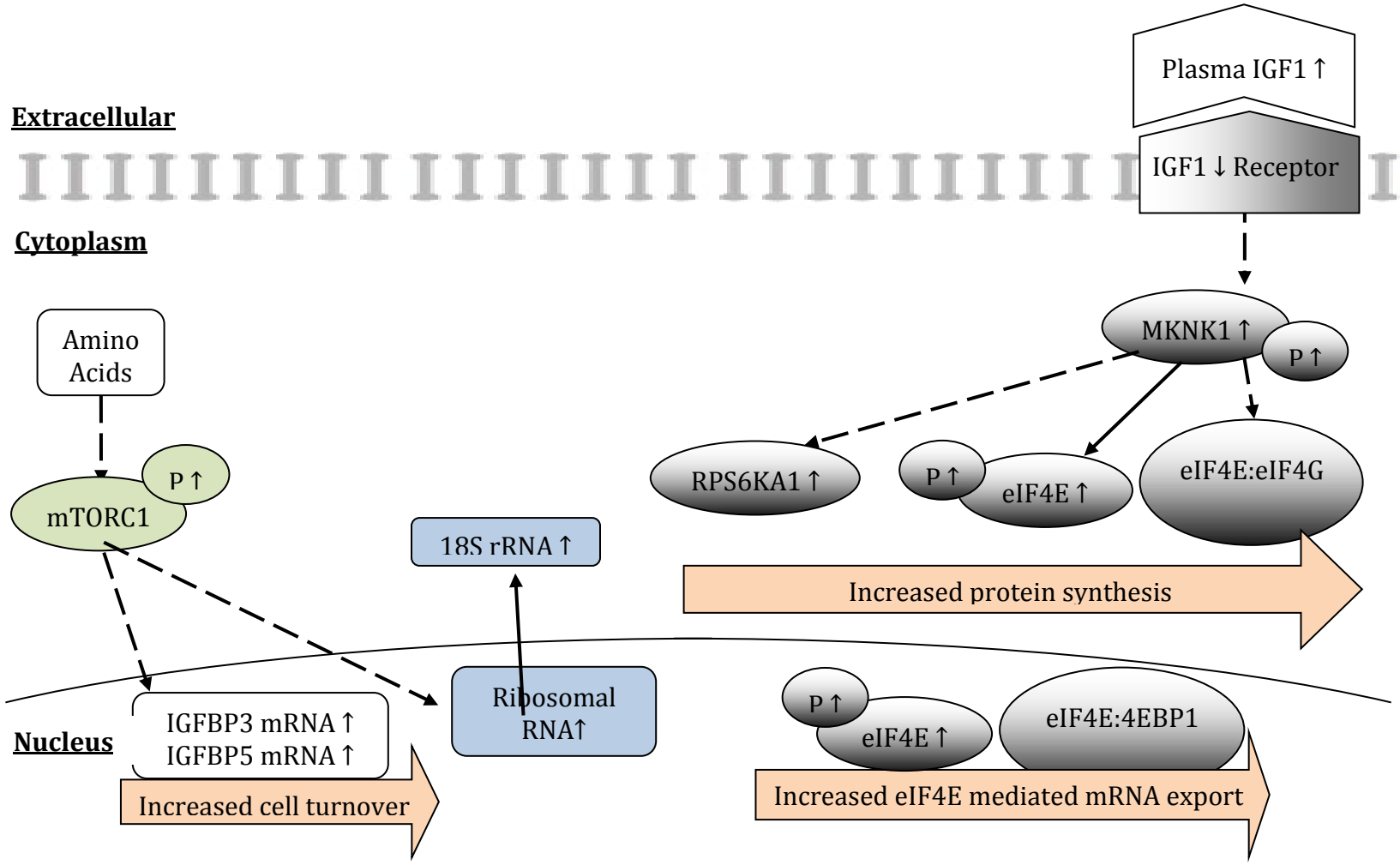
3.5.4 Changes in IGF1-IGF1R signalling potentially regulate the MAPK and mTORC1 pathways

Previously, researchers have reported that circulating levels of IGF1 increase in response to GH treatment (9, 157), which suggests that GH may exert its influence on the mammary gland by utilising the IGF1-IGFR signalling cascade, The IGF1R auto-phosphorylates and activates downstream signalling pathways in response to IGF1 binding (158). The two classical signalling pathways activated by IGF1 are the phosphatidylinositol-3 kinase (PI3K) and MAPK pathways. The IGF1-IGFR-MAPK signalling cascade regulates nucleocytoplasmic export and translation initiation of mRNA, while the IGF1-IGFR-PI3K-mTOR signalling cascade regulates IGFBP3 and IGFBP5 expression (158). Increased IGFBP3 expression aids cell survival and proliferation, whilst increased IGFBP5 has been linked to apoptosis and remodelling of the mammary gland (159). In vitro studies with the bovine mammary epithelial cell line MAC-T show that IGF1 stimulates mTOR pathway activation and protein synthesis (90). We observed that, expression of both IGFBP3 and IGFBP5 were increased in the mammary gland of GH-treated cows, suggesting that mTOR signalling may play a role in maintaining cell number in the mammary gland through increased cell turnover and renewal. This agrees with our biochemical indices results which showed cell number was unaffected by GH treatment. Gene expression analysis also showed that IGF1R mRNA was decreased whereas autocrine expression of IGF1 was unchanged. This may indicate that IGF1 signalling did not occur through an autocrine mechanism but rather a para- or endocrine. IGF1R expression decreased when the levels of IGF1 increased, an action that stimulated apoptosis and was associated with increased IGFBP3 expression (160). It is important to note that previous reports state that activation

of the IGF1-IGFR-MAPK signalling cascade reduced the expression of IGFBP3 and IGFBP5 (161). This reduction could indicate a potential uncoupling of mTORC1 and MAPK signalling via regulation from another pathway and warrants further investigation.

The findings from this study indicate that increased milk protein production in response to GH is potentially mediated by mTORC1 and MAPK signalling, stimulated by IGF1-IGF1R activation (Figure 4.6). Increased abundance of total and mTOR^{Ser2448} is associated with elevated total parenchymal RNA, an indicator of protein synthetic capacity. Increased abundance of total and mTOR^{Ser2448} is also linked to the maintenance of cell number (indicated by total parenchymal DNA content) potentially operating via regulation of increased expression of IGFBP3 and IGFBP5, which are measures of cell renewal and turnover. Increased abundance of total and phosphorylated components of the MAPK signalling pathway are associated with elevated eIF4E-mediated nucleocytoplasmic mRNA export and the initiation of mRNA translation in the cytoplasm. While decreased expression of IGF1R indicates increased levels of circulating IGF1 are directly signalling to the lactating gland through the MAPK and mTORC1 pathways. While the MAPK pathway principally responds to mitogens and hormones, mTORC1 has a far wider range of signalling inputs, ranging from energy, hypoxia and amino acids (107). Further research will be required to determine the potential role IGF1-IGF1R activation plays in regulating mTORC1 and MAPK signalling and to identify if other signalling pathways stimulate mTORC1 and MAPK.

Figure 3.6. Proposed model of the results presented in this study. Solid arrows represent data with direct links to results generated in this study, while dashed arrows represent data with indirect links supported by the literature.



CHAPTER 4: TREATMENT WITH EXOGENOUS GROWTH HORMONE IS ASSOCIATED WITH CHANGES IN PLASMA AND MAMMARY AMINO ACID PROFILES AND THE EXPRESSION OF MAMMARY GLAND AMINO ACID TRANSPORTERS.

The material presented in this Chapter will be submitted to the Journal PLOS ONE.

4.1 Abstract

The objectives of this study were to (1) to identify changes in plasma and mammary gland intracellular amino acids (AA) during increased milk protein synthesis observed in dairy

cows treated with growth hormone (GH), and (2) evaluate the expression of mammary gland genes involved in the transport and utilization of AA identified in (1). To investigate our objectives, plasma and mammary gland AA concentrations and the mRNA abundance of genes involved in mammary gland AA transport and utilization were measured in eight non-pregnant lactating dairy cows. Four cows in each group were treated with a single subcutaneous injection of either a slow-release formulation of commercially available GH (Lactotropin®) 500 mg or physiological saline solution. Six days after treatment, cows were milked and blood collected from the jugular vein for analysis of plasma free amino acids (FAA). Cows were euthanized and mammary tissue harvested. GH treatment decreased absolute plasma concentrations of lysine and tyrosine, and tended to decrease arginine. Absolute concentrations of intracellular glycine, serine and glutamate decreased significantly in the mammary glands of GH-treated cows. A trend for decreased arginine was also observed. Treatment with GH significantly reduced the variance of mammary intracellular but not plasma FAA concentrations. Amino acid changes were associated with increased mRNA abundance of the amino acid transporter SLC3A2, glycolytic/gluconeogenic enzymes PGK1 and GPI, and genes associated with fatty acid metabolism FABP3, FADS2. We propose that these changes occur to support elevated milk production in the mammary gland of GH-treated cows by increasing fatty acid synthesis, glycolysis, synthesis of milk protein AA and mTOR pathway activation.

4.2 Introduction

Amino acids (AA) are used by tissues both as building blocks for protein synthesis and as signalling molecules to regulate the protein synthetic machinery (86). During lactation the

ruminant mammary gland must import from the plasma and endogenously synthesize sufficient quantities of AA to support cell turnover, differentiation and elevated milk protein synthesis (162).

Changes in the concentration and transportation of AA are an important part of a wider, more complex network of events, which includes factors such as rumen digestion, absorption, differential hepatic metabolism and tissue competition for available AA. Identifying and understanding changes in plasma and mammary gland intracellular AA concentrations and AA transport into the lactating mammary gland may provide knowledge regarding this complex system (163).

Growth hormone (GH) is a well-established treatment model used by researchers to study mechanisms involved in the regulation of increased milk protein synthesis by the lactating ruminant mammary gland (40). Treatment of mid-lactation Rahmani ewes with exogenous GH can yield milk protein increases of 7% (164), and 12% in late-lactation Comisana ewes (165). Increased (42%) milk protein yield has also been observed in mid-lactation Alpine and Saanen goats treated with GH (166). Holstein cows treated with exogenous GH in early and late-lactation can yield protein increases of between 14% and 18%, respectively (167), 11% in mid-lactation Friesian cows (135) and 30% in mid-lactation Jersey cows (9). The mammary glands of cows treated with GH, elevate arteriovenous extraction of a range of AA from the blood to support increased milk protein production (168). Increased mammary gland arteriovenous AA extraction also occurs in goats, where increased milk yield is stimulated by altered frequency of unilateral milking (169).

Specific transporter families situated in the plasma membrane surface of mammary epithelial cells (MEC) mediate the extraction of AA from blood. Studies from a range of species (170), including bovine (171) indicate that the mammary gland may contain members of every known AA transporter family and that the expression of these transporters is regulated by stage of lactation, metabolic need, nutritional supply and specific hormonal changes (172).

The nutrient-sensing mechanistic target of rapamycin (mTOR) pathway is known to link AA sufficiency, hormonal signalling and AA transporter gene expression (64). The core of the mTOR pathway is the mTOR protein, which exists in two complexes mTORC1 and mTORC2 (173). Each complex plays a role in the regulation of protein synthesis with mTORC1 playing the major function via direct regulation of cellular machinery involved in nucleocytoplasmic export of growth-promoting mRNA and protein synthesis (173). We have previously reported that increased abundance of mTOR and phosphorylated at Ser2448 in the mammary glands of cows treated with exogenous GH occurred independently of GH induced IGF-1 signalling (105). *In vitro* and *in vivo* studies show activation of mTOR signalling can be stimulated by AA sufficiency and/or specific AA accumulation (84, 85). Inactive mTORC1 relocates from the cytoplasm to the lysosome in response to AA sufficiency or specific AA accumulation within the lysosomal lumen (86). Once in the lysosome, mTORC1 is activated via phosphorylation at mTOR (Ser2448) (87, 88). The precise role of individual AA transporters is yet to be fully elucidated, however the expression of a number of AA transporter genes has been linked to mTORC1 activation.

Increased expression of the L-glutamine transporter SLC1A5 elevates mTORC1 activation in both human hepatoma cells (174) and the mammary epithelial cell line MCF-7 (64), via interactions with the heterodimeric L-leucine / L-arginine transporter SLC3A2/SLC7A5 (64)

The aims of this study were to (1) identify changes in plasma and mammary gland intracellular AA during increased milk protein synthesis observed in dairy cows treated with growth hormone (GH), and (2) evaluate the expression of mammary gland genes involved in the transport and utilization of AA identified in (1).

4.3 Materials and methods

4.3.1 Animals and treatments

Described in the Materials and methods of Chapter 3.

4.3.2 Free amino acid profiles

Mammary tissue and plasma free amino acids (FAA) were analysed using High-Performance Liquid Chromatography (HPLC), as previously described (175), and reported as μmol per gram of mammary tissue or per litre of plasma. Differences between GH-treated and control animals were assessed via two-sample *t*-tests for individual AA using the TTEST procedure in SAS 9.1 (SAS Institute Inc., Cary, North Carolina, USA). Initial data exploration indicated that for some AA the variances were not homogenous between treatments. Thus, the analysis was done using the Cochran option to adjust the probability values for those AA with unequal variance per treatment. Both the probabilities for the null hypotheses of equal mean and equal variance are reported.

4.3.3 Quantitative real-time PCR

RNA Extraction, cDNA Synthesis and Primer Design **(Table 4.1)**

Described in the Materials and methods of Chapter 3.

qPCR Assay

Described in the Materials and methods of Chapter 3.

Table 4.1. Name, symbol, accession number, primer sequence and amplicon size of genes analysed by qPCR.

Gene Name	Symbol	Accession number	Hybridisation	Primers (5′ to 3′)	Amplicon Size (bp)
Reference Genes					
Mitogen-activated protein kinase 1	MAPK1	NM_175793.2	Forward Reverse	TCGCAGGAAGACCTGAATTG TCCTCTTGTGAGGGTTGAACG	165
(Src homology 2 domain) transforming protein 1	SHC1	NM_001164061.1 NM_001075305.2	Forward Reverse	CAGTCCATCTCGTTTGCATC GGCTCTTCCTCCTCCTCATC	260
Amino Acid Transporters					
Solute carrier family 1, member 1	SLC1A1	NM_174599.2	Forward Reverse	TCTGGTGGATTTCTTCAATGC T TAAAGGCCCAGTTTGCGG	151
Solute carrier family 1, member 5	SLC1A5	NM_174601.2	Forward Reverse	AGGAGAGATTGTTCAACGGC GAAGAAGCGAATGAGCAGC	152
Solute carrier family 3, member 2	SLC3A2	NM_001024488.2	Forward Reverse	AGCTGAGTGGCAGAACAT TTAAGCTGGAGTGTGACAGG TA	152
Solute carrier family 6, member 14	SLC6A14	NM_001098461.1	Forward Reverse	CTGATTGACCACTTCTGTGC GCAAGTTCTCCACCATAGCC	161
Solute carrier family 7, member 5	SLC7A5	NM_174613.2	Forward Reverse	TCTGCAGCACAGCGTTC GCTTTAAGGCAACGAGACGTA	154

4.4 Results

4.4.1 Effect of GH treatment on plasma and intracellular mammary tissue amino acid profiles

Growth hormone (GH) treatment decreased absolute plasma concentrations of the AA lysine, with a trend for decreased arginine, tyrosine and methionine (Table 4.2). Absolute intracellular concentrations of the L-amino acids glutamate, glycine and serine increased significantly in the mammary glands of GH-treated cows (Table 4.3). A trend for decreased arginine and increased total AA was also observed (Table 4.3). GH-treated cows had significantly reduced variance in the absolute concentrations of plasma AA methionine, valine, tyrosine and alanine, and an observed trend for leucine, asparagine and ornithine (Table 4.2). GH-treated cows had decreased variance in the absolute concentrations of mammary gland intracellular AA lysine, valine and glutamine with observed trends for tyrosine, phenylalanine, isoleucine, histidine, aspartate and alanine (Table 4.3).

Table 4.2. Growth hormone (GH) treatment of mid-lactation Jersey cows decreased absolute plasma concentrations of free L-amino acids lysine, and tended to decrease arginine and tyrosine. The table shows the mean (µmol per litre of plasma), the standard error of the mean (SE), and the P value of difference between treatment means, adjusted for those amino acids with unequal variances. Significance (P ≤ 0.05); trend (P ≤ 0.15).

Plasma Free Amino Acids						
Amino acid	Treatment				P-value	
	Control		Growth Hormone		Null Hypothesis of Equal Means	Null Hypothesis of Equal Variance
	Mean	SE	Mean	SE		
Essential						
Arginine	65	4.9	48	5.5	0.06	0.84
Histidine	42	3.6	37	4.8	0.42	0.67
Isoleucine	94	5.1	90	8.0	0.69	0.48
Leucine	71	4.0	58	10.8	0.30	0.13
Lysine	60	4.1	44	4.5	0.04	0.91
Methionine	17	4.0	9	0.3	0.14	<0.01
Phenylalanine	37	5.4	34	3.1	0.68	0.39
Tyrosine	48	9.8	23	2.0	0.09	0.03
Valine	163	3.3	163	17.1	0.98	0.02
Non-essential						
Alanine	166	6.9	101	35.4	0.17	0.02
Asparagine	26	2.6	29	8.3	0.71	0.09
Aspartate	3	0.4	4	1.1	0.26	0.16
Glutamate	30	2.8	34	3.1	0.32	0.85
Glutamine	252	10.6	228	13.2	0.21	0.74
Glycine	305	32.1	286	36.8	0.71	0.83
Proline	50	4.9	39	5.2	0.19	0.91
Serine	112	8.5	116	11.1	0.80	0.67
Others						
Ornithine	45	1.6	52	10.7	0.56	0.01
Total	1745	57.4	1604	73.8	0.18	0.69

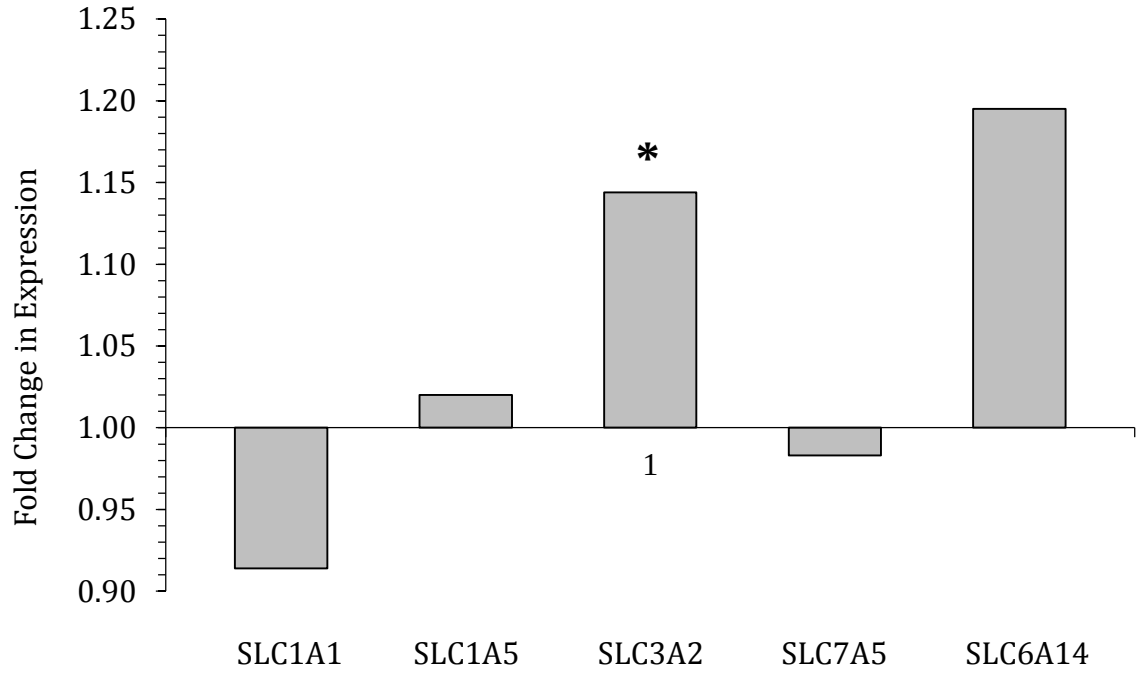
Table 4.3. Growth hormone (GH) treatment of mid-lactation Jersey cows increased absolute concentrations of mammary intracellular free L-amino acids glutamate, glycine and serine, and tended to decrease arginine and increase total free amino acids. The table shows the mean (µmol per gram of tissue), the standard error of the mean (SE), and the P value of difference between treatment means, adjusted for those amino acids with unequal variances. Significance (P ≤ 0.05); trend (P ≤ 0.15).

Mammary Gland Intracellular Free Amino Acids						
Amino acid	Treatment				P-value	
	Control		Growth Hormone		Null Hypothesis of Equal Means	Null Hypothesis of Equal Variance
	Mean	SE	Mean	SE		
Essential						
Arginine	0.06	0.023	0.01	0.009	0.10	0.14
Histidine	0.05	0.011	0.04	0.003	0.34	0.09
Isoleucine	0.06	0.025	0.08	0.008	0.37	0.09
Leucine	0.12	0.034	0.11	0.011	0.80	0.11
Lysine	0.13	0.043	0.09	0.004	0.49	<0.01
Methionine	0.02	0.014	0.02	0.006	0.74	0.18
Phenylalanine	0.05	0.030	0.06	0.009	0.79	0.08
Tyrosine	0.04	0.025	0.05	0.007	0.78	0.08
Valine	0.16	0.055	0.19	0.014	0.65	0.05
Non-essential						
Alanine	1.90	0.438	1.91	0.157	0.98	0.13
Asparagine	0.09	0.008	0.11	0.007	0.20	0.78
Aspartate	0.57	0.101	0.64	0.029	0.54	0.07
Glutamate	4.51	0.487	7.17	0.353	<0.01	0.61
Glutamine	1.11	0.789	0.18	0.034	0.33	<0.01
Glycine	1.78	0.101	2.28	0.109	0.02	0.91
Proline	0.21	0.030	0.21	0.031	0.99	0.99
Serine	0.41	0.040	0.56	0.026	0.02	0.51
Others						
Ornithine	0.06	0.008	0.06	0.003	0.56	0.16
Total	12.39	1.049	15.25	0.727	0.07	0.56

4.4.2 Effect of GH treatment on amino acid transporter gene expression

Treatment with GH increased mRNA abundance of the amino acid transporter SLC3A2 by 1.15-fold (Figure 4.1). The abundance of SLC1A1, SLC1A5, SLC7A5 and SLC6A14 mRNA did not change significantly.

Figure 4.1. Growth hormone (GH) treatment increased the expression of the heterodimeric heavy-chain L-arginine transporter SLC3A2. No significant gene expression changes were detected for any of the other transporters measured. MAPK and Src homology 2 domain containing 1 (SHC1) had the most stable expression of all the genes analyzed using Normfinder and were used as reference genes. The figure shows the fold change in gene expression in GH animals compared with control animals (n = 4/treatment) ± SE (represented by the lines), *P ≤ 0.05.



4.5 Discussion

In this study we identified three plasma FAA (lysine, tyrosine and arginine) and four mammary gland intracellular FAA (glutamate, glycine, serine and arginine) whose concentrations changed in response to increased milk protein synthesis observed in dairy cows treated with GH. We propose that these changes occur to support elevated milk production in the mammary gland of GH treated cows by increasing fatty acid synthesis, glycolysis, synthesis of milk protein AA and mTOR pathway activation.

The lactating mammary gland synthesizes protein in response to a wide range of external stimuli, and modulates the uptake of plasma FAA to support changing protein output (176). Treatment with growth hormone (GH) increases milk protein synthesis in sheep, goats and cows. In this study, treatment with GH caused reductions in the variance of eight of the nine essential AA in mammary tissue and only four of the nine essential AA in the plasma. We propose that the greater variance reduction in mammary tissue essential AA compared to plasma indicates a direct effect of GH on the lactating gland and could be an orchestrated event by the lactating gland to optimize AA supply relative to protein synthetic activity and the energy status.

Circulating plasma is the primary source of AAs for the production of milk protein by the lactating ruminant mammary gland (168). Amino acids extracted from circulating plasma by the lactating ruminant mammary gland can be classed into three groups. Group 1 AA are those essential amino acids (EAA) that are transferred from plasma to milk protein in a ratio close to 1:1, and include Tyr (+Phe). The remaining EAA taken up in excess of milk

protein output constitute group 2, foremost of which are Arg and Lys. The remaining AAs form group 3 and are generally not taken up from plasma in sufficient quantities to account for their secretion in milk and include the non-essential AA. In this study, we demonstrate that treatment of mid-lactation cows with GH decreases the concentration of plasma Lys, Arg and Tyr. This result partly agrees with the results by Hanigan et al (1992) who reported that Holstein cows treated with exogenous GH increased mammary gland arterio-venous extraction of Lys and Arg to support elevated milk protein synthesis. The decrease in plasma Tyr observed in this study was not described in the study by Hanigan et al (1992) or any previous studies investigating the effect of exogenous GH administration on lactation performance, nor has it been identified as a potentially limiting AA as it is readily produced from the metabolism of Phe.

The net removal of Lys and Arg by the ruminant mammary gland from circulating plasma is in excess of its requirements for milk protein synthesis (177). This indicates that Lys and Arg may serve role(s) within the gland additional to substrates for milk protein synthesis. A considerable amount (20 to 35%) of Lys taken up by the lactating ruminant mammary gland is oxidized (178). In mammalian cells the oxidative metabolism of Lys ends in the production of acetyl-CoA, an essential precursor of fatty acid synthesis and component of the Krebs's cycle production of NEAA and lactose (179). In another study, (McCoard, *unpublished*) microarray and qPCR validation data collected from the same tissues, shows expression of two key enzymes involved in converting acetyl-CoA into fatty acid precursors, FABP3 (95) and FADS2 (180) were increased in the mammary glands of GH-treated cows. In this study, intracellular levels of glutamate, a by-product of Lys

metabolism to acetyl-CoA (181) were elevated in response to GH and expression of the primary glutamate AA transporter SLC1A1 was unaffected. These results suggest that the mammary glands of GH-treated cows increase the extraction of plasma Lys to maintain an intracellular supply of acetyl-CoA to support increased milk fat production, (9) and as a by-product, elevate intracellular glutamate concentrations.

Glutamate was not the only mammary intracellular AA affected by GH treatment. Concentrations of Ser and Gly, both of which are not taken up in sufficient quantities to account for the AA in secreted milk protein from goats and cows (182), were also increased in response to GH. Analysis of microarray and qPCR validation data collected from another study, using the same tissues, shows that expression of the glycolytic pathway genes phospho-glycerate kinase 1 (PGK1) and glucose-6-phosphate isomerase (GPI) were increased in the mammary glands of cows treated with GH (McCoard et al, *unpublished*). A function of glycolysis in the lactating ruminant mammary gland is the production of pyruvate. Pyruvate and other amino acids can be used as carbon skeletons for the synthesis of AA required to support lactation via the Krebs cycle. The ruminant mammary gland endogenously synthesizes Ser using pathway intermediates of glycolysis (3-phosphoglycerate) and the Krebs cycle (α-ketoglutarate), whilst Gly is directly synthesized from Ser (183). Interestingly, PGK1 catalyses the glycolytic production of 3-phosphoglycerate, the direct precursor of Ser biosynthesis. Collectively, these results suggest the mammary glands of GH-treated cows increase glycolysis to provide carbon skeletons for the production of AA via the Krebs cycle, and the glycolytic pathway precursor 3-phosphoglycerate for the production of Ser and Gly.

Arginine was the only AA whose plasma and intracellular mammary gland concentrations were affected by treatment with exogenous GH. The observed decrease in plasma Arg coincided with increased expression of the AAT SLC3A2, which co-transports plasma Arg into cells and forms the heavy chain of a heterodimeric complex that has a reciprocal regulatory connection with mTOR (64). Studies show that SLC3A2 activity can be driven by SLC1A5 import of Gln, which is rapidly effluxed by SLC3A2 when complexed with SLC7A5 (64). In this study, the expression of SLC7A5 and SLC1A5 was unaffected by GH treatment, indicating other regulatory partners of SLC3A2 such as SLC7A1, A2, A6 or A7 may be involved in Arg uptake from circulating plasma.

Of all the AA extracted from circulating plasma, Arg has the highest uptake relative to milk protein outputs (200 - 300% (67)). Excess Arg (not incorporated into milk protein) is catabolized via two pathways, nitric-oxide synthesis or ornithine production (184). The production of nitric oxide by mammary epithelial cells harvested from milk (185) and glandular tissue (186) aids in regulating their own local nutrient environment through alteration of the capillary blood supply and can be used to modulate immune function of the mammary gland. The production of ornithine feeds into the synthesis of proline, an AA which is not taken up in sufficient quantities from circulating plasma to support both milk protein synthesis and polyamine synthesis, the latter involved in the regulation of cellular differentiation and proliferation (184).

The diversity of roles attributed to Arg in the lactating gland fit with its proposed function as an activator of the nutrient sensing, mechanistic target of rapamycin (mTOR) pathway, in a wide range of tissues and species: *in-vitro* (71, 187-189) *in-vivo* (85). Supplementation with Arg increases milk protein synthesis *in vitro* (190) and *in vivo* (191), and previously published results using the same tissues show the abundance of mTOR and (phosphorylated) mTOR (Ser2448) increased in the mammary glands of cows treated with exogenous GH, independently of GH induced IGF-1 signalling (105). These findings suggest Arg may directly or indirectly regulate mTOR abundance and activation in the mammary glands of GH treated cows via SLC3A2. Thus, in Chapters 6 and 7 of this thesis, the objective was to establish an *in vitro* model that reproduced the chronic (long term) nature of bovine lactation. The resultant model could then be used to study the potential role SLC3A2 and Arg play in regulating mTOR signalling, and the effect on milk protein synthesis.

CHAPTER 5: EFFECT OF PARENTERAL L-ARGININE ADMINISTRATION ON MATERNAL MAMMARY DEVELOPMENT AND FUNCTION

The material presented in this Chapter will be submitted for publication in the Journal of Nutrition

5.1 Abstract

Arginine is one of the most metabolically versatile amino acids and has been reported to improve mammary gland function and development in a number of species. The objective

of this study was to determine if L-arginine supplementation during mid-late pregnancy in twin-bearing ewes influenced maternal mammary gland development, mTOR abundance and activation pre-partum, and lactation performance in the early post-partum period. Two cohorts of animals were used in this study. Cohort 1 was administered L-arginine from d 100 of pregnancy until d 140. At d 140 ewes were euthanized and maternal mammary tissue collected for analysis of biochemical indices and mTOR pathway activation. Cohort 2 was administered L-arginine from d 100 until parturition. At parturition, treatment ceased and ewes allowed to lamb naturally. Milk was collected over a 14 day period (day 1, 4, 7, 10, 14) to assess milk yield and composition. Arginine administration decreased milk somatic cell count (d 7 – 14), the associated effects of high milk somatic cell count on crude protein percentage (d 7 – 10) and absolute concentrations of some free amino acids. Increased maternal mammary hyperplasia was observed at d 140 of pregnancy, this was associated with increased plasma insulin and no change in mTOR protein abundance or activation. In conclusion, our results demonstrate that parenteral L-arginine administration (d 100 – parturition) improves maternal mammary gland health and hyperplasia.

5.2 Introduction

A critical development phase of the ruminant mammary gland is during pregnancy (192). The size of the mammary gland precipitously increases (193) to support an elevation in the number, size and milk production potential of secretory cells (192). At parturition, the

numbers of secretory cells in the mammary gland are close to their maximum complement, while secretory cell milk production potential remains plastic (194). Thus, pregnancy provides an opportune timeframe to develop intervention strategies aimed at increasing subsequent lactation performance. Supplementation with specific nutrients, in particular amino acids (AA), may play an important role in stimulating mammary gland development and function (195). Of particular interest is the AA L-arginine. L-arginine is essential for mammary gland development in pregnant rats (196), increases milk yield when supplemented during late-pregnancy in Holstein cows (191) and is reduced in the plasma and mammary gland of mid-lactation Jersey cows with increased milk production after growth hormone administration (Chapter 4).

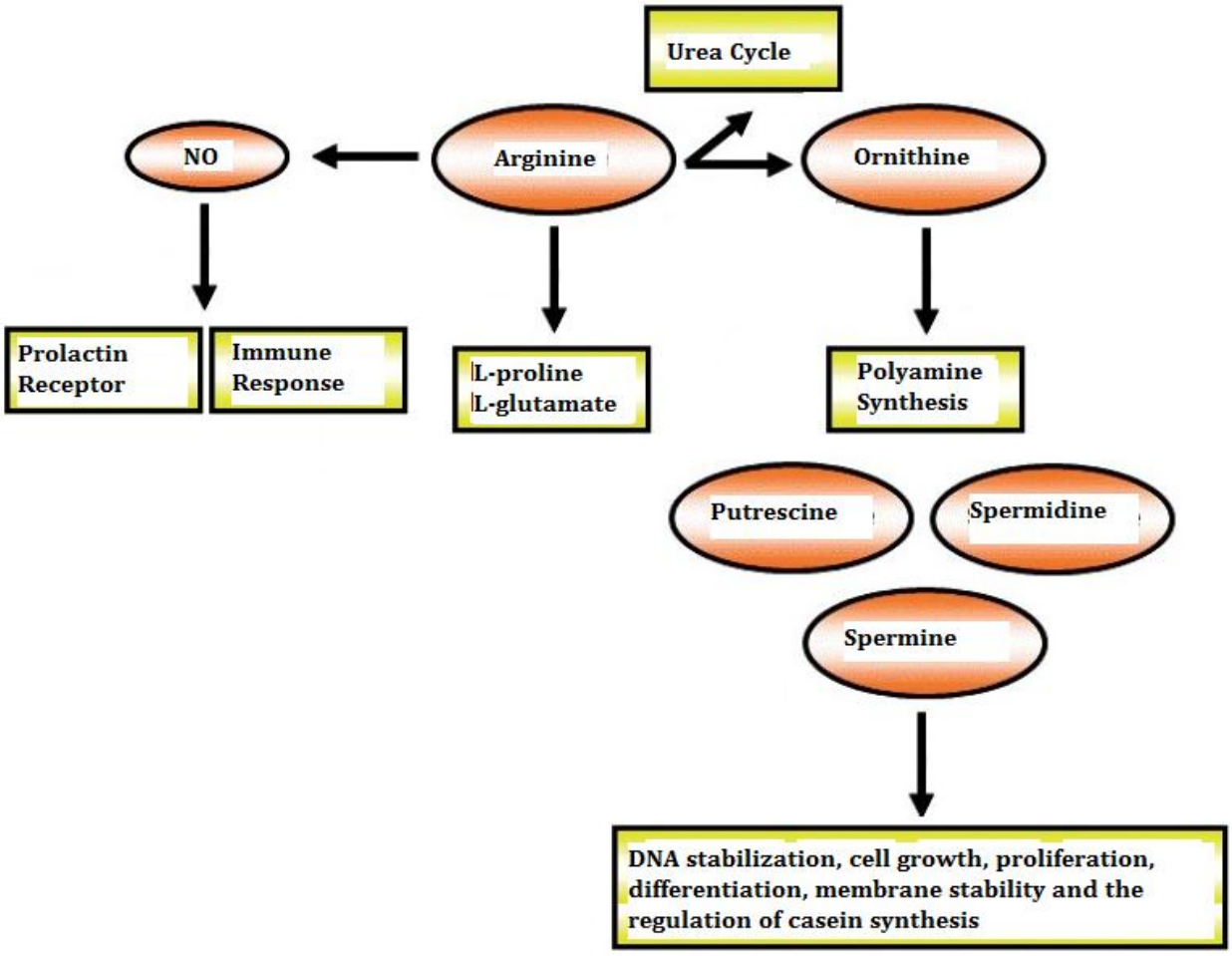
L-arginine is utilised by the mammary gland (Figure 5.1) to synthesise polyamines, nitric oxide (NO) and the AAs L-proline and L-glutamate (66, 67). Polyamine (putrescine, spermidine and spermine) synthesis supports a large number of cellular processes within the mammary gland, such as DNA stabilisation (68), cell growth, proliferation, differentiation (69), membrane stability (70) and the regulation of casein synthesis (197). L-proline and L-glutamate production supports increasing lactation as L-proline is not taken up in sufficient quantities by the lactating gland to support casein synthesis (198), while L-glutamate is used as an energy source and in the production of L-glutamine, the most abundant AA in mammalian milk protein (199). Nitric oxide is produced by nitric oxide synthase (NOS) an enzyme which is activated by prolactin and converts L-arginine to NO and L-citrulline (200). Mammary gland intracellular NO has been shown to aid prolactin receptor release from internal compartments so that it can move to the cell surface and

elevate prolactin signalling (200). Nitric oxide also provides support for the immune system (185) in response to intra-mammary infections (IMI). A pathway activated by polyamine and NO synthesis in response to L-arginine supplementation (71), and supplementation with specific AA (63, 64) is the mechanistic target of rapamycin (mTOR).

At the core of the mTOR pathway is the mTOR protein, an evolutionarily conserved serine threonine kinase that exists in two known complexes, mTORC1 and mTORC2 (173). In all mammalian species both complexes regulate cell growth, proliferation, differentiation and protein synthesis, with mTORC1 serving a more dominant role (173, 201). *In vitro* and *in vivo* studies of mammary gland development and function show specific AA (84), growth hormone (105) and lactogenic hormones (104, 202) stimulate the abundance and activation of factors belonging to the mTOR pathway.

The purpose of this study was to determine if L-arginine supplementation during mid-late pregnancy in twin-bearing ewes influences the abundance of total and activated mTOR, mammary gland biochemical indices and lactation performance in the early post-partum period.

Figure 5.1. Outline of the diverse role L-arginine and its metabolic products play within the ruminant mammary gland



5.3 Materials and methods

5.3.1 Animals and treatments

The trial and treatment methodologies have been described in detail elsewhere (Appendix A). Briefly, twin-bearing, multiparous Romney ewes were housed in separate stalls and fed once daily, a lucerne-based pellet diet (University B mix, Camtech Nutrition, Cambridge NZ) formulated to meet 100% of the NRC-recommended maintenance requirements for twin-bearing pregnant and lactating ewes throughout the trial. The diet contained 6.69 mg/g of arginine (6% of total AA). Gross composition of the diet is described in Table 5.1.

Table 5.1. Composition of the concentrate diet fed to the ewes from d 100 of gestation to d 140 of lactation.

	Content
Metabolisable energy (MJ/kg dry matter)	10.5
Gross Composition (g/100g dry matter)	
Dry matter	86.8
Crude protein	17.0
Lipid	3.4
Ash	3.8
Acid detergent fibre	20.1
Neutral detergent fibre	40.8
Soluble sugars and starch	13.8
Macro-element Composition (g/100g dry matter)	
Potassium	2.01
Magnesium	0.29
Phosphorus	0.38
Calcium	1.25
Sulphur	0.33
Sodium	0.40

Ewes were weighed and body condition scored weekly, and feed allowances adjusted according to live weight changes. From d 100 to d 140 of gestation, ewes received either a bolus of L-arginine-mono-hydrochloride (L-arginine-HCL; Merck KGaA, Darmstadt, Germany; 345 μmol/kg BW) or the equivalent volume of sterile saline three times daily (0800, 1600, 2400 hrs). Cohort 1: at d 140 ewes were euthanized (0.5 mL/kg BW Pentobarb 300, Provet NZ Pty Ltd, Auckland, NZ). Following euthanasia mammary glands were trimmed of extra parenchymal tissue, weighed, sampled, and snap frozen in liquid nitrogen (−80°C) within 5 min, for subsequent analysis of biochemical indices and mTOR pathway activation. From the 23 ewes available in the d 140 gestation group, three were omitted from the study. One ewe had a defective catheter and two ewes aborted their lambs prior to d 140 of pregnancy for reasons not related to the treatment. In total 20 ewes were used (arginine group n = 9; control group n = 11). Cohort 2: at the first sign of labour

induction, which was between 1 and 10 hours prior to birth, L-arginine treatment ceased and ewes were allowed to lamb naturally. One ewe was omitted from the study due to aborting her lambs (unrelated to the treatment regimen). In total 25 ewes were used (arginine group, $n = 13$; control group $n = 12$).

5.5 Milking and sample analysis

Cohort 2: Ewes were assigned to milking groups based on parturition date. The animals in this cohort were part of wider study and collection of a colostrum sample within the 12-hours post partum could have compromised animal behaviour and ewe-lamb bond formation. Therefore, a range of 0.58 – 1.22 days in lactation was produced when the "colostrum" sample was collected. Ewes were then milked at approximately d 1, 4, 7, 10 and 14 of lactation, using the oxytocin method (99). At each milking ewes were milked by machine followed by hand-stripping, after an intravenous injection of 1 IU of synthetic oxytocin (Oxytocin V, 10 IU/mL, PhoenixPharm, Auckland, NZ). The time when the udder was empty was recorded. Animals were milked again (machine and hand-stripping) approximately 4 h later, with the time and weight of the milk recorded. Lambs were separated from the ewes and bottle-fed as required during the intervening period, using the milk collected from their respective dam. The same operators milked all ewes on each occasion in this study using a mobile milking machine (DeLaval, Hamilton, NZ). Milk obtained after the 4 h intervening period was subsampled for analysis of milk composition (% fat, % crude protein (CP), % lactose and somatic cell count). Milk samples were preserved with bronopol and refrigerated at 4°C until analysis using a FT120-FTIR, calibrated for sheep milk (DairyNZ, Hamilton, NZ). Two reference checks (fat and CP) were taken during lactation to account for changes in the matrix of the milk due to stage of lactation. Somatic cell counts were determined using a bromide based fluorescent dye and a FOSS flow Cytometer (MilkTestNZ, Hamilton, NZ) and values log transformed prior to statistical analysis. Ewes were weighed and body condition scored (scale of 1 to 5; Jefferies, 1961) once a week. Throughout milking dams and offspring were housed in individual

pens immediately adjacent to each other. Daily milk yield was calculated using the formula published by van der Linden. 2009 (99). Repeated-measures analysis of milk yield and composition using the MIXED procedure (SAS Inst. Inc., Cary, NC, USA) with a linear model that included the fixed effects of dam, dam treatment group, and the covariates average dam live weight, average dam body condition score and days in lactation were included in the model. From the 25 ewes available only 18 were used for statistical analysis (arginine group n = 8; control group n = 10). One ewe had an infected udder and was removed from the trial after Day 1, while six had blind teats which significantly impacted milk yield.

5.6 Biochemical indices

Cohort 1: Total RNA, DNA and protein were extracted, purified and quantified as previously described (105). Differences in the biochemical indices of cell size (protein:DNA ratio), protein synthetic efficiency (protein:RNA ratio), protein synthetic capacity (RNA:DNA ratio), DNA, RNA, protein concentration, and total parenchymal DNA, RNA and protein content between treatment groups were determined using the MIXED procedure in SAS (SAS). All data were normally distributed as per Kolmogorov-Smirnoff analysis. Differences between means were considered significant at $P \leq 0.05$.

5.6.1 Immunoblotting

Cohort 1: All ewe mammary tissue protein samples were separated via reducing SDS-PAGE. Protein targets mTOR and mTOR^{Ser2448} (Cat No: 2672, 2971; Cell Signaling Technologies, Boston, MA, USA) were separated on 3-8% Tris-acetate gels (Invitrogen, Auckland, NZ), according to the manufacturer's instructions. Proteins were then transferred using an iBlot® Gel Transfer Device to a polyvinylidene difluoride membrane (Invitrogen). The membrane was then blocked according to the antibody manufacturer's instructions (Cell Signaling Technologies), incubated with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody (Cat No: 7074S; Cell Signaling Technologies). Proteins were then visualised using SuperSignal West Pico enhanced chemiluminescence reagents (BioRad, Auckland, NZ) and Kodak Biomax XAR film (Rochester, NY, USA). At least three repeat films were scanned using an HP Photosmart B110 (Hewlett Packard, Auckland, NZ) set at 300 dpi, and the peak area of individual bands determined by using the image analysis software ImageJ (National Institutes of Health, Bethesda, MD, USA). Within each film individual peak areas were calculated per μg of DNA. These values were then expressed as a percentage of the total area from all bands on the film to reduce variation between repeat films (abundance of total and phosphorylated protein). Differences between treatment groups were analysed using the MIXED procedure (SAS) and reported as a normalised peak area per μg of DNA. Differences between means were considered significant at $P \leq 0.05$.

5.6.2 Profiles of free amino acids in milk

Cohort 2: Milk free amino acids (FAA) were analysed using High-Performance Liquid Chromatography (HPLC). Briefly, skim milk (500 µL) was mixed with 500 µL lithium extraction buffer (14 g/L lithium chloride, 3 g/L lithium hydroxide, 1 g/L phenol, 50 g/L sulphosalicylic acid) and placed on ice for 15 to 20 min. Volumes of 10 µL lithium hydroxide (5.88 M) were added to the sample to obtain a final pH of 1.5 to 2. This was followed by centrifugation at 13,680 × g for 5 min at 4°C, and the resulting supernatant was filtered through a 4 mm 0.2 µ syringe filter. The filtered sample was analysed for free AA using a Shimadzu LC10Ai HPLC (Shimadzu Oceania Ltd., Auckland, NZ), fitted with a high-efficiency lithium-ion exchange column (3 mm i.d. × 150 mm; Shimadzu Oceania Ltd.). Injected volumes were 10 µL, the reagent flow rate was 0.3 mL/min, and the run was 162 min between injections, using lithium buffers as eluants and ninhydrin post column derivatisation. Free amino acid values are reported as µmol per litre of milk. Differences between treatment groups were analysed using the MIXED procedure (SAS) with a linear model that included the fixed effects of dam, dam treatment group, and the covariates average dam live weight, average dam body condition score and days in lactation were included in the model.

5.6.3 Profiles of free amino acids in plasma

Cohort 1 and 2: Plasma samples from randomly selected arginine and control ewes from cohort 1 (d 140: n = 6/group) and cohort 2 (2 hours post-birth: n = 5/group) were selected for analysis. Plasma free amino acids (FAA) were analysed using High-Performance Liquid Chromatography (HPLC), as previously described (203) and reported as μmol per litre of plasma. Differences between treatment groups were analysed using the NLME package in R (R Core team 2012) with a linear mixed effects model which included the fixed effect of dam treatment group.

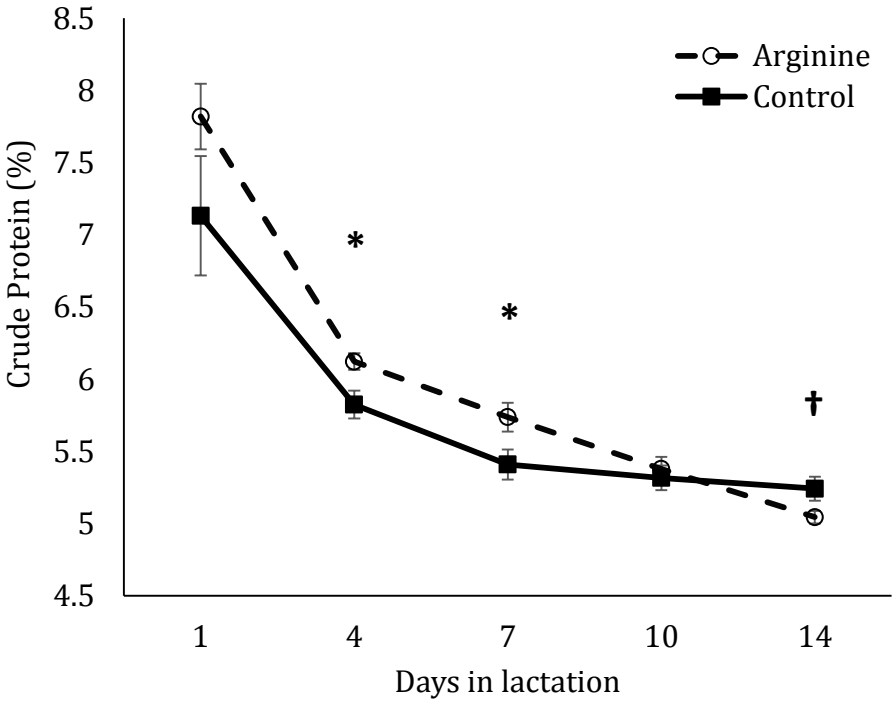
5.7 Results

5.7.1 Milk composition (crude protein, somatic cell count, fat and lactose) and milk yield

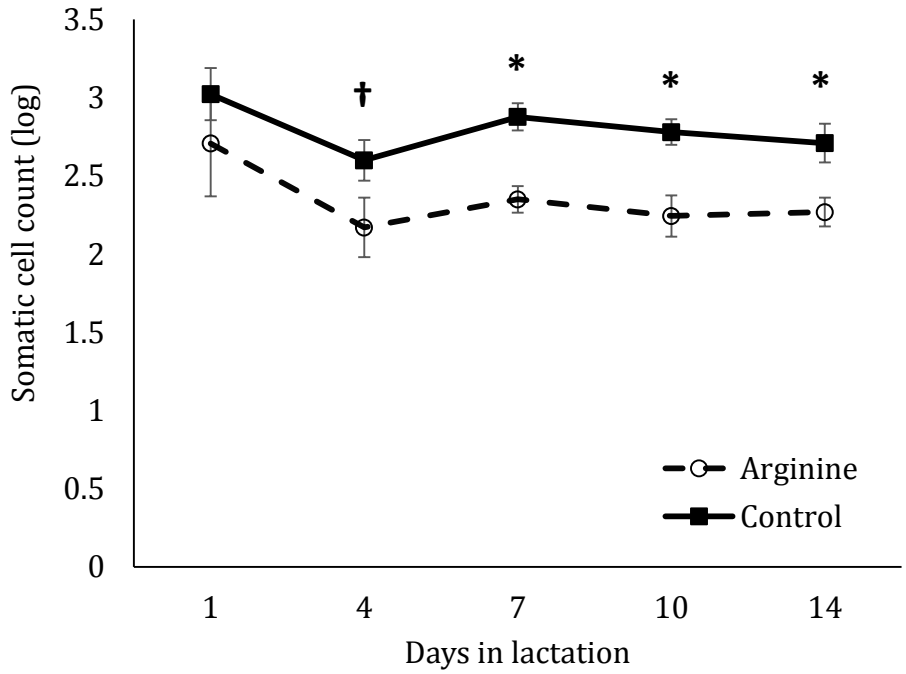
Cohort 2: Increased crude protein percentage from d 4 - 7 was observed in L-arginine treated ewes, compared to saline-treated controls; *P ≤ 0.05 (Figure 5.2*A*). A trend for increased crude milk protein percentage was observed in saline-treated controls at d 14 compared to L-arginine-treated ewes; †P ≤ 0.10 (Figure 5.2*A*). Decreased milk somatic cell counts from d 4 - 14 were observed in L-arginine treated ewes, compared to saline-treated controls (Figure 5.2*B*). There was a trend for increased fat percentage at d 1 in saline-treated controls (Figure 5.2*C*). No effect on lactose percentage (Figure 5.2*D*), or milk yield (Figure 5.2*E*) was observed.

Figure 5.2. Increased crude protein percentage (*A*) was observed in ewes administered L-arginine (day 4 – 7), but tended to be higher in control ewes at day 14. Decreased milk somatic cell counts from d 4 to d 14 were observed in ewes administered L-arginine (*B*). Fat percentage was increased in controls at d 1, compared to treated ewes (*C*). No effect on lactose percentage (*D*) or milk yield (*E*) was observed. **P* ≤ 0.05; †*P* ≤ 0.10.

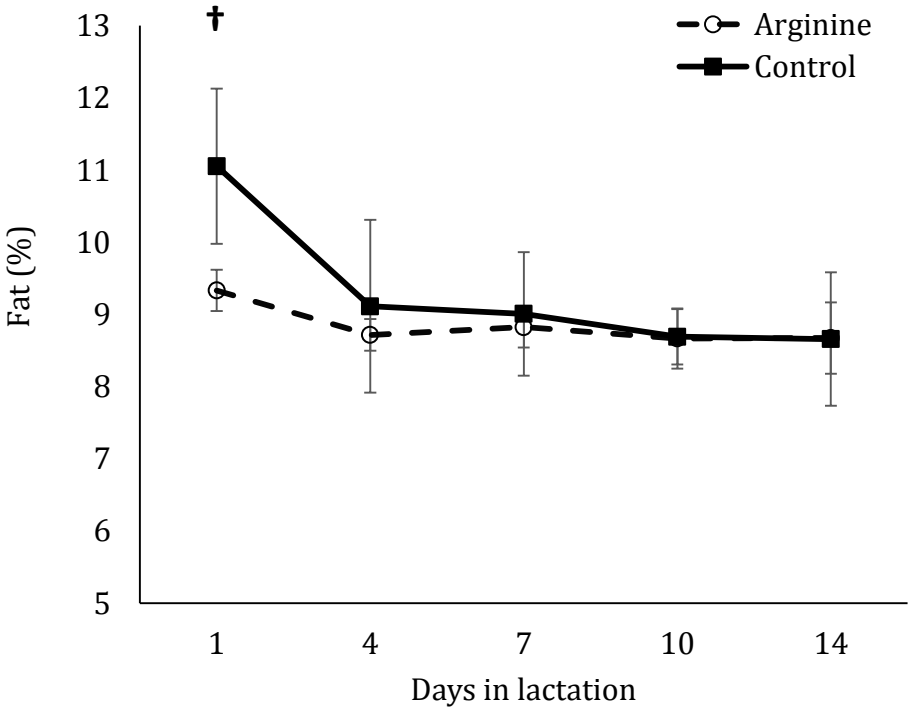
(*A*)



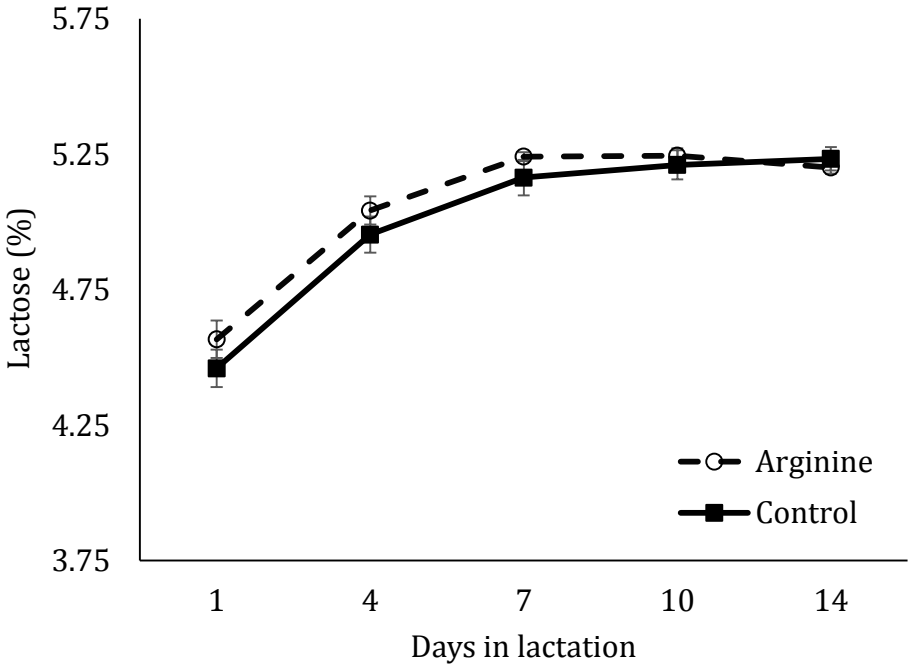
(*B*)


3.5
3
2.5
2
1.5
1
0.5
0
Somatic cell count (log)
†
*
*
*
Arginine
Control
1
4
7
10
14
Days in lactation

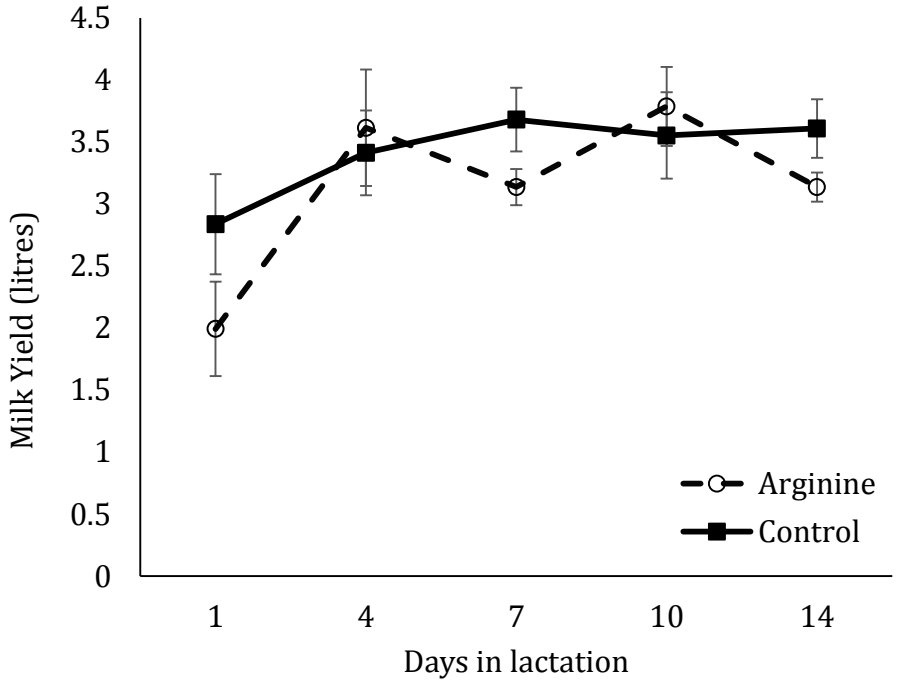
(*C*)


13
12
11
10
9
8
7
6
5
Fat (%)
†
Arginine
Control
1
4
7
10
14
Days in lactation

(*D*)


5.75
5.25
4.75
4.25
3.75
Lactose (%)
1
4
7
10
14
Days in lactation
Arginine
Control

(*E*)


4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Milk Yield (litres)
1
4
7
10
14
Days in lactation
Arginine
Control

5.7.2 Plasma free amino acid profile

(Data sourced from another study within the same trial)

Cohort 1: L-arginine supplemented ewes had increased concentrations of arginine and ornithine, while concentrations of serine, glycine and methionine were reduced relative to saline-treated controls (Table 5.2). Cohort 2: (2-hours post-birth), L-arginine supplemented ewes had decreased concentrations of methionine and histidine, while ornithine was increased relative to saline-treated controls (Table 5.2).

Table 5.2. Concentrations (µmol/L) of amino acids in the plasma of control and arginine-treated ewes 1 h following bolus administration at d 140 of pregnancy and 2 hours post-partum. Data are presented as means and average standard error of the means (SEM)

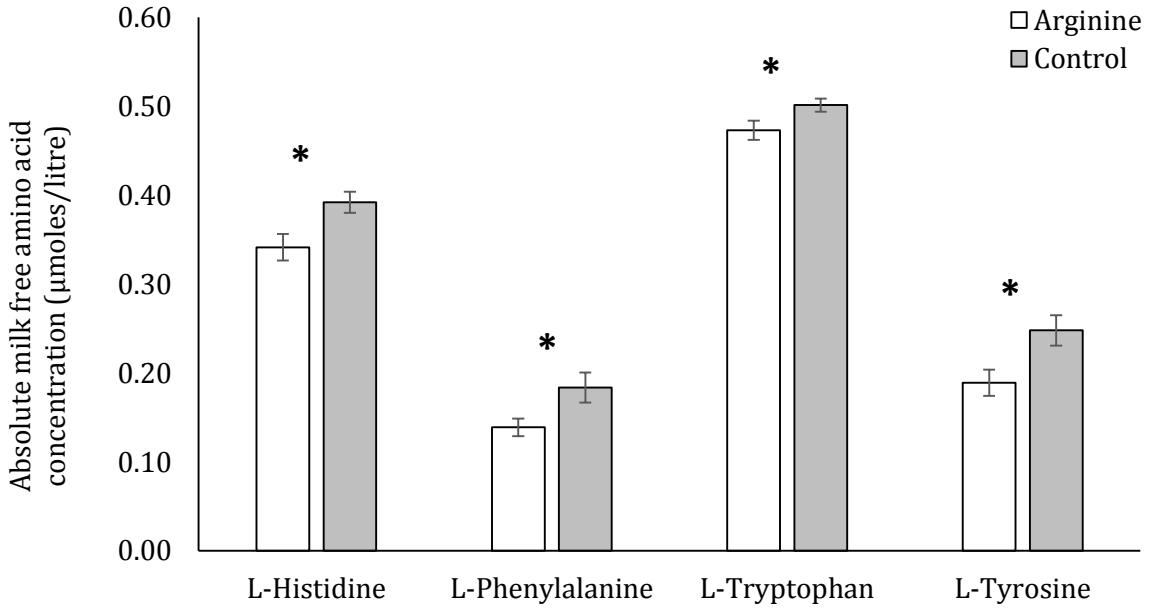
	Day 140 gestation				2 hours post-birth			
	Arginine	Control	SEM	P-value	Arginine	Control	SEM	P-value
Taurine	61	77	12	0.34	63	74	15	0.63
Aspartic acid	6	7	1	0.56	6	5	1	0.71
Threonine	148	235	30	0.07	91	130	17	0.15
Serine	66	91	8	0.05	52	53	7	0.88
Asparagine	24	44	8	0.13	13	17	2	0.36
Glutamic acid	80	65	13	0.40	53	63	10	0.50
Glutamine	257	266	13	0.60	191	218	13	0.19
Glycine	516	795	58	<0.01	379	396	36	0.74
Alanine	169	167	14	0.86	200	247	27	0.24
Citrulline	211	262	32	0.29	194	156	23	0.27
Valine	227	223	27	0.91	143	168	33	0.61
Methionine	26	38	2	<0.01	20	29	2	0.01
Isoleucine	113	114	11	0.88	105	78	42	0.67
Leucine	161	164	18	0.92	84	124	25	0.29
Tyrosine	57	64	4	0.27	49	39	10	0.50
Phenylalanine	55	62	5	0.30	40	54	11	0.38
B-Alanine	1	0.5	0.5	0.64	200	247	27	0.24
Tryptophan	59	63	5	0.63	55	48	12	0.66
Ornithine	394	137	38	<0.01	113	62	12	0.01
Lysine	212	192	17	0.44	121	119	14	0.94
Histidine	91	103	9	0.37	94	118	6	0.02
Arginine	992	109	84	<0.01	127	110	14	0.40
Proline	93	101	7	0.43	66	98	38	0.56

5.7.3 Milk free amino acid profile

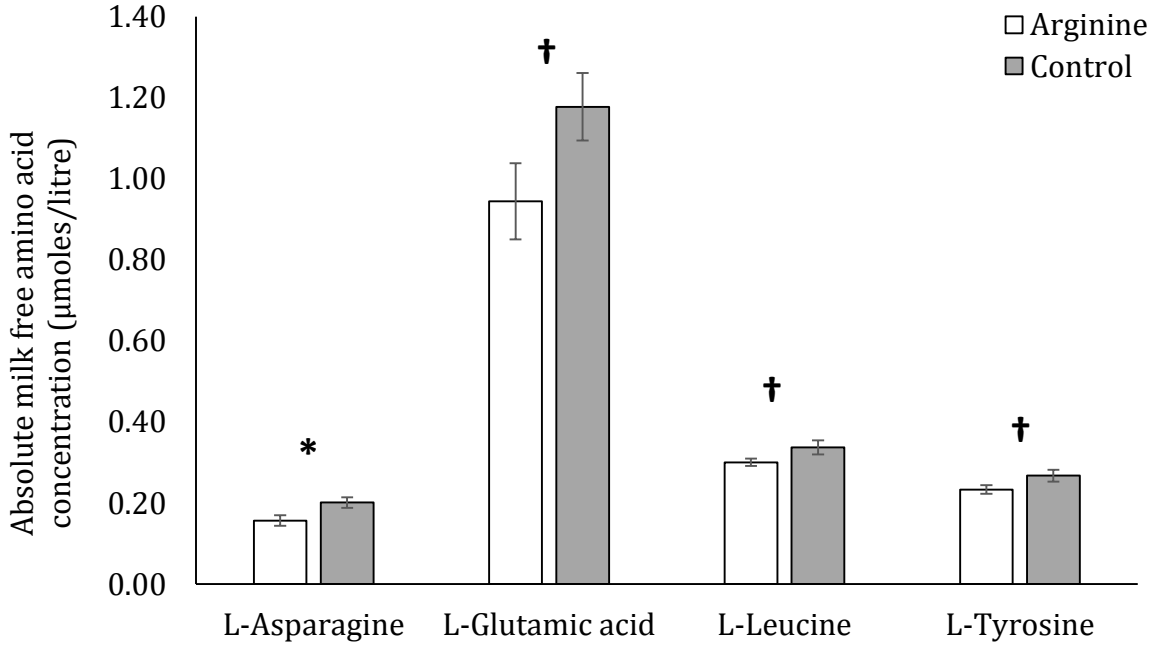
Cohort 2: At d 7 of lactation, absolute concentrations of the milk amino acids histidine, phenyalanine, tryptophan and tyrosine were significantly higher in saline-treated controls compared L-arginine treated ewes; *P ≤ 0.05 (Figure 5.3*A*). At d 14 of lactation (Figure 5.3*B*) asparagine was significantly decreased whilst trends; †P ≤ 0.10, for decreased glutamate, leucine and tyrosine were also observed in saline-treated controls (Figure 5.3*B*).

Figure 5.3. Parenteral L-arginine administration is associated with changes in milk free amino acid profiles. ****P* **≤ 0.05; †*P* **≤ 0.10.**

(*A*)



(*B*)



5.7.4 Mammary gland weight, biochemical indices and mTOR pathway activation

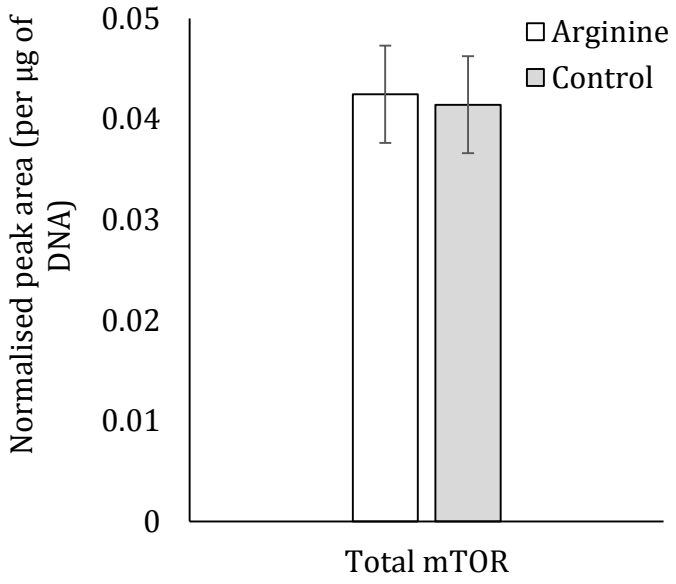
Cohort 1: Total mammary DNA content tended to be greater (P = 0.07) in ewes administered L-arginine compared to controls (Table 5.3). No differences were observed in any of the other biochemical indices measures or mammary gland weight (Table 5.3). The abundance of total, ser-2448 phosphorylated or relative mTOR protein activation was unaffected by L-arginine supplementation in the mammary glands of ewes at d 140 (Figure 5.4).

Table 5.3. Mammary glands of ewes administered L-arginine showed a trend towards increased total mammary DNA content tended compared to saline-treated controls. †$P \leq 0.10$.

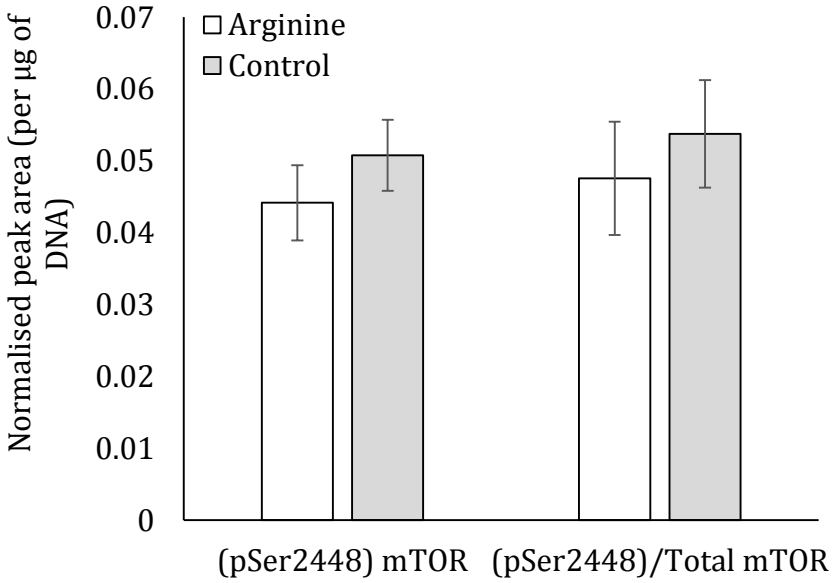
	Control ± SEM (n = 10)	Arginine ± SEM (n = 8)	P value
Mammary gland weight, kg	1.70 (0.161)	1.95 (0.180)	0.35
Total parenchymal content, mg			
DNA	0.10 (0.030)	0.18 (0.034)	0.07†
RNA	1.38 (0.134)	1.51 (0.149)	0.50
Protein	160.11 (22.268)	165.31 (24.896)	0.88
Parenchymal concentration, µg/mg			
DNA	0.78 (0.046)	0.81 (0.052)	0.71
RNA	0.81 (0.036)	0.78 (0.400)	0.58
Protein	91.96 (9.267)	88.41 (10.360)	0.80
Parenchymal ratio			
RNA:DNA	1.03 (0.025)	0.98 (0.028)	0.22
Protein:DNA	10.91 (0.587)	10.33 (0.656)	0.52
Protein:RNA	10.56 (0.489)	10.52 (0.547)	0.96

Figure 5.4. Abundance of total (A), Ser-2448 phosphorylated or activated (Ser-2448/total) (B) mammary mTOR protein was unaffected by L-arginine treatment, compared to saline-treated controls at d 140 of pregnancy.

(*A*)



(*B*)



5.8 Discussion

A surprise finding of this study was parenteral administration of L-arginine during mid-late pregnancy, in twin-bearing sheep, reduced milk somatic cell count and was associated with changes in milk free amino acid concentrations. Parenteral administration of L-arginine also had no negative effect on lactation performance.

5.8.1 L-arginine supplementation improves mammary gland health

Intramammary infections (IMI), collectively known as mastitis, are one of the most costly diseases in meat and dairy farming. Mastitis can be acute and severe or chronic and subclinical. Acute/severe mastitis is easily recognised by the farmer and mostly cured by intramammary antibiotic treatment. Chronic/subclinical mastitis is often not recognised by the farmer and can lead to progressive loss of animal productivity and fertility (204). In addition, chronic/subclinical mastitis is often difficult to cure due to extensive fibrosis and microabscess formation present in the infected gland, and culling is increasingly the final decision. The end result of both forms of mastitis is reduced animal welfare and economic losses through reduced milk yields and, most importantly, reduced milk quality (205).

In the udder of healthy sheep, the milk somatic cell (MSC) count is low (<500,000/ml), and the population is almost exclusively made up of inactive macrophages (MΦ) (206), with the remaining cell population consisting of polymorphonuclear leukocytes (2 – 28%) and lymphocytes (11 – 20%). At the onset of an IMI MSCs provide the first line of defence (207). If this first line of defence is breached cytokine signalling is utilised to activate the

production of nitric oxide (NO) in mammary epithelial cells, increasing gland vascularity and allowing the recruitment of other immune cells, mainly neutrophils, into the milk to fight the infection (185, 208), leading to increased numbers of MSCs. At this stage of infection the MSC count can range from medium (500,000 - 1,000,000/ml) to high (1,000,000 - 2,000,000/ml), with a population consisting of activated neutrophils, MΦ and other immune effector cells (209). In this study, the MSC count for untreated ewes was higher than 500,000, while ewes treated with L-arginine had MSC counts below 300,000. Multiple non-infectious factors can also increase MSC counts in sheep, with the most significant being parity, stage of lactation, season, flock, flock management and diurnal variation (210). Non-infectious factors were discounted from contributing to the observed increase in MSC count because all animals were sourced from the same flock, of similar parity, identically managed and milked at the same stage of lactation. Also, unlike cows and goats, sheep do not experience a post-partum surge in MSC numbers; counts are generally stable throughout lactation (211).

Milk MΦ are commensal residents of the mammary, where they play a role in removing cellular debris, modulating gland development and protecting the uninfected mammary gland from IMI pathogens (212). When IMI pathogens invade the gland, milk MΦs phagocytise the pathogens (213) and destroy them with proteases and NO (212). If the milk MΦs cannot effectively defend the gland they use chemoattractants to recruit other immune cells from the plasma (214). Neutrophils are the predominant immune cell type mobilised to fight both acute/clinical and chronic/subclinical IMI (214). Ruminant neutrophils are unable to produce NO like MΦs (215), instead they produce other cytotoxic

reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCL) and tyrosyl radical to kill invading pathogens (216). The production of ROS by MΦs and neutrophils requires L-arginine and L-ornithine as substrates. L-arginine is metabolised in MΦs by inducible nitric oxide synthase to produce the potent cytotoxic ROS, NO and by arginase I and arginase II to synthesize L-ornithine and urea (217). The production of H_2O_2 by neutrophils is catalysed by super oxide dismutase (SOD), whilst the production of HOCL and tyrosyl radical are catalysed by myeloperoxidase (MPO) from H_2O_2 (218). L-arginine, L-ornithine and L-citrulline provide the direct precursors necessary for the production H2O2, HOCL and tyrosyl radical by neutrophils (217). Exogenous L-ornithine and L-arginine supplementation to whole human blood stimulates unactivated neutrophil production of H_2O_2, increases MPO activity, and elevates intracellular concentrations of arginine and citrulline, whilst only arginine supplementation elevates intracellular ornithine (219, 220).

Treated ewes had increased levels of plasma L-arginine and L-ornithine at all-time points tested during pregnancy, and elevated plasma L-ornithine 2 hours post-partum. We propose that ewes administered L-arginine were able to resist infection and keep MSC low during the length of the trial because plasma MΦs and neutrophils were able to utilise the extra L-arginine and L-ornithine to increase intracellular concentrations of AAs required to produce cytotoxic ROS. When MΦs and neutrophils migrated to the gland increased intracellular concentrations of ROS substrate AAs “primed” the MΦs and neutrophils, making them more cytotoxic than those from control ewes. The observation that L-arginine administered ewes had significantly lower SCC than control ewes between d 1 and d4,

suggests that “primed” MΦs may have been sufficient to eliminate the IMI before the recruitment of neutrophils was necessary. Or, “primed” neutrophils were recruited during the d1 – d4 period and were able to rapidly respond to the IMI and be cleared from the gland before SCC measurement at d4.

When IMI bacteria invade the lactating mammary gland they require a sufficient source of AA to survive and multiply. Free AA concentrations in ruminant milk are low and deficient for some essential AA required for IMI pathogens to survive and multiply (221). In order to obtain adequate AA’s IMI bacteria hydrolyse milk proteins, this leads to a decrease in crude milk protein levels, similar to those identified in this study. It is worth noting that MSC have been implicated in the hydrolysis of milk proteins during IMI, and could also be responsible for the observed decrease in crude milk protein percentage (222). As the IMI progresses, crude protein levels in milk can increase above uninfected levels as serum albumin proteins leak in through damaged tight junctions and into the gland (223). The hydrolysis of proteins in milk also leads to the release of small peptides and free AA. Results from this study are consistent with these observations and confirm mammary glands of untreated ewes had subclinical IMI, whilst treated ewes did not.

The results of this study show absolute concentrations of milk free AA differ between treated and untreated ewes at d 7 (histidine, tryptophan, tyrosine and phenylalanine) and d 14 (asparagine, glutamate, lysine and tyrosine) of lactation. A study by Andrei et al (2011) is the only other published study looking at changes in L- and D- free AA in milk. The authors of that study show IMI increases the percentages of some free AA (224). While

the results from our study agree with those from Andrei et al (2011), neither study was able to ascertain the significance of the observed changes in milk free AA changes. This is deserving of further investigation, as increased milk free AA during IMI may be a by-product of bacterial and / or leukocyte hydrolysis, increased leakage of plasma free AA into the infected gland or increased secretion of free AA by epithelial cells into the gland as they switch from production to protection mode.

In addition, fat, lactose and protein percentages in L-arginine administered ewes are similar for Romney ewes (225), confirming the observation that treatment with L-arginine has no adverse impact on milk quality or yield (225).

5.8.2 L-arginine administration increases total mammary gland DNA content independent of mTOR signalling

Mammary gland growth and development during pregnancy is crucial for subsequent lactation performance (23). Two of the most potent stimulators of mammary gland development during pregnancy are insulin and prolactin, which increase cell number (226, 227) through the regulation of polyamine production (197, 228) and cellular function via mTOR pathway stimulation (229, 230).

The polyamine synthesis (PAS) pathway regulates cellular differentiation and proliferation via consumption of L-methionine and L-ornithine (231). The activity of the PAS pathway is controlled by lactogenic hormones. Ornithine decarboxylase (ODC), which catalyses the decarboxylation of L-ornithine to form the polyamine putrescine, is activated by both prolactin (232) and insulin (233). In this trial, L-arginine supplementation increased L-ornithine and insulin (ng/mL: arginine 0.33 vs. control 0.05 P=0.02), and decreased L-methionine plasma concentrations in treated ewes at d 140 of pregnancy. Prolactin was not measured; however, Chew *et al* (1984) showed supplementation with L-arginine in late-pregnant Holstein cow's increased plasma concentrations of both insulin and prolactin. As lactation progresses, the gradual decrease in mammary epithelial cell numbers within the gland, accounts largely for the decline in milk production (33). Results from this study show treated ewes tended to have higher total mammary DNA content, suggesting elevated plasma prolactin and insulin levels increased cellular proliferation within the gland. Increased mammary epithelial cell number may have implications for lactation persistency which should be investigated further. A pathway activated by polyamine and NO synthesis

in response to L-arginine supplementation (71) is the mechanistic target of rapamycin (mTOR). In this study the abundance of mTOR or mTOR^{Ser2448}, or relative activation was unaffected in treated ewes, indicating the increase in total mammary DNA content occurred independently of mTOR.

In summary, parenteral L-arginine administration from d 100 of pregnancy to parturition was associated with decreased milk somatic cell count in twin-bearing Romney ewes. Furthermore, a relationship was demonstrated between L-arginine administration from d 100 to d 140 of pregnancy, and mammary gland DNA content, which occurs independently of mTOR pathway signalling. These results showed that the positive effects attributed to parenteral L-arginine administration, in terms of reduced MSCC, provide a protective response up to d 14 post administration and as a consequence, potentially maintain uninfected gland milk composition. These findings have important implications for the meat and dairy industries where high MSCC impacts negatively on neonatal growth and the quality of products produced from milk.

CHAPTER 6: MILK SOMATIC CELLS ARE NOT SUITABLE FOR MEASURING BIOCHEMICAL INDICES AND GENE EXPRESSION IN THE LACTATING RUMINANT MAMMARY GLAND.

The material presented in this Chapter has been published as a paper:

Sciascia Q, Pacheco D, Senna Salerno M, Blair HT, McCoard SA. (2012). Milk somatic cells are not suitable biomarkers of lactating ruminant mammary gland function. Proceedings of the New Zealand Society of Animal Production, Volume 72, pp 3-7

Chapter Emendations

Additional explanations as to why differences in the biochemical indices and real time PCR were evident between milk somatic cells and mammary tissue.

Secretory cell heterogeneity: The alveolus is a functional unit of production within the lactating mammary gland in which a single layer of milk secretory cells are grouped. It has been shown that the activity and function (cell cycle, senescence, and differentiation state) of the secretory cells varies across the ruminant mammary gland (234). Thus, the observed differences between milk somatic cells and mammary tissue in this study, may be due to variations in activity and function at the two different collection points.

Milk RNases: Ruminant milk is a rich source of ribonucleases (RNases), where they play a role in the innate immune response (20). In this study, the milk somatic cells were centrifuged at 600 g, to pellet as many cells as possible, and the resultant pellet snap frozen in phosphate buffered saline (PBS) and no RNAse inhibitors. It is possible that centrifugation at 600 g caused some somatic cells to burst and without the presence of RNAse inhibitors in the PBS buffer, some RNA would have been digested. This would explain the observed differences in RNA biochemical indices and real time PCR, between milk somatic cells and mammary tissue.

6.1 Abstract

Understanding the molecular regulation of milk production by the ruminant mammary gland may help farmers meet increasing production milestones. Investigating these mechanisms currently relies upon invasive sampling by post-mortem or biopsy. The objective of this study was to determine if milk somatic cells, shed by the gland during milking, could be used as a non-invasive source of cells to measure these mechanisms. To answer this we employed two widely used molecular techniques, biochemical indices and quantitative PCR (qPCR). Experiment 1: Biochemical indices measures of cell size, protein production capacity and efficiency were determined in milk somatic cells and mammary tissue harvested from lactating dairy goats (n=6). Results showed protein production capacity (*P=0.03*) and cell size (*P=0.03*) were higher in mammary tissue compared to somatic cells while protein production efficiency was unaffected (*P=0.55*). Experiment 2: Milk protein and ribosomal RNA gene expression were measured in milk somatic cells and mammary tissue collected from lactating dairy cows treated with growth hormone (n=3) or saline (n=4), using qPCR. Results showed expression of all genes differed between milk somatic cells and mammary tissue. Taken together, these results indicate milk somatic cells are not suitable for measuring biochemical indices or gene expression in the lactating ruminant mammary gland, and would be poor indicators of mechanistic target of rapamycin (mTOR) pathway protein abundance.

6.2 Introduction

The growing nutritional significance of ruminant milk has increased global demand, placing pressure on New Zealand farmers to make constant productivity increases through the application of novel environmental, genetic and nutritional intervention strategies (3). Understanding how the mammary gland regulates milk production at the molecular level may be crucial to helping farmers meet increasing performance milestones.

Currently, the majority of studies investigating molecular regulation of milk production rely upon the collection of tissue harvested from biopsies or at slaughter (9, 235). Collection at slaughter has the advantage of harvesting large samples; however the requirement to euthanize animals provides samples from only one time point during lactation. Biopsies partially address this deficiency by allowing multiple sample collections during lactation; however they are invasive and can require long recovery times between sample collections (235).

A potential solution lies in the mammary epithelial cells that, along with immune cells, are shed into milk during lactation. Previous studies in a wide range of species show that milk somatic cells are viable and exhibit characteristics of fully differentiated alveolar cells (236, 237). Ruminant studies using milk somatic cells collected from goats (238) and purified mammary epithelial cells from bovine milk (239), show they can be used to determine the expression of key genes within the mammary gland.

Two techniques used to study molecular regulation of milk production are biochemical indices and quantitative PCR (qPCR). Biochemical indices are measured using the DNA, RNA and protein extracted from cells or tissue (240). When they are quantified, the ratio of nucleic acids and protein can be used to indirectly measure cell size, using the ratio of protein to DNA; protein production capacity, the ratio of RNA to DNA; and protein production efficiency, the ratio of protein to RNA (240). To date, no studies have been conducted to determine if milk somatic cells can be used to determine biochemical indices of the lactating ruminant mammary gland. Measurement of gene expression is routinely performed using qPCR (241). This sensitive technique is employed throughout the dairy industry to determine changes in a wide range of factors affecting milk production, such as the presence of mastitis causing bacteria to the level of milk gene expression (242).

The objective of this study was to determine if milk somatic cells could be used to measure biochemical indices and gene expression in the lactating ruminant mammary gland.

6.3 Materials and methods

6.3.1 Animal trial procedures

All procedures involving animals were carried out in compliance with the guidelines of the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand), in accordance with the 1999 Animal Welfare Act of New Zealand. Experiment One: Six dairy goats in the mid to late stages of their second-lactation were milked and cells harvested from the milk collected immediately prior to slaughter. At slaughter, lobular-alveolar mammary tissue was collected and immediately frozen in liquid nitrogen for subsequent analysis. Experiment Two: The trial design and sample collection methodology has been described in the Materials and methods of Chapter 3. Milk somatic cells were collected immediately prior to slaughter, at which time mammary tissues were collected, and both snap frozen in liquid nitrogen. RNA from the milk somatic cells was extracted and stored at -80^{o}C at the time of the trial for use in future experiments, therefore biochemical indices could not be measured using these samples.

6.3.2 Collection of milk somatic cells

Milk somatic cells were harvested from goat and cow's milk as follows. The mammary gland was first milked using a milking machine until no further milk was recovered. Residual milk (~50-150-ml) was removed by hand milking, collected into sterile 50-mL conical tubes and centrifuged at 600 × g for 10 min at 4°C. Preparations from preliminary samples indicated that the collection of cells from residual milk yielded a much greater proportion of viable milk cells (~60-70% based on trypan blue viability staining). Cream and skim milk were removed and cell pellets were washed twice in 5 mL of ice-cold

phosphate buffered saline (pH = 7.2) and centrifuged at 600 × g at 4°C for 10 min. The supernatant was discarded and the pellet was snap frozen in liquid nitrogen and stored at -80^{o}C.

6.3.3 Biochemical indices

Experiment 1: Archived (stored at -80^{o}C) milk somatic cells and mammary tissue, harvested from lactating goats, were used to extract total DNA, RNA and protein using TRIzol® reagent (Invitrogen, Auckland, NZ), according to the manufacturer's instructions. DNA and RNA were quantified using a NanoDrop Spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE). Protein was quantified using the Bradford method (141). The ratio of RNA to DNA, a measure of protein production capacity, ratio of protein to DNA, a measure of cell size and ratio of protein to RNA, a measure of protein production efficiency, were calculated. To compensate for variations in cell number total DNA, which is a measure of cell number, was used to normalise between milk somatic cells and mammary tissue. Differences between milk somatic cells harvested from lactating goats and mammary tissue were analysed using MIXED procedure (SAS Inst. Inc., Cary, NC), with a linear model that included the fixed effects of mammary tissue. Log transformed data was back-transformed and presented with 95% confidence intervals (CI). Differences between means were considered significant at $P < 0.05$.

6.3.4 Quantitative PCR

Experiment 2: Archived bovine somatic cell RNA extracted from a previously published trial was used for this study (9). Mammary tissue RNA was extracted from the same cows

using an RNeasy mini kit (Qiagen, San Diego, CA, USA), with on-column DNase I treatment (Qiagen), according to the manufacturer's instructions. RNA integrity was assessed using gel electrophoresis in a 1% non-denaturing agarose gel, stained with SYBR® Safe (Invitrogen). All milk somatic cell RNA samples for one GH treated animal were degraded so it was excluded from the analysis, leaving 3 GH animals and 4 controls. The remaining milk somatic cell samples were purified using an RNeasy mini kit with on-column DNase I treatment (Qiagen), to remove any residual DNA contamination. Purified RNA (500 ng) was reverse transcribed to make complementary DNA (cDNA) using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Quantitative PCR (qPCR) was performed using the cDNA as a template and the SensiFAST™ SYBR No-ROX Kit (Bioline, London, UK) as per the manufacturer's instructions in a Rotor-Gene™ 6000 (Qiagen). All primers used have been previously published (McCoard et al. 2010). The PCR efficiency and quantification cycle (Cq) values were obtained for each sample using LinRegPCR (143). Reactions were performed in triplicate and all values entered into REST 2009 (145) and the fold change in expression determined.

6.4 Results

Experiment 1: Results showed protein production capacity was 294% (*P=0.03*) and cell size was 223% (*P=0.03*) higher in mammary tissue compared to milk somatic cells collected from goats, while no difference (*P=0.55*) in protein production efficiency was observed (Table 6.1).

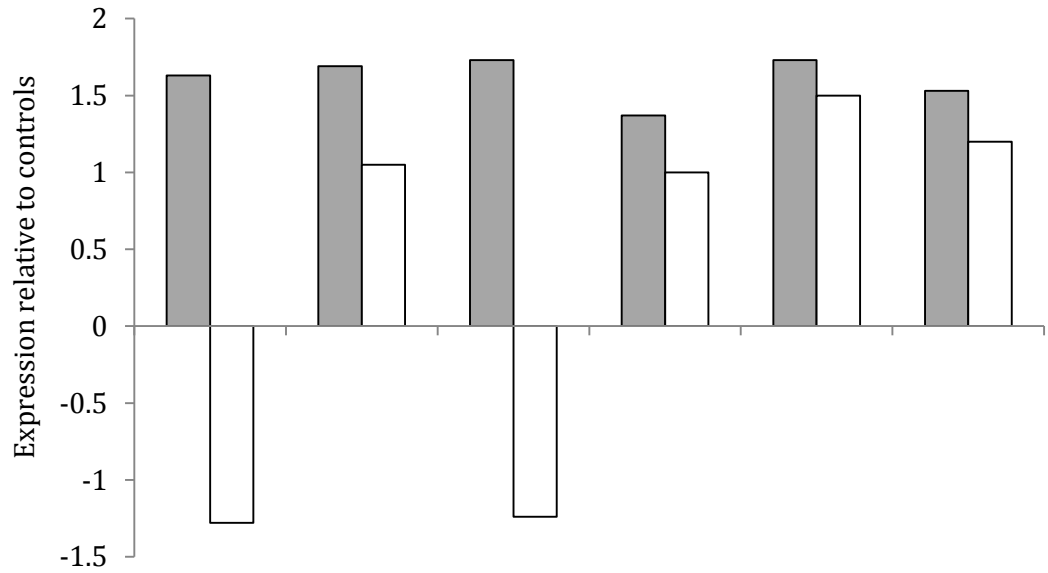
Table 6.1. Biochemical indices measurements from milk somatic cells and mammary tissue (n=6) collected from lactating goats. Back transformed data are represented as least squared means (95% CI).

Biochemical Index	Milk Somatic Cells	95% CI	Mammary Tissue	95% CI	p-value
Protein production capacity	0.17	0.14 - 0.22	0.50	0.39 - 0.63	0.03
Protein production efficiency	90.13	71.87 - 113.02	70.42	56.16 - 88.31	0.55
Cell Size	15.68	13.20 - 18.63	34.94	29.41 - 41.50	0.03

Experiment 2: Results show that milk somatic cell expression of the major milk protein genes, alpha-S1-casein, alpha-S2-casein, beta-casein, kappa-casein, alpha-lactalbumin and the ribosomal RNA gene 18S, between GH treated and control animals (Figure 6.1) was not significant (*P>0.05*). While expression significance was not detected in milk somatic cells, the direction of alpha-S2-casein, kappa-casein, alpha-lactalbumin and 18S ribosomal RNA matched that of mammary tissue. The expression of alpha-S1-casein (-1.28-fold) and beta-casein (-1.24-fold) decreased in milk somatic cells from GH-treated animals, where expression in mammary tissue increased (1.63- and 1.73-fold, respectively). Overall, milk somatic cell expression of the major milk proteins and 18S ribosomal RNA was lower than that of mammary tissue.

This result is consistent with previous research where northern blots were used to measure the expression of three milk genes in milk cells harvested from goat's milk. The authors of that study were able to show that when compared to mammary tissue, milk somatic cell hybridisation signals for alpha-S1-casein, kappa-casein and alpha-lactalbumin were always lower (238).

Figure 6.1. Gene expression profiles of the major milk proteins and 18S ribosomal RNA in somatic cells and mammary tissue collected from lactating dairy cows treated with saline (n=3) or growth hormone (n=4). Columns represent relative expression in GH-treated animals compared to saline-treated controls, with *p-values* noted below.



	alpha S1-casein	alpha S2-casein	beta casein	kappa casein	alpha lactalbumin	18S rRNA
Mammary Gland	***0.018***	***0.013***	***0.001***	***0.092***	***0.001***	***0.005***
Milk Somatic Cells	*0.575*	*0.929*	*0.639*	*0.996*	*0.458*	*0.402*

6.5 Discussion

This study was conducted to determine if milk somatic cells could be used as non-invasive measures of the lactating ruminant mammary gland. To answer this question we employed two techniques widely used in molecular biology, biochemical indices and qPCR.

Biochemical indices are a quick and relatively inexpensive set of measurements that are made using the ratios of DNA, RNA and protein extracted from milk somatic cells and mammary tissue. Analysis of these ratios can be used to determine if a treatment, or nutritional regime, has altered levels of the components necessary to support milk production in the mammary gland (153). Our research shows that the biochemical indices measures of cell size, protein production capacity and protein production efficiency differ between milk somatic cells and mammary tissue harvested from lactating goats.

Gene expression analysis using qPCR is a method routinely employed to study how the lactating mammary gland controls and supports milk production (243). It amplifies the RNA template used by milk secreting cells to manufacture milk components and the factors necessary to support their production (241). This study shows that milk somatic cell expression of the major milk proteins and 18S ribosomal RNA was lower than that of mammary tissue.

We propose two potential explanations for why the biochemical indices and gene expression profiles differ between somatic cells and mammary tissue in the lactating ruminant. First, studies show that during lactation the ruminant mammary gland expresses

high levels of the apoptosis markers BCL-2 and BAX around the acinar cell lining (244) and present fragmented DNA laddering consistent with on-going apoptosis (245). This suggests that milk somatic cells from this study may be entering apoptosis resulting in the observed decreases in protein production capacity, cell size and gene expression. Second, cells harvested from ruminant milk are a heterogeneous population of immune cells and milk secreting cells, with goat's milk also containing cytoplasmic particles (238). Whilst the residual milk collected in both experiments increases the percentage of milk somatic cells, studies show that residual milk still contains immune cells, albeit at a lower percentage compared to other fractions. This suggests that the remaining immune cells and cytoplasmic particles may contribute to the differences found in the biochemical indices measures and gene expression profiles between the milk somatic cells and the mammary tissue. To prevent this, it has been shown in several studies that milk secreting cells should be purified from milk using immuno-magnetic separation (239, 246-248). This technique exploits the presence of cytokeratin 8 and other unique markers found on the surface of milk secreting cells (249). Once purified, the milk secreting cells can be used as measures of changes occurring within the mammary gland.

This study has shown milk somatic cells harvested from residual milk cannot be used to assess biochemical indices or gene expression changes in the lactating ruminant mammary gland. Results from the literature suggest this may be due to apoptosis or the presence of immune cells and cytoplasmic particles. These studies advocate the purification of milk secreting cells from milk before any molecular analysis is undertaken. This is important as milk somatic cells may be an inexpensive and non-invasive tool that can be used to

understand the molecular regulation of milk production, and provide novel production technologies to dairy farmers. Therefore, the use of milk somatic cells to measure molecular changes within the lactating mammary gland warrants further investigation.

CHAPTER 7: ASSESSMENT OF BOVINE MAMMARY EPITHELIAL CELL MODELS FOR LACTATION RESEARCH

7.1 Abstract

The objectives of this study were to (1) develop a chronic, lactogenically differentiated *in vitro* model of bovine lactation, and utilise this model (2) to identify the potential role L-arginine plays in stimulating mTOR pathway signaling and milk protein synthesis (Chapter 4). In this study two well established cell lines were employed the immortalised bovine mammary epithelial cell line MAC-T and primary bovine mammary epithelial cells (pbMECs). MAC-T cells were incubated in differentiation medium, supplemented with growth hormone (GH), in the presence or absence of fetal bovine serum (FBS) and alternative culturing surfaces. Cultured pbMECs were incubated in differentiation medium on Matrigel. The development of a lactating phenotype was assessed by visual changes in monolayer morphology, quantitative PCR measurement of the milk protein gene β-casein and biochemical indices. MAC-T cells: absence of FBS produced monolayers that were less confluent than controls, with thinner and more elongated cells. The presence of Matrigel resulted in MAC-T monolayers that were thinner than controls and grew in "rivers" around zones of low to no cell density, this effect more evident in the 1:10 Matrigel. In areas of high density cells had begun to grow on top of each other. Further, addition of GH to the differentiation medium reduced protein production capacity (*P=0.09*) and cell size (*P=0.03*) compared to control, while cell number tended to increase (*P=0.10*). No significant levels of β-casein mRNA could be detected in any of the MAC-T experimental conditions, indicating differentiation had not occurred. Mammosphere formation and β-casein mRNA abundance increased two- and four-hundred-fold in pbMECs treated with differentiation medium, compared to pre-treated cells. Taken together, the results from this study indicate that

whilst pbMECs can be lactogenically differentiated (acute not chronic) and utilised to measure the expression of milk protein genes, the requirement for the use of Matrigel and low cell numbers means they cannot be used to measure the abundance of mTOR pathway proteins, or assess the effect amino acids have on lactation potential. Whilst MAC-Ts are unsuitable due to their inability to lactogenically differentiate, preventing assessment of any characteristics of lactation.

7.2 Introduction

Animal models have been widely-used over the years to understand how the ruminant mammary gland responds to interventions designed to increase milk production (9, 243). However, animal models have high financial and ethical costs due to the large numbers of animals required for time course experiments and requirement for either slaughter or repeated biopsies. A potential solution lies in the development of *in vitro* lactation models using mammary epithelial cells (MECs). Mammary epithelial cells are responsible for the production and secretion of milk components in the lactating ruminant mammary gland and therefore are ideal models for the study of ruminant lactation.

Two commonly used *in vitro* MEC models used to study bovine lactation are the mammary alveolar cell-T (MAC-T) and primary bovine mammary epithelial cells (pbMECs). MAC-T and pbMECs (41, 90) are two of the most utilised cell lines because they retain many of the morphological and biochemical characteristics of MECs (250). These cell lines have been used as models for MECs regarding responses to different stimuli such as IGF1 (90), GH (41), nutrition (154, 251) and for investigating and identifying molecular components regulating milk protein production and secretion. MAC-T is an immortalised (can be cultured indefinitely) bovine cell line specifically developed for *in vitro* studies of bovine lactation (250), while pbMECs are isolated from parenchymal tissue harvested at biopsy or after slaughter and are not immortal (252).

To accurately model bovine lactation *in vitro,* cultured MECs must meet two pre-requisites: be capable of consistent differentiation (produce a lactating phenotype) and survival in a differentiated state for long periods (days to weeks). Studies show that whilst pbMECs are able to differentiate (produce a lactating phenotype) when harvested at the correct developmental stage and secrete milk specific proteins when cultured with lactogenic hormones (154), MAC-T do not consistently differentiate (253). Attempts to develop a MAC-T culture system capable of consistent differentiation have utilised growth hormone supplemented media, transfected cells (41) or growth on different extracellular matrices (254), but to date, results have been varied. These studies have also focused on the short term (minutes to hours) effects of hormones and mitogens (90, 255) on the activation of pathways linked to milk production, such as the mechanistic target of rapamycin (mTOR). However, lactation is not an acute process thus current *in vitro* studies may not accurately reflect what occurs *in vivo*.

The objective of this study was to determine if a chronic, lactogenically differentiated, *in vitro* model of bovine milk production could be developed using two well established cell culture models, MAC-T and pbMECs, to study the potential Arg – mTOR signalling changes identified in Chapter 4.

7.3 Materials and methods

7.3.1 Cell culture and treatments

MAC-T cells were grown to confluence on NUNCLON-treated plastic surfaces (ThermoFisher, Auckland, NZ) using proliferation medium [Dulbecco's Modified Eagle's medium (DMEM), 4-mM GlutaMAX™, 1% Penicillin/Streptomycin and 10% fetal bovine serum (all sourced from Invitrogen, Auckland, NZ; FBS)] (255). Cells were then washed twice with phosphate buffered saline (pH 7.4; Invitrogen) to remove any residual proliferation medium and incubated with differentiation medium as outlined below.

The differentiation capacity of MAC-T cells was determined by incubating monolayers in proliferation medium containing [dexamethasone (10-µg/ml), insulin (5-µg/ml), prolactin (5-µg/ml); DIP] with GH (500-ng/ml) for 2-days (255) and without GH for 10-days. The effect of FBS on MAC-T differentiation was determined by incubating cells for 7-days in proliferation medium containing DIP with or without 10% FBS (41). The effect of cell culture surface on MAC-T differentiation was assessed by incubating cells in proliferation medium with DIP and GH for 7-days on NUNCLON-treated (ThermoFisher) plastic. Plates were uncoated, or coated at 1:1 or 1:10 in Matrigel (BD Biosciences, Auckland, NZ) or Synthemax-coated (purchased pre-coated: Corning, Auckland, NZ).

Primary bovine MECs were generously provided by Dr Kuljeet Singh (AgResearch Ruakura, Hamilton, New Zealand). Briefly, pBMECs were harvested from dairy cows 30 days prior to parturition at AgResearch Ruakura, Hamilton, New Zealand. The collection was timed to be before the 'springing' of the udder to maximise the chances that that harvested pBMECs would be able to differentiate. Cells were grown to confluence on NUNCLON-treated plastic (ThermoFisher) using culturing media [20% horse serum, 5% FBS, 1% penicillin/streptomycin, insulin (1 mg/ml), cortisol (5 mg/ml) and EGF (10 μg/ml)]. Six well plates were coated with 1:1 Matrigel (BD Biosciences), seeded with 6 x 10^5 cells per well and incubated in attachment media [20% horse serum, 5% fetal bovine serum, 1% penicillin/streptomycin, insulin (1 mg/ml), cortisol (5 mg/ml) 5% Transferrin (1 μl/ml) and prolactin (1 mg/ml)] for 24-hours. Attachment media was removed and pbMECs treated with differentiation media: 1% penicillin/streptomycin, insulin (1 mg/ml), cortisol (5 mg/ml) 5% Transferrin (1 μl/ml) and prolactin (1 mg/ml) for 10-days. All pbMEC media formulations contained a liquid base of two-times concentrated, pre-made culture base [M199, Hams/F12, 1M HEPES (20 μl/ml), NaHCO3 (7.5 mg/ml) and sodium acetate (32.5 μg/ml)]. Unless stated, all reagents were purchased from Sigma Aldrich (Auckland, NZ). To ensure that any effect of serum was consistent between treatments, all FBS used was from a single batch.

7.3.2 Gene expression

Total RNA was extracted using TRIzol® Reagent (Invitrogen) and quantified using a NanoDrop Spectrophotometer (ND-1000; Nanodrop Technologies). RNA integrity was assessed using non-denaturing gel electrophoresis in a 1% agarose gel, stained with SYBR® Safe (Invitrogen). Total RNA was purified using an RNeasy mini kit with on-column DNase I treatment (Qiagen), according to the manufacturer's instructions, to remove any residual DNA contamination. Purified RNA (2-µg) was reverse transcribed to make complementary DNA (cDNA) using the transcriptor first strand cDNA Synthesis Kit (Roche, Auckland, NZ). Quantitative PCR (qPCR) was performed using the cDNA as a template and the LightCycler® FastStart DNA MasterPLUS SYBR Green I kit (Roche) as per the manufacturer's instructions in a LightCycler® instrument (Roche). The milk protein gene β-casein was used as a marker of MAC-T and pbMEC differentiation and glycerol-3-phosphate acyltransferase 1, mitochondrial 1 (GPAM) used as a reference gene. Primer sequences are published elsewhere (9). The PCR efficiency and quantification cycle (Cq) values were obtained for each sample using LinRegPCR (143). Reactions were performed in triplicate and fold change in expression determined using REST 2009 (145). Experimental conditions where the Cq value was > 30 were determined to have an mRNA abundance below detection and not analysed for expression differences.

7.3.3 Biochemical indices

Total DNA, RNA and protein was extracted from MAC-T cells incubated in proliferation medium supplemented with DIP and GH using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. DNA and RNA were quantified using a NanoDrop Spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE). Protein was quantified using the Bradford method (141). The ratios of RNA to DNA, protein to DNA and protein to RNA were calculated as indicators of protein production capacity, cell size and protein production efficiency, respectively. Differences between Day-0 (untreated) and Day-2 (treated) were analysed using the T-TEST procedure (SAS Inst. Inc., Cary, NC). Differences between means were considered significant at $P < 0.05$. Biochemical indices were not investigated in subsequent MAC-T differentiation experiments (without GH, or the presence / absence of FBS) as increased β-casein mRNA abundance replaced biochemical indices as the initial measure of MAC-T differentiation. Biochemical indices could not be determined for pbMECs or MAC-T cells grown on Matrigel as TRIzol reagent dissolves the Matrigel, which is a gelatinous protein mixture. Matrigel protein contaminated the protein extracted from cultured cells, preventing accurate quantification of cellular protein. Methods for isolating cells cultured on Matrigel have been published (256), but were not able to be used in the timeframe of this study.

7.3.4 Monolayer morphology

MAC-T and pbMECs monolayers were visualised on an inverse microscope (Leica DMIL). Photographs were taken on a Nikon Coolpix900 digital camera at 20x magnification.

7.4 Results

Primary bovine MECs: In the presence of differentiation medium, pbMECs formed mammospheres, three dimensional multi-cell structures indicative of differentiation (Figure 7.1; *A* - *C*). Abundance of β-casein mRNA increased two-fold by Day-5 (*P=0.01*) and four hundred-fold by Day-10 (*P<0.01*) in cells treated with differentiation medium compared to pre-treated cells (Figure 7.2).

MAC-T: Monolayer morphology was different in cells grown in the absence of FBS and on 1:1 and 1:10 Matrigel, compared to control (Figure 7.1; *D* - *H*). Cells treated with proliferation medium containing DIP and no FBS were less confluent, thinner and more elongated (Figure 7.1; *F*). Cells grown on 1:1 and 1:10 Matrigel (Figure 7.1; *G,H*) were thinner and grew in "rivers" around zones of low to no cell density, this effect more evident in the 1:10 Matrigel. In areas of high density cells had begun to grow on top of each other. No difference in monolayer morphology was observed in cells grown on Synthemax (Figure 7.1*E*) compared to control (Figure 7.1*D*). Culturing on alternative surfaces or the absence of FBS did not produce significant levels of β-casein mRNA (Cq > 30). In all experiments reference gene GPAM was detectable (Cq < 30).

Figure 7.1. Treatment of pbMECs with differentiation medium and grown on Matrigel induced the formation of mammospheres at Day-5 and Day-10, post-treatment. Photographs of cultured pbMECs: *A*. pre-treatment (no differentiation medium, no Matrigel) *B*. 5-days and *C*. 10-days post-treatment. In the absence of FBS, and on 1:1 and 1:10 Matrigel, MAC-T monolayer morphology changed compared to control. Photographs of cultured MAC-Ts: *D*. control (no differentiation medium) *E*. Synthemax *F*. no FBS *G*. 1:1 Matrigel *H*. 1:10 Matrigel.

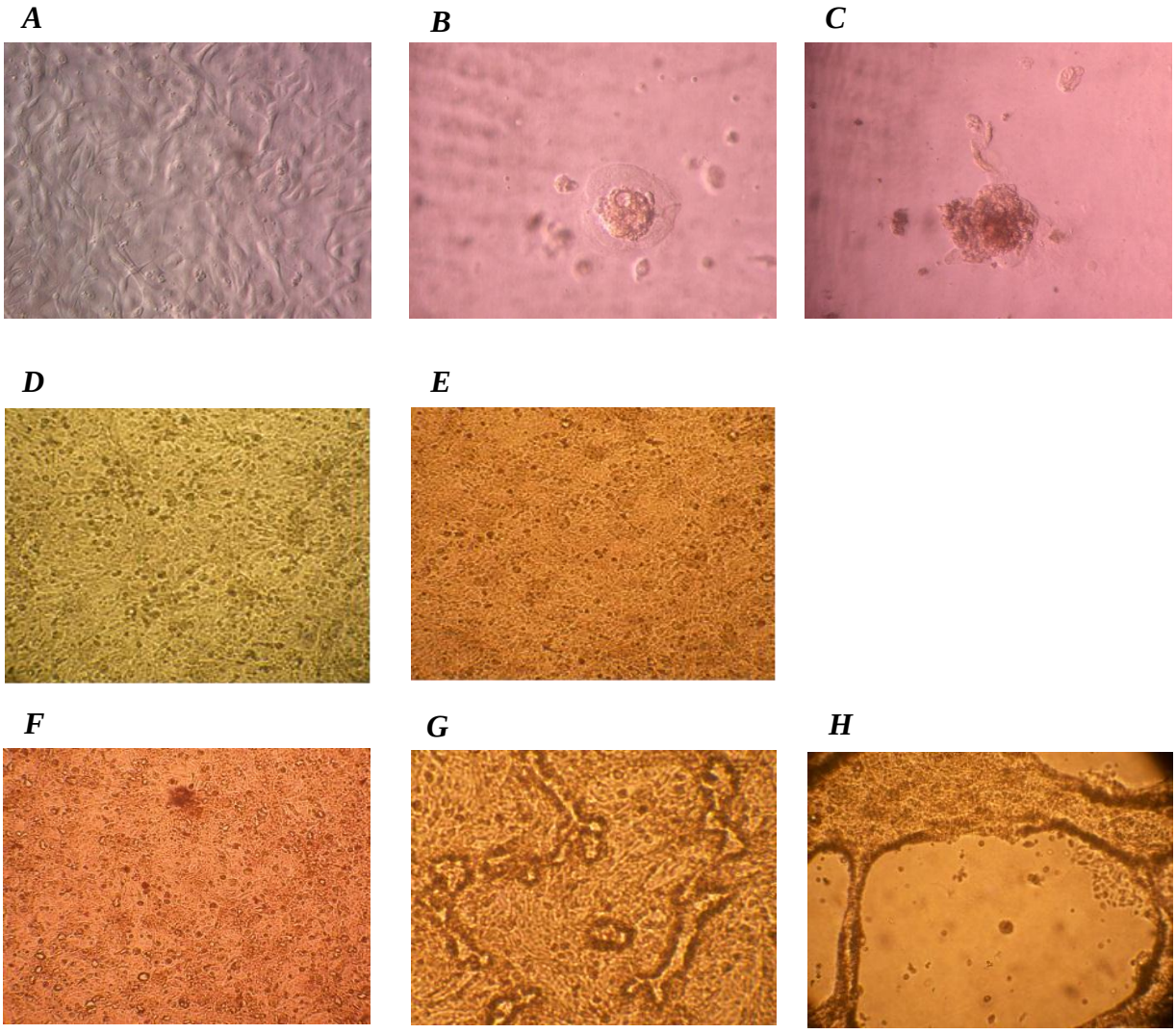
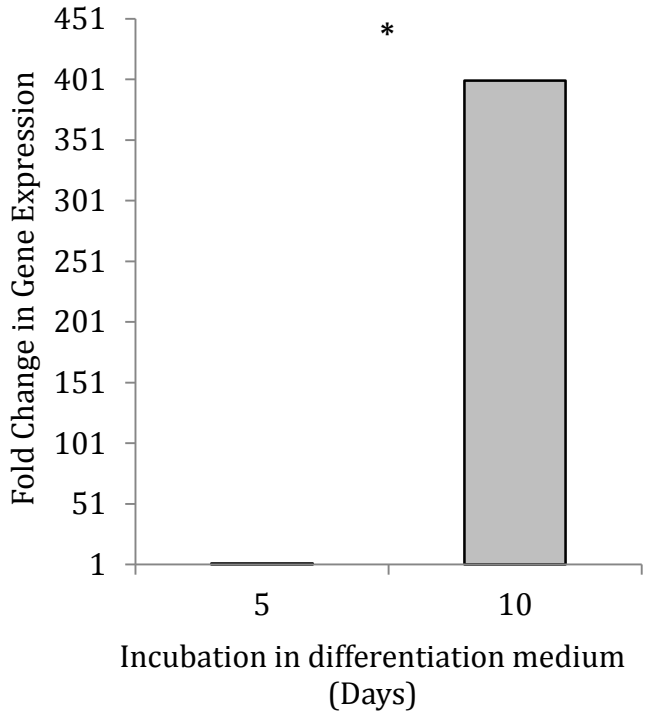


Figure 7.2. Treatment of pbMECs with differentiation medium on 1:1 Matrigel increased the abundance of β-casein mRNA. The figure shows the fold change in gene expression in pbMECs treated with differentiation medium compared to Day-0, untreated controls, n=2/treatment, *$P \leq 0.01$.



Biochemical indices results from cells incubated in proliferation medium supplemented with DIP and GH show a trend for increased total DNA (*P=0.10*), decreased protein production capacity (*P=0.09*) and significantly decreased cell size (*P=0.03*), in treated cells compared to untreated (Table 7.1). Quantitative PCR results from the same experiment show the abundance of β-casein mRNA was below the detection limit of the assay (Cq > 30), while the reference gene GPAM was detectable (Cq < 30).

Table 7.1. Biochemical indices (total DNA, RNA, protein and relative ratios) from MAC-T incubated in proliferation medium with DIP and GH. P ≤ 0.05 = significant; P ≤ 0.10 = trend.

	Day-0 (*N*=2)	Day-2 (*N*=2)	SEM	P-value
Total MAC-T cellular content, µg				
DNA	39.40	88.61	17.32	0.10
RNA	112.86	152.96	16.42	0.14
Protein	1504.00	1844.00	268.33	0.33
MAC-T cellular ratio				
Protein synthetic capacity (RNA:DNA)	2.90	1.76	0.37	0.09
Cell size (Protein:DNA)	38.49	21.00	3.08	0.03
Protein synthetic efficiency (Protein:RNA)	13.33	12.01	0.71	0.21

7.5 Discussion

This study was conducted to determine whether MAC-T or pbMECs could be used to establish an *in vitro* chronic, lactogenically differentiated model of bovine lactation. Biomarkers of lactogenic differentiation we employed were β-casein mRNA abundance and changes in monolayer morphology. Biochemical indices were used to measure potential changes in cell number, cell size, protein synthetic capacity and efficiency.

Primary bovine MECs are routinely used as models of ruminant lactation because they consistently differentiate and retain many of the physiological characteristics of *in vivo* MECs. In this study pbMECs formed mammospheres and increased β-casein mRNA, consistent with lactogenic differentiation (257), indicating that pbMECs can be used to model how potential nutritional or hormonal interventions may effect MEC gene expression during differentiation. How such interventions may affect differentiated pbMECs in a model of chronic (long-term) lactation is yet to be investigated. This is primarily due to the finite lifespan of pbMECs (20 -26 doublings before entering apoptosis; (258)). Also, how such interventions may affect milk protein synthesis cannot be investigated using the method outlined in this study. TRIzol reagent dissolves Matrigel, contaminating any extract with Matrigel associated proteins.

The inability of MAC-T cells to differentiate in the presence of DIP and GH is inconsistent with previous studies (255). Modification of the differentiation protocol (absence of FBS and culture on Matrigel) changed monolayer morphology, but did not increase MAC-T β-casein abundance, indicating differentiation did not occur. Zavizion et al (1995) reported

that the MAC-T cell line was a heterogeneous population with three different mammary epithelial cell types, labelled CU-1, CU-2 and CU-3, each with distinct morphologies, growth rates and chromosome makeup. Cytogenetic analysis of each cell showed they differed in the frequency, presence or absence of different chromosomal defects and unidentified DNA fragments. The authors of that study conclude that the MAC-T cell line is phenotypically unstable, and each new passage will produce a new array of subclonal lines, each with a different new combination of chromosome rearrangements and lactation potential phenotypes. This may explain why MAC-T cells seem unresponsive to the presence of Matrigel and the lactogenic hormones prolactin and dexamethasone used in this study, while pbMECs exposed to the same Matrigel and hormone stocks were able to differentiate.

The results from both *in vitro* lactation studies (Chapter 6 and 7) show none of the cell culture models or differentiation methods employed produced a system that could be used to study changes in protein and gene expression. New MEC lines are being developed to fill this critical deficiency in ruminant lactation research, and aid our understanding of the molecular mechanisms that regulate milk production by MECs (259-261). To date, these cell lines have seen limited use, with researchers still preferring to use MAC-T for *in vitro* investigation of MEC function.

CHAPTER 8: GENERAL DISCUSSION

8.1 Methodological considerations

In this section aspects of the key methodologies used in this thesis are discussed and considerations for future studies proposed.

8.1.1 The problem

The ruminant mammary gland is not a homogenous tissue. The composition (fat pad, parenchyma, skin, cellular matrix) and the activity of individual cells (cell cycle, senescence, and differentiation state) vary widely across the gland, yet many of the molecular techniques utilised in this thesis did not account for this variation. The primary limitation was how the samples were stored. In each trial the samples used had been dissected out and frozen at -80^{o}C as an entire gland (chapter 2), or parenchymal slices (chapter 3, 4, 5). Nucleic acids and protein were then extracted from these samples to determine nucleic acid / protein ratios and abundance, the expression of selected genes and the abundance of total and activated mTOR pathway signalling proteins. Thus, the results obtained within this thesis, except components of chapter 2, encompass all the cell types and states found within the fetal (chapter 2) and maternal (chapters 3, 4, 5) mammary glands. A solution to the problem of cell type / state variation is histology.

8.1.2 The solution

Histology is a powerful technique that allows researchers to visualize and identify microscopic tissue structures, through the use of differential stains; and to quantify and localise specific proteins, through the use of antibodies linked to chemi- or fluorescent tags.

The use of differential stains would allow future researchers to discriminate between cell types and states, and directly determine cell number, within the ruminant mammary gland. Through immuno-histochemical techniques researchers could determine receptor / target protein localisation and measure the abundance of specific proteins within cell types through the use of different antibody-probe combinations. In addition, histological samples could be used to employ even more powerful molecular techniques such as in-cell or single-cell real time PCR to measure the expression of genes within and across cell types. However, as powerful as these techniques are, they could not have been employed in a study of the scope outlined in this thesis. These techniques are very expensive and require many years of training to develop basic proficiency. That said, I suggest that future lactation researchers focus on these techniques to unravel the molecular events that underpin phenotypes produced from animal trials involving the mammary gland.

8.2 General discussion

The aim of this thesis was to measure mTOR pathway activation in the ruminant mammary gland and identify the potential association of mTOR pathway activation with lactation performance. This is an important research field for the New Zealand dairy industry as studies in other species and tissues show the mTOR pathway can be manipulated to increase the synthesis of protein, a key economic component of ruminant milk and enhance tissue development.

Archived mammary tissue collected from pregnant sheep (Chapter 2) and mid-lactation dairy cows (Chapter 3) were used to identify if the abundance of mTOR protein was linked to the improved lactation phenotype observed in each trial. Each trial used well-established treatment models to modify ruminant mammary gland lactation potential; nutrition (Chapter 2) and exogenous growth hormone (GH) administration (Chapter 3). Results from both studies show increased total and activated mTOR pathway protein abundance and biochemical indices changes are associated with improved lactation performance.

Results from Chapter 2 show fetal mammary glands from lambs carried by maintenance fed dams had increased abundance of total and activated mTOR pathway signalling proteins, compared to fetuses carried by *ad libitum* fed dams. In Chapter 3, dairy cows administered exogenous GH had increased milk protein yield and abundance of total and activated mTOR pathway signalling proteins, compared to saline treated controls. Increased abundance of total and activated mTOR pathway proteins was associated with increased protein synthetic capacity (Chapter 2), total protein content, ribosome number

and cell size (Chapter 3), factors all known to be regulated by mTOR signalling. A surprise finding in Chapters 2 and 3 was increased abundance of signalling proteins belonging to the MAPK pathway. The MAPK pathway responds to hormones and mitogens to co-regulate signalling proteins downstream of the mTOR pathway, suggesting that mTOR and MAPK signalling converge to regulate fetal mammary lactation potential in response to altered maternal nutrition and maternal mammary lactation potential in response to exogenous GH. While it was proposed that activation of MAPK signalling was being mediated by IGF1 (Chapter 2 and 3), the basis for mTOR pathway activation was hypothesized to occur via amino acid (AA) signalling. Unfortunately, investigating potential AA-mTOR pathway signalling in Chapter 2 could not be facilitated as maternal plasma samples were discarded prior to the start of this study and fetal plasma samples were not collected. In addition, fetal mammary tissue had not been separated into the individual gland components (parenchyma and fat pad). The importance of separating the fetal mammary gland into component tissues, such as parenchyma and fat pad, was highlighted in Chapter 2, where results showed greater abundance of $mTOR^{Ser2448}$ in the fat pad, compared to parenchyma. The greater abundance of $mTOR^{Ser2448}$ in the fat pad may have prevented accurate detection of $mTOR^{Ser2448}$ in fetal mammary tissues that were not separated in the same chapter (2).

In Chapter 3 it was proposed that the increased abundance of total and activated mTOR protein was mediated by AA signalling. To investigate this proposal the absolute free amino acid (FAA) concentration in plasma and mammary gland tissue harvested from the trial described in Chapter 3 was measured (Chapter 4). In the same study (Chapter 4), mammary gland AA transporter mRNA abundance was also measured.

Results from Chapter 4 showed GH administration decreased the absolute plasma concentrations of lysine and tyrosine, and tended to decrease arginine. Absolute concentrations of intracellular glycine, serine and glutamate decreased significantly in the mammary glands of GH-treated cows. A trend for decreased arginine was also observed. Thus, L-arginine was the only AA whose absolute concentrations decreased in response to exogenous GH administration, in plasma and mammary tissue. Studies show that the administration of L-arginine to late-pregnancy dairy cows and *in vitro* cultures of cells harvested from the lactating bovine mammary gland increases milk production and the abundance of milk gene αs1-casein and β-casein mRNA, respectively. L-arginine has also been identified in porcine muscle, ovine trophectoderm cells and rat intestinal epithelial cells to increase the abundance of total and / or activated mTOR pathway signalling proteins. In addition, expression of the AA transporter SLCA2 increased. SLC3A2 is the heavy chain of a dimeric AA transporter complex that involves many small chain components, some of which are involved in the transport of L-arginine. Thus, L-arginine was identified as a prime candidate for a future targeted nutritional intervention trial (Chapter 5). A problem that cannot be adequately addressed from this thesis is the conflicting data from Chapters 3 and 4. The administration of GH to lactating dairy cows

increases the abundance of activated and total mTOR (Chapter 3), after six days, whilst decreasing the absolute concentration of plasma and mammary gland L-arginine (Chapter 4). It is possible that the results observed in Chapters 3 and 4 highlight the disconnection between current literature on mTOR pathway regulation, which involves the use of acute models (short term pathway activation) and what is occurring *in vivo*, chronic, sustained signalling. However, this is speculative and cannot be definitively answered without repeating the experiment and including more sampling time-points, or the development of an *in vitro* model of lactation.

Results from Chapter 5 show that L-arginine administration between d 100 and d 140 of pregnancy had no effect on the abundance of total or activated mTOR, but did increase mammary gland cell number. L-arginine supplementation from d 100 of pregnancy to parturition had no significant effect on milk yield or composition, but did lower milk somatic cell count (MSCC), compared to saline treated controls.

The reduction in MSCC was a completely unexpected result, and is currently the only known nutritional intervention shown to reduce MSCC. It was postulated that L-arginine increased cell number independently of the mTOR pathway through increased the stimulation of increased insulin production by the pancreas and mammary gland polyamine synthesis. Whilst, decreased MSCC was hypothesized to occur via L-arginine conversion to reactive oxygen species by immune effector cells. However, the mechanisms of action are only proposed this thesis does not identify how L-arginine increases mammary gland cell number or prevents increased MSCC. Both of these results are

important to the ruminant dairy and meat industries as increased mammary gland cell number at the onset of lactation is proposed to increase lactation persistency, whilst increased MSCC through sub- and clinical mastitis negatively impacts lactation performance.

To elucidate the molecular mechanisms underpinning AA regulation of mTOR pathway signalling, in particular L-arginine (Chapter 4), the development of an *in vitro* model of ruminant lactation was attempted (Chapter 6, 7). The model needed to produce a lactating phenotype (differentiate), determined by elevated expression of the milk genes and protein, and the model needed to have a long term experimental window spanning days (chronic) as opposed to hours (acute). These two preconditions would ensure the *in vitro* model reflected, as close as possible, *in vivo* lactation. Mammary epithelial cells of bovine origin were chosen to establish the *in vitro* model for two reasons. 1) The experimental work in Chapters 3 and 4 was conducted using samples harvested from lactating dairy cows and 2) the well-established *in vitro* models of ruminant lactation have been developed using bovine mammary epithelial cells (MEC).

In this thesis, the development of a lactogenically differentiated, chronic *in vitro* model of ruminant lactation was not successful (Chapters 6, 7). Primary bovine MEC lines used were successfully differentiated, however low cell numbers prevented sufficient levels of RNA and protein being extracted for downstream analysis by qPCR and Western blotting. In addition, the immortalised MEC MAC-T could not be differentiated using any of the conditions available in the current literature. Why MAC-Ts could not be differentiated in

this study could not be definitively established, nor did data from published literature provide any conclusive answer. One thing is certain, the absence of an *in vitro* model of lactation that can reproducibly differentiate and be utilised for chronic studies of lactation is a crucial gap in ruminant research that needs to be addressed. A valid criticism of lactation research in ruminants is the reliance on molecular models developed in species such as mouse and rats to explain phenotypic results obtained from animal trials. Literature evidence shows us that cows are not mice (262), and that the so called "model species" do not accurately reflect the molecular changes that occur within ruminants. The New Zealand dairy industry has ambitious goals for increasing the productivity of the New Zealand dairy herd, ambitious goals that cannot be met without first identifying and understanding the fundamental nature of mammary epithelial cells. Identifying the signalling pathways that control milk yield / composition and then understanding how those pathways respond to hormones, mitogens and nutrients. These factors can only be studied using an *in vitro* model that lactogenically differentiates and can be used for chronic lactation studies.

In conclusion, the findings presented in this thesis have provided novel information about the association of mTOR (and MAPK) pathway signalling with ruminant lactation performance via potential control of mRNA translation, nucleocytoplasmic export, cell number and cell size. Whilst the unexpected finding that L-arginine reduces milk somatic cell count provides a potential tool to the New Zealand dairy and meat sectors for improving ruminant mammary gland health, and subsequent animal performance. An *in vitro* model of ruminant lactation still needs to be developed to aid our understanding of milk production regulation and provide tools for industry to meet future demands for increased productivity.

8.2.1 Potential implications and future directions

The mTOR pathway has been identified as one of the key control points of protein translation and cellular differentiation in a number of tissue types and mammalian species. This makes the mTOR pathway an attractive target for interventions designed to improve ruminant lactation potential – via enhanced "pre-lactation" mammary gland development (improved future lactation potential) and/or increased milk production (during lactation). However, this poses the question: at what developmental time-point should an intervention be trialled? The ruminant mammary gland undergoes specific developmental changes (fetal, pre-pubertal, pubertal, pregnancy and lactation) each with differing effects on tissue growth (allometric vs. isometric), cellular differentiation and function. Due to the ubiquitous nature of mTOR in tissue location and function it is not inconceivable that the role mTOR signaling plays at each developmental stage will also differ. Thus, the application of any intervention designed to change mTOR pathway signaling is bound to have differing effects on lactation potential if applied at different developmental stages. This thesis does not "definitively" answer the question when to intervene, but it does provide some evidence that the fetal (Chapter 2: nutrition) and lactation (Chapters 3 and 4: exogenous growth hormone) stages are two potential developmental time-points to be further investigated. The ubiquitous nature of mTOR in tissue location and function poses another important question: What role is mTOR signaling playing? The ubiquitous function(s) of mTOR signaling indicates that, increasing the abundance of mTOR protein may not be the only way to regulate cellular functions, active mTOR complexes could also be retasked, and as such a researcher would only observe changes in the downstream targets of mTOR and not mTOR itself. Thus, it is these target proteins of mTOR signaling

that will be more informative to researchers in identifying the role(s) mTOR signaling may be playing. Inhibition studies using rapamycin (in the presence and absence of mTOR pathway activators) would also aid in identifying the role of mTOR(C1) signaling, however inhibition experiments are typically performed using *in vitro* cell cultures. To accurately reflect *in vivo* lactation *in vitro* cell cultures would need to be chronic (grow for weeks to months) and lactogenically differentiate. Attempts were trialled to develop cell lines that could reflect the *in vivo* environment (Chapter 7) or use somatic cells as *in vivo* markers (Chapter 6), yet neither method was successful. Future investigations may need to probe the relationship of mTOR signaling and its role in lactation performance through the localised infusion / injection of rapamycin. By infusing selected teats (into the gland) or injection (into the blood stream) with rapamycin (in the presence and absence of mTOR activators), effects on milk production and mammary tissue / milk somatic cell gene expression and mTOR pathway activation could be assessed. Identifying the role mTOR signaling plays in regulating lactation potential is a body of fundamental work that needs to be addressed to inform future researchers what potential interventions can be utilised at specific developmental time-points. A potential intervention, identified in Chapter 4, is L-arginine. L-arginine has been shown to increase mTOR protein abundance, *in vitro* and *in vivo* and has been associated with increased milk production. L-arginine is utilised by the mammary gland to synthesise polyamines, nitric oxide (NO) and L-proline. Administration of L-arginine during late-pregnancy (Chapter 5) did not increase milk production or the abundance of mTOR in the mammary gland but it did improve mammary gland health by decreasing milk somatic cell counts. Thus, increasing arterial supply of a single amino acid, L-arginine, improved gland health – underpinning the hypothesis that targeted nutrition

can produce beneficial outcomes for the agricultural industry. As to why elevated L-arginine had no observable effect on milk production or mammary mTOR abundance, it could be a combination of any of the aforementioned questions. L-arginine was identified as a potential intervention candidate from a growth hormone model of increased milk production in dairy cows treated in mid-lactation. The L-arginine intervention trial used sheep in mid-late pregnancy supplemented with L-arginine, thus the species, developmental time-point and intervention were all different – accounting for the possible differences.

The development of interventions that can be utilised on-farm, to increase milk production, are of particular interest to the New Zealand dairy industry. While the majority of the work outlined in this thesis cannot be considered "farm-ready" this body of research does provide some key fundamental sign-posts about which directions future researchers can pursue. Of particular interest to researchers and the dairy industry maybe the use of a single nutrient (L-arginine: Chapter 5) to improve gland health. L-arginine has been shown by other researchers to increase milk production, *in vivo* and *in vitro* so modifications in dose, time-frame for intervention of the use of rumen-protected arginine as a feed-additive could be considered to increase milk production and / or improve gland health.

APPENDIX A

Animal model used to study the effect of parenteral L-arginine administration on mammary gland development and function in twin-bearing sheep

Note: *The PhD candidate was involved in the planning and implementation of this animal experiment. The study outlined in Chapter 5 and part of Chapter 2 was conducted as part of this animal experiment, which was assessing the effect of parenteral L-arginine on early life development of twin born lambs and has been included to provide experimental background to the reader*

Multiparous Romney ewes, previously synchronized with an intravaginal progesterone-containing device (CIDRs; Pharmacia and Upjohn Ltd. Co., Auckland, New Zealand) were naturally mated to one of two Poll Dorset sires in 2 different groups. Ewes in cohort 1 were mated in two sub-groups 3 weeks apart and both sub-groups were euthanized at 140 days of gestation (P140). Ewes in cohort 2 were mated one month later and were allowed to lamb, and lambs were evaluated at 153 days of life, which corresponds with the average market age. At ~60 days of gestation, ewes were pregnancy scanned via transabdominal ultrasonography. Forty nine twin-bearing ewes were selected and maintained under *ad-libitum* grazing conditions. The live weight of the ewes at mating was 65-75 kg with a body condition score of 3-3.5, using a 1-5 scale (263). Two weeks prior to indoor housing, ewes were offered a concentrate diet (University B mix, Camtech Nutrition, Cambridge NZ) while still in grazing (up to 20 % of daily requirements). At P80, ewes were acclimatised to housing indoors for one week in group pens and then in individual pens. Ewes were fed *ad libitum* with same concentrate diet used during grazing period and had water freely available. The diet consisted in a lucerne pellet, offered once a day between 0800 and 0900 which contained 6.69 mg/g of Arg (6% of total AA), 17% crude protein and 10.5 MJ/kg metabolisable energy. The diet was formulated to meet 100% of NRC-recommended requirements for twin-bearing ewes (264). This study and all animal handling procedures were approved by the University of Auckland Animal Ethics Committee, New Zealand in accordance with the 1999 Animal Welfare Act (C889).

At P85, animals were randomly assigned to Arg (cohort 1, *n* = 12; cohort 2, *n* = 13) or control (cohort 1, *n* = 11; cohort 2, *n* = 13) groups. At P97, polyvinyl catheters (0.040 inch internal diameter polyvinyl tubing (Critchley Electrical Products Pty Ltd, Australia) were inserted into the tarsal vein of the hind leg under brief anaesthesia induced by injectable anaesthetic and sedation (combination of 0.5 mg/kg BW Pamlin and 10 mg/kg BW Ketamine, Parnell Technologies, NZ). The catheter was externalised and flushed regularly with heparinised saline (0.9% sodium chloride, Baxters Healthcare Pty Ltd, Australia, 10 U/ml sodium heparin), sealed with stopcocks, secured in a plastic bag and anchored to the mid line of the ewe. The bag and catheter were further secured with tube net. After catheter insertion, the ewes received prophylactic i.m. antibiotics (Duplocillin® LA, Intervet LTD, Newmarket, Auckland, 2 mL /50 kg BW).

From day 100 of pregnancy ewes received three times daily (0800, 0400, 2400 hrs) either a bolus of L-Arg-mono-hydrochloride (L-Arg-HCL; Merck KGaA, Darmstadt, Germany) (345 µmol/kg BW) adjusted according to the live weight gain of the ewe each week, or the same volume of sterile saline. The L-Arg-HCL solution was prepared daily, dissolved using sterile physiologic saline (0.9% sodium chloride, Baxters Healthcare Pty Ltd, Australia) with a final concentration of 1.8g Arg per 5 mL. The pH was adjusted to 7.0 with 1M NaOH and filtered-sterilized using a 0.22 µm PES syringe filter (Jet Biofil, Elgin IL, USA). The dose of L-Arg-HCL supplemented was based on a previous study at a maximum dose-rate known to be safe for use in sheep (265), and the administration route to avoid ruminal (266) and/or intestine (267) Arg degradation.

APPENDIX B

DRC 16 - V3 Online Statement of Contribution to Doctoral Thesis Containing Publications

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Quentin Sciascia

Name/Title of Principal Supervisor: Professor Hugh Blair

Name of Published Research Output and full reference:

Peer Reviewed Publication:
Sciascia, Q., D. Pacheco and S.A. McCoard. (2013). Increased milk protein synthesis in response to exogenous growth hormone is associated with changes in mechanistic (mammalian) target of rapamycin (mTOR)C1-dependent and independent cell signaling. J Dairy Sci. 96(4): p. 2327-38.

In which Chapter is the Published Work: Three (3)

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Candidate completed all the laboratory work, real time PCR and 4E-SE data analysis. Western blotting and biochemical indices data analysis was performed by the candidate and assisted by the coauthors. The candidate wrote the manuscript and draft revisions were made in conjunction with listed coauthors.

Quentin Sciascia Digitally signed by Quentin Sciascia DN: cn=Quentin Sciascia, o=Leibniz-Institut für Nutztierbiologie, ou=Nutritional Physiology "Oskar Kellner", email=sciascia@fbn-dummerstorf.de, c=DE Date: 2013.11.27 21:17:43 +01'00'

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27-11-2013

Date

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Principal Supervisor's signature

28 November 2013

Date

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Quentin Sciascia

Name/Title of Principal Supervisor: Professor Hugh Blair

Name of Published Research Output and full reference:

Peer Reviewed Publication:
Sciascia Q, Pacheco D, Senna Salerno M, Blair HT, McCoard SA. (2012). Milk somatic cells are not suitable biomarkers of lactating ruminant mammary gland function. Proceedings of the New Zealand Society of Animal Production, Volume 72, pp 3-7

In which Chapter is the Published Work: Six (6)

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Candidate completed all the laboratory work and real time PCR data analysis. Biochemical indices data analysis was performed by the candidate and assisted by the coauthors. The candidate wrote the manuscript and draft revisions were made in conjunction with listed coauthors.

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Principal Supervisor's signature — 28 November 2013 — Date

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