The Mechanistic Target of Rapamycin Complex 1 Controls Lipid and Lactose Synthesis in Bovine Mammary Epithelial Cells

Marc-Antoine Guesthier

Department of Animal Science
McGill University, Montreal

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ABSTRACT

The objective of this study was to assess the role of the mechanistic target of rapamycin complex 1 (**mTORC1**) on lipid and lactose synthesis in bovine mammary epithelial cells (**BMECs**). Cells isolated from the mammary tissue of 3 lactating Holstein cows by enzymatic digestion were expanded and induced to differentiate with lactogenic hormones (**LH**; insulin, prolactin and hydrocortisone) for 4 d. Relative mRNA levels were measured by real-time quantitative PCR (**RT-qPCR**). Protein abundance and site-specific phosphorylation were measured by immunoblotting. Lipid and lactose synthesis were assessed by the incorporation of radiolabeled acetate and glucose, respectively. Statistical analyses were conducted by ANOVA using PROC MIXED in SAS. To ascertain the role of mTORC1 on milk component synthesis, we combined pharmacological and genetic manipulation approaches. We first confirmed that LH treatment induced lactogenic differentiation of BMECs, as measured by increased expression of mammary-specific milk protein genes *CSN1S1* (2-fold; $p = 0.02$) and *LALBA* (1.7-fold; $p = 0.03$). Then, lactogenic differentiated BMECs were treated with either 100 n*M* rapamycin (**RAP**) or vehicle control (**Veh**) for 16 h. Compared to vehicle-treated cells, phosphorylation of 3 important markers of mTORC1 activity; S6K1 Thr389 ($p = 0.001$), rpS6 Ser240/244 ($p = 0.009$), and 4E-BP1 Thr70 ($p < 0.0001$) was lower in BMECs treated with rapamycin. Lipid synthesis was 24% ($p = 0.001$) lower in rapamycin-treated BMECs, which was accompanied by significantly lower mRNA expression of *FASN* ($p = 0.04$) and nominal reductions in *DGAT1* ($p = 0.07$) and *FABP3* ($p = 0.08$) expression, indicative of coordinated transcriptional regulation. Indeed, the expression of *SREBF1,* the gene encoding for the master regulator of lipid synthesis SREBP1, was 31% lower ($p = 0.04$) in BMECs treated with rapamycin. However, there was no difference in SREBP1 protein abundance suggesting mTORC1 may affect lipid synthesis by other mechanisms. In addition, lactose synthesis was 71% lower ($p < 0.001$) in rapamycin-treated BMECs, which was

accompanied by reduced expression of *PGM1* (p = 0.03) and *B4GALT* (p=0.003), two genes involved in lactose synthesis. To complement our pharmacological approach, we used lentivirus-delivered CRISPR-Cas9-mediated knockout (**KO**) of TSC2, a critical upstream regulator of mTORC1 activity, as a genetic model of mTORC1 hyper-activation. BMECs transduced with guide RNAs (**gRNA**) targeting the *TSC2* locus had 95% lower (p <0.0001) TSC2 protein abundance. As expected, TSC2 KO BMECs had higher phosphorylation of rpS6 Ser240/244 (p=0.004) compared to cells transduced with non-targeting gRNAs, demonstrating mTORC1 hyper-activation. Importantly, TSC2 KO cells had higher expression of genes involved in lipogenesis including *SREBF1* (1.6-fold; p = 0.001) and *FABP3* (1.4-fold; p = 0.01) as well as lactose synthesis, *PGM1* (1.3-fold; p = 0.03) and *B4GALT1* (1.8-fold; p = 0.006). In conclusion, these results demonstrate a critical role for mTORC1 in the regulation of both lipid and lactose synthesis in BMECs.

RÉSUMÉ

L'objectif de cette étude était d'évaluer le rôle de *mechanistic target of rapamycin complex 1* (**mTORC1**) sur la synthèse des lipides et du lactose dans les cellules mammaires épithéliales bovines (**CMEBs**), Les cellules de tissues mammaires de 3 vaches de race holstein en lactation ont été isolées par digestion enzymatique et induits à se différencier par des hormones lactogéniques (**HL**) (insuline, prolactine et hydrocortisone) pendant 4 jours. Les niveaux relatifs d'ARNm ont été mesurés par PCR quantitatif en temps réel (**RT-qPCR**). L'abondance en protéines et la phosphorylation au site spécifique ont été mesurées par immunoempreinte. La synthèse des lipides et du lactose a été évaluée par l'incorporation d'acétate et de glucose radioactifs, respectivement. Les analyses statistiques ont été faites par ANOVA en utilisant la fonction PROC MIXED dans le logiciel SAS. Pour déterminer le rôle de mTORC1 sur la synthèse des composants du lait, nous avons combiné des approches pharmacologiques et de manipulations génétiques. Premièrement, nous avons confirmé que le traitement d'HL induisait la différenciation des CMEBs. Pour ce faire nous avons mesuré l'expression des gènes de protéines spécifiques à la production de lait, soit l'ARNm de *CSN1S1* (2 fois ; $p = 0,02$) et *LALBA* (1,7 fois, $p = 0,03$) qui était tous deux augmenté par le traitement aux HLs. Ensuite, des CMEBs différenciées avec des HLs ont été traitées avec de la 100 n*M* de rapamycin (**Rap**) ou avec un véhicule (**Veh**) pendant 16 heures. Comparativement aux cellules traitées avec le véhicule, la phosphorylation de 3 importants marqueurs de l'activité de mTORC1 : S6K1 Thr389 ($p = 0,001$), de rpS6 Ser240 / 244 ($p = 0,009$) et de 4E-BP1 Thr70 ($p < 0,0001$) était plus faible dans les cellules CMEBs traitées à la rapamycin. La synthèse lipidique était inférieure de 24 % ($p = 0,001$) chez les CMEBs traités à la rapamycin, accompagnée d'une expression plus faible du gène *FASN* ($p = 0,04$), et d'une réduction nominale des gènes : *DGAT1* ($p = 0,07$) et de *FABP3* ($p = 0,08$), ce qui laisse croire une coordination dans la régulation de la transcription des gènes impliqués dans la synthèse des lipides par mTORC1. De

plus, l'expression du gène *SREBF1,* responsable de la transcription de l'importante protéine impliquée dans la synthèse des lipides : SREBP1, était 31 % réduite (p = 0,04) dans les CMEBs traitées à la rapamycin. Cependant, aucune différence dans l'abondance de la protéine SREBP1 n'a était observé avec le traitement pharmacologique ce qui suggère que mTORC1 influence la synthèse des lipides par un mécanisme différent. De plus, la synthèse du lactose était inférieure de 71 % (p < 0,001) dans les CMEBs traités à la rapamycin, ce qui était accompagné d'une expression réduite de *PGM1* (p = 0,03) et de *B4GALT* (p = 0,003), deux gènes impliqués dans la synthèse de lactose. Pour compléter notre approche pharmacologique, nous avons procédé à l'inactivation du gène *TSC2* en utilisant la méthode CRISPR-Cas9 avec une livraison lentivirus. Le complexe protéique TSC2 est un important régulateur négatif de l'activité de mTORC1. Ceci nous a permis de créer un modèle génétique d'hyper-activation de mTORC1. Comparés aux cellules transduites avec un lentivirus ARN simple guide (**gRNA**), les CMEBs transduites avec le gRNA ciblant *TSC2* présentaient une abondance en protéines de TSC2 inférieure de 95 % (p < 0,000 1). La phosphorylation de la cible de mTORC1 rpS6 Ser240/244 (p = 0,004) était aussi plus élevée dans les CMEBs transduites avec le gRNA ciblant TSC2, démontrant l'inactivation du complex TSC2 et l'hyper-activation de mTORC1. Les cellules avec TSC2 désactivées avaient aussi une expression plus élevée de gènes impliqués dans la lipogenèse, soit *SREBF1* (1,6 fois ; p = 0,001) et *FABP3* (1,4 fois; p= 0,01), et dans la synthèse du lactose, soit *PGM1* (1,3 fois; p= 0,03) et *B4GALT1* (1,8 fois; p= 0,006). En conclusion, ces résultats démontrent un rôle critique pour mTORC1 dans la régulation de la synthèse des lipides et du lactose dans les CMEBs.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

The use of the genomic editing tool Clustered Regularly Interspaced Short Palindromic Repeats and its associate endonuclease Cas9 in differentiated bovine mammary epithelial cells was considered as an original contribution to knowledge. In addition, evidence that mTORC1 is involved in lactose synthesis in regulation in bovine mammary epithelial cells was also considered as original contribution to general knowledge.

CONTRIBUTION OF THE AUTHORS

General Introduction and Literature Review: Edick, A. Guesthier, S.

Role of mTORC1 in lipid and lactose synthesis in Bovine Mammary Epithelial cells (Article): Huang, J., Burgos, S., Edick, A.

Discussion: Burgos, S., Edick, A.

GENERAL INTRODUCTION

Agriculture is an important industry that accounted for 6.6% of the total Canadian gross domestic product in 2016. According to Agriculture Canada (2017), Food-related production in Canada corresponds to 1.5% of the global food production even though the Canadian population represents less than 0.5% of the world population. Dairy production is an important pillar of the Canadian agriculture industry. In 2016, the Canadian Dairy Commission (2017) ranked the dairy sector second in terms of importance in agriculture activities; outranked only by red meat production. The dairy cow population in Canada was estimated to be 1.4 million head (dairy cows and heifers) producing more than 84.7 hectolitres of milk per year. The number of dairy farms was estimated to be 11,280 according to the Canadian Dairy Commission (2017). The Canadian dairy industry operates under a strict supply management agreement based on planned domestic production.

The chemistry and physico-chemical properties of milk have been studied rigorously throughout the last two centuries and are now understood in considerable detail. Due to the prominence of this industry in Canada, dairy science research is an important aspect of Canadian agriculture development programs. A vast array of research concentrating on genetics, welfare, reproduction and nutrition allow the Canadians dairy research programs to be some of the most comprehensive in the world.

Several metabolic changes and mechanisms underlie the milk production processes. Fluctuations in nutrients and energy supply to the mammary gland are known to influence milk synthesis in dairy cows. In dairy nutrition, yield and milk constituent variation have traditionally been explained by endocrine regulation and fluctuation in nutrient availability to the mammary gland. Nutrients serving as substrates for milk components were identified to be partially

responsible for certain variations in milk component synthesis. Recent studies suggest that the regulation mechanisms involved in nutrients metabolism are more complex that previously suggested. Thus, nutrients may not only serve as substrates or building block for milk synthesis but also as signalling molecules. Now defined as functional nutrients, they are believed to be involved in signalling cascades that communicate information to the cells and engage specific transduction pathways. However, the mechanism by which the cell relay that information remains unclear.

The mechanistic target of rapamycin is an evolutionary conserved, nutrient-sensing protein complex that has emerged as a central regulator of nutrient metabolism in various tissues. The mechanistic target of rapamycin integrates signals indicating nutrient levels including amino acid availability, cellular energy status and hormones levels. This information conveyed by nutrient signalling molecules is later used by the mechanistic target of rapamycin to control a multitude of catabolic and anabolic cellular processes. The mechanistic target of rapamycin is the major catalytic subunit of two distinct complexes, the mechanistic target of rapamycin complex 1 and 2. The discovery of the pharmacological agent named rapamycin has allowed its partial manipulation. Rapamycin was first isolated from the soil bacterium *streptomyces hygroscopicus* initially collected on the Easter Island (Rapa Nui) in the early 1970s. Primary used as an antifungal drug, it was later found to act has an immunosuppressant and to have antiproliferative properties (Sabatini et al., 1994). Rapamycin has the capacity to inhibit certain functions of the mechanistic target of rapamycin complex 1, although, its efficacy as an inhibitor may be compromised in the case of chronic usage due to rapamycin resistance.

Even though rapamycin has shown to be capable of inhibiting certain functions of mTORC1, some remain active after treatment. An amalgamation of studies has led to the

understanding that mTORC1 has both rapamycin-sensitive and rapamycin-insensitive substrates. Thus, to fully understand the role of mTORC1 in nutrient metabolism, novel genomic manipulation methods need to be employed to create specific and complete alteration of the mTORC1 protein complex activity. Recently, the discovery of the precise and powerful tool named: Clustered Regularly Interspaced Short Palindromic Repeats and its associated endonuclease protein Cas9 has opened the door to a new set of possibilities in the field of genomic editing. The development of this innovative, efficient and reliable tool allows the induction of specific targeted changes in the genome of living cells. This new biotechnology, based on the adaptive bacterial immune response, was first isolated from *Streptococcus pyogenes*. It enables the introduction of mutations creating specific genes knock-outs facilitating the study of complex mechanisms involved in animal biology.

1. LITERATURE REVIEW

1.1. MAMMARY GLAND ANATOMY AND PHYSIOLOGY

The class Mammalia is distinguished by the presence of a mammary gland, a specific organ possessed by the female of the species, which produces and secretes milk for the survival of her offspring. Milk synthesis occurs in the tissue of the mammary gland, which is made by the secretory cells of this tissue. Dairy cattle are well known for their high milk production capacity and have been utilised by humans for their ability to produce large quantities of nutritious milk for human consumption.

The mammary gland of dairy cows is a milk-secreting structure which includes the teat, ducts, and lobules of secretory tissues encompassed in the utter of the cow. The milk generated in the mammary gland tissues is then drained by the duct system of the tissue, a process that has been thoroughly described in the literature. This system allows the production, accumulation and secretion of milk. The mammary gland is an accessory organ of the reproductive system and is closely related to the reproductive cycle of the cow while developing. It is one of the few tissues capable of undergoing repeated cycles of growth, functional differentiation and regression (Hurley and Loor, 2011). As a cycling organ, the mammary gland is influenced by endocrine, nutritional, environmental and management factors before, during, and after lactation cycles. A comprehensive understanding of the developmental stages and lactation cycles of the bovine mammary gland (**BMG**) are fundamental to the study of the effects of nutrition and physiological factors on lactation and milk components synthesis.

The external appearance of the BMG has been thoroughly studied and is defined as the udder of the cow. It is formed by four mammary glands joined together. The external structure of the BMG is supported by the lateral suspensory ligament and the skin. Each mammary gland

functions independently and they are often referred to as individual quarters as described by Akers (2002). The right and left halves of the udder are supported internally by the medial suspensory ligament and are separated by the intermammary groove. The lobules of the udder, composed of alveoli, are surrounded by connective tissue capsules. Each quarter is composed of the body of the gland, the secretory tissues transporting the milk to the internal reserved called the gland cistern as described by endoscopic examination by Vangroenweghe et al. (2006) and as shown in Figure 1.1. Milk is secreted into the lumen of the alveoli called the milk space or the lumen in the **BMG**. When the milk is expulsed it goes through the teat's cistern before reaching the streak canal as described by Paulrud (2005).

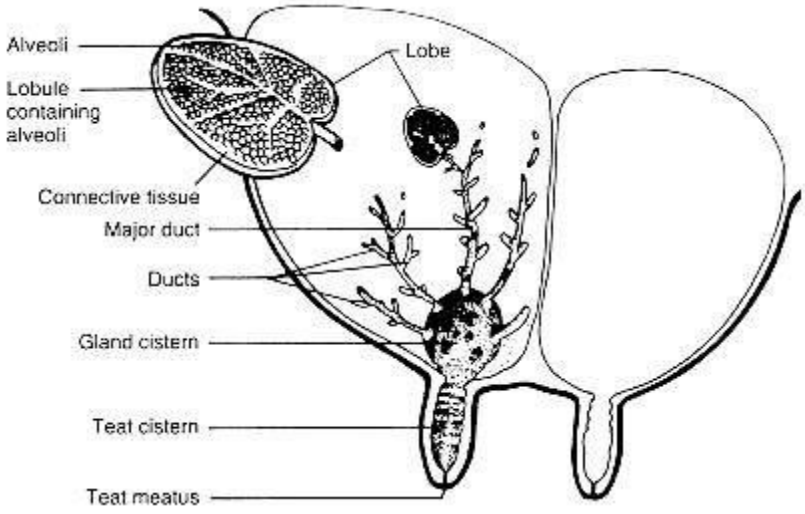


Figure 1.1: *Schematic Representation of the Cow Mammary Gland and its Anatomy. With permission from: Cortes (2010)*

The secretory tissues are composed of clusters of alveoli that are separated by fibrous connective tissue to form lobules that are joined together by a common intralobular duct system. A schematic representation of alveoli is show in Figure 1.2. The clusters of lobules, referred to as lobes or acini, drain into a joint duct system primarily formed by a bilayered epithelium. The

bilayered structures surrounded by a basement membrane of cells support the secretion of milk components (Faraldo et al., 2006). Adriance et al. (2005) showed that myoepithelial cells not only serve as a protective layer to the secretory cells, they also harbor the epithelial progenitors. The secretory epithelial cells are polarized, with the nuclei clustered in the basal region. Cellular organelles responsible for milk component secretion occupy a significant proportion of the intracellular space in secretory cells during lactation. The Golgi apparatus, mitochondria and secretory vesicles become abundant during secretion.

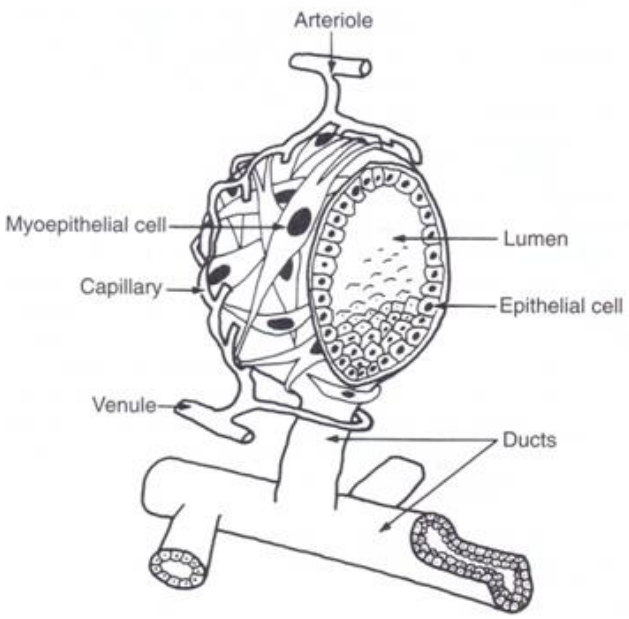


Figure 1.2: *Schematic Representation of Alveoli in BMG. With permission from: (Pond and Bell, 2005)*

Milk components are secreted towards the apical region. Alkafafy et al. (2012) concluded that the basal side of the cells are anchored in collagen structures, in agreement with Ditcham et al. (1993) who showed that collagen coating plates is a sustainable matrix to support culture of cells derived from secretory alveoli in BMG. Epithelial cells are linked together by tight junction structures at the apical portion of the cells creating a tight barrier preventing the passage of

undesirable materials under normal condition (Stelwagen and Singh, 2014). Epithelial cells are also linked by gap junctions allowing transport of low molecular weight molecules between cells. During the lactation phase, the epithelial cells become active secretory structures. This change occurs only if the appropriate endocrinological, nutritional and physiological conditions are met as shown in numerous reports (Shirley et al., 1973, Kim et al., 1997, Ohtani et al., 2011). Hormonal regulation has a potent effect on the cellular changes that increase secretory capacity. These endocrines signaling events lead to significant increases in both mRNAs and proteins abundance and cause a marked increase in substrate requirement and energy supply demand to BMECs (Finucane et al., 2008, Wang et al., 2017).

1.2. MAMMARY GLAND DEVELOPMENT AND LACTATION CYCLES

The mammary gland begins its development at the early embryonic stage from the ectoderm and the mesoderm germ layers in the embryo. During this stage, the development is minimal and gives rise to the early structure named the mammary band. As described by Capuco and Ellis (2013), development of the mammary gland slows shortly after birth and remains so until the calf approaches puberty when it increases rapidly. Once the calf reaches puberty, the mammary gland is fully functional and ready to go through the final stage of the development cycle during pregnancy. Mammary development during fetal growth, puberty, pregnancy and lactation has been described in detail (Sheffield, 1988, Knabel et al., 1998, Akers et al., 2000, Silva et al., 2002). These studies have identified hormonal regulation to be responsible for most of the metabolic and secretory changes in the BMG. More specifically, progesterone levels prior to lactation play an important role in the regulation of the menstrual cycle of the cow as described by Wang et al. (2007a). Lactogenesis then begins with the secretion of estrogen and prolactin hormones (Karg

and Schams, 1974). The expression of the local estrogen and progesterone receptors are drastically increased during the development and throughout lactation stages in the BMG (Schams et al., 2003). At physiological level, glucocorticoids have been shown to stimulate milk production. These hormones have also been shown to play an important role in the occurrence and regulation of lactation by affecting mRNA abundance and protein phosphorylation in the BMG (Accorsi et al., 2002). However, the mechanisms regulating these transcription and translation processes remains unclear.

To define physiological changes leading to milk synthesis, different stages from pregnancy to lactation have been used. These stages are mammogenesis, lactogenesis, galactopoesis and involution. Functional differentiation of BMECs is also divided into four phases: the proliferative phase of early pregnancy; the secretory differentiation phase during late pregnancy and the secretory activation that occurs at the beginning of lactogenesis and lactation. While markers for each phase of development have been characterized, the mechanisms that regulate the transition between them are not well understood.

1.2.1 Mammogenesis

As briefly described above, development of the mammary gland begins during the early fetal stage and proceeds beyond the initiation of lactation of the adult dairy cow. Mammogenesis is defined as the process of mammary development (Erb, 1977). Primary development occurs in the embryo and allows the formation of the fat pad and early secondary sprout. From birth to puberty, there is isometric growth of the mammary tissue. Hormonal changes during puberty lead to allometric mammary growth (Akers et al., 2000). The hormones responsible for the development of the mammary gland at this stage are follicle-stimulating hormone, luteinizing

hormone and estrogen (Neville et al., 2002). Cowie et al. (1980) classified lactogenic hormones nto three categories: reproductive hormones (estrogen, follicle-stimulating hormone, luteinizing hormone and progesterone), metabolic hormones (growth hormones and glucocorticoids) and mammary hormones (prolactin and leptin). Reproductive hormones go through cyclic patterns controlled by the anterior pituitary gland stimulating the ovarian cycle and releasing female sex steroid hormones. Metabolic hormones control metabolic responses to nutrient intake and stress factors. Finally, mammary hormones are involved in the induction of lactogenic differentiation and secretory activity. Mammary development continues past the initiation of lactation.

1.2.2 Lactogenesis

The transition from pregnancy into lactation represents a critical stage for the BMG. Lactogenesis, defined as the initiation of lactation, is a series of cellular and metabolic changes that occur in the BMG leading to the synthesis and secretion of milk components. Lactogenesis requires biochemical changes that transform the BMG from an inactive to an active state by causing epithelial cells to differentiate from non-secretory cells to secretory cells leading to the production and secretion of milk components into the lumen of the mammary gland (Kensinger and Magliaro-Macrina, 2011). There is a marked increase in the mRNAs abundance of key proteins involved in milk components synthesis in the epithelial cells leading to synthesis of lactose, mammary-specific milk proteins, milk lipids and other milk components in mammalian mammary gland during lactogenesis. The change in mRNA abundance indicates an increase in the secretory activities of the cells at the time of parturition and the subsequent milk secretion. Lactogenesis can be divided into two stages. It begins with the cytological and enzymatic differentiation of epithelial cells. This stage is followed by the second stage of lactogenesis that is defined by copious secretion of milk that occurs when the inhibition effect of progesterone is reduced and blood concentrations

of both LH prolactin and glucocorticoids become elevated. This second stage of lactogenesis is accompanied by a significant increase in uptake of metabolic substrates from the blood by the mammary gland (Williamson et al., 1995).

An abrupt increase in secretion of milk components as well as an upsurge in LH, prolactin, estrogen, adrenal steroids and growth factors are all defining characteristics of the second stage of lactogenesis (Jacobs, 1977, Stiening et al., 2008). At this stage, secretory epithelial cells represent approximately 50% of the total cell count of the tissue during lactation (Yart et al., 2013). These cells are responsible for converting most macromolecular precursors into milk constituents. LH in combination with a sufficient nutrient supply to BMECs are required to induce lactogenic differentiation of the mammary tissues (Goodman et al., 1983). Traditionally, regulation of metabolic pathways necessary for lactogenesis have been explained by hormonal status and substrate availability to BMECs. However, accrued evidence now suggests that more complex regulation factors might be involved in these lactogenic mechanisms.

1.2.3 Galactopoeisis

Once milk secretion has been established, maintenance of lactation is defined as galactopoeisis. This step is characterized by cell growth and an increased number of epithelial cells in the BMG (Yart et al., 2013). Regulation of galactopoeisis is strongly influenced by galactopoeisis-related hormone status and nutrient availability. For example, insulin-like growth factor and insulin have been shown to significantly increase during this stage leading to a significant increase in mRNA abundance of milk related proteins in BMG (Prosser et al., 1989, Molento et al., 2002).

Another important factor, described by Stiening et al. (2008), is the rate of emptying of the milk space, which plays an important role in milk synthesis regulation. Indeed, it has been suggested that milk constituents act as inhibitor of milk synthesis and that removal of these inhibitors during milking or nursing influences the rate of milk secretion. One potential explanation to describe this negative feedback loop was proposed by Huang et al. (2013), who showed that cytokine-induced negative feedback loop regulation affects milk secretion. The suppression of cytokines signaling 3 could be an important inhibitor of the signal transducers and activators of transcription 5 (**STAT5**) proteins that plays an important role in cytosolic signaling. This negative feedback loop on milk synthesis was exemplified by the signaling effect of milk serum (or whey), an important bovine milk protein. Thus, presence of whey protein in high abundance in the milk space would results in the reduction of milk synthesis (Kensinger and Magliaro-Macrina, 2011). This has also been used to explain the negative feedback inhibition signaling.

1.2.4 Involution

The involution stage is marked by a significant decline in milk synthesis and secretion (Holst et al., 1987). Involution can either be gradual, as it would be observed under natural conditions, or abruptly initiated as seen in commercial dairy production. During involution, the mammary gland undergoes several morphological changes. Singh et al. (2008) showed a marked decrease in milk protein gene expression, an increase in BMECs apoptosis as well as an increase in cell survival signaling that are associated with multiple protective responses to oxidative stress during involution in BMG. This period is also characterized by the degeneration of epithelial cells leading to a reduction in alveoli size and a significant decrease in the number of cells in the mammary gland of the cow (Hernandez et al., 2011). The involution process begins shortly after

cessation of milk removal and the start of negative feedback loops responsible for regulating the mammary gland as described above.

1.3. SYNTHESIS OF MILK COMPONENTS AND MAMMARY NUTRIENT METABOLISM

The metabolic factors influencing milk component synthesis have been thoroughly studied over the course of the last century leading to important discoveries pertaining to the molecular pathways involved in milk synthesis. However, the molecular mechanisms that control these processes remain elusive. It has been observed that the nutrients supplied to the mammary gland have an important influence on milk yield and composition; however, more complex mechanisms integrating nutrient signaling pathways have been proposed (Bionaz and Loor, 2012, Piantoni et al., 2012, Zhou et al., 2015).

1.3.1. Milk Precursors

Precursors of milk constituents and energy substrates are taken up by the BMG through the blood circulation. These substrates are used to form the main milk constituents including: milk proteins, lipids and lactose (Zhou et al., 2015). The amount of metabolites available to the mammary gland also depends on the blood flow through the cow's udder. The main precursors for milk components are in Table 1.1.

Table 1.1: Milk Components and their Blood Precursor[1].

Milk components		Blood precursors
Proteins		
	Caseins	Amino Acids
	β-Lacto-globulin	Amino Acids
	α-Lactalbumin	Amino Acids
	Milk serum albumin	Amino Acids
	Immunoglobulin	Immunoglobulin
Fat		
	Fatty Acid	Acetate, β-hydroxybutyrate, Circulating FA, triglycerides
	Glycerol	Glucose, glycerol from triglycerides
Lactose		Glucose
Minerals		Minerals
Vitamins		Vitamins
Water		Water

[1] *Adapted from: (Thompson et al., 2009)*

The most important precursors of milk for ruminants, shown in Figure 1.3, are water, glucose, volatile fatty acids (**VFA**), triglycerides (**TG**) and amino acids (**AA**). Some of these precursors are directly absorbed by the BMECs, transported and released into the lumen. Water, minerals and vitamins remain unchanged from the blood to the bovine milk (Bösze, 2008, Park, 2009). Conversely, other metabolites are absorbed in the digestive system and are transformed either by the microbe populations present in the rumen of the cow or by the liver before being routed to the BMG via the circulatory system. Most of the water required for milk synthesis is drawn by osmotic pressure into the BMECs and released in the milk space with lactose and other hydrophilic milk components (Bösze, 2008).

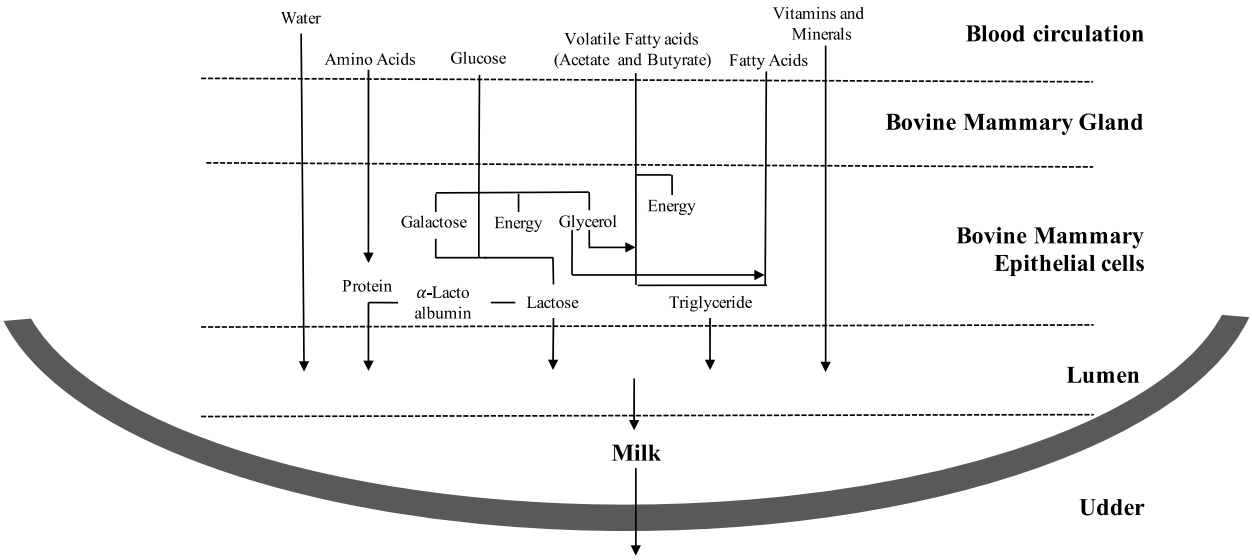


Figure 1.3: *Precursor of the Main Milk Components in BMECs*

1.3.2. Cellular Mechanisms for Milk Secretion and Component Transport

BMECs utilize five different processes to transport the precursors of milk across the apical and basal membranes as shown in Figure 1.4 (Shennan and Peaker, 2000). Milk components can be secreted through exocytosis from the basal membrane of the BMECs into the milk space. Lipids are individually transported in the form of milk fat droplets containing other milk components such as Ca and P (Mani et al., 2009, Ontsouka and Albrecht, 2014). Ions and other specific molecules appear to be passively transported across the cell into the lumen and are directly affected by neurotransmitters and LH signaling, as described by Schmidt et al. (2001). Such components include the ions K, Cl and sodium Na. Certain monosaccharides are believed to use this transmembrane transport system to be delivered to the lumen, drawing small amounts of water with them (Xiao et al., 2004). Other molecules can also pass through the epithelial cells remaining intact. Proteins taken up from interstitial fluids are transported by transcytosis through the lumen and controlled by complex gene expression changes in BMECs in the case of immunoglobulins.

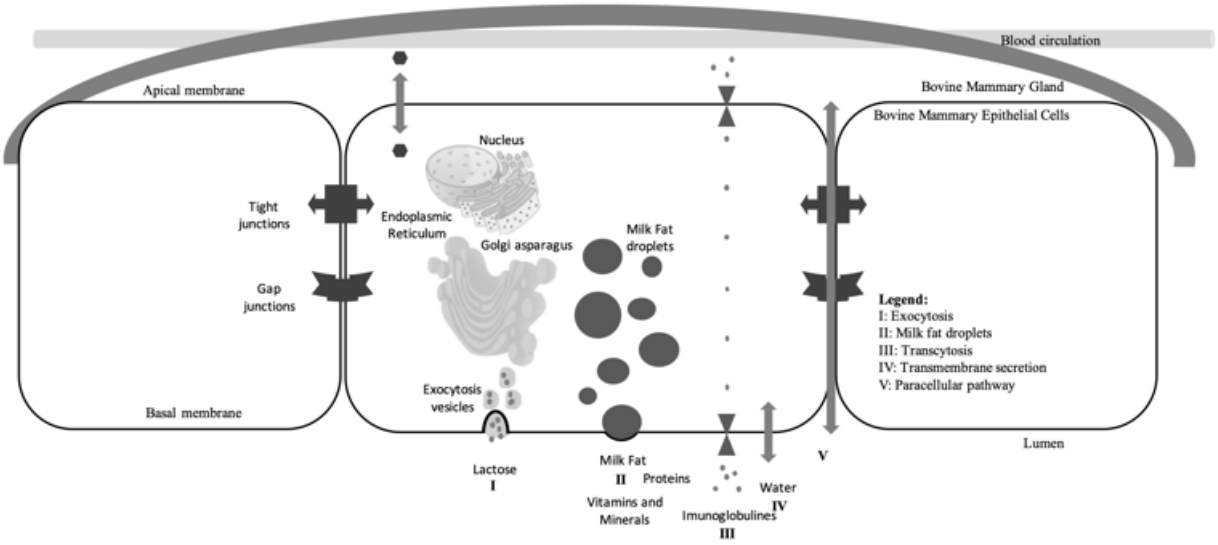


Figure 1.4: *Milk Precursors Transport into the Lumen*

This process required energy. The last transport mechanism used by epithelial cells for milk components transport is the paracellular pathway and was described by Shennan and Peaker (2000). This transport mechanism allows the passage of substances between epithelial cells. The passage of molecules through this transport system fluctuates depending on the stage of the cow and its health status (Stelwagen and Singh, 2014).

1.3.3. Dairy Milk Component

Milk is a very complex fluid composed of hundreds of different constituents. Milk composition can vary between mammalian species or even between breeds of the same species reflecting variation in the evolutionary requirements of the offspring. The concentration of the principal constituents normally varies according to the energy needs (lipid and lactose) and growth rates (protein) of the species' offspring (Thompson et al., 2009). The average composition of dairy cattle milk is in Table 1.2. Dairy milk is mostly composed of water, with solid particles representing less than 13% of the total composition of milk (Park, 2009). The main solid constituents of cow milk are lactose, fat and protein. The remaining solid particles are enzymes,

minerals, vitamins and miscellaneous compounds, which are recorded as a part of the ash component. Milk also contains small amounts of immunological and endocrinological components such as immunoglobulins and hormones.

Table 1.2: Average Composition of Dairy Cattle Milk[2].

Components	Percentage (%)	
Protein		
	Casein	2.5
	Whey	0.5
	Non-Protein Nitrogen	0.2
	Total Protein	3.2
Fat		3.6
Lactose		5.0
Ash		0.7
Total Solids		12.5
Water		87.5

[2] *Adapted from: (Park, 2009)*

1.3.3.1. Milk Protein

Essential amino acids (**AA**) and a large proportion of the non-essential AAs needed for milk protein synthesis come from the free AA pool absorbed from the blood by the mammary gland (Manjarin et al., 2014). Dairy cow milk proteins have been divided into five main fractions: caseins, α-lactalbumin (**α-LA)**, β-lactoglobulin, peptides and non-protein nitrogen (**NPN**) which represent approximately 78, 12, 5, 2 and 3%, respectively, of the total N content in bovine milk (Thompson et al., 2009). The true milk protein content can be estimated by adding the total of the main milk proteins, casein and whey protein (which mainly consists of α-LA and β-lactoglobulin) (Fox and Kelly, 2003). Important properties of the principal milk proteins are in Table 1.3. Most of these proteins are specific to milk and are only synthesised by the mammary gland of the cow.

Table 1.3: Properties of the Mammary Specific Milk Proteins

	Casein Proteins				β-Lactoglobulin	α-Lactalbumin
	αs1-Casein	**αs2- Casein**	**β- Casein**	***k*- Casein**		
Gene Name	*CSNS1*	*CSNS2*	*CSN2*	*CSN3*	*LGB*	*LALBA*
mRNA Accession No.	NM_181029.2	NM_174528.2	NM_181008	NM_174294.2	XM_010819323	NM_174378.2
Molecular weight	23,612	25,228	23,980	19,005	18,362	14,174
Number of Amino Acids	199	207	209	169	162	123

Milk also contains lactoferrin, a multifunctional protein, that is found in high concentrations in the colostrum of certain mammalian species including bovids. This protein is mainly present in numerous secretory fluids such as saliva and tears. In milk, lactoferrin is a globular glycoprotein that mainly serves as an iron-binding protein. It is also involved in the innate immune system and exhibits antimicrobial activity. Bovine serum albumin (**BSA**) is a serum protein also present in milk. Onset of lactation has been shown to be marked by an important increase in genes expression of specific milk proteins in BMECs as described by Sigl et al. (2012).

1.3.3.1.1 Caseins Proteins

There are four main types of caseins in bovine milk: α-subunit 1 casein, α-subunit 2 casein, β-casein, and κ-casein and they represent 38, 10, 35 and 12%, respectively, of the whole bovine casein pool (Fox and McSweeney, 2009). They are distinct phosphoproteins but are similar in structure. Once grouped together, casein molecules form casein micelles (Moon et al., 2009). These micelles also contain other constituents such as Ca, P and water. The micellar structure of milk caseins is an important aspect for milk digestion and the basis of milk products such as cheese.

Casein proteins are encoded by their respective genes: *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3* (Qian and Zhao, 2014). Expression and regulation of these genes is tightly controlled by hormones such as prolactin, glucocorticoids and progesterone in the mammary gland (Wartmann et al., 1996, Castro et al., 2016). Casein proteins are only produced in the mammary gland and are presumably designed to meet the AA requirement of the neonate.

1.3.3.1.2 Whey Proteins

Milk serum or whey proteins are encoded by the genes *LALBA* and *BGL* for α-LA and β-lactoglobulin, respectively. *LALBA* encodes the gene for the protein involved in lactose synthesis. This heavily studied protein is composed of 123 AA, with a molecular weight of 14.2 kDa. Albumin proteins are rich in essential AA, such as α-LA, forming approximately 58% of their total constituents (Jouan, 2002). ß-lactoglobulin is the principal whey protein in cow's milk and is abundant in the mammary gland during pregnancy and lactation (Fong et al., 2008). *BGL* encodes the elements of a three-dimensional protein structure, similar to that of retinol binding proteins, with a weight of 18.36 kDa (Gaye et al., 1986).

1.3.3.1.3 Milk Protein Synthesis

Milk protein specific synthesis occurs in BMECs. In ruminants, protein synthesis in the mammary gland is tightly coordinated by complex endocrine, mitogen and nutritional signaling cascades (Wartmann et al., 1996, Brisken and O'Malley, 2010, Castro et al., 2016). Caseins and whey proteins are synthesized exclusively by the mammary secretory cells and are soluble in the aqueous phase of milk. Essential AA required for the formation of milk proteins originate from dietary undegradable proteins or from proteins generated by the microbial population of the rumen

that are transported through blood circulation to the mammary gland. The AA uptake occurs through the basal membrane of the epithelial cells of the mammary gland (Baumrucker, 1985). AA transport is facilitated by specific transmembrane transporter proteins that require energy to undergo active transport, as previously described. AAs enter the cytoplasm and are eventually assembled to form specific proteins made by the polyribosomes present on the rough endoplasmic reticulum (**RER**). BSA and immunoglobulins compose the remaining true milk proteins and are not synthesized by the epithelial cells of the mammary gland.

1.3.3.1.4 Synthesis of the Mammary Specific Proteins

Regulation factors from both nuclear and cytoplasmic provenance are involved in milk protein synthesis (Culjkovic et al., 2008). Some of these nuclear factors described below regulate the export of the specific mRNA transcripts needed for the cell to grow, proliferate and function whereas cytoplasmic components are needed for mRNA translation and protein formation (Singh et al., 2010). These factors provide instruction and infrastructure, so that once grouped in the cytoplasm, AAs can covalently bind to form proteins. The RER facilitates the synthesis of milk protein such as casein, α-LA and ß-lactoglobulin. These proteins then travel from the RER to the Golgi apparatus to be processed and transported into the milk space of the cells. Most milk proteins undergo post-translational processing while in the Golgi and are then subsequently transported to the apical membrane and secreted by secretory vesicles into the lumen.

LH such as glucocorticoids, insulin, and prolactin stimulate transcription of milk protein genes (Doppler et al., 1991, Shao et al., 2013). Wartmann et al. (1996) showed that numerous transcription factors (**TF**) are capable of binding the β-casein promoter. One such factors, which is indispensable for the hormonal induction of β-casein transcription, binds to a conserved

sequence present in the promoter region of casein genes in bovine genome. This factor is the mammary gland factor or Stat5, a member of the Stat family (Wartmann et al., 1996). Stat family members are activated in response to cytokines whose receptors are associated with tyrosine kinases of the Janus kinase (**JAK**) family. Following ligand binding and receptor aggregation, Stats are phosphorylated on tyrosine residues by the receptor-associated JAKs. Tyrosine phosphorylation mediates the specific binding of Stats to IFN-γ activated GAS-like sites, leading to activation of target genes. Regulation of the transcriptional activation by LH in the mammary gland is not yet clear. Many signaling cascades involving LH, cytokines, growth factors, mitogens and more are involved in the regulation of specific milk proteins and are yet to be fully understood.

1.3.3.2. Milk Fat

Fat is a major energy component in milk and accounts for many of its physical properties. It is also known to be the most variable component in cow milk as a direct consequence of its biological origin. Milk fat is of economic importance to dairy producers and research specialized in understanding the biosynthesis of this component has long been encouraged in the dairy industry. Milk fat is composed of various mixtures of lipids. It is present as an oil-in-water emulsion that forms fat droplets. Milk fat is mainly composed of TG (95-97%) which are made of three fatty acids (**FA**) bound to one molecule of glycerol. The other sources of fat are diacylglycerol (1.5%), phospholipids (1%) cholesterol (0.5%) and a small portion (less than 0.1%) of free FA (Park, 2009). Jensen (2002) estimated that there are at least 400 different types of FA used to form TG or present as free FA in bovine milk.

In many species, FA composition is greatly influenced by diet composition. However, ruminants are quite different due to dietary lipid alteration by bacterial metabolism in the rumen. Rumen microorganisms are responsible for a process called biohydrogenation of polyunsaturated

fatty acids (**PUFA**) that greatly affect the FA composition of their milk. In ruminants, the FAs in milk are known to equally arise from two sources, which are direct uptake from blood circulation or *de novo* lipid synthesis within the BMECs (Dils, 1986, Neville and Picciano, 1997).

1.3.3.2.1 Milk Fat Synthesis

Nutritional regulation of milk fat synthesis was previously reviewed by Bauman and Griinari (2003). Whereas glucose is used as the main sources of carbon for *de novo* lipid synthesis in non-ruminants, ruminants utilize acetate and β-hydroxybutyrate, VFAs produced in the rumen. Acetate results from fermentation of carbohydrates and is a major source of carbon for FA used in milk fat synthesis. β-hydroxybutyrate, produced by the rumen epithelium from absorbed butyrate also provide carbons sources used for *de novo* synthesis of FA. Another source of precursors for milk fat comes from lipoprotein lipase (**LPL**) that hydrolyzes TG in circulation to form FA and gycerol. Hydrolysis of these FAs produces either diacylglycerides, monoacylglycerides or glycerol that are made available for BMECs (Park, 2009).

Once in the BMG, FAs, VFAs and glycerol are used as major substrates in the formation of TG that is later secreted in milk spaces. Glycerol is either metabolised by the BMEC or absorbed through the blood circulation. TG formation occurs in the smooth endoplasmic reticulum of BMECs. Once formed, TGs later coalesce into large droplets that are drawn to the apical membrane of the cell as described in early research by Luick (1961). The lipid droplets progressively fuse together and gradually become enveloped by the apical plasma membrane, finally separating from the cell as milk fat globules. Phospholipids and cholesterol are also components of the membrane surrounding the milk fat globules. The surrounding membrane

prevents the fat globules from coalescing into fat droplets that would be too large to be efficiently secreted by the cells. Fat globules can range from 0.1 to 15 μm in diameter (Park, 2009).

1.3.3.2.2 Pre-Formed Fatty Acids

Pre-formed FA are taken up by the BMG and used as a carbon source for TG formation, as a source of energy, or directly secreted by the BMEC into the milk space. Long-chain FAs ($\geq$16 carbons) account for a largest proportion of the pre-formed FA (Bauman and Griinari, 2003, McSweeney and Fox, 2009). Pre-formed FAs are absorbed through the basal membrane of the BMEC. They are derived from circulating lipoproteins and non-esterified FA that originate from the absorption of lipids in the digestive tract or from the mobilization of body fat reserves (Barber et al., 1997). The diverse population of microorganisms present in the rumen also greatly influence the FA profile that is available for BMECs uptake and present as free FA in milk (Jenkins et al., 2008). Lipids circulating in the blood taken up by the BMEC can also come from very low-density lipoproteins that originate from the digestive system or the liver of the cow. They form chylomicrons, which contain the ingested FAs and are later absorbed by the BMEC and used to compose milk TGs (Park, 2009). Composition of FAs in milk has been shown to be influenced by genetic factors, such as breed and various genotypic features, stage of lactation, health status, ruminal fermentation, nutritional factors, as well as seasonal environmental effects (Jensen, 2002, Lindmark Månsson, 2008).

1.3.3.2.3 *De novo* Fatty Acid Synthesis

De novo lipid synthesis by BMECs is the other source of of fat in milk. The FA synthesis pathway in ruminant metabolism is shown in Figure 1.5. Short-chain FAs (4 to 8 carbons) and medium chain FAs (10 to 14 carbons) arise mostly from *de novo* lipid synthesis in BMECs. FAs are pre-formed through the malonyl-CoA elongation pathway. This pathway allows the *de novo* synthesis of FAs through a stepwise elongation that requires a carbon source and reducing equivalents (Smith et al., 2003). NADPH and H+ are used as reducing factors and mostly come

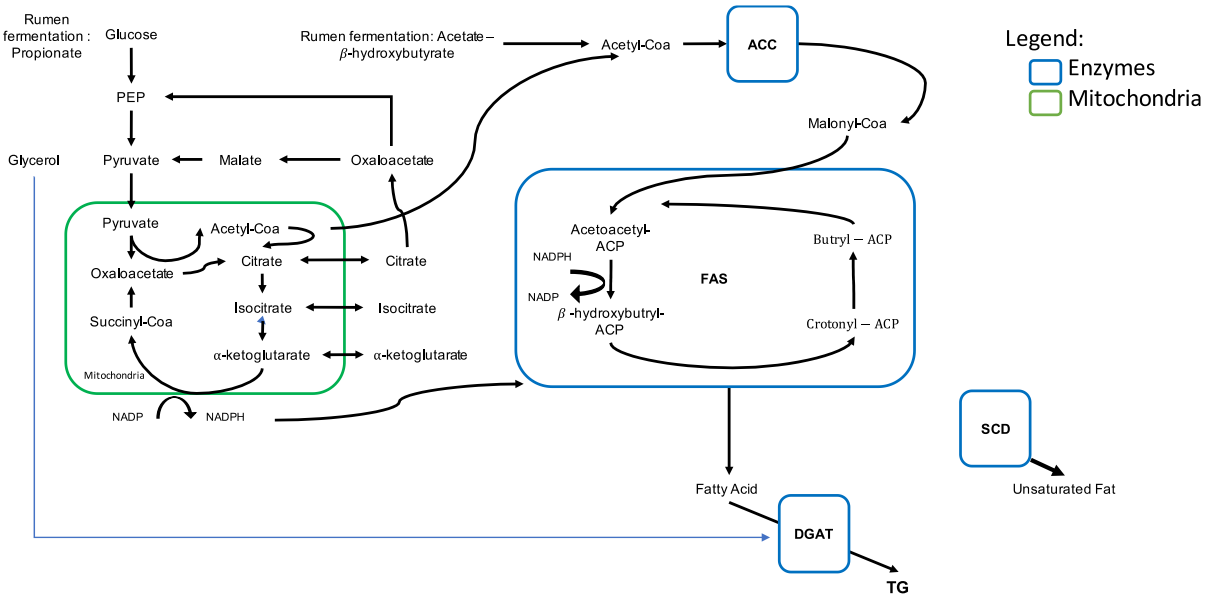


Figure 1.5: *Milk Fat Synthesis in BMECs*

from the oxidation of glucose in the pentose phosphate pathway (**PPP**) (Palmquist, 2009). The cytosolic reducing equivalents used in FAs synthesis can also come from the isocitrate cycle, which catalyzes the oxidative decarboxylation of isocitrate producing the reducing equivalent $NADPH_2$ (Bauman and Griinari, 2003).

As aforementioned, acetate and β-hydroxybutyrate compose the main sources of carbon used to form the acetyl-CoA molecules needed for *de novo* FA synthesis in BMECs. However, the VFA propionate, also produced by rumen fermentation, can served as energy sources for peripheral

organs after being transformed into glucose by the liver of the cow. The FA synthesis process occurs in the cytoplasm of the BMEC. This process involves a complex pathway in which FA synthase (**FAS**), encoded by the *FASN* gene, and acetyl-CoA carboxylase (**ACC**), encoded by the *ACACA* gene, are considered to be rate-limiting enzymes. FAs synthesis occurs via six major recurring reactions ceasing once the FA is produced. Acetyl-CoA is transformed into Malonyl-CoA by the Malonyl-CoA: Acyl Carrier Protein (**ACP**) transacylase. The 3-Ketoacyl-ACP reductase catalyse the reduction of the carbon's 3 ketone groups to a hydroxyl group. The 3-hydroxyacyl: ACP dehydrase removes the water molecule formed and finally the Enoyl-ACP reductase is responsible for the reduction of the C2-C3 double bond. The glycerol needed to form TGs can be synthesized in the BMG by the tricarboxylic acid (**TCA**) cycle or comes from free circulating glycerol absorbed by the BMEC. It is used as precursors of glycerole-3-phosphate, a product of the glycolysis pathway, that is acylated with acyl-CoA to formed diacylglycerol. The enzymes involved in this mechanism are glycerol-3-phosphate acyltransferase (**GPAT**), acylglycerophophate acyltransferase (**AGPAT**), phosphatidic acid phosphohydrolase (**PAP**). The enzyme diglyceride acyltransferase (**DGAT**), encoded by the *DGAT* gene, then catalyze the ormation of TG from diacylglycerol and fatty acyl-CoA. Figures 1.6 shows a map of the key enzymes (and the genes encoding them) that are involved in milk fat synthesis in BMECs.

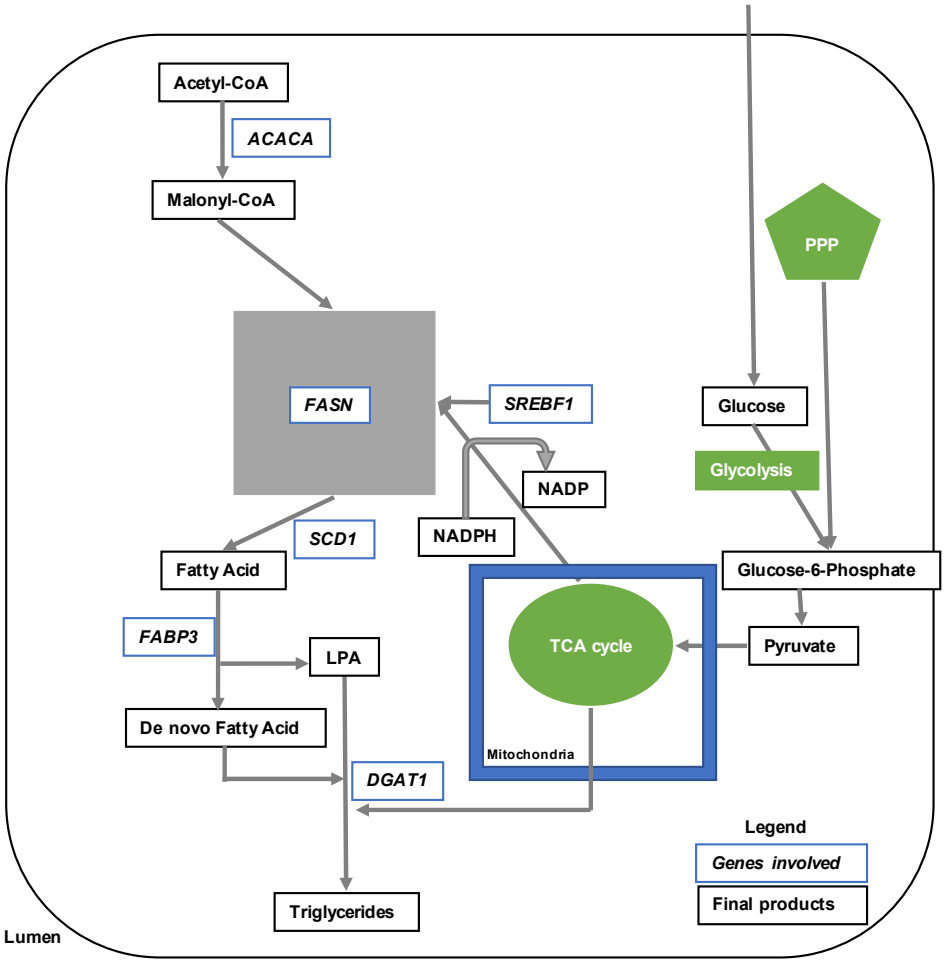


Figure 1.6: *Key Genes Involved in Milk Fat Synthesis in BMECs*

1.3.3.3. Milk Lactose

Lactose, the major carbohydrate in milk, functions as a source of energy for the offspring. Lactose, or 4-0-ß-D-galactopyranosyl-D-glucopyranose, is a reducing disaccharide composed of one molecule of D-glucose and one molecule of D-galactose joined by a ß -1-4 glyosidic linkage. Lactose is a non-permeable solute. As such, this carbohydrate is crucial in maintaining the osmotic equilibrium of milk (McSweeney and Fox, 2009). Lactose concentration in milk is the least variable due to its close relationship with the milk volume. For this reason, the concentration of

lactose in milk is inversely related to the concentration of milk lipids and proteins as a consequence of the osmotic effects of lactose in the Golgi vesicles (Jenness and Holt, 1987).

1.3.3.3.1 Glucose Uptake and Transport

Glucose transport has been described by Xiao et al. (2004). Glucose taken up from the blood circulation provides a portion of the substrates needed for both the glucose and the galactose unit of lactose. An early study by Keenan et al. (1970) described lactose synthesis in the mammary gland of rats. Glucose is absorbed from the blood circulation through the basal membrane of the epithelial cell via specific transporters such as the solute carrier family 2 enzymes (**SLC2**) (Lin et al., 2016). More recent studies have shown the expression and key role of this glucose transporter family in BMECs (Bionaz and Loor, 2008, 2011). An important glucose transporter of this family, present in the BMG, is member 1 encoded by the *SLC2A1* glucose transporter gene, also known as GLUT1 (Eger et al., 2016). Intracellular glucose transporters do not require energy and the presence of SLC2 transporter in the Golgi membrane is specific to epithelial cells of the mammary gland.

Once in the epithelial cells, glucose can be used for lactose synthesis, completely oxidized through glycolysis and the TCA cycle for adenosine triphosphate (**ATP**) production, used by the non-oxidative PPP for production of reducing equivalents for FAs synthesis, or used to synthesize glycerol (Fox and McSweeney, 2009). However, a fraction of the processed glucose transformed into galactose molecules is actively transported into the Golgi lumen where lactose is formed. This step is a potentially rate limiting process for the synthesis of lactose (Bentley et al., 2012).

Additionally, an increase in glucose availability, or blood glucose tends not to lead to an increase in milk production as reviewed by Liu et al. (2013). However, other research has

suggested that insufficient glucose availability tends to decrease lactose synthesis (Wang et al., 2016). Regardless, small decreases in blood glucose has not been shown to affect milk production, as other substrates can be used as sources of energy in ruminants, such as VFAs (Frobish and Davis, 1977, Kim et al., 2001).

1.3.3.3.2 Lactose Synthesis and Secretion

Formation of lactose in mammary epithelial cells requires two molecules of glucose, one of which is converted into galactose-P via the Leloir pathway. In this pathway, glucose is transformed into uridine diphosphate (**UDP**)-glucose by the UDP-glucose pyro-phosphorylase and later into UDP-galactose. The enzyme UDP-galactose 4'-epimerase or GALE allows the formation of the transformed glucose into galactose. The assembled glucose and UDP-galactose are then used to form lactose through a condensation reaction releasing a molecule of water in the process. The UDP molecule generated during the synthesis of galactose is hydrolyzed into uridine monophosphate (**UMP**) and inorganic phosphate by nucleoside diphosphatase (**NDPase**) as described by Keenan et al. (1970). The resulting UMP molecule is actively transported out of the Golgi while the remaining inorganic phosphate diffuses into the cytoplasm.

The enzyme phosphoglucomutase-1, encoded by the gene *PGM-1*, catalyzes the transfer of phosphate between the carbon in position one to the carbon in position six of the glucose molecule. This reaction produces the UDP-glucose required for the synthesis of lactose (Zimin et al., 2009). Lactose synthesis is a one-way reaction leading to the condensation of two simple sugars (Fitzgerald et al., 1970, Keenan et al., 1970). The resulting lactose cannot be hydrolyzed to reform glucose and galactose, and the reaction is not subject to end-product feedback inhibition, as such the accumulation of lactose does not inhibit its formation. The condensation process of the two

simple sugars occurs through the action of a unique enzyme: lactose synthetase (McSweeney and Fox, 2009). This enzyme is composed of two subunits, β -1.4-galactosyltransferase (GT) and α-LA. GT is a glycoprotein that acts as the catalytic subunit of lactose synthase. The enzyme permits the addition of UDP-galactose to oligosaccharides with terminal N-acetyl-glucosamine residue with a β-1-4 linkage. GT is encoded by *B4GALT1* gene, a member of the type II transmembrane family of proteins called glycosyltransferases, which are present in the Golgi apparatus in the BMG (Shahbazkia et al., 2012). In presence of α-LA, GT has been found to be capable of changing its substrate, instead, using glucose as a replacement for glycosylated proteins. α-LA is only expressed in the mammary gland which can explain why lactose production is unique to this tissue (Shahbazkia et al., 2012). As mentioned earlier, α-LA expression is tightly regulated by LH. Thus, lactose synthesis is only active during lactation in dairy cows (Sciascia et al., 2013). Lactose does not diffuse out of the Golgi, but rather is transported through the apical membrane by secretory vesicles (Sharer et al., 2003). The amount of water drawn into these vesicles to balance the osmotic pressure in large part determines the volume of milk, as the water is released by exocytosis into the lumen of the acini as described above. α-LA can be found in milk as its soluble in water and is discarded into the lumen of the epithelial cells as the secretory vesicle fuses with the apical surface of the BMEC. GT has been shown to be mostly retained by the apical membrane, but some GT enzymes can be found in milk (Shahbazkia et al., 2012).

1.3.3.4. Minerals and Vitamins

Bovine milk contains most of the important minerals and vitamins required for animal nutrition (Porter, 1978, Weiss, 2017). The most abundant minerals present in bovine milk are Ca and P which are required for the rapid growth of the offspring of the cow and to support bone

growth and development of soft tissues. They are also involved in the secretion of TGs and milk proteins. Milk contains all major cations (K, Na and Mg) and anion (Cl) (Moreno-Fernández et al., 2016). Micro-minerals known to be involved in enzymatic reactions as cofactors are also present in milk in lower quantities, often bound to milk proteins. Iron is bound to lactoferrin as well as transferrin, xanthine oxidase and casein proteins in bovine milk (Park, 2009). Fe is essential for hemoglobin formation and function in neonate and is found in low quantities in the milk. Zinc is an important cofactor of several enzymes and is mostly bound to casein during its secretion in the lumen; however, Zn can also bind to lactoferrin (Bösze, 2008). Copper can bind to casein but is also capable of binding to β-lactoglobulin, lactoferrin and other milk fats membrane. Molybdenum binds to enzymes associated with cell membranes, to milk fat globules and to xanthine oxidases. Additionally, Manganese is associated with the milk fat membranes in the lumen and Cobalt is secreted as a part of vitamin B_{12} in aqueous phase of milk. In addition, minerals help to maintain ionic strength and milks osmotic pressure. Milk also contains trace elements that have either been absorbed and secreted during milk synthesis or come from external contamination. Traces of B-complex vitamins can be found in milk in the aqueous phase as described by Darke (1976). Most water-soluble vitamins are synthesized by ruminal microflora and absorbed in the intestine of the cow. Fat-soluble vitamins (A, D, E and K) found in milk are associated with the milk fat globules (Park, 2009). Although present, vitamins and minerals represent a small proportion of milk components.

1.4. PHYSIOLOGICAL AND NUTRITIONAL REGULATION OF MILK SYNTHESIS

Recent studies have shown that complex mechanisms involving nutrients as key signaling molecules in the regulation mechanism of milk synthesis in the BMG. Previous research on the mammary gland have cultivated a thorough understanding of the physiological, endocrinological, and metabolic mechanisms influencing bovine lactation. This knowledge provided the necessary foundation to support further investigations in cellular mechanisms involved in milk component formation with potential nutrients playing a key role as signaling molecules influencing milk component synthesis through unclarified pathways.

1.4.1. Energy Metabolism for Milk Synthesis

Milk synthesis and secretion require sufficient energy supply to the mammary gland, as the energetic demand is significantly increased during lactation. Animals obtain the energy requirements necessary for lactation through absorbed energy sources present in the diet. These energy sources are transformed into a combination of simple sugars, such as glucose, the primary energy source used to meet lactose synthesis requirements. VFAs supplied from the digestive system in the case of ruminants are another important source of energy utilized by bovids to meet energy requirements. Major VFAs, acetate and butyrate are both used to meet the energy demands and macronutrient synthesis requirements in the BMG as reviewed by Kristensen (2005).

1.4.1.1. Volatile Fatty Acids

The three major VFAs are acetate, butyrate and propionate (Bergman and Wolff, 1971). Propionate and a small proportion of butyrate are redirected to the liver of the cow where they are

either converted into glucose or used as other carbon sources needed for the metabolism of the cow (Lauryssens et al., 1957, Yonezawa et al., 2009). Glucose is then released into the blood circulation of the cow where it can be absorbed by various peripheral organs. Butyrate can also be converted by the liver but is instead transformed into ketone bodies that can also be used as carbon source and energy supply in several tissues including the BMG (Krehbiel et al., 1992). Finally, acetate is immediately directed to peripheral tissue from the digestive tract to meet energy requirements and to be used as precursors for metabolic reactions as first described by Emmanuel et al. (1974).

VFAs are produced by microorganisms present in the rumen through fermentation of fibers and other dietary elements consumed by the bovid (Yonezawa et al., 2009). This process is made possible by maintaining homeostatic conditions conducive to microbial health, diversity, and enzymatic activity in the rumen. The microorganisms inhabiting the rumen have a synergistic relationship with the bovid. The cow consumes substrates that can be converted into energy dense molecules by the microbial population inhabiting the rumen. Various types of microorganisms such as anaerobic bacteria and fungi, archaea, and ciliated protozoa allow the production of VFAs and other important milk precursors such as FAs and essential AAs. As one of the main precursors to milk components and the main carbon source, used to meet energy requirements, VFAs play an important role in bovine metabolism.

1.4.1.2. Energy Production in Bovine Mammary Epithelial Cells

Once the major VFAs reach the BMEC, they can be used in glycolysis to be converted into Acetyl-CoA, the main precursor of the TCA cycle also known as the Krebs cycle. This series of chemical reactions produce the chemical energy used by the cow in the form of ATP, and release

energy in the form of CO_2 and are significantly up-regulated during lactation-related metabolic changes (Sun et al., 2017). The TCA cycle also provides nicotinamide adenine dinucleotide (**NADH**) and various AA precursors that are needed for subsequent biochemical reactions. In parallel, the PPP is used by BMECs to generate 5-carbon sugars (pentose) in addition to ribose 5-phosphate and NADPH. Pentose sugars are later used by the cow in nucleotide formation or in glycolysis and the reducing agents are used in other metabolic pathways.

1.4.2. Molecular and Genetic Regulation of Metabolism in Bovine Mammary Epithelial Cells

Coordinated changes in the expression of genes encoding for regulatory enzymes are vital for the regulation of lipid, energy, and glucose metabolism. In BMECs, the regulation of the molecular pathways underlying changes in nutrient metabolism affect both the quantity of milk produced and the constituents it contains. Transcriptional regulations of lipid synthesis in BMECs has been shown to be influenced by the action of sterol regulatory element-binding protein 1 (**SREBP1**) encoded by *SREBF1* gene in bovids (Ma and Corl, 2012).

Düvel et al. (2010) identified specific genes involved in energy metabolism in mammals that respond to various internal and external stimuli that can be easily measured by modern techniques. 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-1 encoded by *PFKFB1*, Glucose-6-biphosphate dehydrogenase encoded by *G6PD* gene, Phosphoglycerate kinase 1 encoded by the *PGK1* gene and vascular endothelial growth factor A encoded by *VEGF-A* gene have all appear to play important rate-limiting roles in energy metabolism. However, the control mechanisms influencing their variation in expression and abundance in BMECs remains unclear.

PFKFB1 is a member of the bifunctional 6-phophofructo-2-kinase enzyme family that catalyzes the synthesis and the degradation of Fructose-2-6-biphosphate during glycolysis. As an activator of glycolysis, Fructose-2-6-biphosphate has been identified as a rate-limiting enzyme that can also inhibit the gluconeogenesis pathways in mammalian metabolism as described by (Pegoraro et al., 2013). The *G6PD* gene encodes an important enzyme of the PPP. This enzyme is required to produce the reducing equivalent NADPH needed by the cell for metabolic reactions. *G6PD* catalyses the chemical reactions leading to the formation of 6-phospho-D-glucono-1-5-lactone and NADPH + H^+ from D-glucose 6-phosphate and $NADP^+$. The final products of this reaction are then used either in the PPP (6-phospho-D-glucono-1-5-lactone) or to facilitate other cellular processes (NADPH + H^+) such as FA synthesis as previously described. The *PGK1* gene encodes another important glycolytic enzyme that has been identified as a potential rate-limiting in glycolysis as described by Li et al. (2016). The PGK1 enzyme catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerates, an important intermediate of glycolysis. This enzyme has also demonstrated involvement in other mechanisms such as angiogenesis or in acting as a cofactor for polymerase alpha. However, its potential application in energy metabolism in BMECs, as an intermediate in the glycolysis pathway, is in the transformation of glycerol molecules. As a member of the vascular endothelial growth factor family, *VEGF-A*, encodes a glycosylated mitogen protein that acts on endothelial cells. Its effects include regulation of the increase in vascular permeability as well as promoting cell migration and mediating other cellular processes (Yang and Fortune, 2007). *VEGF-A* has also been shown to be mediated by the TF hypoxia-inducible factor 1-alpha (**HIF-1$\boldsymbol{\alpha}$**). This master transcriptional regulator is known for its role in the cellular response to hypoxia as described by Hu et al. (2003). However, accrued studies show

that HIF-1α can be activated by stimuli other than hypoxia and that it plays an important role in the regulation of energy metabolism pathways.

Molecular regulation of gene expression plays an important role in orchestrating glucose metabolism, and thus plays an important role in the synthesis of lactose in BMECs. The enzyme phosphoglucomutase-1, encoded by the gene *PGM-1*, catalyzes the transfer of phosphate between the carbon in position one to position six of glucose. This reaction produces UDP-glucose required for synthesis of lactose (Zimin et al., 2009). Lactose synthesis is a one-way reaction leading to the condensation of two simple sugars (Keenan et al., 1970) as previously described. The condensation process of the two simple sugars occurs through the action of a unique enzyme: lactose synthetase. This enzyme is composed of two subunits, β -1,4-galactosyltransferase (**GT**) and α-lactalbumin (**α-LA**). GT is a glycoprotein that acts as the catalytic subunit of lactose synthase as describe above. GT is encoded by *B4GALT1*, a member of the type II transmembrane family proteins. α-LA is only expressed in the mammary gland, hence, lactose production is unique to the mammary gland (Shahbazkia et al., 2012). Interestingly, very little is known about the mechanisms involved in the regulation gene expression of lactose synthase components in BMECs.

As the literature demonstrates, milk component synthesis is tightly regulated by the expression levels of key genes encoding specific enzymes involved in these mechanisms. Explanations behind the regulation of mRNA expression and activation of these regulatory enzymes are only partially understood. Apart from the substrate availability and endocrine regulation, more evidence now suggests the possible involvement of other important nutrient-sensing protein complexes involved in the regulation of milk component synthesis.

1.5. mTORC1 IN CELLULAR METABOLISM

mTORC1 is a protein complex known to act as a nutrient and energy sensing complex in mammalian cells. mTORC1 acts on cellular growth and metabolism by modulating DNA transcription, mRNA translation and enzyme activity (Ma and Blenis, 2009, Laplante and Sabatini, 2013). Through these mechanisms, mTORC1 functions to regulate nutrient metabolism in several tissues.

1.5.1. mTORC1 Components

mTORC1 is a large protein complex defined by five main protein components. The first of which is the catalytic component mTOR, a Ser/Thr protein kinase (Sabatini et al., 1994). The second and defining component of mTORC1 is the regulatory protein associated with mTOR (**Raptor**). Raptor acts as a scaffolding protein that facilitates substrate recruitment to mTORC1 by the binding of the TOR signaling motif found on most canonical substrates of mTORC1. Raptor is essential for the activation and subcellular localization of mTORC1 (Sancak et al., 2008). The mammalian lethal with Sec13 protein 8 (**mLST8**) also known as GβL is a component of both mTORC1 and the mTORC2 complexes (Kim et al., 2003). The role of mLST8 is not well characterized, but it is believed to be involved in the stabilization of the kinase activation loop of the protein complex (Kakumoto et al., 2015). There are also two main inhibitory subunits within the mTORC1 protein complex which are the proline-rich Akt substrate of 40 kDA (**PRAS40**) and DEP domain containing mTOR interacting protein (**Deptor**) (Peterson et al., 2009).

1.5.2. The mTORC1 Pathway

mTORC1 regulates various functions of cellular metabolism such as cell growth and proliferation, autophagy, angiogenesis, senescence, innate and adaptive immune responses and cell development as recently reviewed by Saxton and Sabatini (2017). The mTORC1 pathway senses the environmental energy status of the cell and the cellular nutrient availability, consequently causing the activation or repression of specific targeted cellular mechanisms. Several factors such as cytokines, growth factors, AAs, insulin, and Toll-like receptor ligands have been shown to activate mTORC1 and increase the phosphorylation status of downstream targets including eukaryotic translation initiation factor 4E-binding protein (**4E-BP1**) and ribosomal protein S6 kinase beta-1 (**S6K1**) (Tang et al., 2001, Zhang et al., 2006). Several growth factors, nutrients and mitogens are believed to be involved in the stimulation and activation loop of the two mTOR complexes; mTORC1 and mTORC2. mTORC1 is also involved in pathways that are of interest in the regulation of nutrient synthesis and nutrient metabolism specific to the mammary gland. Previous work has implicated mTORC1 in the control of protein synthesis in BMECs and its role was described by Burgos and Cant (2010) demonstrating that mTORC1 could directly influence milk component synthesis.

1.5.3. mTORC1 Regulation

Copious studies have concentrated on the manipulation of mTORC1 activity in various tissues. The early discovery of mTORC1 as the target of the highly specific inhibitor rapamycin allowed investigation into its cellular function (Brown et al., 1994, Sabatini et al., 1994). Figure 1.7 shows the mTORC1 signaling pathway effectors that are involved in nutrient metabolism.

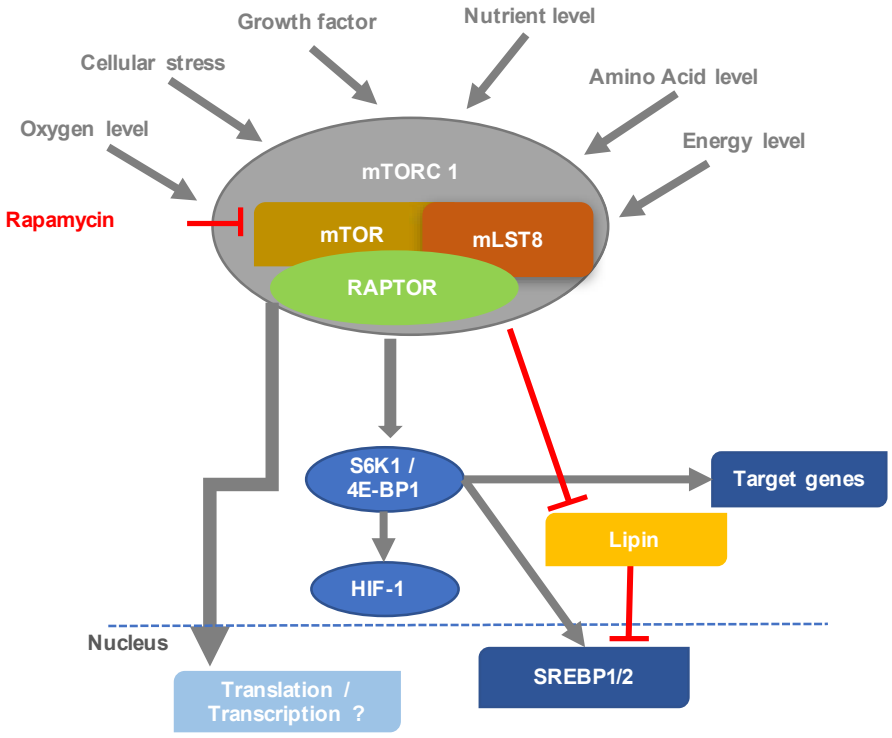


Figure 1.7: *mTORC1 Signalling Pathways in Nutrients Metabolism*

Rapamycin was first isolated from the soil bacterium *streptomyces hygroscopicus* of Easter Island (Rapa Nui) in the early 1970s. Primarily used as an antifungal drug, it was later found to act as an immunosuppressant and to possess antiproliferative properties in mammals. Genetic screens using yeast as a model allowed the discovery of its effects on two specific genes called target of rapamycin 1 and 2 (Cafferkey et al., 1993). Homologous studies in mammals showed that rapamycin also acts on one specific gene: mTOR also known as FK506-binding protein 12-rapamycin-associated protein 1. In mammals, rapamycin does not directly inhibit mTOR, it binds to the FK506-binding protein 12 leading to the inhibition of mTORC1 through a gain-of-function complex (Brown et al., 1994, Sabatini et al., 1994). mTORC2 has been shown to be insensitive to the use of rapamycin. However, Thoreen and Sabatini (2009) demonstrated that the chronic use of rapamycin leads to a partial inhibition of mTORC2.

Despite the important role of rapamycin on early discoveries in the field, recent studies have revealed some function of mTORC1 are rapamycin-insensitive. Indeed, as reviewed by Yoon and Roux (2013), some functions and substrates of mTORC1 are insensitive to rapacmyin,. In addition, it has become evident that chronic use of rapamycin may lead to rapamycin resistance, as demonstrated by a long-term study in mice liver cells that became insensitive to rapamycin treatments (Kang et al., 2013). In another study by Thoreen and Sabatini (2009), mTORC1 activity was re-established after chronic use of the agent during the course of the study. This suggests the existence of numerous activation loops able to counter the inhibitory effects of rapamycin in several cell types.

The activity of mTORC1 is regulated by the action of various growth factors such as insulin and insulin-like growth factor 1 (**IGF-1**) via different effectors (Wang et al., 2007b, Burgos and Cant, 2010, Saxton and Sabatini, 2017). Binding of insulin and IGF-1 to its cognate receptors activate their intrinsing tyrosine kinase activity resulting in the phosphorylation of adaptor molecules such as the insulin receptor substrate 1 leading to the recruitment of the class I family of the phosphatidylinositol-3-kinase (**PI3K**) to the cell membrane (Zinzalla et al., 2011). These events lead to the activation of the PI3K which phosphorylates phosphatidylinositol 4,5-biphosphate (**PIP2**) and generates phosphatidylinositol-3,4,5-triphosphate (**PIP3**) that acts as a second messengerthat then recruits and activate downstream targets such as the Ser-Thr protein kinase Akt (Sarbassov et al., 2005).

A main effector of Akt is the tuberous sclerosis complex (**TSC**) 2 (**TSC2**), a GTPase-activating protein (**GAP**) (Zhang et al., 2006). This tumor suppressor forms a heterodimeric complex with TSC 1 (**TSC1**). When activated, TSC2 acts as an upstream negative regulator of mTORC1. TSC2 can also be phosphorylated by the action of Akt leading to its inactivation. Ras

homolog enriched in brain (**Rheb**) is a GTPase that directly activate the kinase activity of mTORC1 (Sancak et al., 2007). As a GAP, TSC2 negatively regulates mTORC1 by converting Rheb into its inactive GTP-bound state (Inoki et al., 2006). The TSC1/2 complex conveys many upstream endocrine, nutrient and energy signals that in turn affect mTORC1 activity. The upstream kinases that phosphorylate TSC2 to modulate its activity include Akt, extracellular-signal-regulated kinase 1/2 (**ERK 1/2**), AMP-activated protein kinase (**AMPK**) and ribosomal S6K1 (Inoki et al., 2002, Manning et al., 2002, Ma and Blenis, 2009). These effector proteins directly phosphorylate the TSC1/2 complex causing its inactivation and thus preventing it from suppressing mTORC1 activity. Other TSC1/2 independent pathways have been identified in the regulation of mTORC1 activation. Akt is also phosphorylates PRAS40, a substrate of mTORC1 that acts as an inhibitor by binding to Raptor, in addition to its role as an upstream kinase for the TSC1/2 complex. In this pathway, Akt phosphorylates PRAS40 leading to the dissociation of PRAS40 from Raptor causing mTORC1 activation, as described by Sancak et al. (2007).

mTORC1 activity can also be modulated by other molecular mechanisms. The canonical Wnt pathway is a major regulator of cell proliferation and cell growth and acts on mTORC1 activity through the TSC1/2 pathway. This mechanism acts through the inhibition of the signaling pathway for glycogen synthase kinase 3 β (**GSK3-β**), that normally promotes TSC2 activity, inhibiting mTORC1 functions (Inoki et al., 2006). Proinflammatory cytokines like tumor necrosis factor-α influence mTORC1 activity via mechanisms that resemble growth factor activation. IκB kinase β can directly phosphorylate TSC1 leading to the inactivation of the TSC1/2 complex (Lee et al., 2007). These independent mechanisms have all been shown to act on mTORC1 activity through the regulation of TSC1/2 complex or PRAS40 function.

mTORC1 senses the availability of specific AA such as leucine and arginine (Bar-Peled and Sabatini, 2014). AA act through the Rag GTPases pathway, affecting lysosomal localization, by binding to distinct complexes capable of sensing AA that are involved in mTORC1 signaling. mTORC1 localization and subsequent translocation at the lysosome surface are necessary for activation, as it is required for interaction with its kinase activator Rheb (Chantranupong et al., 2016). Rag GTPases, regulated by upstream AA sensors, are necessary for mTORC1 movement toward this organelle. The Rag GTPases form heterodimeric complexes are RagA or RagB bound to RagC or RagD, resectively (Lawrence et al., 2018). The regulation of this pathway is complex and depends on numerous factors influenced by the Ragulator, which serves as a lysosomal scaffold for RagA and RagB. Other GAPs like folliculin-folliculin interacting protein 2 are involved in the regulation of the binding of both RagC and RagD (Tsun et al., 2013). The GTP loading states of the Rags are regulated by AA availability as the binding of AAs to AA sensors such as Sestrins and Castors enable Gap activity towards rags 2 (**GATOR2**) activity, which inhibits negative regulator of Rags Gap activity towards rags 1 (**GATOR1**), a GAP (Parmigiani et al., 2014). GATOR1 is a critical negative regulator of mTORC1 due to its inhibitory effect within the mTORC1 AA signaling pathway. In contrast, GATOR2 is a known positive regulator of mTORC1, due to its inhibition of GATOR1, in response to specific AA binding to upstream sensors, Sestrin and Castor (Wolfson and Sabatini, 2017).

Sestrin 1/2, cytosolic sensors of leucine, are known to interact with GATOR2 when they are not bound to this essential AA (Budanov, 2015). Sestrin1/2 are capable of sensing leucine availability but have also been shown to bind to AA with similar conformation, such as isoleucine and to a lesser extent, methionine. Recent studies have shown that specific AAs leucine and arginine, involved in mTORC1 signaling, are sensed by Sestrin1/2 and Castor2 proteins,

respectively, and subsequently effect Rag-dependent mTORC1 localization (Budanov and Karin, 2008). In the absence of leucine, Sestrins inhibit GATOR2 which causes inhibition of mTORC1 activity through GATOR1. Thus, leucine is a positive regulator of mTORC1 activity and acts via binding Sestrin1/2, to remove the inhibitory binding of GATOR2 allowing it to supress GATOR1, a negative regulator of Rag activity. Solute carrier family 38 member 9 (**SLC38A9**) is a putative lysosomal arginine sensor and may be involved in mTORC1 essential AA dependent signaling pathways as suggested by Rebsamen et al. (2015). A Recent study by Lei et al. (2018) have shown that CASTOR1 functions in parallel with SLC38A9 to influence mTORC1 responses to arginine. In this scenario, Castor1 forms a heterodimeric complex with Castor2. Both Sestrin1/2 and Castor1/2 complexes interact with GATOR2 to negatively influence mTORC1 activity as it was also described by Chantranupong et al. (2016).

1.5.4. mTORC1 in Milk Component Synthesis

In order to grow, cells need to increase the synthesis of macronutrients such as protein, lipids and nucleotides. In addition, cells need to suppress catabolic pathways such as autophagy. To meet these demands, cells need to maintain the balance between anabolic and catabolic responses to environmental and intrinsic factors. As described above, mTORC1 plays a crucial role in the regulation of macronutrient synthesis in numerous cell types. Recent evidence suggests that mTORC1 also plays an important role in regulating milk component synthesis in the BMG, however, the underlying mechanisms and pathways mTORC1 participates in to affect this organ have yet to be elucidated.

1.5.4.1. mTORC1 in Protein Synthesis

mTORC1 controls proteins synthesis through phosphorylation of multiple protein involved in mRNA translation (Ma and Blenis, 2009). Among these, the eukaryotic initiation factor 4E-BP1 and the ribosomal protein S6K1 (Zid et al., 2009) are the best characterized mTORC1 targets. mTORC1 promotes protein synthesis primarily through the phosphorylation of these two effectors. S6K1 is phosphorylated at its hydrophobic motif site, Thr389, which enables its subsequent phosphorylation and activation by phosphoinositide-dependent kinase-1 (**PDK1**) (Peterson et al., 2009). When activated, S6K1 promotes mRNA translation initiation through the phosphorylation and activation of several substrates such as eukaryotic translation initiation factor 4B (**eIF4B**), a positive regulator of the 5'cap binding eukaryotic translation initiation factor 4F (**eIF4F**) complex (Ma and Blenis, 2009). S6K1 can also promote the degradation of an inhibitor of eIF4B by the phosphorylation of programmed cell death protein 4 (**PDCD4**). This leads to the improved translation efficiency of spliced mRNAs via its interaction with S6K1 Aly/REF-like target (**SKAR**), a component of the exon junction complexes (Richardson et al., 2004). 4E-BP1 can inhibit translation by binding and sequestering eIF4E which prevents the assembly of the eIF4F complex. mTORC1 is responsible for its phosphorylation at multiple sites which promotes its dissociation from eIF4E.

Studies have shown that even though mTORC1 is involved in the regulation of mRNA translation, it primarily affects mRNAs containing pyrimidine-rich 5' top or TOP-like motifs (Thoreen et al., 2012, Fonseca et al., 2015). mRNAs containing TOP-like motifs include most important genes involved in protein synthesis. AAs act as the building blocks of proteins but also as signaling molecules regulating protein synthesis as described by Dong et al. (2018). Alteration of the phosphorylation status of mTORC1 demonstrated involvement in the regulation of protein

synthesis through AA signaling. Increased levels of methionine, while lysine and other AA ratios were maintained, increased the concentration and utilization of essential AAs. Dong et al. (2018) determined that this could be due to the improvement of the activity of AA transporters that are, in part, controlled by mTORC1 signaling.

S6K1 phosphorylation of BMG in dairy cows was found to be influenced by nutrient levels (Toerien et al., 2004); but whether these changes were mediated by mTORC1 remains unclear. mTORC1 has been shown to play an important role in the regulation of protein synthesis in BMECs. Burgos and Cant (2010) showed that MAC-T cells treated with IGF-1 increased protein synthesis through mTORC1 activation. Downstream targets of mTORC1, 4EBP1 and S6K1, showed important increases in phosphorylation following insulin-like treatment leading to an increase in protein synthesis in this cell model. This research also demonstrated that the IGF-1 activated the PI3K-Akt pathway as described above and led to the activation of mTORC1 and ultimately the phosphorylation of its targets involved in protein synthesis mechanisms in BMECs. Recently, the role of mTORC1 in protein synthesis of BMECs has also been shown to act through the activation of the protein Menin encoded by the *MEN1* gene (Li et al., 2017). mTORC1 signaling pathways sense nutrient and hormone levels and could regulate the phosphorylation of specific proteins involved in the protein synthesis pathway through this mechanism. Additionally, overexpression of Menin leads to a significant suppression of numerous factors involved in the mTORC1 signaling pathway by Li et al. (2017). These studies demonstrated a key role of mTORC1 in the regulation of protein synthesis in BMECs.

1.5.4.2. mTORC1 in Lipid Synthesis

Meeting the increasing need for lipid synthesis during lactation is driven by complex regulatory mechanisms that integrate various signals from the endocrine system but also from key nutrient signaling molecules. Various TFs have been identified as playing a key role in these lipid synthesis pathways leading to the activation of a vast network of genes encoding lipogenic enzymes.

Lamming and Sabatini (2013) recently reviewed the role of mTORC1 in lipid homeostasis. A schematic of the role of mTORC1 on lipid synthesis is in Figure 1.8. It was hypothesis that d*e novo* lipid synthesis can be enhanced by mTORC1 through the activation of SREBPs TF (Porstmann et al., 2008). SREBPs controls the expression of metabolic genes involved in FA and

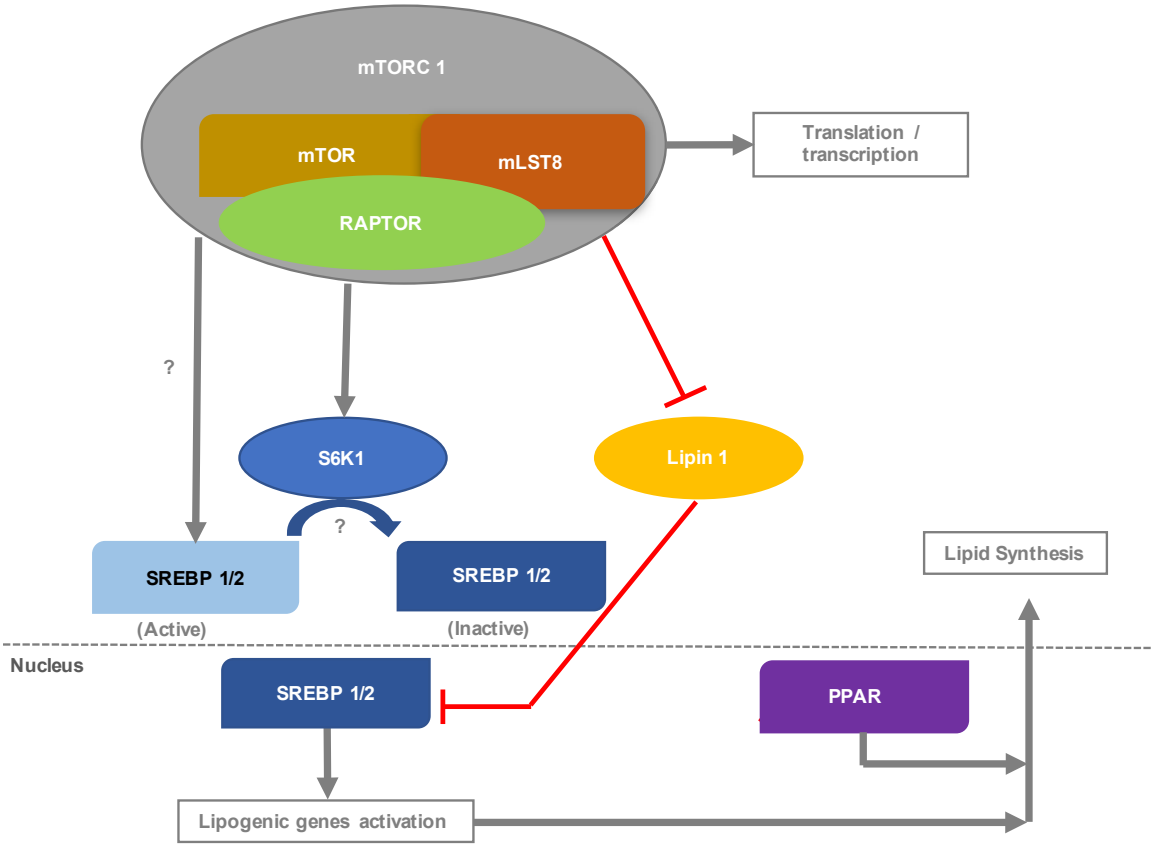


Figure 1.8: *mTORC1 in Lipid Synthesis*

cholesterol synthesis as described by Horton et al. (2002). mTORC1 can activate SREBPs through wo-main pathways. The first pathway involved the phosphorylation of the effector S6K-dependent mechanisms as described by Owen et al. (2012). The second pathway suggested by Peterson et al. (2011) showed that mTORC1 promotes lipid synthesis by the phosphorylation and inactivation of Lipin 1. Lipin 1 can inhibit SREBP in the absence of mTORC1 signaling. These research have shown the key role of mTORC1 in SREBP1 signaling and milk synthesis but the mechanisms by which it might influence milk fat synthesis in BMECs remains to be fully understood.

1.5.4.3. mTORC1 in Energy Metabolism

Efficient glucose supply and energy metabolism are essential for tissues with high macromolecule production capacity, such as the mammary gland of lactating dairy cow. mTORC1 signaling pathway in energy metabolisms is shown in Figure 1.9. mTORC1 is also involved in the

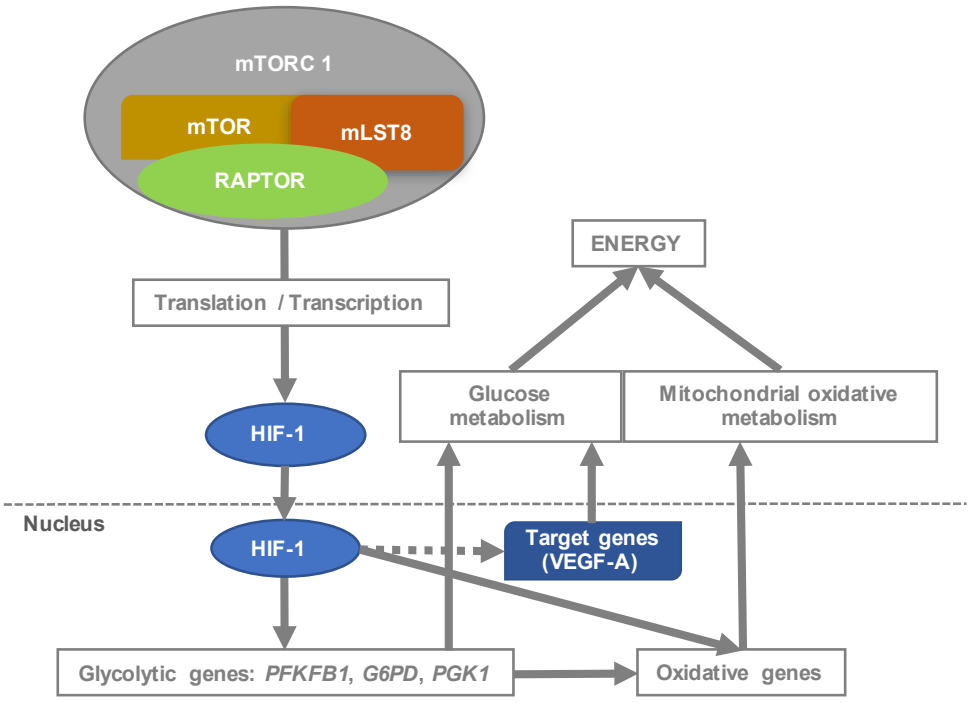


Figure 1.9: *mTORC1 in Energy Metabolism*

regulation of energy balance by promoting a shift in energy metabolism from oxidative phosphorylation to glycolysis via various molecular mechanisms (Inoki et al., 2006, Burgos et al., 2013, Dunlop and Tee, 2013). This shift facilitates the incorporation of nutrients into energy sources that are usable by the cells. It has been suggested that mTORC1 influences energy metabolism by increasing the translation of the TF HIF1-α in murine liver cells by Düvel et al. (2010). Whether HIF1-α is involved in energy metabolism under non-hypoxic conditions in BMECs remains unclear. During variable oxygen availability, cells respond through a transcription cascade primarily mediated by HIF1-α. This mediator is a heterodimer of HIF- α and the aryl hydrocarbon receptor with nuclear translocator subunits: HIF- 1α and HIF- 2α (Hu et al., 2003). HIF- 1α has been shown to be a master TF involved in the regulation of energy metabolism leading to the stimulation of glycolytic gene expression as described by Hu et al. (2003). HIF- 1α also plays an important role in vascularization, explaining its close relationship with the expression of *VEGF-A* (Brugarolas et al., 2003). In addition, this versatile TF is also implicated in angiogenesis and cell survival mechanisms. Studies have shown that HIF-1α can respond to various cellular environment changes but the mechanisms that lead to its activation still need to be explained. Many studies have found HIF-1α to act downstream of the protein complex mTORC1 and have identified mTORC1 as a potential regulator of its activity (Laughner et al., 2001, Hudson et al., 2002, Düvel et al., 2010).

These findings on role of mTORC1 in protein, lipid synthesis and energy metabolism suggest that mTORC1 potentially play a key role in the regulation of milk component synthesis in BMECs and that research should focus on determining by which potential mechanisms mTORC1 could influence milk fat and lactose synthesis in BMECs.

1.6. GENOMIC EDITING USING CRISPR-Cas9

The development of targeted genetic biotechnologies allow specific gene modification, and can facilitate precision functional studies of complex biological systems and mechanisms, as was first described by Cong et al. (2013). Clustered regularly interspaced short palindromic repeats (**CRISPR**) and its associated endonuclease *Cas9* genes are features of the prokaryotic genome that encode an adaptive immunological mechanism in bacteria and archaea. The CRISPR-Cas9 system allows these organisms to respond to and eliminate invading genetic material introduced by bacteriophages (Garneau et al., 2010, Deltcheva et al., 2011, Cong et al., 2013). Cas nucleases used with CRISPR can vary in size and type. The key discovery of the naturally occurring bacterial endonuclease, Cas9, that is a distinguishing feature of the Type II CRISPR immune system began a wave of biotechnologies adapting this system for use in the genomic manipulation of eukaryotic cells (Garneau et al., 2010). The CRISPR-Cas9 nuclease system is now operational in the mammalian genome allowing gene knock-outs (**KO**) or knock-ins (**KI**) of specific genes making the study of specific mechanisms or protein complexes more powerful and precise. (Garneau et al., 2010, Deltcheva et al., 2011, Cong et al., 2013). This is possible due to the guideline of the trans-activating CRISPR RNA (**tracrRNA**): CRISPR RNA (**crRNA**) duplex. In this complex, tracrRNAs was shown by Chylinski et al. (2013) to help RNA to induce the maturation of the crRNAs by host RNase III and the CRISPR-associated Csn1 protein. Genome editing allows the modification of the genetic loci in the organism, often leading to changes in the observable phenotype.

This CRISPR-Cas9 biotechnologies, consisting of a gRNA and Cas9, can be harnessed to inactivate specific genetic elements in target mammalian cells by creating a genomic mutation. The use of a gRNA in place of the CRISPR-RNA simplified the CRISPR-Cas9 system. Indeed,

the use of a simple enzymatic protein and one RNA molecule is all that is needed to produce a RNA-programmed DNA cleavage leading to the development of precise gene KO (Hale et al., 2009). To design a successful gRNA, it is important to respect the relatively simple protospacer adjacent motif (**PAM**) requirement of the NGG sequence at the beginning of the guide RNA (Cong et al., 2013). This process was recently described by Jinek et al. (2013) in human cells. The design of the gRNA is critical to the efficacy of the gene KO. The PAM is necessary for the binding of the gRNA/Cas9 complex, and subsequent cleavage inducing double-stranded break (**DSB**) as it was first described by Bolotin et al. (2005). First, a DSB in the DNA is introduced by Cas9, at a specific site designated by the gRNA, leaving behind an insertion/deletion (**indel**) mutation.

The CRISPR-Cas9 system can be used in research to execute functional gene studies by creating both loss and gain of function mutations in genes encoding proteins in complexes of interest, such as mTORC1, targeting the genes responsible for its activation and inhibition. Thus, this biotechnology allows the study of the role of mTORC1 in nutrient metabolism and milk synthesis in mammalian cells, specifically in BMEC cultures. Even though there are various CRISPR-Cas systems, the most frequently used system in mammalian cells is the Type II which allows the degradation of specific nucleic acid targets. When CRISPR-Cas9 is employed as a biotechnology, the gRNA determines the genomic location where cleavage will occur; however, first the system must be delivered, using viral or non-viral methods, to the host cell.

1.6.1. CRISPR-Cas9 Viral Delivery Methods

Viral delivery of genome-editing agents has been explored in the deployment of CRISPR-Cas9 using lentivirus, adenovirus, and adeno-associated virus vectors as described by (Gori et al., 2015). The gene specific sequence 20 nucleotides in length is encoded at the 5' end of the gRNA

and can be integrated into an expression vector for viral delivery. The lentivirus delivery system is capable of infecting non-dividing cells. It can transduce a variety of specific target cells of the host and target different locations in the host genome. Lentiviruses that can infect non-dividing cells and have been used *in vivo* murine cells to efficiently transduce a variety of cells from specific target organs (Cockrell and Kafri, 2007). However, improvements can still be implemented within this system allowing the prediction and reduction of off-target effects. Furthermore, the packaging limit of lentiviruses is 8.5 kb (although inserts larger than 3 kb are packaged less efficiently), sufficient to package most Cas9 genes, gRNA expression constructs, and the required promoter and regulatory sequences (Kumar et al., 2001; al Yacoub et al., 2007).

CRISPR-Cas9 delivered using a lentiviral vector facilitates the elucidation of underlying molecular mechanisms by means of gene perturbation studies (Ortinski et al., 2017). Gene perturbation studies have been conducted on a variety of mammalian cell types including those of humans, mice, and bovids. This method has been employed in the reprogramming of bovine somatic cells by Heo et al. (2015). Lentiviral vectors are an innovative biotechnology that have also been instrumental in graduating from single gene studies to genome-wide screens intended to identify genes that influence a particular phenotype of interest. There are two main screens employed in high-throughput genomic studies both of which are facilitated by lentivirus delivery systems: pooled and arrayed screens. In sum, lentiviral delivery methods expand the capabilities and scale of CRISPR-Cas9 genetic modification studies and applications in several industries by increasing the power and precision of functional genomic manipulations.

1.6.2. CRISPR-Cas9 Applications in Bovine Mammary Epithelial Cells

As briefly described above, the CRISPR-Cas9 system has been employed in the gene perturbation of a variety of mammalian cells. These methods coupled with the lentivirus delivery system has been used successfully in the delivery of Cas9 and specific gRNA expression constructs in mice and other organisms and will be used in BMECs in our research. CRISPR-Cas9 has recently been used in KI fibroblasts and transgenic blastocysts in the bovine β-casein gene locus (Jeong et al., 2016). Ikeda et al. (2017) also showed the use of the CRISPR-Cas9 genomic editing tools to repair a specific mutation in bovine fetal fibroblast (**BFF**) cells. This research concentrated on the modification of a single nucleotide in BFF. The use of CRISPR-Cas9 genomic editing to the study the molecular mechanisms of the bovine mammary gland by the generation of specific gene KOs in primary BMECs has not yet been demonstrated. Liu et al. (2018) recently compared the use of CRISPR-Cas9 editing to zinc finger nucleases (**ZFN**) and transcription activator-like effector nucleases (**TALENs**) methods. The results of this study demonstrated that the CRISPR-Cas9 system was more precise and efficient than previous methods used in bovine fibroblast editing. Other research has concentrated on the application of genomic editing in bovine embryos or somatic cells (Choi et al., 2015, Heo et al., 2015, Daigneault et al., 2018). To this day, very few studies have used CRISPR-Cas9 genomic editing to study mammary biology in BMECs. Thus, the use of this novel tool, allowing BMECs genome engineering, still needs to be carefully evaluated and developed further.

2. THE MECHANISTIC TARGET OF RAPAMYCIN COMPLEX 1 CONTROLS LIPID AND LACTOSE SYNTHESIS IN BOVINE MAMMARY EPITHELIAL CELLS

2.1. INTRODUCTION

mTORC1 pathway is a nutrient-sensing pathway that has emerged as a central regulator of nutrient metabolism and macromolecular synthesis in mammals (Saxton and Sabatini, 2017). mTORC1 controls metabolism by phosphorylation of several downstream targets including eukaryotic initiation factor 4E-BP1 and S6K1. Rapamycin is a highly specific inhibitor of mTORC1 (Sabatini et al., 1994). However, rapamycin is only capable of inhibiting certain functions of mTORC1 (Thoreen et al., 2009). Early studies revealed that mTORC1 is a downstream mediator of numerous mitogen- and growth factor-dependent signaling pathways (Sabatini et al., 1994). These factors all inhibit the tuberous sclerosis complex, a heterotrimeric complex comprised of TSC1, TCS2 and TB1D7, that acts as an upstream negative regulator of mTORC1 (Dibble et al., 2012).

Several reports have demonstrated the fundamental role of mTORC1 in promoting *de novo* lipid synthesis in lipogenic tissues such as liver and adipose tissues by regulating the activation of TFs such as SREBP1 (Soliman et al., 2010, Laplante and Sabatini, 2013). SREBP1 directly acts on the expression of important rate limiting genes involved in *de novo* lipid synthesis (Horton et al., 2002). In order to influence transcription, the terminal domain of SREBP must be released so it can be activated and enter the nucleus (Brown and Goldstein, 1997). Once activated, cleavage of SREBP1 allows its translocation into the nuclei to regulate gene expression (Li et al., 2014). mTORC1 positively regulates SREBP1 by promoting its transformation into its mature form and

movement to the nuclei. Li et al. (2014) have characterized the function of SREBP1 in fat synthesis occurring in BMECs. This study suggests that mTORC1 downstream targets, such as S6K1, are important promoters of SREBPs maturation, and may play a role in modulating lipid synthesis. Once phosphorylated by mTORC1, S6K1 promotes the activation of SREBP1 by inducing its nuclear accumulation. In addition, mTORC1 can regulate transcription of SREBP1 through S6K1-independent mechanisms (Düvel et al., 2010, Yecies et al., 2011, Owen et al., 2012). mTORC1 has been shown to promote lipid synthesis by the phosphorylation of Lipin 1 which inhibits SREBPs in the absence of mTORC1 signaling (Peterson et al., 2011). Although the regulatory roles of mTORC1 in SREBP1 activation have been confirmed, mTORC1 independent mechanisms in murine liver cells for the activation of SREBP1 have also been reported by Yecies et al. (2011). This study suggested that more complex mechanisms are involved in the regulation of lipid biosynthesis and that mTORC1 stimulates SREBPs activation. Another study by McFadden and Corl (2010) proposed other possible regulators of *de novo* lipid synthesis including the liver X receptor (**LXR**) which was shown to influence SREBP1 regulation in BMECs. Thus, the involvement of mTORC1 in lipid biosynthesis in differentiated primary BMECs has not yet been described. Our project concentrated on the characterization of mTORC1's potential roles in *de novo* lipid synthesis in differentiated primary BMECs.

Molecular pathways that control glucose utilization and lactose synthesis in BMECs are not well characterized. Düvel et al. (2010) showed that mTORC1 signaling pathways influence the expression of genes encoding important enzymes for glycolysis in murine liver cells. This research has identified potential rate limiting genes for these mechanisms in murine liver cells. 6-phosphofructo-2-kinase/fructose -2.6-biphosphatase-1 encoded by the *PFKFB1* gene catalyzes the synthesis and degradation of fructose-2-6-biphosphate during glycolysis (Lange et al., 1991).

The *G6PD* and *PGK1* genes encoding the enzymes Glucose-6-biphosphate dehydrogenase and Phosphoglycerate kinase 1 both play a role in glucose metabolism regulation. HIF-1α is a master TF known for its role in the cellular response to hypoxia. The vascular endothelial growth factor A protein encoded by the *VEGF-A* genes is a known marker of HIF-1α activity (Deudero et al., 2008). Studies show that HIF-1α can be activated by stimuli other than hypoxia and plays an important role in the regulation of energy metabolism pathways (Düvel et al., 2010, Mattmiller et al., 2011). In addition, there is emerging evidence that HIF-1α is involved in the regulation of the physiological event occurring in the development and onset of lactation (Shao and Zhao, 2014). This TF is involved in the regulation of energy metabolism leading to the stimulation of glycolytic genes expression (Hara et al., 1999, Hu et al., 2003, Gao et al., 2007, Zimin et al., 2009, Shahbazkia et al., 2012). It has also been shown to regulate two members of the glucose transporter family GLUT1 and GLUT8 (Shao et al., 2014). The objective of this study is to examine the role of mTORC1 in the synthesis of both lipid and lactose in BMECs.

2.2. MATERIAL AND METHODS

2.2.1. Materials

Chemicals and cell culture reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) or ThermoFisher Scientific (Waltham, MA, USA), respectively, unless otherwise stated. Catalogue numbers are listed in Appendix Table 3.1. Collagenase type 3 was from Worthington Biochemical Corp. (Lakewood, NJ, USA). Rapamycin was purchased from BioShop. Primary antibodies were from Cell Signaling Technology (Beverly, MA, USA) unless otherwise stated. Reagents and materials for immunoblotting and real-time quantitative PCR (qPCR) were from Bio-Rad Laboratories (Hercules, CA, USA). MCDB170 was from US Biological (Salem, MA, USA). Radiochemicals were from PerkinElmer (Waltham, MA, USA).

2.2.2. Isolation of Bovine Mammary Epithelial Cells

Mammary gland tissue from lactating Holstein cows was aseptically collected at the time of slaughter from a local abattoir and placed into 50 mL tubes containing ice-cold Ham's F12 medium supplemented with 1× antibiotics/antimycotics (100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, and 50 µg/mL gentamicin) for transport into the laboratory. The characteristics of the donor cows are in Appendix Table 3.2. After removal of visible fat, connective tissue and blood vessels, mammary tissue was minced into ~1 mm^3 pieces using scalpels and then rinsed 5× using ice-cold Ham's F12 medium supplemented with antibiotics/antimycotics to remove residual milk and blood. The minced tissue was enzymatically digested in Dulbecco's Modified Eagle's Medium (**DMEM**)/F12 containing 300 U/mL Type-3 collagenase, 400 U/mL of hyaluronidase, and 1 mg/mL DNase I supplemented with antibiotics/antimycotics at 37 °C with constant shaking (80 rpm) for 4 h. The tissue digest

was filtered through a 200-µm mesh sieve and then centrifuged at 80×*g* at room temperature for 30 s. The resulting pellet was highly enriched in mammary epithelial organoids (acini). The pellet was either resuspended in BMEC growth medium for outgrowth of mammary epithelial cells or cryopreserved. The medium is composed of 1:1 DMEM/F12:MCDB170, 0.25% (vol/vol) fetal bovine serum, 0.1% (wt/vol) albumax II, 7.5 µg/mL bovine insulin, 0.3 µg/mL hydrocortisone, 5 ng/mL recombinant human epidermal growth factor, 2.5 µg/mL bovine apo-transferrin, 5 µ*M* isoproterenol, 5 p*M* 3,3′,5-triiodo-L-thyronine, 0.5 p*M* β-estradiol, 0.1 n*M* oxytocin, and 1× antibiotics/antimycotics. The medium was originally developed by Garbe et al. (2009) for selective growth of human mammary epithelial cells under serum-reduced conditions. Primary mammary epithelial cells from acini outgrowths were passaged once for expansion and cryopreserved.

2.2.3. Design of TSC2 gRNA and lentiviral CRISPR-Cas9 Vector

Guide RNAs (gRNA) targeting the *bos taurus* genome (Btau 5.0) were designed using CHOPCHOP (http://chopchop.cbu.uib.no/) (Gagnon et al., 2016). The gRNAs were designed to target the coding region of exons 2 and 3 of *TSC2* (NCBI Gene ID: 504985). We selected gRNAs with the highest predicted efficiency scores according to Doench et al. (2016), lowest predicted off-targets (up to 3 mismatches in the protospacer), 40-60% GC content and ≤ 1 self-complementarity. We selected two non-targeting (**NT**) control gRNAs from a universal NT control gRNA library (Doench et al., 2016). We computationally validated that the nucleotide sequence of the selected NT control gRNA lacked sequence homology with the bovine genome using BLASTn (Boratyn et al., 2012). The sequences of the gRNAs used are in Table 2.1.

The gRNAs were synthetized as single-strand DNA oligonucleotides by ThermoFisher Scientific (Burlington, Canada). The 24-bp forward and reverse oligonucleotides including the 20-bp target sequence and BsmBI cohesive end were annealed and the cloned into the

lentiCRISPR_V2 vector (Sanjana et al., 2014), a gift from Feng Zhang (Addgene; 52961), according to the method of Shalem et al. (2014).

Table 2.1: gRNA sequences

gRNA name	Target Sequence	Exon
NT[3]	GTATTACTGATATTGGTGGG[4]	-
TSC2-1	GTGGCCTCAATAATCGCATC	3
TSC2-2	ATAATCGCATCCGGGTGATA	2
TSC2-3	TTTTGGGACTGGGGACTCCA	3

[3]NT: Non-targeting sequence
[4]NGG: PAM sequence

2.2.4. Production of lentiviral particles

To produce lentivirus, HEK293T cells (Genhunter; Q401) seeded in 100-mm plates were transfected with LentiCRISPR_v2 plasmid cloned with either NT or TSC-targeting gRNAs, psPAX2 (Addgene; DP12260), and pMD2.G (Addgene; DP12259), using transfection-grade polyethylenimine 'Max' (MW 40000) (PEI MAX, Polysciences, Inc.), in accordance with the manufacturer's instructions. After a 24 h incubation, the media containing lentiviral particles was harvested, centrifuged at 800×*g* for 5 min to remove any cells and debris and stored at -80°C.

2.2.5. Viral Transduction and Selection of BMEC

First-passage BMECs seeded into 60-mm collagen-coated plates and grown to 50% confluence in antibiotic-free BMEC growth medium were transduced with lentivirus in the presence of 8 µg/mL polybrene (Sigma) and then cultured for an additional 24 h. After 2 d, transduced BMEC were passaged and seeded into 60 mm collagen-coated plates. For selection of stably-transduced cell, BMECs were subculture in antibiotic-free BMEC growth medium

containing 8 µg/ml of puromycin (Sigma). The puromycin containing BMEC growth medium was replaced every 2 d. Transduced BMECs were allowed to grow to 50-60% confluence collection for cryopreservation or re-seeded for experiments. The GeneArt™ Genomic cleavage assay detection kit (Thermo Fisher) was used to determine the efficiency of genomic cleavage in the genome of primary BMECs. Primer design was made adhering to the GeneArt™ Genomic cleavage assay detection kit protocol using Primer Blast (Ye et al., 2012). Primers used are shown in Appendix Table 3.3.

2.2.6. Culture and Treatment of Bovine Mammary Epithelial Cells

For experiments, wild-type and gene-edited BMECs were seeded into collagen-coated dishes at a density of 200 cells/cm^2 and grown to confluence. To induce lactogenic differentiation, BMEC were incubated in medium composed of DMEM containing 3.5 m*M* D-glucose, 1 m*M* sodium acetate and 200 µ*M* L-glutamine supplemented with lactogenic hormones (**LH**; 5 µg/mL of bovine insulin, ovine prolactin and hydrocortisone), 5 µg/mL bovine apo-transferrin, 0.5 mg/mL bovine serum albumin and 1× antibiotic-antimycotics for 4 d. For lactogenic differentiation experiments, control (**Ctrl**) cells were incubated in differentiation medium without LH. The medium was changed every 2 d. On day 4 of differentiation, cells were treated with 100 n*M* rapamycin (BioShop) or 0.01% of dimethylsulfoxydel used as a vehicle (**Veh**) control for 16 h, unless otherwise stated.

2.2.7. RNA Extraction and Quantitative Real-Time PCR

Total RNA from BMEC was extracted using the TRI-Reagent (Sigma), according to manufacturer's instructions. RNA yield was quantified by measuring absorbance at 260 nm in a spectrophotometer (BioTek; Winooski, VT). RNA integrity was determined by bleach agarose gel

electrophoresis as described in (Aranda et al., 2012). Representative gels are shown in Appendix Figure 3.1. Total RNA (1 µg) was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories) in a T100 thermal cycler (Bio-Rad Laboratories), according to manufacturer's instructions. cDNA was used fresh or stored at -80℃. Quantitative real-time PCR (**qPCR**) was performed using SsoAdvanced Universal SYBR green (Bio-Rad Laboratories) and gene-specific primers in a CFX96 Touch Real Time PCR System (Bio-Rad Laboratories). Information on primers used are shown in Appendix Table 3.4. Samples for each experimental condition were run in duplicate. Relative gene expression was determined using the geometric mean of the reference gene used (*GAPDH*, *ATCB* or *PPIA*) and calculated using the $\Delta\Delta C_q$ method. Experiments were conducted using the CFX Maestro Software (Bio-Rad Laboratories).

2.2.8. Immunoblotting

BMECs were rinsed twice with ice-cold PBS before being lysed in a buffer containing 50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1% (vol/vol) Triton X-100, 1 m*M* EDTA, 50 m*M* β-glycerophosphate, 50 m*M* NaF, 10 m*M* Na_4PO_7, 10 m*M* Na_3VO_4 and supplemented with a protease inhibitor cocktail on ice for 15 min. The cell lysates were centrifuged at 15,000×*g*, 4 °C, for 15 min. A portion of the cleared cell lysates was combined with Laemmli sample buffer and incubated at 95 °C for 5 min. BCA protein assay using BSA as Standard was used to determine protein concentration. SDS-PAGE was used to resolve equal amounts of protein lysate (50 µg) and then transferred onto a PVDF membrane. The membranes were blocked in 5% (wt/vol) non-fat milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (**TBS-T**) at room temperature for 1 h and then incubated primary antibodies raised against phospho-specific proteins diluted in 5% non-fat milk in TBS-T at 4 °C with constant rocking overnight. The antibodies used are also listed in Appendix Table 3.5. After washing 6× in TBS-T for 5 min, membranes were incubated with

secondary antibodies diluted 1:10,000 in 5% non-fat milk in TBS-T at room temperature for 1 h with constant shaking. After washing in TBS-T, the bound horseradish peroxidase-linked secondary antibodies were visualized by chemiluminescence (Clarity ECL; Bio-Rad Laboratories). The signal intensity was quantified using the Image Lab Software (Bio-Rad Laboratories). After detection of the phospho-specific signal, the antibodies were stripped-off the membranes by incubation in 62.5 m*M* Tris-HCl, pH 6.8, 2% (wt/vol) SDS, and 100 m*M* 2-mercaptoethanol at 50°C for 30 min with constant rocking. The membranes were washed, blocked, and re-probed with primary antibodies that recognized the proteins irrespective of their phosphorylation state. The signal intensity of phospho-specific proteins was normalized to total protein levels. For determination of protein abundance, total protein levels were normalized to α-tubulin. For SREBP-1c processing, the mature (cleaved) SREBP-1c was normalized to total SREBP-1c (precursor + mature).

2.2.9. *De novo* Fatty Acid Synthesis

De novo FA synthesis was determined by quantifying the incorporation of [^{3}H]-labeled acetate into total lipids. Lactogenic differentiated cells were treated with rapamycin or vehicle control for 16 h. After 12 h of treatment, [^{3}H]-labeled acetate (0.37 μCi/μmol; PerkinElmer; Waltham, MA) was added to the cells at a final concentration of 1 μCi/mL and the incubation continued for 4 h. Cells were lysed in 0.1% SDS in PBS. A portion of the cell lysate was retained for measurement of protein concentration. Total lipids were extracted from cell lysates using hexane:isopropanol (3:2 vol/vol). The solvent layer was combined with scintillation cocktail (Universol, MP Biomedical; Solon, OH) for quantification of radioactive label incorporation into lipid using a Tri-Carb liquid scintillation counter (Perkin-Elmer). FA synthesis was expressed as *p*mol of acetate incorporated per μg of protein.

2.2.10. Lactose Synthesis

Lactose synthesis was measured by incorporation of D-[U-^{14}C]-glucose into lactose in spent medium (Mellenberger et al., 1973). Lactogenic differentiated cells were treated with rapamycin or vehicle control for 16 h. After 12 h of treatment, D-[U-^{14}C]-glucose (0.37 μCi/μmol; PerkinElmer; Waltham, MA) was added to the cells at a final concentration of 1 μCi/mL and the incubation continued for 4 h. At the end of the incubation, the spent medium was collected. Cells were lysed in 0.1% SDS and a portion of the cell lysate was retained for measurement of protein concentration. Lactose monohydrate (Sigma) was added to the collected medium as a carrier. Lactose was then precipitated using magnesium chloride hexahydrate and sodium hydroxide according to Kwon et al. (1981). The lactose precipitate was resuspended in water and combined with a scintillation cocktail (Universol, MP Biomedical; Solon, OH) for quantification of radioactivity using a Tri-Carb liquid scintillation counter (Perkin-Elmer). The radioactive counts were corrected by protein amount in the cell lysate.

2.2.11. Statistical Analyses

Data are presented as mean ± SEM. Unless otherwise stated, results are from 6 replicates per treatment group using cells derived from 3 independent cows. Data were analyzed by ANOVA using PROC MIXED in SAS Version 9.4. The statistical model included the fixed effect of the treatments, random effect of cow, all-way interactions and the residual random error. For genomic editing experiments, the statistical model included the fixed effect of the gRNA (**NT** or **TSC2**), and the fixed effect of the medium (**CTR** or **LH**), the random effect of cow, as well as all 2- and 3-way interactions. None of the interactions were significant and were therefore removed from the model. A p-value of <0.05 was considered significant.

2.3. RESULTS

2.3.1. Lactogenic Hormones Induce the Expression of Genes Involved in Milk Component Synthesis and Activate mTORC1 Signaling

To study the role of mTORC1 on milk component synthesis, we first measured the effect of LH on established markers of lactogenic differentiation in BMECs. The mRNA abundance of *CSNS1* (p =0.02) and *LALBA* (p =0.03), which encode for the mammary-specific milk proteins α-s1 casein and α-LA, respectively, were higher in LH-treated cells compared to non-treated control BMECs (Figure 2.1a). These results confirm that our BMECs are capable of recapitulating key features of lactogenic differentiation.

Having validated our cellular model, we tested the effect of lactogenic differentiation on the expression of genes involved in lipid and lactose synthesis. The expression of *ACACA* gene, which encodes for acetyl-CoA carboxylase, was 74% higher (p = 0.05) in LH-treated cells. There was numerical increase *DGAT1* expression by 20% (p = 0.07) and *FABP3* by 90% (p = 0.08) but did not significantly affect the mRNA abundance of *SREBF1*, *FASN* or *SCD1* (Figure 2.1b). Furthermore, the mRNA abundance of *SLC2A1* that encodes for GLUT1, the major glucose transporter in mammary cells (Zhao, 2014), increased by 72% (p = 0.007) in LH-treated compared to non-treated control cells. However, the expression of phosphoglucomutase-1 (*PGM1*), which converts glucose-6-phosphate into UDP-galactose, and β -1,4-galactosyltransferase (*B4GALT1*), that forms lactose synthase in a complex with α-LA, were not affected by LH treatment (Figure 2.1c). Thus, lactogenic differentiation induced the expression of selected genes involved in lipid and lactose synthesis in BMECs.

To assess whether mTORC1 was activated during lactogenic differentiation in BMECs, we measured the phosphorylation status of mTOR and its downstream effectors, 4E-BP1 and rpS6, as

markers of mTORC1 activity. LH treatment activated mTORC1 signaling, as judged by a 33% increase in phosphorylation of mTOR Ser2448 (p = 0.02) as well 4E-BP1 Thr70 (p =0.04) and rpS6 Ser240/244 (p = 0.03) by 36% and 46%, respectively (Figure 2.1d). Thus, LH induced the expression of genes involved in lipid and lactose synthesis while concomitantly enhancing the activity of mTORC1 in, suggesting mTORC1 may play a role in milk component synthesis in BMECs during lactogenic differentiation.

2.3.2. Rapamycin Inhibits De Novo Fatty Acid Synthesis and Alters the Expression of Lipogenic Genes in BMECs

Before assessing the role of mTORC1 on milk component synthesis, we first confirmed inhibition of mTORC1 signaling by rapamycin in our cellular model. Lactogenic differentiated BMECs were treated with 100 n*M* rapamycin or vehicle control for 16 h. Phosphorylation of mTOR at Ser2448, rpS6 at Ser240/244, 4E-BP1 at Thr70 and S6K1 at Thr389 was 36% (p = 0.04), 86% (p = 0.009), 60% (p <0.0001), and 55% (p = 0.001) lower, respectively, in rapamycin-treated cells compared to vehicle-treated cells. As prolonged rapamycin treatment can disrupt mTORC2 assembly thereby inhibiting its function in a cell-type specific manner (Sarbassov et al., 2006), we monitored the phosphorylation of its downstream target Akt Ser473. We found that chronic rapamycin exposure did not affect mTORC2 signaling in BMECs, as Akt Ser473 phosphorylation did not differ from vehicle-treated BMECs (Figure 2.2a). Taken together, these results demonstrate chronic (16 h) rapamycin inhibits mTORC1, but not mTORC2, in BMECs.

To examine the role of mTORC1 on *de novo* fatty acid synthesis in BMECs, we measured the effect of rapamycin on incorporation of radioactive acetate into total cellular lipids. In comparison to vehicle-treated cells, *de novo* fatty acid synthesis was 24% (p = 0.005) lower in

BMECs treated with rapamycin (Figure 2.2b). Thus, as in the case for other lipogenic cell-types in non-ruminants, mTORC1 controls lipid synthesis in BMECs.

We next assessed whether mTORC1 regulates the expression of lipogenic genes in BMECs. Compared to vehicle-treated cells, the expression of *FASN* (p = 0.04) was lower in rapamycin-treated BMECs. The expression of *DGAT1* and *FABP3* were also numerically lower in rapamycin-treated cells, albeit not significantly, p = 0.07 and p = 0.08, respectively. This result raised the possibility that mTORC1 may regulate lipid synthesis in BMECs by small but coordinated transcriptional changes. Interestingly, *SREBF1* expression was also 30% (p = 0.02) lower in rapamycin treated BMECs (Figure 2.2c), suggesting that this master TF of lipogenesis may mediate the effect of mTORC1 on lipid synthesis.

Since the mRNA abundance of *SREBF1* was affected by mTORC1, we investigated the effect of rapamycin on SREBP1 protein abundance and proteolytic processing in BMEC. We used an antibody that detects both the precursor and mature forms of SREBP1. As shown in Figure 2.2d, rapamycin treatment did not significantly affect SREBP1 abundance (sum of the precursor and the mature forms normalized to tubulin).

2.3.3. Rapamycin Reduces Lactose Synthesis and Expression of Lactose Related Genes in BMECs

To determine the role of mTORC1 on lactose synthesis, we measured incorporation of D-[U-^{14}C]-glucose into lactose in conditioned medium of lactogenic differentiated BMEC treated with 100 n*M* of rapamycin or a vehicle control for 16 h. As shown in Figure 2.3a, lactose synthesis was 71% lower (p <0.001) in rapamycin-treated BMECs compared to vehicle control cells, demonstrating that mTORC1 controls lactose synthesis.

To investigate the mechanisms by which mTORC1 controls lactose synthesis, we measured the expression of genes involved in glucose uptake and lactose formation in lactogenic differentiated BMECs treated with rapamycin. The expression of *SLC2A1* was not affected by the rapamycin treatment. However, the expression of two key genes involved in lactose synthesis *PGM1* (p = 0.003) and *B4GALT1* (p = 0.003) were lower in rapamycin-treated cells compared to vehicle controls (Figure 2.3b). Düvel et al. (2010) implicated the TF HIF-1α as a mediator of mTORC1 control of glycolysis. Therefore, we measured the expression of glycolytic genes and *VEGF-A,* a marker of HIF-1α activity, in rapamycin- vs. vehicle-treated BMEC (Figure 2.3c). We found that although rapamycin did not affect the expression of the glycolysis genes *PGFK1, PFKFB1* and *G6PD, VEGF-A* expression was lower (p <0.001) in cells treated with rapamycin. Taken together, these results suggested mTORC1 might regulate lipid synthesis by modulating the expression of genes involved in lactose synthesis; however, a role for HIF-1α in mediating this effect remains to be established.

2.3.4. CRISPR-Cas9-mediated Knock Out of TSC2 Induces the Expression of Genes Involved in Lipid and Lactose Synthesis

To confirm the role of mTORC1 in lipid and lactose synthesis, we used CRISPR-Cas9-mediated KO of TSC2 in BMEC. Previous studies have shown that genetic ablation of TSC2, an upstream negative regulator of mTORC1, in mouse embryo fibroblasts leads to its constitutive hyper-activation (Goncharova et al., 2002, Jaeschke et al., 2002). We designed 3 specific gRNAs targeting exons 2 and 3 of the bovine *TSC2* gene (Figure 2.4a). Of these, BMECs transduced with gRNAs 1 and 2, but not gRNA 3, showed evidence of DNA cleavage when compared with two non-targeting gRNAs (Figure 2.4b). Importantly, BMECs transduced with lentiCRISPR_V2 expressed Cas9 and those infected with gRNAs 1 and 2 targeting *TSC2* had 94 % (p =0.008) and

96% (p =007) lower TSC2 protein abundance, respectively, than the BMECs infected with non-targeting gRNAs (Figure 2.4c). These results confirmed that lentiviral delivered CRISPR-Cas9 systems can efficiently KO TSC2 in primary BMECs. Importantly, as shown in Figure 2.4d, phosphorylation of rpS6 at Ser240/244 was 87% (p = 0.004) higher in BMECs treated cells compared to the NT gRNAs. These results confirmed functional hyper-activation of the mTORC1 signaling pathway in our TSC2 KO BMECs.

Having established our genetic model of mTORC1 hyper-activation in BMECs, we measured the expression of genes involved in lipid and lactose synthesis that were affected by rapamycin treatment. We found that the expression of *SREBF1* and *FABP3* were 56% (p =0.001) and 44% (p =0.01) higher, respectively, in BMECs infected with gRNA targeting TSC2 compared to NT (Figure 2.5a). The expression of both *FASN* and *DGAT1* was also numerically higher in TSC2-KO cells, but the differences were not statistically significant. Similarly, *PGM1* (p =0.03) and *B4GALT1* (p = 0.006) expression was higher in TSC2-KO cells compared to NT (Figure 2.5b). These results confirmed that mTORC1 selectively regulates the expression of genes involved in lipid and lactose synthesis in BMECs.

2.4. FIGURES

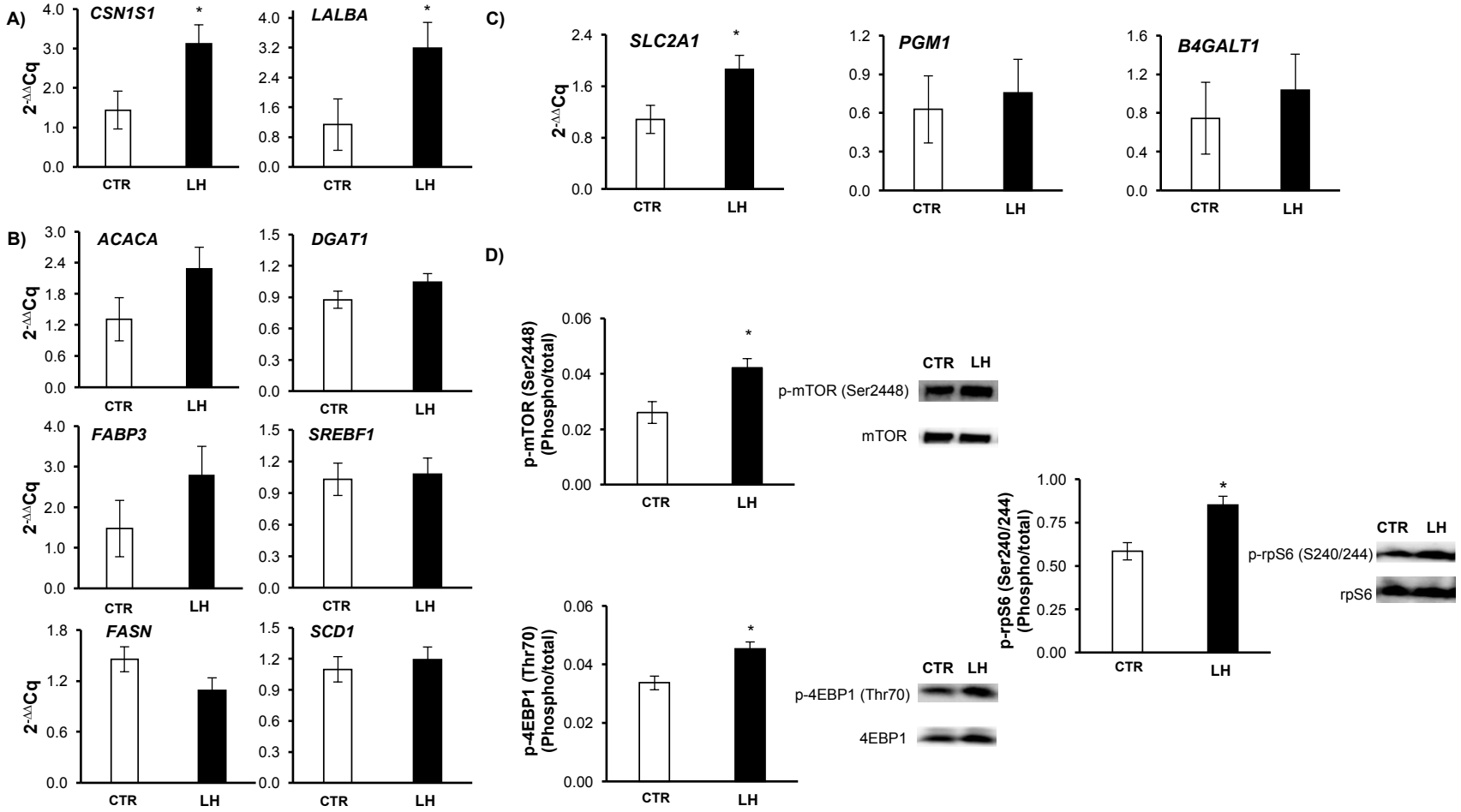


Figure 2.1: Lactogenic hormones induce the expression of genes involved in milk component synthesis and activate mTORC1 signaling. Cell were treated for 4 d with lactogenic hormone (**LH**) media or control media (**CTR**). Media was change after 2 d. Gene expression of key milk protein genes *CSN1S1* and *LALBA* (**A**), lipid synthesis related genes *ACACA, DGAT1, FABP3, SREGBF1, FASN* and *SCD1* (**B**) and glucose and lactogenic genes *SLC2A1, PGM1* and *B4GALT1* (**C**) were measure by RT-qPCR. Phosphorylation of mTOR, 4E-BP1 and rpS6 **(D)** were measure by immunoblot analysis. Results are presented as the mean ± SE of the sample mean (n=6). * indicate a *p* value <0.05.

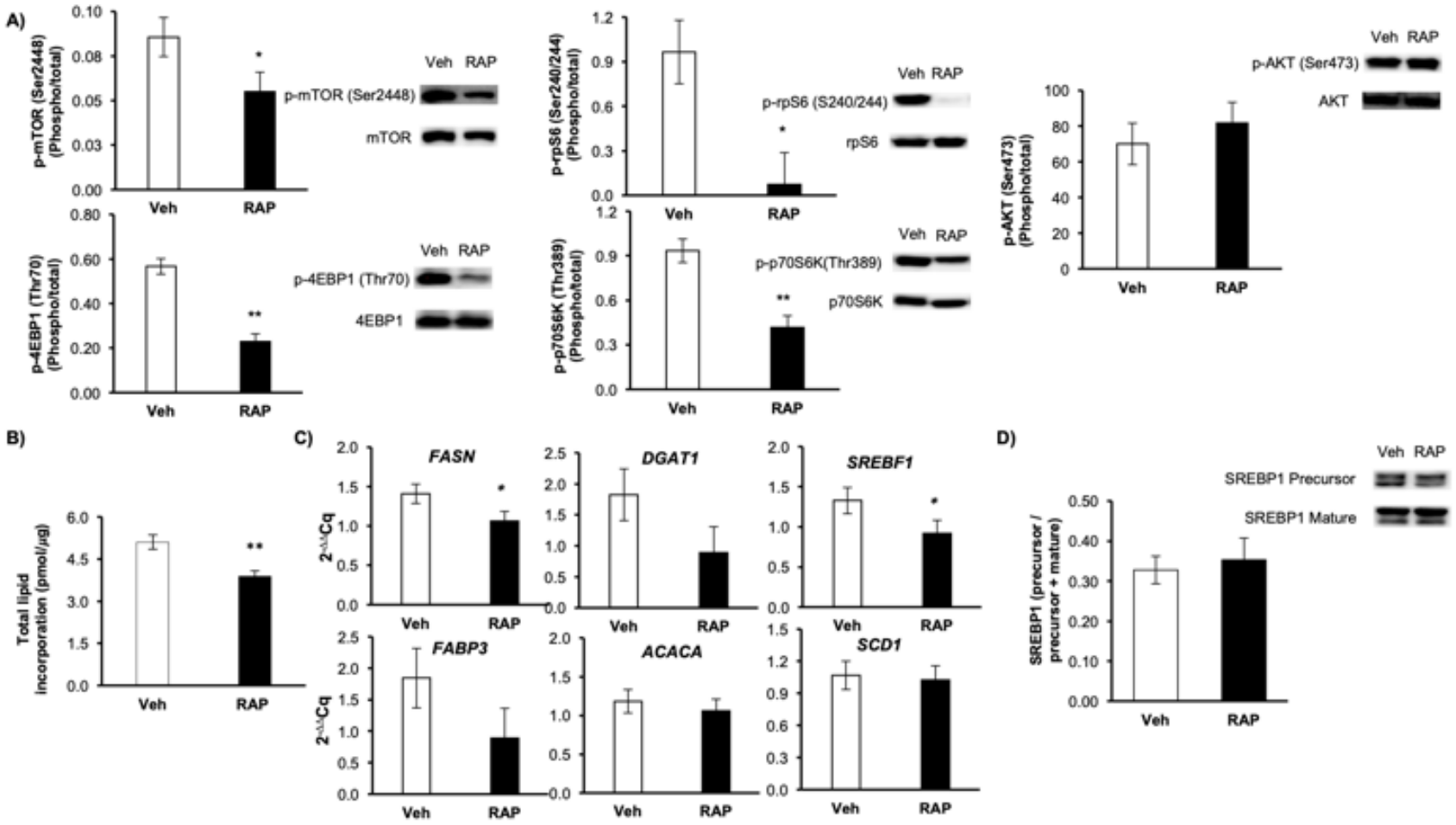


Figure 2.2: Rapamycin inhibits *de novo* fatty acid synthesis and alters the expression of lipogenic genes in BMECs. Cell were treated with 100 μ*M* Rapamycin (**RAP**) or vehicle control (**Veh**) for 16 h. Phosphorylation of mTOR at Ser2448, 4EBP1 at Thr 70, rpS6 at Ser 240/244 and p70S6K at Thr389 (**A**) were measured by immunoblot analysis. Incorporation into ^{3}H-acetate into total lipids (**B**) was measured by liquid scintillation counting. Expression of key the gene involved in lipid synthesis: *FASN, DGAT1, SREBF1, FABP3, ACACA* and *SCD1* (**C**) was measured by RT-qPCR**.** Phosphorylation of SREBP1 (precursor/precursor + mature) (**D**) was measured by immunoblot analysis. Results are presented as the mean ± SE of the sample mean (n=6). * indicate a p value <0.05. ** indicate a p value <0.001.

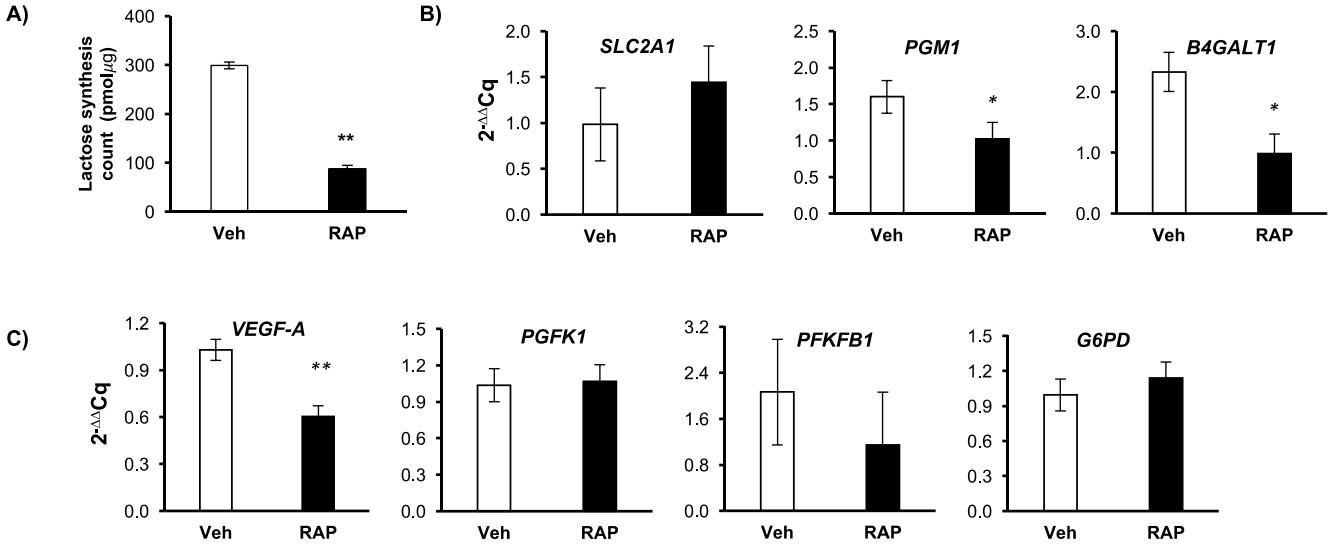


Figure 2.3: Rapamycin reduces lactose synthesis and expression of lactose related genes in BMECs. Cell were treated with 100 μ*M* Rapamycin (**RAP**) or vehicle control (**Veh**) for 16 h. Incorporation of ^{14}C-glucose into total lactose (**A**) was measured by liquid scintillation counting. Expression of key genes involved in glucose transport and lactose synthesis: *SLC2A1, PGM1* and *B4GALT1* (**B**) genes involved in energy metabolism: *VEGF-A, PGFK1, PFKFB1* and *G6PD* (**C**) was measured by RT-qPCR**.** Results are presented as the mean ± SE of the sample mean (n=6). * indicate a p value $<$ 0.05. ** indicate a p value $<$ 0.001.

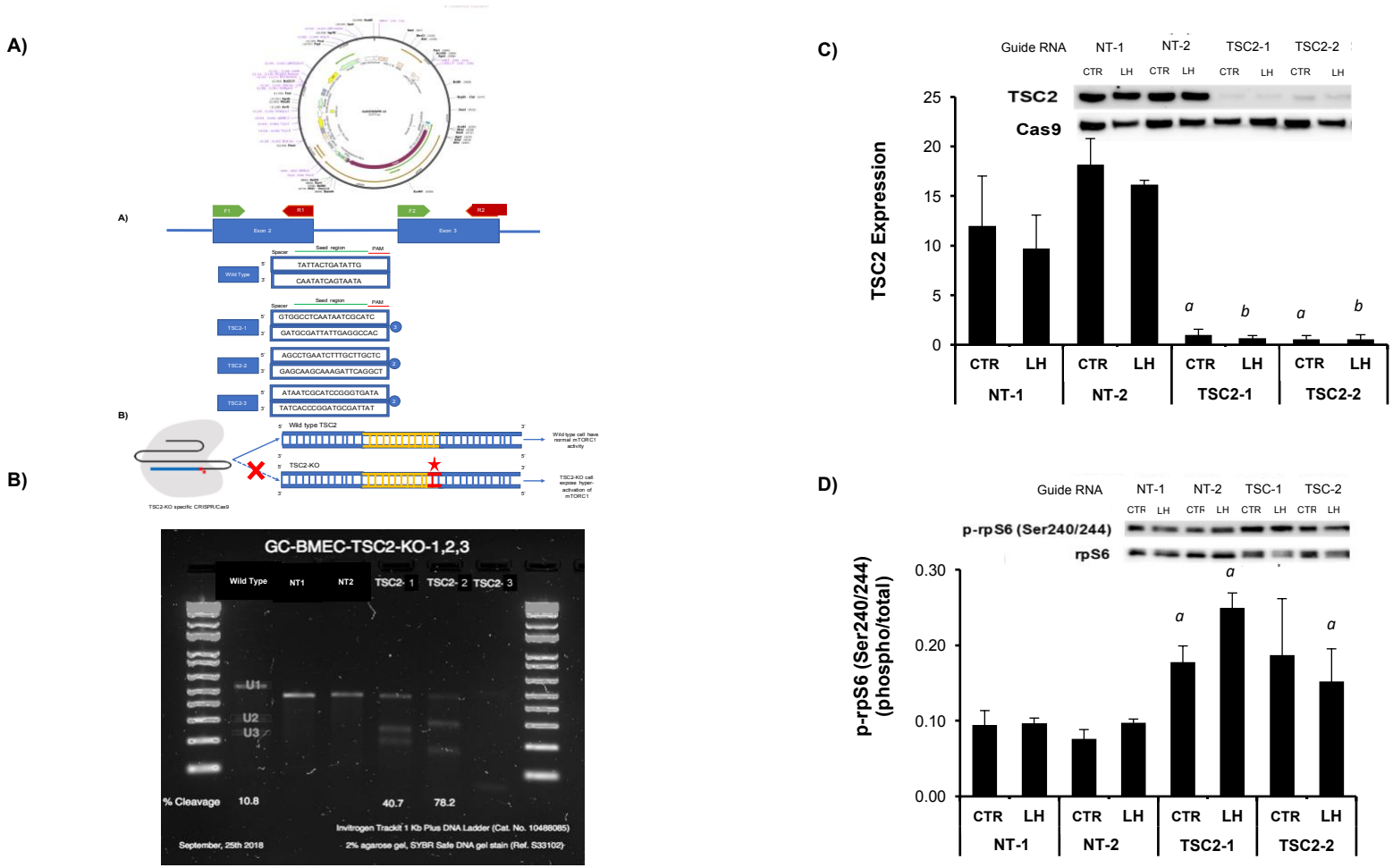


Figure 2.4: CRISPR-Cas9-mediated Knock Out of TSC2 in BMECs. Non-targeted (**NT**) and gene-edited (**TSC2**) cells were treated with lactogenic hormones (**LH**) or control media (**CTR**) for 4 d. gRNAs were designed to target TSC2 exon 2 and 3 (**A**). DNA cleavage assay (**B**) confirmed TSC2 knock out. Expression of Cas9 and TSC2 (**C**), phosphorylation of rpS6 at Ser 240/244 (**D**) was measure by immunoblot analysis. Results are presented as the mean ± SE of the sample mean (n=4). Letters a-b indicate a p value <0.05.

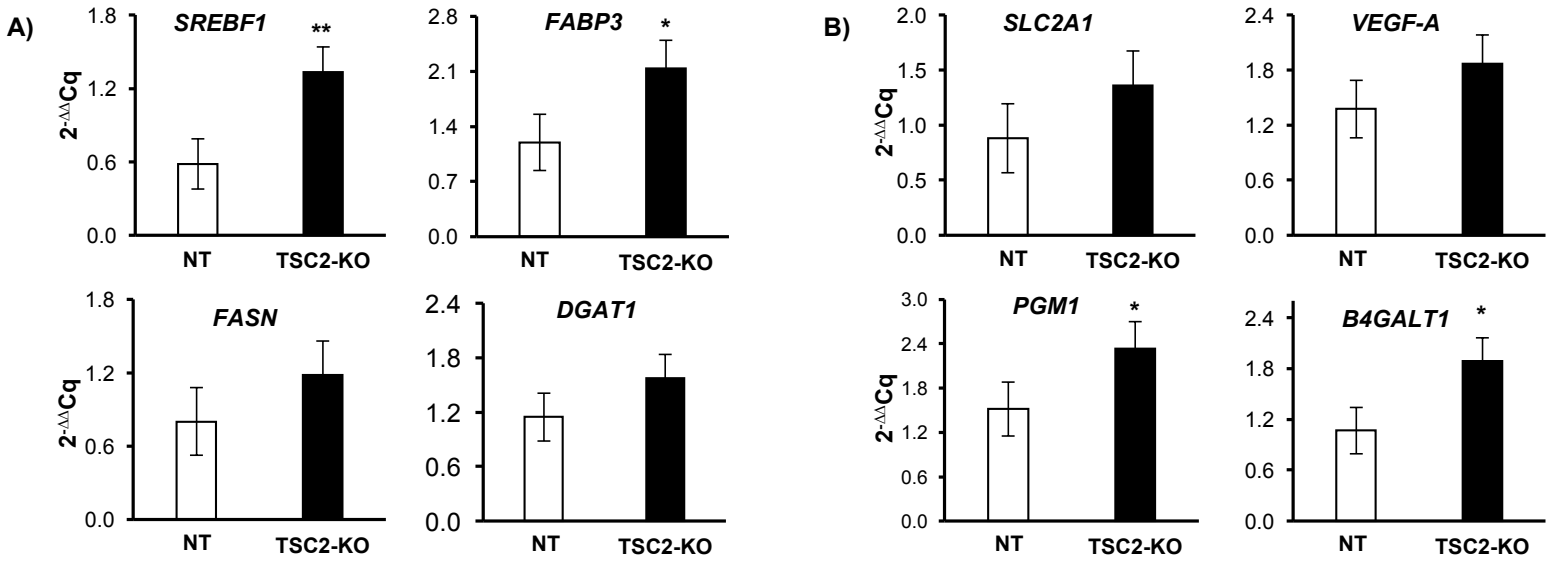


Figure 2.5: CRISPR-Cas9-mediated Knock Out of TSC2 Induces the Expression of Genes Involved in Lipid and Lactose Synthesis. Non-targeted (**NT**) and TSC2 knock out (**TSC2-KO**) differentiated cells were grown to confluency. Expression of key genes involved in lipid synthesis: *SREBF1, FABP3, FASN* and *DGAT1* (**A**), in glucose metabolism and lactose synthesis: *SLC2A1*, *VEGF-A, PGM1* and *B4GALT1* (**B**) were measured by RT-qPCR. Results are presented as the mean ± SE of the sample mean (n=4). * indicate a p value < 0.05. ** indicate a p value < 0.001.

2.5. DISCUSSION

To study the role of mTORC1 in milk component synthesis, we established a cellular model of lactogenic differentiation in primary BMECs. Jedrzejczak and Szatkowska (2014) demonstrated that early passage BMEC cultures appeared to be the best material to study mammary gland function and gene expression activity. In our study, we obtained primary BMECs from outgrowths of isolated mammary organoids cultured in a medium containing designed to promote the selective growth of epithelial cells (Garbe et al., 2009). Whereas many studies have used immortalized cell lines with limited differentiation capacity such as MAC-T, or undifferentiated primary cells, lactogenic differentiation of BMECs more closely resembles the cellular phenotypes present during lactation. Indeed, Neville et al. (2002) described the importance of inducing lactogenic differentiation of BMEC using a cocktail of insulin, prolactin and hydrocortisone when studying the mechanisms and regulation of milk synthesis. We demonstrated that lactogenic differentiation of primary BMECs using our model led to increased expression of mammary-specific milk proteins genes *CSN1S1* and *LALBA*. In addition, we found that mTORC1 activity was elevated in lactogenic differentiated BMECs. This result is consistent with a previous study by Burgos et al. (2010), which showed that LH treatment of BMECs induced mTORC1 activation to regulate components of the translational machinery in freshly isolated mammary acini. These results demonstrated great potential for the use of lactogenic differentiated BMEC in elucidating the role of mTORC1 on milk component synthesis and more specifically on the regulatory mechanisms governing lipid and lactose synthesis.

In order to characterize the role of mTORC1 in lipid and lactose synthesis, we first measured the effect of LH on lipid and lactose related genes in differentiated BMECs. Our results showed a tendency to increase lipogenic gene expression after differentiation. Recent studies

conducted by Shao et al. (2013) demonstrated that LH stimulates expression of lipogenic genes, such as *SREBF1*. Contrary to these findings, LH treatment did not significantly affect *SREBF1* mRNA abundance in our study. Although, our results partially agree with Shao et al. (2013) that also showed that BMECs treated with LH displayed an increased in expression of *FASN*, *ACACA* and *SCD1* genes. Interestingly, our treatment similarly tended to affect *ACACA* and *DGAT1*. In addition, our result showed a tendency to increase *FABP3* expression but had no effect on *FASN* or *SCD1*. Another study conducted by Zhao et al. (2012) also reported similar results on BMECs treated with LH, either alone or with a combination of three LH, at near physiological concentrations. At this stage, whether these changes were modulated by the mTORC1 signaling pathway remains unclear.

To study the effect of LH on lactose we measured the expression of genes involved in lactose synthesis, on which substantially less is known. Early research conducted by Mellenberger et al. (1973) found that the activity of the enzymes lactose synthase and α-LA concentration in bovine mammary-tissue slices increased roughly 2-fold between 7 days pre-partum and 7 days postpartum. In contrast, phosphoglucomutase activity increased by ~25% during the same period. Consistent with these results, we found that LH increased gene expression of α-LA, but not phosphoglucomutase or β-1, 4-galactosyltransferase. Available evidence suggests that α-LA expression plays a crucial role in the synthesis of lactose during lactogenic differentiation. Shao et al. (2013) studied the effect of LH on lipogenic genes in addition to glucose transporters in the BMG. In contrast with our findings, their results showed no significant changes on *SLC2A1* expression in bovine mammary explants using LH. Interestingly, another study conducted by Zhao et al. (2012) showed that the combination of insulin and prolactin had no effect on glucose transport whereas insulin alone up-regulated GLUT8 expression. GLUT8 is another glucose

transporter known to be expressed in BMG (Shao et al., 2014). Hydrocortisone at 10 ng/mL increase expression of both GLUT1 and GLUT8 but combination of hydrocortisone and insulin had no effect. The reason for the discrepancy among studies is not immediately obvious but these results confirm that complex regulation mechanisms are involved in the regulation of glucose transport and metabolism in the BMG. Further research is required to understand how the heightened demand of glucose for lactose synthesis is met by the mammary gland at the onset of lactation and the potential role of mTORC1 in the regulation of lactose synthesis.

As previously mentioned, mTORC1 has been identified as a regulator of protein synthesis in BMECs, but its role in the control other milk components was unknown. In this study, we combined a pharmacological approach with gene editing tools using CRISPR-Cas9 to generate TSC2-KO BMEC to demonstrate a critical role of mTORC1 in milk component synthesis. First, we used a highly-specific pharmacological inhibitor of mTORC1, rapamycin, to demonstrate that inhibition of mTORC1 function reduces lactose and lipid synthesis in lactogenic differentiated BMECs. Using a similar experimental approach, Düvel et al. (2010) who studied the role of mTORC1 in the activation of metabolic gene regulatory network in mice liver cells. Soliman et al. (2010) also used rapamycin as mTORC1 inhibition agent to study role of this protein complex in TG metabolism murine adipocytes. Therefore, our results agree with other studies that have suggested a direct link between mTORC1 activity and lipid production in various tissues (Soliman et al., 2010, Wang et al., 2011).

Despite the importance of rapamycin in defining mTORC1 functions in a myriad of cellular processes in a variety of cell types, we also considered that some functions of mTORC1 are rapamycin-resistant. Using the ATP-competitive inhibitor Torin1, Thoreen et al. (2009) found that some mTORC1 functions necessary for cap-dependent translation and autophagy are resistant to

rapamycin inhibition. These findings were confirmed by Yoon and Roux (2013), who demonstrated that some phosphorylation sites in mTORC1 substrates are insensitive to the drug. Therefore, we also used lentivirally-delivered CRISPR-Cas9-mediated KO of TSC2, upstream negative regulator of mTORC1, to confirm the role of mTORC1 in the regulation of genes involved in lipid and lactose synthesis. A recent study by Tian et al. (2018) introduced CRISPR-Cas9 mediated KOs by transfection into goat mammary epithelial cells to study the implication of *SCD1* expression for milk production. In another study by Zhu et al. (2017) buffalo mammary epithelial cells with targeted KOs were generated using CRISPR-Cas9 delivered with an adenovirus system. Collectively, these studies demonstrate the potential of this technology for the study gene function in ruminant mammary cells to better understand mammary gland biology and molecular regulation of milk synthesis.

Lipids are a key component of milk that derive from the diet or are produced *de novo* by the mammary gland. The molecular mechanism by which nutrients and hormones regulate lipid synthesis have been subject of intensive research interest in recent years. As summarized by Osorio et al. (2016), this research has focused in the regulation of milk fat synthesis by TFs including PPARs, SREBP1 and LXR. In our study, we were specifically interested in assessing the role of SREBP1 in mediating the effect of mTORC1 on lipid synthesis. Down regulation of mTORC1 using rapamycin was accompanied by a clear decrease in *SREBF1* gene expression in BMECs. Consistent with this finding, hyper-activation of mTORC1 in TSC2-KO BMECs showed an increased *SREBF1* expression, there by demonstrating the key role of mTORC1 in regulation of this TFs expression in BMECs. Reduction of *SREBF1* expression would have affected the transcription of related *de novo* lipid synthesis genes. Düvel et al. (2010) also reported similar findings in specific TSC2 KO murine cell lines. Han et al. (2018) previously shown that *SREBF1*

expression directly regulates *FASN* expression in BMECs. In our model, hyper-activation of mTORC1 led to a 32% increase in *FASN* expression in TSC2-KO cells compared to NT cells, but the results were non-significant. Taken together, we propose that mTORC1 play a critical role in the control of lipid synthesis, likely through changes in the expression of key lipogenic genes.

The mechanism by which mTORC1 controls lipid synthesis in BMEC remains to be fully elucidated. Although *SREBF1* gene expression was reduced in rapamycin treated cells, its protein abundance remained unchanged. The mechanism most often proposed as an explanation for mTORC1's effects on SREBP1 maturation involves S6K1-mediated phosphorylation, leading its transport into the nucleus where it can influence the transcription of key genes involved in lipid biosynthesis (Porstmann et al., 2008). This explanation has been frequently suggested by other findings (Düvel et al., 2010, Li et al., 2010, Wang et al., 2011). In contrast, our results did not display significant change in SREBP1 maturation in mTORC1 inhibited BMECs. Another potential mechanism of action for mTORC1 to control SREBP1 maturation could be via the phosphorylation of Lipin 1 mechanism as suggested by Peterson et al. (2011). Lipin 1 acts as a transcriptional coactivator. Phosphorylation of Lipin 1, by mTORC1, leads to its exclusion from the nucleus. Accumulation of Lipin 1 in the nucleus promotes the association of SREBPs to the nuclear matrix which affects its ability to bind to target genes. Further investigation should be conducted in a mTORC1 up-regulated model to determine potential implications of Lipin 1 in the SREBP1 maturation mechanism. Also, considering our previous findings in partially inhibited BMECs that suggested that *SREBF1* reduction did not produce significant changes in the SREBP1 maturation status, similar results were expected in our hyper-activated model. This would agree with the suggestion of McFadden and Corl (2010) that nuclear receptor LXR could potentially play a key role in the regulation of SREBP1 maturation. These alternative mechanisms could

potentially compensate for the down regulation of mTORC1 in our model. Thus, we concluded that mTORC1 controls expression of important TF genes involved in *de novo* lipid synthesis in combination with other regulation loops in BMECs as illustrated in Figure 2.6.

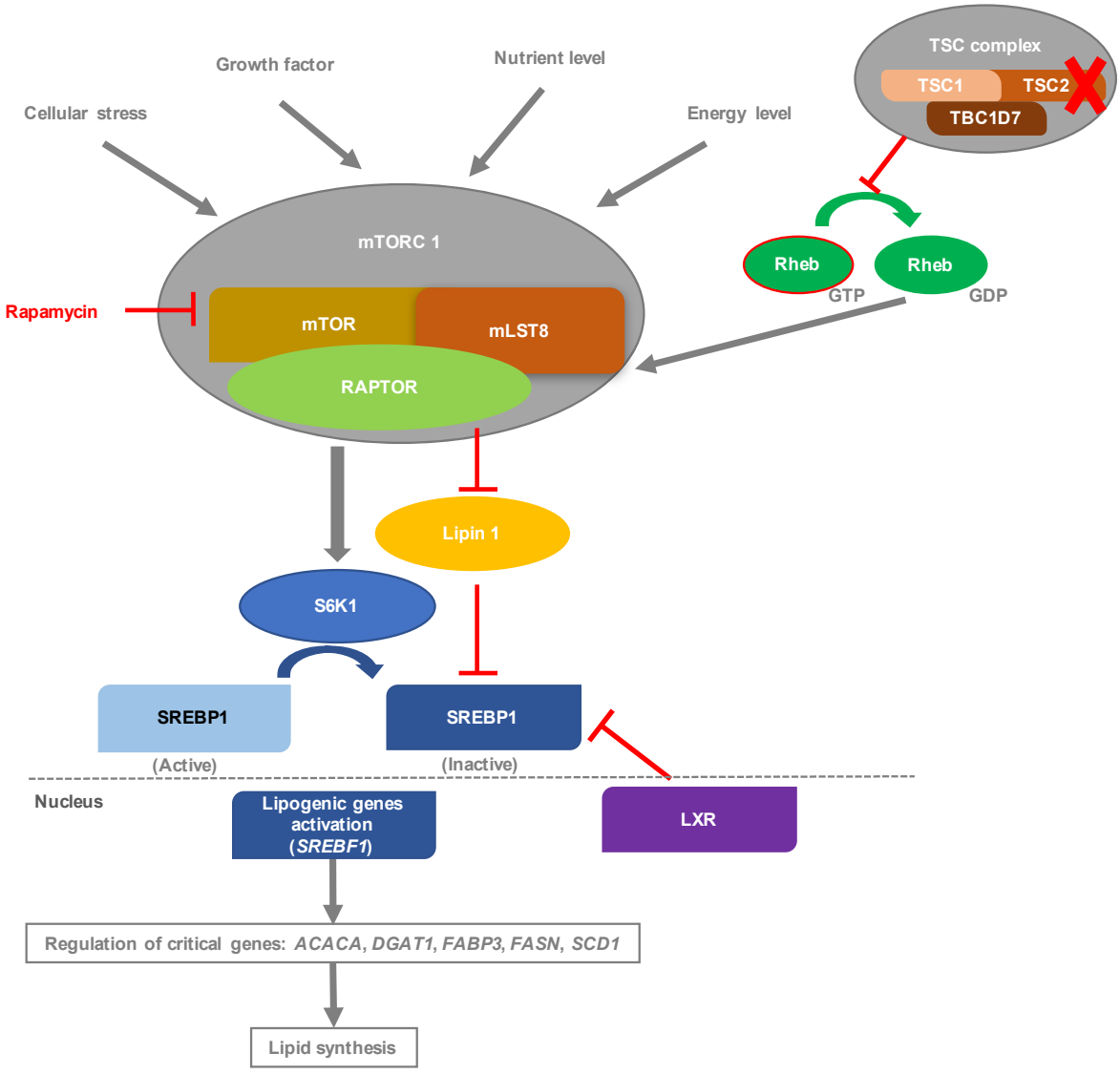


***Figure 2.6**: mTORC1 Mechanisms in Lipid Synthesis in BMECs*

The onset of lactation is marked by the synthesis and secretion of lactose, which results in a dramatic increase in demand for glucose by the mammary gland. For this reason, most prior studies on the regulation of glucose metabolism during lactogenic differentiation have focused on the expression of glucose transporters. Mammary cells are unique in their ability to synthesize lactose, but surprisingly little is known about how this process is regulated in dairy cows. Studies in other cell types have revealed an important role for mTORC1 in the control of glucose metabolism (Bachar et al., 2009, Düvel et al., 2010, Veilleux et al., 2010). Thus, we hypothesized that mTORC1 could be involved in the control of lactose synthesis. In this study, we found that pharmacological inhibition of mTORC1 by rapamycin reduced lactose synthesis. This effect was associated with lower expression of phosphoglucomutase and β-1, 4-galactosyltransferase. However, the expression of the glycolytic genes was not affected by the rapamycin in our study. All *PGFK1*, *PFKFB1* and *G6PD* glycolytic genes remained non-significantly disturbed by mTORC1 partial inhibition. This discrepancy may relate to the fate of glucose in different tissues. While in most tissues glucose is primarily oxidized, 55-70% of glucose flux is directed to lactose synthesis in the BMG (Guinard-Flament et al., 2006).

The mechanism by which mTORC1 controls lactose synthesis has yet to be fully elucidated, but transcriptional regulation through HIF-1α is a likely candidate. The HIF-1α TF has been implicated in the regulation of glucose metabolism downstream of mTORC1 (Düvel et al., 2010). We found that the expression of a marker of HIF-1α transcriptional activity, *VEGF-A*, was reduced by rapamycin in lactogenic differentiated BMECs. This TF has been previously implicated in the regulation of glucose metabolism during lactation. Shao and Zhao (2014) suggested HIF-1α may be implicated in the control of glucose uptake at the onset of lactation. Furthermore, there is a link between hypoxia-associated gene expression and glucose metabolism in mammary gland

during the transition period as described by Mattmiller et al. (2011). In addition to hypoxia, the expression of key genes involved in glycolysis and lactose synthesis as well as lactose content in BMECs is sensitive to glucose availability (Liu et al., 2013, Lin et al., 2016).

As suggested by Liu et al. (2013) the reduction of glucose transport could be directly linked with the regulation of expression of key genes involved in lactose synthesis in BMECs. We found that glucose availability influenced mTORC1 in BMECs, thus, providing a potential pathway by which glucose availability affects lactose synthesis. In addition to mTORC1, other kinases such as protein kinase C could be involved in the regulation of glucose (Zhao et al., 2014). Recently, Lohakare et al. (2018) demonstrated that peroxisome proliferator-activated receptor β/δ (**PPARβ/δ**) does not regulate glucose uptake and lactose synthesis in BMECs letting the mTORC1 signaling pathway to be a potential candidate as we illustrated in Figure 2.7. Their research demonstrated that no effects on glucose uptake or lactose synthesis were detected by modulation of PPARβ/δ activity using MAC-T cell line. These results suggest that PPARβ/δ does not play a major role in glucose uptake and lactose synthesis in BMECs. We suggest that HIF-1α potentially a key role in regulation of lactose via the mTORC1 signaling pathway in BMECs and more research should focus on determining potential mechanisms involved.

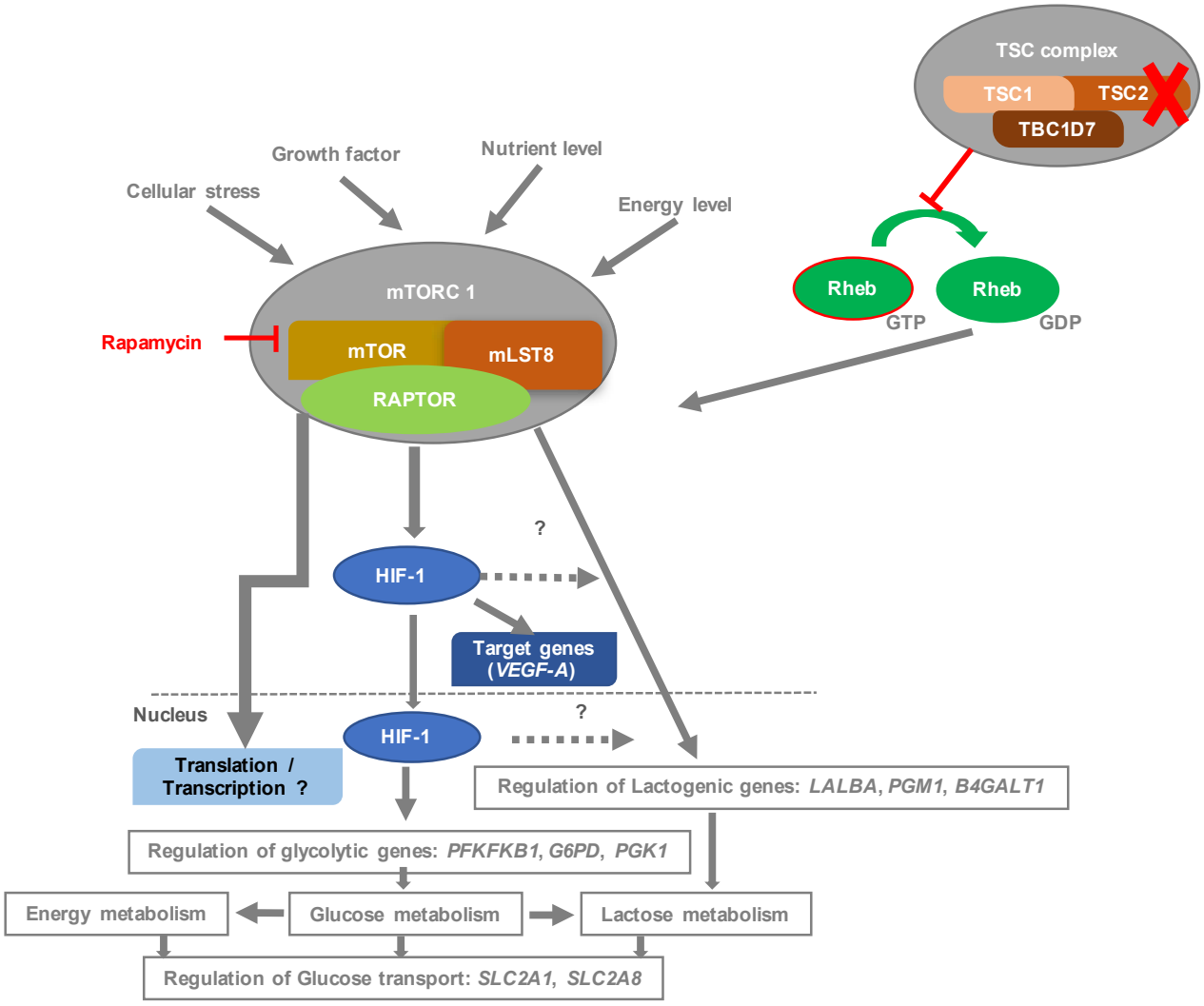


Figure 2.7: *mTORC1 Mechanism in Lactose Synthesis BMECs*

2.6. CONCLUSION

We established and characterized an *in vitro* model to study gene function using lactogenic differentiation of primary BMECs. By combining pharmacological and genetic tool to modulate mTORC1 function in lactogenic differentiated BMECs, we demonstrated a critical role for this signaling pathway in both lipid and lactose synthesis. Specifically, we showed that mTORC1 inhibition by rapamycin led to a decrease in total synthesis of lipid and lactose in BMECs. The rapamycin-induced changes and TSC2-KO alterations in lipid and lactose synthesis were associated with coordinated changes in key gene expression. Our results identified SREBP1 as a target and potential mediator of mTORC1 effects on lipid synthesis, but the exact mechanism has yet to be elucidated. We also established that mTORC1 controls lactose synthesis in BMECs. This effect was associated with changes in the expression of key genes involved in this pathway, but the TF that mediates the effect of mTORC1 on lactose synthesis genes remain to be elucidated. Importantly, we showed the potential of CRISPR-Cas9-mediated KO biotechnology in the study of milk component synthesis in BMECs.

3. APPENDICES

Table 3.1: List of Reagents

Tissue Digestion:

Reagent	Source	Catalog No.
Hyaluronidase from bovine testes, Type 1A	Sigma	H3506
Collagenase, Type 3	Worthington	LS004182
Deoxyribonuclease I, from bovine pancreas (DNAse I)	Sigma	DN25
Hank's Balanced Salt Solution (HBSS) with calcium and magnesium, no phenol red	ThermoFisher Scientific	14025
100 x Antimycotic-Antibiotic	ThermoFisher Scientific	15240-062
50 mg/ml Gentamicin	ThermoFisher Scientific	15750

Cell culture:

Reagent	Source	Catalog No.
Mammary Epithelial Basal Medium MCDB170, powder	US Biological	M2162
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12)	ThermoFisher Scientific	11330
Fetal bovine serum, qualified (FBS)	ThermoFisher Scientific	12483
Oxytocin acetate salt hydrate	Sigma	O6379
10 mg/ml Insulin solution, from bovine pancreas	Sigma	I0516
3,3′,5-Triiodo-L-thyronine sodium salt (T_3)	Sigma	T6397
β-Estradiol	Sigma	E2758
Isoprenaline hydrochloride (Isoproterenol)	Sigma	I5627
Hydrocortisone	Sigma	H0888
apo-Transferrin bovine	Sigma	T1428
Prolactin, from Sheep Pituitary	Sigma	L6520
AlbuMAX II Lipid-rich bovine serum albumin	ThermoFisher Scientific	11021
Distilled water	ThermoFisher Scientific	15230
DMEM without glucose, L-glutamine, phenol red, sodium pyruvate and sodium bicarbonate, powder	Sigma	D5030
D-(+)-Glucose	Sigma	G7021
6 mg/ml Collagen solution, from bovine skin, sterile-filtered	Sigma	C2124
Bovine Serum Albumin, fatty acid-free, low endotoxin,	Sigma	A8806

Pharmacological Agents

Reagent	Source	Catalog No.
Dimethyl Sulfoxide (DMSO)	Sigma	D2650
Rapamycin	BioShop	RAP004.10

RNA Isolation

Reagent	Source	Catalog No.
TRI-Reagent	Sigma	T9424
Chloroform	Sigma	C2432
Isopropanol, Molecular Biology Grade, Fisher BioReagents	Fisher Scientific	BP2618
Anhydrous ethyl alcohol (absolute ethanol)	Commercial Alcohols	P016EAAN

Agarose Bleach Gel

Reagent	Source	Catalog No.
Agarose	Fisher Scientific	BP160
Tris-Borate EDTA buffer 10x	Sigma	T4415
Bleach	Selection	N/A
SYBR™ Safe DNA Gel Stain	ThermoFisher Scientific	S33102
TrackIt Cyan/Orange Loading buffer 6x	ThermoFisher Scientific	10482-028
TrackIt™ 1 Kb Plus DNA Ladder	ThermoFisher Scientific	10488085

cDNA synthesis and RT-qPCR

Reagent	Source	Catalog No.
iScript™ cDNA Synthesis Kit	BioRad Laboratories	1708891
Supermix (2 x) Sso EverGreen	BioRad Laboratories	1725271

Radiochemicals

Reagent	Source	Catalog No.
Acetic acid, Sodium Salt, [^{3}H]	Perkin Elmer	3661
Glucose, D-[^{14}C(U)]	Perkin Elmer	3676

Genomic Cleavage Assay

Reagent	Source	Catalog No.
GeneArt Genomic Cleavage Detection Kit	Thermo Fisher	A24372

Table 3.2: Donor Cow Information

Cow ID	Description	Mammary Quarter Sample	ATQ Number	Experiment used
BMEC-03	Lactating Holstein (< 3rd. parity)	Q3	107 723 452	Lactogenic differentiation and Rapamycin
BMEC-09	Lactating Holstein (< 3rd. parity)	Q1	117 577 997	Lactogenic differentiation and Rapamycin
BMEC-10	Lactating Holstein (< 3rd. parity)	Q2	107 097 043	Rapamycin and TSC2-KO
BMEC-12	Lactating Holstein (>3rd. parity)	Q4	107 327 262	Lactogenic differentiation and Rapamycin and TSC2-KO
BMEC-14	Lactating Holstein (< 3rd. parity)	Q1	107 463 936	Rapamycin and TSC2-KO
BMEC-16	Lactating Holstein (>3rd. parity)	Q3	107 571 786	Lactogenic differentiation and Rapamycin
BMEC-17	Lactating Holstein (< 3rd. parity)	Q4	109 069 292	TSC2-KO

Table 3.3: Genomic Cleavage Assay Primers

Pair	Primer Name	Forward Primer	Position	Reverse Primer	Position	Product size
1	NT-1	CACCACCTACCTTGCTGCTC	F1071	CTCACCTTCTGGATGGAGGA	R1336	265
2	NT-2	CCCAGAGAGCGTCACTCATT	F1602	AATCCTTTGGGAACGAATCC	R2209	607
3	TSC2-1	CGTGGTGTTCTGGTCACATC	F6609	GTGGGAGGAAGAAAGGTCGT	R7098	489
4	TSC2-2	TTCACGGGTTTATTTCTGGC	F6751	TCCTGAGGAAAACCATCACC	R6991	440
5	TSC2-3	CCTGGTTTCAAGAGGTCTGC	F8053	TCTTCCCAACCCAGAGATTG	R8502	449

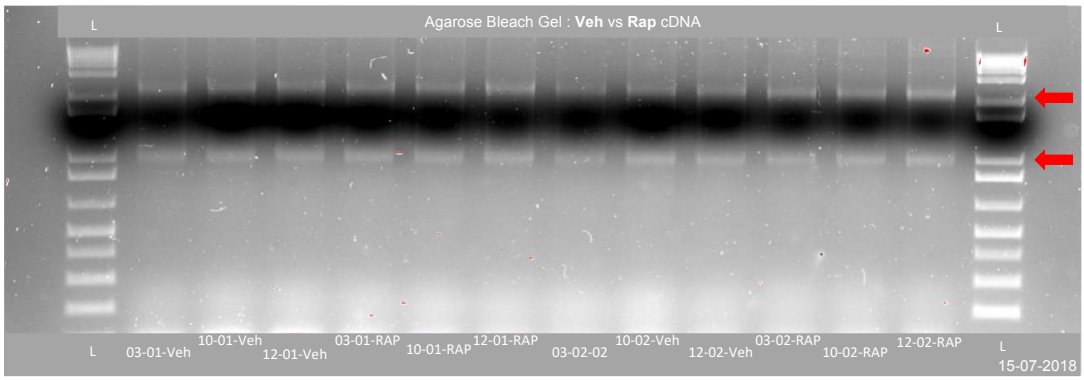
Table 3.4: RT-qPCR Primers Information

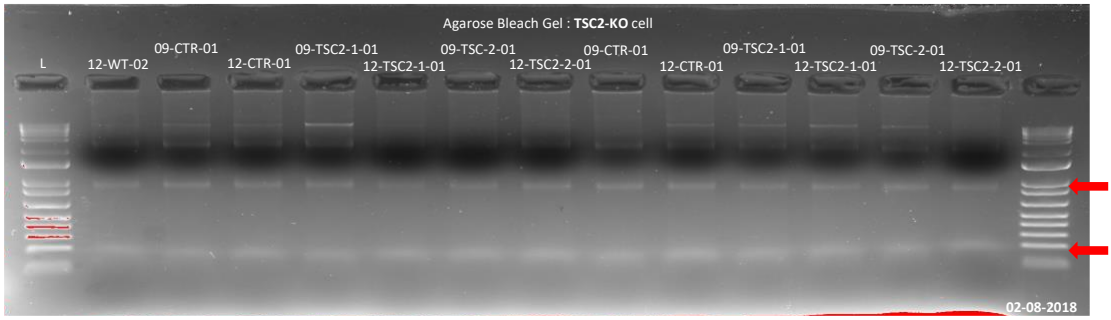
	Gene name	Gene Symbol	Gene ID	mRNA Accession No.	Forward Primer	Reverse Primer	Primer size (Bp)	Start Forward / Reverse	Exon Junction
Reference genes	Actin beta	*ACTB*	280979	NM_173979.3	GACCCAGATCATGTTCGAGA	CTCATAGATGGGCACCGTGT	145	F449 / R593	455 / 456
	Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	281181	NM_001034034.2	GAGGGGCTGCCCAGAATATC	CCAGTGAGCTTCCCGTTGAG	91	F654 / R754	742 / 743
	Peptidylprolyl isomerase A	*PPIA*	281418	NM_178320.2	GGCAAGTCCATCTATGGCGA	GCCATCCAACCACTCAGTCT	150	F234 / R383	373 / 374
Milk protein genes	Casein alpha s1	*CSN1S1*	282208	NM_181029.2	AGTGCTGAGGAACGACTTCA	CCAGGCACCAGATGGATAGG	150	F488 / R637	496 / 497
	Lactalbumin alpha	*LALBA*	281894	NM_174378.2	TTGCCTGAATGGGTCTGTACC	TTCTGGTCGTCTTTGCACCA	131	F151 / R281	160 / 161
Lipogenic genes	Sterol regulatory element binding transcription factor 1	*SREBF1*	539361	NM_001113302.1	CCGAGAGGCTGTACCCATTG	CTGGGTAGGGGTTTCTCGGA	70	F2727 / R2796	2781 / 2782
	Acetyl-CoA carboxylase alpha	*ACACA*	281590	NM_174224.2	CTTCTGTGATTCCCCACCCC	GTTCATCCCTGGGGACCTTG	89	F4105 / R4193	4177 / 4178
	Fatty acid binding protein 3	*FABP3*	281758	NM_174313.2	AGAGACATCACTTGTGCGGG	AGGAGTAGCCCACTGACAGA	148	F349 / R496	-
	Fatty acid synthase	*FASN*	281152	NM_001012669.1	CCGAAGACAGGGATTGTGCT	GGGTTGGGATCTTCCCACTG	122	F2866 / R2987	-
	Diacylglycerol O-acyltransferase 1	*DGAT1*	282609	NM_174693.2	CGAGTACCTGGTGAGCATCC	CCGATGATGAGTGACAGCCA	147	F1261 / R1407	-
	Stearoyl-CoA desaturase (delta-9-desaturase)	*SCD1*	280924	NM_173959.4	CTGGTGAATAGTGCTGCCCA	TGGTGGTAGTTGTGGAAGCC	119	F931 / R1049	-
Glycolytic genes	6-Phosphofructo-2-kinase/frutose-2,6-biphosphatase-1	*PFKFB1*	282304	NM_174572.4	TCGTTAGCTGTGAGGCCCAA	GTAAACTGTGGTATGGATGAGCCC	137	F156 / R292	273 / 274
	Phosphoglycerate kinase 1	*PGK1*	507476	NM_001034299.1	GCTCCTGGAAGGTAAAGTGCT	GTGGAGATGCAGACAGTGCT	118	F1320 / 1437	1330 / 1331
	Glucose-6-phosphate dehydrogenase	*G6PD*	281179	NM_001244135.1	CGCGATGAGAAGGTCAAGGT	GGGGTCATCCAGGTACCCTT	126	F943 / R1068	954 / 955
Glucose transport gene	Solute carrier family 2 member 1	*SLC2A1*	282356	NM_174602.2	CGTGCTCCTGGTTCTGTTCT	GGAACAGCTCCTCAGGTGTC	137	F1484 / R1620	-
HIF-1α marker gene	Vascular endothelial growth factor A	*VEGF-A*	281572	NM_001316992.1	GCCCACTGAGGAGTTCAACA	CCCACAGGGATTTTCTTGCC	148	F1329 / R1476	-
Lactogenic genes	Phosphoglucomutase 1	*PGM1*	534402	NM_001076903.1	CTCCACGCCTGCTGTATCAT	GGCAGGACCTCCATTGGAAA	136	F381 / R516	508 / 508
	Beta-1-4-galactosyltransferase 1	*B4GALT1*	281781	NM_177512.2	TCTCGCCCAAATGCTGTGAT	GTGCAATTCGGTCAAACCTCTG	97	F1180 / R1276	373 / 374

Table 3.5: Antibodies Information

Antibodies	**Source**	**Catalog No.**
Phospho-tuberin/TSC2 (Ser1387) antibody	Cell Signaling Technology	5584
α-Tubulin (11H10) rabbit monoclonal antibody	Cell Signaling Technology	2125
GAPDH (D16H11) XP® rabbit monoclonal antibody	Cell Signaling Technology	5174
Phospho-mTOR (Ser2448) rabbit antibody	Cell Signaling Technology	2971L
P-4E-BP1 (S65) rabbit monoclonal antibody	Cell Signaling Technology	9451S
P-4E-BP1 (T37/46) (236B4) rabbit monoclonal antibody	Cell Signaling Technology	2855S
S6 ribosomal protein (54D2) Mouse monoclonal antibody	Cell Signaling Technology	2317S
P-S6 ribosomal protein (S240/244) XP® rabbit monoclonal antibody	Cell Signaling Technology	5364S
P-S6 ribosomal protein (S235/236) (D57.2.2E) rabbit monoclonal antibody	Cell Signaling Technology	4858S
P-p70 S6 Kinase (T389) (108D2) rabbit monoclonal antibody	Cell Signaling Technology	9234S
P-AMPK alpha (T172)(40H9) rabbit monoclonal antibody	Cell Signaling Technology	2535S
SREBP1 (2A4) mouse monoclonal antibody	Thermo Fisher Scientific	MA5-11685
p44/42 MAPK (Erk1/2) Rabbit Antibody	Cell Signaling Technology	9102
Phospho-AKT (S473) rabbit antibody	Cell Signaling Technology	9271L
Tuberin/TSC2 (D93F12) XP rabbit monoclonal antibody	Cell Signaling Technology	4308

A)

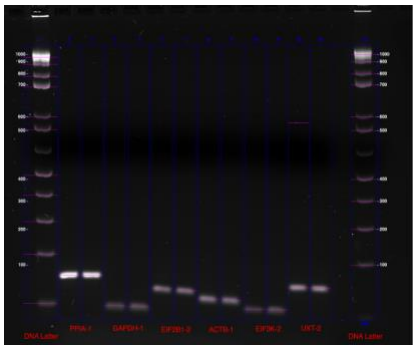




GAPDH ***ACTB*** ***PPIA*** ***DGAT1*** ***FABP3*** ***ACACA*** ***FASN***

CSN1S1 ***LALBA*** ***SLC2A1*** ***VEGF-A*** ***PGM1*** ***B4GALT1*** ***SCD1***

A)



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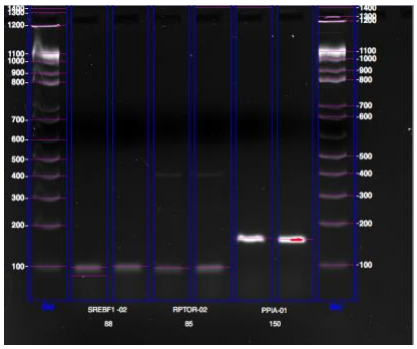

C)

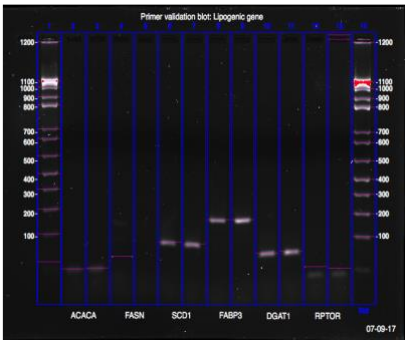

Figure 3.2: RT-qPCR Validation Information. Amplicon length was measure for each primer used during experiments. Amplicon length of reference genes: *PPIA, GAPDH, EIF2B1, ACTB, EIF3K, UXT* (**A**), target genes: *SREBP1, RPTOR, PPIA* (**B**) and lipogenic genes *ACACA, FASN, SCD1, FABP3, DGAT1, RPTOR2* (**C**) were measure using ChemiDoc™ imaging system.

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