Biosynthesis of milk fat, protein, and lactose: roles of transcriptional and posttranscriptional regulation

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Osorio JS, Lohakare J, Bionaz M. Biosynthesis of milk fat, protein, and lactose: roles of transcriptional and posttranscriptional regulation. Physiol Genomics 48: 231–256, 2016. First published January 26, 2016; doi:10.1152/physiolgenomics.00016.2015.—The demand for high-quality milk is increasing worldwide. The efficiency of milk synthesis can be improved by taking advantage of the accumulated knowledge of the transcriptional and posttranscriptional regulation of genes coding for proteins involved in the synthesis of fat, protein, and lactose in the mammary gland. Research in this area is relatively new, but data accumulated in the last 10 years provide a relatively clear picture. Milk fat synthesis appears to be regulated, at least in bovines, by an interactive network between SREBP1, PPARγ, and LXRα, with a potential role for other transcription factors, such as Spot14, ChREBP, and Sp1. Milk protein synthesis is highly regulated by insulin, amino acids, and amino acid transporters via transcriptional and posttranscriptional routes, with the insulin-mTOR pathway playing a central role. The transcriptional regulation of lactose synthesis is still poorly understood, but it is clear that glucose transporters play an important role. They can also cooperatively interact with amino acid transporters and the mTOR pathway. Recent data indicate the possibility of nutrigenomic interventions to increase milk fat synthesis by feeding long-chain fatty acids and milk protein synthesis by feeding amino acids. We propose a transcriptional network model to account for all available findings. This model encompasses a complex network of proteins that control milk synthesis with a cross talk between milk fat, protein, and lactose regulation, with mTOR functioning as a central hub.

milk synthesis; transcription regulation; dairy cows; mTOR pathway
IMPORTANCE OF TRANSCRIPTOMICS IN IMPROVING MILK SYNTHESIS

Transcriptomics Technologies to Understand Milk Production

The discovery of DNA and its role in biology gave rise to the genomics era. The invention and improvement of quantitative polymerase chain reaction (qPCR) (152, 153, 193), followed by the discovery of the synthesis of complementary DNA (cDNA) from mRNA using a reverse-transcriptase (RT) (228), was essential to study expression of mRNA, usually present in cells in extremely low amounts. The major limitation with RT-qPCR is the relatively high cost (several $/gene per sample in terms of reagents and time) in obtaining information about the expression of relatively few genes for any given sample. In contrast to RT-qPCR, microarray and RNA sequencing technology were developed with the capacity to measure from a few thousand transcripts to the whole transcriptome (131, 132). Unlike RT-qPCR, microarray and RNA sequencing have a higher overall cost per sample, but the cost per each gene per each sample is in the order of a few cents (>1,000-fold less compared with RT-qPCR).

The use of large transcriptomic analysis (i.e., microarray or RNA sequencing) by which to study the adaptation of mammary gland to lactation and/or change in milk production under specific conditions has been carried out in mouse (4, 54, 122, 123, 143, 189, 191, 217, 236, 256), rat (3, 47, 187), bovine (31, 36, 56, 75, 96, 144, 183, 209, 218, 233, 239), sheep (209), goat (71, 163, 164), human (121, 139, 147), pig (204), kangaroo (120), and seal (144). It is beyond the scope of the current review to present a thorough discussion of the results from those studies. However, a common feature in the aforementioned studies was the extreme change in the transcriptome from pregnancy to lactation, indicating the large importance of transcriptomic changes in the mammary gland to initiate and maintain milk synthesis. Consequently, a change in the transcription of genes coding for proteins directly or indirectly related to the synthesis of milk components has been posited as one of the major driving factors in maximizing the efficiency of milk synthesis in the mammary gland. For this reason, a detailed understanding of the transcriptional and posttranscriptional regulation of genes coding for main proteins involved in the synthesis of milk constituents can provide a way to control and improve yield and quality of milk.

Main Factors Controlling Transcription of Genes

The transcriptome is tightly controlled by several factors to maintain homeostasis and facilitate a proper response to environmental challenges. The long-term effect on gene expression is controlled by epigenetic factors, whereas short- to mid-term effects are controlled by transcription factors (TF) (1, 63, 213). The TF are proteins that, when activated by hormones, nutritional molecules, or other factors, are able to recognize specific regions of the DNA referred as the response element (RE) and trigger all events necessary for enhancing or inhibiting the expression of target genes (213). The ligand-dependent nuclear receptors (LdNR) are TF-present in the nucleus (or possess the capability to enter the nucleus upon activation) and can be activated by specific exogenous or endogenous ligands, especially food-derived compounds (42).

Moreover, LdNR are central for nutrigenomics, where protein abundance of the LdNR can govern the sensitivity of the cell to the specific food-derived agonists. However, their activity is primarily determined by the abundance and potency of the agonist(s) (42).

REGULATION OF MILK FAT SYNTHESIS

Process of Milk Fat Synthesis: What We Know Today

The synthesis of milk fat has been extensively studied in the second half of the 20th century (15), where major metabolic aspects of mammary lipid metabolism, including de novo synthesis and FA uptake from blood, were defined and quantified. The advent of RT-qPCR was an essential step toward a better understanding of the transcriptional control of milk fat synthesis. This was exemplified by a study where the expression of several genes thought to be involved in all steps of milk fat synthesis was measured with high precision in mammary tissue of dairy cows from pregnancy to the end of subsequent lactation (29). The knowledge produced in the last 50 yr, in addition to the data from that study, informed the rationale for proposing an all-encompassing model (Fig. 1).

Transcription Factors and the Control of Milk Fat Synthesis

Milk fat depression and the role of sterol regulatory element-binding transcription protein 1. The milk fat depression phenomenon in dairy cows has been known since the beginning of 20th century. It was originally observed by Powell in 1938 (175) and confirmed afterward in many studies (13, 226). Since the beginning, it was evident that high-grain, low-roughage diet in high-producing dairy cows substantially decreases milk fat percentage. Several theories were put forward to explain such phenomenon, as reviewed by Van Soest et al. in 1963 (226). Among the most important theories were the decreased availability of acetate for the de novo FA synthesis in the mammary gland, the hypoketogenic effect of high propionate being β-hydroxybutyrate (BHBA) used as a precursor of de novo FA synthesis, and the endocrinological control of fat mobilization with the evident competition between milk fat synthesis and accumulation of fat in adipose tissue. In the same review, Van Soest and coworkers point out that these theories, however, were unable to fully explain the milk fat depression phenomenon. Furthermore, Van Soest et al. also suggest that the intermediate products of the oxidative-reductive activity of the rumen might play a role.

A combination of high fat in the diet of ruminants, particularly containing polyunsaturated LCFA (PUFA), with high grain (i.e., increased rumen fermentation with consequent high biohydrogenation of PUFA) is conducive to milk fat depression (38). Milk fat depression was originally explained as being a function of an inhibitor effect of PUFA on the rumen bacteria responsible for the production of acetic acid from fiber fermentation (137). It was also very well known that such a condition was accompanied by an increase of CLA and t10-monoenoioc acid in milk fat. The existence of a link between CLA and milk fat depression was demonstrated more than 10 yr ago by the abomasal infusion of a supraphysiological dose of t10,c12-CLA (17) and has been confirmed multiple times in bovine (reviewed in Ref. 16) and other species, including sheep (101, 129, 207), goat (9, 73, 79, 80), mouse (91, 110), and rat (92, 184).
Fig. 1. Milk fat, protein, and lactose synthesis in mammary cells. Milk fat synthesis: long-chain fatty acids (LCFA), short-chain fatty acids (SCFA; prevalently acetic acid), and ketone bodies (prevalently β-hydroxybutyrate or BHBA) are actively or passively taken up by the mammary gland. Very-low-density lipoprotein receptor (VLDLR) cooperates with lipoprotein lipase (LPL) to hydrolyze the triacylglycerol (TAG) and free the LCFA from VLDL. Once the LCFA are freely present in the extracellular milieu, they are actively taken up and activated (i.e., adding a coenzyme A) by a coordinate activity between the fatty acid translocase (CD36), the fatty acid transporter (SLC27A), and the acetyl-CoA synthetase (ACSL, likely the isoform ACSL1). The LCFA that enter the cells through passive diffusion (or flip-flop) are either activated or readily bound to fatty acid binding proteins (FABP), whereupon they are transported to the active metabolic enzymes. Most of the acetic acid and a small part of BHBA are used as precursors for de novo FA synthesis. The de novo FA synthesis is carried out in the cytosol through the activity of fatty acid synthase (FASN) and acetyl-CoA carboxylase alpha (ACACA) after activation by acyl-CoA synthetase (ACSS), at least for the acetic acid. The outputs of the de novo FA synthesis are both medium-chain FA (MCFA) and LCFA. These are immediately activated by an ACSL in order to enter the TAG synthesis pathway. The TAG synthesis is carried out in the smooth endoplasmic reticulum membrane, partly facing the cytosol and partly the lumen. One of the initial steps of a large amount of LCFA (mostly saturated) is the unsaturation prevalently by Δ9 stearoyl-CoA desaturase (SCD).

The actual first step of the TAG synthesis is the activation of the LCFA through addition of a phosphate group by a glycerol-3-phosphate acyltransferase (GPAM) forming a lysophosphatic acid that becomes the substrate for the 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT), forming a phosphatidic acid. The latter is then dephosphorylated by a phosphatidate phosphatase (lipin or LPIN, likely the isoform LPIN1), forming a 1,2-diacyl-sn-glycerol. To this, a 3rd LCFA is added by a diacylglycerol O-acyltransferase (DGAT1) with the formation of a molecule of TAG. The TAG is directly formed between the 2 leaflets of the endoplasmic reticulum membrane with an important role of adipophilin (or perilipin 2; PLIN2) for the formation of the lipid droplet. As a result of the interaction between the xanthine dehydrogenase (XDH), the PLIN2, and the butyrophilin (BTN1A1), active exocytosis of the lipid droplet into the lumen of the alveolus occurs. Lastly, the lipid droplet surrounded by the apical membrane containing cytosolic factors is secreted. Protein synthesis: amino acids are imported into the cytosol. The insulin signaling is required for the activation of mTOR pathway and enhanced by amino acids, such as Leu. The regulatory mechanism of mTOR is primarily posttranscriptional via phosphorylation of intermediate regulatory proteins, which ultimately results in the activation of the ribosomes located in the membrane of the rough endoplasmic reticulum (RER) to initiate translation of mRNA into nascent proteins that are secreted in the RER lumen. Newly formed proteins are shuttled to the Golgi apparatus by vesicular transport, where further posttranslational modifications are applied to the new proteins. The mature proteins are packed within exocytotic vesicles within the Golgi apparatus and transported toward the apical membrane, where they are secreted in the milk. Lactose synthesis: glucose after absorption by the basolateral membrane of mammary epithelial cells is phosphorylated to glucose-6-phosphate and then joined with uridine-di-phosphate (UDP) to form UDP-glucose. The UDP-glucose is then converted to UDP-galactose by the UDP-glucose pyrophosphorylase 2 (UGP2) and phosphoglucomutase 1 (PGM1) in the cytoplasm and enters the Golgi, through the UDP-galactose transporter 2 (SLC35A2), where the lactose is synthesized by combining one molecule of UDP-galactose with one of glucose by lactose synthase composed by β1,4-galactosyltransferase 1 (B4GALT1) and alpha-lactalbumin (LALBA). Protein structures, when available, are from ModBase http://modbase.compbio.ucsf.edu.
Sterol regulatory element-binding transcription protein 1 (SREBP1, gene name SREBF1) is a TF that plays a central role in the regulation of cholesterol biosynthesis and FA metabolism, particularly the biosynthesis of fat (63). Originally isolated in human liver cells (237), SREBP1 resides as inactive precursor on the endoplasmic reticulum membrane and is transported to the Golgi for proteolytic cleavage (i.e., activation) prior to entering the nucleus where it activates expression of sterol response element (SRE)-containing genes.

In monogastrics, unsaturated LCFA and CLA inhibit the proteolytic cleavage of SREBP1 negatively affecting its activity, as observed in vitro with c9,t11-CLA in human embryonic kidney cells (87) and in vivo with unsaturated LCFA in mice (186). The same negative effect on proteolytic cleavage of SREBP1 was demonstrated in bovine mammary cells for one of the products of rumen biohydrogenation of PUFA, the t10,c12-CLA, revealing the molecular mechanism of milk fat depression (169). It was observed that the decreased activity of SREBP1 was associated with decreased expression of key genes involved in de novo FA synthesis and LCFA desaturation, such as fatty acid synthase (FASN), acetyl-CoA carboxylase alpha (ACACA), and stearoyl-CoA desaturase (SCD). It was demonstrated in the same study that the promoter region of bovine FASN and SCD contains an SRE. It was later demonstrated that exogenous t10,c12-CLA reduces the SREBF1 expression in bovine mammary tissue (89), a fact that was not observed in the original in vitro study (169). The decrease in SREBF1 expression was explained by the fact that the SREBF1 gene contains an SRE in its promoter region (89), indicating that SREBP1 has the ability to regulate its own transcription. As reviewed recently, further studies have confirmed a central role of SREBP1 in milk fat depression (14).

Overall, the data from the above studies indicate that the effect of CLA on SREBP1 activity occurs mostly at the posttranscriptional level due to CLA inhibiting the proteolytic cleavage of SREBP1 (169), which, in turn, decreases the expression of its own gene SREBF1 and related target genes involved in de novo FA synthesis.

SREBP1 and the regulation of milk fat synthesis. The studies conducted on milk fat depression reveal only indirectly the essential role of SREBP1 in regulating milk fat synthesis, and none of the studies demonstrate a direct effect. That is, the decrease in activity and expression of SREBP1 could have been concomitant and unrelated to the decrease in expression of genes related to milk fat synthesis. A more direct demonstration of a key role of SREBP1 in controlling the expression of milk fat-related genes was recently provided by a series of experiments by suppressing expression of SREBF1 in bovine mammary epithelial cells (i.e., siRNA technology) (134, 165). In addition, overexpression of SREBF1 in cultured bovine and goat mammary epithelial cells increased the expression of several genes related to milk fat synthesis and increased secretion of triacylglycerol (TAG) (125).

There are, however, data indicating a relatively limited role of SREBP1 in regulation of milk fat synthesis in vivo. For instance, in bovine mammary tissue expression of INSIG1 (insulin induced gene) increased >12-fold from pregnancy to lactation, whereas the expression of SREBF1 only increased twofold (29). The major function of INSIG1 protein is to retain the immature SREBP1 in the endoplasmic reticulum, thus inhibiting its function. In mouse mammary tissue, the increase in expression of Insig1 during lactation was >40-fold (85), whereas Srebf1 increased approximately twofold (191). As previously discussed (29), the large increase in abundance of INSIG1 protein should decrease the cleavage and thus the activity of SREBP1, suggesting that the activity of SREBP1 is strictly controlled and maybe even partially inhibited in the lactating mammary gland.

Mechanistic target of rapamycin and SREBP1. The activity of SREBP1 in bovine mammary is also potentially under the control of the mechanistic target of rapamycin (mTOR) (173, 174). In mouse, it was demonstrated that mTOR regulates SREBP1 activity by controlling the nuclear entry of Lipin 1 via phosphorylation (170). Unphosphorylated Lipin 1 enters into the nucleus and, through a still unknown mechanism, inhibits the activity of the mature SREBP1, reducing lipogenesis. However, when the Lipin 1 is phosphorylated by mTOR, the entry of Lipin 1 into the nucleus is blocked, avoiding the inhibition of SREBP1 (170). It is interesting that Lipin 1 is a target gene of the peroxisome proliferator-activated receptors (PPAR) in monogastrics and in bovine (26). Consequently, activation of PPAR could inhibit SREBP1 activity through Lipin 1 gene upregulation in the absence of mTOR activity. Data from a recent study on bovine mammary cells indicated a potential negative effect of SREBP1 on PPAR but also indicated a role for SREBP1 in controlling the expression of MTOR (125). In that study, overexpression of SREBF1 in bovine mammary epithelial cells increased expression of MTOR and synthesis of TAG but decreased the expression of the PPARγ gene (gene symbol PPARG). The opposite was observed when SREBF1 expression was inhibited. The results of that study are not supported by other studies in which the expression of PPARγ was not affected by the decrease in expression of SREBF1, for instance during milk fat depression or t10,c12-CLA treatment (108, 231).

Is SREBP1 amenable to nutrigenomic approaches? SREBP1 is not known to be directly activated by any nutritional molecule, including LCFA. In contrast, inhibition of this TF by t10,c12-CLA with consequent decrease in expression of SREBP1-target genes (including SREBF1) has been clearly demonstrated. Thus, nutrigenomic approaches involving SREBP1 can be proposed as indirect rather than direct. Besides the inhibition by PUFA, saturated LCFA also can affect the activity of SREBP1. This was recently demonstrated in bovine mammary cells where stearic acid treatment increased the expression of PPAR activation, as observed in bovine mammary tissue (26). The authors did not provide any mechanistic explanation for such effect. However, stearic acid is a (potent) PPAR agonist in ruminants (26). Thus, the observed increase in the expression of SREBF1 is potentially a consequence of PPAR activation, as observed in bovine mammary cells (108). Unfortunately, the discovery of an essential role of SREBP1 in milk fat synthesis has not yet produced any practical application. The only conclusion from the discovery of the SREBP1 role in milk fat depression is that the synthesis of milk fat can only be “preserved” at the transcriptomic level by providing a diet that minimizes the production of CLA. The inability of SREBP1 to bind and to be directly activated by nutritional molecules suggests that this TF may not have any nutrigenomic applications. One application might be the repartitioning of energy for milk synthesis. It has been suggested...
and demonstrated in lactating sheep and cows that the t10,c12-CLA can help improve negative energy balance (61, 196, 238).

In summary, the scientific evidence supporting a role of SREBP1 in controlling milk fat synthesis is overwhelming. It remains to be determined if the SREBP1 is only essential to maintain milk fat synthesis or if it is amenable to manipulation in order to augment its expression or activity to increase milk fat synthesis through dietary approaches. It is unclear what upstream regulators can control the expression of SREBF1. For instance, the regulatory factor(s) involved in controlling the increase in SREBF1 expression during onset of lactation in mammary tissue of bovine (29) and mouse (4, 191) are still unknown. However, a role for prolactin and serine/threonine protein kinase AKT1 in controlling the expression of SREBF1 was suggested (4). Additionally, the energy level in the diet may affect expression of SREBF1 as indicated by a recent study in mouse (68).

**LCFA-activated TF and Milk Fat Synthesis**

The LCFA can differentially affect milk fat synthesis, with saturated LCFA increasing synthesis and unsaturated LCFA mostly decreasing it [e.g., (52, 215, 216)]. The increase in milk fat synthesis by LCFA is partly due to the augmented availability of LCFA precursors for the TAG synthesis in mammary tissue and possibly the regulation of gene expression involved in milk fat synthesis through the activation of TF (30). As discussed earlier, the SREBP1 cannot be considered the TF targeted directly by LCFA to increase milk fat synthesis. For this reason, other TF able to bind and be activated by LCFA are likely to be responsible for the increase in milk fat synthesis by LCFA.

To date, very few TF are known to be activated by LCFA or their metabolic derivatives. Among those, the most important are the three PPAR isotypes, α, β/δ, and γ (63, 115), and the liver X receptor (LXR) isotypes, α and β. PPAR and LXR belong to the ligand-dependent nuclear receptor superfamily (114). Among these, the PPAR, and especially PPARα and PPARγ, have been well studied in monogastrics and are known to play a pivotal role in controlling several biological functions, especially lipid metabolism, which is also apparent in ruminants (26). In rodents and humans, the activation of PPAR by LCFA was greater compared with other tested nuclear receptors, such as the LXR and retinoic X receptor (227).

**Role of LXR.** The two LXR isotypes, LXRα (gene symbol NR1H3) and LXRβ (NR1H2), are activated by oxysterols and other derivatives of cholesterol metabolism and regulate important aspects of cholesterol and FA metabolism primarily by controlling the expression of SREBF1 and SREBF2 (63). In monogastrics, NR1H3 is highly expressed in the liver, and NR1H2 is ubiquitously expressed (63). The same pattern of expression between isoforms was observed in bovine as well, with the mammary tissue having a relatively low expression among several tested tissues (90). Both LXR isotypes are known to control the expression of SREBF1 in nonruminants (203). This has been also confirmed for LXRα in bovine mammary cells in several independent studies (90, 141). The in vitro activation of LXRα by a synthetic agonist in bovine mammary cells increased expression of SREBF1 and FASN and led to an overall increase in de novo FA synthesis (141) and lipogenesis (90). Interestingly, in bovine mammary epithelial cells LXRα is not essential for basal expression of SREBF1, but activation of this TF increases the expression of SREBF1 (90, 165). The effect of LXRα on mammary lipogenesis is not strictly via SREBF1 expression because activation of LXRα in bovine mammary cells increased expression of other genes related to milk fat synthesis independently of SREBF1 (165); this was also confirmed in monogastrics (reviewed in Ref. 20).

Oxysterols are oxygenated metabolites of cholesterol and the main natural agonist of LXR (20). In monogastrics, LXR can bind and be activated by LCFA; however, the activation by LCFA can be considered modest if not negligible, particularly compared with PPAR isotypes, at least in monogastrics (227). In liver tissue of monogastrics, LXR activity is inhibited by several unsaturated LCFA, especially t10,c12-CLA and linoleic acid (55), and by very-long-chain PUFA. However, the inhibitor effect of PUFA on monogastrics LXR activity is currently being debated because the above findings were not confirmed by subsequent studies (reviewed in Ref. 67). In bovine mammary epithelial cells, LXRα does not respond to t10,c12-CLA (90, 238). Consequently, LXRα does not participate in t10,c12-CLA transcriptional inhibition in bovine (90).

Overall, the data suggest some role of LXR in controlling milk fat synthesis, but the role appears relatively minor. From our perspective, the most interesting findings about LXRα in bovine mammary tissue/cells pertain to the transcriptional upregulation of milk fat synthesis-related genes independent of SREBP1 (165) and the capacity of increasing expression of SREBF1 upon activation. The latter might be a way to exploit a nutrigenomic effect by upregulating SREBF1 expression via activation of LXR in order to increase milk fat synthesis. However, it would be essential to test if bovine LXR can bind and be activated by LCFA.

**Role of PPARα.** The role of bovine PPARα in controlling expression of genes involved in milk fat synthesis and its activation by several LCFA in mammary cells was previously reviewed (26). It is, however, noteworthy that the expression of PPARG in bovine mammary tissue/cells pertain to the transcriptional upregulation of milk fat synthesis-related genes independent of SREBP1 (165) and the capacity of increasing expression of SREBF1 upon activation. The latter might be a way to exploit a nutrigenomic effect by upregulating SREBF1 expression via activation of LXR in order to increase milk fat synthesis. However, it would be essential to test if bovine LXR can bind and be activated by LCFA.
albumin, although more similar to the in vivo situation, can be problematic because BSA tends to retain higher affinity for the remaining fatty acids (212), making them less available to the cells. It is, however, interesting that Jacobs and collaborators were not able to detect the expression of PPARγ in MAC-T cells. Although they concluded that the lack of effect of palmitate and stearate might be due to the absence of PPARγ, the expression of PPARγ was previously detected, although at low level, in MAC-T cells by Kadegowda and collaborators (108). The difference in the detection of PPARγ expression in the two studies may be due to a difference in RT-qPCR sensitivity but is more likely a result of an episodic difference in MAC-T cell batches used.

The proposed role of PPARγ in controlling milk fat synthesis has raised some concerns that have been debated in previous publications (14, 26). The primary concern pertains to the absence of PPARγ role in milk fat depression (14). The absence of a role of bovine PPARγ on milk fat depression, especially a lack of change in its activity or expression due to t10,c12-CLA treatment, is not surprising. As previously demonstrated, PPARγ activity in bovine mammary epithelial cells is not affected by t10,c12-CLA (30, 108). This is contrary to monogastric’s cells where t10,c12-CLA is an activator of PPARα and γ (149, 249). The activation of PPARα by t10,c12-CLA in monogastrics is consistently observed, but not always for PPARγ (12, 81). An activation of PPARα in bovine by t10,c12-CLA was also indicated by a modest, although statistically significant, increase in expression of carnitine palmitoyl transferase 1A (CPT1A) by augmented doses of a mixture of CLA in immortalized bovine kidney cells (25). The lack of bovine PPARγ activation by t10,c12-CLA is supported by recent data obtained in goat and bovine mammary epithelial cells (243, 255). The difference in the response of PPARγ to t10,c12-CLA is likely due to a different structural conformation of the ligand pocket of the PPAR between cow and mouse, as observed for PPARα (26). Although t10,c12-CLA activates PPARα and γ in monogastrics, a downregulation would be expected in activity and/or expression of these PPAR isoforms if they are in fact involved in the milk fat depression condition associated with t10,c12-CLA.

Despite the demonstrated inability of the t10,c12-CLA to regulate the activity of bovine PPARγ, if this TF is involved in milk fat depression, the activation of PPARα by synthetic agonists should attenuate the decrease in milk fat synthesis. This was not the case in mouse (232), where treatment with rosiglitazone failed to prevent CLA-induced milk fat depression in lactating animals. The authors concluded that PPARγ has a zero-to-negligible role in controlling milk fat synthesis. These conclusions are supported by the large decrease in expression of PPARα from pregnancy to lactation in mammary tissue of mouse (70) and human (147) but more convincingly by the demonstrated lack of transcriptional response to rosiglitazone treatment in mouse immortalized mammary epithelial cells (26). A study conducted in the mouse indicated that the deletion of a PPARγ RE in the promoter region of phosphoenolpyruvate carboxykinase (Pck) reduced milk TAG (99), demonstrating that PPARγ plays a crucial role in mouse mammary tissue. However, that effect was likely due to the crucial glyceroenogenic role Pck plays in the white adipose tissue of the mammary gland (98).

Overall, the data reviewed above indicate that the role of PPARγ in milk fat synthesis might be ruminant specific. Furthermore, if PPARγ really plays an important role in controlling milk fat synthesis in ruminants, then using mouse as a model to study milk fat synthesis in ruminants is problematic. To provide support for the lack of effect of PPARγ activation in preventing milk fat depression, the same experiment carried out in mouse (232) should be performed in cows or goats. It can also be argued that the role of PPARγ in controlling expression of milk fat synthesis-related genes in ruminants is SREBP1 dependent, as suggested by the positive effect of PPARγ activation on the expression of SREBF1 (125). Because PPARγ only works at a transcriptional level, the decrease in milk fat synthesis by t10,c12-CLA, which physically impedes the activation of SREBP1, might not be recapitulated by the activation of PPARγ.

The findings from studies carried out to date provide support for a role of PPARγ in regulating expression of genes coding for proteins involved in milk fat synthesis in ruminants. However, a conclusive demonstration has not been provided yet. Considering the nutrigenomics potential of PPARγ, a conclusive demonstration of PPARγ role in milk fat synthesis would be pivotal (26).

Other TF

Spot14. It has been demonstrated that the thyroid hormone responsive protein or THRSP (i.e., Spot14) has a role in controlling lipid metabolism with respect to monogastrics. Its activity is modulated by the thyroid hormone, glucose, and PUFA (reviewed in Ref. 58). A role for Spot14 in controlling the expression of genes related to milk fat synthesis in bovine is emerging. Spot14 expression is decreased in bovine mammary tissue during milk fat depression and in bovine mammary epithelial cells after treatment with t10,c12-CLA (14, 89). Very recently, it was demonstrated in bovine mammary epithelial cells that the overexpression of Spot14 increases TAG synthesis and the expression of FASN, SREBF1, and PPARγ (57). Opposite results were obtained from the inhibition of Spot14 expression (57). It remains to be determined whether Spot14 is able to bind directly to the promoter region of any milk-fat related genes, including SREBF1 and PPARγ, and if it can bind and be directly activated or inhibited by nutrients, including LCFA. In the mouse, the role of Spot14 in mammary tissue is not as clear. In Spot14-null and Spot14-overexpressing mice, it was observed that only a decrease of milk fat synthesis in the former and an increase in the proportion of medium-chain FA in milk TAG in the latter (but no change in total milk fat) occurred. In addition, the main finding was that there was increased activity of fatty acid synthase without any effect on expression of proteins involved in milk fat synthesis (57).

Carbohydrate-responsive element-binding protein. The carbohydrate-responsive element-binding protein (ChREBP, gene symbol MLXIPL) is a TF able to respond to glucose and is involved in both regulation of glucose metabolism and de novo FA synthesis in liver of monogastrics (74). Its main agonist is glucose-6-phosphate, but its activity is also stimulated by high intracellular Ca2+ (74). A role for ChREBP in milk fat synthesis was originally proposed in a study carried out in the mouse (109). This was confirmed more recently in bovine mammary epithelial cells where downregulation of MLXIPL in

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response to $t_{10,c12}$-CLA treatment was observed (90). However, because of its low expression in bovine mammary epithelial cells and the absence of its expression in purified murine mammary epithelial cells (192), it was suggested that ChREBP role in mammary tissue might be minimal. However, as indicated above, the importance of a TF may be independent from its expression level and may rely more on the presence/absence of its agonist(s). Therefore, a clear role for ChREBP in milk fat synthesis still needs to be established. The fact that ChREBP is activated by glucose-6-phosphate and can control FA synthesis makes it a very interesting candidate for evaluating the co-regulation of milk fat and lactose synthesis.

**Specificity protein 1.** Very recently it was demonstrated that the Specificity protein 1 (Sp1) also plays a role in controlling milk fat synthesis in goat mammary cells (257). The overexpression of Sp1 increased the transcription of several genes involved in milk fat synthesis, including $\text{PPARG}$, whereas the $\text{SREBF1}$ remained unaffected and the $\text{NR1H2}$ was downregulated (257).

**In silico analysis of potentially new TF.** To reveal potentially new TF involved in controlling the expression of milk fat synthesis, we have performed an in silico analysis of the promoter region of milk fat synthesis-related genes in bovine (29). The analysis was performed using the newly developed LASAGNA software (119), which allows an automatic retrieval and analysis of transcription factor binding sites (TFBS) and related TF in the promoter region of genes in several species. Considering all the TFBS with a hit significant at $P < 0.001$, we detected a very large number (>200) of putative TF that can control expression of milk fat synthesis-related genes (results not shown). When a very stringent $P$ value cut-off was applied ($P < 0.00001$), we still found a large number of TF for each of the evaluated genes (Fig. 2). According to LASAGNA, the genes tested are simultaneously affected by very few TF (i.e., common upstream regulators). Interestingly, none of the above-discussed TF, with the exception of a very significant TFBS for the SREBP1 in the promoter region of $\text{FABP3}$, were uncovered. This in silico analysis can be considered indicative only because the availability of the TFBS is highly determined by the epigenetic status (prevalently methylation of the DNA). Consequently, a high hit does not guarantee a high functionality. Additionally, the TFBS sequences and matrices used by LASAGNA were developed for mouse and human (i.e., TRANSFAC and JASPAR). Thus, it may be not as relevant for bovine. Furthermore, the software only analyzes the promoter region up to $-5,000$ to $+1,000$ nt relative to the transcription start site (TSS), whereas functional TFBS can be present up to $100,000$ nt upstream or in the coding region, as with the case for SREBP-1c, an isoform of SREBP1 (124), and as previously shown for the PPAR RE in several bovine genes (33). Despite these limitations, it is clear that a large number of TF are involved in controlling the expression of milk fat-related genes. This observation is also supported by the data on the experimentally tested TF discussed above, in which it is obvious that more than one TF is involved in the control of the
milk fat synthesis, and a large interaction between TF likely occurs, as previously proposed (29).

Proposed model of transcriptomic regulation of milk fat synthesis and potential for nutrigenomic approaches. In accordance with what has been previously suggested (29), we propose that the expression of milk-fat-related genes is controlled chiefly by an interactive network of TF, which includes SREBP1, PPARγ, and LXRα. To visualize the potential of SREBP1, PPARγ, and LXRα to regulate the transcriptome related to milk fat in bovine and mouse, we have performed an in silico analysis of the presence of putative TFBS for the three TF in the promoter region (−950 to +50 nt relative to the TSS) of several of the most important genes involved in milk fat synthesis using LASAGNA (119) in bovine and mouse (Fig. 3). The LASAGNA software uses databases containing TFBS matrix for PPARγ, SREBP1, Sp1, and LXRβ but does not have TFBS matrix for LXRα, Spot14, and ChREBP. The results in bovine underscored the importance of TFBS for PPARγ in the promoter region of all tested genes. With the exception of LPIN1, LPL, ACSL1, and AGPAT6, all the genes tested contain a TFBS for SREBP1, and only ACACA, GPAM, LPIN1, and AGPAT6 contain a significant hit for the TFBS of LXRβ. In mouse, the promoter region of all evaluated genes has at least one TFBS for PPARγ. With the exception of Scd1, Lpl, and Fasn, all genes tested have a TFBS for LXRβ. Only the promoter region of Acaca, Scd1, and Acsl1 has a TFBS for SREBP1. These data seem to support a more important role of PPARγ in controlling the expression of milk fat-related genes in bovine compared with SREBP1 and LXR (n.b., the TFBS used for LXR is specific for LXRβ not α). The important role of PPARγ in the mouse indicated by the in silico analysis is not supported by experimental data, as discussed above. At least one TFBS for the Sp1 was present in all milk-fat-related genes, supporting a role for this TF, as recently demonstrated (257).

Using the same in silico analysis as above but with a larger up- and downstream region relative to the TSS (i.e., −5,000 to +1,000 nt), we have evaluated the potential transcriptional regulation (i.e., cross talk) between PPARγ, LXRβ, SREBP1, and Spot14 in bovine and mouse (Fig. 4, A and B). The analysis indicated that the region examined for all the TF tested has at least one significant TFBS for all the evaluated TF. Therefore, a strong cross-regulation between those TF exists. In addition, from examination of the two results, it is obvious that a strong conservation of the promoter region between the mouse and the bovine for the four TF exists. When the same analysis was performed restricting the region from −950 to +50 nt relative to the TSS, a significantly lower number of TFBS were uncovered (Fig. 4, C and D), but a more prominent role of PPARγ emerged (Fig. 4, C and D). Overall, the in silico analysis supports the concept of a strong cross-regulation between the four TF, all of which are known to constitute the central hub in the regulatory network of milk fat synthesis.

Overall, our data allow us to establish a model in which SREBP1 plays a central role in maintaining the basic transcription of genes involved in milk fat synthesis. The PPARγ also appears to play a central role, especially in the response to saturated LCFA. The transcriptional interaction between SREBP1 and PPARγ in bovine mammary epithelial cells is complex and unidirectional, in that PPARγ controls the expression of SREBF1. The SREBP1 does not seem to control the expression of PPARG in bovine and ovine, if one considers that inhibition of SREBP1 activity by t10,12-CLA does not affect PPARG expression, as observed in sheep in vivo (101) and bovine mammary cells (108). However, it is possible that SREBP1 controls expression of PPARG in other tissues or in other species, such as mouse (110). The control of expression of SREBF1 by LXRα has been clearly demonstrated. In addition, LXRα might control expression of genes related to milk fat synthesis independently of SREBP1 (165). The interaction between LXR and PPARγ has not been tested in ruminants. Nevertheless, the potential for a strong interaction exists and is worth assessing (250) (Fig. 4). Lastly, data from previous work, both in dairy cows (33, 108) and mice (26), strongly suggested that the activation of lipogenic-related genes in mammary epithelial cells is under control of additional TF, likely including Spot14, ChREBP, and Sp1.

![Fig. 3](http://physiolgenomics.physiology.org/)

**REGULATION OF MILK PROTEIN SYNTHESIS**

**Overview of Milk Protein Synthesis**

Milk protein is affected by energy content of the diet and the availability of amino acids (AA). A review of the milk protein synthesis and potential regulation in dairy cows was recently published (27), and that information was used to construct the milk protein synthesis part in Fig. 1. In that review, there was a discussion of the role of energy and AA, as well as AA transporters, in controlling milk protein synthesis through post-transcriptional regulation by an insulin-mTOR signaling pathway. A detailed model of the insulin-mTOR pathway in the
regulation of milk protein synthesis has been previously proposed (28) and will be used in the discussion of this section. A review on the translational regulation of milk protein synthesis, including historical overview of the progress toward understanding posttranscriptional regulation, is available elsewhere (179).

Transcription Factors Controlling Expression of Casein Genes

Among TF important for regulation of casein genes are the CCAAT/enhancer binding protein (C/EBP) beta, the glucocorticoid receptor Signal Transducer and Activator of Transcription 5 (STAT5), and the octamer binding transcription factor 1 with the Yin and Yang 1 protein being an inhibitor (27). Among them, STAT5 is the most important due to its role in controlling expression of various caseins, genes, and lactalbumin (27). Activated STAT5 binds to DNA sites known as GAS (interferon-gamma activated sequence) elements and upregulate target genes (reviewed in Ref. 83). Deletion of STAT5 in the mammary gland of mouse compromised the expression of whey acidic proteins and lactalbumin but had no effects on beta casein (Csn2) (178). Although these data indicate that STAT5 might not be necessary to the basal expression of Csn2, others have reported a significant and numerical decrease in Csn2 expression under both reduced phosphorylation and increased methylation of the binding site for STAT5 (49, 159). Overall, there exists substantial evidence that STAT5 plays a central role in mammary gland development and function by interacting with several signaling pathways controlling milk protein gene expression and cell survival (50, 83). STAT5 activity increases during lactation mostly due to phosphorylation (246, 247). Although insulin plays an important role in the activation of STAT5, other hormones, such as prolactin and glucocorticoids, can also regulate the activity of STAT5 (reviewed in Ref. 83). Interestingly, the level of regulation of STAT5 through Jak/Stat signaling by hormones may vary across species, which has been reviewed previously (27). For instance, prolactin in rats increases before parturition and remains high during the entire lactation; in contrast in dairy cows this hormone increases sharply at the onset of lactation and decreases afterwards to prepartal levels. Bionaz and collaborators (27) suggested that this difference between species might be associated to the high positive correlation between prolactin and suckling or milking, which is considerably higher in rodents than ruminants.

AA Transporters and Milk Protein Synthesis

The transport of AA is one of the major limitations for milk protein synthesis. In fact, the low concentration in plasma, coupled with high extraction rates by the mammary gland, identified Methionine (Met) and Lysine (Lys) as the most-limiting AA for milk protein synthesis in dairy cows (53, 84) as well as in humans and other species (60). For other AA, in addition to the provision of building blocks for proteins, the transport from blood into the mammary epithelial cells has a regulatory role as well. For instance, the larger intracellular concentration of Leu can increase overall protein synthesis, Leu being a well-known activator of mTOR, the master regulator of the translation of mRNA into proteins (113).

The L-type AA transporter is a well-studied system in lactating mouse, guinea pig, bovine, and rat mammary tissue for transport of neutral AA (e.g., Met) (202). LAT1, the most abundant L-type AA transporter in mammary gland, has been identified as the primary transporter of Met into the mammary
crement of mechanism may partially explain the large transcriptional in-
tensation/availability and their final impact in milk protein syn-
thesis are warranted. For instance, during glutamine depletion 
transient or prolonged periods of AA imbalance between the 
extracellular and intracellular compartments may occur. Mech-
anism that respond to intracellular fluctuations of AA concen-
tration/availability and their final impact in milk protein syn-
thesis are warranted. For instance, during glutamine depletion 
in rat kidney cells, the general AA control (GAAC) pathway is 
induced through the activating transcription factor 4 (ATF4) in 
order to maintain intracellular AA levels (48). ATF4 upregu-
lates AA biosynthesis and indirectly induces protein synthesis 
by increasing activation of mTOR via upregulation of several 
AA transporter genes including SLC7A5 (LAT1), the major 
AA transporter for Leu, that, in turn, activates mTOR (88, 
138). The role of GAAC in controlling intracellular AA avail-
ability might also be present in the mammary gland. Such a 
system (i.e., GAAC-ATF4 pathway) can be an important driver 
of milk protein synthesis during adverse periods where supply 
of AA may be limited, such as the transition between preg-
nancy and early lactation. During the first week of lactation, 
high-yielding dairy cows need to mobilize as much as 1 kg of 
tissue protein daily to satisfy the demand of the mammary 
gland for AA (19), substantially altering the extracellular and 
intracellular AA pools. We speculate that dairy cows rely on 
this GAAC-ATF4 pathway during this period. In fact, this 
mechanism may partially explain the large transcriptional in-
crement of SLC7A5 in the mammary gland of dairy cows 
during the onset of lactation (28).

AA transporters may also act as sensors of intracellular and 
intracellular AA abundances regulating directly or indirectly 
protein synthesis. This is supported by the idea of AA trans-
porters functioning as “transceptors” (receptor-transporter) 
(221), which has been demonstrated for SNAT2 (SLC38A1) in 
breast cancer cells (172). The implications and the extent of the 
AA sensing by transceptors in milk protein synthesis are 
currently unknown but may be a phenomenon worth investi-

gating.

In summary, the extraction of AA from blood into the 
mammary gland via cell membrane transporters is a complex 
topic because of the variety of systems (e.g., L, ASC, SNAT) 
and interaction between AA transporters (e.g., LAT1/4F2hc 
and ASC2). The ability of certain AA transporters to be 
transceptors adds an additional variable to our current under-
standing of regulation of milk protein synthesis.

Regulation of Protein Synthesis by the Energy Content in the 
Diet: via Insulin and Glucose

In an organism, the protein synthesis/turndover is the highest 
energy demanding process next to ion transport. In fact, in 
mammals, the basal energy expenditure for maintenance of 
protein synthesis and ion transport has been estimated to be 
~10 and 35%, respectively (10). For instance, from pregnancy 
to lactation, the mammary gland of dairy ruminants ex-
periences approximately fivefold increase in energy utilization for 
milk protein synthesis due to a four- to sevenfold increase in 
mRNA translation (reviewed in Ref. 27). In addition, dairy 
cows have a relatively low efficiency (~25–30%) in the 
transformation of dietary nitrogen into milk proteins (23), with 
a high rate of protein turnover (22). In the goat mammary 
gland, the daily tissue protein synthesis (i.e., nonmilk protein in 
mammary tissue) can account for as much as 88% of the total 
protein synthesized, which represents half of the available ATP 
supply generated in the lactating udder (86). Considering all 
these factors, it is not surprising that the milk protein yield is 
relatively proportional to the energy content of the diet in dairy 
cows, as already highlighted more than 30 yr ago (69). Dietary 
energy stimulates milk protein yield in monogastrics (225) 
and ruminants (224). The agonistic effect of dietary energy on 
milk protein synthesis is not only related to availability of 
more energy for the translation machinery, but also appears to be driven by the increase in hematic insulin and, 
likely, glucose availability. The positive role of insulin on 
milk protein synthesis was demonstrated in mice and cows 
(reviewed in Ref. 27).

With respect to energy availability for protein synthesis, data 
indirectly seem to indicate a competition between milk protein 
synthesis and milk fat synthesis in mammary gland. Such 
competition is supported by the consistent increase in milk 
protein content in animals receiving a milk fat-depressing diet 
or t10,c12-CLA (e.g., Ref. 129). When one observes the 
relationship between SREBP1 and mTOR (174), it appears that 
a coordinated regulation between SREBP1 and mTOR during 
cell growth allows for simultaneous production of proteins and 
lipids. This relationship is likely disrupted during milk fat 
depression but may be important during normal situations. It 
would be interesting to determine if such a relationship is 
maintained in the fully developed lactating mammary gland 
where proliferation is greatly reduced (31, 45, 132).

Via insulin. The mammary gland is known to be hypersen-
sitive to insulin during lactation, at least in rodents (41), and at 
the onset of pregnancy, with an increased sensitivity until the 
end of pregnancy, primarily due to an augmented kinase 
activity of the insulin receptor (46). One of the primary 
functions of insulin in mammary gland is the control of milk

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protein synthesis by inducing translation via activation of mTOR pathway and activation of STAT5 through phosphorylation (reviewed in Ref. 27). The insulin effect on mTOR pathway is twofold (27). Namely, it directly stimulates mTOR protein by phosphorylation and prevents mTOR inhibition by blocking via phosphorylation the two tuberous sclerosis proteins (i.e., TSC1 and TSC2), the main inhibitors of mTOR. Insulin can also affect milk protein synthesis by increasing uptake of AA, particularly the branched-chain and the essential AA (27, 82). In fact, insulin upregulates the expression of y+ transport system for cationic AA, such as Lys and Arg, in mouse mammary cells (200). In a recent study carried out by RNA sequencing of milk fat layer during colostrogenesis, transition, and mature milk in two groups of women based on lactation capability, it was proposed that protein tyrosine phosphatase receptor type F (PTPRF) might be responsible for mammary insulin resistance, resulting in decreased milk yield and poor lactation (121). The study did not consider if the expression of PTPRF had any relationship with milk protein synthesis. However, because insulin in mammary tissue is important for milk protein synthesis, PTPRF may be an interesting candidate for study concerning milk protein synthesis. In mammary tissue of dairy cows, the expression of PTPRF increases 2 wk prior to parturition to slowly but significantly decrease until peak lactation, and it increases again after peak lactation (32). The expression pattern of PTPRF in the bovine mammary is somewhat opposite to the curve of lactation and milk protein yield. No other data are available in the literature on the role of PTPRF in milk synthesis. It should be noted, however, that milk synthesis is controlled by a complex network of factors. For this reason, it is unlikely (although possible) that a single protein would determine the insulin sensitivity status of the mammary gland.

Via glucose. Increase in intracellular glucose can enhance protein synthesis by increasing ATP production with a subsequent inhibition of 5' AMP-activated protein kinase activity, one of the main negative regulator of mTOR (194). In addition to playing an indirect role on mTOR activity, glucose can enhance translation by reducing phosphorylation of Eukaryotic Initiation Factor 2 a. This was demonstrated in mammary tissue of feed-restricted dairy cows infused intravenously with glucose (224). Despite evidence of glucose being a promoter of protein synthesis, a contrasting effect was observed by Curtis and collaborators (59). In their study, intravenous glucose infused in lactating dairy cows increased milk yield and lactose yield but only numerically increased milk protein yield and decreased milk protein percentage. The decrease in milk protein percentage is likely due to a dilution effect as consequence of increased milk yield. The authors also observed a decrease in phosphorylated 4EBP1 and S6K in glucose infused cows, which indicates a lower activation of the translation machinery.

Role of AA

The availability of AA is essential for milk protein synthesis (66, 82). The AA are not only the "building blocks" of the proteins, but they can also activate the translational machinery through mTOR pathway (27) and affect the expression of milk protein genes (39, 64).

AA effect on transcription of milk protein synthesis-related genes. It is becoming evident that the AA can also regulate directly the expression of target genes in mammals through nutrient/gene interactions (64, 107). Most of the current data regarding AA direct regulation of gene expression in monogastrics were generated under scenarios of AA or nutrient deprivation in mouse and human mammary cells, as well as human cancer cell lines (39, 64). Nan and collaborators (155) demonstrated a strong nutrigenomic effect of AA in bovine mammary epithelial cells. It was observed that Lys and Met at 1.2 and 0.4 mM, respectively, resulted in significantly higher expression of all genes coding for major milk proteins (e.g., CSN1S1, CSN1S2, CSN2, CSN3, LALBA), genes related to translation (ELF5, MTOR), and JAK-STAT signaling (JAK2, STAT5), but expression of genes coding for AA transporters was not affected. The authors also reported a downregulation in expression of Eukaryotic Translation Initiation Factor 4E Binding Protein 1, one of the major inhibitors of the mTOR pathway. Huang and collaborators (100) confirmed an increase in mRNA expression of MTOR and CSN2 by 0.6 mM of Met in bovine mammary epithelial cells, but they also observed a decrease in expression of SOCS3 (suppressor of cytokine signaling 3) (100). The SOCS proteins are known to be inhibitors of JAK/STAT5 pathway; thus, the downregulation of SOCS3 could potentially be translated as an increase in protein synthesis. Furthermore, bovine mammary epithelial cells treated with Met had increased mRNA expression of ribosomal protein S6 kinase (RPS6KB1), AKT1, MTOR, and CSN2 (252). All the above data strongly support an active nutrigenomic role of Met in controlling milk protein synthesis, which may help explain the positive effect of Met on milk protein synthesis observed in vivo (166, 168) and in vitro (155). A nutrigenomic role of Arg in controlling milk protein synthesis in bovine mammary epithelial cells was recently demonstrated (235). It remains to be determined if the nutrigenomic effect of AA is direct (i.e., via binding and activating a specific transcription factor) or indirect (e.g., through activation of mTOR pathway). Overall, the above data are of great interest because they open up the possibility for fine-tuning the milk protein synthesis through nutrigenomic approaches.

AA as activators of translation. In dairy cows, the AA transporters with the largest upregulation in gene expression from late pregnancy to lactation in mammary tissue were the ones related to the mTOR pathway (28). Among AA capable of increasing translation, the most well known are Leu, Lys, and Met. The role of Leu in controlling translation in the bovine mammary was recently reviewed (27). Leu affects translation by enhancing mTOR activity indirectly and the translation initiation complex directly. Lys appears to augment translation through the phosphorylation of Mitogen-activated Protein Kinase that in turn enhances the expression and phosphorylation of mTOR and STAT5a, as recently demonstrated in bovine mammary cells (133). Met is well known for its essential role in the initiation of mRNA translation, which is the binding of methionyl-tRNA to the 40S ribosomal subunit followed by the joining of a 60S ribosomal subunit to form a translationally competent 80S ribosome (112). In addition to functioning as initiator of translation, Met increases protein expression and phosphorylation of STAT5a and mTOR and protein expression of β-casein (100). An increase in activation of mTOR pathways by Met and by increasing the phosphorylation of the mTOR inhibitor Glycogen Synthase Kinase 3 beta

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was observed in bovine mammary epithelial cells (252). Taken together, these results suggest that Met activates the mTOR and STAT5 signaling pathways to upregulate milk protein synthesis. In addition to Leu, Met, and Lys, other AA, such as Tryptophan, Arginine, and Isoleucine, have positive effects on milk protein synthesis via phosphorylation of mTOR pathway-related proteins in bovine mammary epithelial cells (6).

**REGULATION OF LACTOSE SYNTHESIS**

**Overview of the Lactose Synthesis**

An overview of the main proteins involved in lactose synthesis is presented in Fig. 1. Lactose, a disaccharide composed of glucose and galactose, is the major carbohydrate found in milk of most species. Milk yield greatly depends on mammary lactose synthesis due to its osmoregulation of milk, one that induces mammary uptake of water. Therefore, the rate of lactose synthesis in the epithelial cells of the mammary gland serves as a major factor influencing milk volume production.

The supply of glucose for lactose synthesis increases dramatically in the mammary gland of lactating animals. It has been estimated in lactating mammary in goats utilizes 60–85% of the total glucose produced in the body (5). Mammary tissue of dairy cows extracts ~20% of glucose from blood (44, 181). In the presence of α-lactalbumin, β1,4-galactosyltransferase present in the Golgi apparatus transfers galactose from UDP-galactose to glucose instead of the usual substrate N-acetylgalactosamine (177). Specific transporters, solute carrier family 2A1 (GLUT1) and solute carrier family 35 (UDP-galactose transporter), member A2 (SLC35A2), pump glucose and galactose into the Golgi apparatus (136, 157).

There is only one gene encoding α-lactalbumin (LALBA), but there are seven different β1,4-galactosyltransferase genes. Only the β1,4-galactosyltransferase 1 (B4GALT1) is expressed abundantly in the mammary gland and forms a complex with α-lactalbumin (2, 177). Removal of either Lalba (219) or B4gal1t1 (7) by gene knockout in mice prevents the synthesis of lactose. The conversion of glucose to UDP-galactose is catalyzed by the UDP-glucose pyrophosphorylase 2 (gene symbol UGP2) and phosphoglucomutase 1 (PGM1). The UDP-galactose is then transported into the Golgi by SLC35A2. The latter might be a rate-limiting process in lactose synthesis (146). The pattern of expression of the above genes from late pregnancy to lactation in dairy cows and mouse are presented in Fig. 5 (27, 191). All, except the PGM1, display a significant increase in expression during lactation in both species, supporting the observation that they play an important role in lactose synthesis.

**Effect on Milk Components by Overexpression of α-Lactalbumin**

Lactose is required for normal milk production due to its osmoregulatory effects, and lack of lactose substantially reduces milk production and the milk produced is more viscous with less water. This was demonstrated in mice with a homozygous Lalba gene deletion (214). Normal milk production was re-established in those mice when human LALBA was introduced in the mouse genome (214), demonstrating the essential role of α-lactalbumin in lactose synthesis. Interestingly, the insertion of the homozygous human LALBA gene increased milk production by 30% compared with mouse wild type, but the milk composition was moderately affected, with only a significant decrease in the percentage of milk fat. Similarly, insertion of bovine LALBA in mice increased milk production by 14%. However, excluding a 5% decrease in protein compared with their wild-type counterpart, the insertion had only a minor effect on milk composition (35). Transgenic pigs expressing bovine LALBA had increased milk production with high lactose content (especially during the first 10 days post-partum) and litter growth, without any effects on the proportion of other milk components (140, 161).

The essential role of LALBA in driving milk synthesis is also supported by a positive correlation observed between the expression of LALBA and milk yield in a large transcriptomics analysis of the bovine mammary tissue from late pregnancy to end of lactation (27). In the same study, the functional analysis of the data uncovered lactose synthesis as the top function among the differentially expressed genes during lactation. Recently, a comparison in expression of LALBA from pregnancy to lactation between bovine, mouse, goat, pig, and

**Fig. 5.** Expression of lactose synthesis-related genes during lactation. Expression of genes related to glucose transport (SLC35A2), conversion of glucose to UDP-galactose (UGP2 and PGM1), and lactose synthase (B4GALT1 and LALBA) in bovine (31) and mouse (191) mammary tissue from pregnancy to lactation.
kangaroo was performed (27). In all tested species except kangaroo, the expression of LALBA increased greatly at the onset of lactation and persisted until decrease or cessation of lactation. Therefore, increasing the expression of LALBA can be an effective way to increase milk yield improving the efficiency of milk production but without affecting milk quality. Because of this, understanding the transcriptional control of LALBA appears critical.

**Transcriptional Regulation of the Lactose Synthase Enzymes LALBA and B4GALT1**

Compared to milk fat and protein synthesis, lactose synthesis and regulation have received little attention. It is known that lactose synthesis is predominantly subjected to hormonal control with very few dietary factors influencing its production (15, 104, 158).

It has been observed that LALBA expression is strongly induced by the onset of lactation in several species (27), decreases in late lactation in bovine (27), and further decreases at the onset of involution as observed in bovine and kangaroo (111, 171). The expression of LALBA increases upon treatment with a cocktail of lactogenic hormones (i.e., insulin + hydrocortisone + prolactin + 17β-estradiol or growth hormone), as observed in mouse and bovine mammary cells in vitro (105, 199). The expression of LALBA is also induced by increased milking frequency in bovine and caprine (21, 154) and by the AA Lys and Met in primary bovine mammary cells (155), but it is decreased by mastitis in dairy cow and yak (126, 150) and by feed restriction in the cow (62). It is well established that the promoter region of LALBA contains a STAT5 binding site, and the presence of STAT5 is essential for the expression of LALBA in mouse (178, 245).

As for LALBA, the expression of B4GALT1 increases at the onset of lactation in bovine (27) but only during established lactation in mouse (191) (Fig. 5). The B4GALT1 isoform involved in the lactose synthesis in the mammary gland appears to be an evolutionary adaptation through a change in the transcription starting site. This was demonstrated in mouse where the shorter isoform of B4GALT1 (i.e., ~3.9 kb instead of ~4.1 kb) is highly abundant in mammary tissue and contains in its promoter a number of TFBS that are absent or not as abundant in the promoter region of the longer isoform (176).

Among transcriptional regulators of B4GALT1, it was observed that p16, a tumor suppressor protein, decreases expression of B4GALT1 in adenocarcinomic human cells (251); however, this effect has not been evaluated in nontumorigenic mammary epithelial cells. Recently, in human mammary cancer cells, it was demonstrated that estrogen controls the expression of B4GALT1 by binding three different estrogen response elements in the promoter region of the gene (51). We are not aware of any study performed to uncover the main TF controlling the expression of B4GALT1 in ruminants; however, analysis using LASAGNA (119) uncovered a large number of TFBS for estrogen at ~5,000 nt upstream the TSS for bovine and ovine B4GALT1 (data not shown).

Few nutritional factors can affect the expression of the two lactose synthase genes. In a recent study, the effect of glucose on mRNA expression of genes coding for key proteins in milk fat, lactose, and glucose metabolism was assessed in primary bovine mammary epithelial cells (128). The authors detected a greater expression of B4GALT1 with 5 or 10 mmol/l of glucose concentration in the medium compared with 2.5 mmol/l. No effect of high glucose was observed on the expression of LALBA despite an overall increase in activity of lactose synthase. The authors also observed that higher glucose concentration increased the activity of pyruvate kinase, a key glycolytic enzyme, and glucose-6-phosphate dehydrogenase, a key enzyme in the pentose phosphate pathway and production of ATP, indicating that the excess glucose is mostly used for energy production. Unfortunately, in that study, lactose and/or glucose were not measured in the media, so no definitive conclusion can be made. The practical relevance of the findings from that study is questionable based on ruminant physiology. A level of glucose of 10 mmol/l in plasma is not physiological. Level of glucose in blood of dairy cows is normally between 3.5 and 4.5 mmol/l (or 63 and 81 mg/dl) in the fasting state during transition from pregnancy to lactation (34) and midlactation (160), respectively, and can reach a level of 5 mmol/l only during disease and/or stress conditions (151) with a maximum of 4.6 mmol/l 6–10 h after meal (160). The 2.5 mmol/l tested and reported in the above study can be reached only when animals are in a serious hypoglycemic condition (151). In contrast, it is also difficult to increase the availability of glucose in vivo in the mammary epithelial cells to the extent reported in the above study because plasma glucose levels are tightly regulated by homeostatic mechanisms.

The effect of glucose in the expression of genes is likely direct through MLXIPL (ChREBP) but can also be indirect, as reviewed previously (145). In bovine mammary tissue, the MLXIPL expression is ~100-fold and 10-fold lower than liver and adipose, respectively, but higher than muscle (90). Its expression is more than twofold higher in lactating vs. nonlactating bovine mammary tissue when measured by RT-qPCR (90). However, in a large transcriptomics study of bovine mammary tissue during lactation, its expression was significantly downregulated during lactation compared with pregnancy (see File S3 in Ref. 27). In addition, as discussed earlier in this review, the importance of ChREBP in mammary tissue is also questioned by the absence of this TF in purified epithelial cells of mouse (192), but no data about expression of MLXIPL in purified bovine mammary epithelial cells are available.

Overall, a role of glucose in controlling expression of genes involved in lactose synthesis is possible but seems unlikely for the above reported reasons. Nevertheless, further investigations are worth conducting in order to assess whether glucose has a nutrigenomic role in controlling milk synthesis.

**Regulation of Glucose Transporters and Their Role in Lactose Synthesis**

The mammary gland is unable to synthesize glucose from other precursors because it lacks glucose-6-phosphatase. This has been demonstrated in lactating bovine mammary gland (198) (but activity of this enzyme has been reported in mammary tissue of the mouse, Ref. 205). Blood glucose levels are tightly regulated in all mammals. The intracellular glucose concentrations are within 0.1–0.5 mM in mammary epithelial cells in most species where this was evaluated (72), a much lower range compared with plasma glucose levels. These data also indicate that lactose synthesis is not saturated by glucose.

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Therefore, the active uptake of glucose by the mammary epithelial cells is rate limiting, playing a major role in regulating the final milk volume and consequently improving dairy productivity and efficiency.

The change in the availability of glucose in the blood does not seem to consistently affect milk lactose synthesis or milk yield. This has been demonstrated in several studies. Cant et al. (44) observed a 40% increase in glucose uptake and increase in lactose percentage in milk but not significant change in milk yield or lactose yield in cows infused with 90 g/h of glucose into iliac artery. Rigout et al. (181) detected a quadratic but not a linear increase on uptake of glucose by the mammary gland with a consequent increase in milk yield and lactose yield in cows receiving duodenal infusion of increased doses of glucose. In that study the authors reported decreased mammary tissue extraction rate of glucose with increased dose of infused glucose, indicating a lack of change in active glucose import. Galindo et al. (78) detected a linear increase in milk and lactose yield in cows receiving duodenal infusion of increased doses of a mixture of amino acids. In the latter study, the authors reported an increase in hepatic net flux of glucose. In another study, the abomasal infusion of nonessential AA increased mammary glucose uptake, but the percentage of milk lactose only increased numerically (65).

Glucose enters the mammary gland through active glucose transporters (253), most of which belong to the solute carrier family 2 (facilitated glucose transporter). In bovine mammary tissue, the most important are GLUT1 (SLC2A1), GLUT3 (SLC2A3), GLUT8 (SLC2A8), and GLUT12 (SLC2A12), with SLC2A1 and SLC2A8 having the larger increases in expression from pregnancy to lactation (27, 254). Additionally, through RNA sequencing analysis of human milk fat layer it was detected that the SLC2A9 was the highest expressed glucose transporter during lactation. However, further investigation is needed to determine whether this transporter is exclusive for glucose (121).

Contrary to peripheral tissues, whereas insulin-dependent GLUT4 is the main glucose transporter, the main glucose transporters in mammary gland are insulin independent (253). This feature of the mammary tissue appears important when considering the whole physiology of lactation. Early lactation in dairy cows is characterized by a large decline of hematic insulin and a decrease in insulin sensitivity in most tissues but not in mammary tissue. Therefore, the presence of insulin-independent glucose transporters in mammary tissue appears critical to support the large increase in uptake of glucose for lactose synthesis (253). The importance of glucose transporters in mammary is supported by a study where it was demonstrated that overexpression of SLC2A1 and SLC2A12 genes in goat mammary epithelial cells increases uptake of glucose. However, an increase in lactose synthesis was observed only in conjunction with an overexpression of SLC2A1 (248). This finding supports the prominent role of GLUT1 in lactose synthesis, likely by importing glucose into the Golgi, as demonstrated in lactating rat (157). The lack of a consistent increase in lactose synthesis upon augmented availability of glucose in the study presented above may be associated with a lack of increase in activity of GLUT1 and/or SLC35A2.

Potential role of AA, Glycerol, and PEPCK in Lactose Synthesis

In addition to the essential role of glucose in lactose synthesis, glycerol contributes to the de novo galactogenesis in the human mammary gland (220). In a recently published study performed in humans, glycerol contributed 10% and 69% of de novo synthesized glucose and galactose, respectively, for lactose synthesis (148). In 1958, it was demonstrated by an elegant unilateral glycerol-1,3 C14-labeled injection experiment that glycerol contributes significantly to the galactose produced in the mammary gland of dairy cows, in addition to contributing to fatty acid and casein syntheses (242). Similar to glycerol, the AA can also contribute to the synthesis of lactose, as suggested by recent studies (24, 117). For both glycerol and AA, the synthesis of hexose, such as glucose and galactose, is through the gluconeogenic pathway. Within the gluconeogenic context, the phosphoenolpyruvate carboxylase enzyme (the cytosolic isofrom or PEPCK-C; gene symbol PCK1) is crucial for the indirect entrance of AA into the gluconeogenic pathway via the Krebs cycle. Data generated in bovine mammary tissue are indicative of an increased activity of the Krebs cycle during lactation (31). The glycerol kinase (gene symbol GK) is the key enzyme for the entrance of glycerol in the gluconeogenesis.

In dairy cows gluconeogenesis in the mammary gland appears to be extremely low, if not virtually absent (198). However, evidence of gluconeogenic activity in the mammary tissue of guinea pigs was observed as being primarily mitochondrial due to PEPCK, a gluconeogenesis-related enzyme, where a large increase in activity of both Pck1 and Pck2 (but with a larger increase for Pck2) from pregnancy to lactation was detected (106). Increased expression of Pck1 in mammary tissue from pregnancy to lactation was also observed in rats (230). In a large transcriptomics study of the mammary tissue of dairy cows, the expression of PCK1 was not affected by lactation, whereas the expression of PCK2 was decreased (see File S3 in Ref. 32). In contrast to PCK2, the expression of GK significantly increased from pregnancy to lactation in mammary tissue of dairy cows (32). The expression of PCK1 and PCK2 was also assessed in mammary tissue of lactating dairy goats where a negative role of insulin on the expression of PCK1 was demonstrated (135). More recently, a role for the PEPCK-C in mammary epithelial and adipose cells in mouse was assessed in a study using stable isotope-labeled compounds (98). In that study, the expression of Pck1 decreased in epithelial and increased in adipose cells during lactation compared with pregnancy, and the same pattern was observed with prolactin treatment. Overall, the authors from that study concluded that mammary epithelial cells from mouse relied on precursors from liver and mammary adipocytes to produce lactose and TAG but also use glycerol for lactose synthesis. The Pck1 is likely controlled by PPARγ in mouse mammary tissue and plays a crucial role in providing glycerol for TAG synthesis (99).

All the above data indicate that in addition to uptake of preformed glucose, lactose synthesis is also affected by de novo synthesized glucose and galactose via glycerol and AA precursors in the mammary gland. The role of PEPCK in the mammary gland is not fully understood, especially in ruminants. Further investigations may help clarify PEPCK role.
Potential TF Involved in the Regulation of the Lactose Synthesis Gene Network in Bovines

With the purpose of uncovering the main potential TF involved in controlling glucose transport and lactose synthesis, we have performed our analysis of the promoter region of main genes involved in those functions in bovine using the LASAGNA software (118) (Fig. 6). Using the most stringent \( P \) value available (i.e., \( P < 0.00001 \)), the analysis identified a complex network of TF potentially involved in controlling glucose uptake and lactose synthesis. Among these TF, the presence of a very significant TFBS for SREBP1 in the promoter region of LALBA is of interest. This is probably not relevant in vivo, considering that milk fat depression in dairy cows inhibits and decreases the activity and expression of SREBP1 while increasing milk lactose synthesis rather than diminishing it (95). Other TF uncovered by LASAGNA analysis that may play a role in controlling lactose synthesis-related genes are Hepatic Nuclear Factor 4 (HNF4) and STAT5A. The former might control the expression of UGP2 and SLC35A2 and may potentially bind and be activated by fatty acids, at least in monogastrics (93). STAT5A is central in controlling expression of milk protein genes and may control the expression of UGP2. The genes involved in lactose synthesis do not seem to be highly coordinated, at least considering the existence of relatively few TFBS with high significance in more than one gene (Fig. 6). The data are indicative of a very large number of highly significant TFBS for the glucose transporters, especially with respect to SLC35A2, which is responsible for UDP-galactose import into the Golgi, and the UGP2, which is responsible for the production of UDP-glucose and an essential intermediate for galactose synthesis. The two genes also share the highest number of common TFBS among the genes tested.

In part, this supports the similar increase in expression observed from pregnancy through lactation in the mammary tissue of bovine (Fig. 5). Conversely, LALBA has only four highly significant TFBS, and B4GALT1 has no common highly significant TFBS with the other tested genes, despite displaying a very similar pattern of expression with UGP2 gene in bovine mammary tissue (Fig. 5). Overall, the analysis indicates that the coordination of expression of genes involved in lactose synthesis in bovine is not very high, considering the low number of common potential upstream TF and, in some ways, confirmed by longitudinal transcriptomic data (Fig. 5). The results of this in silico analysis can be considered only indicative at best, knowing all the limitations of this approach (discussed above). However, it can be used as starting point to investigate the transcriptional network of lactose synthesis in mammary.

Effect of mTOR on Expression of GLUT Genes

mTOR is known to increase the expression of SLC2A1 (40). In contrast, increased glucose uptake via GLUT1 can increase the activity of mTOR. The latter effect could be associated with AMPK, which is inhibited by ATP and is known to inhibit mTOR by activating TSC1 and TSC2 (244). Therefore, high availability of glucose increases production of ATP and prevents the inhibition of mTOR (244). The inhibition of glycogen synthase kinase-3 (GSK-3) activity, an activator of TSC2, increased glucose uptake and Slc2a1 expression via mTOR in rat cells (40). A role for GSK3 in controlling GLUT1 activity was also demonstrated in a murine pro-B-cell lymphoid cell line (240).

All the above observations clearly provide support for the existence of a potential cross talk between milk protein and...
lactose synthesis through mTOR via increase glucose transport through GLUT1. This would indicate a positive relationship between level of glucose and milk protein synthesis. However, the positive relationship between mTOR and glucose import has not been confirmed in bovine mammary tissue. In a recent work, intravenous injection of glucose in lactating dairy cows increased lactose synthesis, decreased the phosphorylation of mTOR pathway, numerically increased the yield of protein, and decreased the percentage of milk proteins (59). In that study, glucose transport and expression of glucose transporters were not measured.

**Hormones and Regulation of GLUT Genes in Mammary**

Despite the low hematoconcentration of insulin in early postpartum cows, an effect of insulin in the expression of SLC2A1 in mammary can be inferred by the increased expression of Slc2a1 via insulin induction of Akt1 in mouse hepatoma cells (11). Similarly, a role for Akt1 in promoting glucose transport and Glut1 activity through mTOR was demonstrated in murine cells (240). The importance of Akt1 in lactose synthesis is supported by a study where the overexpression of Akt1 in mouse mammary gland increased uptake of glucose and lipid synthesis (197). Furthermore, Akt1−/− mice failed to lactate primarily due to a failure in upregulating expression of Slc2a1 (37). The role of Akt1 in lactation has also been supported by a greater than twofold up-regulation in transcript abundance in mammary tissue at the onset of lactation in mouse (4). In bovine mammary, AKT1 is upregulated approximately twofold in lactation compared with late pregnancy but only during established lactation (28). A role for insulin in the expression of GLUT1 was also demonstrated recently in bovine mammary explants (199). Based on the above data, a role for insulin, likely through AKT1, in controlling SLC2A1 expression in mammary gland appears deserving further attention.

Other hormones do not seem to play a role in the expression of GLUT genes because the use of lactogenic hormones in cultured bovine and murine mammary epithelial cells and explants did not affect the expression of SLC2A1 and SLC2A8 and down-regulated the expression of SLC2A12 (199). The same study also uncovered a lack of in vitro effects of lactogenic hormones in the expression of GLUT genes in bovine mammary epithelial cells, which was partially confirmed in vivo by a lack of an effect on expression of SLC2A1 after prolactin infusion in early lactation dairy cows.

**Cross Talk between Glucose and AA Transporters**

There is an increasing evidence of cross talk between glucose and AA transporters, which adds another dimension of complexity to milk synthesis. Goat mammary epithelial cells overexpressing SLC2A1 or SLC2A12, which are glucose transporter genes, had significant or numerically greater expression of SLC1A5, SLC3A2, and SLC7A5 coding for AA transporters (248). Relevant data support the existence of a positive interaction between glucose transport and availability of AA for milk protein synthesis. In the same study, the overexpression of GLUT proteins also increased expression of milk protein-related genes (248). The increase in expression of mTOR-related AA transporters SLC3A2 and SLC7A5 by overexpression of SLC2A12 provides support for a reciprocal regulatory connection of GLUT proteins with mTOR (76). This is supported by the positive effect of 4F2hc (SLC3A2) in the activation of GLUT1 and AA transporters, leading to simultaneous increase of glucose and AA transport, as observed in human embryonic kidney cells, HeLa cells, and mouse brain cells (162).

**Potential Role of PPARβ/δ in Regulation of Lactose Synthesis**

Among the three PPAR isotypes, the PPARβ/δ has been the least studied. However, the importance of this PPAR isotype in monogastrics has gained attention, as extensively reviewed recently (156), but relatively little information is known about the role of PPARβ/δ in ruminants (reviewed in Ref. 26). In monogastrics, PPARβ/δ is the chief regulator of lipid catabolism in skeletal muscle and heart but also regulates glucose metabolism and is involved in insulin activity (156). With respect to mammary tissue, relatively few studies examining the role of PPARβ/δ have been conducted in relation to cancer (156). The main finding from those studies is that PPARβ/δ is involved in the regulation of proliferation of cancerous mammary cells.

In addition to playing a role in the proliferation of mammary cells, PPARβ/δ may also play a role in milk synthesis via regulation of glucose uptake. A role of PPARβ/δ in glucose uptake was demonstrated in bovine aortic endothelial cells where activation of PPARβ/δ by peroxides decreased the expression of SLC2A1 (180). In that experiment, the peroxides were produced via 12-lipoxygenase enzyme as consequence of high glucose in the media. The decreased expression of SLC2A1 was a consequence of an increase in expression of calreticulin via activation of PPARβ/δ. Calreticulin is a protein that increases degradation of SLC2A1 mRNA. Therefore, the inhibition of glucose uptake was due to an increased degradation of SLC2A1 mRNA by calreticulin. If this is also true for ruminants and if the inhibition of PPARβ/δ decreases the use of glucose via glycolysis in mammary gland, the inhibition of PPARβ/δ should increase the amount of glucose for the synthesis of lactose.

The demonstrated negative role of PPARβ/δ in glucose uptake is very interesting when examined from a milk-lactose synthesis point of view. This is particularly exciting considering the abundant expression of PPARβ/δ gene in bovine mammary tissue (26). Furthermore, the expression of PPARβ/δ is significantly downregulated from late pregnancy to lactation in bovine mammary tissue when there is also an increase in expression of several glucose transporters, including SLC2A1 (31). Contrary to bovine, in mouse mammary tissue, the Ppard expression is not affected by stage of lactation despite having a large increase in Slc2a1 expression from pregnancy to lactation (4, 191). Notwithstanding the difference observed with mouse, it appears possible that in bovine, any natural or synthetic compound that inhibits PPARβ/δ may have a positive effect on milk production via increased glucose uptake and thereby, lactose synthesis. Initial data from our laboratory provided support for such a conjecture (130), but additional experiments yet unpublished (Lohakare J, Osorio J, and Bionaz M.) did not confirm the initial observations.
EPIGENETIC REGULATION OF MILK SYNTHESIS

The epigenome deeply affects the whole transcriptome defining the cell identity, including its function and capacity to respond to external stimuli. Consequently, the study of the epigenome is of utmost importance to understand the medium-to long-term regulation of milk synthesis.

As for other tissues, the functional identity of the mammary gland appears to be highly determined by its epigenetic status during its entire development (116). In a transcriptomic study of the bovine mammary tissue from late pregnancy to end of the subsequent lactation, data associated with changes of chromatin status (i.e., euchromatin or active transcribed chromatin and heterochromatin or tightly packed and transcriptionally unavailable chromatin) indicated a decrease of epigenetic changes as lactation begins and a reactivation of epigenetic changes as milk yield decreases during late lactation (31). This appears essential to have a consistent transcriptome context of the CpG dinucleotide (185). DNA methylation is partly driven by epigenetic effects, particularly by an increase in DNA methylation of the RE for STAT5 in the promoter region of caseins (229). The authors of the study did not investigate the molecular factors responsible for the higher DNA methylation that remain to be determined.

The DNA methyltransferases (DNMT), of which there are several isoforms, including DNMT1b, DNMT2, and DNMT3b, are known to methylate cytosines in DNA and consequently create methylated CpG patterns in the mammalian genome (206). DNMT have been observed to have a high degree of structural and functional conservation among human, mouse, and bovine (188). The DNMT3a and DNMT3b identify unmethylated CpG regions within the DNA and initiate de novo methyltransferase, whereas the activity of DNMT1 is primarily methylation of remaining unmethylated cytosine within CpG regions previously methylated by DNMT3a (97, 206).

In Fig. 7, the expression of DNMT genes in bovine (31) and mouse (191) mammary tissue at the end of pregnancy and during lactation is reported. In both species, there is downregulation of DNMT1 and DNMT3B and upregulation of DNMT3A, suggesting an increase in de novo methylation coupled with a decrease in the maintenance of methylated CpG regions during lactation. The pattern of expression observed is consistent with the epigenetic stability and specialization of the mammary gland appears to be highly determined by its epigenetic status during its entire development (116). In a transcriptomic study of the bovine mammary tissue from late pregnancy to end of the subsequent lactation, data associated with changes of chromatin status (i.e., euchromatin or active transcribed chromatin and heterochromatin or tightly packed and transcriptionally unavailable chromatin) indicated a decrease of epigenetic changes as lactation begins and a reactivation of epigenetic changes as milk yield decreases during late lactation (31). This appears essential to have a consistent transcriptome context of the CpG dinucleotide (185). DNA methylation is partly driven by epigenetic effects, particularly by an increase in DNA methylation of the RE for STAT5 in the promoter region of caseins (229). The authors of the study did not investigate the molecular factors responsible for the higher DNA methylation that remain to be determined.

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tissue toward milk synthesis, as previously suggested (31). Therefore, altering the expression of the DNMT genes might provide a way to improve the mid- to long-term control of the expression of milk synthesis-related genes.

Among epigenetic factors, microRNAs (miRNA) play a major role in controlling the availability of mRNA for translation through posttranscriptional regulation. Studies dealing with “miRNAomics” in dairy cows have been partly reviewed (131). A large miRNA transcriptomics experiment in the bovine mammary tissue from pregnancy to peak lactation was performed (234). Results from that study indicated that most miRNA with large upregulation during lactation are potentially related to the control of expression of genes related to milk fat and milk protein synthesis. The importance of miRNA in the control of milk-related genes was demonstrated in a recent study where the knockdown of the expression of the milk protein β-lactoglobulin was successfully achieved in dairy cows by transgenic overexpression of a specific miRNA (102). Besides demonstrating the important role of miRNA, this study established the potential for changing milk traits through targeted expression of miRNA. From this point of view, it is interesting to note the findings that miR33, which is located within the intron 17 of SREBF1 (190), plays a role in controlling the expression of SREBF1. Even though the SREBF1 is a miR33 target gene, it is likely this miRNA works on specific transcripts of SREBP isofoms because a positive cooperation of miR33 and SREBP-1c might contribute to increase lipogenesis in monogastrics (reviewed in Ref. 190). The cooperation between miR33 and SREBP-1c in bovine mammary samples was also indicated by recent transcriptomic data (234).

Milk is a highly specialized materno-neonatal relay system that functions by transferring not only essential nutrients for growth (e.g., glucose, FA, and proteins), but also other constituents, such as exosomes containing miRNA, most likely representing a genetic transfection system that functions by transferring not only essential nutrients for growth (e.g., glucose, FA, and proteins), but also other constituents, such as exosomes containing miRNA, most likely representing a genetic transfection system delivering dietary messengers such as branched-chain AA and exosomal miRNA produced by the mammary gland. This notion introduces a new complexity in the paradigm of the transcriptomic control of milk synthesis because it opens up the potential not only to affect the production of major milk components, but to also produce milk with the “best” miRNA for humans (or calves).

The field of epigenetic in dairy animals is still in its infancy but holds great promise for a better understanding of the nutritional regulation of milk synthesis. The possibility of affecting epigenetic status via nutrition is of utmost interest considering the potential for mid- to long-term fine-tuning of milk production.

**SUMMARY**

**Disclaimers**

Most of the data presented in this review of the literature were generated by RT-qPCR, the most sensitive technique for measuring abundance of transcripts. Unfortunately, most of the cited papers in this review have not properly provided all the information necessary to evaluate the reliability of the RT-qPCR, as requested by MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (43). Therefore, we have to disclose that the results used to draw conclusions from our survey of the literature might be compromised by the questionable reliability of the RT-qPCR data. We also have to acknowledge that lactogenic complexes were not always used in studies carried out in mammary epithelial cells cited in this review. Lactogenic cocktails should be used when looking at how mammary epithelial cells make milk components because those hormones are key to establishing this effect.

**Milk Fat Synthesis**

Analyses of the data produced so far have started to delineate a clearer picture of what controls milk fat synthesis. Milk fat synthesis is strongly regulated at the transcription level by a network of TF, including SREBP1, PPARγ, LXRα, Sp1, and Spot14. Among these TF, a pivotal role of SREBP1 seems to be established, but SREBP1 appears to play a major role in maintaining the basal expression of milk fat-related genes, and, based on the data analyzed so far, studies do not support a role of this TF in transducing the LCFA signal that leads to increased expression of milk fat-related genes. Data from few studies are indicative of a role for mTOR in controlling milk fat synthesis via cross talk with SREBP1. More promising from a nutrigenomic perspective are PPARγ and LXRα. Among the two, the PPARγ appears to possess the strongest nutrigenomic potential due to the demonstrated capacity of being activated by LCFA in ruminants. In conjunction with the in silico analysis of the TPBS in the promoter region of milk fat-related genes, it is safe to conclude that a complex interactive network of TF orchestrates the transcription of genes coding for proteins related to milk fat synthesis in mammary tissue. In addition for the need to provide stronger evidence (likely in vivo) of a role of LXR and PPARγ, as well as other TF, the following questions pertaining to the regulation of milk fat synthesis remain unanswered: 1) How do the mammary epithelial cells sense whether they have to decrease or increase milk fat synthesis? 2) In what order do the transcriptional regulators respond? 3) Is there a leading TF that senses the need for a change and drives the rest of the TF network?

**Milk Protein Synthesis**

The regulation of milk protein synthesis appears to be more conserved than the regulation of milk fat synthesis, because the former seems to primarily be regulated by posttranscriptional factors rather than at the transcriptional level, with a prominent role of the insulin-mTOR pathway. This pathway is also tightly linked with the availability of AA. Therefore, cross talk between mTOR, insulin, and AA transporters seems to play a major role in controlling milk protein synthesis. In addition to constituting the building blocks for protein synthesis, AA...
affect the transcriptional and posttranscriptional regulation of protein synthesis. Even though the posttranscriptional regulation of milk protein synthesis appears dominant, new data indicate the existence of a nutrigenomic role of several AA, including Lys, Met, and Arg, in controlling milk protein synthesis. It remains to be determined which TF is sensing AA and inducing the change of the transcripts related to protein synthesis. Several AA, including Leu, Met, and Lys, play important posttranscriptional regulatory roles by affecting the activity of STAT5 and mTOR pathway-related proteins. Regulatory mechanisms that respond to changes in extracellular/intracellular AA profiles might provide new insights into possible coping mechanisms that function to maintain milk protein synthesis during adverse conditions. There is strong evidence for the role of DNA methylation in the epigenetic regulation of milk protein synthesis. Based on this evidence, the possibility of modifying the DNA methylation via nutritional approaches (e.g., use of diets enriched with Met), as observed in liver of dairy cows (167), would be of high interest. Although posttranscriptional regulation in the case of milk protein synthesis has received substantial attention, there is growing evidence for the importance of transcriptional and epigenetic regulation of the expression of both milk proteins (i.e., caseins) and components of the translation machinery (i.e., mTOR pathway).

**Milk Lactose Synthesis**

The control of expression of the main genes coding for proteins related to lactose synthesis is poorly characterized. Understanding the transcriptional and translational control of *LALBA* and *B4GALT1* can be critical to improving milk yield.

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**Fig. 8. Known and proposed interactions between principal transcription factors regulating milk fat, protein, and lactose synthesis.** mTOR plays a central hub role in connecting synthesis of protein, lactose, and milk in mammary tissue. mTOR is activated by insulin (through AKT1), several amino acids (Leu, Lys, and Met), and, possibly, glucose. mTOR also appears to activate SREBP1, which, in turn, can affect mTOR activity and expression. Met and Lys can control the expression of mTOR pathway-related genes, casein, and *LALBA*, likely through STAT5. There is a strong signaling and transcriptional network between glucose and amino acid transporters. The GAAC-ATF4 can respond to decrease intracellular amino acids (Gln) by increasing expression of amino acid transporters. There is a complex and interactive transcriptional factor (TF) network controlling the expression of milk related genes with SREBP1 as the central hub. SREBP1 can be inhibited by CLA. PPARy can be activated by LCFA and, in turn, increases expression of milk fat-related genes, including SREBP1. The network encompasses other TF, such as LXR, Spot14, Sp1, and ChREBP. The potential negative effect of PPARy or on expression of glucose transporters genes is also shown. Arrows denote direction of the effect; dashed arrows denote import; bar-headed lines denote inhibition. Letters associated with arrows denote the following: A, activation; A?, activation through phosphorylation; E, positive effect on expression; A? or E?, potential activation or potential positive effect on expression. AKT1, RAC-alpha serine/threonine-protein kinase; ATF4, activating transcription factor 4; ChREBP, carbohydrate-responsive element-binding protein; CLA, conjugated linoleic acids; GAAC, general amino acid control; Gln, Glutamine; GLUT, glucose transporters; LALBA, α-lactalbumin; Leu, Leucine; Lys, lysine; LXR, liver X receptor; Met, Methionine; mTOR, mammalian target of rapamycin; PPARγ, peroxisome proliferator-activated receptor γ; Spot14, thyroid hormone responsive protein; Sp1, specificity protein 1; SREBP1, sterol regulatory element-binding transcription factor/protein 1; STAT5, signal transducer and activator of transcription 5; TF?, unknown transcription factor(s).
and efficiency of milk production. In silico analysis indicates a complex and poorly orchestrated TF network in the transcriptional regulation of those genes. Mammary glucose uptake is an important determining factor for the rate of milk synthesis. With respect to mammary glucose uptake, glucose transporters play an important role. The scientific data reported in literature prompt us to suggest a particular important role of the transporters of glucose and galactose into the Golgi on milk lactose synthesis. There is a paucity of data about the transcriptional regulation of those transporters. Data also indicated the existence of cross talk between glucose and AA transporters and glucose and mTOR signaling, suggesting an orchestrated control of milk protein and lactose synthesis. It remains to be determined what controls the expression of lactose synthesis-related genes (including glucose transporters) and whether there is any possibility of modulating the transcription of these genes through diet modifications. However, increasing milk lactose synthesis through nutrigenomic interventions might be one of the most potent approaches for maximizing milk production.

PERSPECTIVE

Overall, our survey of the literature reveals that the regulation of milk synthesis is governed by complex networks of TF and signaling proteins (Fig. 8). We envisage a future where, as factors involved in the transcriptional and posttranscriptional regulatory network are revealed, a more precise intervention can be made using diet and/or management to exploit TF networks so as to affect milk components and/or increase efficiency of milk synthesis.

Milk synthesis requires an orchestrated complexity of factors that goes beyond the simple expression of the relatively few genes coding for major proteins with the specific and sometimes unique task of synthesizing and secreting milk protein, fat, and lactose. In fact, milk synthesis is the product of complex interactions between several tissues and organs (e.g., mammary, liver, adipose, muscle, and immune cells). Therefore, the use of an integrative systems-biology approach seems essential to make fundamental advances in understanding milk synthesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.S.O. and M.B. prepared figures; J.S.O., J.L., and M.B. drafted manuscript; J.S.O., J.L., and M.B. edited and revised manuscript; J.S.O., J.L., and M.B. approved final version of manuscript; M.B. conceived and designed of research; M.B. analyzed data; M.B. interpreted results of experiments.

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