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# **Regulation of protein synthesis in the mammary gland**

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

**Doctor of Philosophy**

in  
Animal Science

at Massey University  
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This thesis is dedicated to my parents

**Mandi Hayashi and Ivaci Hayashi**

For your unconditional love

*"It is the simple things in life that are the most extraordinary, only wise men are able to understand them."*

*Paulo Coelho*

## ABSTRACT

This thesis examines the signaling pathways involved in the regulation of milk protein synthesis in the lactating mammary gland and their control. The protein synthetic machinery can be regulated during the transcription, translation and degradation stages of mRNA processing. Translation control in eukaryotes involves changes in the activity or other functional properties of the translation factors. These include proteins involved in initiation, peptide-chain elongation and termination of mRNA processing. Changes in the nutritional, physiological and hormonal status of the body are sensed by receptors that signal to a central protein, known as mammalian target of rapamycin (mTOR). The mTOR signaling pathway then activates or inhibits the activity of translation factors and kinases involved in the initiation and elongation stage of translation.

A major objective of this thesis was to elucidate which genes and pathways are involved in the regulation of milk protein synthesis in the mammary gland and the mechanism(s) that regulate their action. The results presented here show that changes in milk protein production occurring during lactation in response to external stimuli are potentially regulated at the level of translation or subsequent processing rather than by transcriptional regulation (mRNA abundance).

The results also show that in response to growth hormone (GH) treatment, which increased the yield of milk protein, the phosphorylation status of the ribosomal protein S6 (S6) is increased as well as the protein abundance of eukaryotic elongation factor 2 (eEF2) and eukaryotic initiation factor 4E (eIF4E). These results suggest an important

relationship between milk protein yield and changes in the initiation and elongation stages of translation.

Another major finding was the elucidation that mTOR is involved in the signaling pathways activated by GH and that this effect involves signaling through the PI-3 kinase pathway. In these experiments, increased protein synthesis was potentially achieved with the use of GH. Thus, this study suggests the mTOR signaling pathway is a key mediator of the GH effects in protein synthesis stimulation.

Finally, the requirement for a functional mTOR signaling (TOS) motif in the eukaryotic initiation factor 4E binding protein (4E-BP1) was identified. This finding could help the identification of other proteins that may be controlled by mTOR and consequently are regulators of mRNA translation.

In summary, this thesis unveils key signaling pathways involved in the regulation of milk protein synthesis and provides further insight into the control of the mTOR signaling pathway. These findings open new frontiers for the manipulation of milk composition.

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## ABBREVIATIONS

<b>4E-BP1</b>	Eukaryotic initiation factor 4E-binding protein 1
<b>ATP</b>	Adenosine triphosphate
<b>BES</b>	N, N-bis [2-Hydroxyethyl]-2 aminoethanesulfonic acid
<b>BSA</b>	Bovine serum albumin
<b>cDNA</b>	Complementary DNA
<b>Ct</b>	Cycles to threshold
<b>DMEM</b>	Dulbecco's modified Eagle's medium
<b>DMSO</b>	Dimethyl sulphoxide
<b>DNA</b>	Deoxyribonucleic acid
<b>dNTP</b>	Deoxyribonucleotide triphosphate
<b>DTT</b>	Dithiothreitol
<b>ECL</b>	Enhanced chemiluminescence
<b>EDTA</b>	Ethylenediamine tetraacetic acid
<b>eEF2</b>	Eukaryotic elongation factor 2
<b>eEF2K</b>	Eukaryotic elongation factor 2 kinase
<b>eIF</b>	Eukaryotic initiation factor
<b>eIF4E</b>	Eukaryotic initiation factor 4E
<b>eRF</b>	Eukaryotic release factor
<b>ERK</b>	Extracellular signal-regulated protein kinase
<b>EST</b>	Expressed sequence tag
<b>FCS</b>	Foetal calf serum
<b>FDR</b>	False discovery rate
<b>GDP</b>	Guanosine diphosphate
<b>GH</b>	Growth hormone
<b>GHR</b>	GH receptor
<b>GO</b>	Gene ontology
<b>GTP</b>	Guanosine triphosphate
<b>IGF</b>	Insulin-like growth factor
<b>IGFBP</b>	Insulin-like growth factor binding protein
<b>IPA</b>	Ingenuity pathway analysis
<b>IPTG</b>	Isopropyl-b-thiogalactopyranoside



<b>IRES</b>	Internal ribosomal entry segments
<b>JAK</b>	Janus kinase
<b>JNK</b>	c-Jun N-terminal kinase
<b>m<sup>7</sup>GTP</b>	7-methyl-GTP
<b>MAPK</b>	MAP kinase
<b>mRNA</b>	Messenger RNA
<b>mTOR</b>	Mammalian target of rapamycin
<b>NADPH</b>	Nicotamide adonine dinuclotide phosphate
<b>NCBI</b>	National Center for Biotechnology Information
<b>PBS</b>	Phosphate-buffered saline
<b>PCR</b>	Polymerase chain reaction
<b>PI 3-kinase</b>	Phosphatidylinositol 3-kinase
<b>PIP<sub>2</sub></b>	Phosphatidyinositol 4,5-biphosphate
<b>PKB</b>	Protein kinase B
<b>PTEN</b>	Phosphatase and tensin homologue deleted on chromosome 10
<b>PVDF</b>	Polyvinylidene difluoride
<b>qRT-PCR</b>	Quantitative real time PCR
<b>REST</b>	Relative expression software tool
<b>RNA</b>	Ribonucleic acid
<b>S6</b>	Ribosomal protein S6
<b>SDS-PAGE</b>	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
<b>STAT</b>	Signal transduction and transcriptional activation
<b>Rheb</b>	Ras homolog enriched in brain
<b>dsRNA</b>	Double-stranded RNA
<b>RNAi</b>	RNA interference
<b>P90<sup>RSK</sup></b>	P90 ribosomal S6 kinase
<b>RNAi</b>	RNA interference
<b>siRNA</b>	Small interfering RNAs
<b>EGF</b>	Epidermal growth factor
<b>CoREs</b>	Composite response elements
<b>C/EBP</b>	CAAT\enhancer binding protein
<b>NF-1</b>	Nuclear factor 1
<b>YY-1</b>	Yin Yang-1

<b>GSK3</b>	Glycogen synthase kinase 3
<b>S6K</b>	Ribosomal S6 kinase
<b>S6</b>	Ribosomal protein S6
<b>P70S6K</b>	P70S6 kinase
<b>TCA</b>	Trichloroacetic acid
<b>GPAM</b>	Mitochondrial glycerol-3-phosphate acyltransferase
<b>MGEA5</b>	Meningioma-expressed antigen 5
<b>KRT15</b>	K15 intermediate filament type I keratin
<b>PKC</b>	Protein kinase C
<b>Raptor</b>	Regulatory associated protein of mTOR
<b>TOS</b>	TOR signaling motif
<b>HPLC</b>	High pressure liquid chromatography
<b>GAPDH</b>	Glyceraldehyde-3-phosphate dehydrogenase
<b>Mac-T</b>	Mammary alveolar cells with large-T antigen
<b>H4IIE</b>	Rat hepatoma cell line
<b>HEK293</b>	Human embryonic kidney cell line
<b>TPA</b>	Phorbol-12-myristate-13-acetate
<b>t-RNA</b>	Transfer RNA
<b>t-RNA<sub>i</sub></b>	Initiator tRNA
<b>TSC</b>	Tuberous sclerosis complex
<b>UTR</b>	Untranslated region

## GENERAL INTRODUCTION

### *Background*

The New Zealand dairy industry is the country's biggest agricultural industry, accounting for 23% of New Zealand's total export earnings ([www.investnewzealand.govt.nz/common/files/Dairy\\_Feb06.pdf](http://www.investnewzealand.govt.nz/common/files/Dairy_Feb06.pdf)). The application of new biotechnologies in the dairy industry is essential for sustaining the competitiveness and profitability of New Zealand in the global market. A number of studies have successfully demonstrated the physiological changes that occur in response to treatments that perturb the milk synthetic machinery. However, the elucidation of the molecular mechanisms, specifically those of the signaling pathways and genes, involved in the regulation of protein synthesis in the mammary gland are still being explored.

Protein synthesis is mainly regulated through the phosphorylation or binding ability of the initiation and elongation components of the mRNA translational machinery. Although there is ample evidence that certain exogenous stimuli change milk protein synthesis in lactating cows and it is clear that mRNA translation is important for the regulation of protein synthesis, there are no studies demonstrating that these stimuli affect the translation initiation and elongation factors and hence protein synthesis in the mammary gland.

During protein synthesis a tightly regulated step occurs during the initiation and elongation of the mRNA translation which involves the mammalian target of rapamycin (mTOR) signaling pathway. The start of initiation is controlled by the formation of the eukaryotic initiation factor (eIF) 4F complex, which requires the eIF4E protein. The

biological activation of the eIF4E protein is regulated by a family of translation repressor proteins, the eIF4E binding proteins (4E-BPs) proteins. mTOR regulates the phosphorylation of 4E-BP1, which occurs in multiple sites. However, the residues involved in the interaction between mTOR and 4E-BP1 and the nature of the interaction are still unclear.

### ***Objectives***

The main objectives of this thesis were to identify the pathways and genes involved in the regulation of milk protein synthesis and to understand their roles in the mammary gland during lactation in response to exogenous stimuli like growth hormone and atropine. A second objective was to study the regulation and interaction of 4E-BP1 and mTOR, the main identified regulators of protein synthesis in other tissues. A better understanding of the 4E-BP1-mTOR interaction will help to identify new downstream targets for the mTOR signaling cascade and consequently new proteins involved in the protein synthesis machinery.

It is anticipated that the knowledge gained from this research will open up possibilities to develop new technologies to manipulate milk composition and yield with potential economic benefits to the dairy industry.