STEROL REGULATORY ELEMENT BINDING PROTEIN (SREBF-1) AND DIETARY LIPID REGULATION OF FATTY ACID SYNTHESIS IN THE MAMMARY EPITHELIUM

Michael C. Rudolph², Jenifer Monks¹, Valerie Burns¹, Meridee Phistry¹, Russell Marians¹, Monica R. Foote³, Dale E. Bauman³, Steven M. Anderson²*, and Margaret C. Neville¹*

Department of Physiology and Biophysics¹, University of Colorado Denver, School of Medicine, Aurora, CO 80045
Department of Pathology and Program in Molecular Biology², University of Colorado Denver, School of Medicine, Aurora, CO 80045
³Department of Animal Science, Cornell University, Ithaca, NY 14853.
*Co-Senior authors

Running Head: Regulation of de novo lipogenesis in the mammary gland

Address correspondence to:
Margaret C. Neville, Ph.D.,
Mail Stop 8309,
12800 E. 19th Ave,
Aurora, CO 80045,
Fax: 303 724-3512,
email: peggy.neville@ucdenver.edu

Abbreviations and gene symbols: Acaca, ACACA Acetyl-CoA carboxylase 1α; Acly, ACLY ATP citrate lyase; Adfp, ADFP adipophilin; CHREBP, carbohydrate response element binding protein; Fasn, FASN fatty acid synthase; MEC, mammary epithelial cell; MUFA, monounsaturated fatty acids; PGC-1, ; peroxisome proliferator-activated receptor-gamma-coactivator; PUFA, polyunsaturated fatty acids; qPCR, quantitative real time polymerase chain

Copyright © 2010 by the American Physiological Society.
reaction; QTL, quantitative trait locus; Thrsp, THRSP Thyroid hormone responsive Spot 14 homolog; Srebfl, SEBF1 sterol regulatory element binding transcription factor 1; SCAP, SREBF cleavage activating protein; Scd2, SCD2 stearoyl-coenzyme A desaturase 2; SLC25a1, SLC25A1 mitochondrial citrate transporter; Sp1/Sp3, transcription factors Sp1, Sp3; TAG, triacylglycerol; WTL, whole tissue lysate; USF, upstream stimulatory factors. For gene and protein names we used the standard nomenclature established by the International Committee on Standardized Genetic Nomenclature for Mice (http://www.informatics.jax.org/nomen/short_gene.shtml). In this convention gene names are lower case and italicized and proteins are upper case, regular type.
Abstract

The lactating mammary gland synthesizes large amounts of triglyceride from fatty acids derived both from the blood and from de novo lipogenesis. The latter is significantly increased at birth of the young and decreased when additional dietary fatty acids become available. To begin to understand the molecular regulation of de novo lipogenesis we tested the hypothesis that the transcription factor SREBF-1c is a primary regulator of this system. The expression of Srebf-1c mRNA and that of six of its known target genes increased at least 2.5 fold at parturition. However, Srebf-1c null mice showed only minor deficiencies in lipid synthesis during lactation possibly due to compensation by Srebf-1a expression. To abrogate the function of both isoforms of Srebf-1, we bred mice to obtain a mammary epithelial specific deletion of SCAP, the SREBF escort protein. These dams showed a significant lactation deficiency and expression of mRNA for Fasn, Insig1, Slc25a1 and Scd2 was reduced 3-fold or more; however, the mRNA levels of Acaca and Acly were unchanged. Further, a diet with 46% fat significantly decreased de novo fatty acid synthesis and reduced the protein levels of ACACA, ACLY and FASN significantly with no change in their mRNAs. These data lead us to conclude that two modes of regulation exist to control fatty acid synthesis in the mammary gland of the lactating mouse: the well-known SREBP-1 system and a novel mechanism that acts at the post-transcriptional level in the presence of both SCAP deletion and high-fat feeding to alter enzyme protein.

Keywords: Milk triglyceride; mammary gland; de novo lipogenesis; SREBF-1; SCAP, lactation,
The mammary gland is one of three major lipid synthesizing organs in the body, the others being liver and adipose tissue (Wakil and Abu-Elheiga, 2009). This fact was recognized by the early biochemists who used the ready availability of mammary tissue from lactating ruminants and rodents to define many of the pathways of fatty acid biosynthesis (Abraham and Chaikoff, 1959, Bauman and Davis, 1974). However, mammary fatty acid synthesis differs from that in adipose tissue in that saturated fatty acids with a chain length between 6 and 14 carbons are the major product of de novo fatty acid synthesis in mammary tissue (Smith and Dils, 1966). Longer chain fatty acids in milk were shown to originate either from the diet or from mobilization of adipose tissue triglycerides (Smith, 1980); they are generally termed “preformed” fatty acids. Although the presence of medium chain fatty acids in milk had been known since the 1960’s, it was not until 1978 that the mechanism was elucidated: Mammary alveolar cells contain a special enzyme, thioesterase II, that terminates fatty acid synthesis after the addition of 8 to 16 carbons (Libertini and Smith, 1978). Mouse milk was shown to contain between 15% and 40% medium chain fatty acids (MCFA), 20% to 30% C:16 (from both de novo lipogenesis and preformed fatty acids), and 30% to 70% preformed fatty acids with a chain length > C:16 (Smith, et al., 1969) depending on strain and diet. Δ9-fatty acid desaturases present in the gland probably increase the proportion of C16:1 and C18:1 but have little effect on the saturation of MCFA due to desaturase specificity (Ntambi and Miyazaki, 2004).

Fatty acid synthesis in the mammary gland is regulated both developmentally and by dietary fat. In a particularly striking early study of incorporation of C\textsuperscript{14} from glucose-3,4-C\textsuperscript{14} into fatty acids by slices obtained from the lactating rat mammary gland, Abraham and Chaikoff (Abraham and Chaikoff, 1959) were able to show that 40% to 70% of the fatty acid C\textsuperscript{14} was derived from glucose metabolized through the pentose phosphate shunt. Slices from the gland of pregnant or weaned animal did not utilize carbons derived from this pathway to synthesize fatty acids, demonstrating the adaptability of this system. This and many other early studies in various species showed that fatty acid synthesis in the mammary gland turns on abruptly at the initiation of lactation. Other studies show that fatty acid synthesis is reduced by high fat diets, although the data are somewhat confusing because whole tissue, which contains variable amounts of adipose tissue, was used in many of these. A seminal study by Munday and Williamson circumvented this issue, measuring fatty acid synthesis by the incorporation of \textsuperscript{3}H\textsubscript{2}O into mammary lipid in vivo as well as in vitro in isolated mammary
acini from lactating rats. They found that a diet containing 20% fat reduced fatty acid synthesis by 95% compared to the rate observed in rats fed a standard low fat chow (Munday and Williamson, 1987). Our question in the present work is whether SREBF-1 is involved in either developmental or fatty acid regulation of mammary lipogenesis.

In tissues that actively synthesize triacylglycerol (TAG), expression of de novo fatty acid and cholesterol synthesis genes are regulated by a small family of transcription factors called Sterol Regulatory Element Binding Proteins (SREBFs) (Brown and Goldstein, 1997). SREBFs and their regulatory (escort) protein “SREBF Cleavage Activating Protein” (SCAP) combine to regulate hepatic expression of mRNAs for the “de novo” enzymes that synthesize fatty acids and cholesterol from precursor molecules (Goldstein, et al., 2006, Horton, et al., 2003, Osborne, 2001). We previously proposed that the mouse (Anderson, et al., 2007) and bovine (Harvatine and Bauman, 2006) mammary glands use the SCAP/SREBF regulatory system to establish de novo fatty acid synthesis and to integrate dietary regulation of lipogenic gene expression during lactation.

SREBF family transcription factors have been extensively studied in liver and hepatic cells where systemic hormones and plasma glucose levels determine whether fatty acids are mobilized or stored (Horton, et al., 2002). The principle step by which SREBF’s are regulated is migration of full-length SREBF protein from the endoplasmic reticulum to the Golgi. This process is governed by the escort protein SCAP, which shuttles inactive SREBFs to the Golgi where the DNA binding region is released by proteolysis into the cytoplasm, ultimately traveling to the nucleus to regulate transcription of SREBF dependent genes. Importantly, loss of SCAP prevents migration of SREBF’s to the Golgi, restricting release of the DNA binding region and limiting transcriptional induction of SREBF dependent genes (Matsuda, et al., 2001).

Based on our mammary gland gene expression profiles showing a dramatic increase in the mRNA encoding enzymes of de novo fatty acid synthesis at parturition (Rudolph, et al., 2007), we hypothesized that SREBF-1c is a critical regulator of fatty acid biosynthesis in the lactating mammary epithelium. To test this hypothesis, we began our studies with quantitative real time RT-PCR (qPCR) validation of the expression of a suite of genes that lead from mitochondrial synthesis of citrate to fatty acid synthesis and desaturation. Most of these genes are known to be regulated by SREBF-1c in other tissues (Horton, et al., 2003). We then examined the formation of TAG in SREBF-1c null mice, finding very minimal responses possibly due to upregulation of SREBF-1a (see supplement). To obtain a more robust decrease in SREBF activity we utilized mice with a mammary
epithelial specific deletion of SCAP to abrogate the expression of both SREBF-1 isoforms. TAG production in these tissue specific SCAP deficient dams was severely compromised. Finally, we tested the hypothesis that an increase in preformed fatty acids would alter milk lipid synthesis acting through the SCAP-SREBF-1c pathway. Interestingly, this treatment had no effect on the mRNA expression of our lipogenic gene suite, although the level of protein for many of these molecules was decreased. Altogether, our observations suggest that mammary alveolar cells employ at least two mechanisms to regulate de novo fatty acid synthesis during lactation—one at the transcriptional level involving SREBF-1 and one at the post-transcriptional level whose mechanism is not well understood.
Results

Lipogenic gene expression in the normal mammary gland. Milk lipid synthesis increases several fold with the commencement of copious milk secretion following birth of the young (Abraham and Chaikoff, 1959). To examine the role of transcriptional level regulation of enzymes involved in fatty acid synthesis, we examined expression profiles of SREBF family members and several downstream targets with qPCR in whole tissue lysates (WTL) from mammary glands of pregnant and lactating mice. Figure 1A shows the expression of Srebfl-a, Srebfl-c, and Srebfl-2 from pregnancy day 12 through day 9 of lactation. These data show both that Srebfl-c is the most highly expressed of these genes throughout the time course examined and that it increased about 2.5 fold between pregnancy day 17 and lactation day 2. These findings suggest that SREBF-1c is most important of the isoforms in secretory activation.

Over the same time period the expression of several genes that are targets of SREBF-1 in other tissues was upregulated 5-fold or more (Fig 1B), mirroring expression in a previous microarray analysis (Rudolph, et al., 2007, Rudolph, et al., 2003, Stein, et al., 2004) (see also Supplementary Fig 1), again suggesting SREBF-1 activation of the fatty acid switch. Citrate formed in the mitochondria is shuttled to the cytoplasm by the mitochondrial citrate transporter, SLC24A1, where it is acted on by ATP citrate lyase (ACLY) to produce acetyl Co-A. Acetyl-CoA is converted by acetyl-CoA carboxylase 1 (ACACA1, also abbreviated ACC1) to malonyl-CoA in turn used by fatty acid synthase (FASN) to synthesize fatty acids. Downstream of FASN, steroyl-coA desaturase 2 (SCD2) inserts a single double bond into synthesized or preformed fatty acids (Ntambi and Miyazaki, 2004). Two additional mRNA’s were quantitated, thyroid hormone responsive protein (Thrsp), also known as Spot 14, and insulin induced gene 1 (Insig1), a protein that binds SCAP in the endoplasmic reticulum. The function of THRSP is not clear, but genomic loss of Thrsp results in a decrease of de novo fatty acid synthesis specifically in the mammary gland of lactating mice (Zhu, et al., 2005). All these genes showed a 2.5 to 5 fold increase in expression between Preg 17 and Lac 2. Consistent with increased SLC25A1 activity, we previously measured a two-fold increase in the concentration of mammary gland citrate at lactation day two (Rudolph, et al., 2007). Together, these results suggested that coordinate up-regulation of the RNAs of several de novo fatty acid synthesis enzymes by SREBF-1c is an important feature of the “fatty acid switch” occurring during secretory activation in the murine mammary gland (Anderson, et al., 2007).
The proportion of medium chain fatty acids (chain length > 16) in the milk was very low at lactation day 1 in these FVB mice (Fig 1C), possibly reflecting the composition of TAG synthesized during pregnancy and discharged into the lumen of the gland at parturition. Preformed fatty acids derived from the diet or body depots (chain lengths > 16) decreased as lactation progressed with a concomitant increase in the de novo synthesized medium chain fatty acids to about 15% of total on day 4 of lactation and about 25% of total by day 10. The proportion of C16:0 fatty acids, which are derived both from de novo synthesis and preformed fatty acids, remained relatively constant during this period.

Effects of deletion of SREBF-1C and targeted deletion of SCAP on the mammary gland. To test the hypothesis that SREBF-1c is important for de novo lactogenesis in the mammary gland we first investigated lactation in the Ssreb-1c null mouse (see methods). We found only a subtle defect in lactation in these mice, possibly because the expression of Srebf-1a was elevated (Supplementary Figures 2 and 3). It is not clear whether this small defect is local or system since SREBF-1c was knocked out in all tissues. Srebf-1a, which has a much stronger activation domain than Srebf-1c (Horton, et al.,2002), may compensate for the loss of Srebf-1c except under extreme dietary conditions. We therefore adopted a different strategy to determine the role of SREBF’s in regulation of de novo lipogenesis in the lactating mouse mammary gland, namely a mammary specific deletion of the SREBF escort protein SCAP, targeted specifically to the mammary gland. In vivo studies of liver specific SCAP loss demonstrated lack of SREBF activation leading to aberrant lipid homeostasis in mice (Matsuda, et al.,2001, Osborne,2001).

To obtain mammary specific deletion of SCAP, SCAP^{fl/fl} were crossed with transgenic mice expressing Cre recombinase driven by the ovine β-lactoglobulin promoter (BLG), a gene expressed only in the mammary epithelium (Selbert, et al.,1998). The region of the SCAP genomic DNA excised by Cre recombinase contains the proximal promoter and exon 1. In this system, SCAP mRNA transcription continues because expression is driven by a constitutive promoter (Nakajima, et al.,1999); however, Cre disruption of the gene effectively reduces the translation of SCAP protein due to loss of the 5′ untranslated region. To obtain SCAP^{fl/fl} Cre+ female mice we bred SCAP^{fl/fl} Cre- females with SCAP^{fl/fl} Cre+ males. The offspring were evenly divided between SCAP^{fl/fl} Cre+ (designated Δ-SCAP mice) and SCAP^{fl/fl} Cre- (used as controls). Figure 2A shows that SCAP protein is decreased about 70% in isolated mammary epithelial cells (MECs) from the Δ-SCAP mice.
resulting in almost complete loss of both unprocessed and mature SREBF-1 from the mammary epithelial cells and a significant diminution in pup growth rate after day 3 (Fig 2B). Growth rates of pups from SCAP\textsuperscript{fl/fl} Cre- and SCAP\textsuperscript{fl/+} Cre+ dams were similar to wild type (Data not shown). The morphology of the mammary glands in both late pregnancy and early lactation was similar in Δ-SCAP mice and controls (supplementary Figure 4), suggesting that the defect is biochemical rather than developmental. Immunohistochemical analysis of nuclear staining of SREBF-1 in mammary epithelial cells supported the conclusion the SREBF-1 activity was significantly diminished in the Δ-SCAP mice (Supplementary Figure 5). We interpret our data to mean that disruption of SCAP impairs proteolytic release of the DNA binding region preventing SREBF-1 maturation and nuclear accumulation; this result is consistent with results of targeted deletion of SCAP in the liver (11).

On lactation days 3 and 4 the rate of growth of the litters fed by Δ-SCAP dams was about half that of the controls (1.2 ± 0.1 vs 2.3 ± 0.2 g/day) but the glands showed little morphological abnormality by H&E analysis (supplementary fig 4). The discrepancy in growth rate continued throughout the experiment but observation of day 10 glands indicated a significant degree of morphological abnormality (Data not shown). SCAP is necessary for activation of the sterol regulatory pathway through SREBF-2. Loss of this pathway, which is important for cholesterol synthesis (Brown and Goldstein, 1997), may be at least in part responsible for the late lactation defect observed in SCAP deleted mice. We, therefore, confined the remaining experiments to lactation day 4. That no lactation defect was observed in heterozygous SCAP\textsuperscript{fl/-}, Cre + dams (data not shown), indicates that only one allele of SCAP is sufficient to support adequate lactation.

SCAP disruption prevents induction of mRNA for some but not all SREBF target genes. We predicted that SCAP disruption would decrease the proportion of medium chain fatty acids in the milk as well as transcription of SREBF-dependent genes. Table 1 shows that fatty acids of chain length < 16:0 or 16:0 were decreased about 25%, in the Δ-SCAP mice and fatty acids with chain length >16:0 were increased by a similar proportion. For RNA analysis, we avoided complications arising from the mammary gland adipose tissue by preparing mammary epithelial cells (MECs) depleted of adipocytes (Rudolph, et al., 2009). This MEC population contains no more than 2% of adipocyte protein found in whole tissue lysates as shown by the loss of 98% of perilipin (PLIN) from the MEC preparation compared to whole tissue lysates (Supplementary Fig 6). Gene expression copy numbers were determined by qPCR for the Srebf family and SREBF target genes (Fig 3A and 3B). There was no
significant change in the amount of Srebf-1a, Srebf-1c or Srebf-2 mRNA between control and Δ-SCAP MECs (Fig. 3A) despite almost complete loss of protein for SCAP and SREBF-1 (Fig 2A). We did, however, observe a significant reduction for some SREBF target genes in SCAP deficient including Slc25a1 (10-fold), Fasn (five-fold), Insig1 (10-fold), Scd2 (15-fold), and Thrsp (2 fold) and (Fig 3C and D). Surprisingly the mRNA for two other SREBF targets, Acly and Acaca showed no change in expression suggesting their regulation is mammary cells is SREBF independent, although these genes have been shown to be regulated by SREBF-1 in the liver (Horton, et al.,2003). Together, these decreases in Fasn, Slc25a1, Scd2, Thrsp and Insig1 identify key mRNAs whose expression is SREBF-regulated in MECs.

**SCAP deficiency reduces protein levels of lipogenic enzymes in MECs.** The observation that expression of two SREBF-1 target genes, Acaca and Acly, was not altered in Δ-SCAP MECs prompted us to ask whether these enzymes might be regulated at the post-transcriptional level. Immunoblot analysis was conducted on the adipose depleted MEC preparations from both control and Δ-SCAP mammary glands (Fig 3C, 3D). Highly significant decreases in the amounts of ACLY and ACACA1 proteins were observed in Δ-SCAP MEC’s suggesting post-transcriptional regulation of these molecules. As expected from the mRNA, little SLC25A1 was detected in the Δ-SCAP MEC’s; however FASN was reduced more at the protein level than expected from observed mRNA loss. Loss of FASN was also observed in immunohistochemical analysis of mammary glands from SCAP-deleted mice (Supplementary Fig 7). These findings suggest that, like adipose tissue (Sekiya, et al.,2007), MECs also have unique mechanisms to regulate lipogenic enzyme levels.

Cumulatively, SLC25A1, ACLY, ACACA1 and FASN constitute the linear *de novo* fatty acid synthesis pathway; and the coordinate reduction of these proteins likely leads to a reduced ability to produce fatty acids from metabolic precursors. The combination of decreased proteins in the Δ-SCAP mice, whether reduced due to SREBF-dependent expression or post transcriptional regulation, resulted in about 25% decrease in the proportion of *de novo* fatty acids (C < 16:0 and C = 16:0) at lactation day 4 (Table 1).

**The effect of a high fat diet on milk lipid and mammary gene expression.** We previously suggested that SREBF-1 activation might be reduced by high fat diets (Anderson, et al.,2007) since such diets would mitigate the need for the *de novo* lipogenic pathway. If this were the case, a high fat diet might be expected to restore the growth performance of pups nursed on Δ-SCAP dams. We therefore
investigated the effect of a diet containing 46% Kcal as fat on pup growth, milk lipid composition, and the expression of lipogenic genes. Both control and Δ-SCAP dams were given either standard rodent chow diet or the high fat diet starting on pregnancy day 17. In the controls the high fat diet had no effect on litter growth in early lactation (inset), but significantly increased litter growth about 20% after days 5 - 10 (Fig. 4A.). In the Δ-SCAP animals the high fat diet increased the litter growth rate by about 40% overall, but it did not restore litter growth to the rate observed in the controls in either early or late lactation.

The high fat diet significantly depressed the proportion of medium chain fatty acids as well as C16:0 in the milk and increased long chain fatty acids (Table 1; supplementary Fig 8). The desaturase index for C16:0 was also increased suggesting that the activity of SCD2 was depressed. We therefore asked whether the high fat diet altered the mRNA for Srebf’s or SREBF target genes. There was no change in Srebf expression as expected from the result in Fig. 4A (Supplementary Fig. 9) nor, to our surprise, was the level of mRNA for the target genes altered (Fig. 4B). For purposes of comparison the target gene levels from control and Δ-SCAP mice from Fig 3 are repeated with values from mice receiving the high fat diets interpolated in Fig. 4B. Although the mRNA levels are lower in the Δ-SCAP animals as we have already seen, the high fat diet decreased expression of only of SCD2 in controls.

We then asked whether there was any change in the level of the protein for these enzymes. Indeed, as shown in Figure 3C and D, the 46% fat diet led to a substantial reduction of SLC25A1, ACLY, ACACA, and FASN protein in MECs lysates prepared from control mice fed high fat diet compared to mice on the chow diet (Fig 3C, compare control chow versus control 46%). There was, however, no change in the protein for. Essentially, the reductions in these proteins in control mice fed the 46% fat diet were comparable to the reductions observed when SREBF-dependent transcription was lost (Fig 3C, compare control 46% and Δ-SCAP chow). Feeding Δ-SCAP mice the 46% diet gave maximal suppression of de novo fatty acids (Table 1) with concomitant maximal loss of lipogenic enzymes. These results indicate that dietary fat regulates the abundance of lipogenic enzymes in the epithelium of the lactating mammary gland not at the mRNA level but post-transcriptionally. This regulation seems not to be dependent on SREBP-1.
**DISCUSSION**

We began these studies with the hypothesis that SREBF transcriptional regulation was central to regulation of fatty acid biosynthesis in mammary epithelial cells as it is in the liver (Foretz, et al., 2002, Horton, et al., 2002). Many elegant studies detailing SREBF activation and function in the liver and hepatic cell culture systems, coupled with our gene expression profiling studies showing that SREBF-1c mRNA increased dramatically at parturition in the mammary gland, supported this hypothesis. Indeed, confirmation that several known downstream targets of SREBF-1c transcriptional regulation are coordinately upregulated up to 5-fold at the onset of lactation (Fig. 1) was also consistent with the hypothesis. To provide direct confirmation we examined SREBF-1c null mice but found only very minor shortcomings in lactation. However, we observed significant upregulation of Srebf-1a, leading us to postulate that elevated SREBF-1a might compensate for the loss of SREBF-1c.

To determine how complete loss of SREBF-dependent gene expression affected fatty acid biosynthesis during lactation, we then turned to the floxed SCAP mouse. Cre recombinase was driven by a mammary specific promoter to deplete the SCAP molecule specifically in the luminal epithelium of the mammary gland. Disruption of SCAP significantly decreased pup growth rate but pups did not die. The proportion of *de novo* synthesized fatty acids in the milk decreased as well, but only by 25%. Together, these observations suggest that SREBF-dependent gene expression is necessary for optimal *de novo* lipogenesis, but that SREBF’s are not required to establish the basal *de novo* fatty acid pathway in the mammary epithelium. The mRNA and protein levels for the *de novo* fatty acid pathway components during lactation exhibit basal levels in Δ-SCAP MECs. Thus, this basal lipogenic program initiated at the onset of lactation is not completely dependent on SREBF-1 regulation of transcription.

Factors other than SREBF that have been implicated in the transcriptional control of lipogenic genes include upstream stimulatory factors (USF) (Griffin and Sul, 2004), the carbohydrate response element binding protein (CHREBP) (Uyeda, et al., 2002), Sp1 and Sp3 (Daniel and Kim, 1996, Fukuda, et al., 1999) and MIG12 (Kim, et al., 2010). Both USF and CHREBP bind to E-boxes which are known to be present in the promoter regions of Acaca. Sp1 and Sp3 have been found to bind to the promoters of Acly, Acaca, and Fasn with Sp3 stimulating transcription. MIG12 increases the activity of ACACA1 by increasing its polymerization but did not increase protein translation in the
liver (Kim, et al.,2010). While we readily detect the CHREBP protein by Western blot in whole tissue lysates, we found neither protein nor mRNA for CHREBP in late pregnant and lactating MECs (MC Rudolph and SM Anderson, unpublished data), suggesting that it does not regulate lipogenic genes in the lactating alveolar cell. However, lactation performance was diminished in USF-2−/− mice (Hadsell, et al.,2003) and a QTL associated with milk fat content in dairy cows was found to contain a binding site for Sp1 and Sp3 (Ordovas, et al.,2008). Thus both USF-2 and Sp1/Sp3 may be good candidates for the SREBF-independent regulation of de novo lipogenesis in the lactating gland.

MIG12 is 36% homologous to THRSP, which is highly expressed in the lactating mammary gland and appears to be essential for de novo lipogenesis. MIG12 is expressed only at low levels in the mammary alveolar cell of mice (P. Ramanathan and M.C. Neville, unpublished) and cows (Harvatine and Bauman,2006) so does not seem to be a candidate for regulation of de novo lactogenesis. The role of THRSP is unclear but Kim et al (Kim, et al.,2010) found no evidence that it altered polymerization of ACACA1.

A final possibility is regulation of translation of de novo fatty acid synthesis enzymes by microRNA. Very clear evidence indicates that chronic treatment of mice with Mir122 downregulates Srebf-1, Fasn, Acaca, Acacb, Scd1, Acly and other genes in the liver (Esau, et al.,2006). Recent data is beginning to identify miRNAs expressed in the mammary gland (Avril-Sassen, et al.,2009, Sdassi, et al.,2009). Interestingly genes associated with the GO category “metabolic regulation” have been found in miRNA clusters in which expression is increased during gestation and falls or stays the same in lactation. No detailed functional tests of potentially important miRNAs have yet been published for the normal mammary gland in pregnancy or lactation.

Regulation of mRNA vs protein. Our results show that preventing SREBF activation significantly attenuated mRNA levels for three key enzymes along the de novo fatty acid and TAG synthesis pathways, Slc25a1, Fasn, and Scd2. This finding provides strong evidence that SREBF-1 directly regulates expression of these three genes directly in MECs. The protein levels of FASN and SLCs5A1 were reduced in parallel with their mRNAs in the Δ-SCAP mouse (Fig 3C). Conversely, ACLY and ACACA protein levels declined even though Acly and Acaca gene expression were not altered in Δ-SCAP MECs (Fig 3C). This finding suggests an independent regulatory mechanism at the post transcriptional level. These two mechanisms are depicted in Fig. 5: the SREBF-1 dependent arm modulates expression of key biosynthetic mRNAs (Slc25a1, Scd2, and Fasn); a second mechanism regulates protein abundance independently of SREBF-1 transcription (ACLY and
ACACA). One possibility is that SREBF-1 regulates the transcription of microRNAs that modify ACLY and ACACA translation. Whether this mechanism is totally independent of SREBF-1 or whether SREBF-1 acts indirectly to decrease enzyme abundance is a problem for future study.

**Effect of high dietary lipid.** Dietary fat has been shown to regulate hepatic lipogenesis. For example, polyunsaturated fatty acids (PUFA), suppressed the activity of SREBF-1c (Jump, 2008) whereas saturated fats induced SREBF-1c and SREBF-1a, possibly through the transcriptional coactivator PGC-1β (Lin, et al., 2005). More recently SREBF-1c has been implicated in the down-regulation of hepatic SLC25A1 by PUFA (Damiano, et al., 2009). PUFA have also been shown to repress fatty acid synthase, possibly regulating the binding of SREBP-1c and NF-Y through contiguous regions in the promoter (Teran-Garcia, et al., 2007). The high fat diet we fed was formulated with corn oil that has a 62% PUFA content of mostly C18:2 and 25.1% MUFA of mostly C18:1 (Supplementary Table 3). The diet markedly suppressed the proportion of *de novo* synthesized fatty acids in milk fat by 40% in control mice and 30% in the Δ-SCAP mice; however, the mRNA for neither SREBF-1c nor most of its targets was downregulated. In other words, dietary PUFA had no influence on gene regulation of *de novo* lipogenesis in the mammary epithelium as it has in liver. The lack of PUFA regulation of SREBF-1 targets was also observed in the SREBF-1c null mice, which consumed either 3.9% or 61% dietary fat (see supplementary data). Even though mRNA levels were unchanged, high fat feeding decreased MEC abundance of SLC25A1, ACLY, ACACA and FASN proteins in control mice. It is tempting to speculate that the mechanism by which the level of these proteins is decreased in the absence of changes in the amount of their RNA, is the same post-transcriptional process postulated to decrease ACLY and ACACA in the Δ-SCAP mice (Fig 5). Whatever the mechanism, the results of these studies provide a framework for analysis of what appears to be a novel mechanism(s) used by the mammary epithelium to alter lipogenic enzyme levels in the presence or absence of SREBF-1 dependent transcription.

**Conclusion:** Srebf-1c and its target genes are upregulated in the mammary epithelium at the onset of lactation, a time when the *de novo* fatty acid pathway is activated to provide milk fat energy in support of the growth and development of the newborn. Use of the conditional Δ-SCAP mouse and adipose depleted MECs identified Slc25a1 and Fasn as direct SREBF target genes, while Acly and Acaca1α were not. The finding of decreased levels of ACLY and ACACA1 protein, in the presence...
of sustained mRNA levels, suggests a role for additional regulatory mechanism(s) for the mammary epithelial de novo fatty acid synthesis pathway. Our data lead to the novel conclusion that the expression of de novo fatty acid pathway genes in the mammary epithelium is not regulated by diets high in PUFA as observed in the liver, however, diet does regulate the levels of these proteins.

**Experimental Procedures**

*Mouse breeding and pup growth studies.* Animals were maintained in the Center for Comparative Medicine in accordance with Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals; all animal procedures were approved by the Institutional Animal Care and Use Committee of University of Colorado Denver. FVB, SREBF-1c null mice (B6;129S6 Srebf1tmbr/J) and floxed SCAP mice (B6;129-Scaptm1Mbjg/J) were purchased from Jackson Laboratories (www.jax.org). Floxed SCAP mice were mated to transgenic mice expressing Cre-recombinase controlled by the ovine beta-lactoglobulin promoter (Tg(BLG-cre)74Acl (Selbert, et al.,1998), a gift of C. Watson (Cambridge University, Cambridge, UK) to obtain SCAPflox Cre+ and SCAPflox Cre- mice. To obtain pregnant and lactating dams SCAPflox Cre- and SCAPflox Cre+ females were mated with FVB males. Pregnancy day one (P1) was designated as the day of vaginal plug. Lactation day one (L1) was the day that pups were born. Litters were standardized to six pups per litter for all studies. For pup growth studies, litters from both strains were combined and pups randomly assigned to different dams. Litters were weighed daily between 9AM and noon a procedure that is standard for estimating milk intake in mouse lactation studies (Knight, et al.,1986, Nagai and Sarkar,1978). In studies where the glands were harvested for immunostaining or isolation of MEC, FVB pups were used to standardize the litters.

*Dietary conditions used in pup growth studies.* Unless otherwise indicated all animals were fed standard laboratory rodent chow (diet number 2018 from Teklad Animal Diets, Harlan, Madison, WI). This chow contains 6.2% (18% Kcal) as fat. Diets with altered fat contents were obtained from Research Diets (www.researchdiets.com). The composition of the diets containing 3.9% and 60.8% kcal as fat (given starting on day 7 of lactation for the SREBP-1c null experiments) is shown in Supplementary Table 2. For the SCAP experiment the high fat diet was RDI 12344, which is 24.6% fat by weight and 46.1% by calories (see supplementary Table 3 for full composition). In this experiment the dams receiving high fat diet were fed ad libitum beginning on day 17 of pregnancy. The food was replaced every four days to prevent spoilage.
Isolation and enrichment of MEC from whole mammary glands. Mammary digestion buffer (19.5 mL of Ham’s F12 medium (Invitrogen/Gibco #11765), 30 mg Trypsin (Gibco #840-7250), 60 mg collagenase (Boehringer #1088793), 0.5 mL fetal calf serum) was prepared. The 4th and 5th mammary glands were excised and lymph nodes removed. Two portions, approximately 30 mg each were snap frozen for use as whole tissue lysates. The remaining glands were diced into a fine paste using scalpels (Miltex #4-322, York, PA) and added to 5 mL of digestion buffer in a pre-cooled 50 ml conical tube. After incubation for 45 minutes in a 37°C shaker at 200 rpm, the digested cell suspension was brought up to 15 mL with cold PBS and spun at < 300 x G for 10 min. This washing was repeated three times. Half the resulting 500 μL of packed organoids was lysed in 1 mL of Trizol (www.Invitrogen.com) for RNA analysis and half was lysed in 500 μL of ‘mammary gland lysis buffer’ for protein analysis (see below). The isolation procedure reduced the adipocyte marker, perilipin, by 98%, indicating that the MEC’s were highly enriched for the epithelial cell population.

RNA isolation. Trizol samples were homogenized using a Brinkman Polytron (www.brinkmann.com) and lysates were cleared at 13,000 x g for 15 minutes at 4°C. RNA was isolated according to the manufacturer’s protocol with the Qiagen RNeasy mini Plus protocol (www.qiagen.com, # 74134). Total RNA was quantitated using the Nanodrop 1000 spectrophotometer (www.nanodrop.com). RNA integrity was verified using the Agilent Bioanalyzer Nanoscale Microfluidics Chip Assay (www.chem.agilent.com, 2100 Bioanalyzer). All samples of MEC enriched preparations had A260/280 ratios > 1.8.

Quantitative real-time PCR (qPCR) analysis. Supplemental Table 1 lists the primers and probes for genes of interest, obtained from Integrated DNA Technologies (www.IDTDNA.com). The cDNA synthesis reaction consisted of 2.0 μg total RNA in 10 μL of nuclease free water plus 1.0 μL of random hexamers (50μM) and 1.0 μL oligo dT (0.5 μg/μL) incubated at 70°C for 5 minutes with master mix 1 containing 4μL of 5x 1st strand buffer (Invitrogen), 2μL of 0.1 M DTT, 1μL of 10mM dNTP blend (Roche), 1 μL RNase inhibitor (40 units/μL, Promega), and 1μL MuLV reverse transcriptase (50 units/μL, Roche) per reaction. Reaction tubes were chilled on ice and spun down. The cDNA was diluted 1:10 and 5 μL was input into the qPCR reaction (representing 50 ng total RNA/reaction) with master mix 2 containing 12.5 μL of Absolute Fast qPCR Mix-Lox Rox (www.ThermoFisher.com), 2.5 μL of 10x primer/probe mix (5.0 μM forward, 5.0μM reverse, and 2.5μM probe), 5.0 μL of nuclease free water, and 5.0 μL of 1:10 diluted cDNA per reaction. qPCR
data were collected on the Applied Biosystems 7500 Fast Thermocycler (www.appliedbiosystems.com). Copy numbers were calculated using the standard curve method with a known quantity of the amplicon (1.204 e7 stepping down 5-fold to 7.70 e2). All standard curves had near optimal slopes of -3.3, y-intercepts near 40, R correlations near 1, PCR efficiencies near 100%, and each target amplified within the standard curve. We have found that expression of most if not all housekeeping genes changes during the onset of lactation; for this reason our results are standardized to 50 ng total RNA.

Protein Isolation/Western immunoblots. Mammary lysis buffer consisting of 50 mM Tris pH 7.4, 150 mM NaCl, 2.0 mM EDTA, 50 mM NaF, 5.0 mM sodium vanadate, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, to which 0.57 mM PMSF, 20 μL/mL EDTA-free Inhibitor cocktail (Roche), and 1.0 mM DTT was used to extract proteins from both the MEC enriched and whole tissue lysate (WTL). Samples were homogenized using a Brinkman Polytron and lysate was centrifuged at 13,000 x g for 25 minutes at 4°C. Protein concentrations were determined using Pierce Coomassie Plus Protein Assay (Pierce Chemical Co, Rockford, IL; #1856210). Proteins were resolved using glycerol-based acrylamide SDS-PAGE gels (8%-12%). Resolved proteins were transferred to PVDF membranes (Millipore), and immunoblotting was carried out using the SNAP i.d. vacuum system (www.Millipore.com). Antibodies directed against ACACA (#3676) and ACLY (#4332) were obtained from Cell Signaling Technology (www.cellsignal.com); anti-FASN (SC-20140), anti-THRSP (SC-67299), and anti-actin (SC-1616) were obtained from Santa Cruz Biotechnology (www.scbt.com); anti-SREBF-1 (PA1-337) was obtained from Affinity BioReagents/ThermoFisher (www.bioreagents.com); anti-perilipin (70R-PR004) from Fitzgerald (www.fitzgerald-fii.com). Western blots showing the composition of the whole tissue lysate for SCL25A1, ACLY, ACACA1, FASN, DGAT1, Keratin 18 (KRT 18) and perilipin (PLIN) are shown in Supplementary Figure 6.

Collection of milk, milk clots, and analysis of milk components. For SREBF-1c null mice and their controls, milk was collected as described by (Schwertfeger, et al.,2003). Samples were analyzed for crematocrit content and fatty acid composition. Milk clots were collected from the stomachs of the pups after CO2 narcosis and frozen at -80º C. Approximately 20 mg of lyophilized milk clot was weighed into an extraction tube containing 250 μg of C17:0 (triglyceride) and C19:0 (methyl ester) as internal standards. Milk clot lipids were extracted by hexane:isopropanol extraction according to Hara and Radin (Hara and Radin,1978). Milk fatty acids were transmethylated using a dual
methylation procedure; lipid was first acid-methylated overnight at 40°C in 1% methanolic sulfuric acid, and subsequently transmethylated, and fatty acids quantitated as described for milk fat (Perfield, et al., 2006). Milk clot fat concentrations were determined based on dilution of the internal standard.

**Immunostaining of whole tissues.** Mice were anaesthetized with Avertin (1 mg/g body weight) and subjected to intracardiac perfusion with 20 ml ice-cold PBS as described (Monks, et al., 2008). Both #4 mammary glands were removed, weighed, frozen between sheets of aluminum foil in liquid nitrogen cooled isopentane and stored at -80°C until sectioning. Frozen sections were collected onto Cell Tak (BD Biosciences)-coated coverslips, fixed at room temperature inside a chamber humidified with 4% paraformaldehyde for 20 minutes. SREBF-1 immunostaining was performed on frozen, perfused tissue with primary antibody (Santa Cruz K10, sc-367) at 1:20 dilution, after glycine (0.2%), and TX-100 (0.2%) permeabilization, NaBH4 (0.1%) quenching, and blocking with 10% donkey serum. Secondary antibodies conjugated with fluorescent probes were from Invitrogen. FASN immunostaining was performed on frozen sections as above with Rabbit anti-FASN (sc-20104, www.scbt.com) at 1:20.

**Image Capture and Analysis.** Images were captured on an Olympus IX81 inverted motorized microscope using Olympus objectives-TIRFM PLAN APO 60X oil N.A. 1.45 WD 0.15mm or U PLAN APO 100X oil N.A. 1.35, a 100 watt mercury lamp, a Hamamatsu ORCA IIER monochromatic CCD camera 1344X1024 full chip, 6.45X6.45 um pixel, with filter sets: DAPI EX360-70 EM420-60, FITC EX450-80 EM535, TRITC EX535 EM635; 2v2(JP4). The system is controlled by SlideBook software, v.4.067. (Intelligent Imaging Innovations, Inc, Denver, CO), which is also used to normalize and analyze immunofluorescence. Analysis was performed on 2D sections only. A minimum of 8 sections from 3 mice each per condition was used.

**Statistics:** Biochemical data were analyzed using the t-test function of Excel with a one-tailed distribution and equal variance of samples. For the milk composition data analyses were performed using the fit model procedure of JMP® (Version 8, SAS Institute). The statistical model accounted for the fixed effects of genotype, diet, the genotype x diet interaction, and day of lactation. When the effect of genotype, the diet, the genotype x diet interaction, or the day of lactation was significant, individual means were separated by Student’s T test. For all analyses statistical significance was declared at $P < 0.05$. 

18
Acknowledgements:
The authors thank members of the Mammary Gland Biology Program Project Grant at the University of Colorado Denver for on-going discussions regarding this research. SMA and MCN are supported by NIH grant PO1-HD38129 which also contributed to the support of MCR, JM, RM, VB, and MP. MCR is currently supported by Department of Defense Breast Cancer Research Predoctoral Fellowship BC 810596.
References


Figure Legends

Figure 1. Developmental changes in mRNA of de novo lipogenesis genes and fatty acid composition of milk (FVB mice). A. Gene expression of Srebf family members in whole tissue lysate as copy number per 50 ng mRNA (N = 5 mice per time point). B. Expression of Srebf target mRNAs in samples as in panel A, each gene is normalized to the mean expression value for that gene. * P<0.05, ** P<0.005 for differences between expression levels at Preg17 and Lac2. C. Effect of lactation stage on proportion of de novo synthesized (C:8 to C:14) and preformed (>C:16) fatty acids in milk from FVB mice (N = 4 mice per time point). Mean and SEM shown for all graphs. *** P<0.001 compared to both L1 and L4.

Figure 2. Mammary epithelial-specific SCAP deletion decreases SCAP and SREBF-1 protein and reduces pup growth. A. Western blot and quantitation for SCAP and the unprocessed and mature forms of SREBF (u-SREBF and m-SREBF, respectively) in MECS from Control and Δ-SCAP mice. The antibody does not distinguish SREBF-1c and SREBF 1a. ** P<0.005 between control and Δ-SCAP. B. Weights of litters nursed by either control or Δ-SCAP dams, N =3 per strain.

Figure 3. Mammary specific expression of Srebf’s, target genes and lipid biosynthetic proteins in control, Δ-SCAP and high fat diet mice. A. Real time PCR copy numbers of Srebf’s in 50ng total RNA in MECs isolated from control or Δ-SCAP dams on lactation day 4. Mean ± SEM from 5 mice per condition. B. Mean Normalized copy numbers for SREBF targets, as in panel A. C. Immunoblots from MECs isolated from control or Δ-SCAP- mammary glands on day 4 of lactation. Mice were fed chow or 45% kcal fat diets from day 18 of pregnancy. Fractions were probed for keratin 18 (KRT18) as a loading control. D. Quantitation of immunoblots from Fig 4C. b, Protein levels for Δ-SCAP mice were significantly different from control at P < 0.005. For mice on the high fat diet, protein levels were significantly different from mice on control diets, a, P < 0.05; b, P < 0.005. For Δ-SCAP mice, ACLY and FASN were not detectable on the high fat diet (n/d).

Figure 4. Effect of high fat diet on pup growth, mRNA and protein for lipid synthesis enzymes. Lactating dams were provided with laboratory chow or a 46% fat diet starting on pregnancy day 17. A. Mean weights of 7 to 9 litters per condition nursed by control or Δ-SCAP dams fed either chow or a 46% fat diet are shown. Inset: growth rate days 1 – 4 with numbers of mice per condition. a, Different from control, P < 0.001; b, different from control, P < 0.05; c, different from Δ-Scap on chow, P < 0.005. B. MEC specific mRNA on lactation day 4. Means ± SEM for n = 5 mice per condition. Only for Scd2 did the high fat diet have an effect on RNA expression levels in control mice; *, P < 0.05.

Figure 5. Model showing two mechanisms for regulation of fatty acid synthesis enzymes in mammary epithelial cells from the lactating mouse mammary gland. Transcription of mRNA for Slc25a1, Fasn, and Scd2 is increased by SREBF-1c (blue or purple). Increased dietary lipids, while decreasing de novo fatty acids in the milk, have a minimal effect on transcription of the genes.
examined. A high fat diet suppresses the de novo fatty acid synthesis by decreasing ACLY, ACACA and FASN protein (red or purple type).

**Supplemental Tables and Figures.**

A. Extended time course of expression of two lipogenic enzymes:

Supplementary Figure 1. Time course of mRNA expression of *Scd2* and *Slc25a1*.

B. Lactation competency in *Srebf-1c* null mice.

Supplementary Figure 2. *Srebf-1c* null mice: Litter growth, milk lipid content, and Srebf mRNA.

Supplementary Figure 3. Effect of a 4% or 61% fat diet on expression of *SREBF* target genes in Control and *Srebf-1c* null mice in whole tissue lysates at lactation day 12.

C. Mammary histology of Δ-SCAP mice

Supplementary Figure 4. Mammary histology in P14 and L4 Cre- and Cre+ mice. Nuclear SREBF-1 in Δ-SCAP mice.

D. Nuclear SREBF-1 in Δ-SCAP mice.

Supplementary Figure 5. Mammary epithelial-specific SCAP deletion suppresses nuclear accumulation of SREBF-1 during lactation.

E. Fatty acid synthesis enzymes in Whole tissue lysates

Supplementary Figure 6. Western blots for lipid synthesis genes obtained from whole tissue lysates.

F. Immunoblot analysis of FASN in control and Δ-SCAP mice.

Supplementary Figure 7. SCAP deletion decreases FASN in mammary epithelial cells.

G. Effects of a 46% fat diet on expression of Srebf family members, milk fatty acid composition

Supplementary Figure 8. Fatty acid composition of milk clots from pups nursing chow and high fat (46% Kcal as fat) fed Control dams.

Supplementary Figure 9. Effect of a 46% fat diet on expression of *SREBF* target genes in control and *Srebf-1c* null mice.

Supplementary Table 1. Probes and primers for real time PCR.

Supplementary Table 2. Low and high fat diets fed to *Srebf-1c* null mice.
Supplementary Table 3. Hi fat diet for SCAP experiment.
Figure 1

A

B

C

Rudolph
Rudolph--Figure 2

A

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control</th>
<th>Δ-SCAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCAP</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>u-SREBF1</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>m-SREBF1</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Krt18</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B

![Graph](image9.png)

**Litter Weight (g)**

**Day of Lactation**

- CONTROL
- Δ-SCAP
Figure 3

A

B

C

D

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control</th>
<th>Δ-SCAP</th>
<th>Control</th>
<th>Δ-SCAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC25a1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACACA1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FASN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>keratin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diet</th>
<th>Chow</th>
<th>46%KCal sat</th>
</tr>
</thead>
</table>

Table: Genotypes and diets used in the experiment.

Bars with different letters (a, b) indicate significant differences (p < 0.05).
A

Days Postpartum

Litter Weight (g)

CNTL-Chow
•
Δ-SCAP-Chow
○
CNTL-46%
■
Δ-SCAP 46%
□

B

Copy Number in 50 ng Total RNA, Mean Normalized

Slc25a
Acly Acaca1 Fasn Scd2 Insig1 Thrsp

Control, chow
■
Control, 46%
□
Δ-SCAP, chow
■
Δ-SCAP, 46%
□

Rudolph--Figure 4
Table 1. Effect of SCAP and diet on milk fatty acids on lactation day 4.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Genotype&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Genotype&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Genotype&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Genotype&lt;sup&gt;1&lt;/sup&gt;</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Chow</td>
<td>Control 45% Fat</td>
<td>Δ-SCAP Chow</td>
<td>Δ-SCAP 45% Fat</td>
<td>SEM</td>
</tr>
<tr>
<td>Milk Fatty Acid&lt;sup&gt;2&lt;/sup&gt;</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 16 Carbons</td>
<td>15.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.81</td>
</tr>
<tr>
<td>16 Carbons</td>
<td>33.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.66&lt;sup&gt;e&lt;/sup&gt;</td>
<td>24.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.61&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.77</td>
</tr>
<tr>
<td>&gt; 16 Carbons</td>
<td>50.80&lt;sup&gt;d&lt;/sup&gt;</td>
<td>70.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.68&lt;sup&gt;e&lt;/sup&gt;</td>
<td>74.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.05</td>
</tr>
<tr>
<td>16:0/16:1 Desaturase Index</td>
<td>5.91&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.57&lt;sup&gt;f&lt;/sup&gt;</td>
<td>17.54&lt;sup&gt;d&lt;/sup&gt;</td>
<td>31.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.72</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values represent LS Means
<sup>2</sup>Fatty acids < 16 carbons originate from mammary de novo fatty acid synthesis, fatty acids >16 carbons originate from dietary sources, and 16 carbon fatty acids originate from both sources (47).
<sup>abc</sup>Values within a row with different superscripts are different (P < 0.05).