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ORIGINAL PAPER

# Sustained upregulation of stearoyl-CoA desaturase in bovine mammary tissue with contrasting changes in milk fat synthesis and lipogenic gene networks caused by lipid supplements

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**Abstract** Long-term mammary expression patterns of lipogenic gene networks due to dietary lipid remain largely unknown. Mammary tissue was biopsied for transcript profiling of 29 genes at 0, 7, and 21 days of feeding cows saturated lipid (EB100) or a blend of fish/soybean oil (FSO) to depress milk fat. Milk fat yield decreased gradually with FSO and coincided with lower molar yield of fatty acids synthesized de novo, stearic acid, and oleic acid. The PPARγ targets *LPIN1* and *SREBF1* along with *ACSS2*, *ACACA*, *FASN*, and *LPL* increased by day 7 of feeding EB100, but differences between diets disappeared by day 21. Expression of *SCAP* increased markedly over time with FSO and differed from EB100 by approximately sevenfold on day 21. Expression of *THRSP* decreased by day 7 with both diets and returned to basal levels by day 21. *SCD* expression increased linearly through 7 days and remained elevated with both diets, a likely mechanism to ensure the proper level of endogenous oleic acid via desaturation of dietary stearate (EB100) or via more SCD protein to account for the reduction in stearate supply from the rumen (FSO). Despite this response, endogenous oleate was insufficient to restore normal milk fat synthesis. Only 2 of 29 genes differed in expression between diets on day 21, suggesting that transcriptional control mechanisms regulating fat synthesis were established as early as 7 days post-feeding. Gene expression reflected vastly different physiological responses by mammary tissue to adjust its metabolism to the influx of saturated fatty acids, *trans*10-18:1, and/or to the lack of stearic acid.

**Keywords** Mammary tissue · Milk fat · Lactation

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## Introduction

Ruminant mammary metabolic responses to supplemental long-chain fatty acids (LCFA) require particular consideration of those *trans* and conjugated LCFA isomers arising from the biohydrogenation of unsaturated LCFA in the rumen. Studies of diet-induced milk fat depression (MFD), i.e., due to high-grain/low forage, fish oil, or supraphysiological exogenous *trans*10,*cis*12-conjugated linoleic acid (10/12CLA), have shown a downregulation of well-established lipogenic genes in mammary tissue, e.g., acetyl-CoA carboxylase (*ACACA*), fatty acid synthase (*FASN*), and stearoyl-CoA desaturase (*SCD*), as well as the transcription regulator sterol regulatory

element binding factor 1 (*SREBF1*) and thyroid hormone-responsive SPOT14 (*THRSP*; Peterson et al. 2004; Harvatine and Bauman 2006; Shingfield et al. 2010). Less studied is the regulatory gene networks operating in concert with those genes, of which several have been recently identified, e.g., Lipin 1 (phosphatidic acid phosphatase, *LPIN1*) and 1-acylglycerol-3-phosphate-*O*-acyltransferase 6 (*AGPAT6*). Both *LPIN1* and *AGPAT6* were upregulated during bovine lactation when milk fat synthesis is copious, thus appearing to play a role in triacylglycerol (TAG) synthesis (Bionaz and Loor 2008a, b). The protein LPIN1 interacts with PPARγ in adipose tissue and is involved in insulin signaling (Huffman et al. 2002; Reue and Zhang 2008).

Recent work from one of our laboratories provided a more in-depth view of a network of transcription regulators and nuclear receptors including *SREBF1*, *SREBF2*, peroxisome proliferator activated receptor-γ (*PPARG*), insulin-induced gene 1 (*INSIG1*), and PPAR-γ co-activator 1-α that seems to coordinate the activation of the genes driving the lipid synthesizing machinery during the lactation cycle (Bionaz and Loor 2008a, b). Furthermore, a recent in vitro experiment studying the effects of different LCFA on bovine mammary cell transcriptomics underscored the relevance of *PPARG* (i.e., a nuclear receptor with LCFA as natural ligands) in the regulation of the lipogenic program by LCFA in the mammary gland (Kadegowda et al. 2009a). Because LCFA cannot bind directly to transcriptional regulators such as SREBF1 or THRSP (Pegorier et al. 2004), it is likely that they elicit effects in mammary tissue at least in part through PPAR or as yet unidentified nuclear receptors. Previous studies of MFD have been narrow in scope, i.e., few genes have been examined and temporal evaluations in mRNA expression have not been performed. Thus, a large portion of the systematic changes that drive the mammary adaptations to diet-induced MFD are still unknown.

Our general hypothesis was that long-term lipid supplementation to induce MFD or to increase/maintain milk fat concentration/yield would affect the network of genes driving milk fat synthesis in opposite ways. Our objectives were to (1) determine how supplementation with a blend of fish oil (approx. 20% of total LCFA as 20:5 and 22:6) plus soybean oil or saturated lipid containing approx. 40% 16:0 and 40% 18:0 affected fat synthesis and milk LCFA profiles and (2) relate those results to gene expression focusing in particular in understanding how milk lipid synthesis may be regulated at the transcriptional level. Specific objectives were to examine expression patterns of genes involved in fatty acid uptake, intracellular activation and trafficking, de novo fatty acid synthesis, desaturation and esterification, lipid droplet formation, transcription regulation, inflammation, cell growth and proliferation, and retinoic acid signaling over a 21-day feeding period.

## Materials and methods

### Experimental design, animals, and management

All procedures performed on cows in this study were according to a protocol (no. 05142) approved by the University of Illinois Institutional Animal Care and Use Committee. Details of the design and animal management have been published previously (Thering et al. 2009). Briefly, 16 Holstein cows from the University of Illinois dairy herd were used during a 4-week study. Cows averaged 100±13 days in milk, 41.7±6.49 kg milk/day, 3.99±0.66% fat, 2.90±0.27% protein, and 2.70±0.31 body condition score before the beginning of the study. Cows were housed in individual tie stalls bedded with sawdust and were offered a total mixed diet once daily (1100 hours). Cows had continuous access to water and were milked at 0500 and 1600 hours.

### Diets

Diets were based on corn silage and alfalfa silage (Electronic supplementary material (ESM) Online Resource 1 and Table S1). Treatments included a control diet with no added lipid (CTR; $n = 5$ cows), a milk fat-depressing diet (FSO, 3.5% of DM) containing fish oil (1.0%; Omega Protein, Inc., TX, USA) and soybean oil (2.5%; Archer Daniels Midland, IL, USA; $n = 5$ cows), and a diet containing a saturated lipid supplement (Energy Booster 100, Milk Specialties, IL, USA) to enhance or maintain milk fat yield (EB100, 3.5% of DM; $n = 6$ cows). Fatty acid composition of lipid supplements is available in ESM (Table S2). Diets had a forage/concentrate ratio of approx. 70:30 (dry matter basis), with lipid supplements in fish/soybean oil (FSO) and EB100 replacing soybean hulls. Diets were fed ad libitum as a total mixed ration to avoid selection of dietary components. Oils were mixed with concentrate ingredients before corn silage was added to optimize oil dispersal.

### Biopsies

Mammary percutaneous biopsies (Bionaz and Loor 2007) for gene expression profiling were obtained on days 0, 7, and 21 relative to start of treatments in cows fed FSO and EB100 and at 0 and 21 day in control cows. The procedure was conducted under mild general (Xylazine, Phoenix Pharmaceutical, Inc., St. Josephs, MO, USA) and local anesthesia. Complete details are available in ESM Online Resource 1.

### Quantitative PCR, primer design, and primer testing

Complete details of these procedures can be found in ESM Online Resource 1. The final mRNA expression data were normalized using the geometric mean of ubiquitously

expressed transcript (*UXT*), eukaryotic translation initiation factor 3, subunit K (*EIF3K*), and mitochondrial ribosomal protein L39 (*MRPL39*) which we identified as suitable internal controls among several tested (Kadegowda et al. 2009b).

### Microarray data mining

We mined microarray data published recently from one of our laboratories (Invernizzi et al. 2009) using the same animals for qPCR in order to evaluate the relative expression of genes that belong to the endoplasmic reticulum (ER) stress pathway. Details and discussion of results can be found in ESM Online Resource 1.

### Fatty acid analysis

Fatty acids in milk were analyzed by gas liquid chromatography according to the method of Mosley et al. (2006). As described by Bionaz and Loor (2008b), daily yield of fatty acids (moles/day) synthesized de novo was calculated by the sum of 4- to 14-carbon FA and yield of FA taken up from blood by the sum of 18- to 24-carbon FA. The index of acetyl-CoA incorporated during FA elongation (ACE or FA synthesis from acetyl-CoA) was also calculated as (chain length/2–1.5) for FA 4:0 to 14:1 and (chain length/2–1.5)×0.6 for 16:0 and 16:1 (Garnsworthy et al. 2006). Delta-9 desaturase indexes were calculated as the ratio of the product divided by the sum of the substrate and the product (Loor and Herbein 2003).

### Statistical analysis

Data points with studentized residuals >2.5 were considered outliers and excluded from the analysis. Gene expression results after normalization were transformed to *n*-fold change relative to 0 day and then log-transformed (e.g., Bionaz and Loor 2008a, b). A repeated measures model was fitted to the adjusted ratios using Proc MIXED (SAS; SAS Inst., Cary, NC, USA). The model consisted of time, treatment, and time × treatment interaction as fixed effects, as well as cow as the random effect. Differences in body mass, dry matter intake (DMI), milk yield, milk fat percentage and yield, milk protein percentage and yield, lactose, and milk fatty acid molar composition were assessed using the same statistical model. Gene expression network and pathway analysis was performed using Ingenuity Pathway Analysis® (Ingenuity Systems, Mountain View, CA, USA).

## Results

### Animal performance

There was a diet × time interaction ($p<0.05$) for DMI, milk fat percentage, and milk fat yield (Fig. 1) primarily due to temporal decreases in these parameters when FSO was fed. Yields of milk (ESM Online Resource 1 and Fig. S1) and yield and percentage of milk components (ESM Online Resource 1 and Fig. S2) for cows in CTR remained relatively constant throughout the experiment. Except for body weight, all other parameters changed over time ($p<0.05$; ESM Online Resource 1 and Figs. S1 and S2).

### Milk fatty acid synthesis

Temporal changes in molar fatty acid yield in cows fed EB100 and FSO are shown in Fig. 2 (see also ESM Online Resource 1 and Fig. S3). Dietary supplementation with FSO resulted in marked alterations in molar FA yield over time relative to the EB100 or CTR diet (Fig. 2). Beyond day 7 during which there was a similar trend in molar FA yield, total molar FA yield increased or remained constant at days 14 and 21 with EB100 and CTR, while it continued to decrease with FSO. Changes in specific FA were characterized primarily by a gradual reduction ($p<0.05$) in 4:0 to 18:0 yield, with 18:0 and *cis*9 18:1 ($p = 0.08$) reaching very low levels. Yield of *cis*9,*trans*11-CLA, all *trans*-18:1, and total *trans*-18:1 with FSO increased drastically ($p<0.05$) by day 7, followed by slight but gradual reductions at days 14 and 21. The yield of fatty acids synthesized de novo, i.e., sum of 4:0 to 14:0 not including 15:0, decreased ($p<0.05$) gradually with FSO only. In contrast, the yield of fatty acids taken up from blood (i.e., uptake derived) increased ($p<0.05$) dramatically over time with FSO due primarily to *trans*-18:1 isomers derived from incomplete ruminal biohydrogenation. The

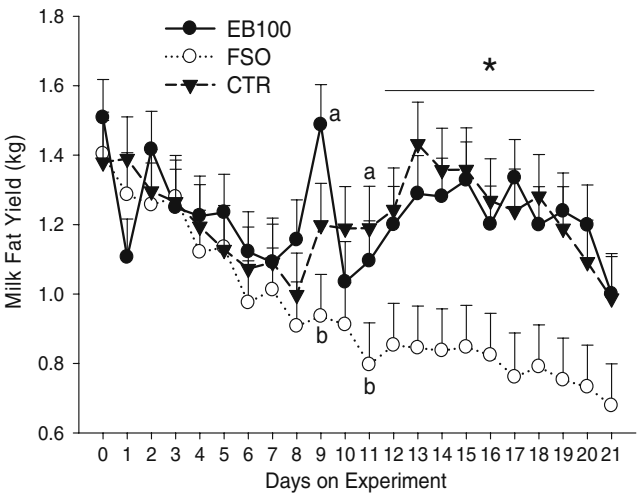



**Fig. 1** Milk fat production in response to saturated lipid (EB100) or a blend of soybean and fish oil (FSO). The number of cows per group was six for EB100 and CTR and five for FSO. *$p\leq0.05$ between EB100 or control (CTR) and FSO diets. $^{a,b}p\leq0.05$ denote differences between EB100 and FSO

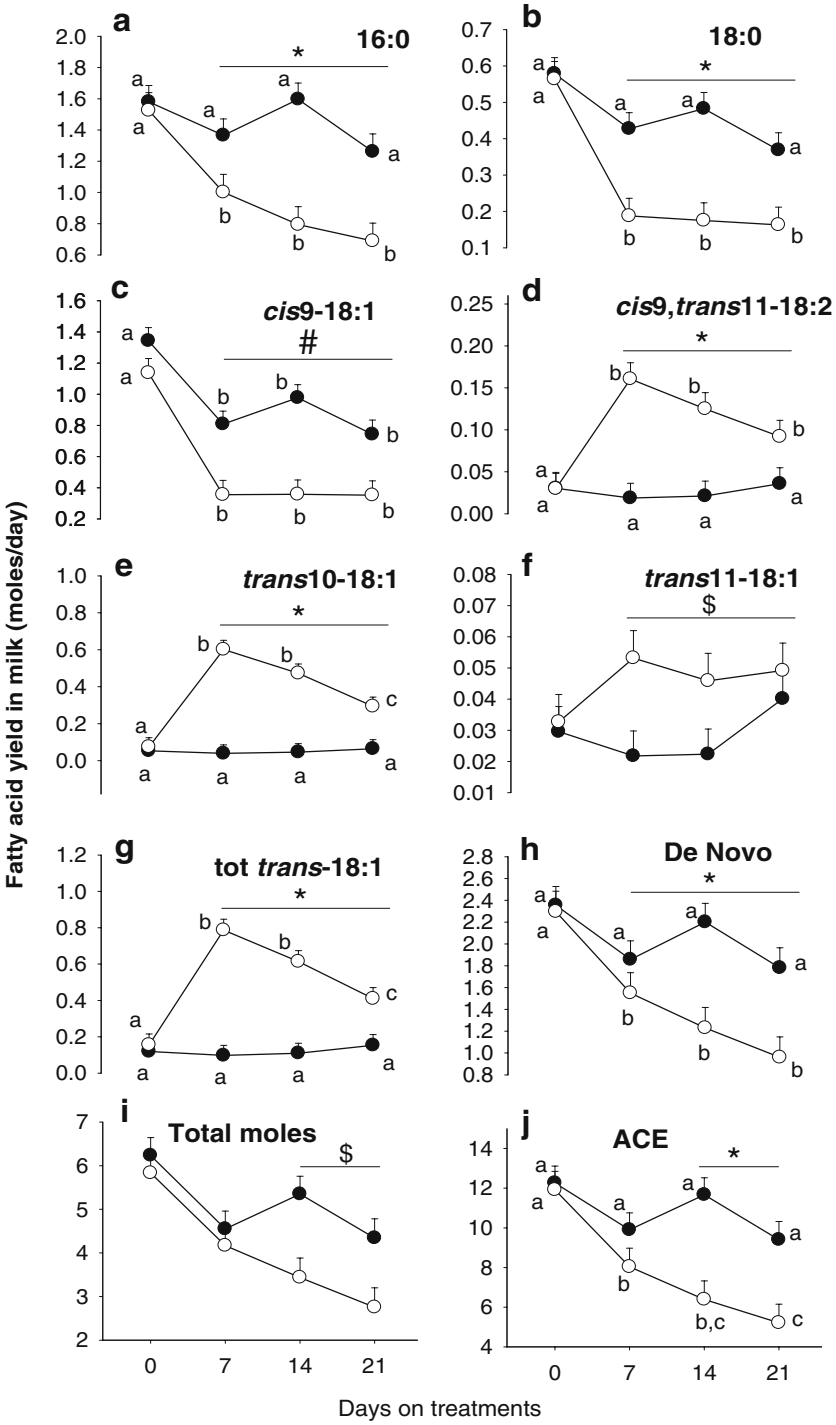



**Fig. 2** Milk fatty acid yield (moles/day) in response to saturated lipid (EB100) or a blend of soybean and fish oil (FSO). Temporal effects of diets on the molar yield of the primary substrates (**a**, **b**) for stearoyl-CoA desaturase (*SCD*), major products of stearic acid (**c**) and vaccenic acid (**d**) desaturation via SCD, two major *trans*-18:1 isomers from rumen biohydrogenation (**e**, **f**), total *trans*-18:1 and de novo-synthesized fatty acids (**g**, **h**), moles of fatty acids (**i**), and moles of acetate used for chain elongation during de novo synthesis of fatty acids (**j**). The number of cows per group was six for EB100 and five for FSO. $^{*}p \leq 0.05$ and $^{\#}p = 0.08$ denote diet×time effects between diets. $^{a,b,c}p \leq 0.05$ denote differences between time points. $^{\$}p \leq 0.05$ denote overall differences between diets during the 21-day period

yield of 10/12CLA was not different due to EB100 or FSO relative to CTR. The pattern of acetate used for chain elongation during de novo synthesis of fatty acids was the same as that of the de novo FA. In general, dietary supplementation with EB100 resulted in responses that were intermediate between CTR and FSO or similar to CTR. Yield of 18:3, 20:5, and desaturation indexes of *cis*9-14:1/14:0 and *cis*9-18:1/18:0 were not affected by diet. However, desaturation index of *cis*9-16:1/16:0 increased ($p<0.05$) between days 7 and 14 with FSO but remained unchanged with EB100, thus leading to differences between diets. Feeding the FSO diet caused a significant reduction ($p<0.05$) in the secretion of total fatty acids in milk and milk fat yield that could be related to the substantial decrease in the amount of short- and medium-chain fatty acid (4- to 16-carbon fatty acids) as well as oleic acid secreted in milk (Fig. 2 and ESM Fig. S4).

Expression of transcription regulators

Genes tested associated with transcription regulation (Fig. 3) responded in different ways to the treatments. *SREBF1* expression was highly influenced by FSO and EB100. At day 7 vs. day 0, in particular, the expression of *SREBF1* increased ($p<0.05$) due to EB100, but it decreased due to FSO. Such response resulted in greater (interaction $p<0.05$) *SREBF1* with EB100 at day 7 relative to FSO. No differences in *SREBF1* expression between treatments were detected at day 21. *SCAP* expression increased (interaction and diet $p<0.05$) markedly with FSO over time and differed from EB100 by approx. sevenfold on day 21. *PPARG* and *SREBF2* had a very similar pattern and did not differ due to treatments. However, an overall effect of day ($p<0.05$) was observed due to an increase of expression at day 21 compared to day 7. In a similar fashion, *INSIG1* expression was not different between treatments, but an overall effect of day ($p<0.05$) revealed an increase at days 7 and 21 compared to its initial expression on day 0. *THRSP* pattern was very similar between FSO and EB100, with an approx. 12-fold decrease in expression at day 7 compared to day 0 followed by an increase to baseline levels.

Expression of fatty acid uptake, intracellular activation, and esterification enzymes

Among genes related with fatty acid transport and esterification (Fig. 4), *FABP3* and *GPAM* increased ($p<0.05$) by day 7 with EB100 followed by a return to basal expression by 21 days, whereas FSO did not alter the expression of either gene at day 7, but increased it at day 21 ($p<0.05$). *LPIN1* expression increased ($p<0.05$) with EB100 at days 7 and 21, whereas FSO decreased *LPIN1* expression at day 7, resulting in treatment differences (interaction $p<0.05$). The pattern of *LPL*

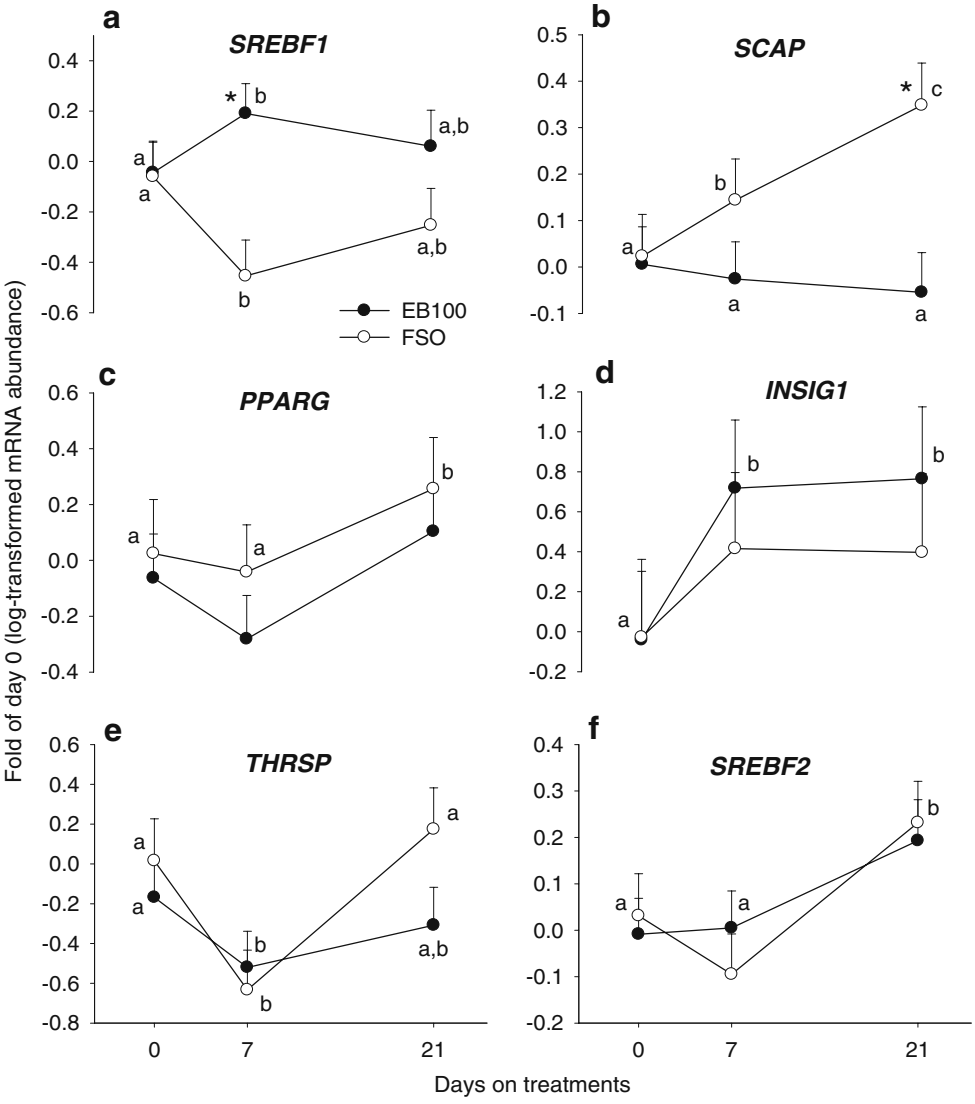



**Fig. 3** Effect of saturated lipid (EB100) or a blend of soybean and fish oil (FSO) on mammary mRNA expression of transcription regulators. Mammary biopsies were collected during a 21-day feeding period and used for mRNA expression profiling of genes associated with transcriptional regulation of lipogenesis (**a**, **b**), ligand-dependent transcriptional regulation of lipogenesis (**c**), regulation of *SREBF1* (**d**), lipid metabolism (**e**), and transcriptional regulation of cholesterol synthesis (**f**). The number of cows per group was six for EB100 and five for FSO. *$p\leq0.05$ denote diet×time differences between diets. $^{a,b,c}p\leq0.05$ denote differences between time points. For *PPARG*, *INSIG1*, and *SREBF2*, there was no interaction effect; $^{a,b}p\leq0.05$ denote overall differences between time points

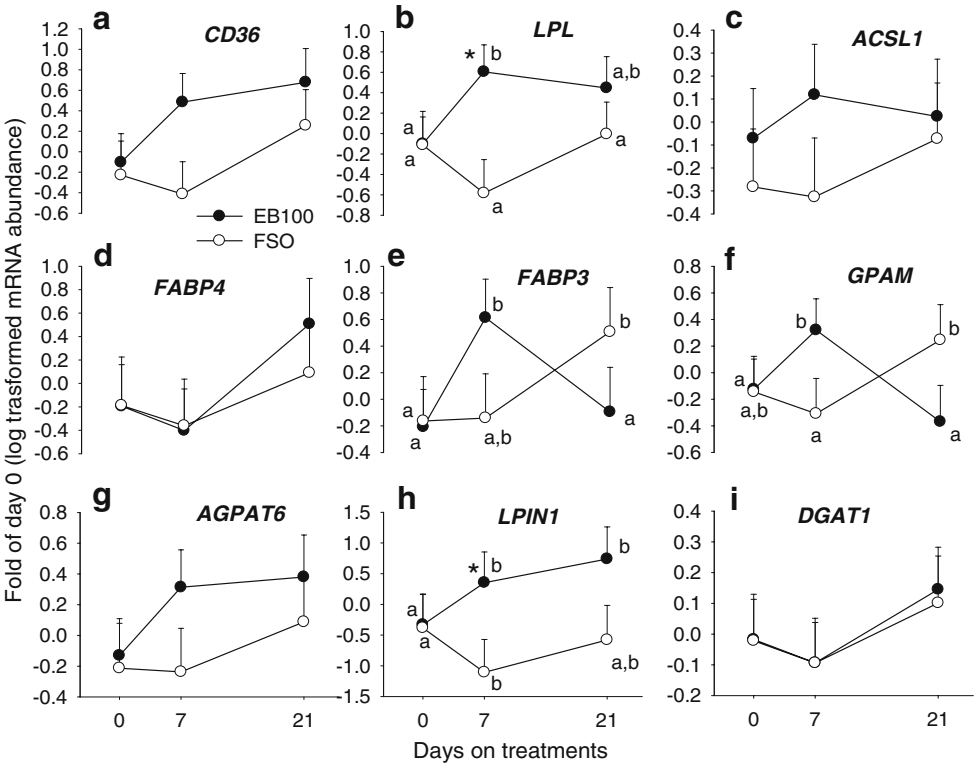



**Fig. 4** Effect of saturated lipid (EB100) or a blend of soybean and fish oil (FSO) on mammary mRNA expression of lipogenic and desaturation enzymes. Mammary biopsies were collected during a 21-day feeding period and used for mRNA expression profiling of genes associated with long chain fatty acid uptake (**a**, **b**), intracellular activation to fatty acyl-CoA (**c**), intracellular transport (**d**, **e**), esterification (**f–i**), and transcriptional co-activation of PPAR (**h**). The number of cows per group was six for EB100 and five for FSO. *$p \leq 0.08$ denote diet×time differences between diets. $^{a,b,c}p \leq 0.05$ denote differences between time points

was characterized by a temporal increase ($p<0.05$) at day 7 with EB100 but a temporal decrease with FSO, resulting in a significant difference (interaction $p<0.05$) between FSO and EB100 at day 7. Both diets resulted in a return to basal expression by day 21. *FABP4* and *DGAT1* had a similar pattern regardless of treatment, with an increase (day $p = 0.02$) in expression at day 21 vs. day 0. *AGPAT6* and *ACSL1* exhibited no significant differences due to treatment or time. There was no interaction effect on the expression of *CD36*, although we found an overall effect of day ($p = 0.05$) characterized by an increase of expression at day 21 vs. day 0 with both treatments.

Expression of lipogenic and desaturation enzymes

Genes related to de novo fatty acid synthesis and very long-chain fatty acid desaturation (Fig. 5) had a similar pattern of expression due to feeding EB100 with *ACACA*, *FASN*, *ACSS2*, and *FADS1* increasing at day 7, followed by a decrease to initial levels by day 21. No changes in the expression of these genes were observed with FSO by day 7; thus, the response with EB100 resulted in significant treatment differences (interaction $p<0.05$ or $p = 0.08$ for *FADS1*) between diets on day 7. Quite unexpectedly, the expression of *SCD* increased by day 7 with both diets (day $P=0.001$) and remained elevated with both diets at day 21 compared to day 0.

Expression of intracellular lipid droplet membrane proteins

Expression of *XDH* did not change with EB100, but decreased ($p = 0.08$) with FSO at day 7 vs. day 0, resulting in differences (interaction) between diets (Fig. 6). Expression of *XDH* with FSO returned to basal levels by day 21. Expression of *BTN1A1* increased ($p<0.05$) at day 7 regardless of diet and remained elevated at day 21 vs. day 0.

Expression of cell proliferation and chromatin remodeling regulators

These results and accompanying discussion are presented in ESM Online Resource 1.

## Discussion

In non-ruminants, transcriptional mechanisms are responsible for the rapid changes in the mRNA expression of some genes implicated in lipogenesis including *ACACA*, *FASN*, and *SCD* (Foufelle and Ferre 2002). Thus, marked alterations in gene expression over time are expected. Results obtained in the present study strongly confirmed this point, revealing two strikingly different scenarios 7 days after the beginning of the trial and another at its end. During the first days after the beginning of the trial, the transcriptional machinery was likely undergoing an adjustment phase, i.e.,

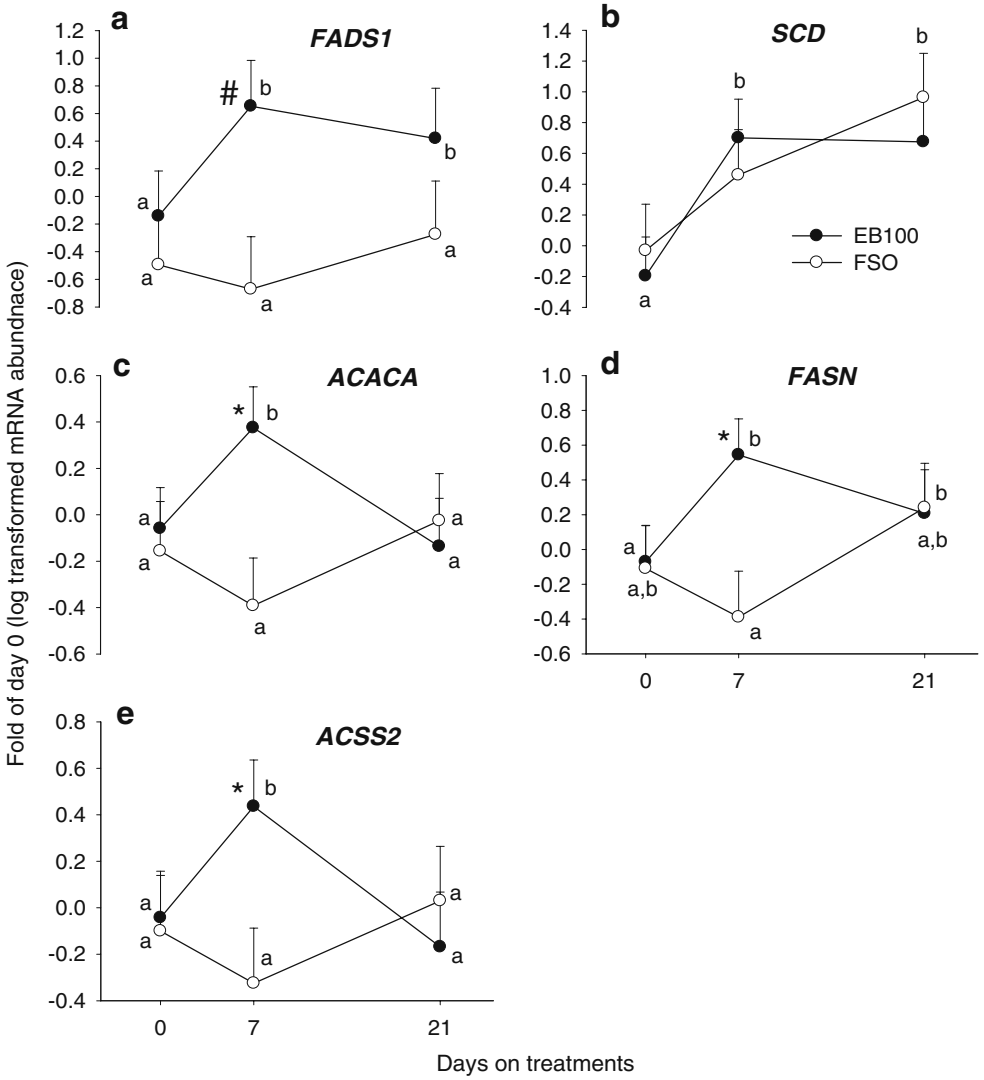



**Fig. 5** Effect of saturated lipid (EB100) or a blend of soybean and fish oil (FSO) on mammary mRNA expression of lipogenic and desaturation enzymes. Mammary biopsies were collected during a 21-day feeding period and used for mRNA expression profiling of genes associated with desaturation of omega-3 and -6 fatty acids (**a**), desaturation of saturated fatty acids (**b**), and de novo fatty acid synthesis (**c**, **d**, **e**). The number of cows per group was six for EB100 and five for FSO. $^{*}p \leq 0.05$ and $^{\#}p = 0.08$ denote diet × time differences between diets. $^{a,b,c}p \leq 0.05$ denote differences between time points. For SCD, there was no interaction effect. $^{a,b}p \leq 0.05$ denote overall differences between time points

a sudden increase in dietary saturated or *trans*-FA from ruminal hydrogenation of dietary polyunsaturated FA (PUFA). Profiles of the major milk FA (16:0, 18:0, and *cis*9-18:1; Fig. 2) as well as milk fat concentration and yield due to treatments were essentially set by day 7 and remained nearly unchanged through day 21. For the most part, our results confirmed work from Shingfield et al. (2006) demonstrating that dietary fish oil induces marked shifts in the production of *trans*-18:1 FA, particularly *trans*10-18:1, in the rumen during the first few days of feeding (see also Shingfield et al. 2010). Changes in the profiles of ruminal microorganisms (Yang et al. 2009), which play a predominant role in PUFA metabolism in the rumen, likely account for the observed patterns of *trans*-FA, CLA, and 18:0 found in milk. Unlike Shingfield et al. (2006), our milk FA yields do not provide evidence that microorganisms adapted to the continued supply of fish oil because 18:0 and *trans*11-18:1 output in milk remained unchanged from day 7 onward (Fig. 2).

The responses of key lipogenic genes in mammary tissue revealed opposite adaptive mechanisms to dietary PUFA from fish oil (FSO) or saturated FA (EB100; Fig. 3). More importantly, the similar trend of expression for a group of metabolically linked genes reinforces the idea that transcription regulators may have an impact on the regulation of bovine mammary lipogenic networks (Bionaz and Loor 2008b). The upregulation of *SREBF1* and several of its targets (at least in non-ruminants; Figs. 2 and 7) by feeding EB100, which contained palmitate and stearate as the primary FA (each accounted for approx. 40% of total FA), was suggestive of an effect on expression and potential activity of this transcription factor. We are unaware of previous in vivo data with ruminants showing upregulation of *SREBF1* and its targets (*ACACA*, *FASN*, *GPAM*) in response to dietary saturated FA, but such a response has been shown in murine liver due to long-term dietary stearate (Sampath et al. 2007). As we observed in vitro, it seems that palmitate and stearate can upregulate *INSIG1* potentially

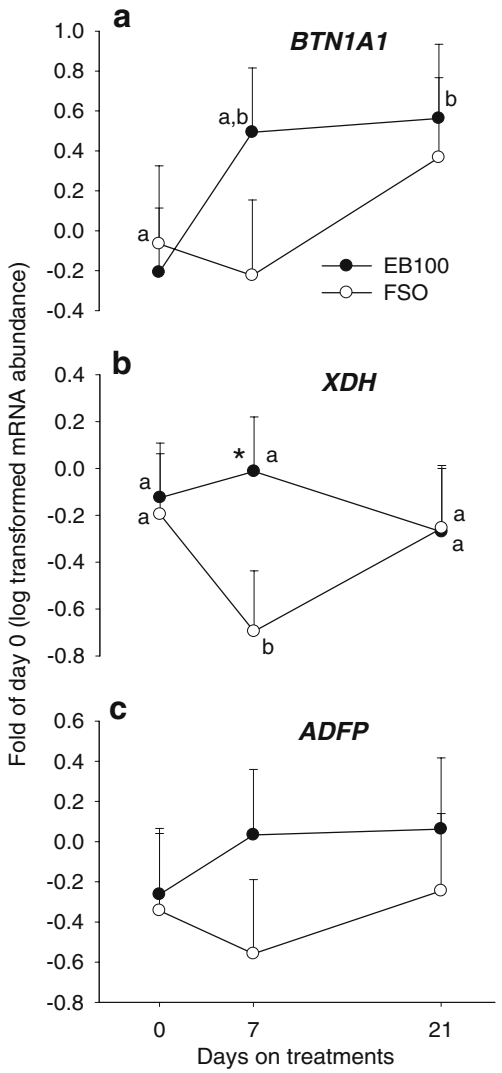



**Fig. 6** Effect of saturated lipid (EB100) or a blend of soybean and fish oil (FSO) on mammary mRNA expression of intracellular lipid droplet proteins. Mammary biopsies were collected during a 21-day feeding period and used for mRNA expression profiling of genes associated with milk fat droplet formation and secretion (**a**–**c**). The number of cows per group was six for EB100 and five for FSO. *$p \leq 0.08$ denote diet×time differences between diets. $^{a,b,c}p \leq 0.05$ denote differences between time points. For *BTN1A1*, there was no interaction effect. $^{a,b}p \leq 0.05$ denote overall differences between time points

through an unidentified nuclear receptor or transcription factor (Kadegowda et al. 2009a). Although the above data support a role of *SREBF1* in milk fat synthesis (Harvatine and Bauman 2006; Bionaz and Loor 2008b; Kadegowda et al. 2009a), the concomitant increase in *INSIG1* expression and the lack of change in *SCAP* with EB100 if it translated to the protein level would have resulted in greater retention of *SREBF1* in the ER, i.e., less mature SREBF1 would have been synthesized (Engelking et al. 2004; Kast-Woelbern et al. 2004; Desvergne et al. 2006).

In contrast with our results, recent data described a decrease of *INSIG1* during milk fat depression in lactating cows induced by fish oil feeding for 14 days or short-term (3 days) intrajugular infusion of supraphysiological amounts of 10/12CLA (Harvatine and Bauman 2006). Recent work from one of our laboratories observed not only a marked upregulation of *INSIG1* relative to *SREBF1* during the lactation cycle but also greater relative abundance of *INSIG1* transcript in mammary tissue. It could be possible that *INSIG1* has some level of control on mammary *SREBF1* (Bionaz and Loor 2008b) as proposed for murine adipose tissue and liver (Engelking et al. 2004; Kast-Woelbern et al. 2004). Our in vitro data with palmitate and stearate clearly revealed the upregulation of *INSIG1*, without effects on *SCAP* or *SREBF1* expression, coupled with greater intracellular lipid droplet formation in mammary cells (Kadegowda et al. 2009a). Thus, it would appear that SREBF1 transcriptional activity might not be essential for the overall process of milk fat synthesis, i.e., other nuclear receptors/transcription regulators could regulate lipogenic gene expression. Judging from the observed patterns with both EB100 and FSO, as well as in vitro data with 20:5 (Kadegowda et al. 2009a), *INSIG1* appears to have a role in milk fat synthesis, and further studies will have to be conducted to clarify such a role.

Although we confirmed (Harvatine and Bauman 2006) a decrease in *SREBF1* and *LPL* (Ahnadi et al. 2002) after 7 days of feeding FSO, differences between treatments essentially disappeared by 21 days (see also ESM Table S5) despite sustained milk fat depression with this diet (Fig. 1). More importantly, dietary FSO had no effect on the expression of *ACSS2*, *ACACA*, and *FASN* over time or when compared at day 21 with EB100 and CTR (ESM Table S5). The response induced by dietary EB100 drove the observed interaction effect for these genes, but only through the first 7 days. These observations (at least for *ACACA* and *FASN*) contrast with previous data obtained after 14 days (Harvatine and Bauman 2006) or 28 days (Ahnadi et al. 2002) of feeding FSO. Previous studies have used greater levels of dietary FSO (i.e., 1.5% to 3.7% of total diet), which could partly explain the differences. In addition, approaches for normalization of mRNA expression data between studies differed. We showed, using mammary samples from this and another study, that *B2M* and *ACTB* (Harvatine and Bauman 2006) were unreliable for normalization (Kadegowda et al. 2009b). Ribosomal proteins (Piperova et al. 2000; Ahnadi et al. 2002) due to their high abundance also are unsuitable as internal controls (e.g., Vandesompele et al. 2002).

Because it cannot bind FA directly (Postic et al. 2007), our network analysis (Fig. 7) suggested that the effect of dietary saturated FA on the expression/activity of SREBF1 might have been indirect, e.g., through an increase in the

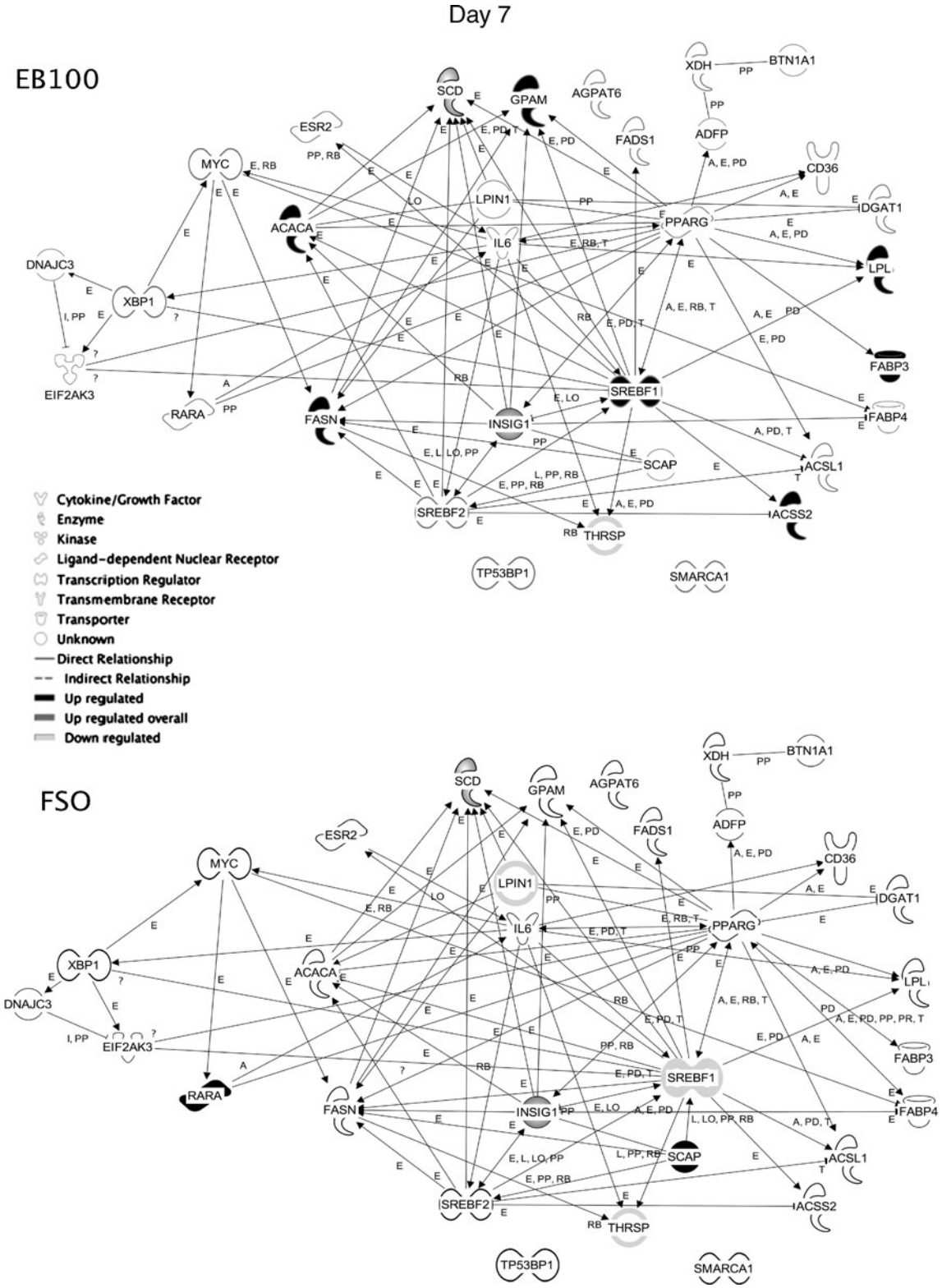



**Fig. 7** Effect of saturated lipid (EB100) or a blend of soybean and fish oil (FSO) on mammary gene expression networks at day 7 relative to day 0. Network created with Ingenuity Pathway Analysis and based on currently known relationships among genes analyzed. The relationships among molecules include activation (*A*), protein–protein interactions (*PP*), localization (*L*), expression (*E*), and transcription (*TR*). Genes are indicated by *capital letters*, and *different shapes* denote the type of protein encoded by the specific genes according to the legend insert

expression of *SCD* (Fig. 5), because it has been previously demonstrated in mouse that the activity of SCD, which produces oleic acid, is essential for the expression of *SREBF1* (Miyazaki et al. 2004); through PPARγ activation of *SREBF1* mRNA expression as we have reported in bovine mammary cells (Kadegowda et al. 2009a); and through the enhanced availability of palmitate for the synthesis of sphingolipids, which are potent regulators of SREBP activity (Worgall 2007). This latter mechanism appears to be supported by the marked increase in *SCAP* with FSO. It is possible that the three mechanisms worked simultaneously in mammary tissue, i.e., the first and second mechanisms explain the effect on the expression of *SREBF1* because *SCD* is a PPARγ target and exogenous 16:0 appears to have a feed-forward effect on *SCD* expression (Kadegowda et al. 2009a). The fact that *PPARG* expression was not affected by diet in our study is not against a role for this nuclear receptor in activating the mammary lipogenic program because we demonstrated that the PPARγ-specific ligand rosiglitazone led to the upregulation of *SREBF1* and a number of lipogenic genes without affecting *PPARG* expression (Kadegowda et al. 2009a).

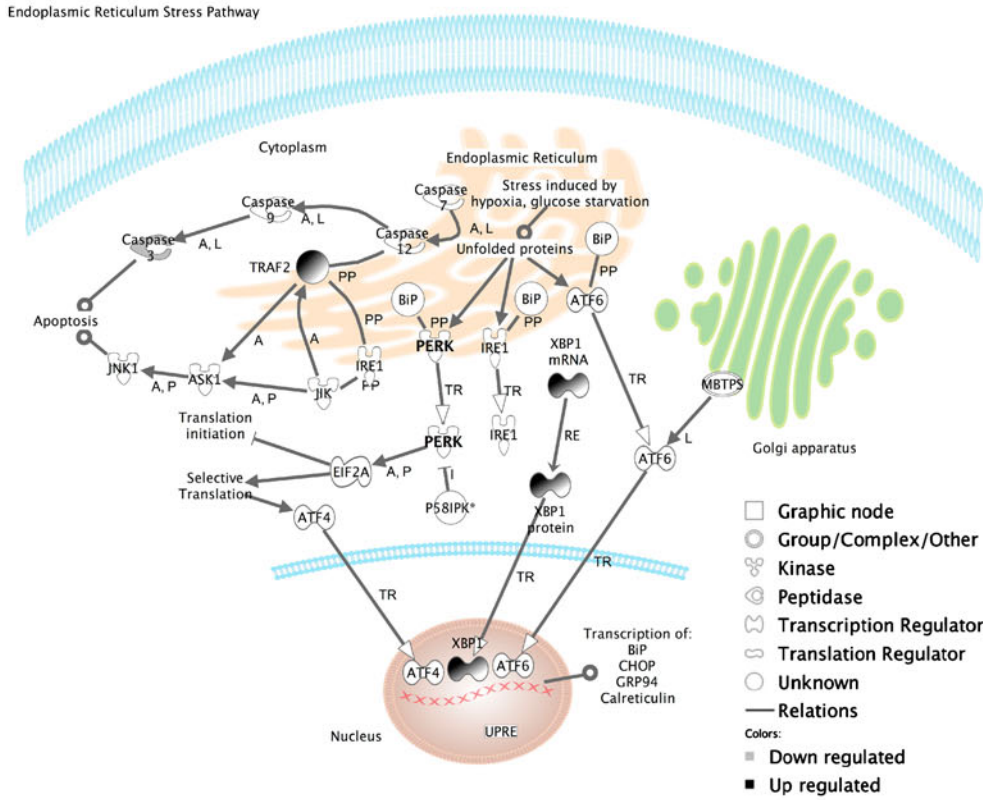
Quite unexpectedly, it is apparent from our study that *SCAP* transcription might be a previously unrecognized regulatory point required for SREBF1 maturation and normal activity in bovine mammary. In fact, it was striking that *SCAP* was one of two genes (along with *SCD*) with a sustained increase in expression in response to dietary FSO (Fig. 3), thus resulting in greater abundance at day 21 even when compared with the control (ESM Table S4). Our results contrast markedly with Harvatine and Bauman (2006) who reported no changes in *SCAP* expression after 14 days of feeding a fish oil-supplemented diet or 3 days of supraphysiological 10/12CLA infusion. We previously observed a downregulation of *SCAP* in mammary cells due to exogenous 20:5 (Kadegowda et al. 2009a), but in the present study, there was no change in milk 20:5 (see ESM Online Resource File 1) despite the fact that it accounted for approx. 14% of total FA in fish oil. Thus, substantial ruminal biohydrogenation of dietary 20:5 in our study likely prevented sufficient influx of this FA into the mammary gland so that it could elicit effects on milk fat synthesis. The well-established mechanism of *SCAP* function in non-ruminant cells involves sterols, and we observed the upregulation of *SREBF2* between days 7 and 21 with both diets. Previous work from one of our laboratories showed that mammary *SREBF2* was upregulated to a greater extent than *SREBF1* during the lactation cycle, suggesting a potentially important function of cholesterogenesis (Bionaz and Loor 2008b). Cholesterol in bovine milk is a minor component of the lipid fraction (Bitman and Wood 1990); thus, the function of *SREBF2* in bovine mammary tissue might be to regulate target genes associated with cholesterol synthesis (Horton et al. 2002) for cellular membrane synthesis and replenishment.

**Fig. 8** Effect of saturated lipid (EB100) or a blend of soybean and fish oil (*FSO*) on mammary mRNA expression of components of the ER stress pathway. Pathways created with Ingenuity Pathway Analysis and based on currently known relationships among genes analyzed. The relationships among molecules include activation (*A*), protein–protein interactions (*PP*), localization (*L*), expression (*E*), and transcription (*TR*). Genes are indicated by *capital letters*, and *different shapes* denote the type of protein encoded by the specific genes according to the legend insert

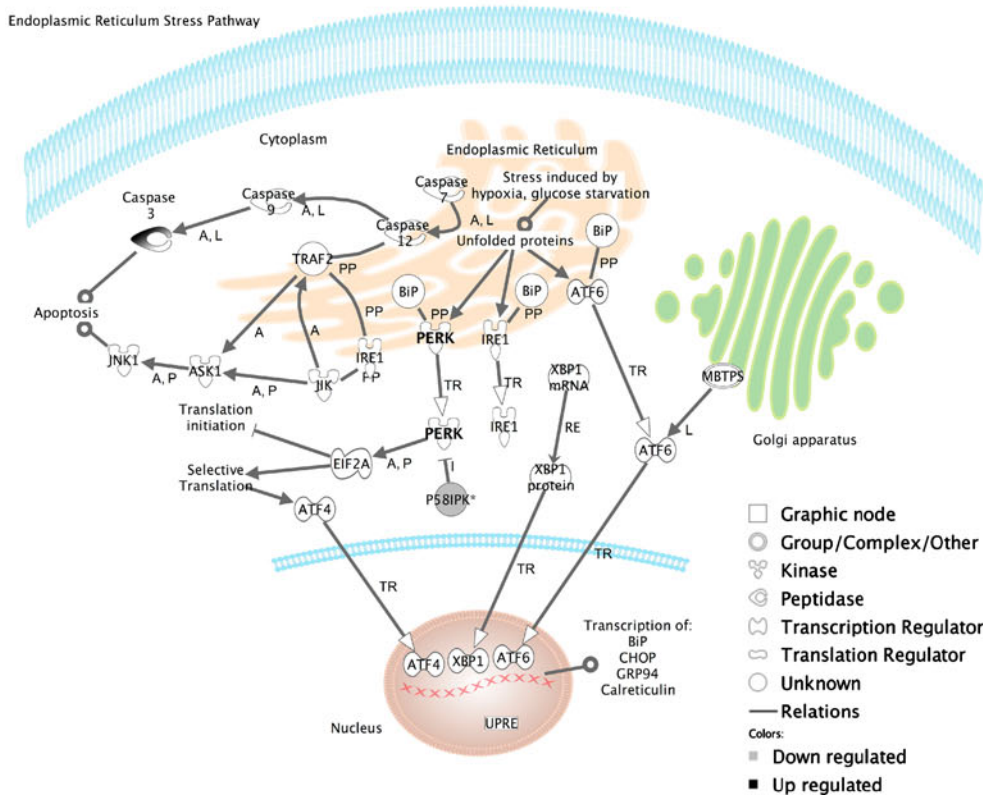
A recent in vitro study revealed an absolute need of *SCAP* for the expression of *SREBF1*, *SREBF2*, *FASN*, and the rate-limiting cholesterogenic enzyme HMGCR in cells used to study the process of myelination, i.e., cellular membrane synthesis (Verheijen et al. 2009). We have previously observed a gradual increase in *SCAP* mRNA expression in bovine mammary tissue during the lactation cycle (Bionaz and Loor 2008b). It is unclear if the observed upregulation of *SCAP* during milk fat depression was an adaptive response by tissue to ensure a proper level of FA for membrane phospholipid synthesis. This could partly explain the fact that EB100 did not affect *SCAP*, i.e., *LPL* and *FABP3* upregulation would have ensured continued uptake and supply of FA for milk fat and phospholipid synthesis. Bovine mammary cell nuclear, ER, and Golgi membranes contain substantial amounts of cholesterol and phospholipids including sphingomyelin (6–14% of total phospholipids), and also the milk fat globule membrane contains substantial amounts of sphingomyelin (approx. 20% of total phospholipids; Keenan and Huang 1972).

As noted in the discussion above, at least in non-ruminant cells, sphingolipids are involved in lipid synthesis regulation through their action on SREBP (Worgall 2007). Mammary tissue synthesizes sphingolipids de novo (Bitman and Wood 1990) from palmitoyl-CoA, leading to ceramide formation and incorporation into sphingomyelin (Bitman and Wood 1990). Thus, palmitic acid used for ceramide synthesis in mammary appears a required step and also might represent a regulatory point for FA synthesis because ceramides can inhibit this process by blocking the activity of AKT/PKB (Zierath 2007). Regulation of FA synthesis by sphingolipids in mammary tissue has never been investigated. However, our previous data on genes associated with ceramide synthesis suggest an increase in synthesis coupled with decreased degradation throughout lactation (Bionaz and Loor 2008a). Based on previous data (Bionaz and Loor 2008a) and current results, we speculate that the sustained increase in *SCAP* expression might have allowed some *SREBF1* as well as *SREBF2* protein to mature in the Golgi (Horton et al. 2002), hence allowing availability of de novo-synthesized FA and cholesterol for milk fat synthesis as well as cellular membrane replenishment. These adaptations,

EB day 21 vs. day 7




FSO day 21 vs. day 7



however, were insufficient to reestablish milk fat synthesis to the level observed with controls.

An additional important finding was that expression of *THRSP*, which has been previously proposed as a protein involved in the regulation of bovine milk fat synthesis (Harvatine and Bauman 2006), decreased with both treatments by day 7 and was numerically greater at day 21 with FSO. Furthermore, no differences between lipid-supplemented diets or the control at day 21 were found (ESM Table S5). This response differed from results of Harvatine and Bauman (2006). It could be possible that *THRSP* independently modulates milk fat synthesis in some fashion, but likely not through direct interaction with FA, i.e., effects might be indirect and probably linked with transcription regulators able to bind FA or with effects on other enzymes (Pegorier et al. 2004; Kadegowda et al. 2009a). Despite previous observations showing modest upregulation of THRSP during the lactation cycle (Bionaz and Loor 2008b), our current data offer no support for an essential role of *THRSP* in the regulation of mammary lipid synthesis.

Upon uptake by mammary gland, the saturated FA indirectly via *SREBF1* or directly via unidentified nuclear receptors (Kadegowda et al. 2009a; Bionaz, Thering, and Loor, unpublished results) might have triggered a set of adaptations which included greater LPL on the mammary endothelium to cope with the release of FA from circulating TAG (Fielding and Frayn 1998); in a similar fashion, intracellular transport of long-chain FA taken up from blood as well as activation of short-chain FA (e.g., acetate, butyrate) appeared to have been enhanced through the upregulation of *FABP3* and *ACSS2* during this adaptation phase. Although we could not detect differences in milk fat yield between EB100 and controls (Fig. 1) in both of those groups, milk fat yield increased between days 7 and 14; the increase in the expression of GPAM might have played a role in promoting TAG synthesis.

Outside the SREBF1 network, but with a potential function in the regulation of transcription of other genes involved in milk fat synthesis (Bionaz and Loor 2008b), *LPIN1* expression in response to the type of dietary fat was completely opposite and followed along with the pattern of *SREBF1*. Although it is tempting to speculate that the observed response agrees with the regulation of *LPIN1* via SREBF1 demonstrated in murine hepatoblastoma cells (Ishimoto et al. 2009), it is important to bear in mind that bovine mammary cells do not appear to synthesize quantitative levels of sterols (Bitman and Wood 1990) which were required to induce SREBF1 activation of *LPIN1* expression. An additional mechanism mediating *LPIN1* expression involves nuclear factor Y (*NFYA*). However, microarray data from this study (Invernizzi et al. 2009) showed (data not presented) an upregulation of this gene in mammary cells with both treatments (+42% with EB100 and +24% with FSO vs. CTR) on day 21. Bovine *LPIN1* and *SREBF1* have a very strong PPAR response element for PPARγ in the promoter/upstream position (M. Bionaz, B. Thering, and J. J. Loor, unpublished results), and both were markedly upregulated by rosiglitazone and palmitate in bovine mammary cells (Kadegowda et al. 2009a, b). Thus, their pattern of expression in the present study suggests they are bovine PPARγ targets.

The expression of *SCD* increased over time regardless of treatment, and at 21 days, there were no differences in expression across all treatments (see ESM Table S5). There are no previous data, to our knowledge, on the longitudinal *SCD* expression in response to lipid feeding or milk fat depression in dairy cows. The lack of differences in *SCD* between treatments at the end of the study partly agree with previous data from cows fed fish oil for 14 or 28 days (Ahnadi et al. 2002; Harvatine and Bauman 2006). However, the conclusions drawn from those single-point observations could lead to the erroneous assumption that *SCD* was not affected and/or had no crucial role in milk fat synthesis as was evidenced predominantly by its sustained upregulation in response of dietary FSO, i.e., when both the availability of stearate for oleate and its subsequent use for milk fat synthesis were dramatically impaired.

In vitro results with bovine mammary cells (Kadegowda et al. 2009a) showing an upregulation of *SCD* with palmitate support the idea that with dietary EB100, it was the availability of precursors (16:0, 18:0) for the enzyme that drove the increase in expression over time. A number of previous studies with dietary fish oil have observed reductions in stearic acid outflow from rumen and secretion in milk fat (e.g., Offer et al. 2001; Ahnadi et al. 2002; Loor et al. 2005a, b; Shingfield et al. 2006; Harvatine and Bauman 2006). Studies with rodent (Miyazaki et al. 2001) and chicken hepatocytes (Legrand et al. 1997) have provided direct evidence for a stringent requirement of endogenous oleic acid for the synthesis of TAG. Because of a proposed stringent requirement for endogenous *cis*-9 18:1 for mammary lipid synthesis (Loor and Herbein 2003), in cows fed FSO, the product of the SCD reaction would have been immediately used for fat synthesis and in this way alleviated the potential for oleic acid to inhibit (i.e., feedback inhibition) mRNA expression, i.e., sustained *SCD* upregulation due to low stearate influx would be required under these conditions. In addition, the amount of dietary oleic acid provided by FSO was approx. 25% of that with EB100. We observed in vitro a dramatic downregulation of *SCD* (−400% from controls) in bovine mammary cells incubated with oleate (Kadegowda et al. 2009a).

Despite the marked upregulation of *SCD* with FSO, the activity of SCD apparently could not overcome the approx. 70% decrease in the yield of oleic acid probably due to the

chronically low availability of stearate (Fig. 2). Clearly, the decrease in oleate accounted for a significant portion of the approx. 56% decrease in the molar yield of total FA (Fig. 2). Under the conditions of our study, it was apparent that adaptations in *SCD* transcription were caused by low stearate influx and were driven by the need to rebound milk fat synthesis to a normal set point. Although not measured in our study, the melting point of the fat produced in response to FSO was likely altered, e.g., more *trans*-18:1 isomers with a greater melting point were available for esterification in mammary cells, thus increasing the need of the cells to synthesize more SCD protein to handle the lower availability of 18:0 for oleic acid synthesis as well as desaturated *trans*11-18:1 to *cis*9,*trans*11-18:2. The latter effect would readily decrease the melting point of the milk fat produced in response to FSO. It was noteworthy that by day 7 in cows fed FSO, delta-9 desaturase indexes decreased along with milk fat yield despite the marked increase in *SCD* expression. During days 7 through 21, desaturase indexes (at least for *cis*9-16:1/16:0; other indexes did not reach significance but followed the same trend as *cis*9-16:1/16:0) were more in line with *SCD* mRNA despite the declining milk fat yield. Similar to our previous long-term study (Bionaz and Loor 2008b), all delta-9 desaturation indexes were poor predictors of *SCD* expression (and likely activity) and fat yield particularly during the early portion of the study. Together, our data cast further doubt on the usefulness of desaturase indexes to infer activity and/or expression of SCD.

Genes in the ER stress pathway that were affected by FSO (Fig. 8) included the ER stress-regulated eukaryotic translation initiation factor 2-alpha kinase 3 (*PERK*; Bobrovnikova-Marjon et al. 2008) and the DnaJ (Hsp40) homolog, subfamily C, member 3 (*DNAJC3*) commonly referred to as *P58IPK*, which interacts with PERK to inhibit its eIF2$\alpha$ kinase activity. The latter is induced during the unfolded protein response by an ER stress response element in its promoter region (Lee et al. 2003). The reduction in *P58IPK* by day 7 of feeding FSO (Fig. 8) might have allowed mammary cells to restore ER homeostasis, e.g., through the upregulation of *SCD* mRNA so that oleic acid could continue to be synthesized. Oleic acid might in turn have had a positive effect on the expression and maturation of SREBF1 (Miyazaki et al. 2004), as discussed above. In contrast, the observed upregulation of *XBP1* by day 7 of feeding EB100 might have been a response to saturated FA in order to maintain the folding capacity of the ER by blocking further buildup of client proteins, which in itself could lead to the activation of apoptotic mechanisms (Mandl et al. 2009). Furthermore, upregulation of *XBP1* agrees with the observed upregulation of *SREBF1* with EB100 and suggests that *SREBF1* as in non-ruminants (Bobrovnikova-Marjon et al. 2008) also is an *XBP1* target gene in bovine mammary. Further studies will have to be conducted to examine the role of ER stress in mammary lipogenesis.

## Summary and conclusions

To our knowledge, this is the first longitudinal analysis of mammary mRNA expression patterns associated with saturated or marine lipid feeding in dairy cows. This approach allowed us to uncover long-term adaptations in expression as the mammary gland adjusted to the influx of saturated FA, *trans*-FA, or decreased availability of stearic acid. Both gene expression and milk FA/fat yield revealed a most drastic response to diets in the first 7 days of feeding and reflected vastly different physiological responses by mammary tissue to adjust its metabolism. The fact that few (2 of 29 genes) differences in mRNA expression between diets were observed on day 21 of feeding suggested that control mechanisms regulating fat synthesis were established as early as 7 days post-feeding. Responses of mammary tissue to dietary saturated FA or marine lipid were very different, with saturated fat upregulating gene networks related to milk fat synthesis. Contrary to most previous data obtained from single-point measurements, milk fat depression was not characterized by lower mRNA abundance of lipogenic enzymes (*FASN*, *ACACA*) but decreased *SREBF1*, while *SCAP* increased markedly through the study and was the only gene with treatment differences at 21 days of feeding. This response provided some evidence of a potentially important role of *SCAP*. However, the sustained upregulation of *INSIG1* regardless of diet indicated that it might be associated with milk fat synthesis regulation as well as inhibition of SREBF1 maturation. Expression of *THRSP* was downregulated during the first 7 days of feeding regardless of diet, providing no support for a central role in milk fat synthesis regulation. A potential role of the ER stress pathway in the adaptations induced by saturated FA or marine lipid was uncovered and, along with *INSIG1* and *SCAP*, deserves further attention for their role in milk fat synthesis regulation.

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**Integrity of research and reporting** The authors declare that the experiment complies with the current laws of the USA. The authors declare no conflicts of interest.

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