Regulation of protein synthesis in the mammary gland

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Amanda Aparecida Hayashi
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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

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

IRES	Internal ribosomal entry segments
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
m^7GTP	7-methyl-GTP
MAPK	MAP kinase
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NADPH	Nicotamide adonine dinuclootide phosphate
NCBI	National Center for Biotechnology Information
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI 3-kinase	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidynositol 4,5-biphosphate
PKB	Protein kinase B
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real time PCR
REST	Relative expression software tool
RNA	Ribonucleic acid
S6	Ribosomal protein S6
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STAT	Signal transduction and transcriptional activation
Rheb	Ras homolog enriched in brain
dsRNA	Double-stranded RNA
RNAi	RNA interference
$P90^{RSK}$	P90 ribosomal S6 kinase
RNAi	RNA interference
siRNA	Small interfering RNAs
EGF	Epidermal growth factor
CoREs	Composite response elements
C/EBP	CAAT\enhancer binding protein
NF-1	Nuclear factor 1
YY-1	Yin Yang-1

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

CHAPTER 1

Literature Review

1.1. Introduction

In this Chapter, an overview of the molecular mechanisms that affect milk production will be presented. In particular, focus will be given to the regulation of transcription and translation due to the nature of the topics discussed in subsequent Chapters.

1.2. Mammary Gland

During the twentieth century important advances in lactation biology were achieved in fields such as the relationship between structure and function in the mammary gland, identification of biochemical pathways for the synthesis of milk components and elucidation of hormonal regulation of development and function of the mammary gland. Features of the last 25 years have been the gain in knowledge of the regulation of milk synthesis and the increasing interest in bioactive components of milk (Bauman *et al*., 2006).

The mammary gland consists of the secreting tissue and a variety of support tissues including nerves, blood and lymph vessels, adipose and connective tissues. The lactation cycle in the adult can be divided into the consecutive stages of mammogenesis, lactogenesis, maintainance of lactation and involution. Each of these phases is strictly controlled by hormones and they have been the focus of numerous studies (Akers, 2006). Being able to support a successful lactation requires the controlled development of the mammary gland in the foetus through to the adult, with major growth and development occurring during pregnancy and at the onset of lactation. In addition, the onset of lactation is accompanied by repartitioning of nutrients from body tissues

towards the mammary gland (Bauman and Currie, 1980). In this Chapter, the maintainance of lactation will be discussed as the regulation of milk protein synthesis is the main topic of this thesis.

1.3. Homeorhesis

The lactation period in dairy cows is usually of 305 days and it can be divided into three phases: early lactation (from 0 to 70 days *postpartum*); mid lactation (usually in the next 10 weeks *postpartum*); mid-late lactation period (usually 140 to 305 days *postpartum*). The peak of lactation happens at 6 to 8 weeks after calving and at this point the energy requirements for milk synthesis can approach 80% of net energy intake (Butte and King, 2005), with all the necessary nutrients derived from either the intake of food or their release from body tissues. This delivery of nutrients to the gland is achieved by co-ordinated changes in various physiological processes orchestrated by the endocrine system. These orchestrated changes to meet particular physiological needs, are known as homeorhesis (Bauman and Currie, 1980). Changes include increased lipolysis and decreased lipogenesis in adipose tissue, elevated production of glucose, more efficient absorption of minerals from the gastrointestinal tract, while the non-mammary tissues use fatty acids as an energy source and redirect nutrients such as amino acids to the mammary gland (Bauman and Currie, 1980).

1.3.1. Hormonal control of lactation

During lactation, the mammary gland is under hormonal control, in particular, prolactin and growth hormone (GH). The role of these hormones varies between species. Prolactin predominates during mammogenesis and lactogenesis in all species and remains so in rodents and humans at least during early lactation. However, its role during lactation in ruminants is unclear. Experiments using drugs to decrease circulating prolactin in cows during established lactation had little effect on milk production or composition (Plaut *et al*., 1987) suggesting that the ruminant mammary gland can produce milk even when exposed to very low concentrations of prolactin. This is in contrast with the lactogenesis period when prolactin is essential for the onset of lactation (Akers *et al.,* 1981).

Similar to the GH effects in the mammary gland, the prolactin effects in the mammary epithelial cells appears to be in part through induction of autocrine insulin-like growth factor I (IGF-1) within the mammary gland (Brisken *et al.*, 2002; Hovey *et al.*, 2003;). Along similar lines, exogenous prolactin was found to suppress the local synthesis of insulin-like growth factor binding protein (IGFBP)-5 in the mammary gland after removal of the sucking stimulus, thereby preventing sequestration of IGF-1 and maintaining cell survival (Flint *et al.,* 1997). In rodents, prolactin has direct and indirect effects on the mammary gland. The role of prolactin signaling in the development of the mammary gland was tested using knock-out mice for the prolactin receptor gene (Brisken *et al*., 1999). In knock-out mice, the lobuloalveolar development was completely inhibited during pregnancy, but the ductal side branching differentiation was still possible via indirect mechanisms, involving the activation of several transcription

factors. Signal transduction and transcriptional activation (STAT) 5 is one of the key signaling molecules activated by prolactin (Lamote *et al*., 2004).

The effects of GH during lactation has been extensively studied, specifically its stimulatory effects on milk production (Bauman *et al*., 1985; Breier *et al*., 1991; Feldman *et al*., 1993; Plath-Gabler *et al*., 2001). In this thesis, the elucidation of pathways involved in GH signaling will be studied, as well as the molecular mechanism(s) of action of GH in the mammary gland (Chapters 2, 3 and 5).

It is still not clear if GH affects milk production by acting directly on the secretory epithelial cells or indirectly either via production of IGF-1 in the mammary gland, paracrine effect, or IGF-1 production in the liver (Flint and Knight, 1997; Hull and Harvey, 2003). Growing evidence has supported the idea that the GH effects on the mammary gland are direct as shown by the presence of GH receptors in this tissue. Chun *et al.* (2005) were able to immunoprecipitate and detect related GH receptor proteins in sheep mammary gland using polyclonal antibody directed against the extracellular domain of GH receptor. Jiang *et al*. (2005) have successfully demonstrated that distinct isoforms of the GH receptor exist in bovine mammary gland. Additionally, GH receptors in the bovine mammary gland are constantly expressed during mammogenesis and lactation (Sinowatz *et al*., 2000) while in liver GH receptor expression is transiently decreased near parturition (Radcliff *et al*., 2003). Collectively, these observations support a role for GH in directly acting in the mammary gland and affecting milk production and secretion as supported by the presence of GH receptors in the mammary epithelium.

The expression of mRNAs for IGF-1 and 2 receptors, IGFBPs have also been demonstrated in primary secretory cells of the bovine mammary gland (Baumrucker and Erondu, 2000). Hadsell *et al.* (2005) showed that direct activation of the mammary gland IGF-1 receptor is involved in enhanced milk synthesis during prolonged lactation. A widely accepted hypothesis is that part of the many actions of GH in the mammary gland are mediated indirectly by IGF-1 as the concentration of this hormone increases in serum after GH treatment in different ruminant species (Davis *et al.,* 1992; Shingu *et al.*, 2004). However the administration of IGF-1 and IGF-2 as well as several IGF analogs were unable to mimic the effect of GH in sustaining milk secretion (Flint *et al*., 1992).

IGF-1 also has alternative roles in the mammary gland. It has a main role in mammary tissue homeostasis, regulating cell proliferation and differentiation during lactogenesis. Studies from Hadsell (2004) using transgenic mice overexpressing IGF-1 demonstrated the importance of IGF-dependent stimulation of cell cycle progression during early mammary gland development. Thus, some of the known effects of GH in mammary cell proliferation might be mediated through IGF-1. GH administration to lactating cows increases the local production of IGF-1 and this local production of IGF-1 potentially increases the cell survival, resulting in increases in milk production and persistency of lactation (Flint and Knight, 1997). Sakamoto *et al*. (2007) have shown that GH effect in bovine mammary epithelial cells enhances phosphorylation of Akt (involved in cellular survival pathways) and this effect is mediated by IGF-1 through IGFBP-5 inhibition. These results further demonstrate that part of the effect of GH on mammary cells are mediated by IGF-1 and provide evidence that GH treatment has a role in cell survival and protein synthesis (supported by the activation of Akt pathway).

The binding of IGF to its receptor is affected by a family of six IGF binding proteins (IGFBPs). IGFBPs have several functions including the transport of IGF to target cells and have been reported to both inhibit and stimulate the actions of IGF (Jones and Clemmons, 1995). Increase in the levels of IGFBP-5 protein has been observed in rat milk 48 hours after the removal of the pups and appears to accelerate the induction of involution (Tonner *et al.*, 1997). These observations further support a role for IGF-1 in the regulation of lactation maintenance in rodents.

Apart from the IGFs, other local growth factors have been inferred in the function of the mammary gland. Epidermal-growth factors (EGFs) and members of the transforming growth factor family (TGF-β) are two examples. It has been shown that these two growth factors are expressed in the ruminant mammary gland (Forsyth, 1996) but most of the studies about these factors have been performed in rodents so their role in ruminant mammary gland are still not clear.

A primary focus of this thesis will be to study the effects of GH in the mammary gland. GH is an important galactopoietic hormone and increasing its concentration is a potential way to explore the molecular mechanisms that regulate milk protein synthesis in the mammary cell.

1.3.1.1. Growth hormone and homeorhesis

Growth hormone is a mediator of homeorhetic control that shifts the partitioning of nutrients in a lactating cow so that more are used for milk synthesis. Treatment of cows with growth hormone increases both the rate of milk synthesis within the mammary gland and orchestrates other body processes in a manner to provide the necessary

nutrients to support this enhanced rate of milk synthesis. This control involves coordinating the metabolism of various body organs and tissues and includes the metabolism of all nutrient classes - carbohydrates, lipids, proteins and minerals. (Baldwin and Knapp, 1993). Many of the effects of GH are indirectly mediated via the actions of the IGF system (Davis *et al.,* 1992; Burton *et al.,* 1994; Bauman, 1999). GH causes changes in the circulating concentrations of IGF-1 and some of the IGFBPs in well fed cows (Baldwin and Knapp, 1993). The role of this system in mammary function is still not fully understood. However, it is apparent that there is a close relationship between the role of these proteins and the maintainance of milk secretion.

1.4. Milk composition

Bovine milk generally contains between 2.5 and 3.7% of protein, 3.5% and 5.0 % of fat, 4.7% and 5.0% of lactose by weight, 85% and 88% of water with wide variation between breeds (Cerbulis and Farrell, Jr., 1975). The primary substrates extracted from the blood by the lactating mammary gland for the synthesis of milk are glucose, amino acids, fatty acids and minerals. Various biochemical pathways are involved in milk synthesis and current understanding of those for lactose and fat synthesis are reviewed elsewhere (Bauman *et al*., 2006). Since the focus of this thesis is on mammary protein synthesis, this process is described in more detail below.

1.4.1. Milk protein composition

The protein fraction in milk consists of proteins synthesised in the gland by the secretory cells and also a small amount of immunoglobulin synthesised by the plasma cells in the interstitial space that are transferred into milk. Other milk proteins include blood serum albumin and immunoglobulins, transferred largely unmodified from the blood. In order to understand protein synthesis in the mammary gland it is necessary to know which proteins are present in milk and their origin, whether they are synthesised in the secretory cells, or by other cells in the gland, or are derived from the blood. Thus, in this section a brief overview of the most abundant proteins present in the milk will be presented.

The milk proteins that are synthetized in the gland are composed of amino acids either delivered from the bloodstream or from the amino acids synthetized by the secretory cells. There are two fractions of milk protein synthesised in the mammary gland, namely the caseins (80%) and the whey proteins. All of the caseins and those whey proteins synthesised in the gland have genetic variants (McLean *et al*., 1984; Boland *et al*., 1992).

1.4.1.1. Caseins

The caseins are phosphoproteins that precipitate at their isoelectric point at approximately pH 4.6, at which pH whey proteins remain soluble (Swaisgood, 1993). There are four groups of caseins, α_{S1}- , α_{S2}- , β- and κ- each with distinctive polypeptide chains and different numbers of phosphorylated serine residues (Whitney *et al*., 1976). The caseins, combined with calcium, phosphate and small amounts of

citrate, form complex structures in the milk known as casein micelles (Swaisgood, 1993).

1.4.1.1.1. α_{S1}-casein

The α_{S1}-casein family constitute up to 40% of the casein fraction in bovine milk. They consist of a single polypeptide chain and the isoforms differ in their degree of phosphorylation (Mercier and Vilotte, 1993). In addition, eight genetic variants, α_{S1}-casein (A to H), have been identified in different breeds and strains of cow (Whitney *et al*., 1976).

1.4.1.1.2. α_{S2}-casein

The α_{S2}-casein family constitutes up to 10% of the casein fraction in bovine milk. This family of protein include proteins with the same amino acid sequence but a different content (13, 12, 11 and 10, respectively) of phosphate groups (Whitney *et al*., 1976). There are four genetic variants (A to D) identified as α_{S2}-casein (Whitney *et al*., 1976)

1.4.1.1.3. β-casein

The β-casein family, which constitutes up to 45% of the casein of bovine milk is complex because of the action of the native milk protease plasmin (Whitney *et al*., 1976). Plasmin cleavage leads to formation of three fragments of β-casein with

different peptide sizes. There are 11 variants of the β-casein identified so far (A to K) (Swaisgood, 1993).

1.4.1.1.4. κ-casein

κ-casein constitutes the minor fraction of the casein of bovine milk. There are two common genetic variants of the κ-casein gene, designated A and B, which differ in substitution of two amino acid residues. In addition, nine other genetic variants have been reported so far (Whitney *et al*., 1976).

1.4.1.2. Whey proteins

The term whey proteins has been used to identify the group of milk proteins that remain soluble at pH 4.6 and 20^{o}C (Whitney *et al*., 1976). Traditionally, β-lactoglobulin, α-lactalbumin, blood serum albumin and immunoglobulins are considered the major components of this fraction.

1.4.1.2.1. β-lactoglobulin

β-lactoglobulin is the most abundant whey protein in milk. There are 11 genetic variants of the β-lactoglobulin gene (Whitney *et al*., 1976), designated A to J and W, which differ in substitution of amino acid residues. While 11 variants of the β-lactoglobulin gene have been identified, the A and B variants occur in high frequency in most breeds

of cows and the presence of one or the other of these two variants is associated with marked differences in the composition of the milk (McLean *et al*., 1984).

1.4.1.2.2. α-lactalbumin

The second most abundant whey protein, α-lactalbumin, has three predominant genetic variants, A to C (Whitney *et al*., 1976), which differ in substitution of two amino acid residues. α-lactalbumin is important in the formation of the lactose synthase complex and consequently the process of lactose synthesis (Stinnakre *et al*., 1994).

1.5. Treatments to affect milk protein synthesis

It is well known that one approach to study the molecular pathways used for controlling mammary protein synthesis is to perturb the physiological system so that protein synthesis is increased or decreased. Some of the treatments used to increase the yield of milk protein of well fed animals include administering insulin (Griinari *et al*., 1997), GH (Bauman *et al*., 1985), or amino acids (Mackle *et al*., 1999) while atropine treatment inhibits milk and protein yield in lactating ewes and cows (Aaron *et al*., 1997; Luimes *et al*., 2002). In the experiments described in this thesis, GH and atropine were used to perturb milk and protein yield and protein composition. Thus, a brief review of the mechanisms of action of these two treatments follows.

1.5.1. Growth hormone

GH is a protein hormone released from the anterior pituitary gland (Lanning and Carter-Su, 2006). It stimulates the secretion of IGFs from the liver. These, along with GH and thyroid hormone, stimulate protein synthesis in the mammary gland in cell culture and the release of fatty acids from adipose tissue cells (Keys *et al*., 1997). GH inhibits uptake of glucose by muscle while stimulating uptake of amino acids. The amino acids are used in the synthesis of proteins, and the muscle shifts to using fatty acids as a source of energy. GH secretion occurs in a pulsatile (short, concentrated secretion) and sporadic manner. In lactating dairy cows, GH exerts a strong galactopoietic effect, leading to a preferential partitioning of nutrients to the mammary gland and the enhancement of milk production (Bauman *et al*., 1982). GH is a key hormone in homeorhesis of the maternal tissues to support lactation. Studies by Cowie and his colleagues (1964) in the mid 60s demonstrated the role of GH during lactation. These authors showed that GH was necessary to maintain milk production for a time or retard the rate at which milk production declines, indicating that GH is necessary for the maintainance of lactation. In accordance with this observation, latter studies from Flint and Vernon (1998) demonstrated that inhibition of GH secretion during lactation in rats decreases milk yield.

The increase in milk production after GH treatment is due either to an indirect effect through changes in nutrient flux to the gland or a direct effect on the secretory epithelium or a combination of both (Peel and Bauman, 1987; Barbano *et al*., 1992). The GH receptor is expressed in both the stroma and secretory epithelium of the mammary gland of mice indicating both direct and paracrine effects (Ilkbahar *et al*., 1999). GH also increases IGF-1 production by the liver (Ilkbahar *et al*., 1999),

potentially providing an endocrine effector that mediates cell survival and indirectly increases milk secretion (Molento *et al*., 2002). Similar to rodents, IGF-1 concentrations in the blood of GH-treated cows are significantly elevated after four to six days of treatment (Molento *et al*., 2002). Furthermore, Shingu *et al*. (2004) have suggested that IGF-1 is more important for milk protein synthesis than GH although other studies (Molento *et al*., 2002) have indicated that milk protein synthesis is dependent on a complex interaction between GH, IGF-1 and insulin.

Many of the studies on the effect of GH in domestic animals address the physiological responses to GH such as milk production and mammary growth with direct practical relevance. However, a few studies with laboratory animals and cell culture models are beginning to unravel details of signal transduction pathways affected by this hormone (Carter-Su *et al*., 1996). Because GH treatment increases milk protein secretion via direct and indirect mechanisms, it is assumed that this treatment is a good model for studying the signaling transduction pathways. In this thesis, further knowledge of the GH effect on gene expression in the bovine mammary gland (Chapter 2), and potential molecular mechanisms mediating the effects of GH on protein synthesis (Chapter 3) will be described. Further, the molecular signaling cascade involved in mediating the effects of GH on protein synthesis within the cell (Chapter 5 and 6 – Note: these are not in the mammary gland) will be presented.

1.5.2. Atropine

Atropine is a muscarinic cholinergic antagonists derived from the plant *Atropa belladonna* (Foley, 2003). In the past, cholinergic antagonists, including atropine, were

used in lactating animals to decrease milk production in ewes at weaning to avoid mastitis problems (Powell and Keisler, 1995; Aaron *et al*., 1997). More recently, muscarinic cholinergic antagonists have been used to study the regulation of milk protein synthesis (Luimes *et al*., 2002). Five distinct muscarinic receptors have been identified in various tissues of the body, with each receptor subtype being the product of a different gene (Brann *et al*., 1993). Each receptor subtype also has a different function. For example the M3 receptor, which is abundantly expressed in smooth muscle throughout the gastrointestinal tract, is important for regulating the permeability of the epithelial cells to macromolecules (Cameron and Perdue, 2007). Atropine and other cholinergic antagonists have an inhibitory effect on all five of these receptors (Racke *et al*., 2006). Inhibition of signaling through these receptors may lead to short-term or long-term desensitization of a particular signaling pathway, including mitogen-activated protein kinase pathway (van Koppen and Kaiser, 2003). To date, there is no research reporting the presence of muscarinic receptor in the mammary gland.

Atropine treatment of lactating cows decreases milk protein percentage and milk yield (Prosser and McLaren, 1997; Luimes *et al*., 2002). This effect caused by atropine was originally attributed to a decrease in nutrient supply to the mammary gland (Roets and Peeters, 1981; Prosser and McLaren, 1997), possibly resultant from reduced gastrointestinal tract motility, salivary secretion and gastric acid and pepsin secretion as these effects have been observed in rats treated with atropine (Korczynski *et al*., 2006). However, an endocrine effect via decreased GH release following atropine administration has also been proposed (Aaron *et al*., 1997). In addition, another study showed that there was a differential response to atropine in the synthesis of individual milk proteins synthesized in the mammary gland. This finding raises the possibility that some milk proteins may be more susceptible to nutritional changes (Prosser and

McLaren, 1997) or that atropine might have a direct effect on the transcription of some milk proteins. Thus, because of the effects of atropine on milk protein production, the experiments described in Chapters 2 and 4 used tissues from animals treated with this drug to study the molecular pathways controlling protein synthesis in the mammary gland.

1.6. Regulation of the milk protein genes

Milk protein synthesis can potentially be regulated at several different points during transcription and translation. In this section, recent discoveries of the control of milk protein synthesis at the transcriptional and translational level will be discussed. However, since there are few reports specifically on the translational control of protein synthesis in the mammary gland, the important control points of protein synthesis in general will be reviewed.

1.6.1. Transcription

The advantage of controlling gene expression at the level of transcription is that the control at this early point avoids the waste of energy that could happen if the regulation happens at later stages e.g. translation. Transcription can be regulated at multiple levels where the main control is typically exerted at the level of RNA polymerase binding**.** Transcription factors promote the binding of RNA polymerase at its binding site, called the promoter region. Proteins can also bind to sites on DNA and inhibit the assembly of the transcription complex and hence transcription (Fig. 1.1). Transcription is a tightly controlled process modified by extra-cellular factors such as hormones, growth factors, differentiation factors and cell-cell interactions. In response to extra cellular stimuli an increase or decrease in the pattern of gene expression will allow the change in the secretory epithelial cells activity. Transcriptional activation may involve chromatin modification, nuclear receptor or transcription factor binding to the response element of the promoter. Despite the advances in the knowledge of molecular mechanisms of gene regulation, overall there is lack of information available on the molecular mechanism(s) for the regulation of milk protein synthesis. The current understanding/knowledge on this topic is described in the following section.

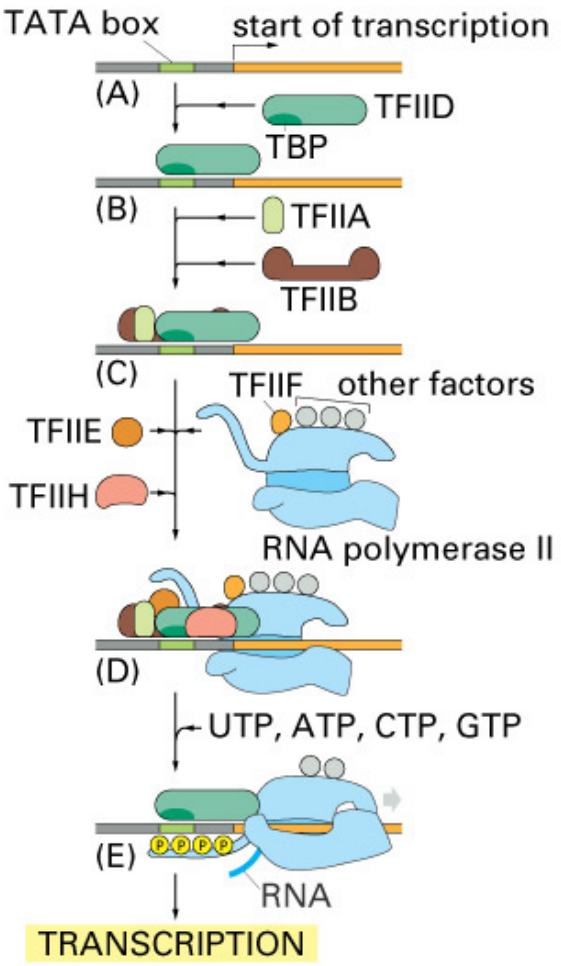

Fig. 1.1: Transcriptional control. To begin transcription, eukaryotic RNA polymerase II requires the general transcription factors. These transcription factors are called TFIIA, TFIIB, and so on. (A) The promoter contains a DNA sequence called the TATA box, which is located 25 nucleotides away from the site where transcription is initiated. (B) The TATA box is recognized and bound by transcription factor TFIID, which then enables the adjacent binding of TFIIB. (C) For simplicity the DNA distortion produced by the binding of TFIID is not shown. (D) The rest of the general transcription factors as well as the RNA polymerase itself assemble at the promoter. (E) TFIIH uses ATP to pry apart the double helix at the transcription start point, allowing transcription to begin. TFIIH also phosphorylates RNA polymerase II, releasing it from the general factors so it can begin the elongation phase of transcription. As shown, the site of phosphorylation is a long polypeptide tail that extends from the polymerase molecule (Alberts et al., 2004)

1.6.1.1. Transcriptional control of milk protein synthesis

Expression of the milk protein genes occurs during late pregnancy, lactation and early involution stages of the lactation cycle. Moreover, the level of expression of each individual gene can vary at different physiological stages (Rosen *et al*., 1999). Juergens *et al*. (1965) were the first investigators to demonstrate that explants of mammary tissue cultured *in vitro* were a suitable model to study the regulation of milk protein genes. However, the milk protein genes were only expressed with lactogenic hormone stimulation, in particular prolactin (Hobbs *et al*., 1982).

Cloning the genes for milk proteins from different mammalian species indicates that the whey proteins, β-lactoglobulin and α-lactalbumin, are encoded individually by single-copy genes (Bawden *et al*., 1994). In contrast, the genes for bovine caseins, α_{S1}-casein, β-casein, α_{S2} -casein and κ-casein are clustered in a 250Kb region on chromosome 6 (Bawden *et al*., 1994). Among all the milk proteins, the regulation of the β-casein gene expression has been the focus of several studies (Altiok and Groner, 1994; Choi *et al*., 2004). One of the main reasons for this is that stable lines of mammary epithelial cells currently available, such as the murine HC11 and bovine Mac-T cells, only produce the β-casein protein and no other milk proteins (Huynh *et al*., 1991; Altiok and Groner, 1994). Non-mammary cells into which the prolactin receptor has been transfected have been used to define regulatory regions in several milk protein genes (Jolivet *et al*., 1996). However, the elucidation of the complex interplay of transcriptional factors and hormone-regulated signaling pathways is still at its infancy but is essential for understanding the regulation of milk protein gene expression.

1.6.1.1.1. Regulatory elements

Transcriptional control of the milk protein gene expression is facilitated by the binding of transcription factors to response elements in the promoter region of the gene. The milk protein genes contain composite response elements (CoREs) which are clusters of transcription factor binding sites that contain both the positive and negative regulatory elements that integrate the signal transduction pathways (Jiang and Levine, 1993). The advantage of this type of gene expression is that it usually confers a higher activation of gene expression than the combined activation of each transcription factor alone (Rosen *et al*., 1998). As an example, STAT5, CAAT\enhancer binding protein (C/EBP)β and glucocorticoid receptor are crucial for β-casein gene expression and the unique combination of these transcriptional factors causes a specific response by the mammary gland. The regulatory factors governing the milk protein gene expression are not limited to these factors. Thus, other transcription factors (e.g. nuclear factor 1 (NF-1), Yin Yang-1 (YY-1)) are also involved but are characterized in less detail (Altiok and Groner, 1994).

The STAT proteins are latent cytoplasmic transcription factors consisting of seven mammalian members. STATs are activated by tyrosine phosphorylation, a post-translational modification that is critical for dimerization, nuclear import, DNA binding and transcriptional activation (Darnell, Jr., 1997). STAT5 is the signal transducer for a range of hormones, growth factors and cytokines (Philp *et al*., 1996). Two isoforms of the STAT5 protein (STAT5A and STAT5B) have important roles in prolactin signaling in the mammary gland including the expression of the β-casein gene (Liu *et al*., 1996). (Teglund *et al*., 1998). However, the expression of the β-casein gene is not altered in knock-out mice, deficient in either STAT5A or STAT5B, indicating that its expression

can be induced in the presence of just one of the activated forms of STAT5 and at lower levels of the other.

Binding of GH or prolactin to their membrane receptors in rabbit primary epithelial cells stimulate the janus kinase (JAK) and STAT signaling cascade by inducing receptor dimerization and subsequent phosphorylation of the STAT5A and STAT5B proteins (Tourkine *et al*., 1995). The physiological consequences of the changes in STAT5 activity have been studied by both gene inactivation and overexpression. For example, STAT5A null mice exhibited decreased lobuloalveolar development and were unable to produce milk after their first gestation (Liu *et al*., 1997). In these mice STAT5B tyrosine phosphorylation was reduced, accompanied by a slight decrease in its concentration. Lobuloalveolar development was also decreased in STAT5B null mice although this phenotype was less severely affected than that of the STAT5A null mice, and lactation was able to be established (Teglund *et al*., 1998).

Of the other six STATs only STAT3 has been directly implicated in the expression of milk protein genes. STAT3 is phosphorylated during all stages of mammary gland development but it is highly phosphorylated during involution, which suggests a key role of this transcriptional factor in the milk protein genes expression at this stage of the lactational cycle (Humphreys *et al*., 2002).

1.6.1.1.2. Hormonal control

Hormones such as insulin, prolactin and GH affect the expression of the milk protein genes through phosphorylation control of the transcription factors. Glucocorticoids are essential lactogenic hormones that regulate milk protein gene expression synergistically

with insulin and prolactin. However, the response to hormonal stimulation varies with different milk protein genes. For example, β-casein gene expression is only slightly induced when mammary epithelial cells are treated with glucocorticoids and insulin but it is markedly induced when prolactin is added to the media. In contrast, the expression of the whey protein genes is induced when the cells are treated with only glucocorticoids and insulin (Hobbs *et al*., 1982; Doppler *et al*., 1991).

Insulin is also required for successful lactation and it increases the transcription of α-lactalbumin and β-casein genes (Prosser *et al*., 1987). The transcriptional activation of the milk protein genes by insulin involves activation of multiple signaling pathways, including mitogen activated protein kinase (MAPK) (Choi *et al*., 2004). The role of insulin in the transcriptional activation of the milk protein genes could be also due to activation of the CEBPα, CEBPβ transcriptional factors, which have been little studied in relation to milk protein synthesis but are clearly involved in adipocyte development (Rosen *et al*., 1998).

1.6.2. Translation

Translation of mRNA is a fundamental process in all living organisms. Translation is the process by which proteins are synthesised from the information encoded by messenger ribonucleic acids (mRNAs). The translation of mRNAs is performed by the ribosome, amino acyl-tRNAs and many associated factors (Table 1.1). These factors associate and dissociate depending on the phase of translation, initiation, elongation or termination, in which the ribosome is engaged. Over the last four decades with the use of new and improved techniques and an expansion of knowledge of gene expression,

and following numerous genome sequencing projects, there has been an explosion of interest and literature concerning the post-transcriptional regulation of gene expression, i.e. translation. The translation of specific mRNA is also subject to sophisticated control mechanisms, allowing the cell to modulate the production of certain proteins. Regulating translation rather than the earlier stages in gene expression (transcription, splicing) confer important advantages to the organism particularly in relation to the rapidity with which it can respond to external conditions and stimuli.

1.6.2.1. General overview of eukaryotic translation

Translation is divided into three phases, initiation, elongation and termination. During initiation, methionyl-tRNA (initiator tRNA) and several initiation factors associate with the 40S ribosomal subunit to form the 43S pre-initiation complex. This complex binds to mRNA and migrates to the correct AUG initiation codon followed by the addition of the 60S ribosomal subunit. This step is then followed by elongation, during which amino acids from amino acyl-tRNA are added to the growing peptide in the order dictated by the mRNA bound to the ribosome. Eventually the termination phase allows the completed protein to be released from the ribosome (Fig. 1.2) (Proud, 2002).

Table 1.1: Eukaryotic translation factors involved in the initiation, elongation and termination

Initiation Factors	Subunits	Function
eIF1	Monomeric	Stimulates formation of 48S pre-initiation complex and movement of the ribosome subunit during scanning
eIF1A	Monomeric	Stabilises Met-tRNA$_i$ binding to the ribosomal P site and enhances the activity of eIF5B
eIF1B	Monomeric	Nucleotide exchange factor for eIF1A
eIF2	α, β and γ	Recruits Met-tRNA$_i$ to the 43S pre-initiation complex in a GTP-dependent manner and positions the Met-tRNA$_i$ at the P site of the ribosome α : Regulatory subunit phosphorylated at Ser51 γ binds Met-tRNA$_i$ and GTP
eIF2B	α, β, γ,δ and ε	Guanine nucleotide exchange factor (GEF) for eIF2
eIF2C	monomeric	Stabilises ternary complexes in presence of RNA
eIF3	11 subunits	Binds RNA and stimulates 43S complex formation. Provides a scaffold for eIFs assembling on the 48S pre-initiation complex
eIF4B	monomeric	Binds RNA, promotes helicase activity of eIF4A
eIF4F	eIF4E	m^{7}GTP cap binding subunit
	eIF4A	ATPase, RNA helicase that binds RNA and unfolds secondary structure in the 5'UTR
	eIF4G	Scaffolding protein that binds eIF4A, eIF4E and eIF3
eIF4H	monomeric	Enhances the RNA helicase activity of eIF4A
eIF5	monomeric	Promotes GTPase with eIF2 and release of eIFs, also has a scaffold function
eIF5B	monomeric	Acts along with eIF5 to induce the binding of the 60S ribosomal subunit to the 48S pre-initiation complex
eIF6	monomeric	Binds to 60S ribosome and promotes dissociation
Poly(A) binding protein (PABP)	monomeric	Binds to the poly (A) tail of mRNA and to eIF4G, causing circularisation of the mRNA, increasing mRNA stability and enhancing translation

Elongation factors	**Subunits**	**Function**
eEF1A	monomeric	GTP-dependent binding of aminoacyl tRNAs; GTPase
eEF1B	A	Possesses GDP/GTP exchange activity
	β	Possesses GDP/GTP exchange activity
	γ	Structural subunit
eEF2	monomeric	Promotes translocation of deacetylated and peptidyl tRNA to the ribosomal E and P sites respectively; GTPase; contains dipthamide
Termination factors	**Subunits**	**Function**
eRF1	monomeric	Recognises UAA, UAG and UGA. Promotes peptide hydrolysis
eRF3	monomeric	GTPase; stimulates eRF1 activity

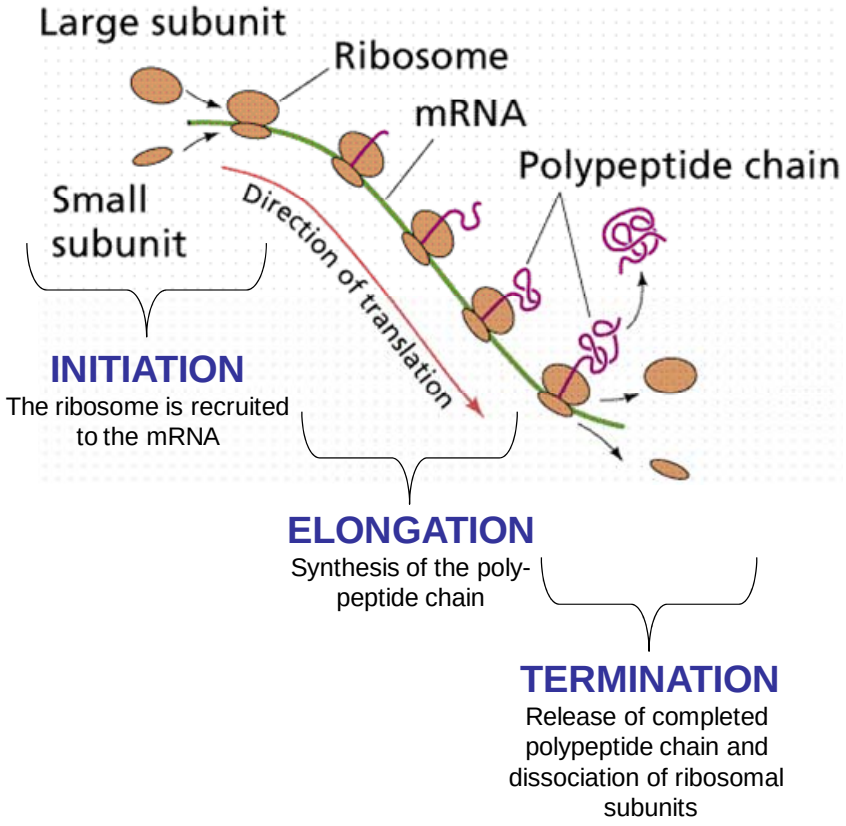

Fig. 1.2: Schematic diagram of the three phases of eukaryotic mRNA translation (adapted from Proud and Denton (1997))

1.6.2.1.1. Eukaryotic ribosome

The mammalian ribosome is built of two unequal sized sub-particles, the large (60S) and small (40S) ribosomal subunits, which are associated with each other in a labile manner. The core of each ribosomal subunit is formed by a high-polymer ribosomal RNA. These subunits are separate prior to the initiation phase of protein synthesis when they are recruited to the start codon and joined together. Ribosomes from all organisms contain three tRNA binding sites (Noller *et al.*, 2002). These sites have been named the A (aminoacyl-tRNA binding site), P (ribosomal binding site of peptidyl tRNA before peptide bond formation) and E (exit site for deacetylated tRNA) sites.

1.6.2.1.2. Initiation

Translation initiation is a complex process requiring numerous translation initiation factors. Initiation commences with the association of the 40S ribosomal subunit with an array of translation initiation factors (eIF1, eIF1A, eIF3 and eIF5). The initiation factor eIF2 forms a stable ternary complex with the initiator methionyl-tRNA$_i$ (met-tRNA$_i$) and the 40S ribosomal subunit, a reaction that results in the formation of the 43S pre-initiation complex (Proud and Denton, 1997).

In the first step of translation, met-tRNA$_i$ binds to the 40S ribosomal subunit as a complex with GDP. For eIF2 to participate in another round of initiation, it must exchange GDP for GTP before formation of a new ternary complex can occur. A second initiation factor eIF2B, mediates guanidine nucleotide exchange with eIF2 (Webb and Proud, 1997). Inhibition of eIF2 activity results in a decrease in the amount of eIF2-GTP available to form the ternary complex, thereby restraining translation initiation. eIF2B activity is regulated reciprocally in part by phosphorylation of eIF2 (Webb and Proud, 1997). Phosphorylation of the α subunit of eIF2 converts eIF2 from a substrate to a competitive inhibitor of eIF2B. Once the eIF2-GTP-Met-tRNA$_i^{Met}$ ternary complex is assembled onto the 40S subunit, it is competent to bind to mRNA. However, the ribosome itself is unable to locate and bind to the 5' region of mRNA, a critical step for the translation of most cellular mRNAs. Instead, the ribosome must recruit the mRNA via the eukaryotic translation initiation factor 4 (eIF4) proteins (Gingras *et al*., 1999b) (Fig. 1.3).

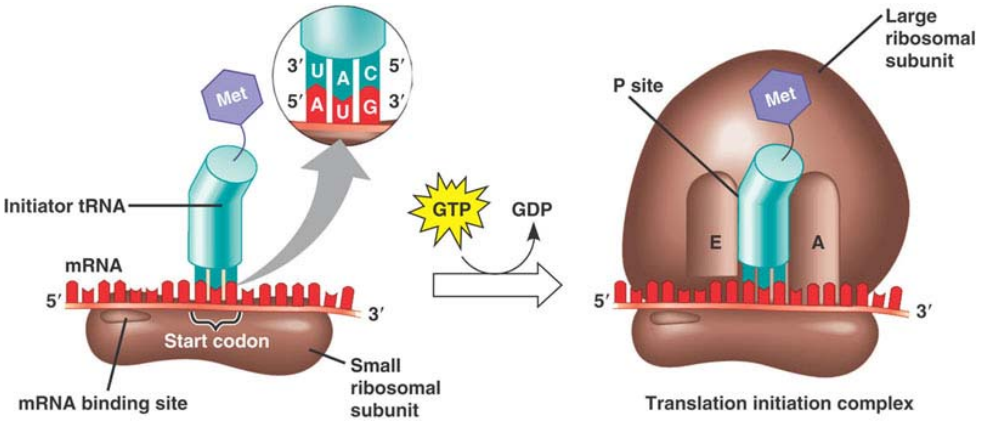

Fig. 1.3: Simplified model for translation initiation complex. Not all steps are shown and several steps have been condensed (http://kvhs.nbed.nb.ca/gallant/biology/translation_initiation.jpg)

Eukaryotic mRNAs possess at their extreme 5' terminus a "cap" structure. The recognition of the 5'cap structure is mediated by the eIF4F complex of proteins (Pestova and Kolupaeva, 2002). Proteins comprising the eIF4F complex are eIF4A, an RNA helicase that functions in conjunction with other proteins: eIF4B, to unwind the secondary structure in the 5'-untranslated region of the mRNAs; eIF4E, the protein that binds to the m^7GTP cap present at the 5'-end of the mRNA; and eIF4G, a scaffolding protein that binds to the 40S ribosomal subunit. The formation of an active eIF4F complex is influenced by alterations in either the phosphorylation state or the availability of eIF4E (Fig. 1.4).

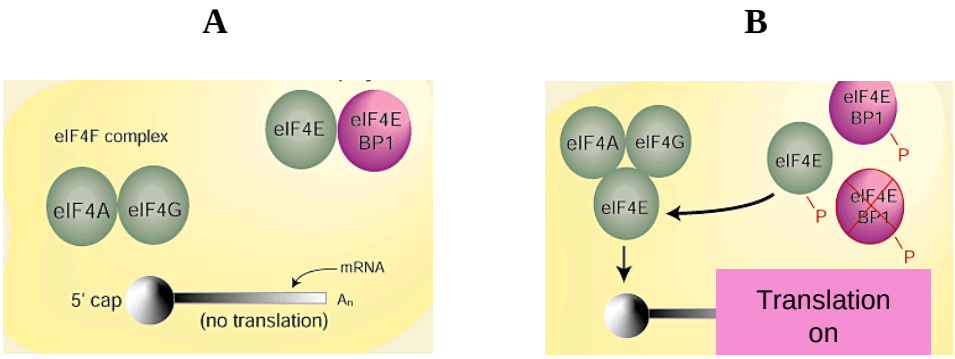

Fig. 1.4: Formation active eIF4F complex is influenced by alterations in either the phosphorylation state or the availability of eIF4E. (A) the basal state, the binding of 4E-BP1 to eIF4E sequesters eIF4E from the eIF4F complex and inhibits cap-dependent translation initiation. (B) The inactivation of 4E-BP1, either by phosphorylation or by gene inactivation, results in dissociation of eIF4E from 4E-BP1 and subsequent activation of the eIF4F complex. Adapted from Chen and Farese (2001)

1.6.2.1.3. Elongation

The speed and accuracy of elongation are regulated by the translation elongation factors eEF1A, eEF1B and eEF2. The process of translation elongation consumes a great deal of metabolic energy, at least four high energy bonds being consumed for each amino acid added to the nascent chain (Browne and Proud, 2002). When protein synthesis is activated (e.g. by insulin, amino acids and other growth factors or mitogens) translation initiation is stimulated and the loading of ribosomes onto mRNAs increases as well. In addition, the rate of elongation by the newly recruited ribosomes should be increased to match their increased rate of attachment to the RNA thus avoiding a limitation in translation rate due to elongation. In contrast, when protein synthesis rates are decreased, inhibition of elongation will ensure that polysomes are retained, even if initiation is also inhibited. This will allow translation to be resumed rapidly when required (Browne and Proud, 2002) (Fig. 1.5).

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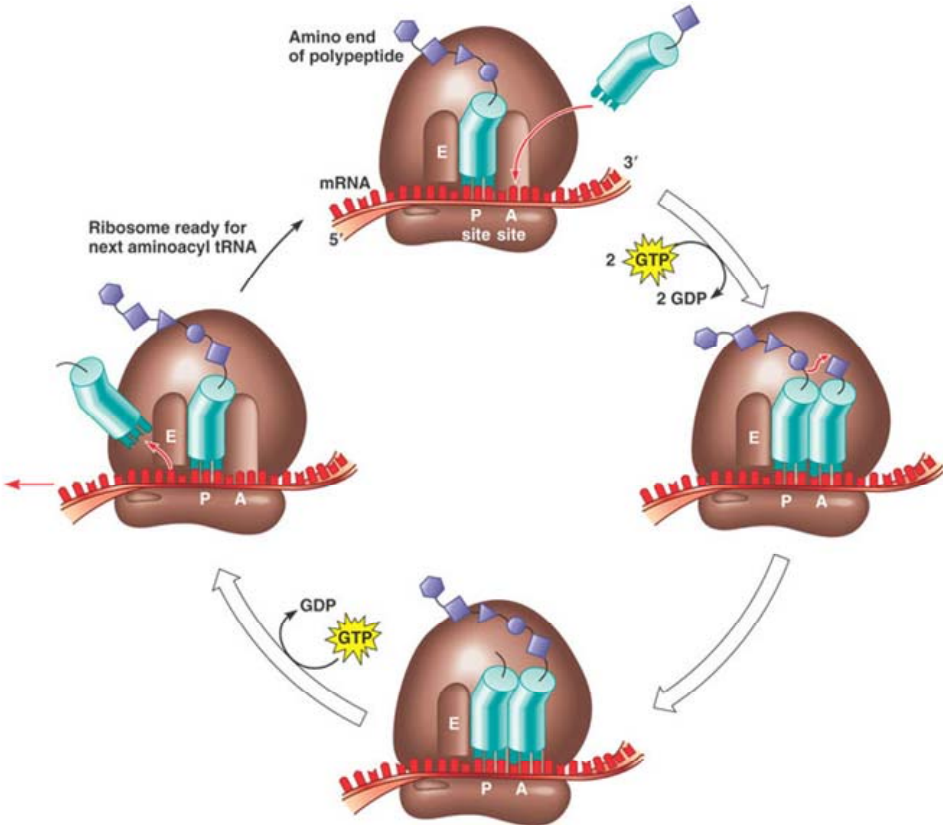

Fig. 1.5: Simplified model for translation elongation. Not all steps are shown and several steps have been condensed (http://kvhs.nbed.nb.ca/gallant/biology/translation_elongation.jpg)

1.6.2.1.4. Termination

The termination of translation occurs in response to the presence of a stop codon in the ribosomal A site. A release factor, eukaryotic release factor 1 (eRF1), enters the ribosome and recognizes the triplet sequence in mRNA known as a stop codon (UGA, UAG or UAA). Following the hydrolysis of the peptide chain from the tRNA, the peptide chain is released, followed by the tRNA and mRNA. The end of the process is marked by the dissociation of the ribosomes into their individual large and small subunits (Fig. 1.6).

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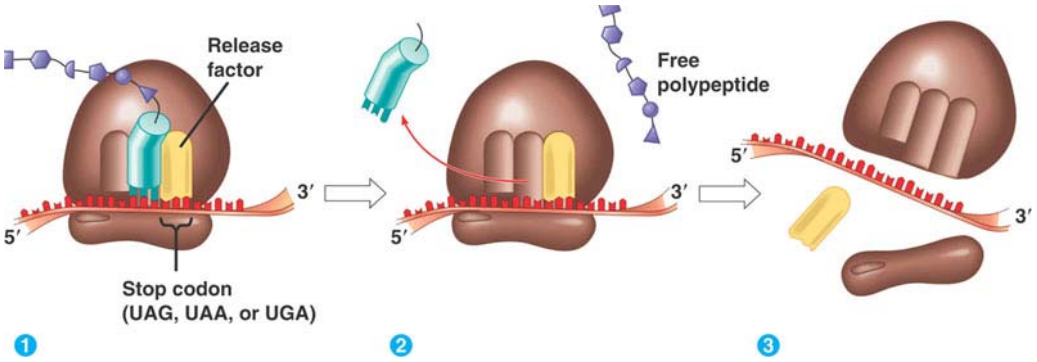

Fig. 1.6: Simplified model for translation termination. Not all steps are shown and several steps have been condensed (http://kvhs.nbed.nb.ca/gallant/biology/translation_termination.jpg)

1.6.2.2. Regulation of eukaryotic mRNA translation

Translation control in eukaryotes plays a key role in the regulation of gene expression. The short term control of translation involves changes in the activity or other functional properties of the translation factors. These include proteins involved in peptide-chain elongation and initiation and proteins that interact with and regulate translation factors. Post-translational modifications of translation factors control both global protein synthesis and the translation of specific mRNAs. Phosphorylation is one of the most common protein modifications that occurs in mammalian cells and is studied in the results Chapters of this thesis.

1.6.2.2.1. Regulation of translation initiation

Translation initiation can be divided into three steps: 1) the binding of the specific initiator Met-tRNA$_i$ to the 40S ribosomal subunit; 2). The binding of this complex to the mRNA and localization of the initiation codon; 3) the formation of the 43S pro-initiation complex (Frederickson and Sonenberg, 1992). Steps 1 and 2 are the two main rate-limiting steps in initiation and require initiation factors. The initiation factors involved in these processes are mainly regulated through phosphorylation.

1.6.2.2.2 Regulation of eIF2 and Met tRNA$_i$ recruitment to the ribosome

eIF2 is a multimeric protein consisting of three dissimilar subunits termed α, β, γ (Proud, 2005a). The primary role of eIF2 in translation is to transfer Met-tRNAi to the 40S ribosomal subunit. Following the association of mRNA with the 40S subunit and location of the subunit at the AUG start codon, eIF5 binds to eIF2 and stimulates the hydrolysis of eIF2-bound GTP (Kapp and Lorsch, 2004). Following GTP hydrolysis, the eIF2-GDP complex is released from the ribosome. Prior to binding Met-tRNAi, the GDP bound to eIF2 must be exchanged for GTP, a reaction mediated by eIF2B (Webb and Proud, 1997). It has always been assumed that one, or more, subunits of eIF2B bind to eIF2α. However, the only detected interaction between eIF2 and eIF2B was the binding of eIF2 β subunit to the eIF2B δ- and ε subunits (Wang *et al*., 2001b).

eIF2B is composed of five subunits termed α-ε in order of increasing size (Proud, 2001). It is a guanidine nucleotide exchange factor and mediates the release of GDP from eIF2 to regenerate active [eIF2-GTP] complex during translation initiation. The rate at which GDP is released from eIF2 is very slow and eIF2B is required to accelerate

the regeneration of active eIF2-GTP. Hence, the level of activity of eIF2B governs the level of active of eIF2 in the cell. The largest and catalytic subunit of eIF2B is the ε subunit. It can catalyze guanidine nucleotide exchange in the absence of the others subunits. The roles of the other four subunits remain less defined. Wang *et al*. (2001b) identified multiple phosphorylation sites in the subunit ε of mammalian eIF2B. These sites are phosphorylated by four different kinases. Two conserved sites (Ser712/713) are phosphorylated by casein kinase II. Glycogen synthase kinase 3 (GSK3) is responsible for phosphorylation of Ser535. This regulatory phosphorylation event requires both the fourth site (Ser539) and a distal region, which acts to recruit GSK3 to eIF2B *in vivo*. The fifth site can be phosphorylated by casein kinase I.

1.6.2.3. Regulation of eIF4E

eIF4E is the smallest translation factor (25KDa) in the eIF4F complex. However, eIF4E appears to be the limiting translation initiation factor in most cell types, present at 0.01-0.02 molecules/ribosome, as compared to 0.5-3 molecules/ribosome for other initiation factors (Raught and Gingras, 1999). Thus eIF4F formation is presumed to be regulated to a large extent by the availability of eIF4E.

eIF4E is the only translation factor from the eIF4F complex which is regulated at multiple levels as follows:

1) Via modulation of its transcription: Upon activation or over-expression of eIF4E, protein synthesis is accelerated. eIF4E preferentially stimulates translation of mRNAs with extensive secondary structure in their 5' untranslated regions (5'UTR), many of

which are related to cell cycle progression (Raught and Gingras, 1999). Therefore, eIF4E is involved in growth regulation of many cell types, including the bovine mammary gland (Long *et al*., 2001b).

2) By phosphorylation: The major phosphorylation site of mammalian eIF4E is Ser209. The exact role of eIF4E phosphorylation is unclear. Lachance *et al*. (2002) have shown that the phosphorylation state of eIF4E is in general correlated with the translation rate and growth status of the cell. These authors reported that phosphorylation of eIF4E is critical for the growth of flies. Flies in which the phosphorylation of eIF4E is prevented are delayed in development and are smaller than control insects, however they are still viable. In contrast, it has been also reported that eIF4E phosphorylation is increased by conditions that activate protein synthesis. Various hypotheses have been put forward to resolve this controversy and to explain the role of phosphorylation of eIF4E in translational control (Scheper and Proud, 2002).

3) Through its interaction with a family of translational repressor proteins (4E-BPs) discussed in detail in the next section (section 1.6.2.4).

1.6.2.4. eIF4E binding proteins (4E-BPs)

The 4E-BPs are a family of three small peptides that inhibit cap-dependent translation by binding to eIF4E and obstructing its interaction with eIF4G (Gingras *et al*., 1999a). The 4E-BPs act as molecular mimics of the eIF4E binding site in eIF4G and thus effectively compete with eIF4G for eIF4E binding (Proud, 2005b).

There are three 4E-BPs in mammals (Poulin *et al*., 1998), the most studied being the eIF4E-binding protein 1 (4E-BP1). 4E-BP1 competes with eIF4G for binding eIF4E and is able to sequester eIF4E into an inactive complex. The binding of 4E-BP1 to eIF4E is regulated by phosphorylation of 4E-BP1, with increases phosphorylation of the protein causing a decrease in the affinity of 4E-BP1 for eIF4E (Gingras *et al*., 2001).

Hypophosphorylated 4E-BPs bind efficiently to eIF4E, but phosphorylation of a critical number of residues in 4E-BP1 abrogates this binding. Numerous types of extracellular stimuli, including serum, hormones, growth factors, mitogens, cytokines and G-protein-couple receptor agonists elicit 4E-BP1 hyperphosphorylation and a concomitant loss of eIF4E activity (Flynn *et al*., 1997). The serum-responsive, rapamycin-sensitive sites from 4E-BP1 (Ser 65 and Thr 70) were identified using mass spectrometry (Gingras *et al*., 2001). 4E-BP1 phosphorylation *in vivo* is achieved through sequential addition of phosphate groups, first added to Thr37 and Thr 46 (primary step), and followed by phosphorylation of Thr70 and Ser65 (Fig. 1.7).

Amino acids and growth factors regulate the 4E-BP1 phosphorylation by the mammalian target of rapamycin (mTOR) signaling pathway (Proud, 2004). eIF4G is also subject to phosphorylation (Gingras *et al*., 1999b) and the phosphorylation states of eIF4G and eIF4B are also regulated by phosphoinositide 3-kinase (PI 3-kinase) and mTOR signaling in mammalian cells. Thus, several translation regulatory proteins are modulated in a concerted fashion by the same intracellular signaling pathways. The dissociation of eIF4E-4EBP1 involves mTOR signaling and the phosphorylation of 4E-BP1 appears to be dependent on the presence of the TOS motif on mTOR complex 1 (mTORC1) (Nojima *et al*., 2003).

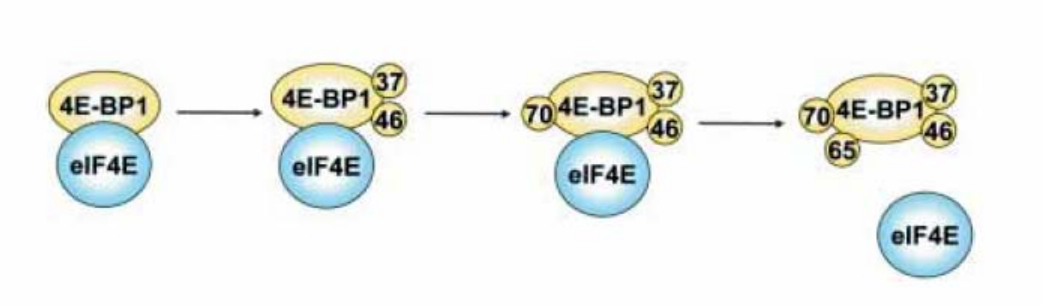

Fig. 1.7: Hierarchical phosphorylation of 4E-BP1 results in its release from eIF4E. Phosphorylation at four sites on 4E-BP1 occurs sequentially (Hay and Sonenberg, 2004)

1.6.2.5. Regulation of translation elongation

The elongation phase of translation is the stage at which the polypeptide is assembled and requires a substantial amount of metabolic energy. Translation elongation in mammals requires a set of non-ribosomal proteins called eukaryotic elongation factors or eEFs. There are three identified elongation factors in mammals, eIF1A, eIF1B and eEF2. Several subunits of eEF1 have been identified as targets for phosphorylation. Phosphorylation of α, β and δ subunits of eEF1 by protein kinase C (PKC) was shown to lead to a 2.0-2.2 fold increase in eEF1 activity. This increase in activity is due to an increased rate of GDP/GTP exchange (Peters *et al*., 1995). The α, β and δ subunits were also shown to be phosphorylated by ribosomal protein S6 kinase (S6K) in response to insulin treatment, resulting again in at least a two fold stimulation of its activity (Chang and Traugh, 1998).

eEF2 is a monomeric protein with a mass of about 93-100 kDa. The function of eEF2 is to catalyse the translocation step in the elongation step during translation. It binds

guanidine nucleosides and is active when bound to GTP. The GTP is hydrolysed late in the translocation process, and the energy released may be coupled to translocation. eEF2 thus leaves the ribosome as inactive eEF2-GDP, but the rate of release of GDP is sufficiently high that no guanidine nucleotide-exchange factor is required to produce active eEF2-GTP (Browne and Proud, 2002). The major physiological phosphorylation site in eEF2 is at Thr56 (Redpath *et al*., 1993).

Phosphorylation of eEF2 inhibits its activity, in translocation and in poly(U)-directed polyphenylalanine synthesis, by preventing it from binding to the ribosome. A study of eEF2 phosphorylation at different stages of the cell cycle found that eEF2 is present mainly in the unphosphorylated state at G_1, S an G_2 of the cell cycle but become significantly phosphorylated during mitosis, that is, the time that protein synthesis declines (Celis *et al*., 1990). eEF2 is phosphorylated by only one known kinase, eukaryotic elongation factor 2 kinase (eEF2K) (Ryazanov *et al*., 1988).

eEF2K is a ubiquitous protein kinase that phosphorylates and inactivates eEF2, and thus can modulate the rate of protein synthesis in animal cells (Ryazanov, 2002). eEF2K can be phosphorylated by several different protein kinases and phosphorylation affects eEF2K activity (Wang *et al*., 2001a).

1.6.2.6. Regulation of the S6 kinases

S6Ks are enzymes that phosphorylate components of the translational machinery such as ribosomal protein S6 (S6), eEF2K and eIF4B (Wang and Proud, 2006). Mammalian cells express two forms of S6 kinases (S6K1 and S6K2), which are encoded by two different genes and share a high level of overall sequence homology. S6K1 has cytosolic and nuclear isoforms (P70 ribosomal protein S6 kinase (P70S6K) and P85 ribosomal protein S6 kinase (P85S6K), respectively) whereas the two isoforms of S6K2 (P54 ribosomal protein S6 kinase 2 (P54S6K2) and P56 ribosomal protein S6 kinase 2 (P56S6K2)) are primarily nuclear (Martin *et al*., 2001). S6K1 and S6K2 are downstream targets of PI 3-kinase and mTOR signaling, and they have been implicated as important positive regulators of cell and body size (Montagne *et al*., 1999).

Activation of P70S6K occurs through a series of phosphorylation events on eight or more serine or threonine resides. P70S6K activation occurs in response to the energy and amino acids status of the cell. For example, in rat adipose cells, insulin rapidly activated P70S6K with a maximal stimulation of approximately 18 fold occurring after 10 min (Diggle *et al*., 1996). In this study, rapamycin totally abolished the stimulation of P70S6K activity by insulin showing an essential role of the mTOR complex 1 (mTORC1) upstream of this protein.

1.6.2.7. Signaling cascades involved in the regulation of translation – PI 3-Kinase signaling

The lipid kinase, PI 3-kinase, plays a key role in signaling downstream of a wide variety of receptors, including those for insulin and other growth factors. Activation of PI 3-kinase leads to increased production of phosphatydylinositol 3,4-biphosphate, which activates proteins such as protein kinase B (PKB, also known as Akt) (Andjelkovic *et al*., 1997). One of the substrates for Akt is GSK3. Akt phosphorylates both GSK3 isoforms (α and β) inhibiting their activity against certain substrates, including eIF2Bε (Welsh and Proud, 1993). The phosphorylation of eIF2B by GSK3 inhibits its activity. Furthermore, the action of insulin is mediated in part by inactivating GSK3 which leads to dephosphorylation of the inhibitory GSK3 site in eIF2Bε and to activation of eIF2B (Welsh *et al*., 1998). In addition, IGF-I regulates the phosphorylation of Ser9 on GSK3β via both PI 3-kinase/Akt and MAPK/Akt/GSK3 pathways (Seimi *et al*., 2004). These authors also examined the effect of over-expression of GSK3 on cardiac hypertrophy *in vitro* and concluded that the unphosphorylatable mutant of GSK3 could inhibit the hypertrophic effects of IGF-I on neonatal rat cardiomyocytes.

1.6.2.8. Signaling cascades involved in the regulation of translation - MAP kinase signaling

The MAPKs are evolutionarily conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells. MAPKs also respond to chemical and physical stresses, thereby controlling cell survival and adaptation. The MAPKs control many cellular events including cell growth, differentiation, proliferation and cell death. In

mammals, there are three main MAPK pathways that have been identified, MAPK/ERK (classical MAPK pathway), SAPK/JNK and the p38 MAPK pathway. In all three pathways, the MAP kinase kinase kinase (MAPKKK) can be activated by small G proteins such as Ras, Rac and Rap1, however, they may also be activated by other enzymes (Seger and Krebs, 1995).

One of the most explored functions of MAPK signaling modules is regulation of gene expression in response to extracellular stimuli (Treisman, 1996). JNKs phosphorylate Jun proteins and thereby enhance their ability to activate transcription without affecting DNA binding (Kallunki *et al*., 1996). MAPKs also regulate gene expression through post-transcriptional mechanisms involving cytoplasmic targets (Kotlyarov *et al*., 1999).

1.6.2.9. Signaling cascades involved in the regulation of translation - mTOR signaling

Rapamycin is a lipophilic macrolide compound originally isolated from a micro-organism indigenous to Easter Island (a.k.a Rapa Nui), and first characterized for its potent antifungal properties (Vezina *et al*., 1975). In the early 1990s, it was discovered that the effects of rapamycin are not limited to fungi and that it inhibited mammalian T cell activation and proliferation, acting as a potent immunosuppressant (Abraham and Wiederrecht, 1996). Later studies demonstrated that the proliferation of many other mammalian cell types is also inhibited by rapamycin (Huang and Houghton, 2003).

The mode of action of rapamycin is conserved from yeast to mammals and rapamycin requires an intracellular cofactor, the FK506 binding protein (FKBP)12, for toxicity. Rapamycin forms a complex with FKBP12, and this complex then binds to and inhibits

the target of rapamycin (TOR). Subsequent biochemical studies in mammalian cells led to the identification and cloning of the mammalian target of rapamycin, mTOR (Fingar and Blenis, 2004; Hay and Sonenberg, 2004). mTOR is a large protein (~280KDa) that *in vitro* exhibits a kinase activity.

The targets of the mTOR pathway include several components of the translation machinery. Downstream targets of mTOR are two well characterized proteins 4E-BP1 and S6K1 (Inoki *et al*., 2005). S6K1 phosphorylates the S6 protein, which is a component of the 40S ribosomal subunit. On the other hand, 4E-BP1 is inactivated by mTOR phosphorylation. When the 4E-BP1 protein is hypophosphorylated it binds to eIF4E protein and prevents the eIF4E protein from binding to eIF4G thus decreasing CAP dependent protein translation (Hay and Sonenberg, 2004).

Research has shown that mTOR forms at least two types of complex involving distinct partner proteins, which account for different sensitivity of TOR signaling to rapamycin (Loewith *et al*., 2002). One complex, mTORC1 contains the partner Raptor (regulatory associated protein of mTOR) and the second one, mTOR complex 2 (mTORC2), contains a different partner, rapamycin-insensitive companion of mTOR (Rictor). Both mTORC1 and mTORC2 contain the protein GβL. The effects of mTOR that are sensitive to rapamycin are mediated through mTORC1 and mTORC2 mediate the effects that are insensitive to this compound (Kim *et al*., 2002; 2003).

mTORC1 signaling also controls the translation elongation process through what appears to be indirect phosphorylation of eEF2K leading to mTOR-dependent regulation of eEF2. Insulin induces the dephosphorylation of eEF2 and the phosphorylation of the 4E-BPs and S6K, and these effects are blocked by rapamycin

(Redpath *et al*., 1996; Wang *et al*., 2000; Everett *et al*., 2001). Recently, new developments into understanding how mTOR signals to its downstream components have been made. Both 4E-BPs and P70S6K contain TOR signaling (TOS) motifs to which Raptor binds and mediates phosphorylation by mTOR (Nojima *et al*., 2003; Schalm *et al*., 2003).

In this thesis, mTOR has been studied as a potent regulatory pathway controlling milk protein synthesis (Chapter 3,4). Also, in Chapter 5 mTOR is shown to be a signaling pathway involved in the overall stimulation of protein synthesis by GH.

1.6.2.10. The role of the translational regulatory factors in the regulation of lactation and milk protein synthesis

Although the role of the translation factors in the overall control of protein synthesis has been studied extensively in the past few years, the role of these factors in the regulation of milk synthesis has just started to be elucidated. Recent work from Dr. Cant's group (Toerien and Cant, 2007) has demonstrated that abundance of total eIF2α and the phosphorylation of S6 were increased in the mammary gland of lactating cows when compared to non-lactating animals. This study was the first one published to establish the possible involvement of mTOR in the regulation of the onset of lactation. Apart from eIF2α, which is also involved in the initiation of translation, eIF4E appears to be involved in mammary gland development and the onset of lactation (Long *et al*., 2001a/b). These authors have shown that the levels of eIF4E were significantly higher in the lactating mammary gland compared to the mammary tissues of non-lactating animals.

In the elongation stage of translation, Christophersen *et al*. (2002) were the first authors to show a close linear relationship between the amount of eEF2 in the mammary gland at the end of lactation and the amount of protein and casein output in milk. In summary, these studies suggest that translational control could be a potential target for the regulation of lactation and milk synthesis.

1.7. Rationale for the study

As presented in this review, there has been considerable research in the field of lactation and mammary biology including investigation of the physiological mechanisms involved in the regulation of the onset (growth and proliferation of epithelial cells) and termination (apoptosis of epithelial cells) of lactation. Other studies examine the hormonal changes that happen during these physiological stages and their influence on lactation and mammary biology. In the medical field, most studies in the mammary gland are focused on cancer research. These studies make extensive use of molecular biology approaches, such as arrays, whilst in the domestic animal field this type of research is still in its infancy.

The advent of the genome sequencing projects was a major achievement in both basic and applied research. Sequencing of the bovine genome began in December 2003 and provided a new tool for agricultural researchers motivated to improve health and disease management of cattle and to enhance the nutritional value of beef and dairy products, such as milk.

Milk proteins are an important source of nutrition for humans and the manipulation of milk composition, through the application of new biotechnologies can sustain and

improve competitiveness and profitability of the New Zealand dairy industry in international markets. Recently, given the importance of the milk for human nutrition and the potential to manipulate this foodstuff, more studies are focusing on the genetic manipulation of milk composition, which will ultimately be important to satisfy the market. Thus, having in mind the importance of studies to unravel the molecular mechanisms involved in the regulation of milk protein synthesis, this study had the following main objectives:

1) To elucidate the pathways and genes involved in the regulation of milk protein synthesis in the dairy cow (Chapter 2).

2) To understand the control of the genes and mTOR signaling pathway involved in the regulation of milk protein synthesis (Chapters 3, 4).

3) To elucidate the participation and involvement of the mTOR signaling pathway in the control of protein synthesis stimulated by GH treatment (Chapter 5 and 6).

4) To study the regulation and interaction of 4E-BP1 and mTOR, the main identified regulators of protein synthesis (Chapter 7).

CHAPTER 2

Elucidation of pathways involved in the regulation of milk protein synthesis

The material present in this Chapter will be submitted as part of a paper to Journal to be confirmed.

2.1. Abstract

Milk protein synthesis is a complex molecular process that is poorly understood. To elucidate the signaling pathways and genes involved in the control of milk protein synthesis, mammary gland tissue samples from growth hormone (GH) and atropine-treated and control cows were collected and total RNA was extracted. Using a microarray of 22,690 bovine cDNAs, the effects of the two treatments (GH and atropine) on gene expression in the mammary gland were examined. In this study, key pathways and genes that are potentially involved in the regulation of milk protein synthesis were identified. The microarray results were validated using quantitative real time PCR (qRT-PCR). The results presented in this chapter show that there were no changes for the mRNA abundance of the milk protein genes, with exception to the α_{s2}-casein gene which, in the GH-treated cows, was differentially expressed in the array (1.4 fold up-regulated) and differentially expressed in the qRT-PCR (1.7 fold up-regulated). Thus, changes in the milk protein genes at the transcriptional level were not the cause of milk protein profile changes observed in response to the treatments, as these changes were not accompanied by changes in the mRNA concentrations of the individual milk proteins. Rather, translational control mechanisms are likely to be involved in the increased output of milk protein in response to GH treatment and the decrease in response to atropine treatment. Furthermore, the results suggest that mammalian target of rapamycin (mTOR) is a potent signaling pathway in the control of milk protein synthesis.

2.2. Introduction

Milk proteins are of central importance to the dairy industry because of their nutritional and functional properties (Boland *et al*., 1992). However, the molecular mechanisms that regulate milk protein synthesis in the dairy cow are not well understood. GH administration in lactating animals increases the yield of milk proteins and the efficiency of milk production (Bauman, 1999). This may be explained by several mechanisms including direct effects in the mammary gland via GH receptors and signaling pathways such as janus kinase (JAK) and signal transducers and activators of transcription (STAT) (Feldman *et al*., 1993; Carter-Su *et al*., 2000). In contrast, atropine, a muscarinic cholinergic antagonist, reduces milk protein concentration (Roets and Peeters, 1981; Prosser and McLaren, 1997). This occurs, at least in part, through a decrease in the concentration of plasma amino acids diminishing their availability (Roets and Peeters, 1981; Prosser and McLaren, 1997). Other data (Powell and Keisler, 1995; Aaron *et al*., 1997) have suggested that atropine can also decrease circulating concentrations of GH, thus a decrease in milk production could be, in part, an endocrine dependent effect. Although GH and atropine treatments have been shown to perturb the milk protein synthetic machinery with differing outcomes, the molecular mechanisms that control these mechanisms remains largely unknown and are the main focus in this thesis.

Major milk proteins are coded by a small group of genes expressed exclusively in the epithelium of the mammary gland during pregnancy and lactation. During lactation, the casein mRNAs account for up to 60 to 80% of the total mRNA in the cell (Kuraishi *et al*., 2000; Malewski *et al*., 2002). Milk protein genes are expressed in the mammary gland epithelial cells during late pregnancy, lactation and early involution, however, the

levels to which individual genes are expressed differ significantly. The expression of these genes is affected by hormones such as insulin, prolactin and GH (Allan *et al*., 2002; Malewski *et al*., 2002). GH activates STAT transcriptional factors in the mammary gland of rabbits (Malewski *et al*., 2002) and ruminants (Plath-Gabler *et al*., 2001; Boutinaud and Jammes, 2004) and these factors have an important role in the onset of lactation as well as in the maintainance and decline of lactation (Philp *et al*., 1996). Post-transcriptional regulation of casein mRNA has also been proposed (Kuraishi *et al*., 2000). Specifically, the poly(A) tail of the casein mRNA is shortened or elongated in lactating mammary tissue depending upon whether milk accumulates or is removed (Kuraishi *et al*., 2000). Casein mRNA stability also changes in response to a variety of stimuli *in vitro* (Zeigler and Wicha, 1992). This suggests that milk protein gene expression may also be regulated through the degradation of the mRNA.

In the post-genomic era, scientists are focusing on the functional analysis of genes and their products, which is achievable, in part, using microarrays. Until recently, few studies have been conducted using microarrays in domestic livestock. Microarray technology enables the survey of the relative activity of thousands of genes in parallel, and has several areas of application such as understanding patterns of expressed genes, which is expected to improve understanding of complex communication networks. This approach was applied in this Chapter in the form of expression monitoring, in which gene transcript levels are measured in different conditions to identify regulatory expression patterns.

It was postulated that using microarrays to compare differential gene expression patterns induced by treating animals with either GH or atropine (Appendix A), could provide insights into the molecular events underlying the effects of these treatments on

the production of milk protein. Thus, the present study was designed to test whether: 1) changes in milk protein output (yield and percentage) are associated with changes in gene expression of the individual milk proteins and 2) to identify pathways and genes that are involved in the control of the milk protein synthetic machinery.

2.3. Materials and methods

The full list of genes differentially expressed at p-value 0.05 and fold change higher than 1.3 (Tables B.1 and B.2) and the full table of significant categories (Tables B.3, B.4, B.5 and B.6) for the GH-treated and atropine-treated cows *vs.* control can be found in Appendix B.

2.3.1. RNA extraction and purification

Total RNA was extracted from samples of mammary gland tissue collected from GH-treated, atropine-treated and control cows (Appendix A). Frozen tissue (300 mg) was wrapped in a double layer of tin foil and pulverised in a French press and then homogenized three times for 10 sec with 3 mL of Trizol Reagent (Invitrogen Life Technologies, Auckland, NZ). The homogenate was allowed to rest for 10 min then 0.6 mL of chloroform was added and the tubes were vigorously shaken by hand for 15 sec. This step allows separation of the protein and DNA fraction (Trizol) from the RNA fraction (chloroform). The mixture was centrifuged at 12,000 *g* for 15 min at 4^{o}C and the upper phase containing the RNA fraction was carefully transferred to a new tube, to which 0.5 mL isopropanol was added to precipitate the RNA. The mixture was incubated for 10 min at room temperature, centrifuged at 12,000 *g* for 10 min at 4^{o}C and the supernatant was removed and discarded. The pellet was washed with 1 mL of 75% ethanol and centrifuged at 7,500 *g* for 5 min at 4^{o}C. The ethanol was removed, and the pellet was air dried for 5 min to 10 min at room temperature. The RNA was resuspended in 20 to 100 μL RNase-free water and quantified by absorbance at 260 nm. The RNA concentration was calculated assuming 1 absorbance unit as 260nm

corresponding to 40 μg/mL of RNA. The ratio of the absorbance at 260:280 nm was higher than 1.8 for all the RNA samples. The quality of the resultant RNA can be seen in Fig. 2.1.

Total RNA was further purified (RNeasy Mini Kits; Quiagen, Victoria, AU) to remove the small RNAs that can create background effects on the arrays, and all samples of RNA were evaluated on a 1% agarose gel using electrophoresis to check for DNA contamination and degradation of the ribosomal RNA.

2.3.2. Microarray experiment

2.3.2.1. cDNA and fluorescent labelling

For all experiments, 10 μg total RNA was used as the template for reverse transcription and fluorescent-labeling reactions using the SuperScript Indirect cDNA Labeling System (Invitrogen Life Technologies, Auckland, NZ). Microcon columns (Millipore, Sydney, AU) were used to concentrate the cDNA to a final volume of 20 μL which were previously labeled in the presence of either mono-functional N-hydroxysuccinimide (NHS)-ester Cy5 or Cy3 dyes (Amersham, Auckland, NZ). Labeled cDNAs were purified to remove unincorporated dyes using the QIA-Quick PCR purification kit (Qiagen, Victoria, AU), concentrated to 10 μL and combined.

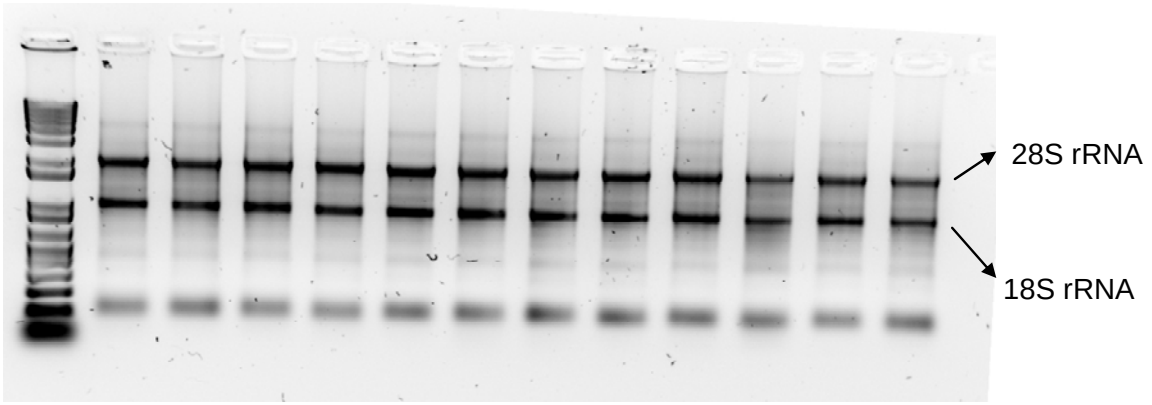

Fig. 2.1: RNA quality after Trizol extraction. Gel represents the 28S and 18S ribosomal RNA bands. From left to right; lanes 1, 2, 3, 5 (GH), lanes 4, 10, 11, 12 (control), lanes 6, 7, 8, 9 (atropine)

2.3.2.2. Array preparation

For generation of AgResearch's proprietary bovine cDNA microarray slides, nine bovine cDNA libraries were generated from a variety of tissues from both dairy and beef breeds. These nine libraries were single pass sequenced, which generated expressed sequence tags (ESTs). A bovine microarray containing 22,690 unique amplified cDNAs, 31 PCR products, and 137 control spots was used. The cDNAs, printed on the array, came predominantly from mammary gland, brain, liver, heart muscle, ovary small intestine mucosa, spleen and testes libraries. The cDNAs were amplified by PCR in a 50 μL reaction volume in 96-well plates containing 55 pmol primers (T7 and T3 for cDNA cloned in pBK-CMV or T7 and Sp6 for cDNA cloned in pCMV-SPORT6), 0.2 mM dNTPs, 1.5 mM MgCl2, and 2.5 units AB Red Hot *Taq* polymerase. After 36 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 60 sec, products were extended at 72°C for 5 min. The control spots on the array consisted of the negative control for each PCR used to amplify the cDNAs. A small aliquot of every PCR product was run on a 1% agarose gel to verify the PCR reaction. Products were precipitated with 0.2 M sodium acetate and an equal volume of isopropanol. The 96-well plates were centrifuged at 3,500 *g* for 1 hour, and the pellets were washed with 70% ethanol and then air-dried. When required for printing, the pellets were resuspended in water, transferred to 384-well plates, dried, and then resuspended in 3XSSC printing solution (0.45 M sodium chloride, 0.045 M sodium citrate). The amplified products were printed onto poly-L-lysine-coated glass slides using the ESI array robot (Engineering Services Inc, Ontario, Canada) with up to 32 split pinheads depositing 0.6 nL with a 100μm spot size. After printing, the slides were UV irradiated to cross-link the DNA to the polylysine coating.

Note: *This section 2.3.2.2 on the microarray preparation was not conducted by the PhD candidate. It was completed by Dr Theresa Wilson's grouping at the Animal Genomics Group, AgResearch Invermay, Mosgiel, NZ.*

2.3.2.3. Slide hybridization and scanning

Slides were pre-hybridized for 1 hour at 42^{o}C in a solution containing 5XSSC, 0.1% SDS, and 0.25% bovine serum albumin (BSA) to avoid non-specific binding of cDNA samples. Subsequently, they were rinsed twice with deionized water and once with isopropanol and air dried. The Cy3- and Cy5-labeled probes were heat-denatured (95^{o}C for 10 min) and combined with 60 µL pre-warmed (68^{o}C) ULTRAhyb (Ambion, Victoria, AU) hybridization buffer number 1, and applied to the slides under lifter coverslips (Erie Scientific, Portsmouth, NH, USA). Slides were incubated for 20 hours at 42^{o}C in humidified CMT-hybridization chamber (CMT Hybridization Chambers, Corning, Acton, MA, USA). After hybridization, slides were washed for 5 min each, first in 2XSSC and 0.1% SDS (Sigma Aldrich, Sydney, AU) then in 1XSSC and finally in 0.1XSSC. All wash buffers were pre-heated (45^{o}C) and filtered through 0.22 µm filters. Before scanning, slides were spin dried at 1,000 *g* for 5 min in a cushioned 50 mL conical centrifugation tube. The slides were stored in the dark to minimize photo-bleaching, and scanned within two days using an Axon Professional 4200A scanner (Molecular Devices, Sunnyvale, CA, USA). The dual images were collected in a TIFF format. The combination and processing of the images were performed using GenePix Pro software (Axon Instruments, Downingtown, PA, USA), which included processing of array images, aligning of spots, integrating of robot-spotting files with the microarray

image and the automated and manual flagging of bad spots. Fig. 2.2 illustrates the microarray experimental steps.

2.3.2.4. Microarray design, normalization and analysis

For this experiment, 18 slides were used in a series of loops design. The loop design method required three aliquots of each test sample that were labeled alternately with the red and green dyes and co-hybridized on arrays with two other test samples. Fig 2.3 illustrates the loop design. In this experiment, 18 slides were used to increase the power of the analyses (increase the detection of small change in gene expression, in this case differences as low as 10% could be detected as significantly different). The only practical limitation of the loop design is that it can only be used if duplicate aliquots of each test sample are available and it requires (at least) the same number of arrays as the common reference design (equal to the number of distinct test samples).

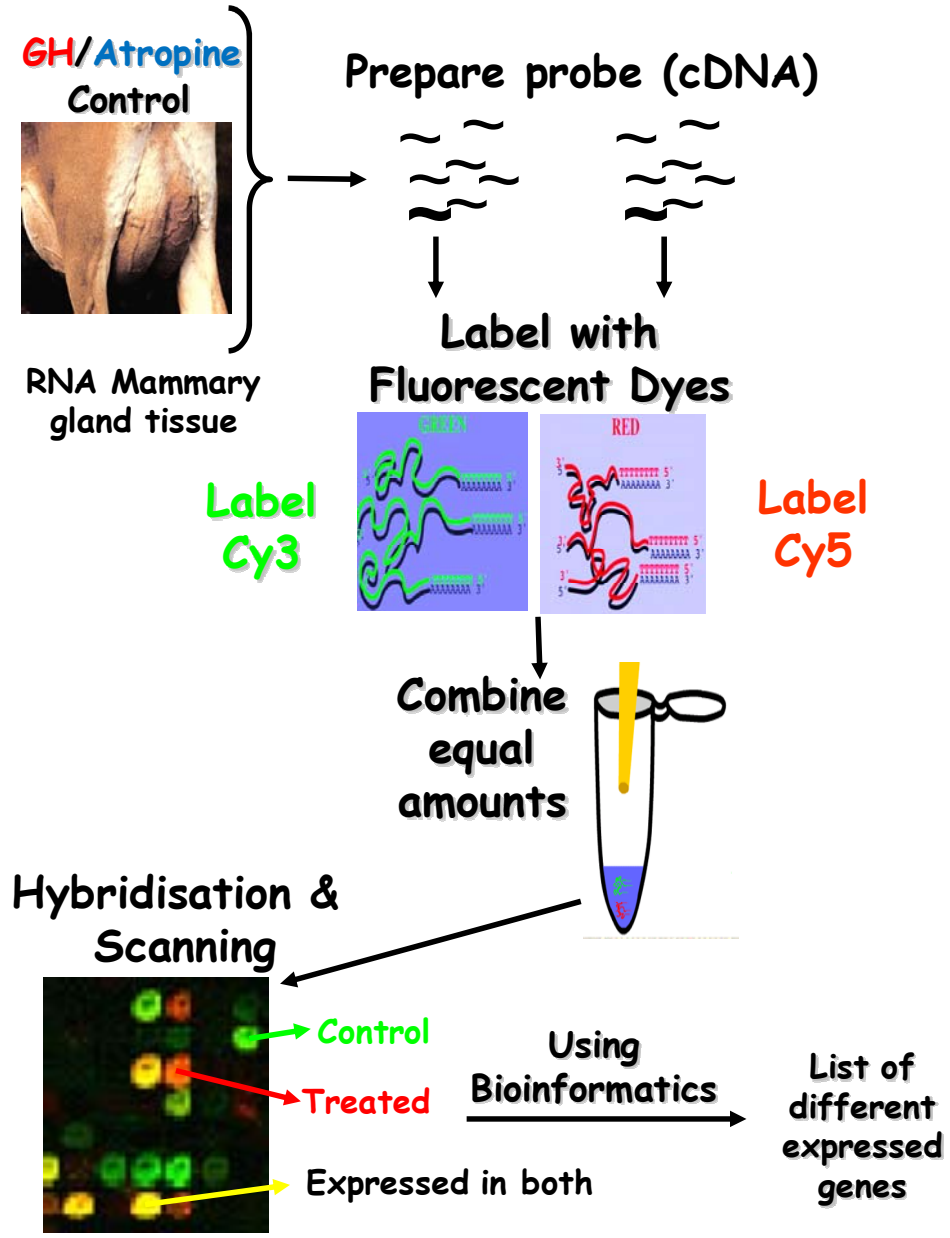

Fig. 2.2: Schematic representation of the steps performed in a microarray experiment

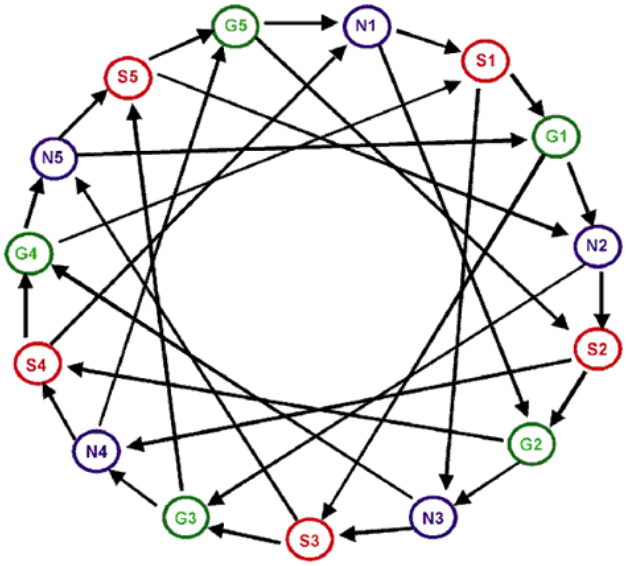

Fig. 2.3: Representation of loop design used in microarray studies. Each arrow represents a microarray slide and connects the two individual samples hybridized to it. On each microarray, arrows indicate labeling (Cy3 or Cy5): one sample is labeled with Cy3 fluorescent dye (base of the arrow) and the other with Cy5 dye (arrow head). N, S, G represent three different treatments (Oleksiak *et al*., 2002)

The analysis of the microarray data was a multiple process. Firstly, the microarray data were normalized for each individual slide following the procedure of Baird *et al*. (2004). The normalization removed noise due to experimental artifacts and corrected for systematic array and dye effects. This procedure corrected for many sources of variation and provided a more robust set of gene expression data. Secondly, the calculation of gene expression statistics was made using the normalized data. To determine which cDNAs were differentially expressed, the values of each corresponding EST were analyzed over the 18 slides of the experiment using analyses of variance, removing dye bias effects (different efficiency of Cy3 and Cy5 dye incorporation). ESTs with a normalized mean log intensity less than 10 were excluded (spots with intensity lower than 10 are not reliably distinguished from noise and background signal) as were EST with more than 9 bad spots out of 16 (not reliable spots). For each remaining EST, the normalized log ratio of the mean was then calculated. This is calculated by dividing the normalized log ratio of the mean of that EST by the standard deviation of the normalized log ratio of mean of all good ESTs on the array. ESTs were counted as significantly differentially expressed if outside the 95% probability thresholds. The false discovery rate (FDR) of the procedure was estimated and it was lower than 5% for the ESTs differentially expressed at a probability value less than 0.05.

To annotate the bovine cDNAs, all the EST sequences, including those publicly deposited in National Center for Biotechnology Information (NCBI), were assembled into contigs using CAP3 program (Huang and Madan, 1999) after an initial clustering step using BLAST (Altschul *et al*., 1990). These contigs were used for all future ontology identification. For the protein annotation, the contig to which each EST belonged was blasted against the GenBank SwissProt database and the top hit extracted.

For the gene ontology (GO) analysis and ingenuity pathway analysis (IPA) the corresponding bovine (or human when bovine was not available) RefSeq for each contig represented on the array was obtained using BLAST ($E<1x10^{-10}$).

Note: *The array statistical analysis, normalization and annotation were not conducted by the PhD candidate. It was completed by Drs. David Baird and Allan McCulloch at the Animal Genomics Group, AgResearch Invermay, Mosgiel, NZ.*

2.3.3. Quantitative real-time polymerase chain reaction

2.3.3.1. cDNA synthesis

Two μg of total RNA from each animal was treated with DNAse I (0.4 units/μg RNA), which digests single- and double-stranded DNA to oligodeoxy-ribonucleotides, according to manufacturer's instruction (Invitrogen, Auckland, NZ). Basically, each RNA sample was prepared in a RNAse-free tube. To each tube, 1 μg RNA sample was added followed by 1 μL 10X DNase I Reaction Buffer, 1 μL DNase I, Amp Grade, 1 U/μL and RNAse free water to complete the volume to 10 μL. The mixes were incubated for 15 min at room temperature, followed by the inactivation of the DNase I by the addition of 1 μL of 25 mM EDTA solution to the reaction mixture. The samples were then heated for 10 min at 65°C and stored at -85^{o}C.

Following the DNAse treatment, the first strand cDNA was synthesized using the First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). For each RNA sample, the following components were mixed in a clean RNAse free eppendorf tube: 2 μL 10X reaction buffer, 4 μL 25mM $MgCl_2$, 2 μL primer oligo-$p(dT)_{15}$, 0.8 μL AMV

reverse transcriptase, 0.6 μg of DNAse-I-treated total RNA and RNase free H_2O up to 20 μL. The reaction was incubated for 10 min and room temperature (primer anneals to the RNA template) and then at 42°C for 60 min (RNA is reversely transcribed, resulting in cDNA synthesis). Following the 42°C incubation, the AMV reverse transcriptase was denatured by incubating the reaction at 99°C for 5 min. At this point the tube was kept at -20°C until ready to use in the qRT-PCR reactions.

2.3.3.2. Primers

Primers for the milk protein genes and reference genes (Table 2.1) were designed using Primer 3.0 (Rozen and Skaletsky, 2000), and publicly available DNA sequences. Preliminary experiments were done with each primer pair to optimize annealing conditions. After PCR conditions of all primers were optimized, amplicons were sequenced at "The Allan Wilson Centre Genome Service" (Massey University, Palmerston North, NZ) to confirm identity (>98% homology with the sequence of interest). Specificities of all reactions were verified by electrophoresis in 1% agarose gels.

Table 2.1: Primers used in quantitative real-time PCR assays

Gene Symbol and description	Gene Bank accession number	Forward Primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
Milk protein genes				
CSN1S1 (Casein alpha s1, mRNA)	NM_181029.2	TGGGAGTGAATCAACTGAGGA	CAGAGCCAATGGGATTAGGGA	434
CSN1S2A (Casein alpha s2-like A, mRNA)	NM_174528.2	GGACGATAAGCACTACCAGA	TGGCTTCATAGCTTTCTGATGC	358
CSN2 (Casein beta, mRNA)	NM_181008.2	GTACCTGGTGAGATTGTGGA	CACAGGGGTTTGAGTAAGAG	225
CSN3 (Casein kappa, mRNA)	NM_174294.1	ACCAACAGAAACCAGTTGCAC	CTACAGTGCTCTCTACTGCTT	303
LALBA (Lactalbumin, alpha, mRNA)	NM_174378.2	ACCAGTGGTTATGACACACAAGC	AGTGCTTTATGGGCCAACCAGT	233
LGB (Lactoglobulin, beta, mRNA)	NM_173929.3	ATCCCTGCGGTGTTCAAGAT	CCATGCAGACGAGGTACT	365
House keeping candidate genes				
GPAM (mitochondrial glycerol phosphate acyltransferase, mRNA)	NM_001012282.1	GACTGACCTCTCTGGACTCTG	CCCCAAAGCATCTGAGTAG	150
MGEA5 (Meningioma expressed antigen 5 (hyaluronidase), mRNA)	NM_012215.1	GCTGACCTTTTCTACCTTCC	CCGCACATCTCTTCAAAC	173
KRT15 K15 intermediate filament type I keratin, mRNA	XM_584069	CGTTGAGGAGTCTGTGGA	CAACTAAGAAACGAAGGACC	195
18S	DQ222453	GAGAAACGGCTACCACATCC	GGACACTCAGCTAAGAGCATCG	338

2.3.3.3. PCR assay conditions

For each sample, 4 μL of DNA master SYBR Green I mix (containing TaqDNA polymerase, dNTP, $MgCl_2$, and SYBR green I dye, Roche Molecular Biochemicals) was mixed with 2 μL of forward and reverse primers (1 pmol/uL) and 9 μL of water (PCR grade). The PCR mix was mixed by carefully pipetting and then 15 μL was added to each capillary (pre-cooled to 4°C) followed by 5 μL of DNA template (5 ng/μL). The amplicon program consisted of 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 sec, 55°C to 67°C (primer specific, optimal annealing temperature) for 15 sec and 72°C for 20 sec. Melting curve analysis was performed by increasing the temperature (0.1°C/sec) from 65°C to 95°C, with continuous fluorescence acquisition.

Cycles to threshold (Ct) values were obtained in triplicate for each sample on the Light Cycler (Roche Diagnostics, Mannheim, Germany). A negative control (no cDNA template) was run with every assay to assess the overall specificity of the assay. PCR efficiencies of each set of primers were calculated for each sample using the LinRegPCR program (Ramakers *et al*., 2003).

Statistical analysis of the differences in expression between control and treated group means were assessed by pair-wise fixed reallocation randomization test, using the relative expression software tool (REST) (Pfaffl *et al*., 2002).

Genes traditionally used as reference genes (to normalize expression changes) for the qRT-PCR method, e.g. 18S, were differentially expressed in response to treatments (microarray data) and confirmed by qRT-PCR. Similar observations, of differential expression of traditional reference genes, were also made by other authors (Dheda *et al*.,

2004; Joyner *et al*., 2005). Therefore a new set of five potential reference genes were selected from the microarray data using three criteria: 1) the candidate reference gene was not differentially expressed in response to treatment; 2) the candidate gene was represented by at least two reliable (similar expression) ESTs in the array; 3) the bovine sequence for the candidate gene was publicly available. The expression profiles of five genes that met these criteria were assessed using qRT-PCR (Table 2.2). The best reference genes were GPAM and MGEA5 for the GH experiment (due to small changes in expression) and GPAM and KRT15 for the atropine experiment (Table 2.2).

2.3.4. Agarose gel

Agarose gels for checking the quality of RNA and DNA fragments were prepared by melting 0.8 to 1.5% (w/v) agarose in TBE buffer (50mM Tris, 40mM Boric Acid, 0.5mM EDTA) in a microwave. After boiling, the mixture was left to cool to approximately 50^{o}C and then ethidium bromide was added (final concentration 5ng/mL) and mixed gently. Gels were run on PowerPac 300 apparatus (BioRad, Hercules, CA, USA) at a constant current (100 mA) in 0.5X TBE buffer. Samples were loaded in 5X DNA loading buffer. After electrophoresis, RNA and DNA were visualized using a gel documentation and analysis system from Kodak (Gel Logic 1500 Kodak New Haven, CT, USA).

Table 2.2: Quantitative real time PCR fold change for the reference genes

Reference genes	**Treatment**			
	GH *vs.* Control		**Atropine *vs.* Control**	
	Fold difference	**P value**	**Fold difference**	**P value**
18S	1.36	<0.001	NA	NA
PGTDS	1.22	0.33	1.3	0.17
GPAM	1.14	0.36	1.02	0.9
MGEA5	0.92	0.65	1.87	<0.05
KRT15	NA	NA	1.09	0.85

NA= Data not available
GH= growth hormone

2.4. Results

2.4.1. Gene expression profiles of mammary gland tissue

The microarray chip used in these experiments (GH *vs.* control and atropine *vs.* control) contained 22,690 ESTs representing 17,307 different genes (Table 2.3). For both experiments approximately 20,000 ESTs were above background (intensity 10 or higher). 31% (6,207) and 40% (7,996) of these genes were differentially expressed ($P<0.05$) in the GH and atropine experiments, respectively (Table 2.3). Overall, similar numbers of ESTs were up-regulated and down-regulated at a probability value lower than 0.05 in response to the individual treatments (approximately 3,000 for GH treatment and approximately 4,000 for atropine treatment) (Table 2.3). ESTs that satisfied the criterion of more than 1.3 fold change in expression were used for IPA analyses and are shown in the Table B.1 and B.2 (Appendix B). On this basis, 208 genes (unique Ref Seq ID) were up regulated and 128 genes were down regulated in response to the GH treatment. For the atropine treatment, 217 genes were up regulated and 124 genes were down regulated (Table 2.3).

Table 2.3: Summary of total gene expression changes in response to growth hormone (GH) and atropine treatment (*vs.* control)

Category	GH *vs.* Control	Atropine *vs.* Control
Total Spots on the array	22,690	22,690
Number of genes represented	17,037	17,037
Spots above background[a]	18,867	18,860
Significant spots (P≤0.05)	6,207	7,996
Number of up-regulated ESTs	3,097	3,891
Number of down-regulated ESTs[b]	3,110	4,105
Number of unique up-regulated genes[c]	208	217
Number of unique down-regulated genes[c]	128	124

[a]Spots whose integrated pixel intensity in channel was greater than background
[b]Significant changes in gene expression at P<0.05
[c]P<0.05, fold difference > 30%; genes representing unique Ref Seq ID

2.4.2. Regulation of milk protein genes

The microarray results show that for all milk proteins represented (α_{S1}-casein, β-casein, κ-casein, α-lactalbumin) there were no changes in expression detected higher than 1.3 fold and at probability lower than 0.05 for either treatment (GH *vs.* control and atropine *vs.* control) (Table 2.4). To validate the microarray data and assess the expression of the β-lactoglobulin gene which was not present on the microarray chip, expression of the individual milk protein genes was evaluated with qRT-PCR. The results show that for the majority of the milk protein genes, including β-lactoglobulin, there was no significant fold change in expression (Table 2.4). These results are in agreement with the microarray results, with the exception of the α_{S2}-casein gene which, in the GH-treated cows, was not differentially expressed in the array but it was differentially expressed in the qRT-PCR (1.7 fold, $P<0.05$). For the atropine-treated animals, there was no difference in expression for all milk protein genes studied for both microarray data and qRT-PCR (Table 2.4).

2.4.3. Functional grouping

To gain insights into the biological processes that changed in response to GH and atropine treatment which may be relevant to milk protein synthesis regulation, the genes were clustered into specific pathways/functions that were affected by either GH treatment or atropine treatment. Two complementary methods were applied to relate changes in gene expression to functional changes in response to the treatments (GH *vs.* control and atropine *vs.* control).

Table 2.4: Microarray and qRT-PCR gene expression of milk protein genes from growth hormone (GH) *vs* control or atropine *vs*. control cows (P<0.05)

Gene Symbol	Fold change in expression (GH *vs* control)		Fold change in expression (atropine *vs* control)		Description	Gene Bank accession number
	Microarray	**qRT-PCR**	**microarray**	**qRT-PCR**		
CSN1S1	NS	NS	NS	NS	α_{s1}-Casein, transcript variant 2, mRNA	NM_001025104.1
CSN1S2A	NS	1.7 (P<0.05)	NS	NS	α_{s2A}-Casein , mRNA	XM_379270.2
CSN2	NS	NS	NS	NS	β-Casein, mRNA	NM_001891.1
CSN3	NS	NS	NS	NS	κ-Casein, mRNA	BC_102120.1
LALBA	NS	NS	NS	NS	α-Lactalbumin, mRNA	NM_005212.1
LGB	NR	NS	NS	NS	β-Lactoglobulin, mRNA	NM_002289.2

NS = not statistically significant at P value lower than 0.05 and fold difference higher than 1.3
NR = not represented in the array

2.4.3.1. Ingenuity pathway analyses

IPA software (http://ingenuity.com) uses defined probability values and fold changes (in these experiments fold change >1.3, $P<0.05$) to identify the most significant pathways affected by the treatment. This method takes into account the broader context in which gene products function, namely in physically interacting networks, such as cellular growth and proliferation which is divided into growth of cells, proliferation of cells and others.

Clustering of the 336 differentially expressed genes between GH-treated and control cows using IPA identified cellular growth and proliferation, cell death and cancer as the most significant pathways that were affected (Fig 2.4). In contrast, cellular growth and proliferation, amino acid metabolism and post-translation modification were the most significant pathways affected in the atropine-treated cows compared to controls (Fig 2.5).

2.4.3.2. FUNC analyses software

FUNC analysis was used to confirm categories differentially expressed using IPA analysis. In response to GH treatment, 45 GO categories were significantly affected ($P<0.05$). For the atropine treatment, 78 GO categories were significantly affected ($P<0.05$). The full table of significant categories can be seen in the Appendix B (Table B.3-B.6). The graphic view of the pathways that were highly differentially expressed in the FUNC analyses and that were within the three top categories listed in the Ingenuity Pathway analyses are presented (Fig. 2.6).

2.4.4. Differentially expressed genes involved in the regulation of protein synthesis

Since this study aimed to elucidate pathways involved in the control of milk protein synthesis, genes involved in the regulation of protein synthesis (according to GO terms) that were up- and down-regulated in response to the GH (Table 2.5) and atropine (Table 2.6) treatments are presented. As previously mentioned, the full list of differentially expressed genes can be found in the Table B.1 and B.2 (Appendix B). However, due to overlap in the source database, several genes are represented in multiple categories, thus, to avoid repetition, the Tables 2.5 and 2.6 only present the expression of genes involved in the process of protein synthesis.

Although the treatments did not change the same group of genes (as expected) a large number of the genes that were differentially expressed in both treatments (Tables 2.5 and 2.6) were involved in translation control (e.g. eEF2- GH and atropine treatment, eEF1A1-GH treatment and eEF2K-atropine treatment); ribosomes (e.g. RPS27-atropine treatment and RPS6-GH treatment), MAPK signaling pathway (e.g. MAP2K5, MAP3K4-atropine treatment); and calcium/calmodulin-dependent protein kinases (e.g. CAMK2D-atropine treatment).

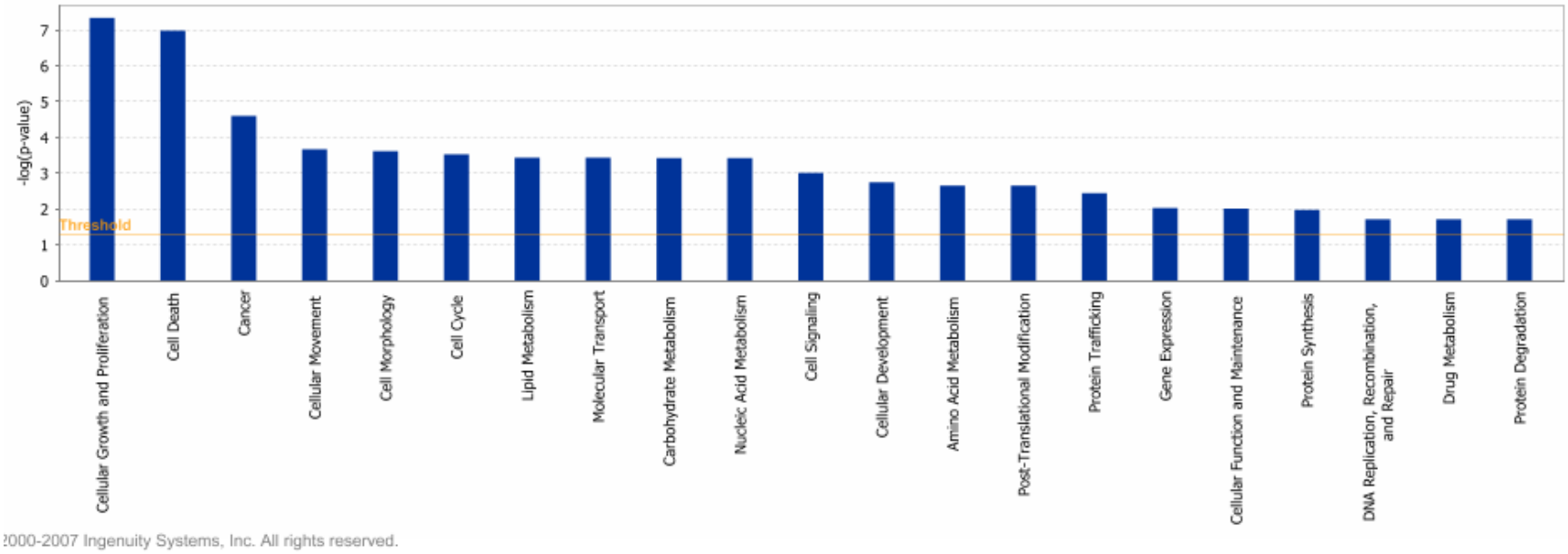

Fig. 2.4: The figure represents the most significant categories of genes differentially expressed ($P<0.05$) in response to growth hormone (GH) treatment. Threshold line is the P-value in a log scale

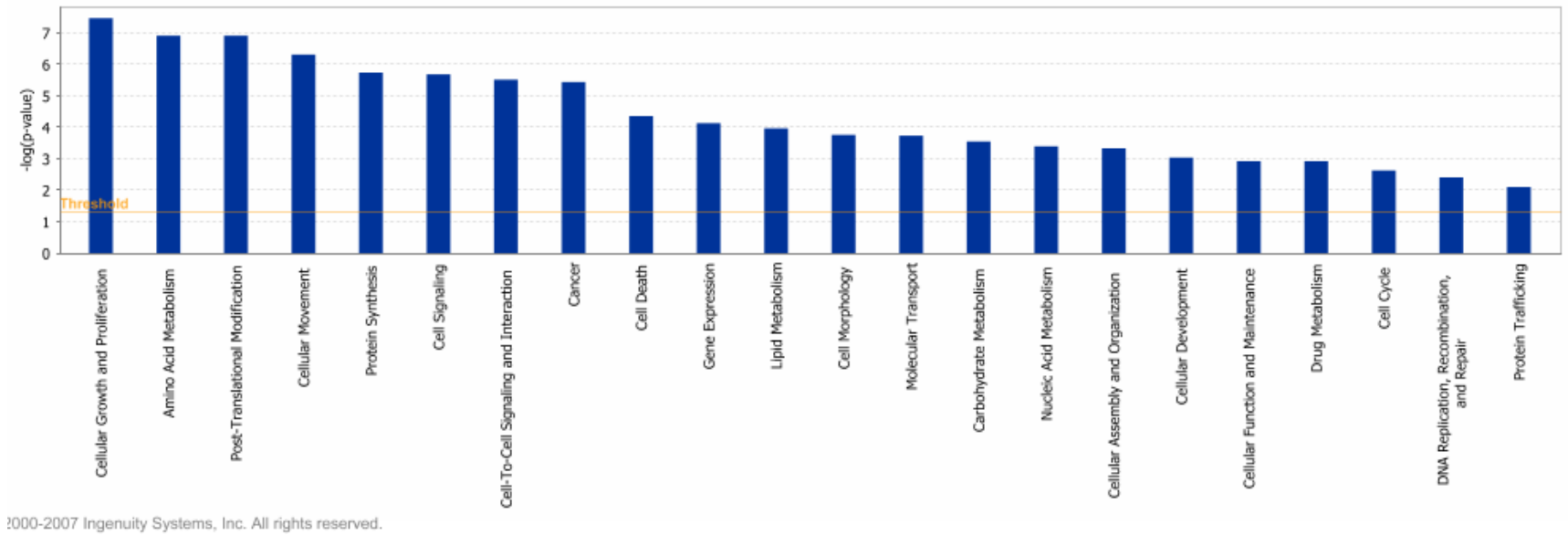

Fig. 2.5: The figure represents the most significant categories of genes differentially expressed in response to atropine treatment. Threshold line is the P-value in a log scale

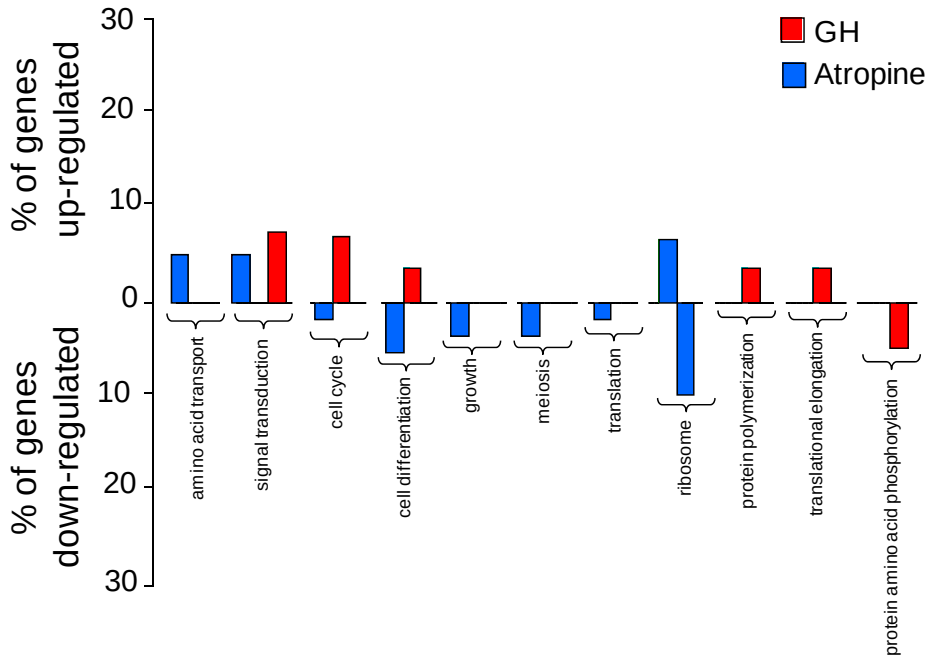

Fig. 2.6: Categories up- and down-regulated in response to the treatments and the percentage of genes differentially expressed that fall inside the categories listed

Table 2.5: Differentially expressed genes involved in protein synthesis (P<0.05) in response to growth hormone (GH) treatment *vs.* control. Genes are ordered in relation to fold difference in expression

Gene Symbol	Acession No.	Gene Name	Fold Change
PKN1	NM_002741	protein kinase N1, transcript variant 2	-1.9
STK6	NM_003600	serine/threonine kinase 6, transcript variant 2	-1.9
MAP3K4	NM_005922	mitogen-activated protein kinase kinase kinase 4, transcript variant 1	-1.5
MAP4K2	NM_004579	mitogen-activated protein kinase kinase kinase kinase 2	-1.5
PRKAR2A	NM_004157	protein kinase, cAMP-dependent, regulatory, type II, alpha	-1.5
RPS6KA1	NM_002953	ribosomal protein S6 kinase, 90kDa, polypeptide 1, transcript variant 1	-1.5
eIF2C4	NM_017629	eukaryotic translation initiation factor 2C	-1.4
eIF3S9	NM_003751	eukaryotic translation initiation factor 3, subunit 9 eta, 116kDa, transcript variant 1	-1.4
PKN2	NM_006256	protein kinase N2	-1.4
RYK	NM_001005861	RYK receptor-like tyrosine kinase , transcript variant 1	-1.4
eEF1A1	NM_001402	eukaryotic translation elongation factor 1 alpha 1	1.3
eEF1A2	NM_001958	eukaryotic translation elongation factor 1 alpha 2	1.3
eEF1G	NM_001404	eukaryotic translation elongation factor 1 gamma	1.3
eEF2	NM_001961	eukaryotic translation elongation factor 2	1.3
eIF3S10	NM_003750	eukaryotic translation initiation factor 3, subunit 10 theta, 150/170kDa	1.3
eIF3S2	NM_003757	eukaryotic translation initiation factor 3, subunit 2 beta, 36kDa	1.3
IMP-3	NM_006547	IGF-II mRNA-binding protein 3	1.3
PPP1R3A	NM_002711	protein phosphatase 1, regulatory (inhibitor) subunit 3A (glycogen and sarcoplasmic reticulum binding subunit, skeletal muscle)	1.3
TUBA6	NM_032704	tubulin alpha 6	1.3
ACVRL1	NM_000020	activin A receptor type II-like 1	1.4
CAMK2G	NM_172170	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	1.4
IKBKAP	NM_003640	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	1.4
PDK1	NM_002610	pyruvate dehydrogenase kinase, isoenzyme 1, nuclear gene encoding mitochondrial protein	1.4
STK11	NM_000455	serine/threonine kinase 11 (Peutz-Jeghers syndrome)	1.4
STK38	NM_007271	serine/threonine kinase 38	1.4
TUBA1	NM_006000	tubulin, alpha 1 (testis specific)	1.4
TUBA3	NM_006009	tubulin, alpha 3	1.4
PRKD1	NM_002742	protein kinase D1	1.5
K-AlPHA-1	NM_006082	tubulin, alpha, ubiquitous	1.7

Table 2.6: Differentially expressed genes involved in protein synthesis (P<0.05) in response to atropine treatment *vs.* control. Genes are ordered in relation to fold difference in expression

Gene Symbol	Acession No.	Gene Name	Fold Change
STK6	NM_003600	serine/threonine kinase 6, transcript variant 2	-2.3
BMPR1B	NM_001203	bone morphogenetic protein receptor, type IB	-1.5
eIF2C4	NM_017629	eukaryotic translation initiation factor 2C, 4	-1.5
PKN1	NM_002741	protein kinase N1, transcript variant 2	-1.5
GSG2	NM_031965	germ cell associated 2 (haspin)	-1.4
KIT	NM_000222	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	-1.4
MAP2K5	NM_145160	mitogen-activated protein kinase kinase 5, transcript variant A	-1.4
NRK	NM_198465	Nik related kinase	-1.4
PRKAR2A	NM_004157	protein kinase, cAMP-dependent, regulatory, type II, alpha	-1.4
RPL15	NM_002948	ribosomal protein L15	-1.4
RPS6KA1	NM_002953	ribosomal protein S6 kinase, 90kDa, polypeptide 1, transcript variant 1	-1.4
STK3	NM_006281	serine/threonine kinase 3	-1.4
TUBA3	NM_006009	tubulin, alpha 3	-1.4
eIF1AX	NM_001412	eukaryotic translation initiation factor 1A, X-linked	-1.3
eIF3S9	NM_003751	eukaryotic translation initiation factor 3, subunit 9 eta, 116kDa, transcript variant 1	-1.3
MAP3K1	XM_042066	mitogen-activated protein kinase kinase kinase 1	-1.3
MAP3K4	NM_005922	mitogen-activated protein kinase kinase kinase 4, transcript variant 1	-1.3
MAP4K2	NM_004579	mitogen-activated protein kinase kinase kinase kinase 2	-1.3
MARK2	NM_017490	MAP/microtubule affinity-regulating kinase 2, transcript variant 1	-1.3
MYLK	NM_053032	myosin, light polypeptide kinase, transcript variant 8	-1.3
RYK	NM_001005861	RYK receptor-like tyrosine kinase, transcript variant 1	-1.3
TUBB	NM_178014	tubulin, beta polypeptide	-1.3
CCL11	NM_002986	chemokine (C-C motif) ligand 11	1.3
CTSD	NM_001909	cathepsin D (lysosomal aspartyl peptidase)	1.3
eIF3S3	NM_003756	eukaryotic translation initiation factor 3, subunit 3 gamma, 40kDa	1.3
eIF4EBP2	NM_004096	eukaryotic translation initiation factor 4E binding protein 2	1.3
ePHB3	NM_004443	EPH receptor B3	1.3
GC20	NM_005875	translation factor sui1 homolog	1.3
IGF1	NM_000618	insulin-like growth factor 1 (somatomedin C)	1.3
IMP-3	NM_006547	IGF-II mRNA-binding protein 3	1.3
MAPK7	NM_002749	mitogen-activated protein kinase 7, transcript variant 3	1.3
PKN3	NM_013355	protein kinase N3	1.3
PRKD2	NM_016457	protein kinase D2	1.3
RPS27	NM_001030	ribosomal protein S27 (metallopanstimulin 1)	1.3
RPS6	NM_001010	ribosomal protein S6	1.3
WARS	NM_213646	tryptophanyl-tRNA synthetase, transcript variant 4	1.3
ACVRL1	NM_000020	activin A receptor type II-like 1	1.4
ADAM10	NM_001110	ADAM metallopeptidase domain 10	1.4
CHUK	NM_001278	conserved helix-loop-helix ubiquitous kinase	1.4

eEF2	NM_001961	eukaryotic translation elongation factor 2	1.4
eEF2K	NM_013302	eukaryotic elongation factor-2 kinase	1.4
eIF5B	NM_015904	eukaryotic translation initiation factor 5B	1.4
INSR	NM_000208	insulin receptor	1.4
MAP4K3	NM_003618	mitogen-activated protein kinase kinase kinase kinase 3	1.4
MKNK2	NM_199054	MAP kinase interacting serine/threonine kinase 2	1.4
SGKL	NM_013257	serum/glucocorticoid regulated kinase-like, transcript variant 1	1.4
TTN	NM_003319	titin, transcript variant N2-B	1.4
CAMK2D	NM_172115	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta, transcript variant 4	1.5
SUI1	NM_005801	putative translation initiation factor	1.6

2.4.5. Potential pathways involved in the regulation of milk protein synthesis

Tables 2.5 and 2.6 show the major genes involved in protein synthesis that changed in response to GH and atropine treatment, respectively. Examples of these genes include: RPS6, eEF2, eEF2K, IGF-I, eEF1. These genes are involved in functions including biosynthesis of protein, translation, metabolism of protein and elongation of protein. Interestingly, most of the genes that responded to the treatments were either directly or indirectly part of the mTOR signaling pathway (Fig 2.7). These results indicate that the mTOR signaling pathway could be involved in the changes in the milk protein yield caused by these treatments.

Downstream genes of the mTOR signaling pathway were also analyzed for changes in gene expression in response to GH and atropine treatments. The microarray results showed that the eukaryotic initiation factor 4E binding protein (4E-BP1) mRNA levels were decreased 1.2 fold ($P<0.05$) after GH treatment and increased 1.3 fold ($P<0.05$) after the atropine treatment. Also, the arrays indicated that mRNA levels of ribosomal protein S6 (S6) were up-regulated 1.2 fold ($P<0.05$) in the GH treatment and down-regulated 1.2 fold ($P<0.05$) in the atropine treatment (Fig 2.7). In contrast, eukaryotic elongation factor 2 (eEF2) transcript level was increased 1.3 fold ($P<0.05$) in response to both GH and atropine 1.4 fold ($P<0.05$) treatments. The expression of eEF2 kinase (eEF2K) was down-regulated (1.2 fold, $P<0.05$) in response to GH treatment and up-regulated (1.4 fold, $P<0.05$) in response to atropine treatment.

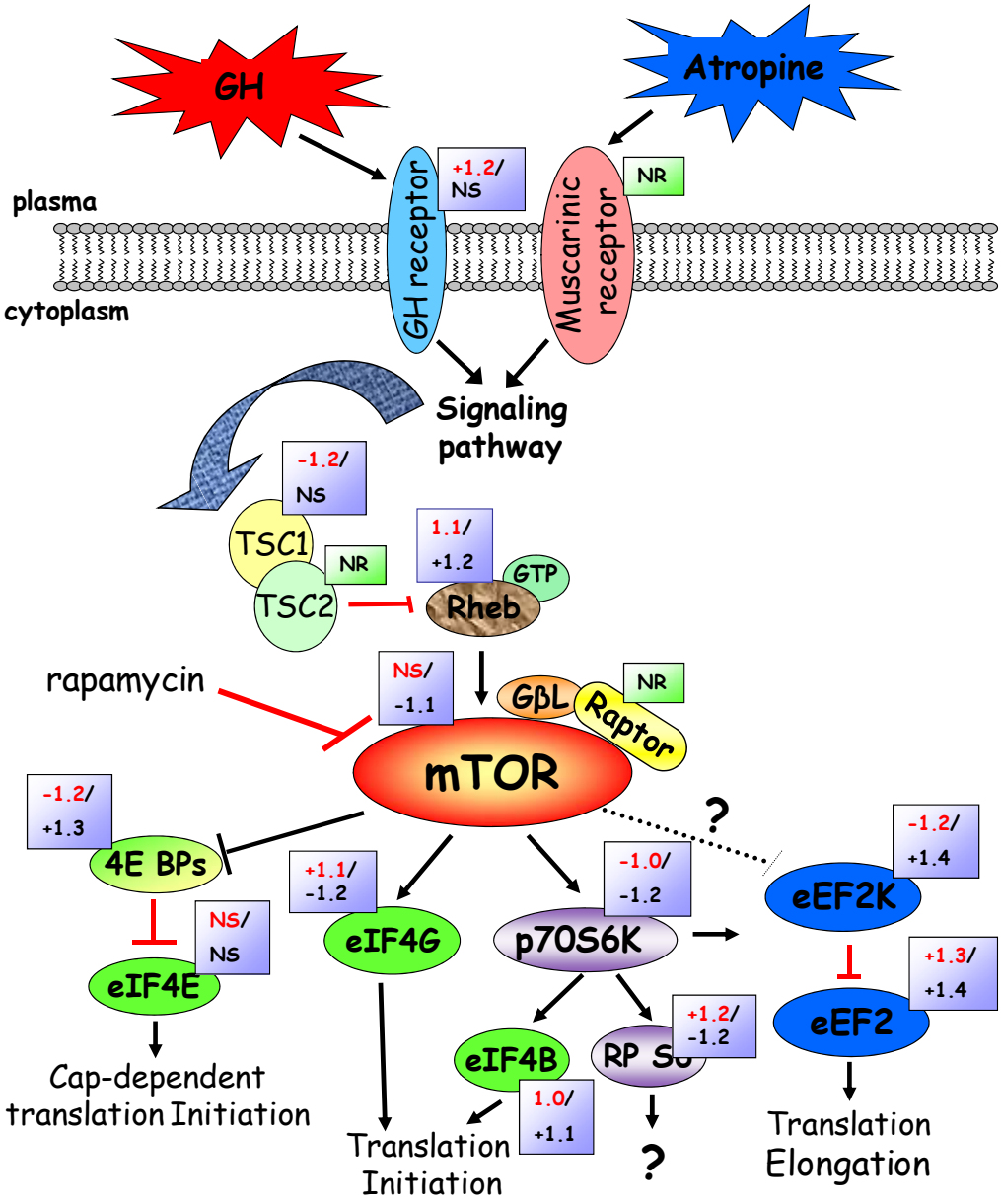

Fig. 2.7: Illustration of the microarray results for members of the mTOR signaling pathway. mTOR signaling regulates translation by integrating several different inputs. GH and atropine activate both the PI3K/Akt signaling cascade and the ERK pathway. These pathways affect TSC1/2 to regulate mTOR activity via Rheb. The raptor/GβL/mTOR complex mediates the phosphorylation of 4E-BP1 and S6K. Pharmacological inhibition of mTOR (rapamycin or its analogue) is indicated. The figure shows the changes in gene expression ($P<0.05$) in response to GH and atropine observed in the members of the mTOR signaling pathway. The values in red represent the GH array results and the values in black represent the atropine array results

NR = not represented

2.5. Discussion

In this study, the effect of the two treatments (GH and atropine) on gene expression in the mammary gland was examined to identify key pathways and genes that were potentially involved in the regulation of milk protein synthesis. A number of criteria were applied for the selection of the components to study in detail. First, it was established whether there had been a change in the transcript levels of the milk protein genes. Second, differentially expressed genes were clustered into different functional groups using two different approaches. Third, a list of genes involved in general protein synthesis was generated and the indirectly, via literature searches, the individual function of some of these genes was assessed. Fourth, a common pathway, of which some of these genes are part, was identified and changes in gene expression of gene members from this pathway were graphically illustrated.

Changes in the milk proteins transcriptional level have been reported before during the onset of lactation in many cases as a response to prolactin acting through STAT5 (Liu *et al*., 1995; Malewski *et al*., 2002). In addition to prolactin, GH has also been shown to stimulate the expression of milk protein genes in primary bovine mammary cell line (Sakamoto *et al*., 2005). A number of studies have been conducted to study the regulation of milk protein genes but to the best of my knowledge, the transcriptional control of milk protein synthesis changes caused by GH or atropine after the onset of lactation have never been studied. To verify if GH and atropine treatment were affecting milk protein synthesis at the transcriptional level, changes in expression of the milk protein genes were assessed. Changes in the milk protein genes at the transcriptional level were not observed. Thus, this is the first study to show that changes in milk protein synthesis after the onset of lactation (Appendix A) are not regulated at

the level of the transcript (Fig. 2.8). Instead, alternate mechanisms such as translational control are likely to be involved in the increased output of milk protein in response to GH treatment and decreased milk protein output in response to atropine treatment (Appendix A) without changing the mRNA concentrations of the individual milk proteins.

Whereas transcriptional regulation of the milk protein genes has been extensively studied, a critical role for translation control has been demonstrated with evidence that the polyadenylation of mRNA (Kuraishi *et al*., 2000) and possibly mTOR signaling might be involved (Toerien and Cant, 2007). The notion that post-translational mechanisms govern milk protein expression is supported by the small increase in β-casein concentration in the milk of transgenic cows having additional copies of β and κ-casein genes (Brophy *et al*., 2003). Indeed, the microarray results support the hypothesis that the genes differentially expressed in response to the GH and atropine treatments are members of the translation machinery (e.g. ribosomal proteins, elongation and initiation factors). Consequently, the microarray data were searched for signaling pathway(s) with differentially expressed genes that link protein synthesis and mRNA translation.

The data set was clustered into specific and important functions and/or pathways with two different software programs (IPA and FUNC). These two approaches were taken to ensure that the resultant list of genes would include those genes that were the most suitable for further analyses. The IPA software uses specific cut off criteria to group genes, and in this experiment these were a probability value of 0.05 and a fold change greater than 1.3 fold, which have been used previously in microarray studies (Keane *et al*., 2006; Mamane *et al*., 2007). This analysis showed that different signaling pathways differentially expressed e.g. cellular growth and proliferation, cell death, cancer, cell

cycle, protein synthesis and post-translational modification, contained genes that were directly or indirectly linked to the mTOR signaling pathway. Similar responses to GH treatment have been reported previously (Boutinaud *et al*., 2003; Sakamoto *et al*., 2005; Akers, 2006) with increases in cell number and cell size in mammary gland of lactating animals. In contrast, atropine administration has been shown to decrease proliferation in a range of cells including neural stem cells (Zhou *et al*., 2004), lung cancer cell lines (Song *et al*., 2003) and mammary adenocarcinoma cells (Rimmaudo *et al*., 2005).

The FUNC analyses confirm the importance of the pathways/functions shown in the IPA analyses. This analysis also showed that different signaling pathways differentially expressed e.g. amino acid transport, signal transduction, cell cycle, growth, meiosis, ribosome contained genes that were directly or indirectly linked to the mTOR signaling pathway. In contrast to the IPA analyses, the FUNC software takes into consideration the false discovery rate of cluster categories that are significantly changed in response to the treatment. The issue of false discoveries (selecting wrong candidate genes for validation) has been discussed previously by bioinformatics groups (Storey and Tibshirani, 2003; Grant *et al*., 2005; Pounds, 2006) and it should be considered even when calculating the sample size in an experimental design (Liu and Hwang, 2007).

Although IPA and FUNC do not use similar category names, the results from the FUNC analyses still validate the results from the IPA. For example, the "post-translation modification" classification using IPA is equivalent to the "translation elongation", "translation" and "protein amino acid phosphorylation" classifications using FUNC. The FUNC software uses terms from the GO annotation (Ashburner *et al*., 2000) while the IPA software uses terms and definitions from the IPA program (http://ingenuity.com).

In the mammary gland of GH-treated cows, there were more genes that were up-regulated than down-regulated which is consistent with the well established understanding that GH has an anabolic effects in various tissues, including mammary gland (Boutinaud *et al*., 2003). In contrast, atropine treatment inhibits the anabolic effects of cholinergic compounds (Racke *et al*., 2006) and consequently it was expected that a larger number of genes would be down-regulated but this assumption proved to be incorrect. However, this assumption is rather general and just looking at up and down genes is not a good indicator of their role and mode of action. Thus, it was necessary to look at genes themselves and their respective roles e.g. stimulatory effect or inhibitory effect on protein synthesis.

The genes that were differentially expressed (fold change >1.3 and $P<0.05$) are involved in several functions and pathways. While the specific roles of all the differentially expressed genes are of interest, the main objective of this study is focused on the genes involved in the regulation of protein synthesis. A large proportion of these genes that are involved in protein synthesis are related to translation control e.g. eEF2, eEF2K, RPS6, eEF1A, P90RSK are components of the ribosome e.g. RPL15, and RPS26. Thus, the results of this study suggest that the mTOR signaling pathway is a possible candidate pathway involved in the control of milk protein synthesis in that activation of the mTOR pathway stimulates protein synthesis (Fig. 2.7 and Fig. 2.8).

In conclusion, it appears that changes in the output of milk protein during lactation period caused by the GH and atropine treatment (Appendix A) are not regulated at the transcript level but possibly at translation, which in turn may be regulated by the mTOR signaling pathway. The link between the mTOR signaling pathway and milk protein synthesis regulation would not be surprising as mTOR is involved in the control of

cellular processes such as proliferation and growth, both requiring tight regulation of the protein synthetic machinery. Thus, in the next Chapters of this thesis, the main objective was to study in more detail the mTOR signaling pathway in the lactating mammary gland of GH- and atropine-treated cows.

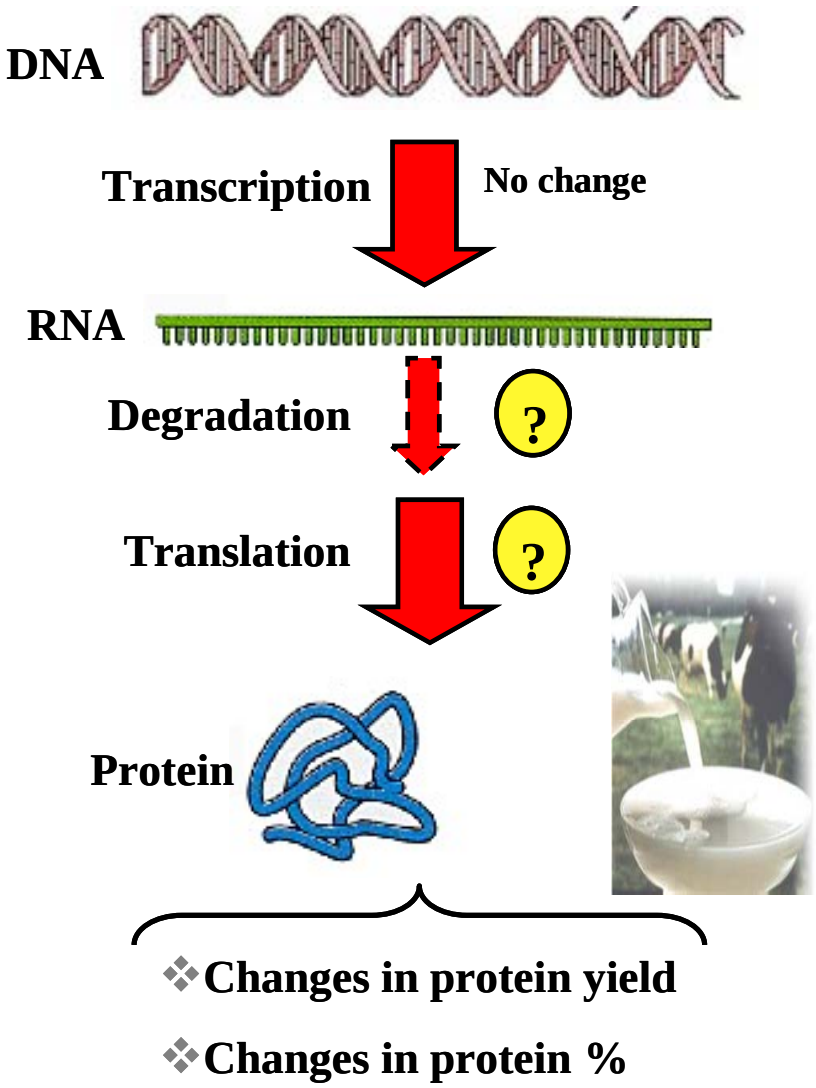

Fig. 2.8: Illustration of the different points of control of milk protein synthesis regulation. No change in the milk proteins was detected at the level of transcription (with the exception of the alpha-S2 casein), but possible regulation at the level of degradation or translation

CHAPTER 3

Ribosomal protein S6 is involved in changes in milk protein yield caused by growth hormone

The material present in this Chapter has been submitted as a paper to: Journal of Dairy Science

3.1. Abstract

Growth hormone (GH) treatment is known to increase milk yield and milk protein yield in domestic animals but the underlying molecular mechanisms by which this occurs are not completely understood. In this chapter, GH treatment was used as an experimental model to establish the role of translation initiation and elongation in the regulation of milk protein synthesis in the mammary gland. A slow-release formula of commercially available GH was administered via a single subcutaneous injection to four lactating cows (GH group). A further four cows were given a single subcutaneous injection of saline (control group). Changes in mRNA transcript level and protein phosphorylation status of key members of the mTOR pathway were assessed in mammary gland tissues of these animals using quantitative real time PCR and western blotting. GH treatment enhanced the phosphorylation of ribosomal protein S6 and increased the protein abundance of eIF4E and eEF2 proteins in the mammary gland of GH-treated animals. These results indicate a link between milk protein synthesis and the regulation of mRNA translation. GH treatment did not change mRNA abundance of ribosomal protein S6, eIF4E and eEF2, neither did it change the mRNA or protein abundance of mTOR and eEF2K. These results demonstrate that GH administration changes mRNA translation initiation and elongation possibly via the mTOR pathway (suggested by the increased levels of S6 phosphorylation), suggesting that the mTOR pathway may be a potential control point in the regulation of milk protein synthesis in the mammary gland.

3.2. Introduction

Milk, especially milk protein, is an important source of nutrition for humans and production needs to increase to match global demand (Huffman and Harper, 1999). However, although the composition and yield of milk protein varies between animals (Jenness, 1979), the concentration of protein in milk is tightly regulated. Furthermore, it is well known, that milk protein yield is increased by GH administration (Bauman *et al*., 1985; Molento *et al*., 2002; Boutinaud *et al*., 2003).

A number of studies have shown that the positive effects of GH on lactation are related to increases in both the proliferation and activity of mammary epithelial cells (Berry *et al*., 2001; Molento *et al*., 2002) either as a result of the direct effect of GH on the mammary gland (Plath-Gabler *et al*., 2001) or an indirect effect via increased secretion of insulin-like growth factor-1 (IGF-1) (Akers *et al*., 2000; Allan *et al*., 2002). Nevertheless, details of the mechanisms by which GH affects the milk protein synthetic machinery in the mammary gland remain largely unknown. GH treatment has previously been shown to stimulate mammary growth in rodents (Feldman *et al*., 1993) at least in part by increasing IGF-1 gene expression (Allan *et al*., 2002). In bovine mammary epithelial cells, GH also decreases the expression of insulin-like growth factor binding protein-5 (IGFBP-5) and induces phosphorylation of the protein kinase B (PKB, also termed Akt) (Sakamoto *et al*., 2007).

GH binds to membrane receptors in target tissues to stimulate tissue growth (Rowland *et al*., 2005) and elicit changes in protein, carbohydrate and fat metabolism. Signaling via the GH pathway is initiated by the binding of GH to its receptor (GHR) followed by phosphorylation and activation of janus kinase 2 (JAK2). JAK2 triggers a cascade of

signaling events that involve the insulin receptor substrate (IRS) proteins, phosphoinositide 3-kinase (PI 3-kinase) and PKB (Souza *et al*., 1994; Carter-Su *et al*., 1996; Costoya *et al*., 1999; Harrington *et al*., 2005). PKB directly phosphorylates tuberous sclerosis complex-2 (TSC2) in response to GH treatment inducing the activation of mTOR (Hayashi and Proud, 2007).

mTOR forms two types of complex, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), of which mTORC1 is sensitive to rapamycin. The activation of the mTOR signaling pathway by insulin and other agents leads to increased phosphorylation and activation of at least three components of the translational machinery. Firstly, mTOR activates 4E-BP1 which binds to and inhibits the mRNA cap-binding protein, eIF4E, thereby blocking the formation of active initiation factor complexes containing the scaffold protein eukaryotic initiation factor 4G (eIF4G) (Gingras *et al*., 1999b). Secondly, mTOR activates the ribosomal protein, S6 kinase 1 (P70S6K1) which phosphorylates and activates S6, a component of the 40S ribosomal subunit complex (Avruch *et al*., 2001). S6 has a role in ribosome biogenesis (Reiter *et al*., 2004) and phosphorylation of S6 plays a role in cell size regulation (Ruvinsky *et al*., 2005), although this mechanism remains to be elucidated. Thirdly, mTOR controls the activity of eEF2K, such that activation of mTOR leads to inhibition of eEF2K, and the dephosphorylation and activation of eEF2 (Browne and Proud, 2002). In hepatoma cells, Hayashi and Proud (2007) have shown that GH treatment induced rapid dephosphorylation of eEF2 in a biphasic manner. Rapamycin treatment completely inhibited this GH effect, indicating that mTORC1 is involved in GH activation of protein synthesis.

Toerien and Cant (2007) have recently reported that the phosphorylation of S6 at Ser235/236 was increased in the mammary gland of lactating dairy cows indicating a potential role of the mTOR signaling pathway in milk production. In many tissues (muscle, liver, adipose tissue) GH influences protein metabolism through changes in the protein translation machinery (Bush *et al*., 2003), specifically through changes in the mTOR pathway signaling (Hayashi and Proud, 2007). A number of studies have demonstrated the role of the mTOR in the regulation of protein synthesis (Wang and Proud, 2006; Proud, 2007) and there is evidence that GH activates protein synthesis both *in vitro* (Hayashi and Proud, 2007) and *in vivo* (Bush *et al*., 2003; Ramsay *et al*., 2004). However, there are no published studies directly relating the effects of GH on milk protein synthesis via the mTOR signaling pathway.

In Chapter 2, it was concluded that the mTOR pathway was a potential point of control in the regulation of milk protein yield but future experiments were required to sustain this hypothesis. Therefore, the aims of this study were to determine whether the GH effects on milk protein synthesis (Appendix A) involved changes in mTOR signaling, confirm whether these effects were caused by changes in transcription (mRNA abundance) or translation (phosphorylation status), and to identify which members downstream of mTOR were involved in the regulation of mammary gland protein synthesis.

3.3. Materials and methods

3.3.1. Animals and experimental design

Note: *Since the design and implementation of the experiment were done by others, these details are presented in the Appendix A.*

3.3.2. Total RNA extraction

Described in the Materials and methods of Chapter 2.

3.3.3. qRT-PCR

Described in the Materials and methods of Chapter 2.

3.3.4. Primers

The primers used in this Chapter and in Chapters 5 are presented in Table 3.1.

Table 3.1: Sequence of primer sets used for quantitative real time PCR

Gene Name[c]	Genebank ID	Forward Primer	Reverse primer	Amplicon size (bp)
Candidate genes				
eEF2[a]	NW_618521.1	5'-CTCTACCAAACCTTCCAGCG-3'	5'-GCTGTTGGCTGACTTGCTGA-3'	318
eEF2K[a]	XM_599997.1	5'-AGTATGATGGGATGCAAGAC-3'	5'-CTCCTCTGCTTTCTCGTAGT-3'	191
eIF4E[a]	AF257235.1	5'-CCCGCCTACAGAAGAAGAGA-3'	5'-CACTTCGTCTCTGCTGTTTG-3'	332
4E-BP1[a]	BC120290.1	5'-GAACTCACCTGTGACCAAGA-3'	5'-CTCAAACTGTGACTCTTCACC-3'	157
P90[RSKb, d]	NM_002953.3	5'-CCCGATGACACCTTCTACT -3'	5'-CCAGGTTCTTCCCATGTAA-3'	204
mTOR[b]	NM_004958.2	5'-CGGGACTACAGGGAGAAAAA-3'	5'-CCTCAAAGCAGTCCCCAAAG-3'	339
Reference genes				
GPAM[a]	NM_001012282.1	5'-GACTGACCTCTCTGGACTCTG-3'	5'-CCCCAAAGCATCTGAGTAG-3'	150
MGEA5[a]	XM_613611.1	5'-GAGGTGCTTTGGACAGGT-3'	5'-CATCCAAACTGAGAAACCTG-3'	173
KRT15[a, d]	XM_584069	5' - CGTTGAGGAGTCTGTGGA-3'	5' - CAACTAAGAAACGAAGGACC-3'	195

The table shows the gene name (symbol)[c], gene bank ID, forward and reverse primer sequences (5'→ 3'), qRT-PCR product length (amplification size) of the investigated genes (candidate genes) and house keeping genes (reference genes)

[a]*Bos taurus* sequence, [b]*Homo sapiens* sequence (The sequence amplified for mTOR in humans was highly homologous to bovine >99%)

[c]eEF2(eukaryotic elongation factor 2) eEF2K (eukaryotic elongation factor 2 kinase), eIF4E (eukaryotic initiation factor 4E), 4E-BP1 (eukaryotic initiation factor 4E binding protein), P90[RSK] (ribosomal protein S6 kinase, 90kDa); mTOR (mammalian target of rapamycin), GPAM (glycerol-3-phosphate acyltransferase, mitochondrial), MGEA5 (meningioma-expressed antigen 5);), KRT15 (K15 intermediate filament type I keratin)

[d]primer used in Chapter 4

3.3.5. Protein extraction from tissues

For western blotting analysis 300 mg of tissue from individual animals were lysed in extraction buffer (50 mM β-glycerophosphate, pH 7.5; 1 mM EGTA; 1 mM EDTA; 1% (v/v) Triton X-100; 1 mM Na_3VO_4; 100 nM microcystin-LR; 0.1% (v/v) β-mercaptoethanol; protease inhibitors (leupeptin, pepstatin and antipain, each 1 μg/ml) and phenylmethylsulfonyl fluoride (200 μM)). Lysates were centrifuged at 13,000*g* for 10 min to remove debris. For the measurement of 4E-BP1, an extra step was performed to enrich for 4E-BP1 which is heat stable. Aliquots of mammary gland homogenates were heated at 100^{o}C for 10 min, cooled at room temperature and then centrifuged at 10,000*g* for 10 min at 4^{o}C.

3.3.6. Determination of protein concentration

Protein content was determined using the Bradford method (1976), using the BioRad Protein Assay kit (BioRad Laboratories Ltd., Hertfordshire, UK) and a 96-well microplate spectrophotometer (SpectraMax, Sunnyvale, CA, USA). Typically samples were diluted 1:10 with H_2O in a volume of 20 μL to which 1 mL of BioRad reagent was added. Samples were gently vortexed and left at room temperature for 10 min. Protein concentrations were calculated with reference to a standard curve constructed by diluting a 2 mg/mL stock of bovine serum albumin (BSA) (BioRad) in water. The absorbance at a wavelength of 595 nm was between 0.06 and 0.07 for a 1 mg/mL solution.

3.3.7. SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by (Laemmli, 1970). SDS-PAGE gels were prepared using Bio-Rad Mini-Protean® II and III gel apparatus (Bio-Rad Laboratories Ltd., Hertfordshire, UK), using 10%, 12% or 15% acrylamide and 0.1% N,N'-methylene-bis-acrylamide (both w/v), polymerised by the addition of 10% (w/v) ammonium persulphate solution. To study the phosphorylation of the S6 protein, 15% (w/v) acrylamide and 0.1% (w/v) methylene bisacrylamide gels were used for better visualization of this protein. All other standard resolving gels contained 12.5% (w/v) acrylamide and 0.1% (w/v) methylene bisacrylamide. Equal amounts of lysate were loaded into each lane and equal loading of the samples was generally further confirmed by western blot analysis with the appropriate antisera. Gels were run in SDS-PAGE running buffer (192 mM glycine, 25 mM Tris-base and 0.1% (w/v) SDS) at 25 mA per gel and 200V. Prior to loading, samples were diluted with 5X sample buffer and boiled for 5 min.

3.3.8. Staining, fixing and drying polyacrylamide gels

To check the quality of the protein lysate, gels were stained in coomassie blue solution (0.13% (w/v) coomassie brilliant blue in 45% (v/v) methanol and 10% (v/v) acetic acid). The destaining solution consisting of 20% (v/v) methanol and 5% (v/v) acetic acid was changed frequently over the period (between 2 and 12 hours). Gels were then dried using a Bio-Rad Model 583 Gel Dryer at 80^{o}C for 2 hours.

3.3.9. Electrotransfer

After SDS-PAGE electrophoresis, proteins on the gels were transferred to Immobilon-P (a polyvinyllidene fluoride (PVDF) microporous membrane, Millipore, Watford, Hertfordshire, UK) using a Bio-Rad Trans-Blot Transfer cell for a period of 1 hour at 100V. Immobilin-P membranes were activated with methanol for 30 sec, reducing their hydrophobicity, and pre-equilibrated in Transfer Buffer (192 mM glycine, 25 mM Tris-base, 0.02% (w/v) SDS and 10% (v/v) methanol) for 10 min prior to electrophoretic transfer.

3.3.10. Western blot analysis

After transfer, immobilon-P membranes were blocked by incubation with Blocking Buffer (PBS, containing 0.2% Triton® X-100 (PBS/Triton) and 15% skim milk) for 1 hour at room temperature to prevent non-specific binding. Membranes were then incubated with the appropriate dilution of antiserum (Table 3.2, section 3.3.11) in PBS/Triton and either 0.5% (w/v) skim milk powder or BSA for 1 to 12 hours. Excess antibody was removed by washing the membrane 3 times, 5 min each, in PBS/Triton. The appropriate peroxidase-conjugated antibody (anti-mouse, -rabbit, -rat or -sheep, Diagnostics Scotland, Carluke, Scotland, UK), diluted 1:10,000 in PBS/Triton, were then applied to the membranes for 1 hour at room temperature. Membranes were then rinsed three times for 15 min with PBS/Triton to remove excess secondary antibodies. All incubations and washes were preformed with constant agitation. Antibody-antigen complexes were detected using Enhanced Chemilumescence (ECL) according to the supplier's instructions (Amersham International, Piscataway, NJ, USA) and visualised following exposure to X-ray film (Konica Corporation, Hohenbrunn, Germany). Film

was developed using an SRX-101A processor (Konica Minolta Medical Imaging USA, Inc, NJ, USA). Each blot was generated at least three times with similar outcomes and the graphs show the densitometry mean ± SE for 4 animals/treatment. Image/J software (available at rsb.info.nih.gov/ij/) was used for quantification where indicated. In the case of western blotting, data from a typical experiment are shown. All data comparisons were analysed for statistical significance using t-test. Differences were considered significant at a P value of < 0.05.

3.3.11. Primary antisera

The list of all antibodies used in the present thesis and their working dilution concentration is presented in Table 3.2.

Table 3.2: List of polyclonal antibodies used

Antibody name	Species in which the antibody was raised	Dilution used in Western Blotting	Reference or manufacturer of the antibody
4E-BP1 Total	Rabbit	1:2000	Cell Signaling
4E-BP1(P) Thr37/46	Rabbit	1:1000	Cell Signaling
4E-BP1(P) Ser65	Rabbit	1:1000	Cell Signaling
4E-BP1(P) Thr70	Rabbit	1:1000	Cell Signaling
Akt Total	Rabbit	1:1000	Cell Signaling
Akt (P) Ser473	Rabbit	1:1000	Cell Signaling
eEF2 Total (Chapters 3 and 4)	Rabbit	1:1000	Cell Signaling
eEF2 Total (Chapter 5 and 6)	Rabbit	1:1000	Dr L.E. McLeod
eEF2 (P) Thr56 (Chapters 3 and 4)	Rabbit	1:1000	Cell Signaling
eEF2 (P) Thr56 (Chapter 5 and 6)	Rabbit	1:1000	Dr L.E. McLeod
eEF2K Total	Rabbit	1:5000	Cell Signaling
eEF2K (P) Ser366	Rabbit	1:1000	Cell Signaling
eIF4E	Mouse	1:1000	Cell Signaling
GST	Rabbit	1:5000	Dr J. L. Parra, University of British Columbia
Myc	Mouse	1:3000	Sigma
Raptor	Rabbit	1:1000.	Cell Signaling
S6 ribosomal protein total	Rabbit	1:1000	Cell Signaling
S6 ribosomal protein (P) Ser235/236	Rabbit	1:1000	Cell Signaling

3.4. Results

3.4.1. Gene expression of mTOR signaling

In this study qRT-PCR was used to validate the results from Chapter 2 and to determine if the GH effects on milk protein synthesis were a result of transcriptional changes in members of the mTOR signaling pathway. Although the 4EBP1 mRNA levels were decreased (1.1 fold), consistent with the 1.32 fold down-regulation observed in Chapter 2, it did not attain statistical significance (P=0.53, Fig 3.1). The second downstream target of mTOR signaling studied was the eEF2 and its regulatory kinase, eEF2K. GH treatment increased the expression of eEF2 (1.35 fold) and decreased the expression of eEF2K (1.1 fold), consistent with the changes observed on the arrays (Chapter 2; i.e. 1.3 fold up-regulated and 1.2 fold down regulated respectively), however, these differences did not attain statistical significance (P=0.76; Fig. 3.1). Similarly, the 1.4 fold increase in the mRNA expression of mTOR in the mammary gland in response to GH treatment, different from the observation in Chapter 2 (no change in gene expression), did not attain statistical significance (P=0.39; Fig. 3.1).

It was also important to assess the GH effect on the expression of eIF4E. The binding of 4E-BP1 to eIF4E prevents eIF4E from binding eIF4G to form complexes that are competent for cap-dependent mRNA translation. The array results from Chapter 2 indicated no difference in expression of this gene; however qRT-PCR was used to validate this observation. There was no change in the concentration of eIF4E mRNA in the mammary gland in response to the GH treatment. Variation in the transcript level between animals in the same treatment can be seen in Fig. 3.2.

These findings suggest that the effect of GH on mammary gland protein synthesis was not mediated through an effect on the expression (mRNA levels) of genes in the mTOR signaling pathway. Therefore, further assessment of the expression of other genes in the mTOR signaling pathway was not progressed.

3.4.2. Effect of GH treatment on phospho-S6 and total S6

The post-translational effects of GH on the S6 protein are shown in Fig. 3.3. Mammary tissue lysates were used to evaluate changes in the total protein and phosphorylation status of this key target. No change was observed in the total abundance of this protein in the mammary gland (164 ± 12 *vs.* 159 ± 30 arbitrary units; P=0.74 for the GH and control group, respectively; Fig. 3.3) but the phosphorylation of S6 at Ser235/236 was increased by GH treatment (1.3 ± 0.08 *vs*. 0.8 ± 0.18, arbitrary units, P<0.001, for the GH and control group, respectively, Fig. 3.3).

3.4.3. Effect of GH treatment on phospho-4E-BP1, total 4E-BP1 and total eIF4E

To study the GH effects on the phosphorylation of this crucial downstream regulator of the mTOR signaling pathway, the protein abundance and phosphorylation status of 4E-BP1 were analysed by immunoblotting. The abundance of 4E-BP1 in the mammary gland was not affected by GH treatment (148 ± 45 *vs*. 184 ± 19, arbitrary units, P=0.20, for the GH and control group, respectively; Fig. 3.4). The phosphorylation status of the

4E-BP1 at Thr70 (4 ± 1.8 *vs.* 2 ± 1.2, arbitrary units P=0.12, for the GH and control group, respectively) was also unaffected by GH-treatment (Fig. 3.4).

eIF4E is involved in growth regulation of many cell types (Sonenberg and Gingras, 1998) and overexpression of eIF4E results in tumorogenic growth in NIH 3T3 fibroblasts (Lazaris-Karatzas *et al*., 1990). Since eIF4E is rate-limiting in translation, increased milk protein synthesis in response to GH treatment may have been associated with increased availability of eIF4E. GH treatment increased the abundance of eIF4E (96 ± 12 *vs*. 50 ± 8, arbitrary units, P<0.01, for the GH and control group, respectively; Fig. 3.4).

3.4.4. Effect of GH on the phospho- and total eEF2 and eEF2K

mTOR signaling also regulates the elongation phase of translation through indirect phosphorylation of eEF2K leading to mTOR-dependent regulation of eEF2 (Browne and Proud, 2002). To examine the effects of GH on the elongation phase of protein synthesis, the protein abundance of eEF2 and eEF2K and the phosphorylation status of eEF2 at Thr56 and eEF2K at Ser366 were analysed by immunoblotting. The protein abundance and phosphorylation status of eEF2K in the mammary gland were not affected by the GH treatment (Fig. 3.5). In contrast, GH treatment significantly increased the abundance of eEF2 (79 ± 4 *vs.* 53 ± 8, arbitrary units, P<0.01, for the GH and control group, respectively). However GH treatment did not alter (P=0.68) the phosphorylation status of eEF2 at Thr 56 (Fig. 3.5).

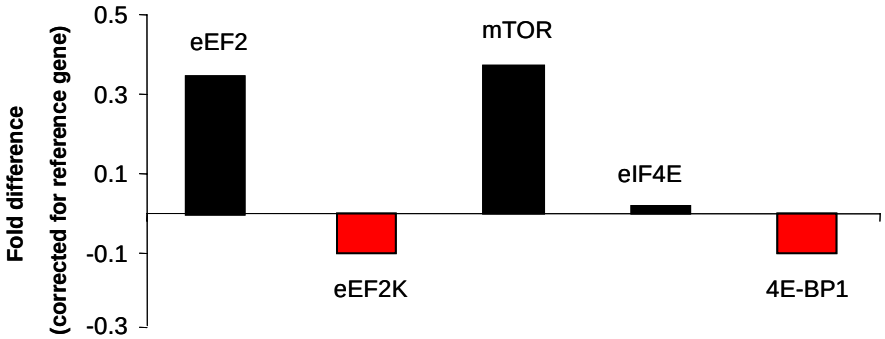

Fig. 3.1: qRT-PCR results comparing growth hormone (GH)-treated and control animals. Total mammary gland RNA from GH-treated and control animals was subjected to qRT-PCR analysis. Differential gene expressions (fold difference), obtained from the qRT-PCR of the individual selected genes (eEF2, eEF2K, mTOR, eIF4E and 4E-BP1) are presented as relative mRNA expression. Black bars show up-regulation and red bars show down-regulation. Data represent means ± SE of 4 animals per group

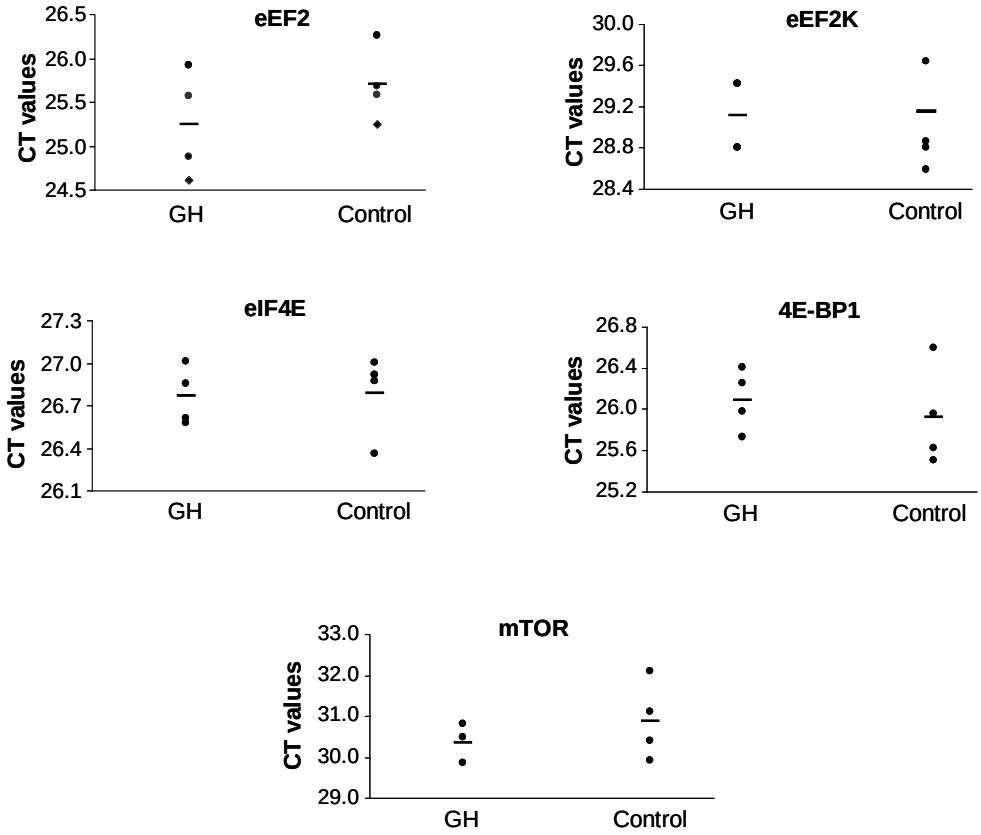

Fig. 3.2: qRT-PCR results comparing growth hormone (GH)-treated and control animals. Total mammary gland RNA from GH-treated and control animals was subjected to qRT-PCR analysis. Cycles to threshold (Ct) values for individual animals (•) separated in the two treatment groups (GH and Control). In some figures, some animals had the same Ct value so less than 4 data points (•) are visible. The horizontal line (–) indicates the average gene expression within each treatment group

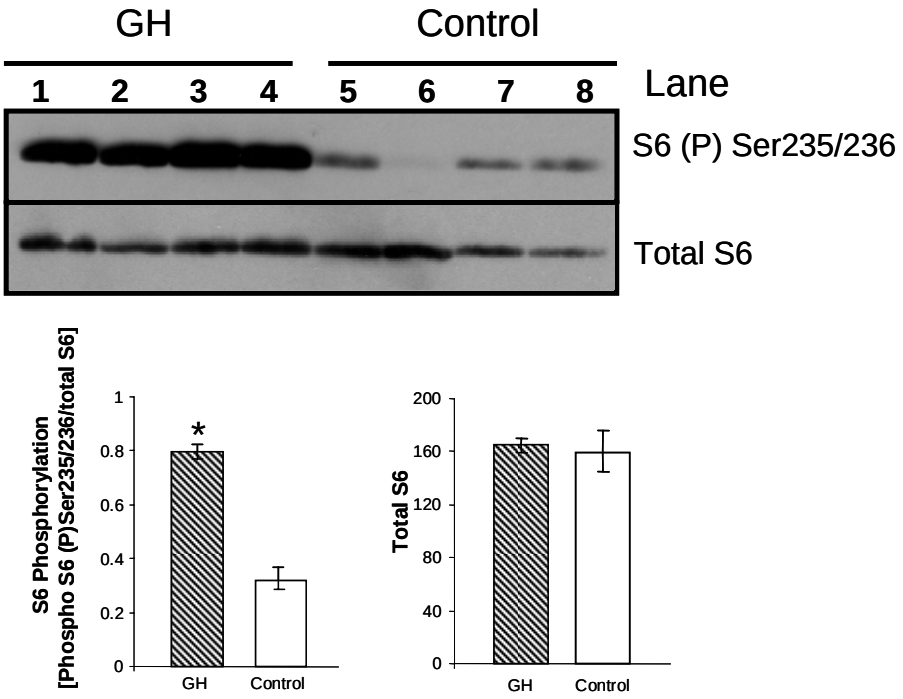

Fig. 3.3: Growth hormone (GH) treatment stimulated phosphorylation of S6 in the mammary gland. Lactating mammary gland lysates from GH-treated and control cows were analysed by SDS-PAGE and western blotting using the phospho-specific (Ser235/236) and total antisera for S6. In all cases, blots represent at least three independent experiments. Values of the phosphorylation of S6 were normalized for total S6 content in the sample. The graph shows the mean ± SE in arbitrary densitometric units, n=4/treatment

*P<0.05

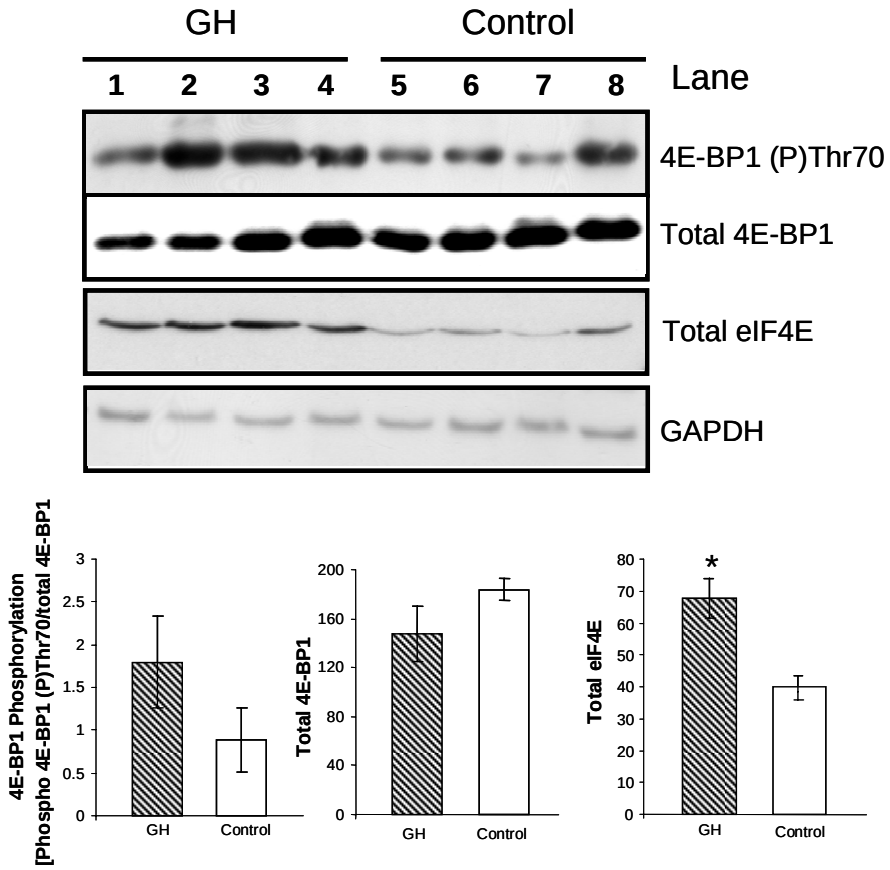

Fig. 3.4: Growth hormone (GH) treatment did not affect the abundance nor the phosphorylation of 4E-BP1 in the mammary gland. However, GH treatment increased the abundance of eIF4E in the mammary gland. Values for the phosphorylation of 4E-BP1 (Thr70) were normalized for total 4E-BP1 content in the sample. Values for total eIF4E were normalized for total GAPDH content in the sample. In all cases, blots are representative of at least three independent experiments. The graph shows the mean ± SE in arbitrary densitometric units, n=4/treatment

* $P<0.05$

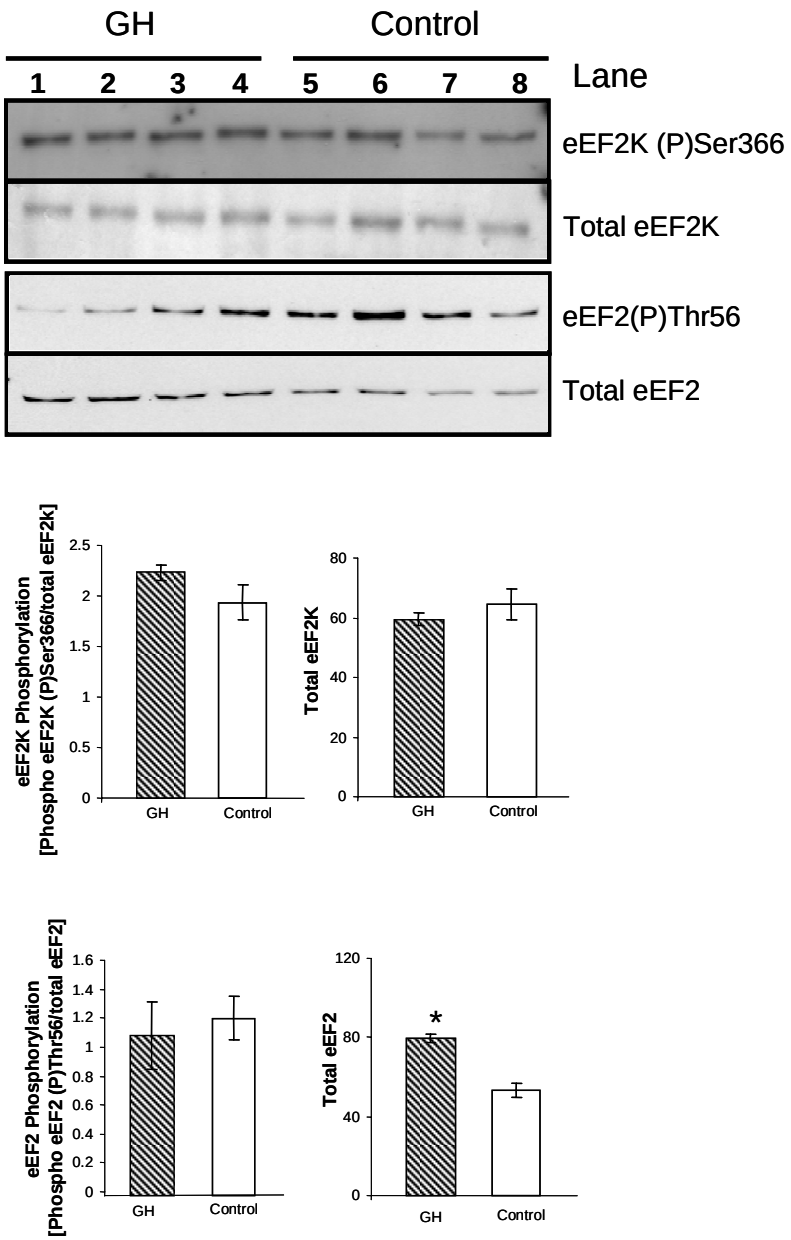

Fig. 3.5: Growth hormone (GH) treatment did not change phosphorylation status of eEF2 and eEF2K nor the abundance of eEF2K but increased total eEF2. Lactating mammary gland lysates from GH-treated and control cows were analysed by SDS-PAGE and western blotting using the phosphor-specific (Ser366) eEF2K and (Thr56) eEF2. Values for the phosphorylation of eEF2K (Ser366) and eEF2 (Thr56) were normalized for total eEF2K and eEF2, respectively. In all cases, blots represent at least three independent experiments. The graph shows the mean ± SE in arbitrary densitometric units, n=4/treatment

* $P<0.05$

3.5. Discussion

Growth hormone, also known as somatotropin, has been used in the milk industry for the past twenty five years and clearly increases the yield and efficiency of milk production (Bauman *et al*., 1985; Bauman, 1999; Akers, 2006). GH is a homeorhetic control that affects numerous target tissues in ways that are highly coordinated to affect marked changes in nutrient partitioning among these tissues (Etherton and Bauman, 1998). Milk production gradually increases over the first few days of GH treatment anreaches a maximum during the first week (Bauman *et al*., 1985). A 32% increase in milk production with a concomitant increase in protein, fat and lactose yield was observed in the lactating cows used in this experiment following six days of GH treatment. The increased milk production after administration of GH occurred without any change in feed intake when GH-treated cows were compared to control cows (Appendix A, McCoard *et al*., 2004). This is in agreement with the results of Etherton and Bauman (1998). Thus increased milk production must have been sustained by a repartitioning of nutrients to the mammary gland and/or through more efficient utilisation of nutrients taken up by the mammary gland in response to GH treatment.

Toerien and Cant (2007) have recently shown that abundance of total eukaryotic initiation factor 2 alpha (eIF2α) and the phosphorylation of S6 were increased in the mammary gland of lactating cows when compared to non-lactating animals. Their study indicated that translation initiation and possibly mTOR (as shown by increased in phosphorylation of S6) were important regulators of lactation. In the present study we evaluated the importance of S6 and other mTOR signaling proteins for milk yield during established lactation. The abundance of mRNA for 4E-BP1, eIF4E, mTOR, eEF2 and eEF2K in the mammary gland were not altered significantly by GH treatment

in this study suggesting that GH does not affect transcript levels of some members of the mTOR signaling pathway. Differences in the transcript levels of eIF4E are associated with cancer (Mamane *et al*., 2004) and a decrease in the mRNA abundance of 4E-BP1 and 4E-BP2 are associated with obesity (Mamane *et al*., 2006; Le Bacquer *et al*., 2007). However, it is possible that the number of animals used in this study were insufficient to find significance for the relatively small changes (<1.5 fold) in gene expression observed. At present, the frequency with which protein expression equates with transcript level is not clear. Chuaqui *et al*. (2002) highlighted that changes in protein expression correlated with changes in mRNA levels for less than 50% of the genes reported. Even though there was no GH treatment effect on the mRNA level of 4E-BP1, eIF4E, mTOR, eEF2 and eEF2K, this is the first study to report the mTOR gene expression in the bovine mammary gland.

The process of mRNA translation is conventionally divided into three main stages, initiation, elongation and termination. Each stage requires a number of translation factors that are regulated by intracellular signaling pathways (Proud, 2007). The advantage of translational control over transcriptional control is that it allows a rapid response with an increase/decrease in protein synthesis without a need for changes in transcription, RNA processing and export. Other studies *in vivo* (Davis *et al*., 2004; Jefferson *et al*., 2003) have reported that GH treatment affects both the initiation and elongation stages of the translational machinery. Thus, it appears that GH regulates mTOR in a manner that is independent of mRNA synthesis (transcription).

S6 is a component of the 40S ribosomal subunit and substrate for the P70S6K1. The role of phosphorylation of S6 by the P70S6K1 is not fully understood. Phosphorylation of S6 is important in regulating the size of at least some cell types, but is dispensable for

translational control of mRNAs with a 5' terminal oligopyrimidine tract (TOP mRNAs) (Pende *et al*., 2004; Ruvinsky and Meyuhas, 2006). However the data reported here are consistent with those of Hang & Rillema (1997) who reported that prolactin and GH stimulated P70S6K1 enzymatic activity in mouse mammary tissue by 98% and 40% respectively after 12 hours of culture *in vitro*. Furthermore, rapamycin treatment inhibited the prolactin stimulation of P70S6K1 activity as well as the synthesis of casein, lipids and lactose (Hang and Rillema, 1997). Thus, the current study and that of Toerien and Cant (2007) show that S6 phosphorylation could be an important control point for the regulation of milk protein synthesis in the bovine mammary gland and that GH activates phosphorylation of S6, consistent with earlier data from Hang and Rillema (1997).

4E-BP1, the best characterized of the direct substrates for mTORC1, is involved in the control of the 43S initiation complex. mTORC1 phosphorylates 4E-BP1 and the hypophosphorylated 4E-BP1 interacts with eIF4E and prevents formation of eIF4F at the 5'-cap (Gingras *et al*., 1999a). The phosphorylation of 4E-BP1 at Ser65 and Thr70 is sufficient to prevent binding to eIF4E (Niedzwiecka *et al*., 2002). Increased phosphorylation of 4E-BP1 at Thr70 following GH treatment has also been observed in muscle and liver of GH-treated pigs (Bush *et al*., 2003) and in muscle of seven day-old pigs (Suryawan *et al*., 2006). Despite this, in the present study, the phosphorylation of 4E-BP1 at Thr70 was not changed in response to GH treatment in the mammary gland and neither was the abundance of 4E-BP1.

A key component in the regulation of translation initiation is the eIF4E protein, which is the limiting component of the eIF4F initiation complex. In this study, the abundance of eIF4E in the mammary gland increased in response to GH treatment which is consistent

with the evidence that eIF4E is involved in mammary gland development and the onset of lactation (Long *et al*., 2001a/b). The increase in eIF4E in response to GH treatment should increase the formation of the eIF4F complex thus stimulating the initiation stage of translation. In accordance with the findings reported here, Long *et al*. (2001a) have shown that the levels of eIF4E were significantly higher in the lactating mammary gland compared to the mammary tissues of non-lactating animals. This group (Long *et al*., 2001b) has also demonstrated that the overexpression of eIF4E induced the proliferation of an immortalized bovine mammary epithelial cell line (Mac-T). The phosphorylation of eIF4E was not measured in this study but it warrants further investigation. However, the biological significance of phosphorylation of eIF4E is not completely clear. Minich *et al*. (1994) suggested that the phosphorylation of eIF4E enhances its binding to capped RNA. Later reports (Scheper *et al*., 2002; Slepenkov *et al*., 2006) showed that phosphorylation actually decreased its affinity for 7-methylguanosine or capped RNA. Additionally, Bush *et al*. (2003) have shown that GH-induced changes in muscle and liver protein synthesis were not regulated by changes in eIF4E phosphorylation.

We further examined the effects of GH on factors involved in the elongation process, such as eEF2. eEF2 dephosphorylation is rapidly induced by insulin and other agents (Proud, 2006). There has been only one kinase identified, eEF2K, that phosphorylates eEF2. The activity of eEF2K is controlled via the mTOR pathway as well as by other signaling pathways (Wang and Proud, 2006). There are several sites in eEF2K that are regulated by mTOR. The Ser366 residue in eEF2K is phosphorylated by the mTOR target, P70S6K1 and by the ERK-activated kinase P90RSK (Wang *et al*., 2001a). In this study, the relative phosphorylation of eEF2K as well as the total abundance of eEF2K did not change in the mammary gland. In the same way, the phosphorylation of eEF2 at Thr56 did not change in response to GH treatment but an increase in the

abundance of this protein was observed in GH-treated cows. This suggests that GH did not affect translation elongation in the mammary gland through phosphorylation, but could have affected elongation through an increase in the abundance of eEF2. In accordance with the data present here, Christophersen *et al*. (2002) have shown that eEF2 levels in mammary gland tissue are 50 times higher than the levels of eEF2 found in muscle, and that there is a close linear relation between the amount of eEF2 in the mammary gland at the end of lactation and the amount of protein and casein output in milk. The current study agrees with these results and supports the hypothesis that mammary eEF2 could be a rate limiting factor for milk protein synthesis.

It is not surprising that GH does not influence the phosphorylation of all the individual proteins downstream of mTOR. *In vitro*, the phosphorylation of 4E-BP1, eEF2 and S6 occur at different times in response to GH treatment (Chapter 5) thus small variations in sampling times during tissue collection could result in phosphorylation of some proteins and not others. Additionally, GH gradually increased milk yield over the first few days, with the maximum milk yield response occurring by about four to seven days after treatment (Appendix A) consistent with other studies (Bauman *et al*., 1985). The samples used in this experiment were collected 6 days after GH treatment. The protein synthetic machinery in the mammary gland had reached a plateau by this time as indicated by milk production parameters (Appendix A). The modification of phosphorylation is a phenomenon that allows a rapid response to external conditions, thus, phosphorylation of some key translation factors might only happen in the mammary gland in the first few days of GH treatment together with an increase in epithelial cell proliferation (Feldman *et al*., 1993). At a later stage, an increase in protein abundance (eEF2 and eIF4E) and an increase in cell size (Boutinaud *et al*., 2003), regulated by the phosphorylation of protein S6 (Ruvinsky *et al*., 2005), as was

observed in the current study, may explain the sustained increase in milk protein production observed in response to GH.

This study has shown that up-regulation of translation might be an important control point for milk protein synthesis in the mammary gland. This is the first time that S6 phosphorylation is shown to be involved in the effects of GH in the mammary gland. Future work aiming to clarify the role of the phosphorylation of S6 on the control of translation and protein synthesis in the mammary gland will enhance our understanding of the role of GH signaling in the regulation of milk protein synthesis.

CHAPTER 4

Study of the molecular mechanism(s) involved in the atropine effects on milk protein synthesis in lactating dairy cows

The material present in this Chapter has been submitted as a paper to: Domestic Animal Endocrinology

4.1. Abstract

Atropine is a muscarinic cholinergic antagonist and the administration of this drug to lactating cows decreases milk yield and protein concentration. The underlying molecular mechanisms by which this occurs are not completely understood. In this chapter, mammary tissue collected from lactating cows in which atropine was injected via subcutaneous injection (atropine group; n=4) or injected with saline (control group; n=4) were evaluated. Changes in mRNA transcript level and protein abundance and phosphorylation status of some key translation regulators were assessed in mammary gland tissues of these animals using quantitative real time PCR and western blotting. Neither the mRNA abundance (mTOR, eEF2, eEF2K, 4E-BP1, eIF4E and $P90^{RSK}$) nor the phosphorylation status (4E-BP1, eEF2K and eEF2) of the translation initiation and elongation factors studied were influenced by atropine treatment. This study suggests that the negative effects of atropine on milk protein synthesis in the mammary gland of lactating cows is not mediated by influencing the initiation and elongation phases of protein translation.

4.2. Introduction

The economic value of milk protein has continued to increase reflecting its nutritional and functional properties. Consequently, understanding how both the concentration and yield of milk protein can be manipulated is of considerable scientific and economic importance. However, milk protein concentration and yield are tightly regulated and to date only small changes have been made outside those caused by varying the plane of nutrition. Thus, increasing either protein concentration or yield in well fed cows has required substantial interventions manipulating physiological processes. As an example, the administration of insulin to lactating cows and goats was only able to increase the milk protein yield by 10 to 20% (Mackle *et al*., 1999; Bequette *et al*., 2002). In contrast, insulin administration to growing pigs can increase the protein synthesis in the muscle by up to 64% (Davis *et al*., 2002), highlighting the difficulty in manipulating milk protein synthesis. Another interpretation of this difference is the possibility that the lactating mammary gland is able to buffer its production against adverse external stimuli.

Atropine is a well known muscarinic cholinergic antagonist and it has been shown previously (Roets and Peeters, 1981; Powell and Keisler, 1995; Prosser and McLaren, 1997) that administering it decreases milk yield and protein concentration (Aaron *et al*., 1997; Prosser and McLaren, 1997; Luimes *et al*., 2002). The mechanisms through which atropine decreases milk production are not well understood. Results from Chapter 2 show that atropine affects important pathways involved in cell growth and amino acid transport. Atropine and other muscarinic cholinergic antagonists decrease the concentrations of amino acids in the plasma thus diminishing their availability to the mammary gland (Roets and Peeters, 1981; Prosser and McLaren, 1997). Others (Powell

and Keisler, 1995; Aaron *et al*., 1997) have shown that atropine can decrease circulating concentrations of growth hormone (GH), which may be expected to decrease milk production by diminishing its effectiveness through its endocrinological and metabolic roles in the maintainance of lactation. Here it is suggested that atropine and other muscarinic cholinergic antagonists may also be acting directly on the secretory cell of the mammary gland.

To determine whether atropine treatment has a direct effect on the milk secretory cells it is necessary to identify the intracellular signaling pathways that are affected in response to the atropine treatment. The signal transduction pathways that have been studied in relation to changes in cell proliferation in a number of primary cell lines, and in other tissues are the proteins, phosphoinositide 3-kinase (PI3-kinase), mitogen activated protein kinase (MAPK) and various down-stream effectors e.g. P70S6 kinase 1 (P70S6K1) (Costa *et al*., 2001). The latter is of particular interest as it provides a link between atropine and the mammalian target of rapamycin (mTOR) pathway, which is known to be important in regulating protein synthesis (Wang and Proud, 2006; Proud, 2007). The stimulation of the muscarinic cholinergic receptors with carbachol (a cholinergic angonist) activates P70S6K1 at Thr389 and this effect is blocked by rapamycin (Tang *et al*., 2003). These observations connect the muscarinic receptor to the mTOR signaling pathway and imply that there are distinct requirements for mTOR to phosphorylate and/or activate P70S6K1 and eukaryotic initiation factor 4E binding protein (4E-BP1), respectively. In Chapter 2 it is demonstrated that atropine treatment changed the mRNA abundance of a few proteins involved in the initiation and elongation phases of translation. Although there is ample evidence that atropine decreases milk protein synthesis (Aaron *et al*., 1997; Prosser and McLaren, 1997; Luimes *et al*., 2002) and that mTOR is important for the regulation of protein synthesis

(Wang and Proud, 2006; Proud, 2007) the results present in Chapter 2 are the first ones relating the effects of atropine on expression and abundance of mTOR itself and downstream targets of this pathway in the mammary gland. In particular, no previous study has tested whether the antagonist effect of atropine on the muscarinic receptors involves inhibition of mTOR signaling to protein synthesis. Such links would be logical and potentially important given the roles of both mTOR and muscarinic receptors in promoting cell growth and proliferation.

Therefore, this study was designed to test whether the detrimental effects of atropine on milk protein synthesis (Appendix A) involve changes in mTOR and in its direct downstream target, 4E-BP1. Additionally, it was of interest to study if atropine has an effect on the cap-dependent translation initiation (eukaryotic initiation factor 4E (eIF4E)) and elongation stage of protein synthesis (eukaryotic elongation factor 2 (eEF2) and eEF2 kinase (eEF2K)) as there is evidence that eIF4E and eEF2 are potentially implicated in the regulation of milk production (Long *et al*., 2001b; Christophersen *et al*., 2002). Mammary gland tissue extracts from atropine-treated and control lactating dairy cows (Appendix A) were used to address this hypothesis using qRT-PCR (qRT-PCR was also used to confirm the microarray results, Chapter 2) and western blotting analysis. Elucidation of the effects of atropine on translation initiation and elongation may open new opportunities to manipulate the milk synthetic machinery in lactating dairy cows.

4.3. Materials and methods

4.3.1. Animals and experimental design

Described in the Materials and methods of Appendix A.

4.3.2. Total RNA extraction

Described in the Materials and methods of Chapter 2.

4.3.3. Quantitative real-time polymerase chain reaction

Described in the Materials and methods of Chapter 2.

4.3.4. Primers

Described in the Materials and methods of Chapter 3, Table 3.1.

4.3.5. Protein extraction from tissues

Described in the Materials and methods of Chapter 3.

4.3.6. Determination of protein concentration

Described in the Materials and methods of Chapter 3.

4.3.7. SDS-PAGE

Described in the Materials and methods of Chapter 3.

4.3.8. Staining, fixing and drying polyacrylamide gels

Described in the Materials and methods of Chapter 3.

4.3.9. Electrotransfer

Described in the Materials and methods of Chapter 3.

4.3.10. Western blot analysis

Described in the Materials and methods of Chapter 3.

4.3.11. Primary antisera

Described in the Materials and methods of Chapter 3.

4.4. Results

4.4.1. Gene expression

In this study qRT-PCR was used to confirm the microarray results (Chapter 2) to determine if the effects of atropine on milk protein synthesis were a result of transcriptional changes of factors and proteins controlling the initiation and elongation stages of the translation. To assess the atropine effects on translation initiation via the mTOR pathway, 4E-BP1 and eIF4E mRNA levels were measured. Although the 4E-BP1 mRNA levels were increased (1.35 fold) in the mammary gland of the atropine-treated animals, consistent with the array results in Chapter 2 (1.3 fold up-regulated), this change did not attain significance (P=0.16; Fig. 4.1). Similarly, the 1.3 fold increase in the mRNA level of eIF4E in the mammary gland of atropine–treated animals was not significant (P=0.58; Fig. 4.1) confirming the array results observed in Chapter 2.

The effect of atropine treatment over the elongation stage of translation was also studied. The qRT-PCR results showed that atropine treatment increased the expression of eEF2 (1.25 fold) and its regulatory kinase, eEF2K (1.46 fold), consistent with the array results in Chapter 2 (1.4 fold up-regulated), but these differences also did not attain statistical significance (P=0.30 and P=0.20, respectively; Fig. 4.1).

mTOR can be controlled through PI3-kinase (Manning *et al*., 2002; Manning and Cantley, 2003a) or through ERK signaling (also known as MAPK) (Wang and Proud, 2002a; Ma *et al*., 2005). Knowing that muscarinic receptors can signal through the ERK signaling (Matthiesen *et al*., 2007) the effect of atropine on p90 ribosomal S6 kinase ($P90^{RSK}$), a member of the ERK signaling pathway was studied. According to the microarray data, the $P90^{RSK}$ was down-regulated in response to the atropine treatment

(Chapter 2). The qRT-PCR results show that atropine decreased the mRNA abundance of the $P90^{RSK}$ but this difference was not statistically different (1.25 fold decrease, P=0.60) (Fig. 4.1). Similarly, there was no change in expression of the mTOR gene (1.1 fold decrease; P=0.90) in the mammary gland of atropine treated cows (Fig. 4.1). Variation in the transcript level between animals subjected to the same treatment can be seen on Fig. 4.2.

It was also important to assess if atropine could be affecting the milk protein synthesis machinery in a manner that requires other mechanisms that are not transcriptional controlled. One mechanism could be the regulation of the post-translational modifications (e.g. phosphorylation) as it has been reported before that these translation factors evaluated are potentially regulated by phosphorylation (Kapp and Lorsch, 2004). Thus, the effects of atropine over the phosphorylation status of initiation factors and elongation factors were evaluated.

4.4.2. Effect of atropine on translation initiation

To determine the post-translational modification effects of atropine on the initiation stage of protein synthesis, the abundance of 4E-BP1 and eIF4E protein and the phosphorylation state of 4E-BP1 at Thr70 were analysed by immunoblotting. Mammary tissue lysates were used to evaluate changes in the total abundance and phosphorylation status of 4E-BP1 and total abundance of eIF4E protein. The hypophosphorylated 4E-BP1 protein negatively regulates eIF4F assembly by sequestering its mRNA cap binding component eIF4E, whereas hyperphosphorylation abrogates this function (Raught and Gingras, 1999). Atropine treatment changed neither the relative

phosphorylation of 4E-BP1 (ratio phospho-4E-BP1 (Thr70): total 4E-BP1; P= 0.90) nor the total abundance of 4E-BP1 in mammary tissue lysates (P=0.30) (Fig. 4.3). The effect of atropine on the abundance of eIF4E protein was also evaluated. The binding of 4E-BP1 to eIF4E prevents eIF4E from binding eukaryotic initiation factor 4G (eIF4G) to form complexes that are competent for cap-dependent mRNA translation. Atropine increased the abundance of eIF4E protein (P=0.01).

4.4.3. Effect of atropine on translation elongation

To determine the effects of atropine on the elongation stage of protein synthesis, the abundance of eEF2 and eEF2K protein and the phosphorylation state of eEF2 at Thr56 and eEF2K at Ser366 were analysed by immunoblotting. Atropine treatment changed neither the relative phosphorylation of eEF2K (ratio phospho eEF2K (Ser366): total eEF2K; P= 0.20) nor the total abundance of eEF2K in mammary tissue lysates (P=0.70) (Fig. 4.4). Similarly, atropine treatment did not change the relative phosphorylation of eEF2 (ratio phospho eEF2 (Thr56): total eEF2; P= 0.50) nor the total abundance of eEF2 in mammary tissue lysates (P=0.90) (Fig. 4.4).

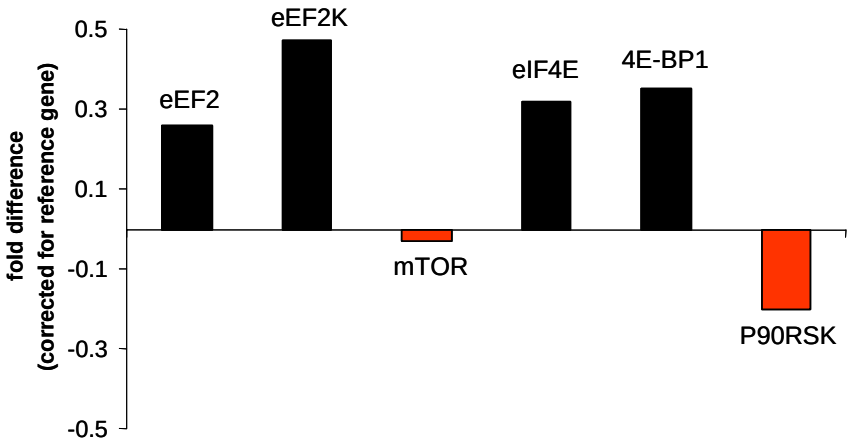

Fig. 4.1: Total mammary gland RNA from atropine-treated and control animals was subjected to qRT-PCR analysis. Differential gene expression (fold difference) of the individual selected genes (eEF2, eEF2K, mTOR, 4E-BP1, eIF4E and $P90^{RSK}$) presented as relative mRNA expression using glyceraldehyde-3-phosphate acyltransferase, mitochondrial (GPAM) and K15 intermediate filament type I keratin (KRT15) as reference genes. Black bars show up-regulation and red bars show down-regulation. Data represent means ± SE of n=4 animals per group

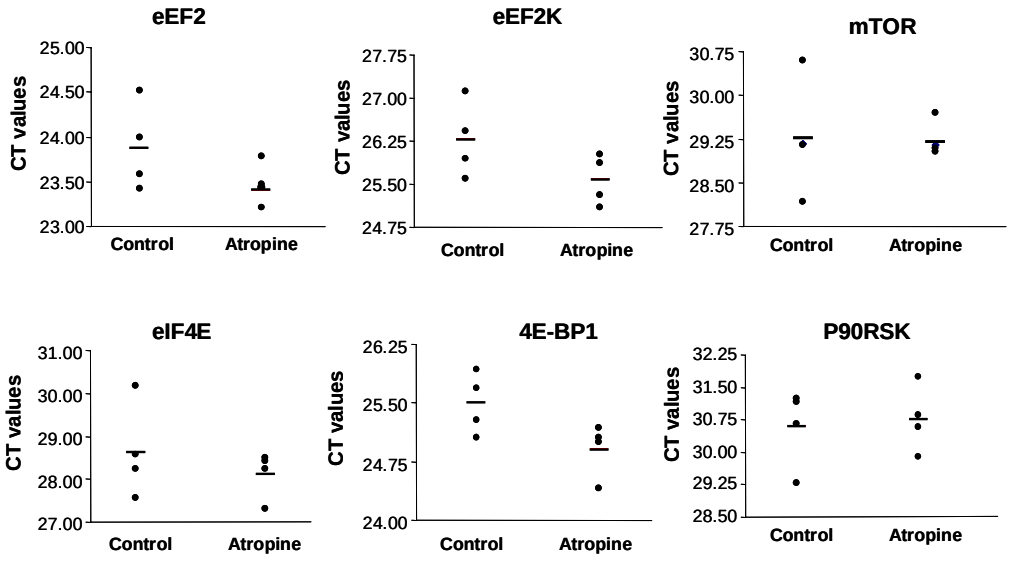

Fig. 4.2: Total mammary gland RNA from atropine-treated and control animals was subjected to qRT-PCR analysis. Cycles to threshold (Ct) values for individual animals (•) separated in the two treatment groups (GH and control). In some figures, some animals had the same Ct value so less than 4 data points (•) are visible. The horizontal line (–) indicates the average gene expression within each treatment group

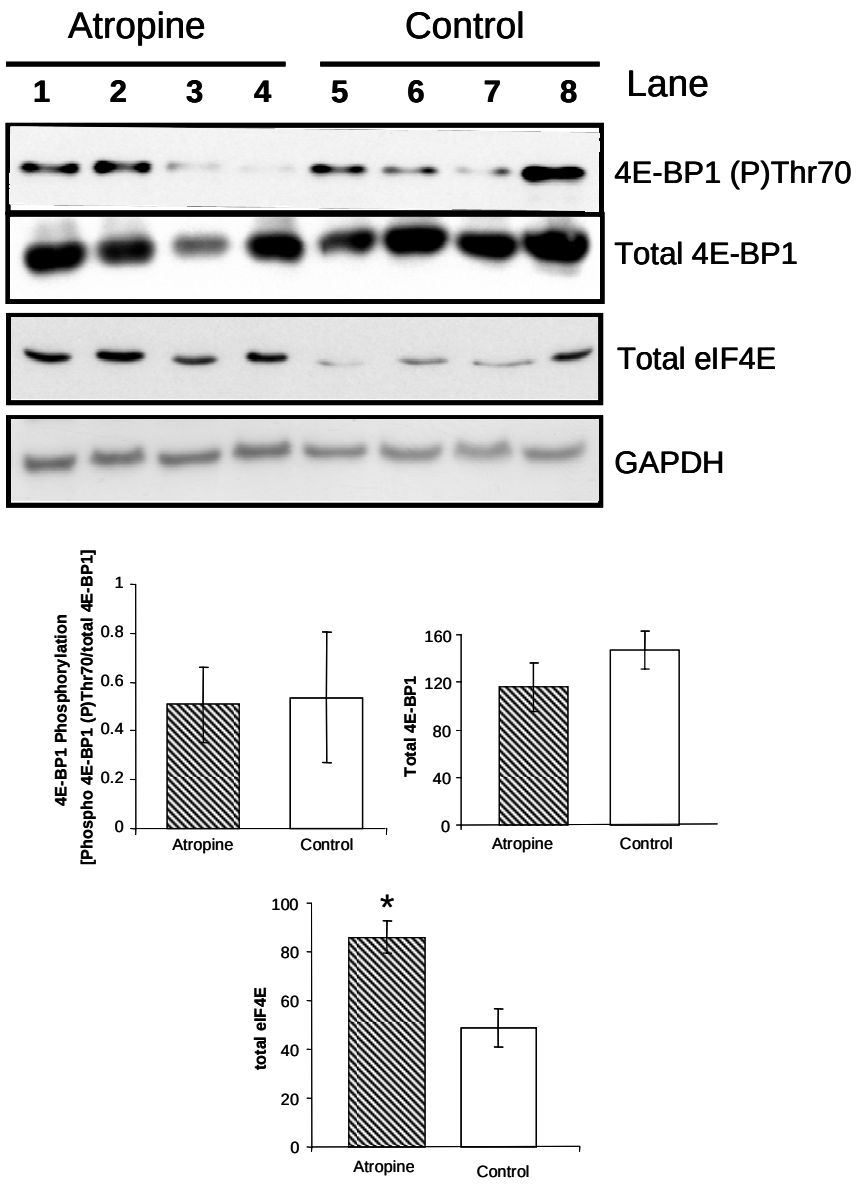

Fig. 4.3: Atropine treatment affects total eIF4E but does not affect the phosphorylation of 4E-BP1. Lactating mammary gland lysates from atropine-treated and control animals were analysed by SDS-PAGE and western blotting using phospho-specific 4E-BP1 (Thr70), total 4E-BP1 and total eIF4E. Values of the phosphorylation of 4E-BP1 (Thr70) were normalized for total 4E-BP1 content in the sample. Values for total eIF4E were normalized for total glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amount in the sample. In all cases, blots are representative of at least three independent experiments. Graph shows the mean ± SE in arbitrary densitometric units, n=4/treatment

*P<0.05

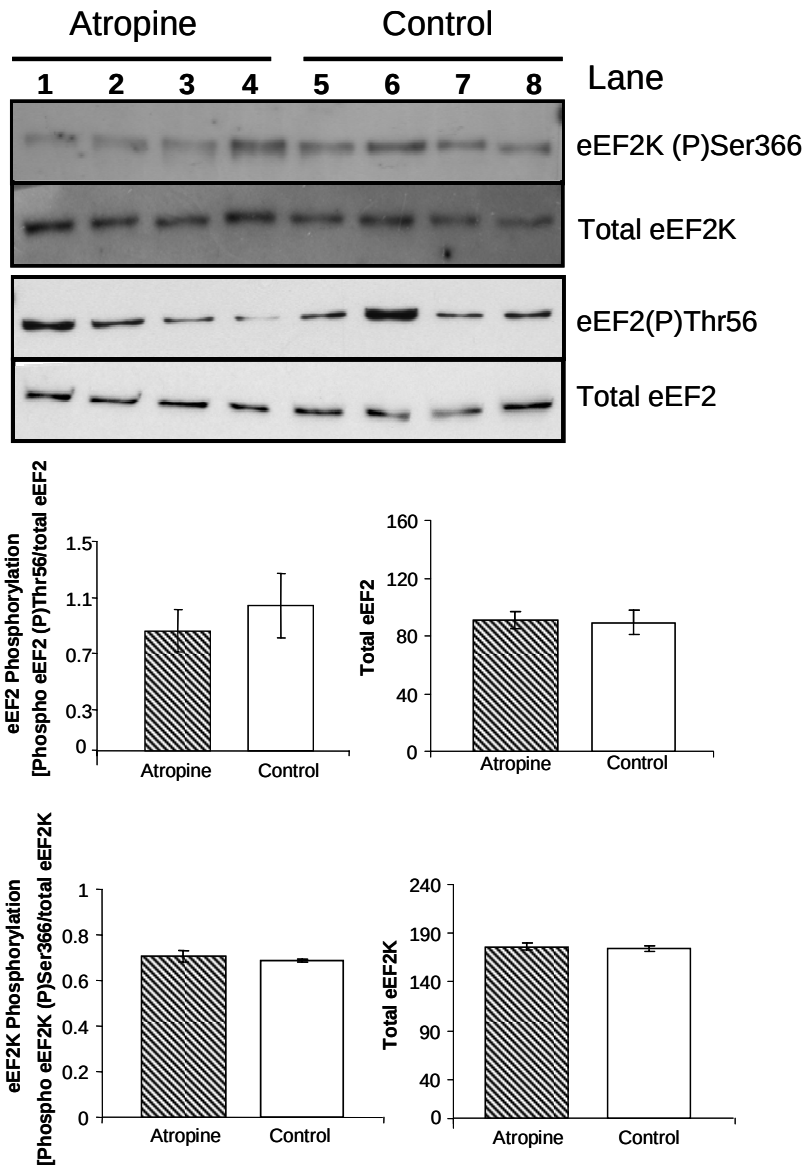

Fig. 4.4: Atropine treatment does not affect the phosphorylation status of eEF2 and eEF2K nor the total amount od these proteins. Lactating mammary gland lysates from atropine-treated and control animals were analysed by SDS-PAGE and western blotting using the phospho-specific eEF2K (Ser366) and eEF2 (Thr56). Values of the phosphorylation of eEF2K (Ser366) and eEF2 (Thr56) were normalized for total eEF2K and eEF2, respectively. In all cases, blots are representative of at least three independent experiments. Graph shows the mean ± SE in arbitrary densitometric units, n=4/treatment

4.5. Discussion

To examine the involvement of translation initiation and elongation factors in the detrimental effects of atropine on milk protein synthesis, mammary gland from atropine-treated and control lactating cows were used in this experiment. This study shows that atropine did not affect the mRNA abundance (transcript) of eEF2, eEF2K, 4E-BP1, eIF4E and mTOR suggesting that mRNA changes in translation initiation and elongation are unlikely to explain the 21% depression of milk protein percentage observed previously (Appendix A). Similar effects of treatment of lactating animals with muscarinic cholinergic antagonists on milk production have been previously observed in ewes (Powell and Keisler, 1995; Aaron *et al*., 1997) and in cows (Luimes *et al*., 2002). One possible reason for this observation is that the genetic variation among the animals used in this experiment (Fig. 4.2) was too large to detect statistically a change in gene expression of less than 1.5 fold. Alternatively, the small difference in expression observed in this study might not be biologically relevant or atropine may not inhibit molecular signaling through the translation initiation and elongation at the transcript level.

The lack of effect of atropine on the phosphorylation of 4E-BP1, which decreases the amount of the inactive 4E-BP1-eIF4E initiation complex (Gingras *et al*., 2001), does not support the present hypothesis that atropine affects the translation initiation step by decreasing phosphorylation of 4E-BP1. 4E-BP1 is a key component in the regulation of translation initiation and binds to the eIF4E protein when hypo-phosphorylated, which is the limiting component of the eIF4F initiation complex. In the current study, the abundance of eIF4E in the mammary gland increased in response to atropine treatment which was unexpected. Previous reports indicate that the concentration of eIF4E in

mammary tissue of lactating cows is considerably greater than that of non-lactating cows (Long *et al*., 2001a; Toerien and Cant, 2007). Long *et al*. (2001b) have also demonstrated that the over-expression of the eIF4E induces the proliferation of the immortalized bovine mammary epithelial cell line (Mac-T). These observations suggest that eIF4E is a positive regulator of protein synthesis in the mammary gland. The increase in the concentration of eIF4E in response to atropine reported here is not consistent with this function, indicating that the negative effect of atropine on milk protein synthesis is not mediated by changes in translation initiation via eIF4E. One possible explanation for the up regulation of eIF4E in response to atropine in this study is that while eIF4E may play a key role in the transition between a lactating and non-lactating state, it may not be a key control point for modulation of milk protein synthesis once lactation is established. Rather, eIF4E may influence the proliferative activity of mammary tissue, however, alternate functional roles for eIF4E require further investigation.

In this study, the effect of atropine over translation elongation was also investigated. The abundance and the phosphorylation status of eEF2 and its kinase (eEF2K), which is controlled via the mTOR pathway as well as by other signaling pathways, was not changed in the atropine treated cows suggesting that atropine may not influence the elongation step of translation. During lactation, the protein synthetic rates in the mammary gland are very high to maintain milk production with increased loading of ribosomes onto mRNAs. The effects of the atropine treatment on milk yield are very rapid, starting within four hours of treatment and quickly recovering following the withdrawal of treatment as observed in the current study (McCoard *et al*., 2003). Thus, an effect of atropine over translation elongation would ensure that polysomes are retained, allowing the translation to be resumed rapidly when atropine treatment is

stopped. The data presented here contradict this hypothesis and show that the atropine treatment does not appear to change translation elongation.

Different authors (Powell and Keisler, 1995; Aaron *et al*., 1997) have attributed the effect of atropine treatment to decreased circulating concentrations of GH. The results present in this study and those presented in Chapter 3 and by Luimes *et al*. (2002) do not support this hypothesis. Luimes *et al*. (2002) have shown that in atropine-treated cows, GH infusions do not rescue milk yield to control levels. Additionally, GH effects on protein synthesis are mediated through changes in mTOR signaling pathway (Chapter 3 and Hayashi and Proud, 2007) and changes on the phosphorylation status of 4E-BP1 (a direct downstream of mTOR) were not observed in this experiment. Thus, it is suggested that the effects of atropine on the mammary gland were not mediated through changes in GH signaling. However, an effect through ribosomal protein S6 (S6) can not be discounted. Because the activation (phosphorylation/ dephosphorylation) of 4E-BP1 and S6 happens at different times following activation or inhibition (Wang *et al*., 2000; Hayashi and Proud, 2007), it is possible that the time when the mammary gland tissues were collected did not coincide with the times of the dephosphorylation/ phosphorylation of 4E-BP1. Thus, future work needs to be done to evaluate if the S6 phosphorylation changes in response to atropine. Additionally, experiments using an *in vitro* model would assist in addressing this question and to help elucidate the molecular pathways mediating the effects of atropine on milk protein synthesis.

In conclusion, this study indicates that the negative effects of atropine on milk protein synthesis in the mammary gland of lactating cows are unlikely to be mediated by influencing the initiation and elongation phases of protein translation via the mTOR

pathway. An alternative mode of action for atropine may be to inhibit the transport of milk proteins from the secretory cells into the alveoli whereby subsequent accumulation of milk protein in the cell decreases further synthesis of protein despite the up-regulation of eIF4E. Another mode of action of atropine could be changing the amino acid use inside the secretory cell. In accordance with that, although atropine decreases the concentrations of amino acids in the plasma (Roets and Peeters, 1981; Prosser and McLaren, 1997) the milk yield in atropine-treated cows does not return to control levels after amino acid infusions (Luimes *et al*., 2002). Thus, amino acid availability in the blood does not appear to be the mechanism by which atropine decreases milk yield but an alteration in the use of these amino acids inside the secretory cells might be the mechanism by which atropine affects milk production. Thus, future work to elucidate if atropine affects the use of amino acids inside the cell is warranted.

CHAPTER 5

The rapid activation of protein synthesis by GH requires signaling through the mammalian target of rapamycin

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

Hayashi,A.A. and Proud,C.G. (2007). The rapid activation of protein synthesis by growth hormone requires signaling through the mammalian target of rapamycin, mTOR. *Am. J. Physiol. Endocrinol. Metab.* 292(6): E1647-55.

5.1. Abstract

An important function of growth hormone (GH) is to promote cell and tissue growth, and a key component of these effects is the stimulation of protein synthesis. In this study, it is demonstrated that, in H4IIE rat hepatoma cells, GH acutely activated protein synthesis through signaling via mammalian target of rapamycin (mTOR), and specifically through the rapamycin-sensitive mTORC complex 1 (mTORC1). GH treatment enhanced the phosphorylation of two targets of mTOR signaling, eukaryotic initiation factor 4E binding protein (4E-BP1) and ribosomal protein S6 (S6). Phosphorylation of 4E-BP1 and S6 was maximal at 10-20 min and 30-45 min after GH stimulation, respectively. Both proteins modulate components of the translational machinery. The GH-induced phosphorylation of 4E-BP1 led to its dissociation from eukaryotic initiation factor 4E (eIF4E) and increased binding of eIF4E to eukaryotic initiation factor 4G (eIF4G) to form (active) eukaryotic initiation factor 4F (eIF4F) complexes. The stimulation by GH of phosphorylation of S6 and 4E-BP1 was blocked by rapamycin. GH treatment also dephosphorylated a third translational component linked to mTORC1, the elongation factor 2 (eEF2). Its regulation followed complex biphasic kinetics, both phases of which required mTOR signaling. GH rapidly activated both the mitogen activated protein kinase (MAPK also known as ERK) and phosphoinositide 3-kinase (PI 3-kinase) pathways. Signaling through PI 3-kinase, alone, was however, sufficient to activate the downstream mTORC1 pathway.

Consistent with this, GH increased the phosphorylation of tuberous sclerosis 2 (TSC2), an upstream regulator of mTORC1, at sites that are targets for protein kinase B (PKB also known as Akt). Finally, the activation of overall protein synthesis by GH in H4IIE hepatoma cells was essentially completely inhibited by wortmannin or rapamycin.

These results demonstrated for the first time that mTORC1 plays a major role in the rapid activation of protein synthesis by GH.

5.2. Introduction

Growth hormone is secreted into the circulation by the anterior pituitary gland, and subsequently binds to membrane receptors in target tissues to stimulate tissue growth (Rowland *et al*., 2005) and elicit changes in protein, carbohydrate and fat metabolism (Carter-Su and Smit, 1998).

Although it plays a key role in controlling growth, details of the mechanism(s) by which GH stimulates protein synthesis remain largely unknown. The rate at which protein synthesis occurs depends in the short term upon the efficiency of the translation process. In the longer term (hours – days) its capacity can also be increased. The protein synthesis process is divided into three main stages: initiation, elongation and termination. Each stage requires a number of translation factors, each controlled through alterations in their phosphorylation status. Several upstream signals, including hormones, growth factors and nutrients activate protein synthesis. In a number of cases, it has been shown that this activation is at least partially inhibited by rapamycin (Wang and Proud, 2006), implying that signaling through mTOR is required for this. mTOR forms two types of complex, mTORC1 and mTORC2, of which mTORC1 is sensitive to rapamycin (Wullschleger *et al*., 2006). Sensitivity to rapamycin thus implies a role specifically for mTORC1.

mTOR controls at least three types of components of the translational machinery (Wang and Proud, 2006). The ribosomal protein S6 kinases (S6Ks) are activated by insulin and other agents, and phosphorylate S6 , a component of the 40S ribosomal subunit complex (Avruch *et al*., 2001). However, despite extensive study over many years, the function of S6 phosphorylation remains obscure (Ruvinsky and Meyuhas, 2006). A second

example of an mTOR target is typified by 4E-BP1 which binds to and inhibits the mRNA cap-binding protein, eIF4E, thereby blocking the formation of active initiation factor complexes containing the scaffold protein eIF4G (Gingras *et al*., 1999b). Activation of mTOR signaling leads to the phosphorylation of 4E-BP1 and to its release from eIF4E, which is thus free to associate with eIF4G. Thirdly, mTOR controls the activity of elongation factor 2 (eEF2) kinase (eEF2K), such that activation of mTOR leads to inhibition of eEF2K, and the dephosphorylation and activation of eEF2 (Browne and Proud, 2002). Thus, mTOR controls both the initiation and elongation steps of translation. Their regulation is sensitive to rapamycin, which inhibits certain functions of mTOR that are mediated through mTOR complex 1 (mTORC1) (Wullschleger *et al*., 2006).

There is now considerable evidence that mTORC1 plays an important role in the control of cell size (Mayer and Grummt, 2006; Wullschleger *et al*., 2006). However, it is not fully clear how cell size is regulated downstream of mTORC1. One candidate for this regulation is S6K. Knock-out studies of this kinase in *Drosophila* and of the S6 kinase 1 gene in mice gave rise to a phenotype characterized by small animal size (Shima *et al*., 1998; Montagne *et al*., 1999). Other data suggest a role for another mTORC1 target, 4E-BP1 in regulating cell size (Fingar *et al*., 2002). In skeletal muscle, recent studies have implied that the mTORC1 pathway may play an important role in skeletal muscle hypertrophy (Bodine *et al*., 2001; Nader *et al*., 2005). Agents such as insulin activate mTORC1 through signaling via PI 3-kinase and PKB (Manning and Cantley, 2003a/b). PKB phosphorylates and apparently inactivates the tuberous sclerosis complex (TSC). This consists of two proteins, TSC1 and TSC2. TSC2 acts as the GTPase activator protein for a small G-protein, Ras homolog enriched brain (Rheb), which is a positive regulator of mTORC1. PKB directly phosphorylates TSC2. An alternative pathway by

which mTORC1 can be activated involves signaling via the ERK (extracellular ligand-regulated kinase) pathway. This again appears to be mediated through phosphorylation of TSC2, but it is unclear whether this is catalysed directly by ERK (Ma *et al*., 2005) or by its downstream effector p90 ribosomal S6 kinase ($p90^{RSK}$) (Roux *et al*., 2004; Rolfe *et al*., 2005).

The binding of GH to the GH receptor increases the affinity of Janus kinase 2 (JAK2) for the GH receptor and activates JAK2 (Argetsinger *et al*., 1993). The phosphorylation of tyrosines within JAK2 and the GH receptor form high-affinity binding sites for a variety of signaling proteins containing Src homology (SH)2 and other phosphotyrosine-binding domains. Recruitment of these signaling molecules to GH receptor/JAK2 complexes activates signaling pathways including the signal transducers and activators of transcription (STAT) (Herrington *et al*., 2000), the MAP kinase (ERK) transduction pathways (Vanderkuur *et al*., 1997) and events that involve insulin receptor substrate (IRS) proteins (Souza *et al*., 1994; Carter-Su *et al*., 1996; Costoya *et al*., 1999).

It has been shown in Chapter 3 that GH increases the phosphorylation of S6 in lactating cows treated with GH. Although there is ample evidence that GH activates protein synthesis (Fryburg *et al*., 1991; Bush *et al*., 2003; Davis *et al*., 2004), and it is clear that mTOR is important for the regulation of protein synthesis (Wang and Proud, 2006; Wullschleger *et al*., 2006), there are no published studies relating the effects of GH to signaling through the mTOR pathway. In particular, it has not previously been tested whether the activation of protein synthesis by GH involves mTORC1, a key regulator of cell growth (Wullschleger *et al*., 2006). Given the roles of both mTOR and GH in promoting growth, such links would be logical and potentially important. The aims of

this study were therefore to determine whether GH activates mTORC1 signaling, how it does this, and the importance of mTORC1 signaling for the activation of protein synthesis by GH.

Here, it is demonstrated that GH activates 4E-BP1 and other targets for mTORC1 signaling in hepatoma cells. Importantly, it is shown for the first time that the short-term activation of protein synthesis by GH is dependent upon mTORC1 signaling.

5.3. Materials and methods

5.3.1. Cell culture (H4IIE)

H4IIE hepatoma rat cells (ATCC, Manassas, VA, USA) were used in the experiments described in this Chapter because they respond to GH stimuli due to the presence of GH receptor. They were maintained in Dubelcco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, New York, USA) supplemented with 25% (v/v) horse serum, 5% (v/v) fetal calf serum, 100 units/mL penicillin and 0.1 mg/mL streptomycin (complete DMEM) and cultured in 10 cm dishes in a 5% CO_2 incubator at 37^{o}C. Cells were grown until 90% confluent and then split every 2-3 days. To split the cells, the culture medium was aspirated, each flask washed first with 10 mL phosphate buffered saline (PBS) which was discarded, and 1 mL of trypsin (Invitrogen, Grand Island, New York, USA) was added. The dishes were returned to the incubator for 5 min. After the cells detached they were resuspended in 5 mL of 'complete DMEM'. 1 mL of a cell suspension was diluted with 9 mL of 'complete DMEM' and used to seed a 10 cm diameter dish either to maintain the stocks or for experiments.

5.3.2. Cell culture and treatment

To nutrient starve the cells, at 50% confluence cells were removed from serum and maintained in serum free medium for 30 hours prior to the start of the experiment treatments. In a number of experiments, specific signaling inhibitors were employed. PD098059 was used to inhibit the ERK pathway, which it does by blocking the activation of the upstream kinase MEK1 (ERK activator kinase) (Alessi *et al.*, 1995).

Wortmannin (Arcaro and Wymann, 1993) was employed as specific inhibitor of PI 3-kinase, and rapamycin was used to inhibit mTORC1 (Sarbassov *et al*., 2005). Cells were pre-treated with these agents before exposure to GH, using the following concentrations and times, which are based upon earlier work in this laboratory (Wang and Proud, 2002a/b; Wang *et al*., 2005): 10μM PD098059 for 30 min; 100nM wortmannin for 30 min; and 50nM rapamycin for 30 min. These concentrations used were chosen because other studies have shown that they completely inhibit the relevant target, whilst being unlikely to interfere with other processes (Wang and Proud, 2002a/b; Wang *et al*., 2005).

As described in greater detail in the results section, it was first necessary to define appropriate conditions for treatment of hepatoma H4IIE cells with GH, and then to explore the time courses over which components of signaling pathways and the translational machinery were regulated. Subsequently, specific signaling inhibitors to examine the signaling events involved in the control of the translational machinery by GH were employed. The design of later studies was, in many cases, prompted by data from initial experiments.

5.3.3. Protein extraction from H4IIE hepatoma cells

For western blotting analysis and protein synthesis assays, cells were washed once with PBS (2 mM KH_2PO_4, pH 7.4, 10 mM Na_2HPO_4, 4 mM KCl, 170 mM NaCl) and harvested in 400 μL of cell extraction buffer (50 mM β-glycerophosphate, pH 7.5; 1 mM EGTA; 1 mM EDTA; 1% (v/v) Triton X-100; 1 mM Na_3VO_4; 100 nM microcystin-LR; 0.1% (v/v) β-mercaptoethanol; protease inhibitors (leupeptin, pepstatin

and antipain, each 1 μg/mL) and phenylmethylsulfonyl fluoride (200 μM)). Lysates were centrifuged at 13,000*g* for 10 min to remove debris. Protein concentrations in the resulting supernatants were determined as described by Bradford (1976) (section 5.3.4). Aliquots of lysate containing equal amounts of protein were used for western blots and for protein synthesis measurements.

5.3.4. Determination of protein concentration

Described in the Materials and methods of Chapter 3.

5.3.5. m^7GTP-Sepharose Chromatography

eIF4E and its associated proteins, e.g., eIF4G and 4E-BP1, were purified by affinity chromatography on m^7GTP Sepharose (Amersham Life Sciences Ltd, Piscataway, NJ, USA). 200 μg of protein from cell lysates and 10 μL of a 2:1 slurry of m7GTP-Sepharose/ Sepharose CL4B were incubated by rotation at 4^{o}C for 1 hour. The eIF4E-m^7GTP-Sepharose complexes were washed three times with cell extraction buffer and finally the beads were resuspended in 30 μL of 1X SDS-PAGE sample buffer (5X SDS-PAGE Sample Buffer; 62.5 mM Tris-HCl pH 6.8, 7% (w/v) SDS, 20% (w/v) sucrose, 0.01% (w/v) bromophenyl blue, 2 mM 2-mercaptoethanol). Samples were subjected to electrophoresis and western blotting for total eIF4G, eIF4E and 4E-BP1.

5.3.6. SDS-PAGE

Described in the Materials and methods of Chapter 3.

5.3.7 Staining, fixing and drying polyacrylamide gels

Described in the Materials and methods of Chapter 3.

5.3.8 Electrotransfer

Described in the Materials and methods of Chapter 3.

5.3.9. Western blot analysis

Described in the Materials and methods of Chapter 3.

5.3.10. Primary antisera

Described in the Materials and methods of Chapter 3.

5.3.11. Production and purification of polyclonal antibodies

Peptides for raising home made antibodies (total eEF2, phospho-eEF2 (Thr56), GST; Table 3.2) were synthesised by Graham Bloomberg (Department of Biochemistry, University of Bristol, U.K.), coupled to keyhole limpet haemocyanin (KLH) using gluteraldehyde. Briefly 500 µL of 10 mg/mL peptide and 300 µL of 33 mg/mL KLH were made up to 1 mL with 200 µL of 0.4 M sodium phosphate pH 7.5. 10X 50 µL aliquots of 20 mM sodium gluteraldehyde were added slowly at room temperature, to the peptide mixture causing the solutions to turn yellow. Unreacted gluteraldehyde was

blocked by adding 150 µL 1 M Tris pH 7.5, mixing well and leaving at room temperature for 1 hour. The KLH conjugated peptides were then dialysed overnight at 4°C against 20 mM sodium phosphate pH 7.5, 150 mM NaCl.

The dialysed KLH coupled conjugates were then pooled together and six 500 µL aliquots were sent to Diagnostics Ltd (Edinburgh, U.K.), where they were injected into rabbits at monthly intervals. Anti-peptide antibodies were purified from antiserum by affinity chromatography against the appropriate peptide-CH-Sepharose-4B.

5.3.12. Protein synthesis measurement

Cells were starved for 30 hours and pre-incubated with fresh serum-free DMEM for 1 hour prior to the start of the treatments. Cells were then incubated, with or without signaling inhibitors, for 30 min. Treated cells were stimulated with GH (500ng/mL) for a further 30 min before the addition of [^{35}S]-methionine (5 µCi/6cm plate) for a further 1 hour. Cells were washed 3 times with cold PBS and were lysed with extraction buffer. Protein was spotted on the 3MM filter paper (Whatman, London, UK) before precipitation with 5% (w/v) trichloroacetic acid (TCA) and measurement of incorporated radiolabel by scintillation counting. All data comparisons were analysed for statistical significance using Anova. Differences were considered significant at a P value of < 0.05.

5.4. Results

5.4.1. GH regulates multiple effectors of mTOR signaling

To study whether GH activates mTOR signaling in H4IIE hepatoma cells, it was first necessary to define conditions under which basal mTOR signaling was low. The cells were therefore starved of serum for a range of times from 6 hours to 30 hours, and the cell lysates subjected to analysis by SDS-PAGE and western blotting for multiple targets for mTOR signaling. As shown in Fig. 5.1, the phosphorylation of both S6 (at Ser235/236) and 4E-BP1 (at Ser65) was decreased as early as 6 hours after serum withdrawal and continued to decline up to 30 hours. The drop in phosphorylation of Ser65 in 4E-BP1 (Fig. 5.1) is likely the cause of the shift from the most highly phosphorylated γ-species to the faster-migrating β-form, which is not phosphorylated at this site. The increase in the signal for total 4E-BP1 suggests its expression is enhanced during serum starvation, and taking this into account, the fall in the relative level of Ser65 phosphorylation is even more pronounced than the change in the signal for the Ser65 phospho-specific antibody alone would suggest.

It was therefore elected to use cells that had been starved of serum for 30 hours in all subsequent experiments. Such cells were treated for various times with GH and cell lysates were again analysed for targets of mTOR signaling, and for potential upstream regulators of mTOR such as Akt/PKB and ERK. The phosphorylation of S6 was markedly increased by GH, although it raised rather slowly, only small increases being observed up to about 20 min after GH treatment. S6 phosphorylation then increased substantially, being maximal by 30-45 min, and falling again thereafter (Fig. 5.2). Some

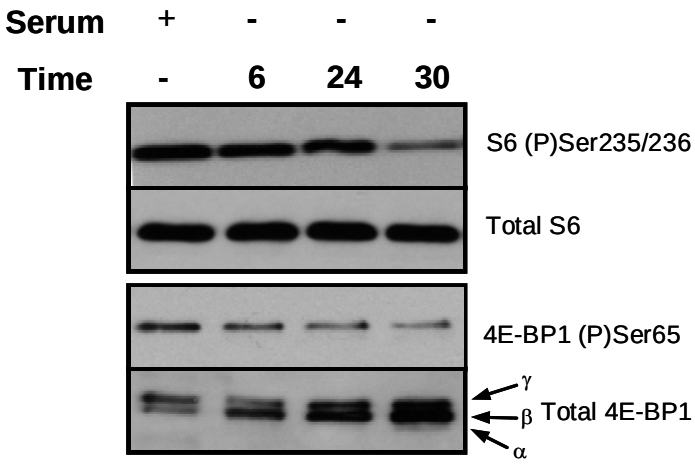

Fig. 5.1: Effects of serum starvation of H4IIE cells on targets of the mTOR pathway. H4IIE cells were grown in medium lacking serum for the times indicated (hours) to define the time point at which phosphorylation of 4E-BP1 (P)Ser65 and ribosomal protein S6 (P)Ser235/236 was decreased. Cells grown in medium with serum were used as a control. For 4E-BP1, labeled arrows indicate the differentially phosphorylated α, β and γ species. Total S6 was used as the 'loading control'

minor variation between the kinetics of these responses was seen across the set of experiments. Therefore, typical data are presented.

GH also enhanced the phosphorylation of a second target for mTOR signaling, 4E-BP1. Its phosphorylation at Ser65 was seen by 10 min (Fig. 5.2), was maximal at 20 min, and fell towards basal levels again by 45 min. The increase in 4E-BP1 phosphorylation is also demonstrated by its shift in mobility on SDS-PAGE from mainly running as the partly phosphorylated β-species in untreated cells to almost entirely as the hyperphosphorylated γ-form after GH treatment (Fig. 5.2). The γ-form is the only one of the three forms resolved on SDS-PAGE that is phosphorylated at Ser65, and it does not bind to eIF4E (Wang *et al*., 2003). GH also caused an initial rapid decrease in the phosphorylation of eEF2 at Thr56 which had returned to basal levels by 20 min only to fall again by 30-45 min, before rising once more by 2 hours (Fig. 5.2). Thus, the time courses for the phosphorylation of S6, eEF2 and 4E-BP1 differ markedly.

The binding of 4E-BP1 to eIF4E prevents eIF4E from binding eIF4G to form complexes that are competent for cap-dependent mRNA translation (Haghighat *et al*., 1995; Mader *et al*., 1995). Thus, by causing the release of 4E-BP1 from eIF4E, the phosphorylation of 4E-BP1 is expected to enhance the binding of eIF4G to eIF4E. To study this, we isolated eIF4E and its binding partners on m^7GTP-Sepharose beads, and analysed the bound material by SDS-PAGE and western blotting, as shown in Fig. 5.3, GH treatment enhanced the association of eIF4G with eIF4E, as assessed by analysis of material bound to m^7GTP-Sepharose, which binds eIF4E and thus also its partner proteins (Fig. 5.3). This effect was blocked by rapamycin, which also decreased the basal level of eIF4G bound to eIF4E and increased 4E-BP1 binding to eIF4E (Fig. 5.3).

GH thus promotes formation of initiation factor complexes that are required for the initiation of cap-dependent translation.

A third translational regulator that is regulated by mTOR, the elongation factor eEF2 was also examined. GH caused a rapid decrease in the phosphorylation of eEF2 at Thr56 (Fig. 5.2), which corresponds to its activation, since phosphorylated eEF2 is inactive as it cannot bind ribosomes (Carlberg *et al*., 1990). However, by about 20 min, eEF2 phosphorylation had returned to basal levels, but fell again by 30-45 min, before rising once more by 2hours. It was previously observed a rather similar biphasic regulation of eEF2 in cardiomyocytes in response, e.g., to insulin (Wang *et al*., 2000). The mechanism that underlies this response to GH is studied in more detail below.

Rapamycin treatment blocked the ability of GH to promote the phosphorylation of S6 and of 4E-BP1 (Fig. 5.4) as judged from analysis with phospho-specific antisera and by its mobility on SDS-PAGE. This indicates that, as anticipated, the GH-induced phosphorylation of S6 and 4E-BP1 requires signaling through mTOR, and in particular, the rapamycin-sensitive type of mTOR complex, mTORC1 (Wullschleger *et al*., 2006).

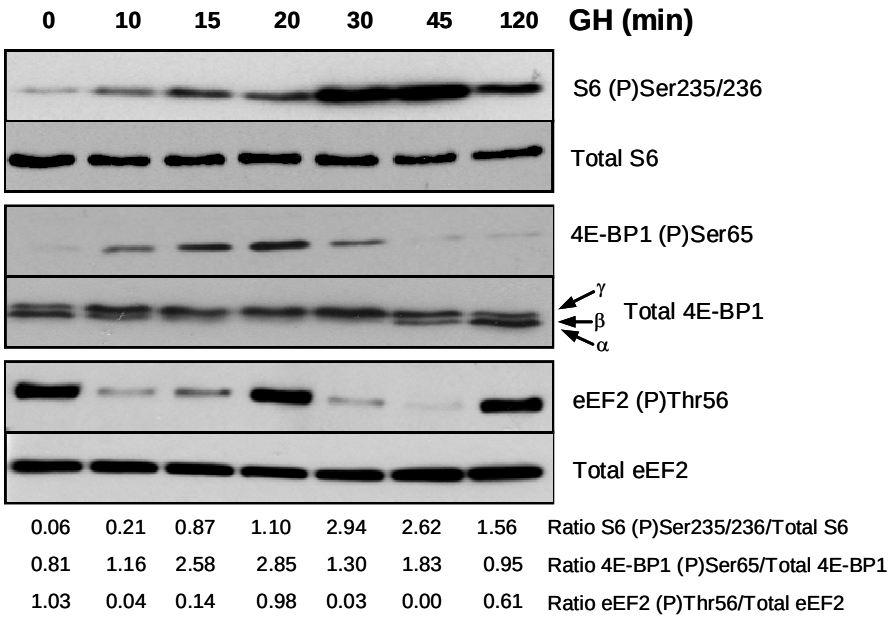

Fig. 5.2: Growth hormone (GH) stimulates targets of mTOR signaling and potential upstream regulators of mTOR. Cells were serum starved for 30 hours and then treated with GH (500ng/mL) for the times indicated (min). Clarified whole cell extracts were analysed by SDS-PAGE and western blotting using the indicated antisera, including antibodies to assess total levels of each protein, to which levels of the phosphospecies should be compared. In all cases, blots are representative of at least three independent experiments. The ratio values represent the individual densitometric analysis of the immunoblots shown in the figures. For 4E-BP1, labelled arrows indicate the differentially phosphorylated α, β and γ species. Ratios represent the signal observed with the phospho-specific antibody normalized to the total amount of the corresponding protein

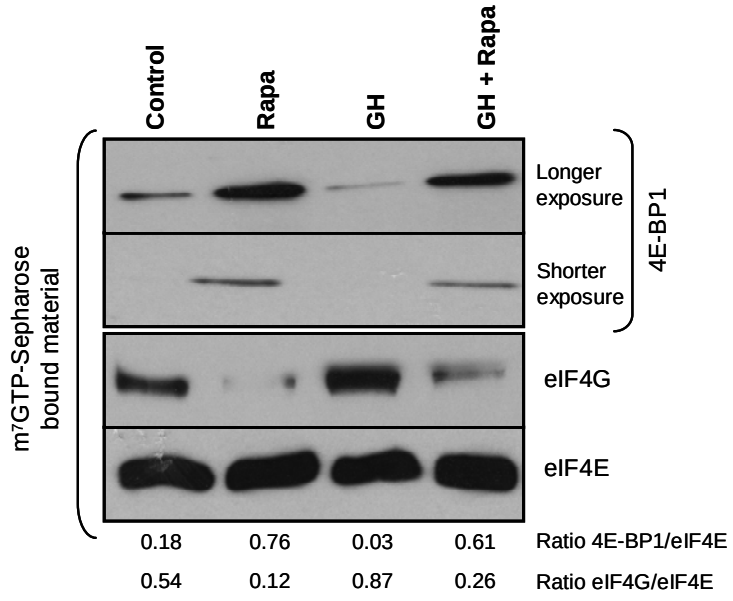

Fig. 5.3: Growth hormone (GH) stimulates targets of mTOR signaling and potential upstream regulators of mTOR. Cells were serum starved for 30 hours and then treated with GH (500ng/mL). Clarified whole cell extracts were analysed by SDS-PAGE and western blotting using the indicated antisera, including antibodies to assess total levels of each protein, to which levels of the phosphospecies should be compared. In all cases, blots are representative of at least three independent experiments. The ratio values represent the individual densitometric analysis of the immunoblots shown in the figures. Cells were pretreated with or without rapamycin (Rapa) prior to GH treatment (30 min). Cell extracts were then subjected to affinity chromatography on m^7GTP-Sepharose as described in the methods and the bound material was analysed by SDS-PAGE and western blotting using antisera against eIF4E, 4E-BP1 and eIF4G. Ratios represent the quantitation of 4E-BP1 and eIF4G relative to total eIF4E

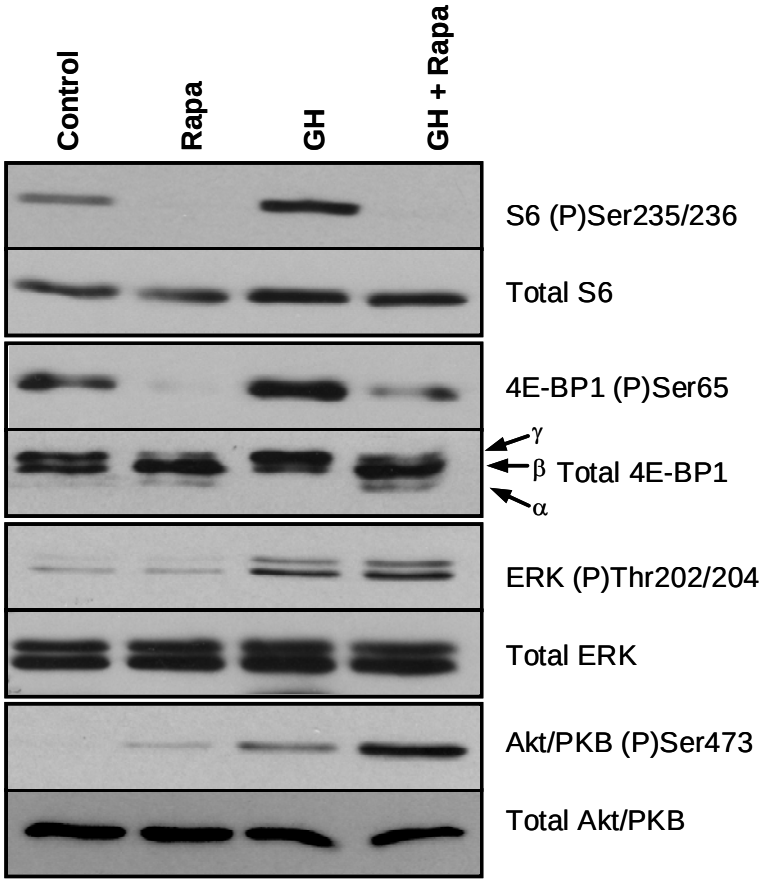

Fig. 5.4: Growth hormone (GH) stimulates targets of mTOR signaling and potential upstream regulators of mTOR. Cells were serum starved for 30 hours and then treated with GH (500ng/mL) for the times indicated (min). Clarified whole cell extracts were analysed by SDS-PAGE and western blotting using the indicated antisera, including antibodies to assess total levels of each protein, to which levels of the phosphospecies should be compared. In all cases, blots are representative of at least three independent experiments. The ratio values represent the individual densitometric analysis of the immunoblots shown in the figures. A summary figure with SEM is not presented because the precise time courses of some responses varied slightly between experiments. Cells were pretreated with or without rapamycin (Rapa) prior to GH treatment (30 min). Cell lysates were analyzed by SDS-PAGE and western blotting using antisera for the indicated proteins

5.4.2. GH activates two pathways that can function upstream of mTOR

mTOR can be controlled by through PI 3-kinase (Manning *et al*., 2002; Manning and Cantley, 2003a) or through ERK signaling (Wang and Proud, 2002b; Roux *et al*., 2004; Ma *et al*., 2005). It was important to check whether GH activated these pathways in H4IIE hepatoma cells. GH elicited the rapid phosphorylation of Akt/PKB, as evidenced by the increased phosphorylation by 10 min of PKB at Ser473, a C-terminal site involved in its activation. Increased phosphorylation was sustained for at least 120 min (Fig. 5.5). Activation of ERK1/2 was also observed, however, the rise in ERK phosphorylation was slower, only seen by 15 min, and transient returning essentially to basal levels by 120 min (Fig. 5.5).

It was important to examine whether rapamycin affected potential upstream signaling pathways, e.g., ERK and PKB. Rapamycin treatment had little effect on GH-induced ERK phosphorylation (Fig. 5.4) and actually enhanced GH-induced PKB phosphorylation (Fig. 5.4; see also Fig. 5.6). This likely reflects inhibition by rapamycin of the negative feedback loop whereby S6K1 phosphorylates IRS1 and impairs signaling through it (Harrington *et al*., 2004; Um *et al*., 2004).

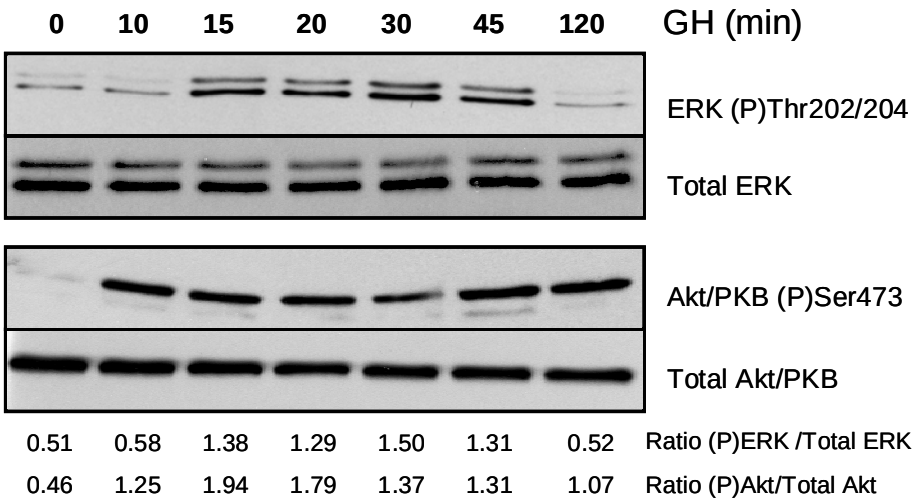

Fig. 5.5: Growth hormone (GH) stimulates targets of mTOR signaling and potential upstream regulators of mTOR. Cells were serum starved for 30 hours and then treated with GH (500ng/mL) for the times indicated (min). Clarified whole cell extracts were analysed by SDS-PAGE and western blotting using the indicated antisera, including antibodies to assess total levels of each protein, to which levels of the phosphospecies should be compared. In all cases, blots are representative of at least three independent experiments. The ratio values represent the individual densitometric analysis of the immunoblots shown in the figures. A summary figure with SEM is not presented because the precise time courses of some responses varied slightly between experiments. Antisera for the phosphorylated, active, forms of Akt/PKB and ERK, and for the total amounts of these proteins, were used to assess activation of potential upstream regulators of mTOR. Ratios represent the signal seen with the phospho-specific antibody normalized to the total amount of the corresponding protein

5.4.3. GH activates mTOR via PI 3-kinase

As reported above, GH activates both PKB and ERK signaling. To study whether either or both of the above pathway(s) mediates the activation of mTOR signaling by GH, pathway specific inhibitors were used. PD098059 is a rather specific inhibitor of ERK signaling which acts at the level of the upstream kinase, MEK (Alessi *et al*., 1995). Wortmannin is an irreversible inhibitor of PI 3-kinase (Arcaro and Wymann, 1993), but may also inhibit mTOR at certain concentrations (Brunn *et al*., 1996). Treatment of cells with PD098059 completely eliminated the phosphorylation of ERK1/2 (Fig. 5.6), confirming its efficacy in blocking this pathway, but had no effect upon the activation of the phosphorylation of S6 or 4E-BP1 by GH (Fig. 5.6), ruling out ERK signaling as a significant component in the control of mTOR by GH. On the other hand, wortmannin, which completely prevented GH-induced phosphorylation of PKB (Fig. 5.6), clearly inhibited the phosphorylation of S6 and of 4E-BP1 that was induced by GH (Fig. 5.6).

This inhibition could reflect a requirement for PI 3-kinase signaling, or might be a consequence of the ability of wortmannin to inhibit mTOR itself. To distinguish between these possibilities, the effect of a range of concentrations of wortmannin on the phosphorylation of the N-terminal sites in 4E-BP1 whose basal phosphorylation requires amino acid-dependent mTOR signaling, but not PI 3-kinase (Wang *et al*., 2005) were examined. At all concentrations tested, wortmannin eliminated the GH-induced phosphorylation of PKB, S6 and Ser65 in 4E-BP1 (Fig. 5.7). In contrast, wortmannin had no effect upon the phosphorylation of Thr37/46 in 4E-BP1, strongly implying that its ability to impair the GH-induced phosphorylation of (other sites in) 4E-BP1 is a consequence of its ability to block PI 3-kinase rather than a direct effect on mTOR. It has been shown earlier that the phosphorylation of these sites in 4E-BP1 is mediated via

mTORC1 (Wang *et al*., 2005), but is not sensitive to inhibition of PI 3-kinase by wortmannin (Wang and Proud, 2002a). At the highest concentration used, wortmannin decreased the phosphorylation of ERK. The reason for this is unknown, but this effect has been noted in other studies (e.g. Welsh *et al*., 1994).

Thus, it appears that GH activates mTORC1 in a manner that requires signaling via PI 3-kinase. This likely corresponds to the widely-accepted model where phosphorylation of TSC2 (a negative regulator of mTOR) by PKB/Akt inhibits TSC2 function allowing the activation of mTORC1 (Manning and Cantley, 2003a/b). One of the sites in TSC2 that is modified by PKB/Akt is Ser939 (Inoki *et al*., 2002; Tee *et al*., 2003), which is not affected by ERK signaling (Roux *et al*., 2004; Rolfe *et al*., 2005). As assessed using a phosphospecific antibody directed against this site, it was clear that GH enhanced the phosphorylation of this site in TSC2 (Fig. 5.8).

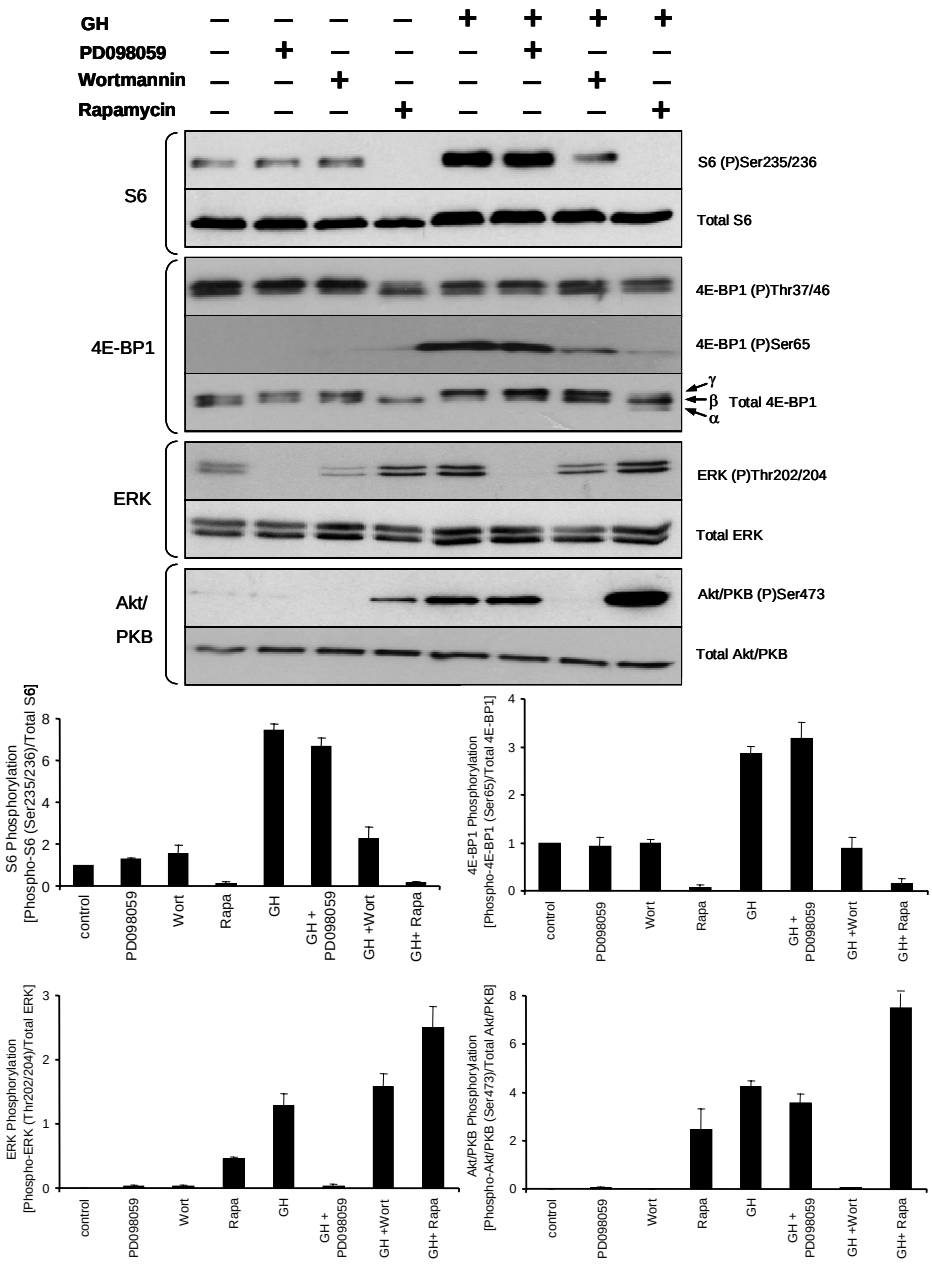

Fig. 5.6: Blocking PI 3-kinase, but not ERK signaling, inhibits the activation of mTORC1 signaling by growth hormone (GH). After 30 hours serum starvation period, cells were treated with 10 μM PD098059; 100 nM wortmannin (Wort); or 50 nM rapamycin (Rapa), as noted, and then treated with GH for 30 min. Clarified whole cell extracts were analysed by SDS-PAGE and western blotting using the indicated antisera. Graphs show the mean ± SE of individual densitometric analyses of several immunoblots for the indicated phospho-antisera normalised to the total amount of the relevant protein

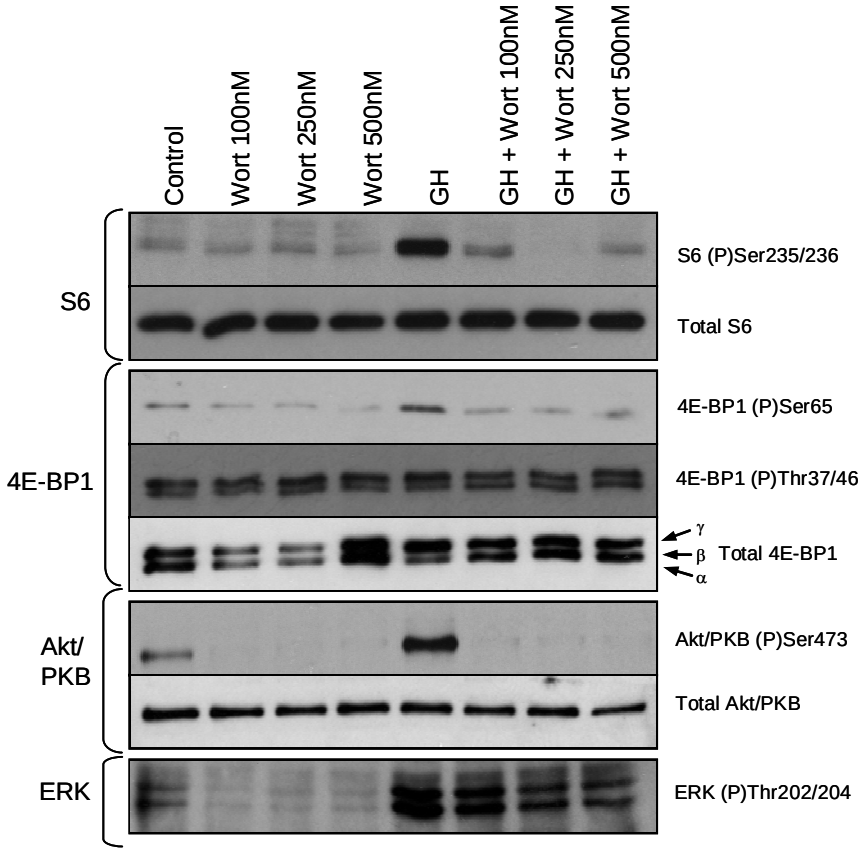

Fig. 5.7: Growth hormone (GH) regulates the mTOR pathway through activation of the PI 3-kinase signaling pathway. H4IIE cells were pretreated with wortmannin at the indicated concentrations (for 30 min) and then treated with GH for 30min (or left for a further 30 min without GH). Clarified whole cell extracts were analysed by SDS-PAGE and western blotting using the indicated antisera. For 4E-BP1, labelled arrows indicate the differentially phosphorylated α, β and γ species

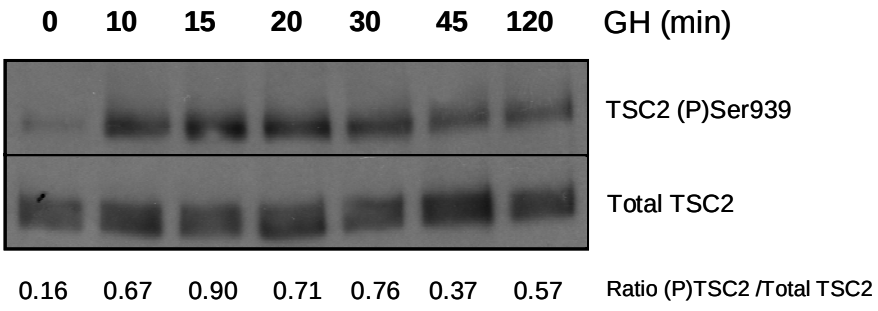

Fig. 5.8: Growth hormone (GH) regulates the mTOR pathway through activation of the TSC2. Immunoblot showing the level of TSC2 phosphorylation at Ser939 assessed using a specific phospho-specific antibody. Anti-TSC2 is used as a 'loading control'. Ratios represent the signal observed with the phospho-specific antibody normalised to the total amount of the corresponding protein

5.4.4. mTOR controls early and late stages of GH-induced eEF2 dephosphorylation

GH caused the dephosphorylation of eEF2 at Thr56 in a biphasic manner (Fig. 5.2). As noted above, the first phase is faster than the changes in the phosphorylation of other targets of mTOR signaling such as 4E-BP1 and S6. In addition, the control of eEF2 kinase and thus of eEF2 phosphorylation is complex (Browne and Proud, 2002; Browne and Proud, 2004), raising the possibility that the first phase of eEF2 phosphorylation might be mediated in a different manner, perhaps independently of mTOR.

To study this, the effect of rapamycin on the early (15 min) and late (45 min) phases of eEF2 dephosphorylation were examined. Rapamycin completely inhibited both, showing that both require mTOR signaling (Fig. 5.9).

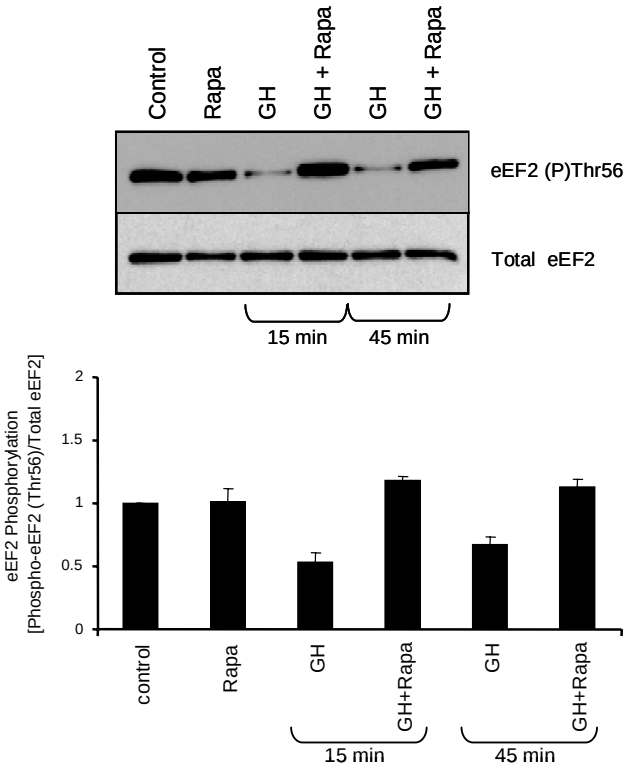

Fig. 5.9: Both phases of the growth hormone (GH) effect on eEF2 phosphorylation are blocked by rapamycin (Rapa). Cells were treated with/without rapamycin for 30 min after serum starvation for 30 hours. Cells were pre-incubated with rapamycin (50nM), and GH was then added for 15 min or 45 min. Phosphorylation of eEF2 at Thr56 was assessed using the phosphospecific antibody. Total eEF2 levels are shown as a 'loading control'. Graph shows the mean ± SE of individual densitometric analysis of several immunoblots of indicated phospho-antisera normalised to the total amount of the relevant protein

5.4.5. Rapamycin inhibits the activation of protein synthesis by GH

The above data show that, via mTOR, GH quickly regulates components of the translational machinery that are associated with its activation. It was important to investigate whether GH also switched on protein synthesis. Treatment of hepatoma cells with GH led to a rapid increase in the rate of protein synthesis (130%, Fig. 5.10), as monitored by following the incorporation of radiolabeled [^{35}S]-methionine into protein.

Given that a number of steps in translation are subject to control, and not all these events are mediated via mTORC1, it was important to test to what extent activation of protein synthesis by GH required mTOR. To test this, the effect of rapamycin on the GH-induced stimulation of protein synthesis was examined. Rapamycin markedly inhibited the increased rate of incorporation of radiolabel (106%, Fig. 5.10). In such experiments, it is critical to take into account any effect of the drug on basal rates of protein synthesis: in fact, rapamycin did have a small but reproducible inhibitory effect on this (85%, Fig. 5.10). However, even when this is taken into account, it is clear that rapamycin significantly inhibits the GH-induced acceleration of protein synthesis (stimulation drops to 106%, $P<0.01$). In this experiment, the effect of the wortmannin on the GH-stimulated protein synthesis was also evaluated. Wortmannin was used at concentrations to specifically inhibit PI 3-kinase. Wortmannin treatment numerically but not statistically reduced the basal rate of protein synthesis (94%, Fig. 5.10). However, the inhibitory effect of wortmannin on the GH-induced stimulation of protein synthesis was significant (stimulation was decreased to 106%, $P<0.01$).

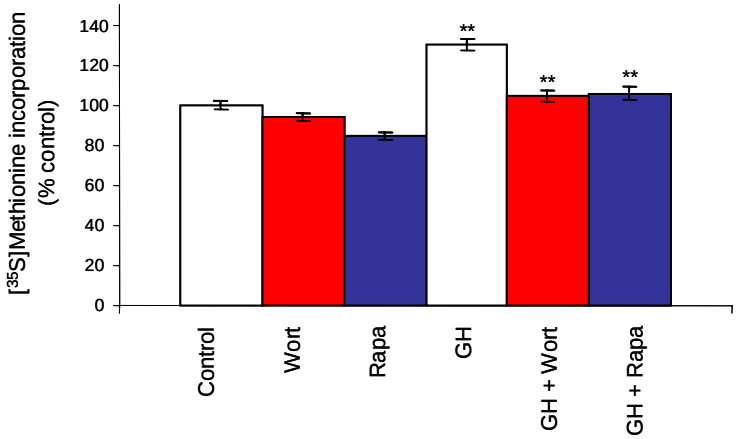

Fig. 5.10: mTOR is required for the activation of protein synthesis by growth hormone (GH) in H4IIE cells. After the 30 hour serum starvation, H4IIE cells were treated with signaling inhibitors as indicated (Wort, wortmannin; Rapa, rapamycin) for 30 min prior to addition of GH. After 30 min later, [^{35}S]-methionine was added and 1 hour later, cells were extracted and the incorporation of radiolabel into TCA-insoluble material was assessed as described in the materials and methods. Since the inhibitors slightly affected basal rates of protein synthesis, data for GH plus inhibitors are shown in relation to control cells treated with the inhibitor, i.e. for GH, control was DMSO (control); for GH + wort, control was wortmannin (wort); for GH + Rapa, control was rapamycin (Rapa). Data are given ± SD, n=4

**P < 0.01

5.5. Discussion

This study aimed to examine how rapidly GH activates protein synthesis. The data clearly show that GH rapidly stimulates protein synthesis (over the min – hour timescale) and that this effect requires signaling through mTOR, as judged by the ability of rapamycin to block completely the GH-induced stimulation of protein synthesis (Fig. 5.10). Both GH and mTORC1 play key roles in regulating cell and tissue growth, so this connection between GH and mTORC1 is not surprising. Nonetheless, this is the first time that GH has been shown, to activate mTORC1-dependent components that regulate the translational machinery and the first time that GH has been shown to turn on protein synthesis in a mTORC1-dependent manner (Fig. 5.10).

GH had been shown previously to stimulate S6 kinase in 3T3-F442A pre-adipocytes (Anderson, 1992a/b). However, the contribution, if any, of S6 phosphorylation to the short-term activation of protein synthesis remains unclear (Ruvinsky *et al*., 2005; Ruvinsky and Meyuhas, 2006). Here it is shown that GH activates two key steps in mRNA translation. These are, firstly, the formation of eIF4F complexes, by the binding of eIF4G to eIF4E, which participate in cap-dependent translation initiation (Fig. 5.3). Secondly, GH also stimulates the dephosphorylation of eEF2, which is associated with activation of translation elongation (Fig. 5.2). Thus, GH activates both the initiation and elongation stages of translation. The effect of GH on both steps was blocked by rapamycin (Fig. 5.4 and 5.9), consistent with the fact that rapamycin also blocked the overall activation of protein synthesis by GH (Fig. 5.10). The data also show that GH enhances the phosphorylation of S6 (Fig. 5.2), consistent with earlier data that it activates S6 kinase.

Wang *et al*. (2005) noted previously that the phosphorylation of Thr37/46 in 4E-BP1 is insensitive to rapamycin. While this is a characteristic of signaling through mTORC2 (Wullschleger *et al*., 2006), the phosphorylation of these sites is dependent upon amino acids (Wang *et al*., 2005), which regulate mTORC1 (Kimball and Jefferson, 2006). This suggests that the phosphorylation of Thr37/46 is likely to be a rapamycin-resistant effect of mTORC1, as mTORC2 is not known to be affected by amino acids.

Interestingly, the GH-induced dephosphorylation of eEF2 was clearly biphasic. GH induced a rapid dephosphorylation of eEF2, such that phospho-eEF2 was almost undetectable 10 min after addition of GH (Fig. 5.2). eEF2 phosphorylation then returned almost to control levels by 20 min, and this was followed by a second dephosphorylation phase, such that was essentially completely dephosphorylated by 45 min. eEF2 phosphorylation returned to control levels by 2 hours after GH treatment. Importantly, both phases were blocked by rapamycin, indicating that mTORC1 drives both.

There are three known inputs from mTOR into eEF2 kinase: phosphorylation at Ser78 (Browne and Proud, 2004) which prevents CaM binding; phosphorylation at Ser359 (Knebel *et al*., 2001), which also inhibits maximal eEF2 kinase activity; and phosphorylation at Ser366, which makes eEF2 kinase less sensitive to activation by Ca/CaM (Wang *et al*., 2001a). Since there was very little change in S6 phosphorylation by 10 min, the first phase of eEF2 dephosphorylation is presumably not due to S6 kinase, and presumably reflects another input. The S6 kinase site, Ser366, can also be phosphorylated by $p90^{RSK}$, which lies downstream of ERK (Wang *et al*., 2001a). However, since ERK activation was slower than the change in eEF2 phosphorylation, the first rapid phase of eEF2 regulation is unlikely to be mediated by ERK/$p90^{RSK}$

signaling. In any case, it was blocked by rapamycin, ruling out a significant role for ERK signaling. The second phase does match the kinetics of S6 kinase activation, as judged by S6 phosphorylation, both being maximal at 45 min (Fig. 5.2). Unfortunately, the phosphospecific anti-eEF2 kinase antisera was not sensitive enough to allow evaluation of changes in the phosphorylation of specific sites in the endogenous eEF2 kinase in hepatoma cells.

How does GH activate mTOR? The best characterized mechanism for the activation of mTOR by hormones involves the phosphorylation of TSC2, a negative regulator of mTOR, by PKB/Akt (Manning and Cantley, 2003b). This is mediated through PI 3-kinase. These data are consistent with GH employing this mechanism. First, the effects of GH on the phosphorylation of 4E-BP1 and S6 were blocked by the PI 3-kinase inhibitor wortmannin (Fig. 5.6), used at concentrations that do not interfere with mTOR function (Wang and Proud, 2002b; Wang *et al*., 2005). Second, these effects of GH were not affected by the MEK inhibitor PD098059, which completely inhibited the activation of ERK by GH (Fig. 5.6). Thus, although wortmannin did interfere with the activation of ERK by GH, the fact that PD098059 did not impair the activation of mTORC1 signaling by GH indicates that this 'side effect' of wortmannin may be ignored in interpreting the present data. It is not known why wortmannin impairs ERK activation, but this effect has been noted in other studies (Welsh *et al*., 1994). Thirdly, GH induced the phosphorylation of TSC2 at Ser939 (Fig. 5.8), a major site of phosphorylation by PKB, but which is not controlled by MEK/ERK signaling (Roux *et al*., 2004; Ballif *et al*., 2005; Rolfe *et al*., 2005). This is completely consistent with the above conclusion that GH signals via PKB/Akt to activate mTOR. Importantly, the change in the phosphorylation of TSC2, an upstream regulator of mTOR, is at least as rapid as the change in eEF2 phosphorylation and faster than the increases in the

phosphorylation of S6 and 4E-BP1. Furthermore, the rapidity of activation of PKB (Fig. 5.5) and of TSC2 phosphorylation (Fig. 5.8) are consistent with one another.

The present data thus demonstrate that GH acts through PI 3-kinase and probably PKB/Akt to activate mTOR signaling, leading to the rapid activation of eEF2 and formation of eIF4F complexes. The fact that the activation of protein synthesis by GH is completely blocked by rapamycin is consistent with the conclusion that these changes in translation factor function contribute to the activation of protein synthesis by GH. Thus, GH activates targets for rapamycin-sensitive mTORC1 signaling, such as eIF4E and eEF2, and treatment with rapamycin or wortmannin inhibits the ability of GH to stimulate protein synthesis. mTOR also positively controls ribosome biogenesis and the synthesis of several components of the translational machinery, thereby increasing the translational capacity of the cell and tissue. It is thus likely that the longer-term effects of GH on protein synthesis and cell growth also involve GH-activated mTOR signaling.

CHAPTER 6

The role of eEF2 in the activation of protein synthesis by GH

6.1. Abstract

Growth hormone (GH) treatment leads to the stimulation of protein synthesis in H4IIE cells. In this study, the role of eukaryotic elongation factor 2 (eEF2) in the stimulation of protein synthesis by GH was evaluated. Knock-down of eEF2 kinase (eEF2K) through small interfering RNA (siRNA) resulted in up to 90% knock down of eEF2K and consequently decrease in eEF2 phosphorylation. The eEF2K knock down also slightly increased the basal protein synthesis but had a negative effect on the stimulation of protein synthesis by GH after 30 min and 45 min. These results suggest that the elongation step of translation appears not to be the primary regulatory step in the GH-induced stimulation of protein synthesis.

6.2. Introduction

Protein synthesis in mammalian cells is a high energy demanding process and most of this energy is used during peptide chain elongation (Browne and Proud, 2002). The elongation stage of protein synthesis in eukaryotes requires two translation factors, eukaryotic elongation factor 1 (eEF1) and eEF2. eEF1 recruits amino-acyl-transfer RNA (tRNA) to the A-site of the ribosomes when eEF1 is in the GTP-bound (active) form. Upon GTP hydrolysis, GDP-eEF1 is released from the ribosome and is recycled. eEF1 is reported to be phosphorylated by S6 kinase and this results in stimulation of its activity (Chang and Traugh, 1997).

eEF2 is also a GTP-binding protein and is required for the second step of elongation, ribosomal translocation. In contrast to eEF1, phosphorylation of eEF2 at Thr56 in its GTP-binding domain (Browne and Proud, 2002) inhibits its activity and protein synthesis, by preventing it from binding to ribosomes (Ryazanov *et al*., 1988). The phosphorylation of eEF2 is catalyzed by a highly specific protein kinase, eEF2K. Insulin and a number of other agents induce a rapid dephosphorylation of eEF2. This results in increased eEF2 activity and accelerated elongation (Redpath *et al*., 1996). The dephosphorylation of eEF2 appears to be due to the inhibition of eEF2K which is controlled by phosphorylation (Browne and Proud, 2002) and involves protein phosphatase 2A (Redpath and Proud, 1990). The mTOR dependent control of eEF2K involves its phosphorylation at several sites (Ser78, Ser359 and Ser366). In Chapter 5 it is shown that GH treatment increased protein synthesis in H4IIE cells and this effect is blocked by rapamycin indicating the involvement of the mTOR pathway in the regulation of GH-induced increase in protein synthesis. In addition, GH treatment

stimulated the dephosphorylation of eEF2 in H4IIE cell in a biphasic manner suggesting the involvement of eEF2 in the response to GH.

To date, there has not been a systematic evaluation of the role of eEF2 in the stimulation of protein synthesis by GH. In this Chapter, it is hypothesized that if eEF2 is a control point for protein synthesis in H4IIE cells, GH treatment may act by reducing the activity of eEF2K, thereby preventing eEF2 phosphorylation and the unphosphorylated eEF2 would stimulate activation of protein synthesis. Further, it is suggested that it should be possible to simulate the effect of GH on protein synthesis by inhibiting eEF2K through the use of RNA interference.

RNA interference is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA that is homologous in sequence to the silenced gene (Hammond *et al*., 2001; Tuschl, 2001). The mediators of sequence-specific messenger RNA degradation are siRNAs of 21-22 nucleotides generated by ribonuclease III cleavage from longer double-stranded RNA (Elbashir *et al*., 2001a). Here, an eEF2K siRNA duplex was used to specifically suppress the expression of the endogenous eEF2K gene in hepatoma rat cell line (H4IIE) to study the effect of eEF2 dephosphorylation on protein synthesis. Therefore, the aim of this study was to determine if phosphorylation of eEF2 has a key role in the stimulation of protein synthesis by GH.

6.3. Materials and methods

6.3.1. Tissue culture cell lines (H4IIE)

Described in the Materials and methods of Chapter 5.

6.3.2. SiRNA molecules

eEF2K gene expression in H4IIE cells was silenced by transfection with siRNAs. Double stranded siRNA for eEF2K was designed according to Terai *et al*. (2005) from the coding region 2116 to 2136 (5'-GGCAGUCCAUGAUUUUAGUdTdT-3') of the target rat eEF2K mRNA (accession number: BC061825) and a universal negative (nonsilencing-NS) control was purchased from Invitrogen (New York, USA).

6.3.3. Transient transfection of H4IIE cells

H4IIE cells were split to a density of $2x10^6$ cells per 10 cm diameter dish and after 8 hours incubation at 37^{o}C in complete Dulbecco's modified Eagle's medium (DMEM), the cells were transfected with a calcium/phosphate (125 mM $CaCl_2$, 50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4)-siRNA (5 – 20 μM in 2.5 – 10 μL) co-precipitate. The calcium phosphate facilitates the binding of the siRNA to the cell surface. The siRNA then enters the cell by endocytocis. After transfection, the cells were incubated at 37^{o}C and 5% CO_2 for 12 hours for siRNA incorporation, after which the medium was aspirated and replaced with 10 mL fresh 'complete DMEM'. This step eliminates dead cells. Silencing was assessed 40 hours and 70 hours after transfection to allow for turnover of the protein of the target gene.

6.3.4. Cell treatment and preparation of cell lysates

At 40 hours or 70 hours after transfection, the cells were treated with either insulin (100 nM) for 25 min or GH (500 ng/mL) for the indicated times in the figures. After treatment, cells were washed once with phosphate-buffered saline (PBS) and harvested in 400 µL of harvesting buffer (20 mM HEPES·KOH [pH 7.5], 50 mM β-glycerophosphate, 0.2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 0.5 mM sodium orthovanadate, 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM benzamidine, 1 µg of leupeptin/mL, 1 µg of antipain/mL, and 1 µg of pepstatin/ mL). Cell debris and nuclei were spun down for 1 min at 12,000 *g*, and the supernatant was transferred to new tubes.

6.3.5. Determination of protein concentration

Described in the Materials and methods of Chapter 3.

6.3.6. SDS-PAGE

Described in the Materials and methods of Chapter 3.

6.3.7 Staining, fixing and drying polyacrylamide gels

Described in the Materials and methods of Chapter 3.

6.3.8 Electrotransfer

Described in the Materials and methods of Chapter 3.

6.3.9. Western blot analysis

Described in the Materials and methods of Chapter 3.

6.3.10. Primary antisera

Described in the Materials and methods of Chapter 3.

6.3.11. Production and purification of polyclonal antibodies

Described in the Materials and methods of Chapter 5.

6.3.12. Protein synthesis measurement

Described in the Materials and methods of Chapter 5.

6.4. Results

6.4.1. eEF2K knock down in H4IIE cells

To determine the concentration of the siRNA to achieve optimal silencing of eEF2K in H4IIE cells, three different concentrations of siRNA were tested (5 nM, 10 nM, 20 nM). eEF2K directly phosphorylates eEF2 at Thr56, thus phosphorylation of eEF2 at Thr56 is an indirect measure of the extent of RNA interference (RNAi) and whether the procedure was successful or not. The reason for using phospho-eEF2 (Thr56) as a redout rather than blotting for total eEF2K is that eEF2K is poorly detectable by western blotting in cell lysates without immuno-precipitation. The dosages of 5 nM and 10 nM had the greatest effect on phosphorylation of eEF2 after 40 hours of transfection (Fig. 6.1; 2.3 vs 1.4 and 1.7 for control and siRNA group (5nM and 10nM, arbitrary units, respectively). The total eEF2 abundance did not change, indicating that the silencing was specific for the target gene. After normalization for total eEF2, the ratios of phospho-eEF2/total eEF2 show that following 40 hours of transfection 10 nM of siRNA was the most effective dose (2.3 vs 1.4 for control and siRNA group, arbitrary units, respectively). Fig. 6.1 also shows that after 25 min of insulin treatment a decrease in phosphorylation of eEF2 was observed in the control cells and in the cells treated with 5 nM and 20 nM of eEF2K siRNA (2.3 vs 2.0, control; 2.1 vs 1.5 20nM). The dephosphorylation of eEF2 at Thr56 in response to insulin treatment was not observed with 10 nM of siRNA.

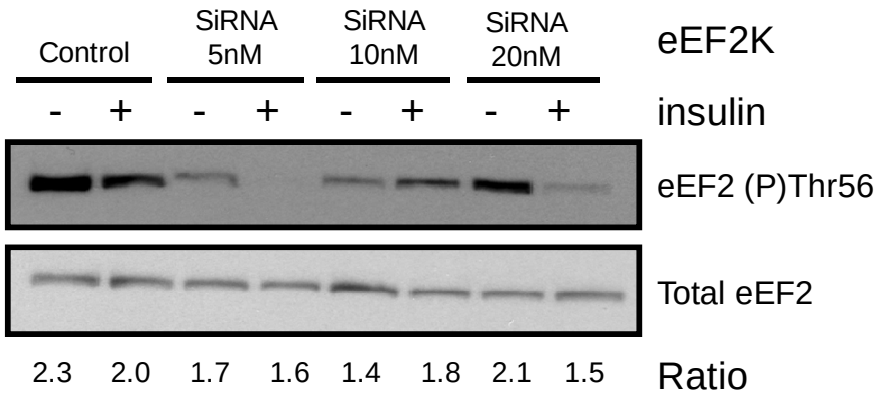

Fig. 6.1: H4IIE cells transfected with different concentrations of eEF2K siRNA molecule (5 nM, 10 nM and 20 nM). 40 hours post-transfection the cells were treated with +/- 100nM insulin for 25 min and the protein concentrations were determined by immunoblotting analysis for eEF2 (P)Thr 56. Total EF2 was used as the loading control. Ratio represents the amount of eEF2(P) corrected to total eEF2 and represents the mean of at least two similar experiments

6.4.2. Transfection and starvation

To assess the optimal transfection period and the serum starvation time (to achieve maximum phosphorylation of eEF2, i.e. maximum eEF2K knock-down) two experiments were performed. In experiment 1, cells were transfected for 40 hours with two concentrations of siRNA eEF2K (5 nM and 10 nM) and cells were incubated for 16 hours in the standard medium in the absence of serum, (Fig. 6.2.A). After 40 hours of transfection with 5 nM or 10 nM of eEF2K siRNA, phosphorylation of eEF2 was reduced by approximately 90% and 80% respectively (Fig. 6.2.B). In the presence of insulin, the phosphorylation of eEF2 was decreased by approximately 80% in the control group (no siRNA), 60% in the cells treated with 5 nM of eEF2K siRNA and by 90% in cells treated with 10 nM of eEF2K siRNA showing that insulin was affecting translation via eEF2 (Fig. 6.2.C).

In experiment 2, the cells were transfected for 70 hours with two concentrations of siRNA eEF2K, 5 nM and 10 nM and then incubated in the absence of serum for 30 hours (Fig 6.3.A). 70 hours of transfection with the two concentrations of eEF2K siRNA produced similar inhibition to that observed in experiment 1 (90% and 80% reduction in phosphorylation for 5 nM and 10 nM treatments, respectively) (Fig. 6.3.B). After 70 hours of transfection and 30 hours of starvation the insulin effect over the phosphorylation of eEF2 Thr56 was 90% in the group with no siRNA (control), 90% in the group treated with 5 nM of siRNA and 80% in the cells treated with 10 nM of siRNA showing that siRNA of eEF2K does not affect the eEF2 dephosphorylation caused by insulin (Fig. 6.3.C).

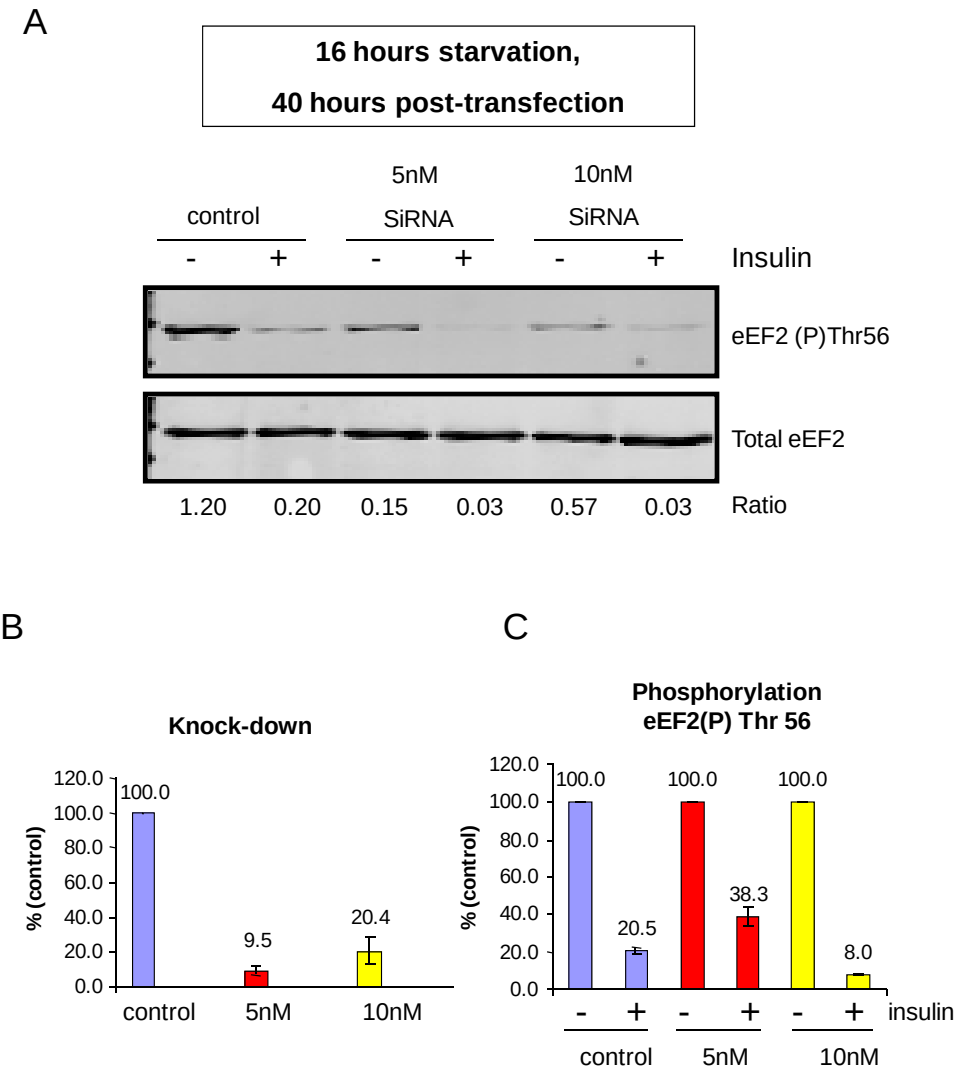

Fig. 6.2: H4IIE cells transfected with two different concentrations of eEF2K siRNA molecule (5 nM and 10 nM) for 40 hours and starved for 16 hours. (A) After the starvation period, cells were treated with +/- 100 nM insulin as indicated for 25 min and the protein concentrations were determined by immunoblotting analysis for eEF2 (P)Thr 56. Total EF2 was used as the loading control. (B) The percentage of inhibition of eEF2 (P) Thr56 after 5 nM or 10 nM of siRNA in relation to the control. (C) The insulin effect over the phosphorylation of eEF2(P) Thr56 is shown in relation to its own control. Mean±SD for duplicate experiments

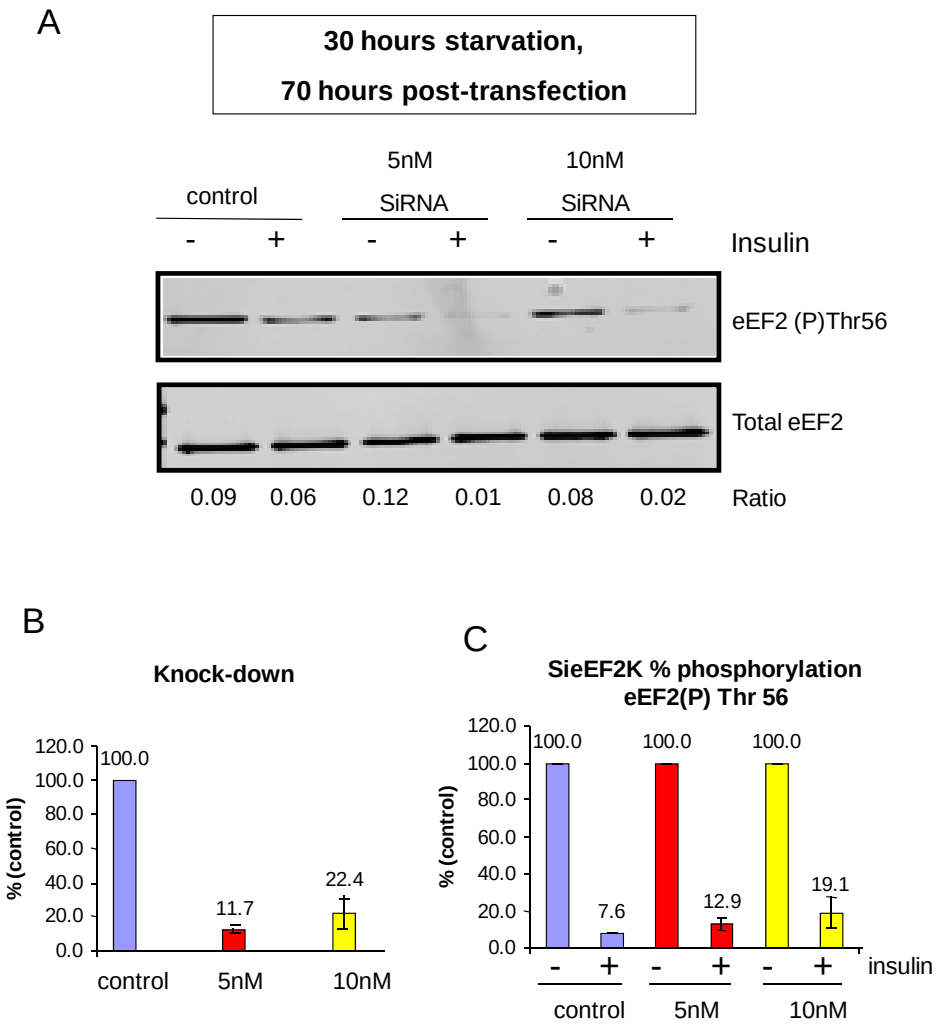

Fig. 6.3: The figure shows H4IIE cells transfected with two different concentrations of eEF2K siRNA molecule (5 nM and 10 nM) for 70 hours and starved for 30 hours. (A) After the starvation period, cells were treated with +/- insulin for 25 min and the protein levels were determined by immunoblotting analysis for eEF2 (P)Thr 56. Total EF2 was used as the loading control. (B) The graph shows the percentage of inhibition of eEF2 (P) Thr56 after 5 nM or 10 nM of siRNA in relation to the control. (C) The graph shows the insulin effect over the phosphorylation of eEF2(P) Thr56 in relation to its own control. Mean±SD for duplicate experiments

6.4.3. Protein synthesis

Given that a number of steps in mRNA translation are subject to control, it was important to test to what extent activation of protein synthesis in H4IIE cells by GH required eEF2 dephosphorylation. To assess this, H4IIE cells were transfected with eEF2K siRNA and the incorporation of [^{35}S]-methionine into protein was measured (section 6.3.12). Cells lysates were produced after 40 hours of transfection and 16 hours of starvation and the level of phosphorylation of eEF2 was determined by immunoblotting analysis (Fig. 6.4.A). As in previous experiments, eEF2 phosphorylation levels were decreased by approximately 90% in cells transfected with eEF2K in relation to the control cells with no siRNA. In addition, the eEF2K siRNA transfection did not affect the total levels of eEF2 for all samples studied (Fig. 6.4.A). These data show that eEF2K was effectively silenced by the treatments. Protein synthesis, as assessed by [^{35}S]-methionine incorporation into intracellular proteins, was increased to 105%, (P=0.10) 15 min and 127% (P=0.02) 30 min after commencing GH treatment. After 45 min, protein synthesis stimulation by GH treatment started to fall (117%, P=0.06). eEF2K silencing tended to increase GH stimulation of protein synthesis after 15 min of stimulation (105 *vs*. 109%, P=0.07). In contrast, eEF2K siRNA markedly inhibited the increased rate of incorporation of [^{35}S]-methionine stimulated by the 30 min and 45 min of GH treatment (stimulation drops to 93%, P<0.01 and 96% P<0.01, for 30min and 45 min, respectively). In such experiments, it is critical to take into account any effect of the silencing on basal rates of protein synthesis. While eEF2K siRNA had a small but reproducible stimulatory effect on this (6%, Fig. 6.4.B), it was not statistically significant. Thus, other components of the

translational machinery other than eEF2 may be involved in growth hormone effects on protein synthesis.

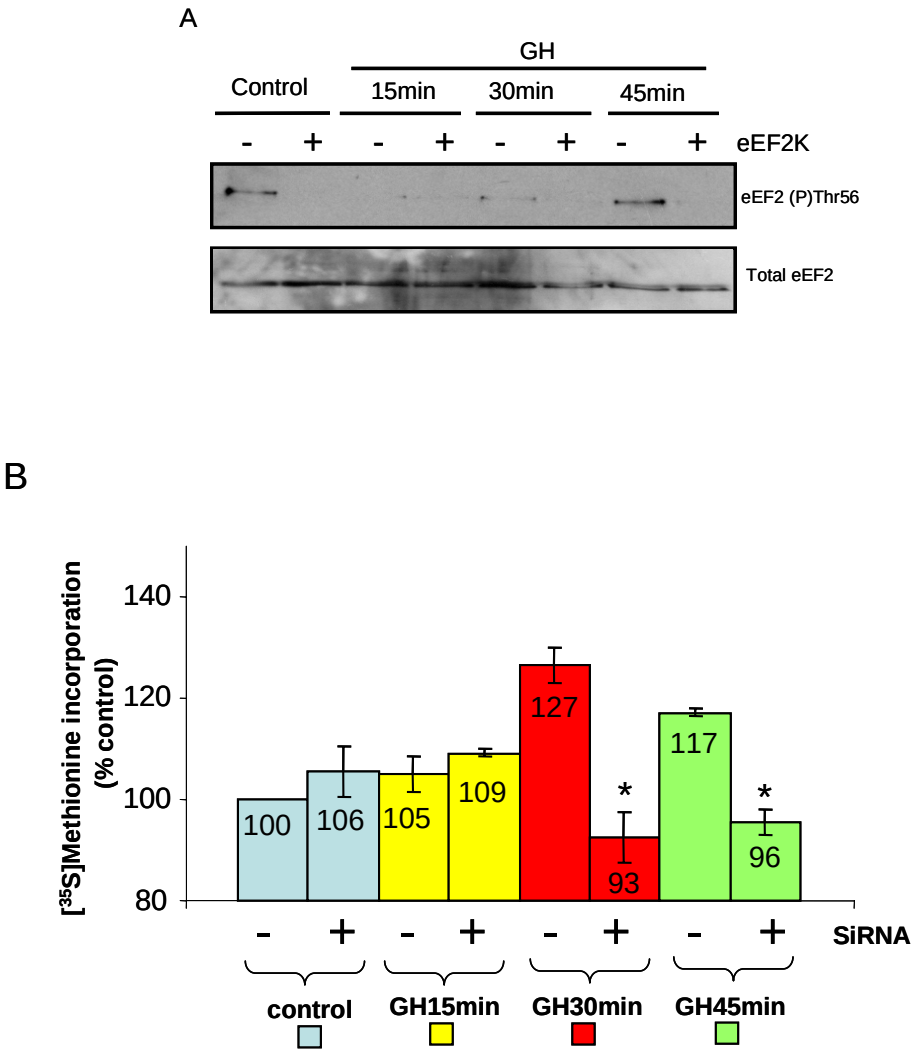

Fig. 6.4: siRNA mediated knockdown of eEF2K in H4IIE cells inhibits the stimulation of protein synthesis in response to growth hormone (GH) treatment. (A) After 16 hours serum starvation, total protein synthesis activity was determined by [^{35}S]-methionine incorporation *in vitro* for 1 hour, followed by trichloroacetic acid precipitation, scintillation counting of the samples, and determination of specific activity of incorporation into protein. After 1 hour incorporation, GH was added for the indicated times (15 min, 30 min and 45 min). Values for GH stimulation are represented in relation to control and values after the knock down are presented in relation to its own control (e.g. GH 30 min SiRNA in relation to GH 30 min). (B) After the protein synthesis assay, protein levels were determined by immunoblotting analysis for eEF2 (P)Thr 56. Data are given mean± SD, n=3

* P<0.01

6.5. Discussion

The aim of this study was to examine the role of eEF2 in the activation of protein synthesis by GH. This was explored by using a chemically synthesized siRNA against eEF2K to reduce eEF2 phosphorylation in H4IIE cells. The data clearly show that eEF2 phosphorylation was specifically inhibited in H4IIE cells treated with siRNA against eEF2K, and the effect of the knock down on eEF2 phosphorylation was maintained for up to 70 hours post-transfection. Braasch *et al*. (2003) have shown that duplexes of siRNA were stable during incubations in serum for up to 72 hours while single strand RNA was completely degraded during incubations that were as short as 30 sec. This study has also shown that concentrations of 5 nM to 10 nM of siRNA were more efficient than higher concentrations of siRNA (20 nM). This has also been observed by other authors (Elbashir *et al*., 2001b) where increased concentrations of siRNA duplexes did not enhance the specific silencing effects but started to affect transfection efficiencies. One possible explanation for such observation is that higher concentrations of siRNA may slightly change the transfection solution pH to basic due to the basic DEPC water present in the siRNA solution.

In the optimization studies, insulin was used instead of GH because insulin stimulation is know to affect the phosphorylation status of eEF2 in a range of studies (Wang *et al*., 2001a; Wang and Proud, 2002a). Additionally, the main objective of the initial studies was to establish optimal conditions (transfection time and siRNA concentration) to further study the role of eEF2 in the GH stimulation of protein synthesis. The transfection of the cells for 70 hours with eEF2K siRNA and the starvation period of 30 hours were apparently more efficient than the transfection for 40 hours and starvation for 30 hours. However, these differences were not statistically significant and a

transfection time of 40 hours and starvation of 16 hours were used in subsequent experiments to avoid extra stress on the cells from long hours of starvation and transfection.

In the previous Chapter of this thesis (Chapter 5) it is shown that GH-induced dephosphorylation of eEF2 is clearly biphasic. Some minor variation between the kinetics of this response was seen across the set of experiments described in Chapter 5. A similar pattern of dephosphorylation of eEF2 was observed here in that GH induced a rapid dephosphorylation of eEF2 at 15 min, which returned to control levels by 45 min after GH treatment. The silencing of these cells was successful as shown by the lack/loss of phosphorylated eEF2 present after the silencing.

It was then hypothesized that the knock down of eEF2K would inhibit the phosphorylation of eEF2 thereby enabling GH to increase protein synthesis if the elongation step of translation was the limiting step for the protein synthesis. The knock down of eEF2K slightly increased the basal levels of protein synthesis, and eEF2K silenced cells tended to increase the GH effect on protein synthesis after 15min of stimulation (P=0.07). However, the expected effect of the silencing over the GH stimulation after 30 and 45 min was not observed. In contrast, the eEF2K siRNA returned the stimulation of protein synthesis to basal levels. A possible explanation for this observation is that the elongation step of translation is not the primary regulatory step in the stimulation of protein synthesis caused by GH. Thus, the initiation of translation is likely alternate key control point (Pestova *et al*., 2001).

The fact that the activation of protein synthesis by GH is completely blocked by eEF2K siRNA at 30 min and 45 min was unexpected. eEF2K is however an unusual calcium

and calmodulin-dependent protein kinase (Browne *et al*., 2004) that is phosphorylated in a mTOR dependent manner on the Ser78, Ser359 and Ser366 phosphorylation sites. The Ser366 is phosphorylated by p90 ribossomal protein S6 kinase and p70 ribosomal protein S6 kinase and the identification of the proteins that phosphorylate the Ser78 and Ser359 remain unknown (Browne *et al*., 2004; Browne and Proud, 2004). The elucidation of the roles of these three phosphorylation sites (and perhaps others) in the control of eEF2K and the identification of the protein kinases that phosphorylate Ser78 and Ser359 in eEF2K may reveal other roles in signaling downstream of mTOR thus demonstrating the importance of eEF2 over protein synthesis regulation under different conditions.

CHAPTER 7

Analysis of the interaction and regulatory motifs in 4E-BP1

Note: *The results present in this Chapter were part of a team research conducted with the students Vivian H. Y. Lee, Timothy Healy and Bruno D. Fonseca at Department of Biochemistry & Molecular Biology, University of British Columbia.*

The material present in this Chapter will be submitted as part of a paper to: FEBS Journal

7.1. Abstract

The mammalian target of rapamycin complex 1 (mTORC1) controls growth and proliferation via the translation regulator eukaryotic initiation factor 4E binding protein (4E-BP1). 4E-BP1 has been shown to be associated with the scaffold protein raptor through its TOS (TOR signaling motif) and RAIP (named after its sequence) motifs to be recognized by mTOR. This study aimed firstly to identify the features of 4E-BP1 that are required for its interaction with raptor and secondly to identify the features of the TOS motif on 4E-BP1 that are essential for its phosphorylation. A series of site direct mutations were used to achieve this objective. The results demonstrate that the TOS motif is essential for interaction with raptor and for phosphorylation of specific sites of 4E-BP1 in HEK293 cells. The results also suggest that the nature of the first and third residues in the TOS motif is essentially important for phosphorylation of 4E-BP1.

7.2. Introduction

Signaling through the mTORC1 plays a key role in the control of a number of cellular functions (Sabatini, 2006; Wullschleger *et al*., 2006). These roles have largely been revealed through the use of rapamycin, an immunosuppressant drug that interferes with signaling through mTORC1.

mTORC1 is a complex comprising several proteins. These include mTOR, a multidomain protein that possesses a kinase domain related to lipid kinases and displays protein kinase activity *in vitro*, and raptor, a scaffold protein that interacts with proteins that are phosphorylated by mTOR (Hara *et al*., 2002; Kim *et al*., 2002; Choi *et al*., 2003; Nojima *et al*., 2003; Schalm *et al*., 2003; Kim and Sabatini, 2004). Ras homolog enriched brain (Rheb), a small G-protein that appears to activate mTOR when it is in its GTP-bound form, also interacts with mTORC1 (Manning and Cantley, 2003b; Long *et al*., 2005). Signaling from cell surface receptors – such as those for insulin, growth factors and mitogens - activates mTORC1, through the inactivation of the tuberous sclerosis complex (TSC), which comprises TSC1 and TSC2 (McManus and Alessi, 2002; Inoki *et al*., 2003; Tee *et al*., 2002; 2003; Zhang *et al*., 2003). In association with TSC1, TSC2 acts as GTPase-activator protein (GAP) which converts Rheb.GTP to its inactive GDP-bound form. For example, agents that activate protein kinase B (PKB, also termed Akt), induce the phosphorylation of TSC2. This is believed to inactivate its GAP function (Manning and Cantley, 2003b; Cai *et al*., 2006), thereby allowing Rheb to accumulate in its GTP-ligand form and switch on mTORC1.

Raptor appears to promote downstream signaling from mTORC1 by binding to short TOS motifs found in proteins whose phosphorylation is positively regulated by mTORC1 (Hara *et al*., 2002; Schalm and Blenis, 2002; Beugnet *et al*., 2003; Choi *et al*., 2003; Schalm *et al*., 2003). The first proteins shown to contain functional TOS-motifs were the ribosomal protein S6 kinases (S6Ks) and the 4E-BPs (Fig. 7.1), each of which is subject to rapamycin-sensitive phosphorylation at multiple sites. The interaction of these proteins with raptor, via their TOS motifs, promotes their phosphorylation by mTOR *in vitro*. Both these types of proteins are implicated in controlling the translational machinery (Wang and Proud, 2006). mTORC1 also controls other cellular functions, although the mTORC1 targets involved in these effects largely remain to be identified (Wullschleger *et al*., 2006).

While the TOS motifs in the S6Ks and 4E-BPs resemble one another, there are also a number of differences between them, and it is not clear what the real requirements are for a functional TOS motif. Defining a 'consensus' TOS motif would help identify such motifs in other proteins that may be controlled by mTORC1 and regulate cellular functions in addition to mRNA translation. It is also not clear whether the TOS motif is sufficient for the interaction with raptor or whether other features are also required.

The *in vivo* phosphorylation of 4E-BP1 requires an additional motif with the sequence Arg-Ala-Ile-Pro, hence, "RAIP motif" (Tee and Proud, 2002), (Fig. 7.1). The phosphorylation of the two N-terminal sites in 4E-BP1 (Thr37/46 in the human protein; Thr36/45 in rat 4E-BP1 requires the RAIP motif (Beugnet *et al*., 2003) and their phosphorylation is needed for the subsequent modification of two sites (Thr70/Ser65) close to the eukaryotic initiation factor 4E (eIF4E) binding motif (Gingras *et al*., 1999b; Mothe-Satney *et al*., 2000a/b;

Beugnet *et al*., 2003; Wang *et al*., 2005). The mTOR-dependent control of 4E-BP1 is thus an example of hierarchical phosphorylation. It is the phosphorylation of Thr70/Ser65 that controls the binding of 4E-BP1 to eIF4E and thus the availability of eIF4E to form functional translation initiation complexes, since 4E-BP1 competes with the scaffolding factor eukaryotic initiation factor 4G (eIF4G) for binding to eIF4E (Mader *et al*., 1995). Earlier work revealed that the RAIP and TOS motifs play distinct roles in regulating the phosphorylation of 4E-BP1 within cells. The phosphorylation of 4E-BP1 is regulated by amino acids and by stimuli such as insulin. The RAIP motif appears to mediate the amino acid input (Tee and Proud, 2002; Wang *et al*., 2005) which promotes phosphorylation of the N-terminal threonines in both 4E-BP1 and 4E-BP2. In contrast, the TOS motif is required for insulin-induced phosphorylation of Ser65 and Thr70.

The (sequence) requirements for a functional RAIP-motif remain to be defined. The roles of the RAIP and TOS motifs in the mTOR-catalysed phosphorylation of 4E-BP1 remain incompletely understood. Here the requirements for a functional TOS motif are addressed.

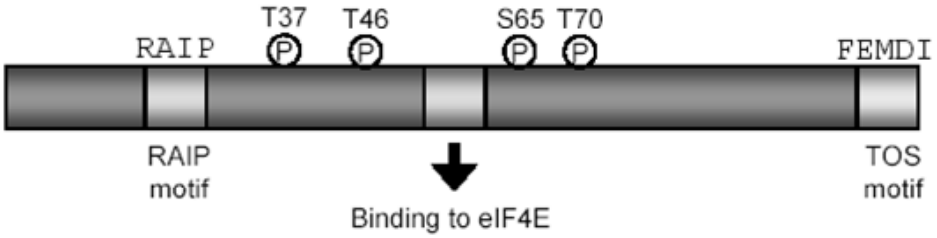

Fig. 7.1: Schematic diagram of 4E-BP1 showing the RAIP and TOS motifs, the region that binds eIF4E and the four phosphorylation sites studied in this report. Numbering is based on the human 4E-BP1

7.3. Materials and methods

7.3.1. Vectors

pRK5 myc-tagged human raptor was a generous gift from Dr. D. Sabatini (MIT, Boston, USA). pcDNA3.1 myc/his-tagged rat 4E-BP1 for mammalian expression and the pGEX-3X human 4E-BP1 for bacterial expression were a generous gift from Dr. A. Tee as used in previous studies (Tee and Proud, 2002; Beugnet *et al*., 2003).

7.3.2. Induction and expression of fusion proteins in *E. coli*

The *E. coli* strain BL-21 (DE3) pLys-S (Invitrogen, Burlington, ON, CA) containing the vectors with appropriate DNA inserts, were diluted 1:100 from overnight cultures into 500 mL Luria-Bertani (LB) (1% (w/v) Bacto-tryptone, pH7.5, 0.5% (w/v) Bacto-yeast extract, 0.5% (w/v) NaCl) containing 100 μg/mL ampicillin). The cultures were allowed to grow at 30^{o}C until the absorbance of the cultures at 600 nm was 0.5 (exponential growth). The expression of recombinant fusion protein was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) 200-500 μM and incubation at 30^{o}C or at room temperature for 2 to 4 hour before harvesting. The optimum conditions for expression were determined by pilot scale experiments at the various IPTG, temperatures and expression times.

7.3.4. Preparation of soluble and insoluble proteins from *E. coli*

E. coli were harvested from 500 mL of liquid culture by centrifugation at 4,000 *g* for 25 min at room temperature. The pellets were resuspended in 10 mL of buffer A (50 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 5% (w/v) glycerol, 0.03% Brij 35, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL antipain, 1 μg/mL pepstatin, 14 mM β-mercaptoethanol) and lysed by sonication using the Bandelin Sonopuls (UW 2070, Progen Scientific Ltd, UK) at 60% power (3x30 sec on ice). Soluble (e.g. proteins in the cytoplasm) and insoluble (e.g. membranes) proteins were separated by centrifugation at 28,000 *g* for 30 min at 4^{o}C.

7.3.5. Purification of soluble GST-fusion protein produced in *E.coli*

The soluble *E. coli* extract containing expressed GST-fusion protein was mixed in a rotating tube for 1 hour at 4^{o}C with 500 mL glutathione-Sepharose swollen and equilibrated in buffer A. The mixture was transferred to a 50 mL Falcon tube and washed 3 times with 10 times the bed volume of glutathione-Sepharose beads. Bound GST-fusion protein was then eluted at room temperature by addition of 10 mM reduced glutathione in buffer A. Fractions of 1 mL were collected and protein concentration determined by method of Bradford (1976). Fractions were pulled and dialysed against 1x3 L buffer A overnight at 4^{o}C to remove gluthatione. Dialised purified GST-fusion protein was frozen in liquid nitrogen and stored at -80^{o}C.

7.3.6. Polymerase chain reaction (PCR)

PCR amplifications were performed in a volume of 50 μL with 50 ng of DNA; 2.5 units of Pfu turbo enzyme (Stratagene, CA, USA) and 1X reaction buffer. Amplifications were carried out in a GeneAmp PCR System 2400 (Perkin-Elmer, Montreal, CA). Temperatures and time on each step was determined by the kind of PCR, annealing temperature of the oligonucleotide, taq polymerase and the size expected for the PCR product.

7.3.7. Determination of nucleic acid concentrations

DNA concentrations were determined by two different methods: direct, by absorbance at 260 nm, and indirect, comparing the DNA sample against a ladder in agarose gel electrophoresis. In the direct method, DNA concentration was calculated assuming 1 absorbance unit at 260 nm corresponds to 50 μg/mL of DNA.

The indirect method to calculate DNA concentrations involves running an agarose gel with different dilutions of the sample against a DNA ladder, where the amount of DNA corresponding to each band is known. Comparing the DNA of the sample with the ladder can indicate approximately the DNA concentration but importantly it indicates the quality of the DNA i.e. if the construct is the correct size and if there is any degradation.

7.3.8. Automated DNA sequence analysis

DNA sequencing was performed by sequencing service facilities (NAPs service, University of British Columbia, BC, CA) using Applied Biosystems methodology and equipment, i.e., Big Dye Terminator chemistry on an automated DNA sequencer. Purified plasmid DNA samples were diluted in water to a concentration of 200 ng/μL and primers were provided at a concentration of 5 pmol/μL in water.

7.3.9. Agarose gel electrophoresis

Agarose gels for separation of DNA fragments were prepared by melting in a microwave 0.8 to 1.5% (w/v) agarose in 0.5 X TBE buffer (0.1 M Tris-borate, pH 8, 2 mM EDTA). After boiling, the mixture was left to cool down to approximately 50^{o}C and then ethidium bromide was added (final concentration 5 ng/mL) and then mixed gently. Gels were run on H2-SET or H3-SET apparatus (Anachem, Bedfordshire, U.K.) at a constant current (100 mA) in 0.5 X TBE buffer. Samples were loaded in 5X DNA loading buffer. After electrophoresis, DNA was visualised using a UVI doc GAS9000 gel documentation and analysis system from Uvitec (Cambridge, UK).

7.3.10. Purification of DNA from agarose gels

After electrophoresis, DNA was extracted from agarose gels using the Gel Extraction kit from Qiagen (CA, USA), following the instructions from the supplier.

7.3.11. Site direct mutagenesis

Mutagenesis of 4E-BP1 at the TOS motif (FEMDI) was performed by PCR using the QuikChange® kit from Stratagene (Stratagene, CA, USA). The template used was the pcDNA3.1 myc/his-tagged rat 4E-BP1 for mammalian expression and the pGEX-3X human 4E-BP1 for bacterial expression vectors (Section 7.3.1). The primer selection was done by inspection of the sequences, and in some cases, internet programs (e.g. Webcutter 2.0, http://www.firstmarket.com/cutter/cut2.html) were used to introduce silent mutations.

7.3.12. Bacterial strains

The *E.coli* strains DH5α (genotype: F^-(ψ80dlacZΔM15), recA1, endA1, gyrA96, thi-1, hsdR17($r_k^-m_k^+$), supE44, relA1, deoR, Δ(lacZYA-argF), U169) and XL10 gold (genotype: $Tet^{r\Delta}$, (mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac Th[F'proAB $lacl^qZ\Delta M15Tn10(Tet^r)$Amy Cam^r]) were used for general transformation of plasmids. The *E.coli* strain BL21(DE3)pLysS (genotype: *E.coli* B F^-, dcm, ompT, hsd(r_B-m_B), gal λ(DE3), [pLysS Cam^r]) was used for expression of recombinant proteins.

7.3.13. Preparation of competent cells

A single colony of *E.coli* was picked from an agar plate and used to inoculate 50 mL of LB broth (1% (w/v) Bacto-tryptone pH 7.5, 0.5% (w/v) Bacto-yeast extract, 0.5% (w/v) NaCl).

After overnight incubation at 37°C at 200 *g*, 1 mL of the culture was then added to 200 mL of fresh LB broth and incubated until the absorbance (OD_{600}) was approximately 0.3 (exponential growth). The culture was then placed on ice for 15 min prior to centrifugation at 5,000 *g* for 5 min at 4°C. The pellet was drained thoroughly and resuspended by mild vortexing, in 16 mL of transformation buffer A (30 mM KAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol, pH 5.8; filter sterilized) per 50 mL of original culture. The cells were then incubated on ice for a further 15 min prior centrifugation at 5,000 *g*. The pellet was drained again and resuspended in transformation buffer B (10 mM 2-[morpholino] propane-sulphonic acid, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% (v/v) glycerol, pH 6.8; filter sterilized). The cells were frozen in liquid nitrogen and stored at -80°C.

Note: *The preparation of competent cells was done by Dra. Yanni Wang (UBC, Vancouver, CA).*

7.3.14. Transformation and selection of competent cells

To transform the competent cells, 0.1 ng of plasmid DNA was incubated in 100 µL of competent cells on ice for 30 min. This step ensures that the plasmid DNA is in close contact with the cell membrane. The samples were then incubated at 42°C for 45 to 60 sec to allow DNA incorporation into the cell and then placed back on ice for 2 min. 1mL of LB broth was then added and the cells were grown for 1 hour at 37°C to allow the expression of the antibiotic resistance genes encoded by the plasmid. The cells were then spread on to LB agar plates (LB medium with 1.5% (w/v) agar) supplemented with the appropriate antibiotic and incubated at 37°C for 16 hours.

7.3.15. Plasmid DNA preparation

Small scale preparation of plasmid DNA was performed using QIAprep® Spin Miniprep (Qiagen, CA USA). This technique was used to prepare DNA for transformation. A single bacterial colony was picked from a freshly streaked selective plate and incubated in 5 mL of LB medium containing the appropriate antibiotic overnight at 37°C with shaking at 250 *g*. Cells were pelleted from 3 mL of the culture and DNA was purified according to the manufacturer's protocol. At the last stage DNA was eluted in sterile distilled H_2O.

Large-scale preparation of plasmid DNA from *E. coli* was performed using Sigma Midi/Maxi Prep kits. A single bacterial colony was picked from a freshly streaked selective plate and incubated in 5 mL of LB medium containing the appropriate antibiotic overnight at 37°C with shaking at 250 *g*. 1 mL of this starter culture was used to inoculate 99 mL of LB medium containing the appropriate antibiotic and cells were grown overnight at 37°C with shaking at 250 *g*. Cells were pelleted and DNA purified following the manufacturer's protocol with one exception - the DNA was eluted in distilled H_20 because the EDTA present in the elution buffer inhibits PCRreactions.

7.3.16. Human embryonic kidney (HEK) 293 cell culture

HEK 293 cells were used as an insulin-responsive cell line that can readily be transiently transfected. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 0.1 mg/mL streptomycin (termed 'complete DMEM') and cultured in 10 cm dishes. Cells were grown

until 90% confluent and then split (every 2 to 3 days). To split the cells, the culture medium was aspirated, each plate was washed first with 10 mL PBS followed by incubation with one ml of trypsin in a 37^{o}C incubator for 5 min. After the cells detached, they were re-suspended in 5 mL of complete DMEM. One ml of a cell suspension was used to seed a 10 cm diameter dish for maintaining stocks or for experimentation, and was added to 9 mL of complete DMEM.

7.3.17. Transient transfection of HEK 293 cells

The calcium phosphate method was used for the transfection (Chen and Okayama, 1988) whereby a calcium/phosphate (125 mM $CaCl_2$, 50 mM BES pH 6.96 (critically important), 280 mM NaCl, 1.5 mM Na_2HPO_4)-DNA co-precipitate is formed which is taken up into the cytoplasm of cells and subsequently transferred to the nucleus. HEK 293 cells were seeded at a density of 0.2x10^{6}/mL cells in 10 cm diameter dishes and after 8 hours at 37^{o}C in complete DMEM, the cells were transfected with 10 μg of DNA per dish. After transfection the cells were placed in the incubator at 37^{o}C and 5% CO_2 for 12 hours, after which the medium was aspirated and replaced with 10 mL fresh complete DMEM.

7.3.18. Cell treatment and preparation of cell lysates

Prior to treatment, 90% confluent HEK 293 cells were starved of serum for 16 hours to reduce the basal levels of phosphorylation. To starve cells, plates were rinsed once in

Dulbecco's phosphate-buffered saline (D-PBS) containing 10 mM D-glucose (D-PBS/glucose) and then incubated in the same solution minus FCS. In some instances, cells were also treated with 100 nM rapamycin for 30 min, followed by stimulation with 100 nM insulin for 25 min. Cells were lysed in 400 μL of extraction buffer containing 50 mM β-glycerophosphate (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM Na_3VO_4, 0.1% (v/v) β-mercaptoethanol, protease inhibitors (leupeptin, pepstatin and antipain, each 1 μg/mL) and phenylmethylsulfonyl fluoride (200 μM). Lysates were pre-cleared by centrifugation at 13,000 *g* for 10 min at 4^{o}C. Typically, 20 μg of total protein lysate were used for analysis by SDS-PAGE/western blotting.

7.3.19. Determination of protein concentration

Described in the Materials and methods of Chapter 3.

7.3.20. SDS-PAGE

Described in the Materials and methods of Chapter 3.

7.3.21. Staining, fixing and drying polyacrylamide gels

Described in the Materials and methods of Chapter 3.

7.3.22. Electrotransfer

Described in the Materials and methods of Chapter 3.

7.3.23. Western blot analysis

Described in the Materials and methods of Chapter 3.

7.3.24. Primary antisera

Described in the Materials and methods of Chapter 5.

7.3.25. Far western blotting using raptor lysate

Far-western blotting is used to identify protein:protein interactions, in this case far-western blotting was used to detect binding of the mTOR-partner protein raptor to 4E-BP1 as it does to other mTOR substrates (e.g. S6K1 (Nojima *et al*., 2003)). HEK293 cells grown as described in section 7.3.16 were split from one confluent plate to four plates 7 hours prior to transfection with a vector encoding pRK5 myc-Raptor. Transfection was performed using calcium phosphate precipitation as described in section 7.3.17. using 5 μg of DNA per 10 cm plate. The transfection mix was vortexed for 1 min and left to stand for 5 to10 min until a cloudy precipitate was formed, then 1 mL of mix added per 10 cm plate. After 24 hours, the medium was changed for fresh medium on each transfected plate. 40 hours after transfection, the cells were lysed with 400 μL of cell extraction buffer. After cell

scraping, the lysates were centrifuged for 10 min at 13,200 *g* at 4ºC, and the supernatants containing over-expressed myc-raptor were collected.

Meanwhile GST fusion proteins of recombinant GST 4E-BP1 were subjected to SDS PAGE, and electrotransferred onto PVDF membrane. The blot was then incubated with 10 mL of lysates (from the raptor-transfected HEK 293 cells) overnight and probed for anti-Myc (Chapter 3, Table 3.2) using secondary anti-mouse HRP conjugated antibody and detected using ECL detection system. The blot was stripped of antibody by incubating at 50°C in stripping solution (Pierce, IL, USA) for 30 min, followed by two rinses in PBS-Tween. The blot was re-blocked in 5% milk and probed using anti-GST antibody for total protein using an anti-rabbit HRP conjugated secondary antibody.

7.4. Results

7.4.1 Regions of 4E-BP1 involved in binding to raptor

This work and previous work (Beugnet *et al*., 2003) showed that mutation of the phenylalanine in the TOS motif to alanine eliminates the binding of raptor to the human 4E-BP1 in the overlay assay (see also Fig. 7.2). This study has not observed binding of raptor to a truncated 4E-BP1 molecule lacking the final six residues that harbour the TOS motif (Δ6; Fig. 7.2). This confirms that the TOS motif is essential for stable binding of raptor to 4E-BP1, but does not show whether it alone is sufficient.

The data presented here (Fig. 7.2) further confirm the key role that the TOS motif in 4E-BP1 plays in binding to raptor in the far-western analysis employed here. So far no detailed analysis has been performed to define which residues are actually required for a functional TOS motif. Such data could be helpful in identifying potential TOS motifs in other proteins. Here, two approaches were employed to study this: (i) the ability of 4E-BP1 variants to bind to raptor and (ii) the ability of a given TOS-like motif to promote the phosphorylation of 4E-BP1 in cells.

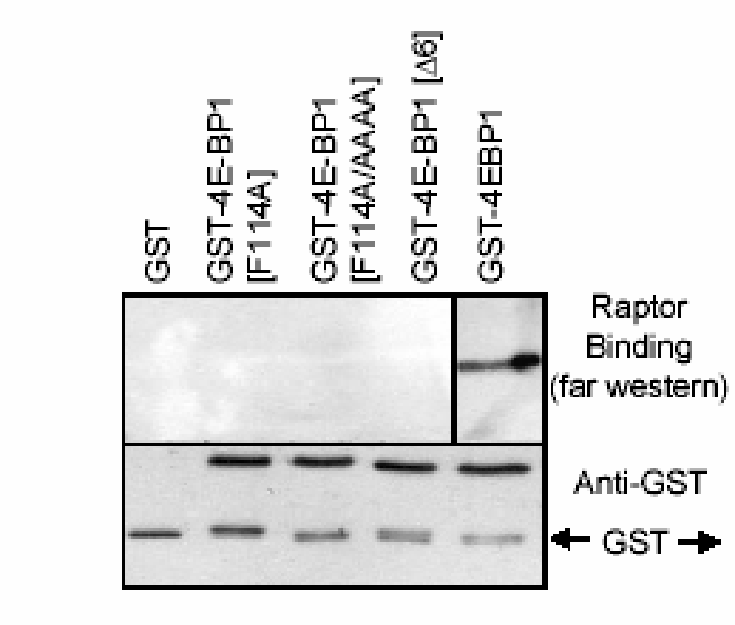

Fig. 7.2: Analysis of binding of raptor to variants based on 4E-BP1. Binding of raptor to variants of 4E-BP1 assessed using the overlay assay ("far western" see material and methods) was assessed using the truncation mutants of 4E-BP1, each expressed as a GST-fusion protein in *E. coli*. The upper section of each panel shows the blot for myc-raptor and the lower sections show a blot with anti-GST to show the amounts of each protein used, as assessed by western blot with anti-GST. From right to left: wild type 4E-BP1; truncated 4E-BP1 molecule lacking the final six residues that harbour the TOS motif; 4E-BP1 with mutate TOS motif (alanines); 4E-BP1 mutation phenylalanine to alanine; GST variant

7.4.2. Defining what constitutes a functional TOS motif

To test the ability of 4E-BP1 variants to bind to raptor it was first necessary to analyze if the TOS motif segment alone was sufficient to confer binding to raptor. To test this, the sequence FEMDI (the TOS motif found in the C-termini of mammalian 4E-BP 1-3) was added to the C-terminus of GST. To obviate possible issues of steric hindrance, a spacer (four alanine residues) between the C-terminus of GST and the TOS motif was provided to create "GST-Ala_4-TOS". As shown in Fig. 7.3, the addition of the TOS motif to GST did not allow raptor binding. This is consistent with the data in Fig. 7.2 and Beugnet *et al*. (2003) which show that additional features in 4E-BP1 are required for raptor binding but that the TOS motif is nonetheless essential.

The effects of altering the TOS motif in 4E-BP1 for both approaches (i) and (ii) above were therefore examined. Phosphorylation of 4E-BP1 involves multiple sites and a rather complex hierarchy (Gingras *et al*., 1999a; Gingras *et al*., 2001; Mothe-Satney *et al*., 2000b; Beugnet *et al*., 2003; Wang *et al*., 2003). To assess the effects of the alterations in the TOS motif, the phosphorylation state of Ser64 was examined, as this site is late in the hierarchy and, hence, 'integrates' the effects of phosphorylation of other sites in 4E-BP1. It has been previously showed that mutation to alanine (A) of the phenylalanine (F) in the 4E-BP1 TOS motif markedly impairs the phosphorylation of Ser64 (Tee and Proud, 2002). The present data also show that the F114A mutation completely blocked the ability to bind raptor in a far-western blot (Fig. 7.3, 7.4A) and almost eliminated the phosphorylation of 4E-BP1 at Ser64 (Beugnet *et al*., 2003) (Fig. 7.5B).

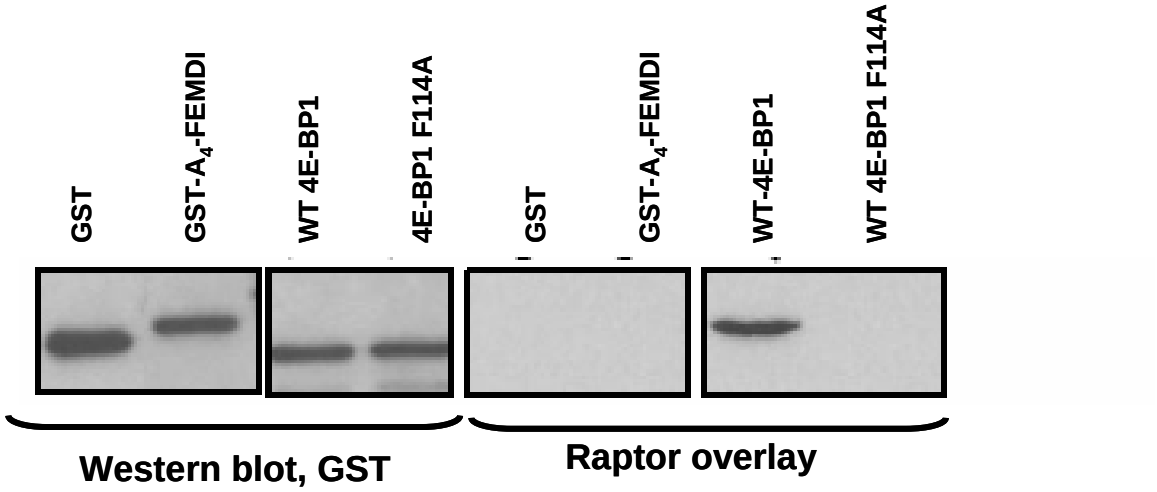

Fig. 7.3: The overlay assay (see materials and methods) was used to assess the binding of raptor to GST or GST to which a four alanine spacer and the TOS motif (FEMDI) was attached. hGST-4E-BP1 and GST-4E-BP1 (F114A) serve as positive and negative controls, respectively. Left side: western blot for GST to assess levels of each protein; right side, raptor overlay, developed with anti-myc

The phenylalanine (F) to alanine (A) change is clearly a major one, and it was tested whether the more conservative mutation of the aromatic phenylalanine (F) to a bulky aliphatic residue also affected function. The F113L ('LEMDI') mutant underwent insulin-stimulated phosphorylation at Ser65 to a similar degree to the wild type protein (Fig. 7.5C). In this, and all other cases, this phosphorylation was blocked by rapamycin, confirming that it requires mTORC1. However, the LEMDI variant failed to bind raptor in the far western assay (Fig. 7.4A). The simplest explanation for this is that the mutation weakens the TOS and raptor interaction to the extent that it is insufficiently stable to 'survive' the washes of the far western procedure, but can still support an interaction *in vivo*. These data imply that merely checking raptor binding in, e.g., a far-western, does not indicate what constitutes a functional TOS motif. In contrast to the LEMDI variant, the IEMDI mutant underwent only a small degree of phosphorylation at Ser64 (Fig. 7.5B). This variant did not bind to raptor in the overlay assay (Fig. 7.4A). It is notable that all the currently known TOS motifs have phenylalanine in the first position (Table 7.1).

A set of other variants based on the FEMDI sequence found in the 4E-BPs were then systematically created. Mutation of the second residue (glutamate (E)) to another acidic one (aspartate (D)) had no effect on raptor binding (Fig. 7.4A) and therefore the effect on the phosphorylation of 4E-BP1 was not examined. Changing the second residue to valine (V) (Fig. 7.5D) or to alanine (A) (Fig. 7.5D) did not discernibly affect the phosphorylation of 4E-BP1 in HEK293 cells. Replacement by proline (P) (Fig. 7.5E) did slightly impair the phosphorylation of Ser64 (Fig. 7.5E). Mutation to arginine (carries

Table 7.1: Known or potential TOS motifs in selected proteins of *H. sapiens*. Bold indicate putative TOS motifs: others have been shown to function in their respective proteins

Protein	Sequence	Residue numbers
S6K1	**F**DI**D**L	5-9 [1]
S6K2	**F**DL**D**L	5-9 [1]
4E-BP1	**F**E**MD**I	114-118
4E-BP2	**F**E**MD**I	116-120
4E-BP3	**F**E**MD**I	86-90
STAT3	**F**P**M**EL **F**D**MD**L	26-30 756-760
PKCδ	**F**V**M**EF	425-429
PKCε	**F**V**M**EY	484-488
HIF1α	**F**V**M**VL	99-103
PRAS40	**F**V**M**DE	129-133

[1] numbering based on the shorter splice variants of these proteins

positive charge; Fig. 7.5F) substantially decreased the phosphorylation of 4E-BP1 when compared to the wild type protein. Raptor binding for all these variants was similar to the wild type protein (Fig. 7.4A,C). Thus, although an acidic residue is present at this position in the 4E-BPs (E) and the S6 kinases (D) (Table 7.1), this feature does not actually appear to be very important for regulation of 4E-BP1 or for raptor binding. Interestingly, the TOS-like motifs in PRAS40 and HIF1α (FVMDE and FVMVL, respectively (Oshiro *et al*., 2007)), each lack an acidic residue at the second position. They have valine (V) in this position instead, which is clearly as effective as an acidic residue in promoting the phosphorylation of 4E-BP1 at Ser64.

In contrast to the high degree of tolerance for variations in the second position, mutation of the third residue (methionine (M), an uncharged, relatively non-polar amino acid) to alanine (A) or glutamate (E) abolished raptor binding (Fig. 7.4B). The methionine (M)→ alanine (A) mutation also severely decreased phosphorylation of Ser64 (Fig. 7.5F), and the phosphorylation of Ser64 was also decreased by placing glutamate (E) or, to a lesser extent, arginine (R) at this position (Fig. 7.5G). Mutation of the methionine (M) to isoleucine (I) (also a non-polar, aliphatic residue) maintained Ser65 phosphorylation at wildtype levels (Fig. 7.5E). It seems likely that the presence of an aliphatic residue with a larger side chain than a methyl group is required for function.

The fourth residue in the motif is an acidic one, aspartate (D). This was mutated to alanine (A) (Fig. 7.5H), asparagines (N) (Fig.7.5H) or arginine (R) (Fig. 7.5I). The substitution by alanine (A) very substantially decreased the phosphorylation at Ser64, but the

phosphorylation of the FEMRI variant was similar to that of wild type 4E-BP1 and that of the FEMNI protein was intermediate between the other two mutants (Fig. 7.5H). None of these three variants was able to bind raptor (Fig. 7.4B,C).

Mutation of the final residue from isoleucine (I) to arginine (R) or alanine (A) reduced raptor binding (Fig. 7.4B), but had little effect on Ser65 phosphorylation (Fig. 7.4C, I). The effect of an acidic residue at this position, i.e., the FEMDE variant was also tested. Phosphorylation of this mutant at Ser65 was slightly reduced compared to the wild type protein (Fig. 7.5D) but it was still able to bind raptor, although less well than wild type 4E-BP1 (Fig. 7.4C). In view of this tolerance for a variety of residues at position five, further mutations were not created here.

It seems surprising that several variants failed to bind raptor in the overlay assay but still underwent substantial phosphorylation within HEK293 cells (e.g., the FEMRI and FEMDR variants). It was possible that the use of denatured 4E-BP1 in the far western assay led to misleading results, although this did not seem likely, as 4E-BP1 reportedly has little if any folded structure (Fletcher *et al*., 1998). For this purpose, dot blot overlay assays in which the GST-4E-BP1 was applied to the membrane without prior resolution on a denaturing gel was also generated. This yielded very similar results to the far western assays, i.e., all of the variants that were negative in the far western, including the two just mentioned were also negative in the dot blot assay, while wild type 4E-BP1 and the FAMDI variants bound raptor in both assays (data not shown).

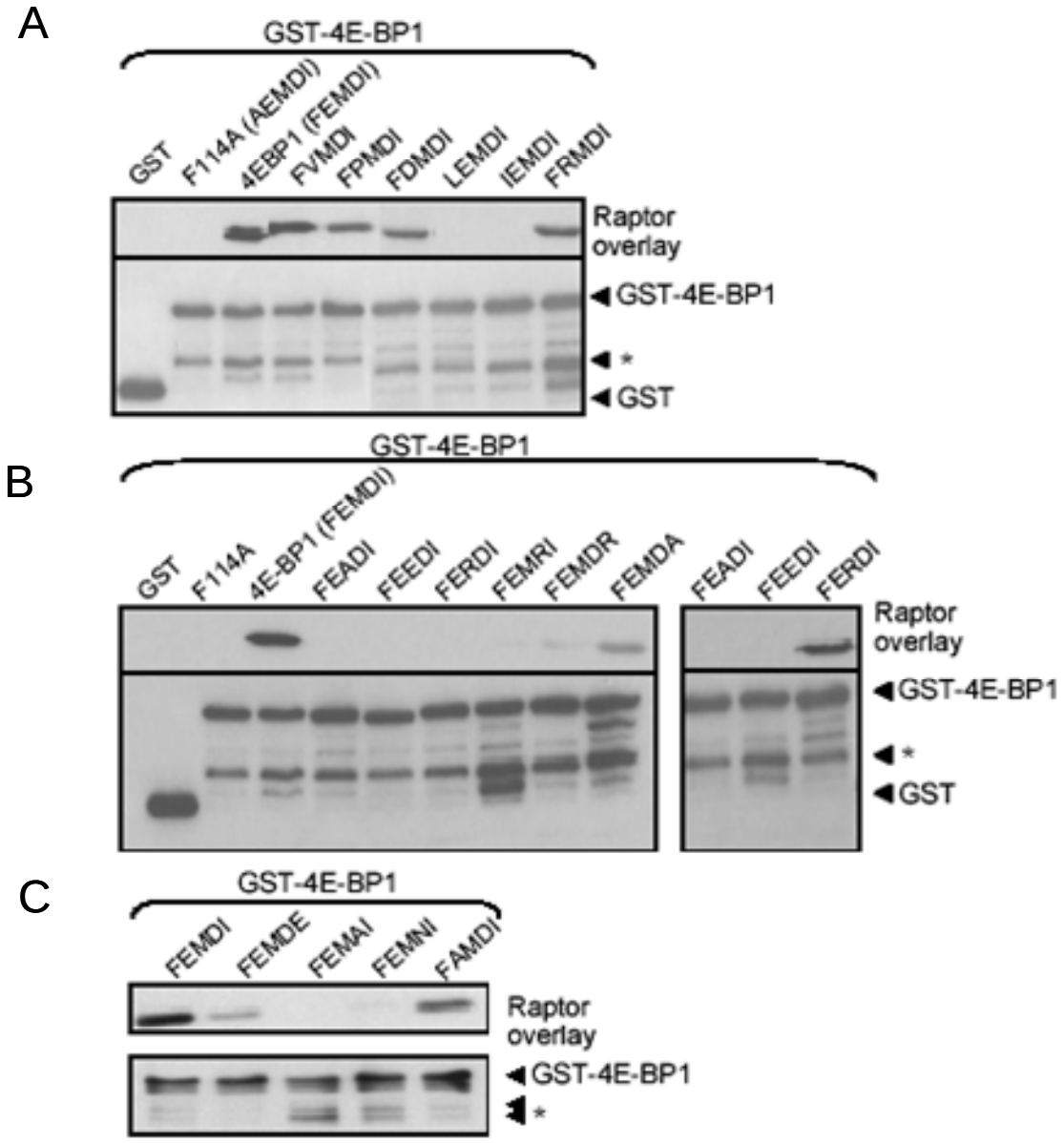

Fig. 7.4: Ability of TOS motifs variants to support the binding of 4E-BP1 to raptor. (A-C) wildtype 4E-BP1 or variants containing the indicated (putative) TOS motifs (as noted) were expressed as GST-fusions in *E. coli*. Binding to raptor was assessed using the overlay assay (upper part).The overlay assay was used to detect binding of raptor to wild type (GST-)4E-BP1 (FEMDI), or mutants with the indicated sequences in the place of the TOS motif. GST and GST-4E-BP1 [F114A] serve as negative controls. Upper section of each panel shows the myc-raptor overlay; lower sections show blots with anti-GST to assess the amounts of each protein used. Arrowheads with asterisks denote degradation products that react with anti-GST (but do not bind raptor)

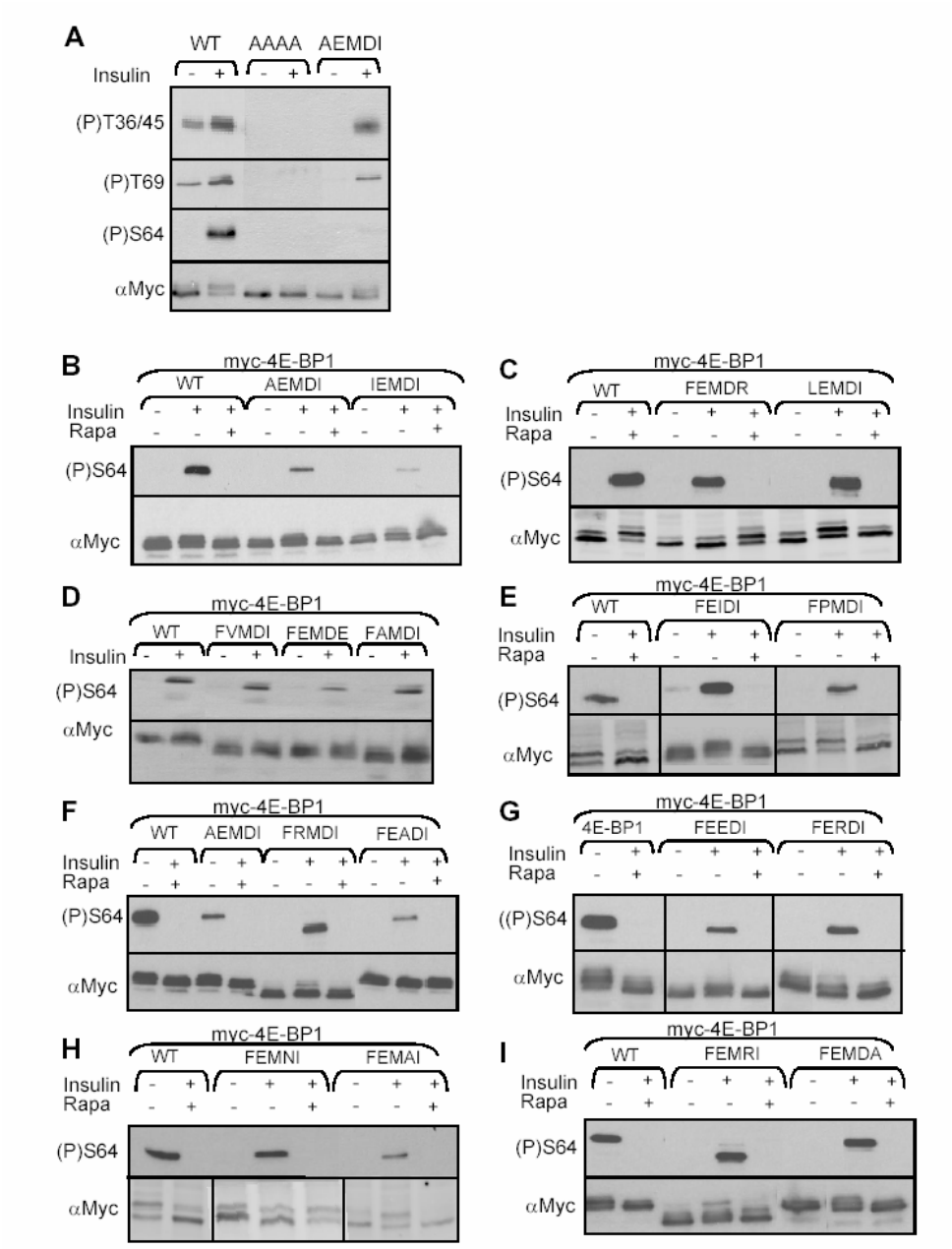

Fig. 7.5: Phosphorylation of 4E-BP1 variants expressed in HEK293 cells. (A-I) wildtype 4E-BP1 or variants containing the indicated (putative) TOS motifs (as noted) were expressed in HEK293 cells. Cells were starved of serum and treated with insulin as indicated and/or rapamycin (Rapa) as indicated (see materials and methods for details). Samples were analysed with the indicated phosphospecific anti-4E-BP1 (P)Thr36/45, (P)Thr69 and (P)Ser64 antibody (upper section of each panel) or anti-myc (bottom section in each case; to assess expression levels of 4E-BP1 variants). Top part of the figures represent mutation sites (e.g. AAAA, AEMDI, IEMDI). As described elsewhere in this Chapter, 4E-BP1 runs as multiple species on SDS-PAGE, the more highly phosphorylated species running more slowly. Only the slowest migrating species is phosphorylated at Ser64

7.5. Discussion

The present study provides new information on the features of 4E-BP1 that are required for its interaction with raptor and/or for its phosphorylation at different sites in living cells. The findings demonstrate that the TOS motif is essential for interaction with raptor and for phosphorylation of specific sites in cells while other features of 4E-BP1 are probably necessary for this interaction. Indeed, the five amino acid-TOS motif are not sufficient to confer binding to raptor. This study represents the first systematic attempt to define the sequence requirements of the TOS motif. Analysis of the TOS motif demonstrates that ability to bind raptor *in vitro* is not a reliable index of function since a number of variants that fail to bind raptor did support phosphorylation of 4E-BP1 within cells. Mutants that bind raptor *in vitro* effectively support phosphorylation within cells, but the converse is not true e.g., the LEMDI mutant which does not bind raptor but is as effective as the wild type sequence at facilitating phosphorylation (Figs. 7.4A and 7.5C). Therefore studying phosphorylation of 4E-BP1 is a more reliable way of assessing TOS-motif function than *in vitro* raptor binding.

The first position in the TOS motif is a critical one: mutation of the phenylalanine (F) residue to another closely similar residue, isoleucine (I), almost abolishes phosphorylation of Ser64. Consistent with this, all the known TOS motifs have phenylalanine (F) at this position. The data indicate that the nature of the second residue in the motif is less critical. Thus, although the first motifs to be identified in the S6Ks and 4E-BPs contain acidic residues, a range of other residues support phosphorylation of 4E-BP1 in cells. The diversity of these 'functional' residues (valine (V), alanine (A), praline (P)) indicates a

range of side-chain is tolerated here. Interestingly, the least effective one tested (arginine (R) (Fig 7.4.A) is positively-charged residue, which is not found in any known TOS motif. Indeed, the known TOS motifs have either an acidic residue or valine (V) (which works well in 4E-BP1, Fig. 7.4.A).

Another aliphatic residue, isoleucine (I), and positively or negatively charged residues, arginine (R) and glutamate (E), were tested in the third position occupied by methionine (M) in the 4E-BPs, HIF1α and PRAS40 (Table 7.1). Isoleucine (I) worked as well as methionine (M), which is consistent with the fact that this position is occupied by isoleucine (I) in S6K1 and leucine in S6K2 (Table 7.1), and suggesting a need for a bulky side chain. Although almost all the known TOS motifs have an acidic residue (glutamate (E) or aspartate (D), Table 7.1) in the fourth position, arginine (R) also functions well in this position (FEMRI, Fig. 7.5I).

Finally, alanine (A) or arginine (R) worked well (Fig. 7.5C,I) in the fifth position where leucine (L) or isoleucine (I) are found in all known TOS motifs, except PRAS40 (Table 7.1). Glutamate (E) was less effective, even though the PRAS40 motif contains glutamate (E) at this position (Table 7.1). The fact that the FEMDE motif was not fully effective in 4E-BP1 may reflect that fact that the final glutamate (E) is also the C-terminal residue of the entire mutant protein and thus actually carries two negatively-charged carboxyl groups, whereas in PRAS40 this is not the C-terminal residue and thus carries only one negative charge.

The present data suggest that the nature of the first and third residues in the motif is particularly important, at least for phosphorylation of 4E-BP1. Hydrophobic residues are required at both positions with phenylalanine (F) or leucine (L) in the first position and methionine (M), isoleucine (I) or probably similar residues in the third. The requirements at the other positions are less strict and a variety of residues seem to be tolerated in the second and fourth positions Based on our data and the recent information of newly-discovered TOS motifs (Fonseca *et al*., 2007) there is no strict need for an acidic motif in the second and fourth positions as deduced from the first TOS motifs characterized (Nojima *et al*., 2003). Although a positively-charged one seems detrimental at position 2 (FRMDI, Fig. 7.5.F), this is not so in position four (FEMRI, Fig. 7.5I). The situation for the final residue is less clear, since an acidic residue is detrimental in 4E-BP1, but functional in PRAS40.

CHAPTER 8

General Discussion

8.1. Methodological Considerations

The objective of the research was to determine the molecular mechanisms that control milk protein synthesis in the mammary gland. The regulation of gene function can occur at many levels including transcription (mRNA) and translation (protein). Consequently, the study of changes occurring during these two steps is critical and equally important. Moreover, interactions between transcripts and proteins levels, represents a complex system and studies in the literature have shown that in some cases the mRNA abundance is a poor indicator of protein expression levels (Chuaqui *et al*., 2002). The advent of high throughput technologies (such as microarrays) for determining the expression patterns of thousands of genes has enabled researchers to study these complex interactions and to unravel the complex molecular mechanisms involved in the coordination of biological processes. Once target genes have been identified or discovered using microarrays, other techniques such as RNAi and cDNA over-expression can be used for evaluating gene function.

In this section, general aspects of some of the key methodologies used in the experiments are considered while more specific analytical methods have already been outlined in each Chapter.

Changes in gene expression are usually observed in response to physiological changes or external stimuli. To adapt to these changes, one of the mechanisms that the cell employs is to adjust mRNA production (transcription). Changes in mRNA abundance are highly complex and tightly regulated in many tissues including the lactating mammary gland (Sakamoto *et al*., 2005). In the past, scientists studied gene expression changes by

comparing one gene at a time. With the advent of microarray technology, scientists can now survey the expression of large numbers of genes simultaneously.

Arrays have been used to study gene expression profiles in multiple tissues and in multiple species (Schmidt and Begley, 2003; Rowland *et al*., 2005; Bose *et al*., 2006) such studies also include those investigating bovine mammary gland growth and development and milk production (Suchyta *et al*., 2003; McCoard *et al*., 2004). When the research presented in this thesis started, this technology was just being developed. Notably, bovine micro arrays slides were not commercially available, largely because this was prior to the sequencing of the bovine genome which started in 2003. The development of AgResearch proprietary bovine microarrays offered a unique opportunity to utilize this technology to study the regulation of milk protein synthesis in the bovine mammary gland. Thus, this research was one of the first studies world-wide using bovine microarrays.

Despite the great potential of microarrays, one limitation of this technology is that it only provides a single picture in time of the changes that are happening in the system, and due to the expense it is not always possible to conduct numerous studies using this technology. Another limitation of microarrays is that following initial screening, the validation of the differential expression of candidate genes is required using quantitative real-time PCR (qRT-PCR). qRT-PCR is a commonly used validation tool for confirming gene expression results obtained from microarray analysis; however, microarray and qRT-PCR data often result in disagreement (Morey *et al*., 2006). Thus, such validation is critical as microarrays have inherent problems leading to statistical errors, which can lead to false positive results

(Grant *et al*., 2005). The problem around false positive discovery has been already discussed in detail in Chapter 2.

Besides the advantage to analyse the expression levels of a large number of genes simultaneously, microarrays are also a useful tool for the discovery of gene function based on similarities in expression patterns with those of known genes. Commercially available arrays are now available from different companies and from a range of different species (examples include human, mouse, rat, bovine and chicken). Because the product of any one gene usually interacts with those of many others (as illustrated by the Ingenuity Pathway Analyses (IPA) software (e.g. see Bunger *et al*., 2007)), pathway analysis is used to help our understanding of gene interactions. Different tools are available for pathway analyses and two of them, IPA and FUNC, have already been discussed in more detail in Chapter 2.

There are different types of microarrays and each one has its own peculiarities. The arrays used here were the two colour arrays which employ clones based on EST sequences that are spotted onto glass slides with two differentially labelled cDNA samples hybridized together on the array (Schena *et al*., 1995). For one-colour platforms such as the Affymetrix GeneChip, a single sample is hybridized to each array (Lockhart *et al*., 1996). In the two colour array, the array-to-array variability of the spotted arrays can be quite large, but the pairing of samples effectively removes this source of variation from treatment comparisons. In the one colour array, the precision of the measurement is achieved by minimizing array-to-array variability. Studies have been conducted to compare different platforms but there is still not a clear consensus as to the most effective platform (Tan *et al*., 2003; Yuen *et al*., 2002). Thus, the success of an array depends on the experimental design and tightly controlled array production and hybridization methods.

The initial hypothesis of this research, supported by current literature (Rosen *et al*., 1999), was that milk protein synthesis was dependent on changes in the transcription (level of mRNA) of the milk protein genes. Thus, the initial objective of this work was to establish the gene pathways that influence the regulation of milk protein gene transcription using microarray technology. However, the array results showed that expression of the milk protein genes after the GH and atropine treatment, were not changed (Chapter 2). Small increases in expression, e.g. 10-30% were observed and coincidently these changes were aligned with the changes observed in milk protein levels (e.g. 30% increase in milk protein yield following GH treatment; Appendix A). These small changes in gene expression could not be validated by qRT-PCR (Chapter 2). In my studies and that of others (Morey *et al*., 2006), qRT-PCR was unable to confirm small changes in gene expression (i.e. less than 1.5 fold differences) which raises the question of whether or not the differences observed in the microarray results were real or simply false positives. Unfortunately, it was not possible to answer this question at the time this research was done because there was no reliable technology available that was more sensitive than qRT-PCR. Future research using new tools, such as Sequenom MassArray Quantitative Gene Expression system (Sequenom, MA, USA) which producers claim sensitivity levels to about 10% differences in expression could potentially help with addressing this issue (Sequenom MassArray, MA, USA). Another point to be considered is the impact of genetic variability between animals which could create sufficient variability and potentially mask changes in gene expression profiles resulting from treatment effects. Thus, it was concluded from the present results that the qRT-PCR results were real and no change in gene expression (except for α_{s2}-casein) is involved in the regulation of milk protein synthesis.

The observation that changes in protein yield and concentration in milk following GH or atropine treatment (Appendix A), were not accompanied by significant changes in the transcript levels of the milk protein genes (Chapter 2), suggested a change in milk protein translation (Chapter 2). The potential involvement of the mTOR pathway, a known regulator of translational control, was evaluated using the microarray results. The arrays data firstly confirmed that this pathway was expressed in the mammary gland and secondly showed that there were changes in expression of some genes of this pathway represented in the array. The array data also provided information of other pathways upstream and downstream of mTOR and the observed gene expression profiles in these pathways supported a potential role for the involvement of the mTOR pathway in mediating the effects on milk protein synthesis.

After the target pathways and specific candidate genes are identified or discovered using microarrays, other tools are employed to study gene function. Several methodologies exist to predict gene function(s) and to establish physiological relevance of a particular gene. Cell assays are useful in that they enable the discovery of causal connections between gene activity and a cellular phenotype of interest. Two very popular tools used for modulating gene activity were employed in this thesis - RNAi (gene silencing) and cDNA over-expression. In cDNA over-expression, the expression level of each gene is increased by transfection of a plasmid containing the cDNA under control of a mammalian promoter. In RNAi knockdown, the expression level of a single gene is reduced by introducing into cells an RNAi "trigger" that specifically interferes with the expression of that gene. In this thesis, RNAi was used to study the role of eEF2 (eukaryotic elongation factor 2) in the stimulation of protein synthesis by GH (Chapter 6) and cDNA over-expression was used to identify the

functional requirements for the TOR signaling (TOS) motif in the eukaryotic initiation factor (eIF) 4E binding protein (4E-BP1). These methods can potentially be used in future studies to evaluate the function of the identified genes in this study (i.e. members of the mTOR signalling pathway) in the lactating mammary gland. To my knowledge, there are no studies published using cDNA over-expression and gene silencing techniques of initiation and elongation translation factors and their effect on milk protein synthesis.

These two different approaches are complementary in practice. For example, cDNA over-expression is used to validate hits from RNAi screens, while RNAi is used to validate hits from over-expression screens. cDNA over-expression and RNAi techniques complement each other in a way that some of the difficulties found in one technique do not apply to the other. Examples of this include: 1) cDNA over-expression requires the whole gene sequence unlike RNAi; 2) RNA is easily degraded, reducing the efficiency of transfection for RNAi, whereas cDNA is more stable; 3) the RNAi molecule represents 'off-site targeting" when it is not totally specific and splice variants represent a particularly difficult design challenge for RNAi, whereas each cDNA codes for exactly one mRNA and exactly one protein; 4) high concentrations of an over-expressed protein may be cytotoxic or may increase the probability of low-affinity binding interactions that stimulate off-target biochemical pathways, an issue not observed with RNAi. The application of both approaches in parallel will yield the most complete understanding of the complexities of gene function. Furthermore, using these two approach in parallel is important because in order to establish the function of a gene it is important to know what happens to the phenotype when a given gene is turned off (down-regulated) and/or turned on (up-regulated) as resulting effects may be different and involve different mechanisms.

As a result of the complementary nature of gene knockdown (using RNAi) and over-expression, it is suggested that for future experiments, both techniques should be used in parallel to confirm the hypothesis that the mTOR pathway, in particular, ribosomal protein S6 (S6), eIF4E and eEF2 are important in the control of milk protein synthesis. Limitations for achieving this objective include the availability of the complete gene sequence for these proteins due to the incomplete nature of the bovine genome. It is suggested that experimental models using primary bovine mammary gland cells should be employed as these reflect more realistically what is happening in the lactating mammary gland when compared to cell lines.

8.2. General discussion

The research presented in this thesis has addressed the identification of specific genes and pathways involved in the regulation of protein synthesis in the mammary gland. This is an important topic for the milk industry because the new knowledge generated may open new opportunities to increase the amount and value of milk products.

The abundance of mRNA for the milk protein genes, with the exception of that for α_{s2}-casein, did not change in response to the GH and atropine treatments described in this thesis. However, the output of milk protein was altered by these treatments, indicating that mechanisms besides changes in the mRNA concentrations are involved in regulating the production of the individual milk proteins, which is probably regulated at the level of translation or subsequent processing rather than at the level of transcription.

Bearing in mind that the mTOR signaling pathway is one of the key control points of protein translation, it was hypothesized that the control of milk protein synthesis occurred at the initiation, especially the formation of the eIF4F complex, and elongation stages of mRNA translation. Based on the array results, GH and atropine treatments changed the expression of genes involved in post-translational mechanisms (ribosomal proteins, elongation and initiation factors - Chapter 2). Additionally, analyses of the signaling pathways (cell proliferation, ribosome, protein synthesis, translation elongation) that were altered in response to these treatments (Chapter 2), link the observed changes in protein synthesis (Appendix A) with mRNA translation. Supporting this hypothesis, the protein expression patterns and modifications (i.e. phosphorylation) of key members of the mTOR

pathway were consistent with the observed changes in milk protein synthesis (Chapter 3). Specifically, GH treatment, which increased the yield of milk protein, also affected the phosphorylation of S6 and the abundance of eEF2 and eIF4E proteins in the mammary gland. These results suggest a relationship between milk protein yield and changes in the initiation and elongation stages of translation.

Atropine treatment, although decreasing milk protein yield, did not appear to affect the initiation and elongation stages of translation. This leaves the question open as to how atropine treatment rapidly reduces milk protein yield and concentration. It is suggested that this involves an amino acid sensor inside the secretory cell that either decreases the use or transport of amino acids in the mammary gland. Another possibility is that because samples were collected at discrete intervals, critical translational changes could have been missed. If that is the case, would short-term changes in phosphorylation of translation factors stimulate sustained physiological changes in the cell that ultimately influence milk protein yield (GH treatment) and percentage (atropine treatment)? There is no evidence that suggest that short-term changes in the functionality (e.g. phosphorylation status) of the translational regulators can have long-term effects on phenotype and this appears to be an important question to be answered. Studies to elucidate the role of translation regulators within the milk secretory cells during different time points for sample collection may shed light on how protein synthesis is regulated in the mammary gland.

Another important finding reported in this thesis was the elucidation of how GH acts through PI 3-kinase to activate mTOR, or more specifically the mTORC1 complex. This discovery is perhaps of more relevance to the medical field where the understanding of

biochemical signaling pathways makes possible the development of drugs to inhibit or stimulate the GH effects in the system. This study also raises the possibility that the longer-term effects of GH such as on milk yield over the whole lactation, and cell maintainance and proliferation, may also involve GH-activated mTOR signaling and consequently the two key steps in mRNA translation, initiation and elongation. There is so far a surprising paucity of information on the control of translation machinery throughout lactation. These data suggest new approaches for future work in this area, for example *in vivo* RNAi and overexpression experiments discussed previously (section 8.1)

Additionally, the GH stimulatory effects on protein synthesis do not seem to be primarily controlled by changes in phosphorylation of eEF2 (Chapter 6) raising the possibility that the initiation of translation is a key control point in the response to GH. However, the regulation of elongation rates during GH stimulation in comparison to initiation rates remain poorly defined, specially the regulation of eEF2 kinase (eEF2K). Studies on the regulation of eEF2K through its phosphorylation and the identification of unknown phosphorylation sites on this kinase may reveal unknown signaling connections in the mTOR signaling cascade.

The TOS motif is essential for the 4E-BP1 interaction with Raptor and for phosphorylation of specific sites in cells. The identification of functional requirements for the TOS motif will provide further insight into the identification of other proteins that may be controlled by mTORC1 and thus, regulate mRNA translation. One important question to be answered in this field is the requirements for a functional RAIP-motif and the possible interaction that the RAIP and TOS motifs play in the mTOR-catalysed phosphorylation of 4E-BP1. This is

a highly competitive field and one anticipates that these questions will soon be answered. Studies are ongoing to identify novel requirements for a functional RAIP-motif and will lead to a better knowledge of the interaction of these two motifs. These studies are still been conducted in a *in vivo* model but it is anticipated that the knowledge gained with this research can elucidate more pathways involved in the regulation of 4E-BP1.

New roles for the mTOR signaling pathway are still being revealed. However, it is well known that mTOR is a key regulator of the nutrient and hormonal status in the cell (Proud, 2004) and of protein synthesis in general (Wang and Proud, 2006). In the lactating mammary gland, mTOR is likely to have a similar role (Toerien and Cant, 2007). It probably regulates proliferation of the secretory cells (as it does with other cell types (Murakami *et al*., 2004)) and controls translation of the milk protein genes. In this study, the mTOR pathway was associated with the effect of GH on milk production in the bovine mammary gland (*in vivo* study) and it was involved in the increase in protein synthesis in hepatoma cells treated with GH (*in vitro* study). After GH treatment to lactating cows, milk yield increases and so does the yield of milk protein. These factors all support the hypothesis that the responses to GH treatment are mediated by the mTOR pathway, either by increasing the protein abundance of eEF2 and eIF4E or through regulation of S6 protein phosphorylation (Chapter 3).

In conclusion, the findings presented in this thesis have provided novel information regarding the regulation of milk protein synthesis, GH signaling and identification of essential motifs for Raptor and mTOR interaction.

8.3. Future directions

To answer the important questions that arose during this PhD research, future experiments would need to characterise further the control of translation in the mammary gland in response to external stimuli. The research presented in this thesis describes for the first time the importance of the mTOR signaling pathway in the regulation of milk protein synthesis. The proteins S6, eIF4E and eEF2 were identified as key targets worthy of further characterisation in the lactating mammary gland. Knockout mice for ribosomal S6 kinase (S6K) and for 4E-BP1 (which inhibits the formation of the eIF4F complex) proteins are available and would represent a complementary model to extend our knowledge of the importance of mTOR signaling in the regulation of milk protein synthesis. Additionally, other methodologies discussed in this thesis such as RNAi and cDNA overexpression are important tools to extend our knowledge of the biological function of these genes.

It is important in the future to resolve the questions as to whether the sampling regimen used in the animal studies was adequate to detect the critical effects of GH and atropine on translation. Thus, more frequent sampling or different sampling times of tissues may be required to detect responses to the GH and atropine treatments *in vivo.*

Also of interest is the negative response observed in protein synthesis after eEF2K knock down. eEF2K knock down decreased protein synthesis - opposite to the expected response. Future work elucidating the roles of newly identified eEF2K phosphorylation sites may reveal other roles for this protein in signaling downstream of mTOR. Additionally, the identification of the essential residues in the TOS motif will elucidate more points of

control and important kinases and factors regulated by mTOR signaling cascade. The discovery of more proteins involved on the mTOR pathway may shed light on other physiological roles where mTOR might be involved and thus open new opportunities for the study of mTOR regulation in the mammary gland.

The regulation of protein synthesis in the mammary gland appears to involve regulation through the mTOR signaling (Chapter 2), specifically through changes in the eIF4E, S6 and eEF2 (Chapter 3). The GH effects in the overall protein synthesis are also mediated through changes in the mTOR pathway (Chapter 5), which reinforces the idea that the changes observed in the present study in milk protein synthesis caused by GH are potentially regulated by changing the mTOR downstream proteins such as 4E-BP1, eEF2 and S6. However, the phosphorylation of the eEF2 protein does not seen to be the key regulator of the increase in protein synthesis caused by GH (Chapter 6) which supports the finding that the abundance of this protein is probably more important to the anabolic effects of GH in the mammary gland than the phosphorylation status (Christophersen *et al*., 2002). Finally, the elucidation of the complex scheme of how 4E-BP1 interacts with the Raptor protein helps the discovery of other potent proteins involved in the mTOR pathway and raises the question of the role of this protein in the mammary gland.

APPENDIX A

Animal models to study the molecular mechanisms involved in the regulation of mammary protein synthesis

Note: *The PhD candidate did not have any part in the design or commissioning of the animal experiments described in this Chapter, however a brief description of the experiment and the major results are presented as tissue samples collected were used in the experiments described in the research Chapters of this thesis.*

A.1. Abstract

In this Chapter, two animal models were used to provide a model system to assess pathways and genes involved in milk and protein yield regulation without the hindrance of different intakes between treatment and control groups. Growth hormone (GH), which increases milk protein yield, and atropine, which decreases milk protein yield and concentration, were employed as the treatments. The data from this study clearly show that GH treatment increased milk production and the yield of the major milk components (lactose, fat, protein). GH treatment also increased the yield of all the individual milk proteins. In contrast, atropine treatment decreased the concentration of total protein in the milk in relation to the control animals. It also decreased the output of κ-casein and β-lactoglobulin B. In this experiment, both atropine and GH treatments successfully modified milk protein synthesis. Thus, mammary gland tissue samples harvested from these experiments were suitable for studying the molecular regulation of milk protein synthesis in the mammary gland (Chapter 2, 3 and 4).

A.2. Introduction

The manipulation of milk composition using nutritional interventions provide only limited opportunities to alter milk composition (Bauman *et al*., 2006). The most sensitive of the major components of milk to dietary manipulations is lipid followed by protein and the least sensitive is lactose (Bauman *et al*., 2006). Many researchers have tried to change milk protein production by enhancing amino acid supply to the mammary gland (DePeters and Ferguson, 1992; Cant *et al*., 1993; Pacheco *et al*., 2003; Johnston *et al*., 2004). However, it has been shown that the mammary gland can adjust mammary blood flow and amino acid extraction to satisfy its requirements (DePeters and Ferguson, 1992; Cant *et al*., 1993; Bequette *et al*., 2002). Thus, changes in milk protein yield and percentage obtained with different stimuli (growth factors and nutrients) are potentially more likely to be due to changes in molecular mechanisms within the mammary epithelial cells, such as intracellular signaling and transcription factors that modulate gene and protein expression.

GH stimulates milk production in lactating cows by enhancing the flow of nutrients towards the mammary gland and by increasing mammary cell proliferation and activity (Boutinaud *et al*., 2003) resulting in increased milk production. Atropine is an anti-cholinergic drug derived from the plant *Atropa belladonna* (Foley, 2003) and it has been used in animal studies to decrease milk production (Aaron *et al*., 1997; Prosser and McLaren, 1997; Luimes *et al*., 2002). While the mechanisms of action of atropine in the regulation of milk synthesis are not well understood, it has been suggested that atropine decreases the concentrations of amino acids in the peripheral blood circulation thus diminishing their availability to the mammary gland (Roets and Peeters, 1981; Prosser and McLaren, 1997). Atropine may also decrease the concentration of GH in the blood,

which would be expected to decrease milk production by diminishing its endocrinological and metabolic roles in the maintainance of lactation (Luimes *et al*., 2002).

Both atropine and GH treatments activate intracellular pathways involved in the regulation of protein synthesis (Chapter 2). These include the phosphoinositide 3-kinase (PI 3-kinase) signaling pathway, the mitogen activated protein kinase (MAPK) signaling pathway, janus kinase (JAK) and signal transducers and activators of transcription (STAT) signaling (Costoya *et al*., 1999; Carter-Su *et al*., 2000; Racke *et al*., 2006; Matthiesen *et al*., 2007). In Chapters 3 and 4, a more in-depth study of some of these intracellular pathways affected by GH and atropine treatment is presented.

In the experiments described in this Chapter, two models were used to modify milk protein synthesis in order to study the molecular mechanisms involved in the control of protein synthesis: GH, which increases milk protein yield (Bauman, 1999) and atropine, which decreases milk protein yield and concentration Thus, these treatments provided a useful model system to study intracellular signaling pathways and mechanisms in the mammary gland controlling milk protein synthesis.

The aim of this experiment was first to establish two animal models with positive or negative changes in milk protein production for studying the molecular regulation of milk protein synthesis in the mammary gland. Secondly, to collect tissue samples at the end of the trials, for undertaking studies to identify the key molecular regulators of milk protein synthesis in the mammary gland.

A.3. Materials and methods

A.3.1. Animals, diets and treatment

All procedures involving these animals were carried out in compliance with the guidelines of the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand). The trial involved 12 non-pregnant second lactation spring-calved Jersey cows that were between 178 to 200 days *postpartum*, sourced from a commercial herd. Cows were weighed twice weekly for one month prior to treatment, and randomly assigned to three groups of four. The mean body weights immediately prior to treatment (±SE) were 300kg ± 9. Cows were housed indoors in separate stalls throughout the experiment to facilitate measurement of feed intake and sample collection. Between the morning and afternoon daytime feeds (0730 hours to 1630 hours), cows were allowed outside onto a sawdust feed pad to allow adequate exercise to avoid lameness.

Cows were offered a diet formulated to exceed requirements for metabolizable energy and protein and essential amino acids. A TMR of 70% as-fed pasture silage and 30% concentrate (Table A.1) was mixed and offered *ad libitum* thrice daily (0700 hours, 1600 hours and 2000 hours) with fresh water available *ad libitum*. Animals were acclimatised to the indoor housing and concentrate feeding conditions for 2 weeks prior to the experiment.

Treatments included a subcutaneous injection of a slow-release formulation (commercially designed for a 2 week slow-release period) of GH (Lactatropin®, Elanco Animal Health, Bryanston, South Africa), or an 8 hours intravenous infusion of atropine (atropine sulphate 120 μg/kg metabolic weight/hour; Sigma Aldrich Sydney, AU).

Atropine sulphate was prepared fresh on the day of the experiment, weighed for each cow and solubilised in sterile 0.9% NaCl (w/v) or vehicle (0.9% NaCl alone) (controls animals).

Table A.1: Diet ingredient and composition (Dry matter basis)

	Unit	Value
Ingredient		
Pasture silage	%	70.00
Barley	%	10.18
Wheat	%	10.20
Soybean EXT 48% CP	%	7.50
Molasses	%	1.20
Limestone 33% Calcium	%	0.30
Dicalcium hydrogen phosphate	%	0.30
Calcined magnesium	%	0.15
Salt	%	0.12
Hi Spec Dairy premix[1]	%	0.0375
Sel-Plex	%	0.009
Total	%	100
Main Composition		
Crude protein	%DM	18.54
Lipid	%DM	3.40
NDF	%DM	31.85
ADF	%DM	22.05
NH_4N	Mg/100g DM	182.17
DCAD	mEq/kg DM	409.5
Metabolizable energy	MJ/kg DM	10.90

[1]Includes vitamins A, D_3 and E

A.3.2. Milking and milk samples

All cows were milked daily at 0730 and 1630 hours; milk yield was recorded during the whole experiment. Milk samples were collected at 0 hours (before the start of the infusion) and 8 hours following the start of the atropine treatment, from both treated and control cows. Milk samples were collected on days 0, 1, 4 and 6 after the GH or vehicle injection. Morning and afternoon milk samples were pooled based on percentage of total daily yield. Aliquots of whole milk samples were analyzed for gross milk composition (Livestock Improvement Corporation, Hamilton, NZ) and stored at -20^{o}C. Skim milk samples containing 0.05% sodium azide (to avoid the growth of bacteria) were prepared by centrifugation at 1200 *g* at 4^{o}C for 15 min, and stored at -20^{o}C for later determination of the five major individual milk proteins (α-casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin) using high pressure liquid chromatography (HPLC).

A.3.3. HPLC method

The HPLC method used resolves the major bovine milk proteins and main casein variants (Carles, 1986). Briefly, 600 μL of dissolution buffer (6moles/L Guanidine HCL; 0.1 moles/L Bis-Tris; 5.5 mmoles/L tri sodium citrate; 1.95 mmoles/L dithiothreitol) were mixed with 200 μL of skim milk and allowed to stand at room temperature for 60 min (mix "A"). Then, in another tube, 490 μL of 4.5moles/L guanidine HCL and 10 μL of mercaptoethanol were mixed together (mix "B"). 500 μL of "mix A" was mixed with the tube "mix B", filtered in a 0.45 μL filter and transferred to a HPLC vial. A HPLC analysis identifying individual milk protein peaks was

conducted using a mobile phase of phosphate-buffered aqueous propan-2-ol containing sodium dodecyl sulphate and an octadecylsilyl stationary phase as described in Carles (1986).

A.3.4. Blood sampling

Catheters were placed in both jugular veins of the cows 2 days prior to sample collection. Animals were injected with 1 mL Rompun (10% xylazine) tranquilizer prior to insertion of the catheters and given a local injection of the analgesic Lopaine (2% lignocaine hydrochloride) around the catheter site. Catheters were kept patent by maintaining them filled with sterile physiological saline with either 200 IU/mL of heparin between experimental days or 50 IU/mL during the sampling period. Blood samples were taken from each cow prior to each milking on day 0 and on days 1, 4 and 6 following GH treatment and at 0 hours (before the start of atropine infusion), 4 hours, 6 hours and 8 hours during the atropine infusion treatment. Circulating concentrations of insulin like growth factor-I (IGF-1) in GH-treated, atropine-treated and control cows were evaluated using the DSL-2800 ACTIVE Non-Extraction Insulin-Like Growth Factor-I Coated-Tube two-site immuno-radiometric assay (Miles *et al*., 1974). All samples were run in duplicate to measure the inter- and intra-assay variation.

A.3.5. Collection of tissue samples

The animals were euthanized with an overdose of sodium pentobarbitone (Provet NZ, Auckland, NZ) and tissue samples collected as outlined in Fig. A.1. On day 6, six days following GH injection, all GH-treated animals were euthanized. On day 7, 8 hours

following the atropine infusion, all atropine-treated animals were euthanized. On day 8, control animals were euthanized. Post-mortem mammary gland samples were collected from the right hind quarter, snap frozen in liquid nitrogen within 5 min and stored at -85^{o}C for later gene and protein expression profiling (Chapters 2, 3 and 4).

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A.3.6. Statistical analysis

Milk composition and yield parameters were analysed using the GLM procedure and blood parameters were analysed using the Mixed procedure in SAS Version 9.3. Treatments were defined as fixed effects, and repeated measurements in time and day were modelled using cows within treatment as subjects. Data are presented as least square means and standard errors of the mean (SEM). Probability values higher than 0.05 were considered statistically significant.

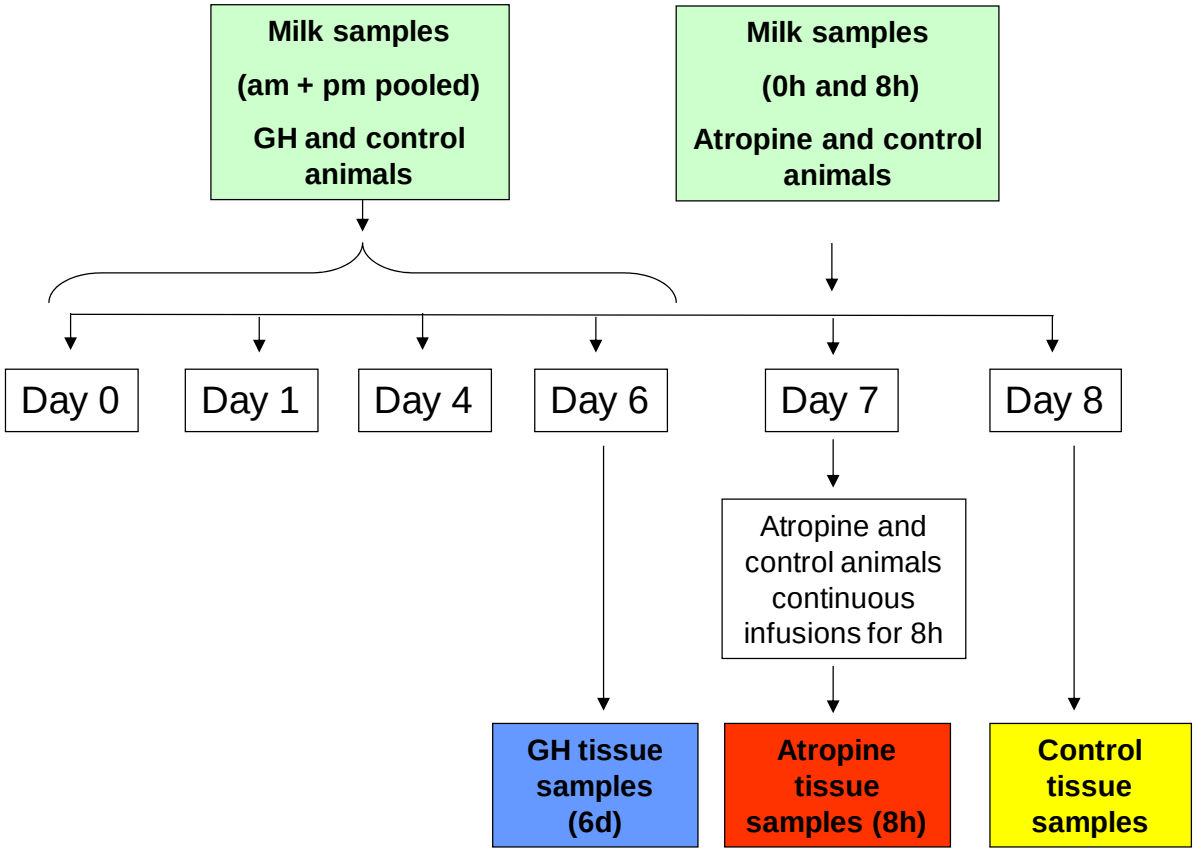

Fig. A.1: Schematic representation of the tissue and milk sampling timing from growth hormone (GH)-, atropine- and control-treated cows. The top part of the figure represents the times of the milk sample collection. The bottom part of the figure illustrates the days of euthanasia and collection of the mammary gland tissue

A.4. Results

A.4.1. GH experiment - Milk yield and composition

Mean daily milk production per cow was 9.9 ± 0.73 L. Six days after treatment, GH had increased milk yield by 32% with corresponding increases in protein, fat and lactose yield (Table A.2). The galactopoietic effect associated with GH treatment became evident after 4 days of treatment (Fig. A.2.A). Fig. A.2.B shows the increase in protein yield following the GH treatment.

Milk composition was unaffected by treatment (Table A.3). GH treatment increased the yield (~30%) but not concentration of all major individual milk proteins (Table A.3).

A.4.2. GH experiment - Hormone concentration

The serum concentrations of IGF-1 were increased after 4 days of GH administration (196.0 ± 26.6 ng/mL *vs.* 928.0 ± 26.6 ng/mL, $P<0.001$, for control and GH group, respectively) and after 6 days of GH administration (74.0 ± 64.2 *vs.* 582.0 ± 64.2, $P<0.001$, for control and GH group, respectively).

Table A.2: Milk yield and composition in control and growth hormone (GH)-treated cows after 6 days of treatment

	Control	GH	SEM	P-value
Yield parameters (kg/d)				
Milk	8.09	11.83	0.65	0.01
Protein	0.35	0.50	0.03	0.01
Fat	0.53	0.75	0.04	0.01
Lactose	0.42	0.61	0.04	0.01
Crude composition (%)				
Protein	4.29	4.26	0.19	0.88
Fat	6.58	6.32	0.30	0.57
Lactose	5.23	5.19	0.07	0.72

Table A.3: Milk protein concentration (skim milk) and yield in control and growth hormone (GH)-treated cows after 6 days of treatment

	Control	GH	SEM	P-value
Concentration (mg/ml)				
κ-casein	6.98	6.60	0.47	0.80
β-casein	17.69	17.38	0.89	0.60
α-casein	22.06	21.58	1.07	0.70
α-lactalbumin	0.92	1.13	0.07	0.10
β-lactoglobulin A	2.56	2.33	0.10	0.20
β-lactoglobulin B	3.21	3.14	0.16	0.80
Total	53.42	52.18	2.52	0.70
Yield (g/d)				
κ-casein	56.33	77.45	5.05	0.02
β-casein	143.94	204.52	13.16	0.02
α-casein	179.23	254.38	15.90	0.01
α-lactalbumin	7.46	13.31	0.85	0.02
β-lactoglobulin A	26.23	37.13	2.64	0.03
β-lactoglobulin B	20.80	27.46	1.81	0.04
Total	433.99	614.27	37.74	0.01

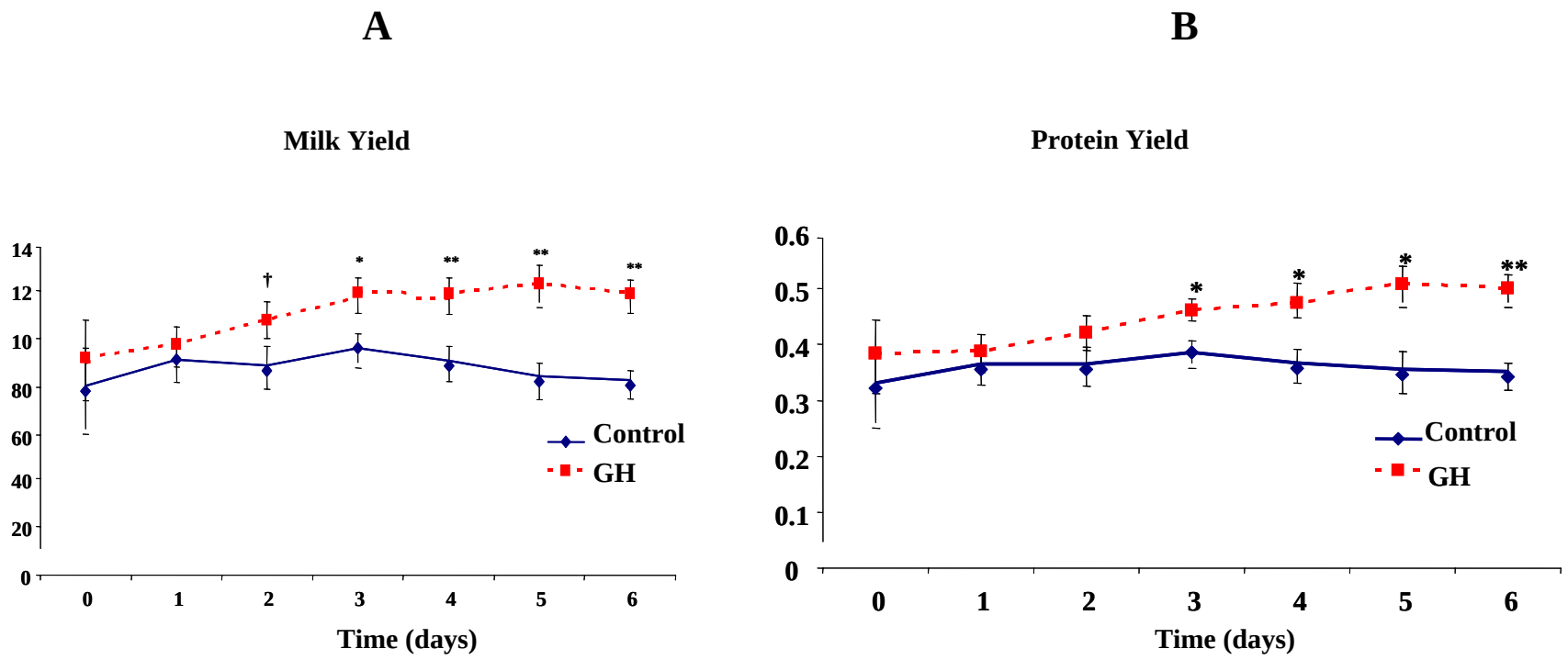

Fig. A.2: Daily milk yield (A) and protein yield (B) in control and growth hormone (GH)-treated cows over 6 days following the injection of a slow release formulation of GH on day 0

A.4.3. Atropine experiment - Milk yield and composition

During the atropine infusion, the milking regimen was not adequate to obtain accurate estimates of milk yield and thus these are not presented but milk samples were collected for analysis of composition. Eight hours after the beginning of infusion, the concentration of milk protein from atropine-treated cows was decreased by 21% (Table A.4). Atropine treatment did not change the concentration of fat but tended to decrease the concentration of lactose (P=0.09) in the milk. HPLC analysis of individual milk proteins indicated that relative to controls, atropine decreased the concentration of κ-casein (25%, P<0.02) and β-lactoglobulin B (35%, P=0.05) and tended to decrease the concentration of α-casein (17%, P=0.06) and β-casein (19%, P=0.09). The concentrations of the other milk proteins did not differ between the treatments (P>0.05, Table A.5).

A.4.4. Atropine experiment - Hormone concentration

The serum concentrations of IGF-1 were significantly decreased after 8 hours of intravenous infusion of atropine sulphate (151.0 ± 11.1 ng/ml *vs.* 91.0 ± 11.1 ng/ml, P<0.05, for control and atropine group, respectively).

Table A.4: Milk composition in control and atropine treated cows 8 hours after intravenous infusion of atropine sulphate

	Control	**Atropine**	**SEM**	**P-value**
Composition (%)				
Protein	3.90	3.10	0.13	0.001
Fat	6.84	6.15	0.52	0.38
Lactose	5.36	5.12	0.08	0.09

Table A.5: Milk protein concentration in control- and atropine-treated cows 8 hours after intravenous infusion of atropine sulphate

	Control	Atropine	SEM	P-value
Concentration (mg/ml)				
κ-casein	6.58	4.96	0.34	0.02
β-casein	15.90	12.90	0.9	0.09
α-casein	19.93	16.62	0.89	0.06
α-lactalbumin	0.74	0.69	0.06	0.55
β-lactoglobulin A	2.91	2.32	0.24	0.16
β-lactoglobulin B	2.79	1.83	0.24	0.05
Total	48.87	39.45	2.29	0.04

A.5. Discussion

In this study, both atropine and GH treatments modified milk protein synthesis. The data from this study clearly showed that GH treatment increased milk production ($P<0.05$) and the yield of the major milk components (lactose, fat, protein). These responses are consistent with the widely reported effects of GH treatment on milk yield in dairy cows (Bauman, 1999). In contrast, atropine treatment decreased the concentration of total protein in the milk by 21% ($P<0.05$) in relation to the control animals. It also decreased the output of κ-casein and β-lactoglobulin. These responses are consistent with the effects of atropine reported by others (Aaron *et al*., 1997; Luimes *et al*., 2002) and observed in a preliminary experiment performed by McCoard *et al*. (personal communication); six hours after atropine infusion, atropine had decreased milk yield by 50% with corresponding decrease in yield of protein (58%), fat (64%) and lactose (55%).

Owing to a technical problem accurate milk yield data were not obtained in this trial but (Luimes *et al*., 2002) and McCoard *et al*. (2003, in a preliminary trial, using the same animals) also reported decreases in milk yield in excess of 50% in response to atropine treatment in similar experimental conditions to those described here.

Due to the labor intensive nature of post-mortem tissue collection from cows, to complete tissue collection from twelve cows would require an entire day. Although it was possible to sacrifice all the cows on a single day, there would be potential for differences in milk production parameters due to differential milk accumulation across the day which would ultimately change gene expression (*e.g.* previous studies show that milk accumulation causes change in casein mRNA poly-A tail (Kuraishi *et al*., 2000)).

It was also not possible to sacrifice one cow per treatment group each day as the treatment regimes would have needed to be staggered across 4 consecutive days which could have resulted in further differences in milk production parameters and gene expression across time. Thus, cows were sacrificed according to treatment group. Using this approach, milk and tissue samples were taken during the same period of the day with only 3-4 hours difference in the sampling period.

Thus the aim of these experiments, which was to establish two animal models with positive and negative changes in milk protein production for studying the molecular regulation of milk protein synthesis, was successfully achieved. Furthermore, due to the significant effect of these treatments on milk protein synthesis, the tissue samples collected at the end of the trials were suitable for undertaking the studies to investigate the regulation of protein synthesis in the mammary gland using gene expression profiling (microarray, quantitative real time-PCR (qRT-PCR)) and proteomic (western blotting) techniques.

APPENDIX B

Table B.1: Complete list of differentially expressed genes in response to growth hormone (GH) treatment versus control. Genes are ordered in relation to fold difference in expression

Gene Symbol	Acession No.	Gene Name	Fold Change
IGLL1	NM_152855	immunoglobulin lambda-like polypeptide 1	-2.0
B3GNT3	NM_014256	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3	-2.0
STK6	NM_003600	serine/threonine kinase 6	-1.9
PKN1	NM_002741	protein kinase N1	-1.9
MGC39518	NM_173822	hypothetical protein MGC39518	-1.8
HEMK1	NM_016173	HemK methyltransferase family member 1	-1.8
CALB1	NM_004929	calbindin 1, 28kDa	-1.8
LOC388078	XM_370835	similar to Ig heavy chain V-I region HG3 precursor	-1.7
DDX3X	NM_001356	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	-1.7
LOC91353	NM_001013618	similar to omega protein	-1.7
IGJ	NM_144646	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	-1.7
DGKD	NM_152879	diacylglycerol kinase, delta 130kDa	-1.7
EMP2	NM_001424	epithelial membrane protein 2	-1.7
MLL	NM_005933	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)	-1.7
PCAF	NM_003884	p300/CBP-associated factor	-1.6
JMJD1C	NM_004241	jumonji domain containing 1C	-1.6
CARD14	NM_024110	caspase recruitment domain family, member 14	-1.6
PON3	NM_000940	paraoxonase 3	-1.6
LAMC1	NM_002293	laminin, gamma 1 (formerly LAMB2)	-1.6
CYB5R1	NM_016243	cytochrome b5 reductase 1	-1.6
TAF6L	NM_006473	TAF6-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor, 65kDa	-1.6
GTF3C1	NM_001520	general transcription factor IIIC, polypeptide 1, alpha 220kDa	-1.6
THBS1	NM_003246	thrombospondin 1	-1.6
HAVCR2	NM_032782	hepatitis A virus cellular receptor 2	-1.6
TIMM50	NM_001001563	translocase of inner mitochondrial membrane 50 homolog (yeast)	-1.6
PSCD4	NM_013385	pleckstrin homology, Sec7 and coiled-coil domains 4	-1.6
ARHGAP26	NM_015071	Rho GTPase activating protein 26	-1.5
COL4A1	NM_001845	collagen, type IV, alpha 1	-1.5
PTGFRN	NM_020440	prostaglandin F2 receptor negative regulator	-1.5
FLJ32363	NM_198566	FLJ32363 protein	-1.5
USP3	NM_006537	ubiquitin specific protease 3	-1.5
ATP5C1	NM_005174	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	-1.5
MAP4K2	NM_004579	mitogen-activated protein kinase kinase kinase kinase 2	-1.5

SRI	NM_198901	Sorcin	-1.5
SLAMF8	NM_020125	SLAM family member 8	-1.5
RPS6KA1	NM_002953	ribosomal protein S6 kinase, 90kDa, polypeptide 1	-1.5
IQGAP1	NM_003870	IQ motif containing GTPase activating protein 1	-1.5
MAP3K4	NM_005922	mitogen-activated protein kinase kinase kinase 4	-1.5
PCGF3	NM_006315	polycomb group ring finger 3	-1.5
WDR75	NM_032168	WD repeat domain 75	-1.5
TRIM35	NM_171982	tripartite motif-containing 35	-1.5
C10orf57	NM_025125	chromosome 10 open reading frame 57	-1.5
SP1	NM_138473	Sp1 transcription factor	-1.5
SAT	NM_002970	spermidine/spermine N1-acetyltransferase	-1.5
PRKAR2A	NM_004157	protein kinase, cAMP-dependent, regulatory, type II, alpha	-1.5
ADAMTS9	NM_182920	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 9	-1.5
CHKA	NM_212469	choline kinase alpha	-1.5
FNDC3B	NM_022763	fibronectin type III domain containing 3B	-1.5
B3GALT2	NM_003783	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	-1.5
ANXA2	NM_004039	annexin A2	-1.5
MGST2	NM_002413	microsomal glutathione S-transferase 2	-1.5
STAT1	NM_007315	signal transducer and activator of transcription 1, 91kDa	-1.4
MTMR12	NM_019061	myotubularin related protein 12	-1.4
PRG1	NM_002727	proteoglycan 1, secretory granule	-1.4
CD74	NM_004355	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	-1.4
FLRT3	NM_198391	fibronectin leucine rich transmembrane protein 3	-1.4
EIF2C4	NM_017629	eukaryotic translation initiation factor 2C, 4	-1.4
CLCA1	NM_001285	chloride channel, calcium activated, family member 1	-1.4
RSU1	NM_152724	Ras suppressor protein 1	-1.4
CNOT8	NM_004779	CCR4-NOT transcription complex, subunit 8	-1.4
C14orf31	NM_152330	chromosome 14 open reading frame 31	-1.4
C2	NM_000063	complement component 2	-1.4
CCNB3	NM_033031	cyclin B3	-1.4
SH3BGRL2	NM_031469	SH3 domain binding glutamic acid-rich protein like 2	-1.4
KRT19	NM_002276	keratin 19	-1.4
UNQ467	NM_207392	KIPV467	-1.4
AKAP13	NM_006738	A kinase (PRKA) anchor protein 13	-1.4
FKBP5	NM_004117	FK506 binding protein 5	-1.4
PKN2	NM_006256	protein kinase N2	-1.4
DMBT1	NM_004406	deleted in malignant brain tumors 1	-1.4
SLC11A1	NM_000578	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	-1.4
ECT2	NM_018098	epithelial cell transforming sequence 2 oncogene	-1.4
MYADM	NM_001020821	myeloid-associated differentiation marker	-1.4

VAT1	NM_006373	vesicle amine transport protein 1 homolog (T californica)	-1.4
RAMP2	NM_005854	receptor (calcitonin) activity modifying protein 2	-1.4
HSF2BP	NM_007031	heat shock transcription factor 2 binding protein	-1.4
EIF3S9	NM_003751	eukaryotic translation initiation factor 3, subunit 9 eta, 116kDa	-1.4
SLC29A1	NM_004955	solute carrier family 29 (nucleoside transporters), member 1	-1.4
FGFBP1	NM_005130	fibroblast growth factor binding protein 1	-1.4
PPAP2C	NM_003712	phosphatidic acid phosphatase type 2C	-1.4
PCDH7	NM_032457	BH-protocadherin (brain-heart)	-1.4
SLC12A2	NM_001046	solute carrier family 12 (sodium/potassium/chloride transporters), member 2	-1.4
FXYD1	NM_021902	FXYD domain containing ion transport regulator 1 (phospholemman)	-1.4
LTBP2	NM_000428	latent transforming growth factor beta binding protein 2	-1.4
AKR1C1	NM_001353	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	-1.4
NMB	NM_021077	neuromedin B	-1.4
CKS1B	NM_001826	CDC28 protein kinase regulatory subunit 1B	-1.4
CR2	NM_001877	complement component (3d/Epstein Barr virus) receptor 2	-1.4
WISP3	NM_130396	WNT1 inducible signaling pathway protein 3	-1.4
RYK	NM_001005861	RYK receptor-like tyrosine kinase	-1.4
SSFA2	NM_006751	sperm specific antigen 2	-1.4
PEX13	NM_002618	peroxisome biogenesis factor 13	-1.4
SCNM1	NM_024041	sodium channel modifier 1	-1.4
CDC14B	NM_033331	CDC14 cell division cycle 14 homolog B (S. cerevisiae)	-1.4
MFAP3	NM_005927	microfibrillar-associated protein 3	-1.3
LOC440891	XM_496578	similar to Ig kappa light chain variable region	-1.3
TACC1	NM_006283	transforming, acidic coiled-coil containing protein 1	-1.3
AZIN1	NM_148174	antizyme inhibitor 1	-1.3
TMSL3	NM_183049	thymosin-like 3	-1.3
MAP4	NM_030884	microtubule-associated protein 4	-1.3
KEL	NM_000420	Kell blood group	-1.3
TRIM28	NM_005762	tripartite motif-containing 28	-1.3
PPARBP	NM_004774	PPAR binding protein	-1.3
TNFRSF21	NM_014452	tumor necrosis factor receptor superfamily, member 21	-1.3
RAB27A	NM_183235	RAB27A, member RAS oncogene family	-1.3
ARTS-1	NM_016442	type 1 tumor necrosis factor receptor shedding aminopeptidase regulator	-1.3
DDX21	NM_004728	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	-1.3
NCOA2	NM_006540	nuclear receptor coactivator 2	-1.3
GM2A	NM_000405	GM2 ganglioside activator	-1.3
WAC	NM_100486	WW domain containing adaptor with coiled-coil	-1.3
CEBPD	NM_005195	CCAAT/enhancer binding protein (C/EBP), delta	-1.3
MBD1	NM_015844	methyl-CpG binding domain protein 1	-1.3

BCLAF1	NM_014739	BCL2-associated transcription factor 1	-1.3
RAB5A	NM_004162	RAB5A, member RAS oncogene family	-1.3
ANXA1	NM_000700	annexin A1	-1.3
ZZZ3	NM_015534	zinc finger, ZZ-type containing 3	-1.3
M-RIP	NM_015134	myosin phosphatase-Rho interacting protein	-1.3
SMARCC2	NM_139067	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2	-1.3
MYH11	NM_022844	myosin, heavy polypeptide 11, smooth muscle	-1.3
WSB1	NM_015626	WD repeat and SOCS box-containing 1	-1.3
CD9	NM_001769	CD9 antigen (p24)	-1.3
SPRR2G	NM_001014291	small proline-rich protein 2G	-1.3
DKFZP434L0117	NM_022778	hypothetical protein DKFZp434L0117	-1.3
SLC27A5	NM_012254	solute carrier family 27 (fatty acid transporter), member 5	-1.3
NDUFS6	NM_004553	NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13kDa (NADH-coenzyme Q reductase)	-1.3
ITGAL	NM_002209	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	-1.3
PSCD3	NM_004227	pleckstrin homology, Sec7 and coiled-coil domains 3	1.2
CTDSP2	NM_005730	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2	1.3
SMNDC1	NM_005871	survival motor neuron domain containing 1	1.3
ABCC8	NM_000352	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	1.3
SPG3A	NM_181598	spastic paraplegia 3A (autosomal dominant)	1.3
EEF1A2	NM_001958	eukaryotic translation elongation factor 1 alpha 2	1.3
SNX2	NM_003100	sorting nexin 2	1.3
GPR39	NM_001508	G protein-coupled receptor 39	1.3
MB	NM_005368	Myoglobin	1.3
CKM	NM_001824	creatine kinase, muscle	1.3
MMP24	NM_006690	matrix metalloproteinase 24 (membrane-inserted)	1.3
C14orf138	NM_024558	chromosome 14 open reading frame 138	1.3
FGF12	NM_004113	fibroblast growth factor 12	1.3
PRKAA1	NM_006251	protein kinase, AMP-activated, alpha 1 catalytic subunit	1.3
PTPN2	NM_080422	protein tyrosine phosphatase, non-receptor type 2	1.3
SLC1A4	NM_003038	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	1.3
GSTP1	NM_000852	glutathione S-transferase pi	1.3
JMJD1A	NM_018433	jumonji domain containing 1A	1.3
EEF1A1	NM_001402	eukaryotic translation elongation factor 1 alpha 1	1.3
MFAP1	NM_005926	microfibrillar-associated protein 1	1.3
EEF1G	NM_001404	eukaryotic translation elongation factor 1 gamma	1.3
PAX9	NM_006194	paired box gene 9	1.3
TUSC4	NM_006545	tumor suppressor candidate 4	1.3
PSMB10	NM_002801	proteasome (prosome, macropain) subunit, beta type, 10	1.3
PPHLN1	NM_016488	periphilin 1	1.3
CCNI	NM_006835	cyclin I	1.3

TIMD4	NM_138379	T-cell immunoglobulin and mucin domain containing 4	1.3
SLC12A4	NM_005072	solute carrier family 12 (potassium/chloride transporters), member 4	1.3
EIF3S2	NM_003757	eukaryotic translation initiation factor 3, subunit 2 beta, 36kDa	1.3
CDS2	NM_003818	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2	1.3
ZNF207	NM_003457	zinc finger protein 207	1.3
NDFIP2	NM_019080	Nedd4 family interacting protein 2	1.3
EIF3S10	NM_003750	eukaryotic translation initiation factor 3, subunit 10 theta, 150/170kDa	1.3
LPO	NM_006151	Lactoperoxidase	1.3
USP7	NM_003470	ubiquitin specific protease 7 (herpes virus-associated)	1.3
KPNA3	NM_002267	karyopherin alpha 3 (importin alpha 4)	1.3
POLR2C	NM_032940	polymerase (RNA) II (DNA directed) polypeptide C, 33kDa	1.3
CDKN2B	NM_078487	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	1.3
GPX1	NM_000581	glutathione peroxidase 1	1.3
G1P2	NM_005101	interferon, alpha-inducible protein (clone IFI-15K)	1.3
CYP2U1	NM_183075	cytochrome P450, family 2, subfamily U, polypeptide 1	1.3
IMP-3	NM_006547	IGF-II mRNA-binding protein 3	1.3
DLG5	NM_004747	discs, large homolog 5 (Drosophila)	1.3
APBA3	NM_004886	amyloid beta (A4) precursor protein-binding, family A, member 3 (X11-like 2)	1.3
RNASE4	NM_002937	ribonuclease, RNase A family, 4	1.3
NR2C1	NM_003297	nuclear receptor subfamily 2, group C, member 1	1.3
F2R	NM_001992	coagulation factor II (thrombin) receptor	1.3
AP3M1	NM_207012	adaptor-related protein complex 3, mu 1 subunit	1.3
PPP1R3A	NM_002711	protein phosphatase 1, regulatory (inhibitor) subunit 3A (glycogen and sarcoplasmic reticulum binding subunit, skeletal muscle)	1.3
SFRP4	NM_003014	secreted frizzled-related protein 4	1.3
HMG20A	NM_018200	high-mobility group 20A	1.3
CAMKK2	NM_006549	calcium/calmodulin-dependent protein kinase kinase 2, beta	1.3
CD14	NM_000591	CD14 antigen	1.3
C1orf27	NM_017847	chromosome 1 open reading frame 27	1.3
EEF2	NM_001961	eukaryotic translation elongation factor 2	1.3
QKI	NM_206855	quaking homolog, KH domain RNA binding (mouse)	1.3
MYO1F	NM_012335	myosin IF	1.3
FKBP10	NM_021939	FK506 binding protein 10, 65 kDa	1.3
NET1	NM_005863	neuroepithelial cell transforming gene 1	1.3
WSB1	NM_134264	WD repeat and SOCS box-containing 1	1.3
BCL7B	NM_001707	B-cell CLL/lymphoma 7B	1.3
IGSF8	NM_052868	immunoglobulin superfamily, member 8	1.3
ITPR3	NM_002224	inositol 1,4,5-triphosphate receptor, type 3	1.3
LIMS2	NM_017980	LIM and senescent cell antigen-like domains 2	1.3
LPL	NM_000237	lipoprotein lipase	1.3

PIGT	NM_015937	phosphatidylinositol glycan, class T	1.3
FTH1	NM_002032	ferritin, heavy polypeptide 1	1.3
UGP2	NM_001001521	UDP-glucose pyrophosphorylase 2	1.3
IDH3B	NM_006899	isocitrate dehydrogenase 3 (NAD+) beta	1.3
TIMP3	NM_000362	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	1.3
CASQ2	NM_001232	calsequestrin 2 (cardiac muscle)	1.3
DOK1	NM_001381	docking protein 1, 62kDa (downstream of tyrosine kinase 1)	1.3
C1S	NM_201442	complement component 1, s subcomponent	1.3
TUBA6	NM_032704	tubulin alpha 6	1.3
PPP5C	NM_006247	protein phosphatase 5, catalytic subunit	1.3
PTPRD	NM_130393	protein tyrosine phosphatase, receptor type, D	1.3
KIAA1467	NM_020853	KIAA1467 protein	1.3
ACADM	NM_000016	acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	1.4
ACVRL1	NM_000020	activin A receptor type II-like 1	1.4
TUBA1	NM_006000	tubulin, alpha 1 (testis specific)	1.4
GNAS	NM_080426	GNAS complex locus	1.4
KLF11	NM_003597	Kruppel-like factor 11	1.4
RYBP	NM_012234	RING1 and YY1 binding protein	1.4
BCL2	NM_000657	B-cell CLL/lymphoma 2	1.4
KIF22	NM_007317	kinesin family member 22	1.4
CIB2	NM_006383	calcium and integrin binding family member 2	1.4
FLJ20345	NM_017777	hypothetical protein FLJ20345	1.4
PAX5	NM_016734	paired box gene 5 (B-cell lineage specific activator)	1.4
FLNC	NM_001458	filamin C, gamma (actin binding protein 280)	1.4
BAX	NM_138761	BCL2-associated X protein	1.4
KIF3A	NM_007054	kinesin family member 3A	1.4
EPS8	NM_004447	epidermal growth factor receptor pathway substrate 8	1.4
C14orf147	NM_138288	chromosome 14 open reading frame 147	1.4
TM4SF4	NM_004617	transmembrane 4 L six family member 4	1.4
KPTN	NM_007059	kaptin (actin binding protein)	1.4
NR5A2	NM_205860	nuclear receptor subfamily 5, group A, member 2	1.4
ZMYND11	NM_006624	zinc finger, MYND domain containing 11	1.4
CRAT	NM_000755	carnitine acetyltransferase	1.4
PD2	NM_019088	hypothetical protein F23149_1	1.4
EPS15	NM_001981	epidermal growth factor receptor pathway substrate 15	1.4
ASB8	NM_024095	ankyrin repeat and SOCS box-containing 8	1.4
PTPRB	NM_002837	protein tyrosine phosphatase, receptor type, B	1.4
TNFSF5IP1	NM_020232	tumor necrosis factor superfamily, member 5-induced protein 1	1.4
CACNA2D1	NM_000722	calcium channel, voltage-dependent, alpha 2/delta subunit 1	1.4
PLCD1	NM_006225	phospholipase C, delta 1	1.4

KLF10	NM_005655	Kruppel-like factor 10	1.4
MYH8	NM_002472	myosin, heavy polypeptide 8, skeletal muscle, perinatal	1.4
MMP19	NM_002429	matrix metalloproteinase 19	1.4
ABLIM3	NM_014945	actin binding LIM protein family, member 3	1.4
TIMP4	NM_003256	tissue inhibitor of metalloproteinase 4	1.4
HSPB8	NM_014365	heat shock 22kDa protein 8	1.4
CAMK2G	NM_172170	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	1.4
GALK2	NM_001001556	galactokinase 2	1.4
ASB11	NM_080873	ankyrin repeat and SOCS box-containing 11	1.4
PSMB2	NM_002794	proteasome (prosome, macropain) subunit, beta type, 2	1.4
MTDH	NM_178812	Metadherin	1.4
PBP	NM_002567	prostatic binding protein	1.4
ALAD	NM_000031	aminolevulinate, delta-, dehydratase	1.4
CTSL2	NM_001333	cathepsin L2	1.4
TUBGCP2	NM_006659	tubulin, gamma complex associated protein 2	1.4
GAS6	NM_000820	growth arrest-specific 6	1.4
SULF2	NM_018837	sulfatase 2	1.4
ITGA2	NM_002203	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	1.4
STK11	NM_000455	serine/threonine kinase 11 (Peutz-Jeghers syndrome)	1.4
GRAP2	NM_004810	GRB2-related adaptor protein 2	1.4
RAB11A	NM_004663	RAB11A, member RAS oncogene family	1.4
CYP26B1	NM_019885	cytochrome P450, family 26, subfamily B, polypeptide 1	1.4
CDCA7	NM_031942	cell division cycle associated 7	1.4
PAPSS1	NM_005443	3'-phosphoadenosine 5'-phosphosulfate synthase 1	1.4
PDK1	NM_002610	pyruvate dehydrogenase kinase, isoenzyme 1	1.4
ACO1	NM_002197	aconitase 1, soluble	1.4
LRPPRC	NM_133259	leucine-rich PPR-motif containing	1.4
SFRS2IP	NM_004719	splicing factor, arginine/serine-rich 2, interacting protein	1.4
ACTA2	NM_001613	actin, alpha 2, smooth muscle, aorta	1.4
DHX30	NM_014966	DEAH (Asp-Glu-Ala-His) box polypeptide 30	1.4
PSME3	NM_005789	proteasome (prosome, macropain) activator subunit 3 (PA28 gamma; Ki)	1.4
HSPA5	NM_005347	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	1.4
GAPDH	NM_002046	glyceraldehyde-3-phosphate dehydrogenase	1.4
GSTM5	NM_000851	glutathione S-transferase M5	1.4
KTN1	NM_182926	kinectin 1 (kinesin receptor)	1.4
UBE2H	NM_182697	ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)	1.4
CLU	NM_001831	clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	1.4
RNASE1	NM_198234	ribonuclease, RNase A family, 1 (pancreatic)	1.4
RAP2A	NM_021033	RAP2A, member of RAS oncogene family	1.4

FGF13	NM_004114	fibroblast growth factor 13	1.4
CPT1B	NM_152246	carnitine palmitoyltransferase 1B (muscle)	1.4
ACSL1	NM_001995	acyl-CoA synthetase long-chain family member 1	1.4
STK38	NM_007271	serine/threonine kinase 38	1.4
ATP2B3	NM_021949	ATPase, Ca++ transporting, plasma membrane 3	1.4
MFGE8	NM_005928	milk fat globule-EGF factor 8 protein	1.4
CREBL1	NM_004381	cAMP responsive element binding protein-like 1	1.4
ALDH1A3	NM_000693	aldehyde dehydrogenase 1 family, member A3	1.4
FLNB	NM_001457	filamin B, beta (actin binding protein 278)	1.4
ATP1A1	NM_000701	ATPase, Na+/K+ transporting, alpha 1 polypeptide	1.4
API5	NM_006595	apoptosis inhibitor 5	1.4
IKBKAP	NM_003640	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	1.4
LOC388610	NM_001013642	hypothetical LOC388610	1.4
PITPNA	NM_006224	phosphatidylinositol transfer protein, alpha	1.4
CYP2C18	NM_000772	cytochrome P450, family 2, subfamily C, polypeptide 18	1.4
FZD4	NM_012193	frizzled homolog 4 (Drosophila)	1.4
FABP7	NM_001446	fatty acid binding protein 7, brain	1.4
RABGAP1	NM_012197	RAB GTPase activating protein 1	1.4
OSGEPL1	NM_022353	O-sialoglycoprotein endopeptidase-like 1	1.4
TUBA3	NM_006009	tubulin, alpha 3	1.4
LIMS1	NM_004987	LIM and senescent cell antigen-like domains 1	1.4
MME	NM_000902	membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)	1.4
ID2	NM_002166	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	1.4
KRT10	NM_000421	keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	1.4
SHC1	NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	1.4
MAN2B1	NM_000528	mannosidase, alpha, class 2B, member 1	1.4
LOC440702	XM_496425	LOC440702	1.4
ANG	NM_001145	angiogenin, ribonuclease, RNase A family, 5	1.5
HIBADH	NM_152740	3-hydroxyisobutyrate dehydrogenase	1.5
IDH1	NM_005896	isocitrate dehydrogenase 1 (NADP+), soluble	1.5
PSMD9	NM_002813	proteasome (prosome, macropain) 26S subunit, non-ATPase, 9	1.5
C5orf12	NM_178276	chromosome 5 open reading frame 12	1.5
SFTPD	NM_003019	surfactant, pulmonary-associated protein D	1.5
HLA-C	NM_002117	major histocompatibility complex, class I, C	1.5
SEPW1	NM_003009	selenoprotein W, 1	1.5
FADS2	NM_004265	fatty acid desaturase 2	1.5
TUBB3	NM_006086	tubulin, beta 3	1.5
CSN1S2A	XM_379270	casein alpha s2-like A	1.5
HLA-DQB1	NM_002123	major histocompatibility complex, class II, DQ beta 1	1.5
SLC35A3	NM_012243	solute carrier family 35 (UDP-N-acetylglucosamine (UDP-GlcNAc) transporter), member A3	1.5

EPB41L1	NM_012156	erythrocyte membrane protein band 4.1-like 1	1.5
HLA-A	NM_002116	major histocompatibility complex, class I, A	1.5
FABP3	NM_004102	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	1.5
BNIP3L	NM_004331	BCL2/adenovirus E1B 19kDa interacting protein 3-like	1.5
DCI	NM_001919	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase)	1.5
PRKD1	NM_002742	protein kinase D1	1.5
PTPRZ1	NM_002851	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	1.5
GAB1	NM_207123	GRB2-associated binding protein 1	1.5
CKS2	NM_001827	CDC28 protein kinase regulatory subunit 2	1.5
VCL	NM_003373	Vinculin	1.5
TF	NM_001063	transferring	1.5
SLC34A2	NM_006424	solute carrier family 34 (sodium phosphate), member 2	1.5
FLJ21908	NM_024604	hypothetical protein FLJ21908	1.6
K-ALPHA-1	NM_006082	tubulin, alpha, ubiquitous	1.7
LTF	NM_002343	Lactotransferrin	1.7
SPARC	NM_003118	secreted protein, acidic, cysteine-rich (osteonectin)	1.7
COL5A2	NM_000393	collagen, type V, alpha 2	1.7
COL1A1	NM_000088	collagen, type I, alpha 1	1.8
HADHA	NM_000182	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit	1.8
ABCG2	NM_004827	ATP-binding cassette, sub-family G (WHITE), member 2	1.8
COL1A2	NM_000089	collagen, type I, alpha 2	1.8
COL3A1	NM_000090	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	2.1

Table B.2: Complete list of differentially expressed genes in response to atropine treatment versus control. Genes are ordered in relation to fold difference in expression

Gene Symbol	Acession No.	Gene Name	Fold change
MGC39518	NM_173822	hypothetical protein MGC39518	-2.4
STK6	NM_003600	serine/threonine kinase 6	-2.3
B3GNT3	NM_014256	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3	-2.3
SAA1	NM_000331	serum amyloid A1	-2.0
PTPRC	NM_080921	protein tyrosine phosphatase, receptor type, C	-2.0
SNX6	NM_152233	sorting nexin 6	-2.0
IGLL1	NM_152855	immunoglobulin lambda-like polypeptide 1	-1.7
CRSP3	NM_004830	cofactor required for Sp1 transcriptional activation, subunit 3, 130kDa	-1.6
UNQ467	NM_207392	KIPV467	-1.6
COL1A1	NM_000088	collagen, type I, alpha 1	-1.5
IDH1	NM_005896	isocitrate dehydrogenase 1 (NADP+), soluble	-1.5
CALB1	NM_004929	calbindin 1, 28kDa	-1.5
BMPR1B	NM_001203	bone morphogenetic protein receptor, type IB	-1.5
SCD	NM_005063	stearoyl-CoA desaturase (delta-9-desaturase)	-1.5
EIF2C4	NM_017629	eukaryotic translation initiation factor 2C, 4	-1.5
LOC388078	XM_370835	similar to Ig heavy chain V-I region HG3 precursor	-1.5
PKN1	NM_002741	protein kinase N1	-1.5
HMGN3	NM_138730	high mobility group nucleosomal binding domain 3	-1.5
SUCLG1	NM_003849	succinate-CoA ligase, GDP-forming, alpha subunit	-1.5
RNASE1	NM_198234	ribonuclease, RNase A family, 1 (pancreatic)	-1.5
HEMK1	NM_016173	HemK methyltransferase family member 1	-1.5
CCNB3	NM_033031	cyclin B3	-1.5
FZD4	NM_012193	frizzled homolog 4 (Drosophila)	-1.5
PTGFRN	NM_020440	prostaglandin F2 receptor negative regulator	-1.5
TAF6L	NM_006473	TAF6-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor, 65kDa	-1.5
HMGCS1	NM_002130	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	-1.4
CYB5R1	NM_016243	cytochrome b5 reductase 1	-1.4
NRK	NM_198465	Nik related kinase	-1.4

ESM1	NM_007036	endothelial cell-specific molecule 1	-1.4
DGKD	NM_152879	diacylglycerol kinase, delta 130kDa	-1.4
PSCD4	NM_013385	pleckstrin homology, Sec7 and coiled-coil domains 4	-1.4
RPL15	NM_002948	ribosomal protein L15	-1.4
PON3	NM_000940	paraoxonase 3	-1.4
GSG2	NM_031965	germ cell associated 2 (haspin)	-1.4
TIMM50	NM_001001563	translocase of inner mitochondrial membrane 50 homolog (yeast)	-1.4
PDIA3	NM_005313	protein disulfide isomerase family A, member 3	-1.4
SAT	NM_002970	spermidine/spermine N1-acetyltransferase	-1.4
IGJ	NM_144646	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	-1.4
STK3	NM_006281	serine/threonine kinase 3 (STE20 homolog, yeast)	-1.4
ABCB4	NM_000443	ATP-binding cassette, sub-family B (MDR/TAP), member 4	-1.4
ATP5C1	NM_005174	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	-1.4
PLEKHA1	NM_021622	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	-1.4
HRB	NM_004504	HIV-1 Rev binding protein	-1.4
GTF3C1	NM_001520	general transcription factor IIIC, polypeptide 1, alpha 220kDa	-1.4
CYCS	NM_018947	cytochrome c, somatic	-1.4
DNAJC10	NM_018981	DnaJ (Hsp40) homolog, subfamily C, member 10	-1.4
TUBA3	NM_006009	tubulin, alpha 3	-1.4
PAPOLA	NM_032632	poly(A) polymerase alpha	-1.4
ALDOC	NM_005165	aldolase C, fructose-bisphosphate	-1.4
SPARC	NM_003118	secreted protein, acidic, cysteine-rich (osteonectin)	-1.4
PSMB5	NM_002797	proteasome (prosome, macropain) subunit, beta type, 5	-1.4
SLC25A12	NM_003705	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	-1.4
HSPA5	NM_005347	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	-1.4
MYO1B	NM_012223	myosin IB	-1.4
EMP2	NM_001424	epithelial membrane protein 2	-1.4
FBXW10	NM_031456	F-box and WD-40 domain protein 10	-1.4
PDCD4	NM_014456	programmed cell death 4 (neoplastic transformation inhibitor)	-1.4
RPS6KA1	NM_002953	ribosomal protein S6 kinase, 90kDa, polypeptide 1	-1.4
ARHGAP26	NM_015071	Rho GTPase activating protein 26	-1.4
JMJD1C	NM_004241	jumonji domain containing 1C	-1.4
KIT	NM_000222	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	-1.4
CANX	NM_001746	Calnexin	-1.4
LAMC1	NM_002293	laminin, gamma 1 (formerly LAMB2)	-1.4

CHKA	NM_212469	choline kinase alpha	-1.4
PRKAR2A	NM_004157	protein kinase, cAMP-dependent, regulatory, type II, alpha	-1.4
USP3	NM_006537	ubiquitin specific protease 3	-1.4
HAVCR2	NM_032782	hepatitis A virus cellular receptor 2	-1.4
THBS1	NM_003246	thrombospondin 1	-1.4
MAP2K5	NM_145160	mitogen-activated protein kinase kinase 5	-1.4
TNFAIP6	NM_007115	tumor necrosis factor, alpha-induced protein 6	-1.4
SASH1	NM_015278	SAM and SH3 domain containing 1	-1.4
KRT1	NM_006121	keratin 1 (epidermolytic hyperkeratosis)	-1.4
SLC12A2	NM_001046	solute carrier family 12 (sodium/potassium/chloride transporters), member 2	-1.4
FLJ32363	NM_198566	FLJ32363 protein	-1.3
FGB	NM_005141	fibrinogen beta chain	-1.3
EIF1AX	NM_001412	eukaryotic translation initiation factor 1A, X-linked	-1.3
FLRT1	NM_013280	fibronectin leucine rich transmembrane protein 1	-1.3
FNDC3B	NM_022763	fibronectin type III domain containing 3B	-1.3
ANXA2	NM_004039	annexin A2	-1.3
SLC30A5	NM_022902	solute carrier family 30 (zinc transporter), member 5	-1.3
MAP3K4	NM_005922	mitogen-activated protein kinase kinase kinase 4	-1.3
SP1	NM_138473	Sp1 transcription factor	-1.3
CKS1B	NM_001826	CDC28 protein kinase regulatory subunit 1B	-1.3
MAP4K2	NM_004579	mitogen-activated protein kinase kinase kinase kinase 2	-1.3
LTBP1	NM_206943	latent transforming growth factor beta binding protein 1	-1.3
PRG1	NM_002727	proteoglycan 1, secretory granule	-1.3
HLA-DRA	NM_019111	major histocompatibility complex, class II, DR alpha	-1.3
A2M	NM_000014	alpha-2-macroglobulin	-1.3
THRB	NM_000461	thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian)	-1.3
ACLY	NM_198830	ATP citrate lyase	-1.3
SLC30A1	NM_021194	solute carrier family 30 (zinc transporter), member 1	-1.3
WDR75	NM_032168	WD repeat domain 75	-1.3
EIF3S9	NM_003751	eukaryotic translation initiation factor 3, subunit 9 eta, 116kDa	-1.3
MGST2	NM_002413	microsomal glutathione S-transferase 2	-1.3
FABP2	NM_000134	fatty acid binding protein 2, intestinal	-1.3
PCAF	NM_003884	p300/CBP-associated factor	-1.3
RSU1	NM_152724	Ras suppressor protein 1	-1.3
NDUFA9	NM_005002	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, 39kDa	-1.3

YWHAB	NM_139323	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	-1.3
MAP3K1	XM_042066	mitogen-activated protein kinase kinase kinase 1	-1.3
SH3BGRL2	NM_031469	SH3 domain binding glutamic acid-rich protein like 2	-1.3
KLF10	NM_005655	Kruppel-like factor 10	-1.3
ATAD1	NM_032810	ATPase family, AAA domain containing 1	-1.3
FABP1	NM_001443	fatty acid binding protein 1, liver	-1.3
MIF	NM_002415	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	-1.3
LTBP2	NM_000428	latent transforming growth factor beta binding protein 2	-1.3
POLR2B	NM_000938	polymerase (RNA) II (DNA directed) polypeptide B, 140kDa	-1.3
TAF6	NM_139315	TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 80kDa	-1.3
RYK	NM_001005861	RYK receptor-like tyrosine kinase	-1.3
C14orf31	NM_152330	chromosome 14 open reading frame 31	-1.3
MRC1	NM_002438	mannose receptor, C type 1	-1.3
MARK2	NM_017490	MAP/microtubule affinity-regulating kinase 2	-1.3
SSFA2	NM_006751	sperm specific antigen 2	-1.3
GPI	NM_000175	glucose phosphate isomerase	-1.3
FGFR1OP2	NM_015633	FGFR1 oncogene partner 2	-1.3
MYLK	NM_053032	myosin, light polypeptide kinase	-1.3
SMC1L1	NM_006306	SMC1 structural maintainance of chromosomes 1-like 1 (yeast)	-1.3
SLAMF8	NM_020125	SLAM family member 8	-1.3
GREM1	NM_013372	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	-1.3
CALR	NM_004343	Calreticulin	-1.3
BF	NM_001710	B-factor, properdin	-1.3
TUBB	NM_178014	tubulin, beta polypeptide	-1.3
NFIA	NM_005595	nuclear factor I/A	1.2
ABCG2	NM_004827	ATP-binding cassette, sub-family G (WHITE), member 2	1.2
PHLDA1	NM_007350	pleckstrin homology-like domain, family A, member 1	1.3
IGFBP5	NM_000599	insulin-like growth factor binding protein 5	1.3
IMP-3	NM_006547	IGF-II mRNA-binding protein 3	1.3
ALOX12	NM_000697	arachidonate 12-lipoxygenase	1.3
PDE7A	NM_002603	phosphodiesterase 7A	1.3
RAMP3	NM_005856	receptor (calcitonin) activity modifying protein 3	1.3
SNAPC2	NM_003083	small nuclear RNA activating complex, polypeptide 2, 45kDa	1.3
ACIN1	NM_014977	apoptotic chromatin condensation inducer 1	1.3
ITGAE	NM_002208	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide)	1.3

GNAO1	NM_020988	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O	1.3
SERPINB2	NM_002575	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	1.3
C20orf72	NM_052865	chromosome 20 open reading frame 72	1.3
MAPK7	NM_002749	mitogen-activated protein kinase 7	1.3
CADPS2	NM_017954	Ca2+-dependent activator protein for secretion 2	1.3
HLA-B	NM_005514	major histocompatibility complex, class I, B	1.3
P4HB	NM_000918	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase-associated 1)	1.3
GMFB	NM_004124	glia maturation factor, beta	1.3
MGC9850	NM_152705	hypothetical protein MGC9850	1.3
SIX5	NM_175875	sine oculis homeobox homolog 5 (Drosophila)	1.3
GFPT2	NM_005110	glutamine-fructose-6-phosphate transaminase 2	1.3
CCNC	NM_005190	cyclin C	1.3
SLC25A3	NM_005888	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	1.3
CSTB	NM_000100	cystatin B (stefin B)	1.3
SOD2	NM_001024465	superoxide dismutase 2, mitochondrial	1.3
CDCA7	NM_031942	cell division cycle associated 7	1.3
RBP4	NM_006744	retinol binding protein 4, plasma	1.3
MADP-1	NM_033114	MADP-1 protein	1.3
ATP5C1	NM_001001973	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	1.3
GHITM	NM_014394	growth hormone inducible transmembrane protein	1.3
CYB5	NM_148923	cytochrome b-5	1.3
IGF1	NM_000618	insulin-like growth factor 1 (somatomedin C)	1.3
RPS6	NM_001010	ribosomal protein S6	1.3
STAT5A	NM_003152	signal transducer and activator of transcription 5A	1.3
UBE2M	NM_003969	ubiquitin-conjugating enzyme E2M (UBC12 homolog, yeast)	1.3
UGP2	NM_001001521	UDP-glucose pyrophosphorylase 2	1.3
CREBL1	NM_004381	cAMP responsive element binding protein-like 1	1.3
THSD6	NM_213604	thrombospondin, type I, domain containing 6	1.3
SCC-112	NM_015200	SCC-112 protein	1.3
DOCK8	NM_203447	dedicator of cytokinesis 8	1.3
SLC7A8	NM_182728	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	1.3
EPHB3	NM_004443	EPH receptor B3	1.3
DUSP7	NM_001947	dual specificity phosphatase 7	1.3
PRKD2	NM_016457	protein kinase D2	1.3
EZH2	NM_004456	enhancer of zeste homolog 2 (Drosophila)	1.3

CTSD	NM_001909	cathepsin D (lysosomal aspartyl protease)	1.3
EIF4EBP2	NM_004096	eukaryotic translation initiation factor 4E binding protein 2	1.3
PKN3	NM_013355	protein kinase N3	1.3
RAB3B	NM_002867	RAB3B, member RAS oncogene family	1.3
NCOA4	NM_005437	nuclear receptor coactivator 4	1.3
FOXP2	NM_148899	forkhead box P2	1.3
GNB4	NM_021629	guanine nucleotide binding protein (G protein), beta polypeptide 4	1.3
PCDH17	NM_014459	protocadherin 17	1.3
RAB35	NM_006861	RAB35, member RAS oncogene family	1.3
SLC15A2	NM_021082	solute carrier family 15 (H+/peptide transporter), member 2	1.3
RBMXL1	NM_019610	RNA binding motif protein, X-linked-like 1	1.3
LAMB1	NM_002291	laminin, beta 1	1.3
ATP2A1	NM_004320	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	1.3
MYST3	NM_006766	MYST histone acetyltransferase (monocytic leukemia) 3	1.3
FABP3	NM_004102	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	1.3
WARS	NM_213646	tryptophanyl-tRNA synthetase	1.3
OAZ1	NM_004152	ornithine decarboxylase antizyme 1	1.3
KRT9	NM_000226	keratin 9 (epidermolytic palmoplantar keratoderma)	1.3
FAM20A	NM_017565	family with sequence similarity 20, member A	1.3
DUSP3	NM_004090	dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related)	1.3
MMP1	NM_002421	matrix metalloproteinase 1 (interstitial collagenase)	1.3
CTSO	NM_001334	cathepsin O	1.3
EIF3S3	NM_003756	eukaryotic translation initiation factor 3, subunit 3 gamma, 40kDa	1.3
ATP6V1D	NM_015994	ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D	1.3
MCM6	NM_005915	MCM6 minichromosome maintainance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	1.3
GC20	NM_005875	translation factor sui1 homolog	1.3
ELL2	NM_012081	elongation factor, RNA polymerase II, 2	1.3
TUBB2	NM_001069	tubulin, beta 2	1.3
ASNS	NM_133436	asparagine synthetase	1.3
SLC39A5	NM_173596	solute carrier family 39 (metal ion transporter), member 5	1.3
RNASE4	NM_002937	ribonuclease, RNase A family, 4	1.3
DLG5	NM_004747	discs, large homolog 5 (Drosophila)	1.3
PLXNC1	NM_005761	plexin C1	1.3
IDH3B	NM_006899	isocitrate dehydrogenase 3 (NAD+) beta	1.3
ACTG1	NM_001614	actin, gamma 1	1.3

ZNF174	NM_003450	zinc finger protein 174	1.3
TNNT3	NM_006757	troponin T3, skeletal, fast	1.3
NFKBIA	NM_020529	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.3
B4GALT1	NM_001497	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	1.3
SH2D3C	NM_170600	SH2 domain containing 3C	1.3
PLEKHA2	XM_496973	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 2	1.3
SERPINB9	NM_004155	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 9	1.3
SLC38A2	NM_018976	solute carrier family 38, member 2	1.3
CEACAM1	NM_001024912	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	1.3
RPS27	NM_001030	ribosomal protein S27 (metallopanstimulin 1)	1.3
G6PC	NM_000151	glucose-6-phosphatase, catalytic (glycogen storage disease type I, von Gierke disease)	1.3
PCK2	NM_004563	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	1.3
IGBP1	NM_001551	immunoglobulin (CD79A) binding protein 1	1.3
CCL11	NM_002986	chemokine (C-C motif) ligand 11	1.3
TIMD4	NM_138379	T-cell immunoglobulin and mucin domain containing 4	1.3
TM4SF4	NM_004617	transmembrane 4 L six family member 4	1.3
MDM2	NM_002392	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	1.3
MMP12	NM_002426	matrix metalloproteinase 12 (macrophage elastase)	1.4
POLR2K	NM_005034	polymerase (RNA) II (DNA directed) polypeptide K, 7.0kDa	1.4
SLC22A17	NM_016609	solute carrier family 22 (organic cation transporter), member 17	1.4
ANG	NM_001145	angiogenin, ribonuclease, RNase A family, 5	1.4
DDB2	NM_000107	damage-specific DNA binding protein 2, 48kDa	1.4
FZD6	NM_003506	frizzled homolog 6 (Drosophila)	1.4
CDC42SE1	NM_020239	CDC42 small effector 1	1.4
BST1	NM_004334	bone marrow stromal cell antigen 1	1.4
SNX5	NM_014426	sorting nexin 5	1.4
SCAMP5	NM_138967	secretory carrier membrane protein 5	1.4
NDUFV3	NM_021075	NADH dehydrogenase (ubiquinone) flavoprotein 3, 10kDa	1.4
ZNF226	NM_016444	zinc finger protein 226	1.4
BID	NM_197967	BH3 interacting domain death agonist	1.4
SREBF1	NM_001005291	sterol regulatory element binding transcription factor 1	1.4
SLC25A11	NM_003562	solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	1.4
ITPR1	NM_002222	inositol 1,4,5-triphosphate receptor, type 1	1.4
KRT17	NM_000422	keratin 17	1.4
L1CAM	NM_000425	L1 cell adhesion molecule	1.4

SLC3A2	NM_001012661	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	1.4
EEF2K	NM_013302	eukaryotic elongation factor-2 kinase	1.4
TTRAP	NM_016614	TRAF and TNF receptor associated protein	1.4
TA-PP2C	NM_139283	T-cell activation protein phosphatase 2C	1.4
CD53	NM_000560	CD53 antigen	1.4
RTN4	NM_007008	reticulon 4	1.4
SAFB	NM_002967	scaffold attachment factor B	1.4
GAS7	NM_201432	growth arrest-specific 7	1.4
CXCL12	NM_000609	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	1.4
PPM1A	NM_021003	protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform	1.4
ARPC1A	NM_006409	actin related protein 2/3 complex, subunit 1A, 41kDa	1.4
GNB2	NM_005273	guanine nucleotide binding protein (G protein), beta polypeptide 2	1.4
ESM1	NM_007036	endothelial cell-specific molecule 1	1.4
EIF5B	NM_015904	eukaryotic translation initiation factor 5B	1.4
UBE1L	NM_003335	ubiquitin-activating enzyme E1-like	1.4
ARIH2	NM_006321	ariadne homolog 2 (Drosophila)	1.4
ACOX1	NM_004035	acyl-Coenzyme A oxidase 1, palmitoyl	1.4
CYP2C18	NM_000772	cytochrome P450, family 2, subfamily C, polypeptide 18	1.4
MAP1LC3B	NM_022818	microtubule-associated protein 1 light chain 3 beta	1.4
PTPRJ	NM_002843	protein tyrosine phosphatase, receptor type, J	1.4
TTN	NM_003319	Titin	1.4
HSPC196	NM_016464	hypothetical protein HSPC196	1.4
BCL11A	NM_022893	B-cell CLL/lymphoma 11A (zinc finger protein)	1.4
KRT19	NM_002276	keratin 19	1.4
MAEA	NM_001017405	macrophage erythroblast attacher	1.4
P8	NM_012385	p8 protein (candidate of metastasis 1)	1.4
GALM	NM_138801	galactose mutarotase (aldose 1-epimerase)	1.4
GALK1	NM_000154	galactokinase 1	1.4
MKNK2	NM_199054	MAP kinase interacting serine/threonine kinase 2	1.4
CHUK	NM_001278	conserved helix-loop-helix ubiquitous kinase	1.4
MAP4K3	NM_003618	mitogen-activated protein kinase kinase kinase kinase 3	1.4
HEBP1	NM_015987	heme binding protein 1	1.4
CSPG3	NM_004386	chondroitin sulfate proteoglycan 3 (neurocan)	1.4
ZNF76	NM_003427	zinc finger protein 76 (expressed in testis)	1.4
TFRC	NM_003234	transferrin receptor (p90, CD71)	1.4

KRT13	NM_002274	keratin 13	1.4
FURIN	NM_002569	furin (paired basic amino acid cleaving enzyme)	1.4
ADAM10	NM_001110	a disintegrin and metalloproteinase domain 10	1.4
NET1	NM_005863	neuroepithelial cell transforming gene 1	1.4
LIPC	NM_000236	lipase, hepatic	1.4
CD74	NM_001025159	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	1.4
RPS26	NM_001029	ribosomal protein S26	1.4
ACO1	NM_002197	aconitase 1, soluble	1.4
HOXA6	NM_024014	homeo box A6	1.4
TNFRSF1B	NM_001066	tumor necrosis factor receptor superfamily, member 1B	1.4
CNNM3	NM_017623	cyclin M3	1.4
GPX3	NM_002084	glutathione peroxidase 3 (plasma)	1.4
CHRNB1	NM_000747	cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)	1.4
ADAM33	NM_153202	a disintegrin and metalloproteinase domain 33	1.4
HLA-C	NM_002117	major histocompatibility complex, class I, C	1.4
EEF2	NM_001961	eukaryotic translation elongation factor 2	1.4
ACVRL1	NM_000020	activin A receptor type II-like 1	1.4
IBRDC2	NM_182757	IBR domain containing 2	1.4
CTDSP2	NM_005730	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2	1.4
INSR	NM_000208	insulin receptor	1.4
ATF4	NM_182810	activating transcription factor 4 (tax-responsive enhancer element B67)	1.4
SGKL	NM_013257	serum/glucocorticoid regulated kinase-like	1.4
HLA-A	NM_002116	major histocompatibility complex, class I, A	1.5
MAN2B1	NM_000528	mannosidase, alpha, class 2B, member 1	1.5
CTNND1	NM_001331	catenin (cadherin-associated protein), delta 1	1.5
F2R	NM_001992	coagulation factor II (thrombin) receptor	1.5
KCNK5	NM_003740	potassium channel, subfamily K, member 5	1.5
RNF130	NM_018434	ring finger protein 130	1.5
FMO3	NM_006894	flavin containing monooxygenase 3	1.5
CD36	NM_000072	CD36 antigen (collagen type I receptor, thrombospondin receptor)	1.5
PRSS16	NM_005865	protease, serine, 16 (thymus)	1.5
INHBA	NM_002192	inhibin, beta A (activin A, activin AB alpha polypeptide)	1.5
RAB18	NM_021252	RAB18, member RAS oncogene family	1.5
ISG20	NM_002201	interferon stimulated exonuclease gene 20kDa	1.5
OGT	NM_181673	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	1.5

THTPA	NM_024328	thiamine triphosphatase	1.5
CAMK2D	NM_172115	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	1.5
ZNF148	NM_021964	zinc finger protein 148 (pHZ-52)	1.5
FDX1	NM_004109	ferredoxin 1	1.5
GTF2B	NM_001514	general transcription factor IIB	1.5
OSBPL10	NM_017784	oxysterol binding protein-like 10	1.5
PTPN12	NM_002835	protein tyrosine phosphatase, non-receptor type 12	1.5
YWHAE	NM_006761	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	1.5
ABCG2	NM_004827	ATP-binding cassette, sub-family G (WHITE), member 2	1.5
LPL	NM_000237	lipoprotein lipase	1.5
ELF5	NM_001422	E74-like factor 5 (ets domain transcription factor)	1.5
PISD	NM_014338	phosphatidylserine decarboxylase	1.5
CD24	NM_013230	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	1.6
CIRBP	NM_001280	cold inducible RNA binding protein	1.6
FZD4	NM_012193	frizzled homolog 4 (Drosophila)	1.6
SCARB2	NM_005506	scavenger receptor class B, member 2	1.6
SUI1	NM_005801	putative translation initiation factor	1.6
COX5A	NM_004255	cytochrome c oxidase subunit Va	1.6
AKNA	NM_030767	AT-hook transcription factor	1.6
AKR1C1	NM_001353	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	1.6
SEPP1	NM_005410	selenoprotein P, plasma, 1	1.7
DHX9	NM_001357	DEAH (Asp-Glu-Ala-His) box polypeptide 9	1.7
SLC25A19	NM_021734	solute carrier family 25 (mitochondrial deoxynucleotide carrier), member 19	1.7
GAB1	NM_207123	GRB2-associated binding protein 1	1.7
PLA2G2A	NM_000300	phospholipase A2, group IIA (platelets, synovial fluid)	1.7
LAS1L	NM_031206	LAS1-like (S. cerevisiae)	1.7
CKLFSF8	NM_178868	chemokine-like factor super family 8	1.8
PABPC1	NM_002568	poly(A) binding protein, cytoplasmic 1	1.8
RBM12	NM_006047	RNA binding motif protein 12	2.5

Table B.3: Significant associations between gene sets and ontological annotations. Categories of genes up-regulated in response to growth hormone treatment

Root name	Node name	GO ID	P value
Biological process	anion transport	GO:0006820	0.0371
	antigen processing and presentation	GO:0019882	0.0000
	cell organization and biogenesis	GO:0016043	0.0367
	fatty acid metabolism	GO:0006631	0.0004
	mitotic spindle assembly	GO:0051227	0.0003
	neuron differentiation	GO:0030182	0.0008
	translational elongation	GO:0006414	0.0003
	protein polymerization	GO:0051258	0.0000
	regulation of cell cycle	GO:0051726	0.0019
	inorganic anion transport	GO:0015698	0.0369
	immune response	GO:0006959	0.0020
	phosphate transport	GO:0006817	0.0000
Cellular component	cell surface	GO:0009986	0.0013
	collagen type I	GO:0005584	0.0001
	collagen type III	GO:0005586	0.0000
	eukaryotic translation elongation factor 1 complex	GO:0005853	0.0022
	Microtubule	GO:0005874	0.0009
Molecular function	ferric iron binding	GO:0008199	0.0007
	GTPase activity	GO:0003924	0.0000
	insulin binding	GO:0043559	0.0023
	metalloendopeptidase inhibitor activity	GO:0008191	0.0001
	MHC class I protein binding	GO:0042288	0.0002
	pancreatic ribonuclease activity	GO:0004522	0.0001
	peptidoglycan receptor activity	GO:0016019	0.0001
	receptor signaling protein serine/threonine kinase activity	GO:0004702	0.0006
	structural constituent of bone	GO:0008147	0.0000
	structural constituent of ribosome	GO:0003735	0.0015
	symporter activity	GO:0015293	0.0008
	transforming growth factor beta receptor binding	GO:0005160	0.0009

Table B.4: Significant associations between gene sets and ontological annotations. Categories of genes down-regulated in response to growth hormone treatment

Root name	Node name	GO ID	P value
Biological process	alcohol metabolism	GO:0006066	0.0025
	carbohydrate metabolism	GO:0005975	0.0002
	cellular lipid catabolism	GO:0044242	0.0204
	fatty acid catabolism	GO:0009062	0.0030
	mitochondrial transport	GO:0006839	0.0047
	negative regulation of angiogenesis	GO:0016525	0.0015
	peptidyl-serine phosphorylation	GO:0018105	0.0021
	platelet activation	GO:0030168	0.0001
	purine nucleotide biosynthesis	GO:0006164	0.0039
	response to extracellular stimulus	GO:0009991	0.0005
Cellular component	fibrinogen complex	GO:0005577	0.0001
	intermediate filament	GO:0005882	0.0005
	intracellular part	GO:0044424	0.0002
Molecular function	catalytic activity	GO:0003824	0.0015
	cation channel activity	GO:0005261	0.0020
	Rho GTPase activator activity	GO:0005100	0.0003

Table B.5: Significant associations between gene sets and ontological annotations. Categories of genes up-regulated in response to atropine treatment

Root name	Node name	GO ID	P value
Biological process	antigen processing and presentation	GO:0019882	0.0000
	biosynthesis	GO:0009058	0.0006
	cellular physiological process	GO:0050875	0.0000
	cytoplasmic sequestering of NF-kappaB	GO:0007253	0.0003
	regulation of muscle contraction	GO:0006937	0.0002
	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	GO:0019219	0.0104
	regulation of transcription, DNA-dependent	GO:0006355	0.0013
	response to wounding	GO:0009611	0.0009
	selenium metabolism	GO:0001887	0.0000
	tryptophan transport	GO:0015827	0.0000
Cellular component	cytoplasmic part	GO:0044444	0.0000
	troponin complex	GO:0005861	0.0009
	cytosolic ribosome (sensu Eukaryota)	GO:0005830	0.0000
	nucleus	GO:0005634	0.0001
	receptor complex	GO:0043235	0.0010
	spliceosome complex	GO:0005681	0.0008
	striated muscle thin filament	GO:0005865	0.0000
Molecular function	calcium:sodium antiporter activity	GO:0005432	0.0003
	interferon-gamma receptor activity	GO:0004906	0.0002
	magnesium ion binding	GO:0000287	0.0006
	peptidyl-prolyl cis-trans isomerase activity	GO:0003755	0.0004
	phosphate carrier activity	GO:0015320	0.0002
	phosphate transporter activity	GO:0015114	0.0361
	poly(A) binding	GO:0008143	0.0000
	structural constituent of cytoskeleton	GO:0005200	0.0000

Table B.6: Significant associations between gene sets and ontological annotations. Categories of genes down-regulated in response to atropine treatment

Root name	Node name	GO ID	P value
Biological process	antigen processing and presentation	GO:0019882	0.0003
	cellular lipid catabolism	GO:0044242	0.0014
	chromatin assembly	GO:0031497	0.0027
	elastic fiber assembly	GO:0048251	0.0023
	meiosis	GO:0007126	0.0192
	mRNA metabolism	GO:0016071	0.0029
	muscle filament sliding	GO:0030049	0.0021
	muscle thick filament assembly	GO:0030241	0.0023
	phosphate transport	GO:0006817	0.0000
	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	GO:0006139	0.0264
	positive regulation of growth	GO:0045927	0.0038
	potassium ion transport	GO:0006813	0.0030
	protein targeting	GO:0006605	0.0010
	protein-nucleus export	GO:0006611	0.0016
	regulation of body size	GO:0040014	0.0048
	regulation of cytokine production	GO:0001817	0.0041
	regulation of meiosis	GO:0040020	0.0012
	RNA processing	GO:0006396	0.0045
	sarcomere organization	GO:0045214	0.0018
	smooth muscle contraction	GO:0006939	0.0033
	smooth muscle fiber development	GO:0048746	0.0023
	tRNA aminoacylation for protein translation	GO:0006418	0.0004
Cellular component	basal lamina	GO:0005605	0.0459
	collagen type III	GO:0005586	0.0001
	cytoplasmic part	GO:0044444	0.0460
	cytosolic large ribosomal subunit (sensu Eukaryota)	GO:0005842	0.0048
	cytosolic part	GO:0044445	0.0479
	cytosolic ribosome (sensu Eukaryota)	GO:0005830	0.0049
	cytosolic small ribosomal subunit (sensu Eukaryota)	GO:0005843	0.0001
	endoplasmic reticulum lumen	GO:0005788	0.0018

	extracellular matrix	GO:0044420	0.0449
	integral to endoplasmic reticulum membrane	GO:0030176	0.0046
	unconventional myosin	GO:0016461	0.0002
	nucleus	GO:0005634	0.0001
	voltage-gated potassium channel complex	GO:0008076	0.0048
Molecular function	aminoacyl-tRNA ligase activity	GO:0004812	0.0002
	ATPase activity, coupled	GO:0042623	0.0019
	calcium ion binding	GO:0005509	0.0002
	cation channel activity	GO:0005261	0.0262
	DNA binding	GO:0003677	0.0208
	fatty acid binding	GO:0005504	0.0009
	structural constituent of cytoskeleton	GO:0005200	0.0000
	structural constituent of ribosome	GO:0003735	0.0000
	structural molecule activity	GO:0005198	0.0375
	transcription factor activity	GO:0003700	0.0019
	intramolecular oxidoreductase activity	GO:0016860	0.0385
	intramolecular oxidoreductase activity, interconverting keto- and enol-groups	GO:0016862	0.0009
	tRNA ligase activity	GO:0004812	0.0002
	motor activity	GO:0003774	0.0043
	nucleic acid binding	GO:0003676	0.0176
	potassium channel activity	GO:0005267	0.0453
	phospholipid-hydroperoxide glutathione peroxidase activity	GO:0047066	0.0015
	voltage-gated potassium channel activity	GO:0005249	0.0027

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