Review

Key stages in mammary gland development

Secretory activation in the mammary gland: it's not just about milk protein synthesis!

Steven M Anderson[1,2], Michael C Rudolph[3], James L McManaman[2,3,4] and Margaret C Neville[3,4]

[1]Department of Pathology, University of Colorado Health Sciences Center, East 17th Avenue, Aurora, CO 80045, USA
[2]Program in Molecular Biology, University of Colorado Health Sciences Center, East 17th Avenue, Aurora, CO 80045, USA
[3]Department of Physiology and Biophysics, University of Colorado Health Sciences Center, East 17th Avenue, Aurora, CO 80045, USA
[4]Department of Obstetrics and Gynecology, University of Colorado Health Sciences Center, East 17th Avenue, Aurora, CO 80045, USA

Corresponding author: Steven M Anderson, steve.anderson@uchsc.edu

Published: 28 February 2007
This article is online at http://breast-cancer-research.com/content/9/1/204

Breast Cancer Research 2007, **9:**204 (doi:10.1186/bcr1653)

Abstract

The transition from pregnancy to lactation is a critical event in the survival of the newborn since all the nutrient requirements of the infant are provided by milk. While milk contains numerous components, including proteins, that aid in maintaining the health of the infant, lactose and milk fat represent the critical energy providing elements of milk. Much of the research to date on mammary epithelial differentiation has focused upon expression of milk protein genes, providing a somewhat distorted view of alveolar differentiation and secretory activation. While expression of milk protein genes increases during pregnancy and at secretory activation, the genes whose expression is more tightly regulated at this transition are those that regulate lipid biosynthesis. The sterol regulatory element binding protein (SREBP) family of transcription factors is recognized as regulating fatty acid and cholesterol biosynthesis. We propose that SREBP1 is a critical regulator of secretory activation with regard to lipid biosynthesis, in a manner that responds to diet, and that the serine/threonine protein kinase Akt influences this process, resulting in a highly efficient lipid synthetic organ that is able to support the nutritional needs of the newborn.

Introduction

Over the past 12 years our understanding of the regulation of milk protein gene expression has improved dramatically. One important advance was the discovery of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway; prolactin (PRL)-induced activation of JAK2 and STAT5 is required to induce expression of most, if not all, milk protein genes [1,2]. Recent advances suggest that the transcription factor Elf5 and the ubiquitin ligase Socs2 (suppressor of cytokine signaling) are important mediators of PRL action. Loss of Socs2, which negatively regulates the PLR receptor (PRLR), or forced expression of the Elf5 transcription factor can restore lactation in mice that fail to lactate due to the loss of one or both alleles encoding the PRL receptor [3]. These findings led the investigators to suggest that Elf5 is encoded by one of the master controller genes that regulate alveolar differentiation (recently termed the alveolar switch in a review by Oakes and colleagues [4] in this series of reviews). Despite these advances, our understanding of the molecular changes that underlie alveolar differentiation and secretory activation (the lactation switch) is relatively unsophisticated. In this review we identify changes that are known to occur in the mouse as a means to identify questions and challenges for the coming decade and suggest that sterol regulatory element binding protein (SREBP)-1c and the serine/threonine protein kinase Akt1 play a major role in the lactational switch.

Morphological differentiation of the murine mammary gland

The morphological changes that occur in the mammary gland during puberty, pregnancy and lactation are well established [5]. A rudimentary mammary ductal structure is established *in utero* [6] and all subsequent developmental events occur after birth. Ductal elongation and branching occur primarily after the onset of puberty under the influence of estrogen, epidermal growth factor, and insulin like growth factor (IGF)-1 [7,8]. The terminal end bud is the primary proliferative

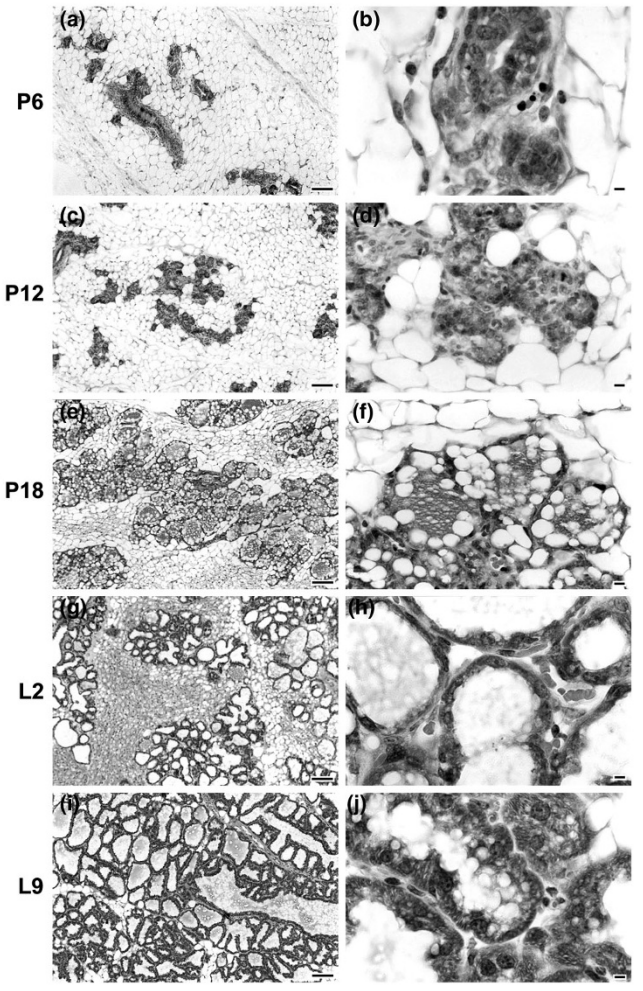
ADRP = adipophilin; BM = basement membrane; CLD = cytoplasmic lipid droplet; DAPI = 4′,6-diamino-2-phenylindole; DGAT = diacylglycerol acyltransferase; FA = fatty acid; FABP = fatty acid bindin protein; GLUT = glucose transporter; GSK-3 = glycogen synthase kinse-3; HK = hexokinase; IGF = insulin-like growth factor; JAK = Janus kinase; mTOR = mammalian target of rapamycin; NF = nuclear factor; PRL = prolactin; PRLR = prolactin receptor; RankL = RANK ligand; SCD = sterol CoA dehydrogenase; SP = specific factor; SREBP = sterol regulatory element binding protein; STAT = signal transducer and activator of transcription; VLDL = very low density lipoprotein; WAP = whey acidic protein; WDNM1 = Westmeade DMBA8 nonmetastatic cDNA1.

structure that directs ductal elongation, which appears to occur maximally between three to six weeks of age. By ten to twelve weeks of age the ducts have reached the margins of the fat pad, the terminal end buds regress to form terminal ducts, and ductal elongation ceases. In contrast to humans, in which ten to fifteen branching ducts connect to the nipple, in the mouse a single primary duct, which can be identified by its proximity to the nipple and the thick sheath of connective tissue, serves as a conduit for the passage of milk to the suckling young. Secondary and tertiary ducts, which contain a single layer of cuboidal luminal epithelial cells surrounded by a layer of basal cells, are formed by branching off the primary duct. Formation of lateral and alveolar buds occurs in the post-pubertal mammary gland following initiation of the estrous cycle [9,10]. These lateral buds are often termed side branches and represent the origin of the alveoli that are the milk producing cells in the lactating mammary gland [5].

In the post-pubertal phase, alveolar buds develop into rudimentary alveolar structures consisting of a single layer of luminal epithelial cells surrounding a circular luminal space. A layer of flattened myoepithelial cells can be seen to surround these structures if specific stains, such as rhodamine phalloidin (an actin stain), are used. During post-pubertal growth, PRL and progesterone are responsible for alveolar bud formation [11-13]. Progesterone is thought to act through the paracrine production of Wnt4 and Rank ligand (RankL) by progesterone receptor-positive cells; these paracrine factors in turn stimulate proliferation of progesterone receptor-negative cells, leading to side branch formation [14]. RankL is thought to stimulate epithelial cell proliferation and alveolar differentiation through activation of cyclin D1 via a pathway that may involve NF-κB [15]. Germline deletion of either RankL or its receptor results in reduced proliferation and increased apoptosis of alveolar epithelial cells, a process that could be modified by activation of Akt [15].

Alveolar differentiation, for example, the formation of lobulo-alveolar structures capable of milk production, occurs during pregnancy and is also stimulated by PRL [16,17]. Transcription profiling studies indicate that PRL stimulates transcription of Wnt4 [18], RankL [18], and cyclin D1 via induction of IGF-2 [19,20]. PRL also induces the expression of two other transcription factors of note: the ETS transcription family member Elf5 [3] and SREBP1 [21]. Harris and colleagues [3] demonstrated that forced expression of Elf5 in mammary epithelial cells from PRLR knockout mice is able to restore morphological differentiation and production of milk proteins. In these experiments it could not be determined whether Elf5 induced a functional restoration since the transfected mammary epithelial cells were transplanted into a recipient host and lactation does not occur in these mice due to the lack of ductal connections with the teat. The role of SREBP1 will be discussed below as it regulates the expression of a number of key lipid metabolism genes [22].

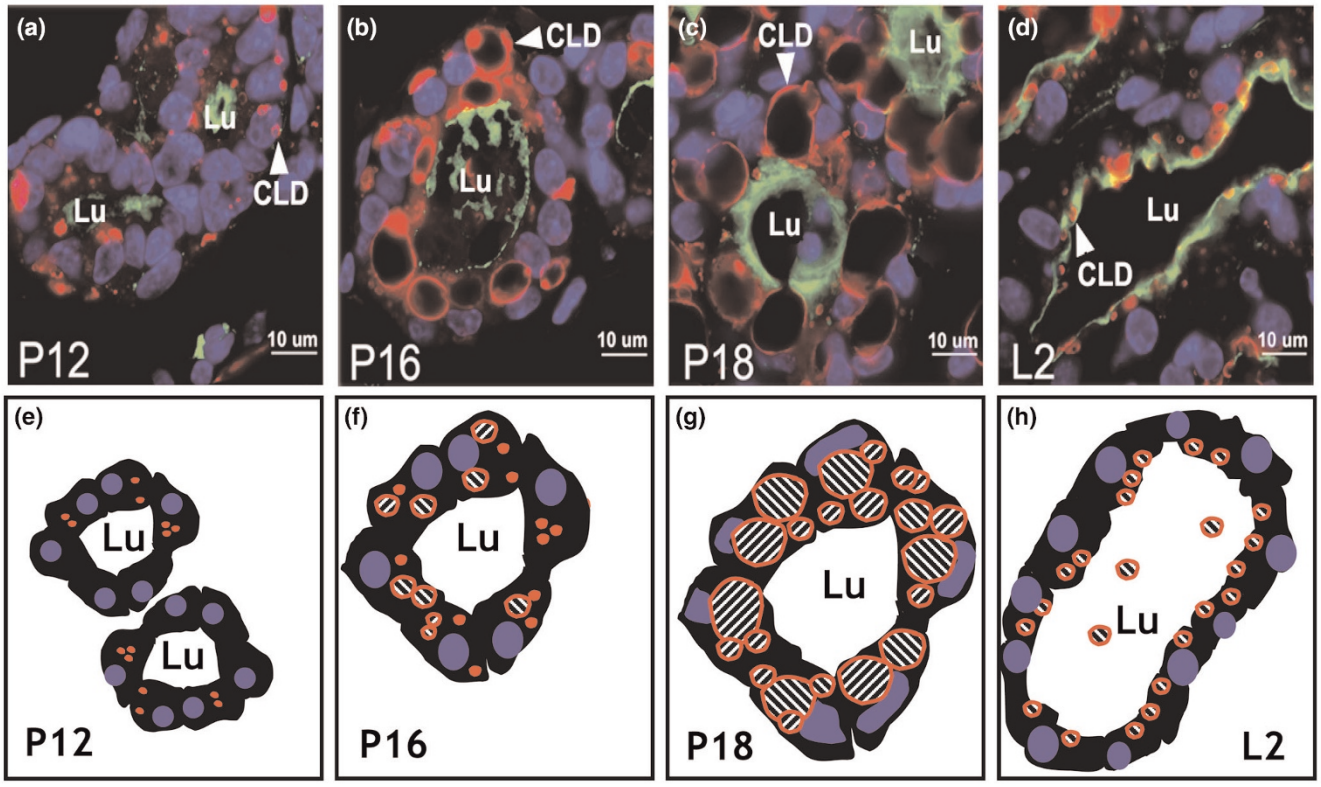
Figure 1



Histological features of the mammary gland of FVB mice during pregnancy and lactation. Mammary glands were isolated from FVB mice on **(a,b)** day 6 (P6), **(c,d)** day 12 (P12), and **(e,f)** day 18 (P18) of pregnancy, and **(g,h)** day 2 (L2) and **(i,j)** day 9 (L9) of lactation, fixed in neutral-buffered formalin, sectioned and stained with hematoxylin and eosin. Scale bars in (a, c, e, g and i) represent 100 μm, while those in (b, d, f, h and j) represent 10 μm.

Histological changes in mammary gland morphology in the mouse during pregnancy and lactation are shown in Figure 1. Initial changes observed during pregnancy include an increase in ductal branching and the formation of alveolar buds (Figure 1a); this phase of differentiation is characterized by the largest increase in DNA synthesis and cellular proliferation during pregnancy [23]. The latter half of pregnancy is characterized by the expansion of alveolar buds to form clusters of lobuloalveolar units, followed by the differentiation of these structures into pre-secretory structures. By day 12 of pregnancy there is a readily apparent increase in the size of the epithelial compartment compared to the adipose compartment (Figure 1c), and expansion of the epithelium continues until the epithelial compartment predominates

Figure 2



The size and location of cytoplasmic lipid droplets (CLDs) changes upon secretory activation. Mammary glands were isolated from FVB mice on pregnancy (P) days **(a)** 12, **(b)** 16, and **(c)** 18, and **(d)** day 2 of lactation (L2). Tissues were fixed in neutral-buffered formalin, stained with anti-adipophilin (ADRP) antibody and Alexa Fluor 594 conjugated secondary antibody to outline the cytoplasmic lipid droplets (appearing in red), Alexa Fluor 488-conjugated wheat germ agglutinin to outline the luminal surface of the luminal space of the secretory alveoli (appearing in green), and 4′,6-diamino-2-phenylindole (DAPI) to stain the nuclei of mammary epithelial cells (appearing in blue). Idealized schematic drawings, not meant to represent the micrographs shown in the top panel, illustrate the positions of the luminal space (labeled LU), nuclei (purple), and CLDs (labeled red) at pregnancy days **(e)** 12, **(f)** 16, and **(g)** 18, and **(h)** day 2 of lactation. The scale bars in (a-d) represent 10 μm. Luminal space is indicated by the letters 'Lu', and the white arrowheads indicate CLDs.

by late pregnancy (Figure 1e). The luminal space is clearly evident by late pregnancy, filled with a proteinaceous substance whose identity is not clear but may represent milk proteins, glycoproteins such as Muc1, lactoferrin, and possibily immunoglobulins (Figure 1f). Large lipid droplets are also present in the cytoplasm of the alveolar epithelial cells and, to some extent, in the luminal space (Figure 1f). Following parturition, the secretory lobuloalveolar structures become more apparent as the luminal space expands, and the epithelial cell layer becomes more prominent against the adipocytes (Figure 1g). The large lipid droplets, which were present at day 18 of pregnancy, are not present, having been replaced by small lipid droplets at the apical surface of the epithelial cells (Figure 1h), and although the luminal space may contain proteinaceous material when it has not been lost during fixation and sectioning, it stains much more lightly than during late pregnancy (Figure 1i versus 1b). By day nine of lactation in the mouse, the mammary gland is producing copious amounts of milk. Examination of the histology of the mammary gland at this stage reveals prominent luminal structures and ducts; however, few adipocytes are visible at this time (Figure 1i). This change is thought to reflect delipidation of adipocytes rather than a decrease in their number [24].

Perhaps the most obvious histological change marking the transition from pregnancy to lactation is the change in the size and cellular distribution of lipid droplets. At mid-pregnancy, small lipid droplets, referred to as cytoplasmic lipid droplets (CLDs), can be seen within luminal mammary epithelial cells. While these are readily apparent in standard histological sections stained with hematoxylin and eosin, we have found that they are better appreciated in sections that are stained with an antibody to adipophilin, which is found at the periphery of all lipid droplets within the alveolar cells (red stain in Figure 2) where DAPI (blue stain in Figure 2) has been used to stain the nuclei, and wheat germ agglutinin (green stain in Figure 2) to identify the luminal surface of the

lobuloalveolar complexes (Figure 2). By the end of pregnancy the CLDs have increased dramatically in size (Figure 2c,g). Following parturition, CLDs are smaller and localized to the apical surface of the alveolar epithelial cells (Figure 2d,h). It is interesting to note that in many genetically engineered mice that exhibit lactation failure, large CLDs remain after parturition and can be observed on the first and sometimes even the second day of lactation. In particular, we have characterized lactation failure in transgenic mice that express activated myr-Akt1 in the mammary gland [25]; prominent CLDs are apparent following parturition, suggesting that secretory activation has not occurred. The presence of large CDLs post-partum is also noted in the following genetically engineered mice: Src null mice (MM Richert and SM Anderson, unpublished data); WAP-human protein C [26]; bovine oxytocin transgenic [27]; oxytocin knockout mice [28]; α-lactalbumin knockout mice [29]; butyrophilin knockout mice [30]; and the xanthine oxidoreductase heterozygous knockout mice [31].

Functional differentiation of the mammary gland

Functional differentiation of the murine mammary gland is divided into four phases: the proliferative phase of early pregnancy; the secretory differentiation phase, which starts during mid-pregnancy; secretory activation, which begins at or around parturition, depending on the timing of the fall of progesterone; and lactation. While markers for most of these phases are recognized, the molecules that regulate the transition between them are not well understood.

As suggested by its name, the proliferative phase is characterized by the extensive proliferation of mammary epithelial cells and begins with conception. *In vivo* labeling of proliferating cells in the mammary gland with ^{3}H-thymidine revealed that 25% of the cells were labeled on day five of pregnancy [23]. Proliferation decreases from this peak in early pregnancy until the tissue reaches quiescence just before parturition [23]. If one can use the changes in the amount of cytokeratin 19 and claudin 7 RNA as a reflection of the increase in the epithelial cell content of the gland, there may be up to a thousand-fold increase in the number of mammary epithelial cells [32,33]. While this increase in epithelial cell markers could reflect an increase in the number of epithelial cells, it might also reflect a change in the size of these cells, or a change in the patterns of genes expressed in these cells. Despite the massive proliferation that takes place, the organization of the epithelium is exquisitely maintained. While the growth factors that regulate the proliferative phase are not clear, the expression patterns of three paracrine growth factors expressed in the mammary gland suggest that they might contribute to this process: RankL, Wnt4, and amphiregulin. The roles of Wnt4 and RankL have been discussed above; these paracrine growth factors appear to be produced by either stromal cells or mammary epithelial cells. Amphiregulin expression is reduced in PRLR null mice, suggesting that its expression may be regulated by PRL [18], and that it may play a role in alveologenesis, as indicated by other studies [34].

Secretory differentiation, which in some literature is referred to as lactogenesis stage I, is defined by several biochemical changes that reflect a change to a pre-lactational state. An increase in the activity of lipid synthetic enzymes was reported in the rabbit mammary gland in a seminal paper by Mellenberger and Bauman [35]. An increase in the expression of adipophilin protein and RNA were correlated with the accumulation of CLDs in the alveolar epithelial cells [25,36], indicating activation of the lipid synthesis function of these cells. The production of milk appears to be blocked by the high plasma concentration of progesterone during pregnancy [37-40]. In fact, it has been suggested that progesterone downregulates expression of the PRLR [41]. Evidence in favor of this notion was obtained by Sakai and colleagues [42], who ovariectomized mice on day 14 of pregnancy, finding a dramatic increase in expression of the long form of the PRLR; an increase in expression of β-casein RNA has also been observed [43]. Ovariectomy on day 17 of pregnancy results in tight junction closure [44] as measured by the sucrose permeability of the epithelium.

Secretory activation, defined as the onset of copious secretion of milk [45], is set in motion by the drop in the level of serum progesterone around parturition [43,44,46,47]. Unlike humans, in which plasma PRL levels are high throughout pregnancy, pituitary PRL secretion increases late in pregnancy in the mouse and rat. At this time PRL appears to stimulate maximal activation of the PRLR and STAT5, leading to a further increase in the transcription of milk protein genes [2,48]; however, unless progesterone action is inhibited, milk secretion is not initiated [37-40]. It has been long appreciated that there is a dramatic increase in the amount of Golgi and endoplasmic reticulum in alveolar epithelial cells at secretory activation [49], and it is generally assumed that these changes are required to support the massive synthesis and secretion of various milk components. While not a functional definition, the absence of large lipid droplets in alveolar epithelial cells provides evidence that this secretory activation has occurred (Figure 2). Both butyrophilin knockout (Btn-/-) and xanthine oxidoreductase heterozygous (XRO+/-) mice accumulate large lipid droplets in the cytoplasm of mammary epithelial cells due to defects in lipid droplet secretion [30,31].

Lactation is defined as the continuous production of milk by the dam. In most species there are two phases: a colostral phase in which the milk contains large amounts of immunoglobulins and other immune defense proteins [50], and the mature secretion phase characterized by the production of large volumes of milk that support the growth of the newborn. Although the colostral phase has not been well-characterized in the mouse, preliminary evidence from the Neville laboratory

suggests that it is brief in this species (Neville MC, unpublished data). Mouse milk contains about 12% proteins (the different caseins, α-lactalbumin, whey acidic protein (WAP), lactoferrin, secretory immunoglobulin A, and others), 30% lipid, and 5% lactose, a disaccharide that is unique to milk. With the closure of the tight junctions there is no transfer of sugars from the blood to the milk. Synthesis of lactose takes place in the Golgi compartment, where the required synthetic enzymes are located. In both mice and rats, lactose is not detected in the mammary gland until the day before parturition [51,52], and thus lactose synthesis may be considered a marker of secretory activation. Furthermore, mice with a null mutation of the gene for α-lactalbumin, an essential co-factor for lactose synthesis, fail to lactate [53].

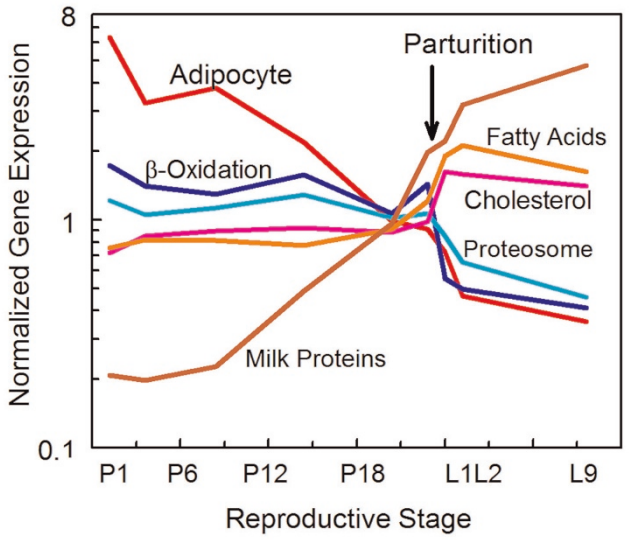
Milk fat is primarily triacylglycerides, made up of a glycerol backbone and esterified fatty acids, which are either derived from the diet, from adipose tissue stores, or synthesized *de novo* in the gland. In mice that are maintained on standard rodent chow, which contains about 8% of its caloric content as fat, a large proportion of the free fatty acid is synthesized in the mammary gland from glucose and, probably, amino acid precursors. To support the synthesis of milk lipids as well as lactose there must be a significant programming of the physiology of alveolar epithelial cells to direct metabolic precursors to the synthesis of these two compounds.

Microarray studies have allowed us to examine temporal changes in mammary gene expression during secretory differentiation and activation in some detail [33]. The results are summarized in Figure 3, which shows mean relative expression levels for a number of classes of proteins through pregnancy and lactation to involution. During pregnancy, adipocyte genes decline about seven-fold, representing dilution by the growing alveoli, with a two-fold drop at the onset of lactation as the alveoli expand further. The overall expression level of milk protein genes increases about five-fold during pregnancy, with a further three- to four-fold increase at parturition. However, these mean values hide a good deal of variation in individual genes within these groups, as discussed in the next section. The other categories shown in Figure 3 remain relatively constant during pregnancy, with a sharp increase (genes for fatty acid and cholesterol synthesis) or decrease (genes for fatty acid degradation and the proteasome) at secretory activation. Examination of numerous mouse models that exhibit lactation failure tends to suggest that, if secretory activation does not occur properly, the mammary gland rapidly undergoes involution [54].

Milk protein synthesis and secretion

Figure 4 shows a summary of array data obtained in both the Neville laboratory [33] and the Gusterson laboratory [55], with expression levels of the 14 major milk proteins plotted as a ratio to their expression on day 17 of pregnancy. Changes in mRNA expression fall into two categories: The first group of proteins is shown in the inset in Figure 4; and the mRNA

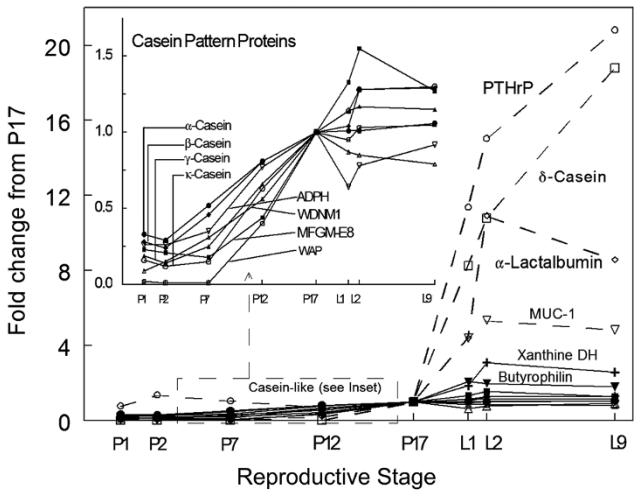
Figure 3



Summary of gene expression during pregnancy and lactation by functional class. Adipocyte specific genes decline throughout pregnancy and early lactation while milk protein genes as a class increase over the same time period. The expression of other classes is stable during pregnancy, possibly representing expression in both the adipose and epithelial compartment and increases two- to three-fold (fatty acid and cholesterol synthesis) or decreases about two-fold (fatty acid and protein degradation) at parturition. Adipocyte genes, red; β-oxidation genes, navy blue; proteosome genes, teal; milk protein genes, brown; fatty acid biosynthesis genes, light brown; cholesterol biosynthetic genes, pink.

expression of this group increases 3- to 50-fold during pregnancy. This category includes most of the caseins, Westmeade DMBA8 nonmetastatic cDNA1 (WDNM1), milk fat globule-EGF-factor-8 (MFGM-E8), WAP and adipophilin. Expression of the mRNA for these proteins is upregulated no more than two-fold at secretory activation. Most of the caseins are expressed in early pregnancy at 30% of the level on day 17 of pregnancy; on the other hand, the expression levels of WAP and WDNM1 mRNAs appear to be insignificant in early pregnancy, turning on between P7 and P12. Despite these differences, expression of most of these proteins has been shown to be regulated by PRL [37] acting through phosphorylation of STAT-5 [56-59]. During pregnancy in the rodent, when PRL levels are known to be low, it is likely that differentiated expression levels respond to placental lactogens [37]. The levels of PRL rise late in pregnancy; however, as noted above, the effect of PRL is suppressed by the high serum levels of progesterone [37-40]. This being the case, synthesis of these proteins, as opposed to transcription of their genes, must be regulated at a level upstream of mRNA transcription. Indeed, in very elegant work in the laboratories of Rhoads and Barash [60,61], both polyadenylation of β-casein mRNA and amino acid availability appear to be involved in the translational regulation of milk protein synthesis. It is tempting to speculate

Figure 4



Expression patterns of milk protein genes. The main graph shows genes whose expression increases more than two-fold at parturition. The inset shows genes with casein-like expression patterns whose mRNA increases mainly during pregnancy. All data are normalized to the level of expression at day 17 of pregnancy (P17). ADPH, adipophilin; MFGM, milk fat globule-EGF-factor; PTHrP, parathyroid hormone related protein; WAP, whey acidic protein; WDNM1, Westmeade DMBA8 nonmetastatic cDNA1; xanthine DH, xanthine oxidoreductase.

that, since the expression of Akt1 increases at secretory activation, Akt could stimulate translation of milk proteins through its ability to phosphorylate 4E-BP1, a negative regulator of translation [62]; however, no evidence exists to support this speculation. Likewise the Akt-dependent activation of mammalian target of rapamycin (mTOR) could stimulate translation through S6 kinase and eEF2 [63,64].

The second category includes a heterogeneous group of proteins whose expression increases up to 20-fold between day 17 of pregnancy and day 2 of lactation, shown in the main graph in Figure 4. Many of these proteins, including α-lactalbumin (lactose synthesis), butyrophilin and xanthine oxidoreductase (lipid secretion), and parathyroid hormone related protein (calcium secretion) turn on secretory processes. In addition, a major milk mucin, MUC1, and one of the caseins, Δ-casein, are significantly upregulated at this time. We understand very little about the regulation of most of these proteins. MUC1, which has been implicated as a signaling molecule [65], is known to be repressed by the ErbB2 pathway acting through the Ras, Raf and phosphatidylonisitol 3′-kinase pathways [66,67]. Parathyroid hormone related protein secretion has been shown to be regulated by calcium availability acting through the calcium sensing receptor [68]. Xanthine oxidase is upregulated in response to hypoxia in some tissues; however, it is not clear that its upregulation at secretory activation is related to changes in the oxidative state. Despite the importance of α-lactalbumin and butyrophilin to synthesis of lactose and secretion of lipid, respectively, little work has been done on the regulation of these genes - a fertile field for investigation.

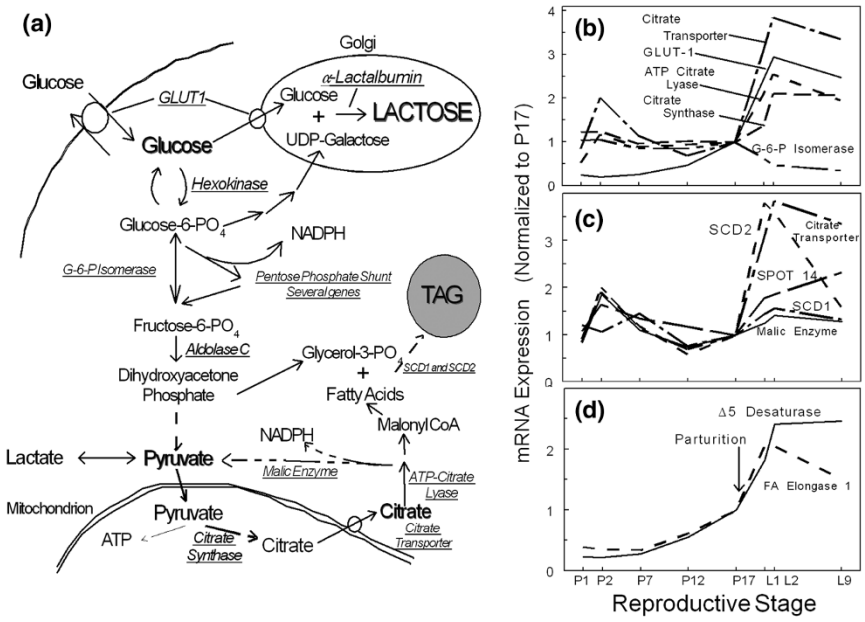
Diversion of glucose for lactose synthesis

The synthesis of lactose from glucose and UPD-galactose is unique to mammary alveolar cells. The reaction is catalyzed by lactose synthase, a combination of β-1,4 galatosyl transferase and the essential cofactor α-lactalbumin in the Golgi compartment (Figure 5). α-Lactalbumin is limiting for lactose synthesis so the very low gene expression during pregnancy (Figure 4) prevents inappropriate lactose formation. The Km for glucose in the lactose synthase reaction is in the millimolar range so it is important that the glucose concentration in the alveolar cells be higher than usual, and, indeed, one of the characteristic features of the lactating mammary alveolar cell is its high cytoplasmic glucose concentration. Most cells maintain glucose concentrations in the range 0.1 to 0.4 mM. As first shown in rats [69] and later in women [70], the concentration of glucose in the milk is thought to be the same as that of the mammary alveolar cell. In women, glucose concentration in milk increases from about 0.34 mM to 1.5 mM during secretory activation [71,72] and decreases in proportion to milk volume during gradual weaning [70]. Comparison of fluxes of stable isotopes of glucose between full lactation and weaning suggested changes in the activity of a glucose transporter in the basal membrane; however, there was no effect of insulin on any milk parameters, including glucose concentration under conditions of a euglycemic clamp [73]. These findings in humans are consistent with the presence of a non-insulin dependent glucose transporter in the plasma membrane and, as described next, subsequent animal work has shown this transporter to be glucose transporter (GLUT)1 [74-76].

As shown in Figure 5b, mRNA for GLUT1 is upregulated significantly at parturition in the mouse, with a 280% increase in its amount. Studies have shown that this finding is reflected in the level of the protein as well and immunohistochemical results suggest localization to both basal and Golgi membranes. Treatment of lactating rats with bromocriptine to inhibit the production of PRL by the pituitary caused a 37% decrease in the level of GLUT1, while a combination of bromocriptine plus anti-growth hormone antibody suppressed the levels of GLUT1 by 90% [77]. Thus, GLUT1 is the major glucose transporter in the basal membrane and its expression is regulated by PRL at secretory activation when the demand for glucose for synthesis of lactose is greatly amplified.

The expression of hexokinase (HK)I was found to be constitutive in the rat mammary gland [78] whereas HKII was found to be present only at lactation. We have recently confirmed this finding at the protein level in the murine mammary gland (SM Anderson, unpublished data). While the differential functions of HKI and HKII are not completely

Figure 5



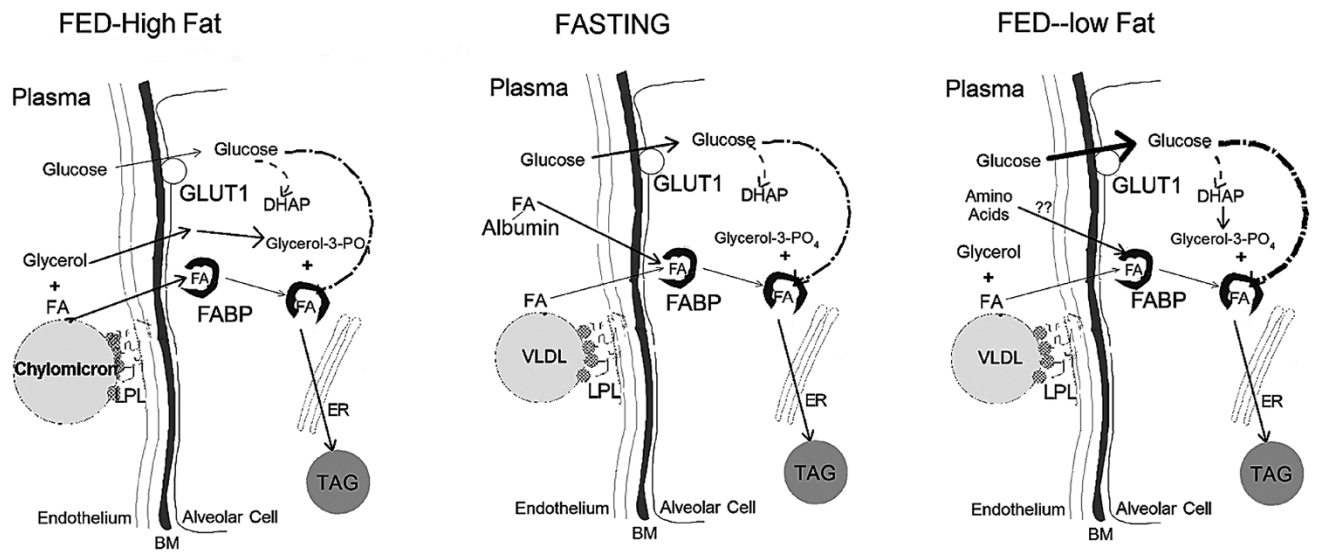
Regulation of glucose entry and utilization in the lactating mammary alveolar cell. **(a)** Glucose enters the cell via glucose transporter (GLUT)1, a non-insulin sensitive transporter. Free glucose enters the Golgi via GLUT1 where it is combined with UDP-galactose, also derived from glucose to make lactose. Since the Golgi membrane is not permeable to disaccharides, lactose draws water osmotically into the Golgi compartment. Glucose is also converted to glucose-6-PO_4 by hexokinase. The glucose-6-PO_4 can be isomerized by glucose-6-PO_4 isomerase to fructose-6-PO_4 from whence it is made into pyruvate or glycerol-3-PO_4. Glucose-6-PO_4 may also enter the pentose phosphate shunt, a major source of NADPH for lipid synthesis. Pyruvate enters the mitochondrion where two major products are ATP, which provides energy to synthetic processes in the cell, and citrate. Citrate has two fates: it serves as the substrate for fatty acid synthesis by conversion to malonyl-CoA and it can be converted to pyruvate through the malate shunt, which provides additional NADPH. NADPH, glycerol-3-PO_4, and pyruvate all contribute to triglyceride (TAG) synthesis. **(b)** Profile of GLUT1, citrate synthase, the citrate transporter, ATP citrate lyase, and glucose phosphate isomerase showing upregulation of the first four and down regulation of the last. **(c)** Profile of enzymes whose mammary expression is downregulated by a high fat diet. **(d)** Profile of enzymes that lead to synthesis of polyunsaturated, long chain fatty acids in the mouse mammary gland. P17, day 17 of pregnancy.

understood [79], the Km for glucose of HKI is 0.03 mM while that for HKII is 0.3 mM. The lower affinity of HKII would allow it to operate in the higher glucose environment of the lactating mammary cell and might contribute to the higher cytoplasmic glucose concentration necessary for lactose synthesis. Furthermore, HKII appears to have two catalytic sites with differential sensitivity to the feedback inhibition by glucose-6-PO_4, possibly allowing it to function under conditions inhibitory to HKI [80]. HKI is thought by Wilson [79] to be best positioned for the catabolic role of introducing glucose-6-PO_4 into the glycolytic chain for generation of ATP in organs like the brain. The type 2 enzyme is thought to be better suited for an anabolic role, being present in insulin sensitive tissues like skeletal muscle and adipose tissue as well as in the liver and lactating mammary gland [78]. In the last three tissues, glucose-6-PO_4 is directed into the pentose phosphate shunt to provide NADPH for lipid synthesis. Thus, the putative switch from HKI as the sole enzyme for glucose phosphorylation to the presence of both HKI and HKII may lead to both an increase in free glucose for lactose synthesis and increased activity of the pentose phosphate shunt.

Milk lipid synthesis and secretion

The mouse mammary gland is quite a unique lipid biosynthetic organ: the FVB mouse has about 2 g of mammary tissue estimated to secrete 5 ml of milk containing approximately 30% lipid, or 1.5 g of lipid per day. Over the course of a 20 day period of lactation, therefore, the dam secretes nearly 30 g of milk lipid, which is equivalent to her entire body weight! Over 98% of the fat in milk is triglyceride, which is synthesized by the condensation of fatty acids with glycerol derived from the plasma as free glycerol or from glucose through the synthesis of dihydroxyacetone phosphate (DHAP) (Figure 6). Fatty acids are either transported from the plasma or derived from the *de novo* synthesis of medium chain fatty acids from glucose in the epithelial cells [81,82]. Importantly, these functions are regulated both at secretory activation (Figure 3) and by the availability of exogenous lipid from either the diet or breakdown of adipose triglyceride (Figure 6). In the fed state under high fat diet conditions, dietary lipid is transferred to the mammary alveolar cell in the form of chylomicrons. The triglyceride in these particles is broken down by lipoprotein lipase into glycerol and fatty acids, both of which are taken up into the alveolar

Figure 6



Sources of substrate for milk lipid synthesis. The substrate for triacylglycerol synthesis depends on plasma sources of substrate. In the high fat fed animal, such as the usual lactating women who consumes up to 40% of her calories as lipid, fatty acids and glycerol for the synthesis of milk triglycerides (TAGs) originate in the chylomicra and very low density lipoprotein (VLDL) of the liver, whereas only about 10% of TAGs are derived from glucose. During a fasting state, fatty acids continue to be derived from the plasma, but now are transported to the mammary gland directly from the adipose tissue bound to albumin or indirectly as VLDL derived from the liver. In the animal fed a low fat diet, such as the laboratory mouse on the usual chow, a much larger proportion of the fatty acids for TAG synthesis are derived from glucose via the fatty acid synthetic pathways shown in Figure 4. BM, basement membrane; DHAP, dihydroxyacetone phosphate; ER, endoplasmic reticulum; FA, fatty acid; FABP, fatty acid binding protein; GLUT, glucose transporter; LPL, lipoprotein lipase.

cell to be used for triglyceride synthesis. The process is augmented by synthesis of dihydroxyacetone phosphate, a precursor of glycerol-3-PO_4, and fatty acids from glucose. During fasting, if adipose stores are replete with fat, fatty acids are transferred from adipose tissue bound to albumin and are available for transport into the mammary alveolar cell. Very low density lipoprotein (VLDL) from the liver also transports fatty acids to the mammary gland; however, the quantitative significance of this process is not well understood. Under conditions of a low fat diet, the type routinely encountered by lactating mice, at least under laboratory conditions, glucose becomes a most important substrate for both glycerol and fatty acid synthesis. In addition, a massive increase in amino acid transport suggests that certain amino acids could also enter the fatty acid synthetic pathway via the citric acid cycle. Milk lipid content data from cows infused with extra methionine support this notion [83].

The expression of several key enzymes for fatty acid synthesis is altered at the mRNA level at secretory activation, supporting the increased formation of fatty acids for triglyceride synthesis [84]. Notably, these findings are supported by a secondary analysis of data from a similar time course study by Stein and colleagues [55]. First, glucose-6-PO_4 isomerase is downregulated (Figure 5b) and the enzymes for substrate entry into the pentose phosphate shunt are upregulated such that glucose-6-PO_4 is shunted toward NADPH production, an essential reducing agent required for fatty acid synthesis. In early biochemical studies the increased activity of the pentose phosphate shunt at the onset of lactation was found to be a major contributor to increased lipogenesis at the onset of lactation [85]. As stated above, the presence of HKII may contribute to this increase. Second, a special form of aldolase, aldolase C, is upregulated to facilitate glycerol formation, similar to the myelinating brain. Expression of this gene product has been shown to be PRL sensitive [86]. Third, at least a portion of mitochondrial activity is subverted by the continued high expression of both pyruvate carboxylase and pyruvate dehydrogenase, leading to the synthesis of large quantities of citrate, which is then exported to the cytoplasm by the mitochondrial citrate transporter, whose expression is also significantly upregulated (Figure 5b). Fourth, increased expression of ATP citrate lyase (Figure 5b) facilitates the formation of acetyl CoA, used both for malonyl CoA synthesis and in the so-called malate shuttle, which leads to additional production of NADPH. Fifth, malonyl-CoA is utilized by fatty acid synthase in a sequence of seven reactions, each requiring two molecules of NADPH to produce fatty acids [87]. Finally, many of the genes that determine the nature of the fatty acids in the triglyceride are upregulated in the mouse mammary gland at secretory activation, including the sterol CoA dehydrogenase (SCD) types 1 and 2, the Δ5 desaturase, and elongase (Figure 5c,d) [84].

Many of these same genes have been found to be down-regulated in other tissues in response to high fat diets [88]. In particular, those genes that responded to a high fat diet in the mammary gland include citrate synthase, the citrate transporter, ATP citrate lyase, malic enzyme and SCD 1 and 2; their profiles are shown in Figure 5b,c. As we shall see below, the transcription factor SREBP1c is an important regulator of this effect.

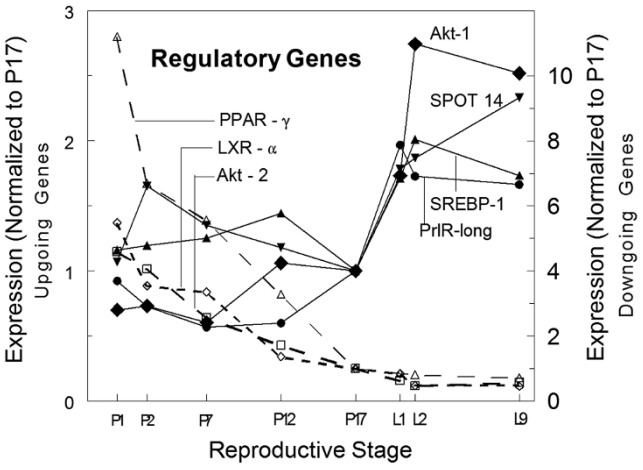
A study by Naylor and colleagues [21] employed three different mouse models that each exhibit failed lactation (PRLR-/- mice, Galanin-/- mice, and mice treated with a phosphomemic mutant of PRL (S179D), which inhibits lactogenesis and lactation), and identified 35 probe sets whose expression was altered in all three models. Consistent with our contention that regulation of lipid synthesis is an important part of secretory activation, a number of lipid synthesis genes were downregulated in this study, including ATP citrate lyase, aldolase C, SCD2, and an elongase, Elov5, suggesting that these genes are very sensitive to the lactation state of the animals, as might have been predicted from studies of their activation during the onset of lactation.

Regulation of lipid biosynthesis in the mammary gland: a central role for SREPB1?

Figure 7 shows the expression of a number of genes known to be involved in the regulation of lipid synthesis in liver, adipose tissue and the mammary gland. Two expression patterns emerge. Several factors, shown by the dotted lines in Figure 7, are downregulated up to ten-fold or more during pregnancy, consistent with the pattern of expression of adipocyte genes shown in Figure 3. These include Akt2, peroxisome-proliferator-activated receptor-gamma (PPAR-γ), and liver X receptor-beta (LXR-β), all known to be involved in regulation of lipid synthesis in adipose tissue. On the other hand, Akt1, the long form of the PRLR, SREBP1, and a protein thought to be important in the regulation of fatty acid synthesis, SPOT 14 [89], are all significantly upregulated between day 17 of pregnancy and day 2 of lactation. We have shown that Akt1 is dramatically upregulated at both the mRNA and protein levels during lactation [25,90] and have previously described the lactation failure that occurs in transgenic mice expressing constitutively activated myr-Akt1 in the mammary gland [25]. CLD formation occurs during early pregnancy in these mice, and they produce milk with an elevated lipid content (25% to 30% in normal FVB mice versus 65% to 70% in the transgenic mice by creamatocrit, a volume/volume method) [25]. Microarray studies comparing myr-Akt1 transgenic mice to FVB control mice indicate that expression of several key regulatory fatty acid biosynthetic enzymes is elevated during pregnancy in these transgenic mice, including SREBP1, Insig1, and Spot 14 (MC Rudolph, MC Neville, and SM Anderson, unpublished data).

Real-time PCR measurements confirm the upregulation of SREBP1 at the transition from pregnancy to lactation and

Figure 7



Expression of regulatory genes during secretory differentiation and activation. Dotted lines show genes that decrease at least ten-fold during pregnancy, consistent with adipocyte localization. The solid lines show genes that increase at least two-fold at the onset of lactation with much smaller changes during pregnancy. These genes are likely to be important in initiating metabolic changes at secretory activation. LXR, liver X receptor; P17, day 17 of pregnancy; PPAR, proliferator-activated receptor; PrlR, prolactin receptor; SREBP, sterol regulatory element binding protein.

show that it is the SREBP1c form that is increased [84], consistent with a role as a regulator of the expression of fatty acid biosynthetic enzymes [22]. The SREBPs are shuttled by the SREBP cleavage activating protein (SCAP), which binds to them at the endoplasmic reticulum and escorts them to the Golgi apparatus where they are proteolytically cleaved to generate active transcription factors. The newly generated fragment of SREBP1 is a member of the basic helix-loop-helix transcription factor family capable of activating the transcription of genes for the synthesis of fatty acids, while SREBP2 activates cholesterol synthesis. SREBP1 is able to activate its own transcription due to the presence of a sterol response element in the promoter region of the gene encoding SREBP1 [91,92]. Insig, an endoplasmic reticulum resident binding protein for the SREPB1s also has a sterol response element in its promoter; our finding that it is also upregulated at secretory activation is additional evidence for a role for SREBP1 at this time. The promoters for many of the genes involved in fatty acid biosynthesis contain sterol response elements in addition to binding sites for nuclear factor (NF)-Y, upstream factor, specific factor (SP)1 and SP3 [93-98]. The exact roles of these transcription factors are not understood but it is interesting to note that upstream factor 2 null mice have lowered production of milk that results in diminished pup weight gain [99]. In these mice the fat content of the milk is normal but the investigators observed a reduction in mammary wet weight, epithelial alveolar luminal area, expression of eukaryotic initiation factors 4E and 4G, and decreased plasma oxytocin.

A potential role for regulation of SREBP by Akt was revealed in a study that demonstrated activation of SREBP in cells expressing activated Akt, resulting in the induction of 24 genes involved in sterol/fatty acid biosynthesis and a statistically significant increase in saturated fatty acids in these cells [100]. Akt-dependent induction of fatty acid synthase, HMG-CoA synthase, and HMG-CoA reductase required the presence of SREBPs since induction of gene transcription was blocked by dominant negative mutants of SREBPs or siRNAs directed against SREBP1a, SREBP1c or SREBP2 [100]. Furthermore, we have observed that expression of SREBP is upregulated in the mammary glands of myr-Akt1 mice (MC Rudolph, MC Neville, and SM Anderson, unpublished data).

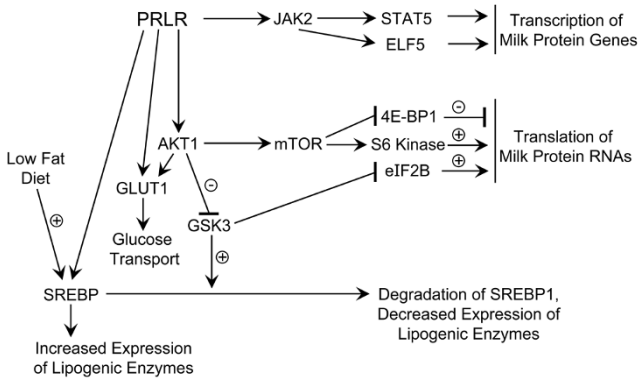
A mechanism by which Akt might regulate activation of SREBPs has been suggested by the recent work of Sundqvist and colleagues [101], who demonstrated that degradation of SREBP is regulated by phosphorylation of Thr426 and Ser430. These phosphorylation sites serve as recognition motifs for the binding of the SCF^{Fbw7} ubiquitin ligase. Binding of SCF^{Fbw7} to SREBP enhances the ubiquitination and degradation of SREBP [101]; thus, phosphorylation of SREBP results in the negative regulation of SREBP transcriptional activity, and the downregulation of SREBP-dependent genes. Phosphorylation of Thr426 and Ser430 is mediated by glycogen synthase kinse (GSK)-3 [101]; the link to Akt is provided by the fact that GSK-3 is the first known substrate of Akt, and phosphorylation of it by Akt inhibits its catalytic activity [102]. Therefore, expression of activated Akt in cells should inhibit the catalytic activity of GSK-3, leading to a decrease in the phosphorylation of SREBP by it and a decrease in the resulting degradation of SREBP; all of these changes should result in the increased transcription of SREBP-dependent genes, as has been observed *in vitro* [100]. Our model proposes that Akt plays a similar function in the *in vivo* mammary gland, acting as a major regulator of fatty acid synthesis at the onset of lactation by stabilizing SREBPs (Figure 8).

A recent study by Boxer and colleagues [103] indicated that Akt1-/- mice, but not Akt2-/- mice, exhibit lactation failure. The absence of Akt1 specifically resulted in a decrease in GLUT1 associated with the baso-lateral surface of mammary epithelial cells during lactation [103]. Milk protein RNA and proteins appeared to be the same in both wild-type and Akt1-/- mice, although there was a marked reduction in total milk volume. Microarray and quantitative RT-PCR analysis revealed decreased expression of RNA for stearoyl-CoA desaturase-2 and stearoyl-CoA desaturase-3 in the mammary glands of Akt1-/- mice compared to wild-type control mice, and an increase in diacylglycerol acyltransferase (DGAT)2 expression [103]. No changes in SREBP1a or SREBP1c expression were detected [103]. These authors note that ATP citrate lyase is an Akt substrate [104] and observed that phosphorylation of ATP citrate lyase was decreased in the Akt1-/- mice. Boxer and colleagues suggest that the decreased phosphorylation results in a lower catalytic activity of ATP citrate lyase to hydrolyze citrate to oxaloacetate and acetyl-CoA; however, it has not been established that phosphorylation of ATP citrate lyase by Akt has any effect upon its catalytic activity [104]. We note in Figure 3 that a decrease in the expression of genes involved in the β-oxidation of fatty acids occurred at secretory activation, and Boxer and colleagues noted that this decrease did not occur in the Akt1-/- mice, suggesting that the failure to suppress transcription of these genes could result in the increased degradation of lipid at the same time that mammary epithelial cells were synthesizing fatty acids for inclusion in the milk [103]. The results of Boxer and colleagues [103] clearly provide support for Akt1 playing an important role in regulating glucose transport and at least some aspects of lipid biosynthesis in mammary epithelial cells.

Future questions and conclusions

In characterizing mammary gland differentiation and lactation, most investigators have focused upon the synthesis of milk proteins, particularly the caseins, since they form a well-defined set of molecules that can be readily examined at either the RNA or protein level. Indeed, analysis of expression of genes such as those encoding caseins and WAP led to the discovery of the PRLR/JAK2/STAT5 signaling pathway that regulates their expression. However, expression of these genes is increased more during pregnancy than at the onset of lactation. It should be clear from the information presented in this review that the expression of another set of genes, a set that includes genes for the enzymes of lipid and cholesterol synthesis, glucose transport, and synthesis of lactose, must be under a different type of regulation since expression is increased only at secretory activation. We suggest a model by which PRL-induced activation of the PRLR in the absence of progesterone results in a dramatic increase in the activation of Akt, which in turn activates a number of milk synthetic programs, including activation and stabilization of nuclear SREBP1c (Figure 8), resulting in the increased expression of lipid biosynthetic enzymes. SREBP1c may be upregulated by a significant increase in demand for fatty acids at the onset of lactation, a demand that is not met by the normal low fat chow (8% of calories as fat) fed to mice. Thus, many of its downstream genes were down-regulated in response to a high fat diet (40% of calories as fat) [84]. A recent study shows a response to lipid feeding in cows that is consistent with this interpretation [105]. It is not clear whether PRL directly regulates expression of SREBP1 during secretory activation, or whether other factors such as IGF-I contribute to this process. Although it does not appear in the model shown in Figure 8, it will be important to determine the role of Spot 14 in regulating lipid biosynthesis; Spot 14 knockout mice exhibit a lactation defect and the milk of these mice have less triglyceride, apparently resulting from reduced *de novo* lipid synthesis [106]. Whether Spot 14 and SREBP independently regulate different aspects of lipid

Figure 8



Model predicting critical regulators of secretory activation in the mammary gland. The transcription of milk protein genes is induced by the binding of prolactin to its receptor (the PRLR) and regulated by the STAT5 and ELF5 transcription factors. Translation of milk protein genes may be enhanced by Akt1 acting on their substrates, such as glycogen synthase kinse (GSK)-3/eIF2B, mammalian target of rapamycin (mTOR)/S6 kinase and mTOR/4E-BP1. Transcription of glucose transporter (GLUT)1 may be induced by the PRLR and Akt1 may contribute to either the expression or localization of GLUT1. The response of the mammary gland to dietary fat is sensed by sterol regulatory element binding protein (SREBP), and the stability of SREBP may be enhanced by Akt1-mediated inhibition of GSK3, since phosphorylation of SREBP by GSK3 enhances the ubiquitination and degradation of SREBP in the nucleus.

biosynthesis or whether crosstalk exists between these molecules remains to be examined.

PRL also stimulates the expression of GLUT1, and it may stimulate the translocation of GLUT1 to various intracellular compartments that support importation of glucose and the subsequent synthesis of lactose. In transformed cells, Akt1 appears to stimulate translocation of GLUT1 to the plasma membrane [107,108]; however, there is currently no indication of what signaling molecules regulate the translocation of GLUT1 to the baso-lateral surface of mammary epithelial cells and to the Golgi at secretory activation. While Akt1 could control the translocation of GLUT1 to both of these cellular locations, it is likely that other molecules, for example, IGF-1, contribute to the differential translocation of GLUT1.

In our model, Stat5 regulates expression of milk protein genes and Elf5 may do the same. Akt1 does not influence the expression of the milk protein genes; however, we believe that it could influence the translation of these genes and possibly other lactose synthesis genes during lactation through known substrates for mTOR and S6 kinase that lie downstream of Akt1 (Figure 8). Such effects might account for the reduction in total milk synthesis in the Akt null mouse [103]. A nice experiment here could be to examine the effect of Akt1 upon the loading of RNAs for milk proteins into polysomes using the tetracycline-regulated expression of Akt1.

This article is part of a review series on *Key stages in mammary gland development*, edited by Charles Streuli.

Other articles in the series can be found online at http://breast-cancer-research.com/articles/review-series.asp?series=BCR_Keystages

Our model predicts profound defects in lipid biosynthesis in mammary epithelial cells from Akt1 null mice in the presence of normal milk protein expression, a prediction that is consistent with the recent analysis of lactation defects in the Akt1 knockout mouse [103]. While the analysis of these mice has confirmed a significant role for Akt in milk lipid synthesis, many questions remain, including how progesterone downregulates milk synthesis during pregnancy, how diet and Spot 14 contribute to the regulation of lipid biosynthesis, how other growth factors such as IGF-1 modulate secretory activation, and how glucocorticoids contribute to the regulation of lipid synthetic enzymes. Clearly, there is still much work to be done.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

The authors would like to thank the members of the Mammary Gland Biology Program Project Grant at the University of Colorado Health Sciences Center for discussions and sharing of ideas. Research in our labs (SMA, JLM, MCN) is supported by PO1-HD38129. JLM was also supported by RO1-HD045962.

References

1. Schmitt-ney M, Doppler W, Ball RK, Groner B: **b-casein gene promoter activity is regulated by the hormone mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor.** *Mol Cell Biol* 1991, **11:**3745-3755.
2. Gouilleux F, Wakao H, Mundt V, Groner B: **Prolactin induces phosphorylation of Tyr694 of STAT5 (MGF), a prerequisite for DNA binding and induction of transcription.** *EMBO J* 1994, **13:** 4361-4369.
3. Harris J, Stanford PM, Sutherland K, Oakes SR, Naylor MJ, Robertson FG, Blazek KD, Kazlauskas M, Hilton HN, Wittlin S, *et al.*: **Socs2 and Elf5 mediate prolactin-induced mammary gland development.** *Mol Endocrinol* 2006, **20:**1177-1187.
4. Oakes S, Hilton H, Ormandy C: **Key stages in mammary gland development. The alveolar switch: coordinating the proliferative cues and cell fate decisions that drive the formation of lobuloalveoli from ductal epithelium.** *Breast Cancer Res* 2006, **8:**207.
5. Richert MM, Schwertfeger KL, Ryder JW, Anderson SM: **An atlas of mouse mammary gland development.** *J Mam Gland Bio Neo* 2000, **5:**227-241.
6. Hens JR, Wyszomerski JJ: **Molecular mechanisms involved in the formation of the embryonic mammary gland.** *Breast Cancer Res* 2005, **7:**220-224.
7. Lyons WR, Li CH, Johnson RE: **The hormonal control of mammary growth and lactation.** *Recent Prog Horm Res* 1958, **14:**219-248.
8. Sternlicht MD: **The cues that regulate ductal branching and morphogenesis.** *Breast Cancer Res* 2006, **8:**201-212.
9. Andres A-C, Strange R: **Apoptosis in the estrus and menstral cycle.** *J Mam Gland Bio Neo* 1999, **4:**221-228.
10. Schedin P, Mitrenga T, Kaeck M: **Estrous cycle regulation of mammary epithelial cell proliferation, differentiation, and death in Sprague-Dawley rat: a model for investigation the roles of estrous cycling in mammary carcinogenesis.** *J Mam Gland Bio Neo* 2000, **5:**211-225.

11. Lydon JP, Demayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA Jr, Shyamala G, Conneely OM, O'Malley BW: **Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities.** *Genes Dev* 1995, **9:**2266-2278.
12. Muldoon TG: **Prolactin mediation of estrogen-induced changes in mammary tissue estrogen and progesterone receptors.** *Endocrinology* 1987, **121:**141-149.
13. Koseki Y, Cole D, Matsuzawa A, Costlow ME: **Prolactin regulation of estrogen and progesterone receptors in normal and neoplastic mouse mammary tissue.** *Jpn J Cancer Res* 1987, **78:**1105-1111.
14. Brisken C, Heineman A, Chavarria T, Elenbaas B, Tan J, Dey SK, McMahon JA, McMahon AP, Weinberg RA: **Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling.** *Genes Dev* 2000, **14:**650-654.
15. Fata JE, Kong Y-Y, Li J, Sasaki T, Irie-Saski J, Moorehead RA, Elliott R, Scully S, Voura EB, Lacey DL, *et al.*: **The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development.** *Cell* 2000, **103:**41-50.
16. Ormandy CJ, Binart N, Kelly PA: **Mammary gland development in prolactin receptor knockout mice.** *J Mamm Gland Biol Neoplasi* 1997, **2:**355-364.
17. Goffin V, Binart N, Clement-Lacroix P, Bouchard B, Bole-Feysot C, Edery M, Lucas BK, Touraine P, Pezet A, Maaskant R, *et al.*: **From the molecular biology of prolactin and its receptor to the lessons learned from knockout mice models.** *Genetic Analysis* 1999, **15:**189-201.
18. Ormandy CJ, Naylor MJ, Harris J, Robertson F, Horseman ND, Lindeman GJ, Kelly PA: **Investigation of the transcriptional changes underlying functional defects in the mammary glands of prolactin receptor knockout mice.** *Recent Prog Horm Res* 2003, **58:**297-323.
19. Brisken C, Ayyannan A, Nguyen C, Heineman A, Reinhardt F, Jan T, Dey SK, Dotto GP, Weinberg RA: **IGF-2 is a mediator of prolactin-induced morphogenesis in the breast.** *Dev Cell* 2002, **3:**877-887.
20. Hovey RC, Harris J, Hadsell DL, Lee AV, Ormandy CJ, Vonderhaar BK: **Local insuln-like growth factor-II mediates prolactin-induced mammary gland development.** *Mol Endocrinol* 2003, **17:**460-471.
21. Naylor MJ, Oakes SR, Gardiner-Garden M, Harris J, Blazek K, Ho TWC, Li FC, Wynick D, Walker AM, Ormandy CJ: **Transcriptional changes underlying the secretory activation phase of mammary gland development.** *Mol Endocrinol* 2005, **19:**1868-1883.
22. Goldstein JL, Bose-Boyd RA, Brown MS: **Protein sensors for membrane sterols.** *Cell* 2006, **124:**35-46.
23. Traurig HH: **A radiographic study of cell proliferation in the mammary gland of the pregnant mouse.** *Anatomical Record* 2001, **159:**239-248.
24. Elias JJ, Pitelka DR, Armstrong RC: **Changes in fat cell morphology during lactation in the mouse.** *Anatomical Record* 1973, **177:**533-547.
25. Schwertfeger KL, McManaman JL, Palmer CA, Neville MC, Anderson SM: **Expression of constitutively activated Akt in the mammary gland leads to excess lipid synthesis during pregnancy and lactation.** *J Lipid Res* 2003, **44:**1100-1112.
26. Palmer CA, Lubon H, McManaman JL: **Transgenic mice expressing recombinant human protein C exhibit defects in lactation and impaired mammary gland development.** *Transgenic Res* 2003, **12:**283-292.
27. Ho MY, Murphy D: **A bovine oxytocin transgene in mice: expression in the female reproductive organs and regulation during pregnancy, parturition and lactation.** *Mol Cell Endo* 1997, **136:**15-21.
28. Wagner K-U, Young WS, Liu X, Ginns EI, Li M, Furth PA, Hennighausen L: **Oxytocin and milk removal are required for postpartum mammary gland development.** *Genes Function* 2000, **1:**233-244.
29. Stinnakre MG, Vilotte J-L, Soulier S, Mercier JC: **Creation and phenotypic analysis of alpha-lactalbumin-deficient mice.** *Proc Natl Acad Sci USA* 1994, **91:**6544-6548.
30. Ogg SL, Weldon AK, Dobbie L, Smith AJH, Mather IH: **Expression of butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets.** *Proc Natl Acad Sci USA* 2004, **101:**10084-10089.
31. Vorbach C, Scriven A, Capecchi MR: **The housekeeping gene xanthine oxidoreductase is necessary for milk fat droplet enveloping and secrection: gene sharing in the lactating mammary gland.** *Genes Dev* 2002, **16:**3223-3235.
32. Blackman B, Russell T, Nordeen S, Medina D, Neville M: **Claudin 7 expression and localization in the normal murine mammary gland and murine mammary tumors.** *Breast Cancer Res* 2005, **7:**R248-R255.
33. Rudolph MC, McManaman JL, Hunter L, Phang T, Neville MC: **Functional development of the mammary gland: Use of expression profiling and trajectory clustering to reveal changes in gene expression during pregnancy, lactation, and involution.** *J Mam Gland Bio Neo* 2003, **8:**287-307.
34. Luetteke NC, Qiu TH, Fenton SE, Troyer KL, Riedel RF, Chang A, Lee DC: **Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development.** *Development* 1999, **126:**2739-2750.
35. Mellenberger RW, Bauman DE: **Metabolic adaptations during lactogenesis: fatty acid synthesis in rabbit mammary tissue during pregnancy and lactation.** *Biochem J* 1974, **138:**373-379.
36. McManaman JL, Palmer CA, Anderson S, Schwertfeger K, Neville MC: **Regulation of milk lipid formation and secretion in the mouse mammary gland.** *Adv Exp Med Biol* 2004, **554:**263-279.
37. Neville MC, McFadden TB, Forsyth IA: **Hormonal regulation of mammary differentiation and lactation.** *J Mam Gland Bio Neo* 2002, **7:**49-66.
38. Bartholomeusz RK, Bruce NW, Martin CE, Hartmann PE: **Serial measurement of arterial plasma progesterone levels throughout gestation and parturition in individual rats.** *Acta Endocrinol* 1976, **82:**2683-2694.
39. Kuhn NJ: **Progesterone withdrawal as the lactogenic trigger in the rat.** *J Endocrinol* 1969, **44:**39-54.
40. Silberstein GB, Van Horn K, Shyamala G, Daniel CW: **Progesterone receptors in the mouse mammary duct: Distribution and developmental regulation.** *Cell Growth Diff* 1996, **9:**945-952.
41. Djiane J, Durand P: **Prolactin-progesterone antagonism in self regulation of prolactin receptors in the mammary gland.** *Nature* 1977, **266:**641-643.
42. Mizoguchi Y, Kim JY, Sasaki T, Hama T, Sasaki M, Enami J, *et al.*: **Acute expression of the PRL receptor gene after ovariectomy in midpregnant mouse mammary gland.** *Endocrine J* 1996, **43:**537-544.
43. Rosen JM, O'Neal DL, McHugh JE, Comstock JP: **Progesterone-mediated inhibition of casein mRNA and polysomal casein synthesis in the rat mammary gland during pregnancy.** *Biochemistry* 1978, **17:**290-297.
44. Nguyen D-A, Parlow AF, Neville MC: **Hormonal regulation of tight junction closure in the mouse mammary epithelium during the transition from pregnancy to lactation.** *Endocrinology* 2001, **170:**347-356.
45. Hartmann P, Trevethan P, Shelton JN: **Progesterone and oestrogen and the initiation of lactation in ewes.** *J Endocrinol* 1973, **59:**249-259.
46. Loizzi RF: **Progesterone withdrawal stimulates mammary gland tubulin polymerization in pregnant rats.** *Endocrinology* 1985, **116:**2543-2547.
47. Deis RP, Delouis C: **Lactogenesis induced by ovariectomy in pregnany rats and its regulation by oestrogen and progesterone.** *J Steroid Biochem* 1983, **18:**687-690.
48. Ball RK, Friis RR, Schoenenberger CA, Doppler W, Groner B: **Prolactin regulation of b-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line.** *EMBO J* 1988, **7:**2089-2095.
49. Hollman KH: **Cytology and fine structure of the mammary gland.** In *Lactation.* Edited by Larson BL, Smith VR. New York: Academic Press; 1974:3-95.
50. Patton S, Huston GE, Montgomery PA, Josephson RV: **Approaches to the study of colostrum: the onset of lactation.** In *Human Lactation 2: Maternal and Environmental Factors.* Edited by Hamosh M, Goldman AS. New York: Plenum Press; 1986:231-240.
51. Jones EA: **Studies on the particulate lactose synthesis if mouse mammary gland and the role of a-lactalbumin in the initiation of lactose synthesis.** *Biochem J* 1972, **126:**67-78.
52. Kuhn NJ: **Lactogenesis in the rat.** *Biochem J* 1968, **106:**734-748.

53. Stacey A, Schnieke A, Kerr M, Scott A, McKee C, Cottingham I, Binas B, Wilde C, Colman A: **Lactation is disrupted by a-lactalbumin deficiency and can be restored by human a-lactalbumin gene replacement in mice.** *Proc Natl Acad Sci* 1995, **92:** 2835-2839.
54. Palmer CA, Neville MC, Anderson SM, McManaman JL. **Analysis of lactation defects in transgenic mice.** *J Mamm Gland Biol Neoplasia* 2006, **12:**269-282.
55. Stein T, Morris J, Davies C, Weber-Hall S, Duffy MA, Heath V, Bell A, Ferrier R, Sandilands G, Gusterson B: **Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3.** *Breast Cancer Res* 2004, **6:**R75-R91.
56. Rosen JM, Wyszomierski SL, Hadsell D: **Regulation of milk protein gene expression.** *Annu Rev Nutr* 1999, **19:**407-436.
57. Kazansky AV, Raught B, Lindsey SM, Wang Y-F, Rosen JM: **Regulation of mammary gland factor/Stat5 during mammary gland development.** *Mol Endocrinol* 1995, **9:**1598-1609.
58. Liu X, Robinson GW, Hennighausen L: **Activation of Stat5a and Stat5b by tyrosine phosphorylation is tightly linked to mammary gland differentiation.** *Mol Endocrinol* 1996, **10:**1496-1506.
59. Liu X, Robinson GW, Wagner K-U, Garrett L, Wynshaw-Boris A, Hennighausen L: **Stat5a is mandatory for adult mammary gland development and lactogenesis.** *Genes Dev* 1997, **11:** 179-186.
60. Choi KM, Barash I, Rhoads RE: **Insulin and prolactin synergistically stimulate b-casein messenger ribonucleic acid translation by cytoplasmic polyadenylation.** *Mol Endocrinol* 2004, **18:** 1670-1686.
61. Moshel Y, Rhoads RE, Barash I: **Role of amino acids in translational mechanisms governing milk protein synthesis in murine and ruminant mammary epithelial cells.** *J Cell Biochem* 2006, **98:**685-700.
62. GIngras A-C, Kennedy SG, O'Leary MA, Sonenberg N, Hay N: **4E-bp1, a repressor of mRNA translation, is phosphorylated by the Akt(PKB) signaling pathway.** *Genes Dev* 1998, **12:**502-513.
63. Redpath NT, Foulstone EJ, Proud CG: **Regulation of translocation elongation factor-2 by insulin via a rapamycin-sensitive signalling pathway.** *EMBO J* 1996, **15:**2291-2297.
64. Jefferies HB, Rumagalli S, Dennis PB, Reinhard C, Pearson RB, Thomas G: **Rapamycin suppresses 5'TOP mRNA translation though inhibition ofp70s6k.** *EMBO J* 1997, **16:**3693-3704.
65. Thompson EJ, Shanmugam K, Hattrup CL, Kotlarczyk KL, Gutierrez A, Bradley JM, Mukherjee P, Gendler SJ: **Tyrosines in the MUC1 cytoplasmic tail modulate transcription via the extracellular signal-regulated kinase 1/2 and nuclear factor-kB pathways.** *Mol Cancer Res* 2006, **4:**489-497.
66. Scibetta AG, Albanese I, Morris J, Cooper L, Downward J, Rowe PP, Taylor-Papadimitriou J: **Regulation of MUC1 expression in human mammary cell lines by the c-ErbB2 and Ras signaling pathways.** *DNA Cell Biol* 2001, **20:**265-274.
67. Adriance MC, Gendler SJ: **Downregulation of Muc1 in MMTV-c-Neu tumors.** *Oncogene* 2004, **23:**697-705.
68. Dunbar ME, Dann P, Brown CW, Van Houton J, Dreyer B, Philbrick WP, Wyszomierski JJ: **The calcium-sensing receptor regulates mammary gland parathyroid hormone-related protein production and calcium transport.** *J Clin Invest* 2004, **113:**598-608.
69. Kuhn NJ, White A: **Milk glucose as an index of the intracellular glucose concentration of rat mammary gland.** *Biochem J* 1975, **152:**118-128.
70. Neville MC, Hay WW Jr, Fennessey P: **Physiological significance of the concentration of human milk glucose.** *Protoplasma* 1990, **159:**118-128.
71. Neville MC: **Lactogenesis in women: Evidence for a cascade of cellular events.** In *Handbook of Composition of Milks*. Edited by Jensen RG. San Diego: Academic press; 2005.
72. Allen JC, Keller RP, Archer PC, Neville MC: **Studies in human lactation: 6. Milk composition and daily secretion rates of macronutrients in the first year of lactation.** *Am J Clin Nutr* 1991, **54:**69-80.
73. Neville MC, Sawicki V, Hay WW Jr: **Effect of fasting, elevated plasma glucose and plasma insulin concentrations on milk secretion in women.** *J Endocrinol* 1993, **139:**165-173.
74. Camps M, Vilaro S, Testar X, Palacin M, Zorzano A: **High and polarized expression of GLUT1 glucose transporters in epithelial cells from mammary gland: acute down-regulation of GLUT1 carries by weaning.** *Endocrinology* 1994, **134:**924-934.
75. Nemeth BA, Tsang SWY, Geske RS, Haney PM: **Golgi targeting of the GLUT1 glucose transporter in lactating mouse mammary gland.** *Pediatr Res* 2000, **47:**444-450.
76. Haney PM: **Localization of the GLUT1 glucose transporter to brefeldin A-sensitive vesicles of differentiated CIT_3 mouse mammary epithelial cells.** *Cell Biology Int* 2001, **25:**277-288.
77. Fawcett HAC, Baldwin SA, Flint DJ: **Hormonal regulation of the glucose transporter GLUT1 in the lactating rat mammary gland.** *Biochem Soc Trans* 1991, **20:**17S.
78. Kaselonis GL, McCabe ERB, Gray SM: **Expression of hexokinase 1 and hexokinase 2 in mammary tissue of nonlactating and lactating rats: evaluation by RT-PCR.** *Mol Genet Metab* 1999, **68:**371-374.
79. Wilson JE: **Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function.** *J Exp Biol* 2003, **206:**2049-2057.
80. Ardehali H, Printz RL, Whitesell RR, May JM, Granner DK: **Functional interaction between the N- and C-terminal halves of human hexokinase II.** *J Biol Chem* 1999, **274:**15986-15989.
81. Mather IH, Keenan T: **Origin and secretion of milk lipids.** *J Mam Gland Biol Neoplasia* 1998, **3:**259-274.
82. Neville MC, Picciano MF: **Regulation of milk lipid synthesis and composition.** *Annu Rev Nutr* 1997, **17:**159-184.
83. Varvikko T, Vanhatalo A, Jalava T, Huhtanen P: **Lactation and metabolic responses to graded abomasal doses of methionine and lysine in cows fed grass silage diets.** *J Dairy Sci* 1999, **82:**2659-2673.
84. Rudolph MC, McManaman JL, Phang T, Russel T, Kominsky DM, Serkova N, *et al*.. **Metabolic regulation in the lactating mammary gland: A lipid synthesizing machine.** *Physiol Genomics* 2007, **28:**323-336.
85. Smith S, Abraham S: **The composition of milk fat.** *Adv Lipid Res* 1975, **13:**195-239.
86. Matsuda M, Lockefeer JA, Horseman ND: **Aldolase C/zebrin gene regulation by prolactin during pregnancy and lactation.** *Endocrine* 2003, **20:**91-100.
87. Smith S: **The animal fatty acid synthase: one gene, one polypeptide, seven enzymes.** *FASEB J* 1994, **8:**1248-1259.
88. Horton JD, Goldstein JL, Brown MS: **SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver.** *J Clin Invest* 2002, **109:**1125-1131.
89. Brown S, Maloney M, Kinlaw W: **"Spot 14" protein functions at the pretranslational level in the regulation of hepatic metabolism by thyroid hormone and glucose.** *J Biol Chem* 1997, **272:** 2163-2166.
90. Schwertfeger KL, Richert MM, Anderson SM: **Mammary gland involution is delayed by activated Akt in transgenic mice.** *Mol Endocrinol* 2001, **15:**867-881.
91. Sato R, Inoue J, Kawabe Y, Kodama T, Takano T, Maeda M: **Sterol-dependent transcriptional regulation of sterol regulatory element-binding protein-2.** *J Biol Chem* 1996, **271:**26461-26464.
92. Memiya-Kudo M, Shimano H, Yoshikawa T, Yahagi N, Hasty AH, Okazaki H, Tamura Y, Shionoiri F, Iizuka Y, Ohashi K, *et al*.: **Promoter analysis of the mouse sterol regulatory element-binding protein-1c gene.** *J Biol Chem* 2000, **275:**31078-31085.
93. Magana MM, Osborne TF: **Two tandem binding sites for sterol regulatory element binding proteins are required for sterol regulation of fatty-acid synthase promoter.** *J Biol Chem* 1996, **271:**32689-32694.
94. Magana MM, Lin SS, Dooley KA, Osborne TF: **Sterol regulation of acetyl coenzyme A carboxylase promoter requires two interdependent binding sites for sterol regulatory element binding proteins.** *J Lipid Res* 1997, **38:**1630-1638.
95. Oh SY, Park SK, Kim JW, Ahn YH, Park SW, Kim KS: **Acetyl-CoA carboxylase b gene is regulated by sterol regulatory element-binding protein-1 in liver.** *J Biol Chem* 2003, **278:**28410-28417.
96. Horton JD, Goldstein JL, Brown MS: **Activators of the complete program of cholersterol and fatty acid synthesis in the liver.** *J Clin Invest* 2002, **109:**1125-1135.
97. Travers MT, Vallance AJ, Gourlay HT, Gill CA, Klein I, Bottema CB, Barber MC: **Promoter I of the ovine acetyl-CoA carboxy-**

lase-alpha-gene: An E-box motif at -114 in the proximal promoter binds upstream stimulatory factor (USF)-1 and USF-2 and acts as an insulin-response sequence in differentiating adipocytes. *Biochem J* 2001, **359:**273-284.
98. Schweizer M, Roder K, Zhang L, Wolf SS: **Transcription factors acting on the promoter of rat fatty acid synthase gene.** *Biochem Soc Trans* 2002, **30:**1070-1072.
99. Hadsell DL, Bonnette S, George J, Torres D, Klementidis Y, Gao S, Haney PM, Summy-Long J, Soloff MS, Parlow AF, *et al.*: **Diminished milk synthesis in Upstream Stimulatory Factor 2 null mice is associated with decreased circulating oxytocin and decreased mammary gland expression of eukaryotic initiation factors 4E and 4G.** *Mol Endocrinol* 2003, **17:**2251-2267.
100. Porstmann T, Griffiths B, CHung Y-L, Delpuech O, Griffiths JR, Downward J, Schulze A: **PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP.** *Oncogene* 2005, **24:**6465-6481.
101. Sundqvist A, Goechea-Alonso MT, Ye X, Lukiyanchuk V, Jin J, Harper JW, Ericsson J: **Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCFFbw7.** *Cell Metabolism* 2005, **1:**379-391.
102. Cross DA, Alessi DR, Cohen P, Andejelkovic M, Hemmings BA: **Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B.** *Nature* 1995, **378:**785-789.
103. Boxer RB, Stairs DB, Dugan KD, Notarfrancesco KL, Portocarrero CP, Keister BA, Belka GK, Cho H, Rathmell JC, Thompson CB, *et al.*: **Isoform-specific requirement for Akt1 in the developmental regulation of cellular metabolism during lactation.** *Cell Metabolism* 2006, **4:**475-490.
104. Berwick DC, Hers I, Heesom KJ, Moule SK, Tavare JM: **The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes.** *J Biol Chem* 2002, **277:** 33895-33900.
105. Harvatine KJ, Bauman DE: **SREBP1 and thyroid hormone responsive Spot 14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA.** *J Nutr* 2006, **136:**2468-2474.
106. Zhu Q, Anderson GW, Mucha GT, Parks EJ, Metkowski JK, Mariash CN: **The Spot 14 protein is required for *de novo* lipid synthesis in the lactating mammary gland.** *Endocrinology* 2005, **146:**3343-3350.
107. Edinger A, Thompson CB: **Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake.** *Mol Biol Cell* 2002, **13:**2276-2288.
108. Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB: **Growth factors can influence cell growth and survival through effects on glucose metabolism.** *Mol Cell Biol* 2001, **21:**5899-5912.