James M. Ntambi *Editor*

Stearoyl-CoA Desaturase Genes in Lipid Metabolism

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Preface

This is the first edition of this book that provides the reader with current information on the stearoyl-CoA desaturase gene family that encode stearoyl-CoA desaturase, a previously unknown enzyme and its role in metabolism in different organisms. Whereas today many genes and gene families are defined and analyzed bioinformatically, early events using the murine 3T3-L1 system provided the foothold that have enabled our current understanding of what has become one of the most interesting and important loci in cellular metabolism. In addition, the reader will learn about the important and widely diversified field of fatty acids and their health implications in humans. However since the precise role of fatty acids in the etiology of the various metabolic disorders has yet to be delineated it is not the intention of the book to present a unified view on the healthy implications of monounsaturated fatty acids or provide guidelines for fatty acid consumption.

Work over the last 20+ years has focused on identifying and analyzing numerous SCD isoforms in a variety of species that are in turn controlled by multiple signaling systems. Given the diverse role of monounsaturated fatty acids in metabolic processes it is not surprising that multiple genes exist and that multiple regulatory elements control isoform expression. In addition, tissue-specific dietary control of isoform expression occurs via a series of complex signal transduction schemes making SCD one of the most highly studied gene families. Conditional alleles and corresponding tissue-specific knockout models for many of the murine SCD genes have provided a wealth of information on not only tissue-specific fatty acid metabolism but also the key transcription factors that regulate SCD expression under a variety of metabolic and genetic backgrounds. Our studies using mice with whole-body and tissue-specific deletions of one of the mouse SCD isoforms (SCD1), together with several human studies have all agreed that the expression of the SCD1 gene isoform represents a key step in partitioning of lipids between storage and oxidation. High SCD1 expression (high oleate levels) favors fat storage leading to obesity while reduced SCD1 (low oleate levels) expression favors fat burning and leanness. However, as we understand more about the detrimental roles of cellular saturated fatty acids, it is becoming clear that unqualified complete inhibition of SCD1 activity, whether systemic or restricted to specific organ systems, may not

come without a plethora of undesirable side effects, including an increased propensity to inflammatory disease. A proper ratio of saturated/monounsaturated fat may be required for normal health and physiological function. Nevertheless, novel insights gained into the multifaceted roles of these intrinsic loci in cellular metabolism may yet lead to new models for pharmacological interventions for the treatment of metabolic disease.

I am very grateful to many people who helped me make possible the creation of the first edition on the role of SCD genes in metabolism. First of all I would like to thank my early research mentors, Dr. Paul Englund and M. Daniel Lane both at the Johns Hopkins University School of Medicine who introduced me to research in biochemistry and molecular biology. David Bernlohr presently at the University of Minnesota handed me the PAL122 cDNA clone that I used as a probe to isolate the first murine SCD gene (mSCD1). I would like to express my sincere appreciation to all the contributors of the diverse chapters on SCD genes for their cooperation and excellent work. Without their participation, this initial project would not have been possible. I would like to thank Anna Marsicek who has edited the various chapters of the book. I would also like to thank Springer for their assistance and support during the course of this project. They saw the potential for this project and helped me in many ways to craft this book. Finally I wish to thank my family for their encouragement, support, and patience over the past many years.

Madison, WI, USA James M. Ntambi, Ph.D.

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Chapter 1
Early Studies on Role of Stearoyl-CoA Desaturase During Preadipocyte Differentiation

David A. Bernlohr and M. Daniel Lane

Background

Having extensively investigated the catalytic mechanism of action of acetyl-CoA carboxylase (ACC) (Polakis et al. 1972, 1974; Guchhait et al. 1971, 1974a, b; Moss and Lane 1971) and its allosteric regulation by citrate (Moss and Lane 1972; Gregolin et al. 1966; Ryder et al. 1967; Beaty and Lane 1983a, b), in the late 1970s and early 1980s, our lab decided to determine how expression of ACC is controlled. To conduct such studies we needed a lipogenic cell line that could be cultured for extended periods of time (i.e., greater than a week), during which gene expression and protein translation could be assessed. About this time Howard Green and colleagues, who had recently moved from the New York University Medical School to the Massachusetts Institute of Technology (MIT), had established several preadipocyte cell lines—notably the 3T3-L1 and 3T3F442 cell lines (Green and Kehinde 1974, 1975, 1976)—for the study of adipocyte biology. These lines could be induced to differentiate into cells with the phenotype of adipocytes including high rates of lipogenesis (Mackall et al. 1976; Mackall and Lane 1977; Coleman et al. 1978; Student et al. 1980) and responsiveness to insulin (Reed et al. 1977, 1981; Reed and Lane 1980). Since then the 3T3-L1 preadipocyte line has become the "gold standard" for studies on adipocyte differentiation. We (Student et al. 1980) and others (Lai et al. 1982) developed protocols to induce differentiation and demonstrated that expression of fatty acid synthase (FAS), ACC, and numerous other lipogenic proteins paralleled the acquisition of adipocyte morphology. These early observations

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verified the system and established the primary parameters linked to the utilization of the 3T3-L1 model including (1) an accumulation of cytoplasmic fat as revealed by Oil Red O staining (Mackall et al. 1976), (2) a dramatic increase in the rate of fatty acid synthesis (Mackall et al. 1976; Student et al. 1980), (3) a coordinate increase in the enzymatic activities of ACC (Mackall et al. 1976), FAS, and complex lipids synthesis (triacylglycerol and phospholipids) (Coleman et al. 1978), and (4) hormonally regulated lipid metabolism including insulin-stimulated glucose uptake and lipogenesis and catecholamine-stimulated lipolysis (Reed et al. 1977, 1981; Reed and Lane 1980).

The most widely used procedure for induction of adipocyte differentiation (Student et al. 1980) involves: allowing 3T3-L1 preadipocytes to proliferate until growth arrest at confluence (G_1 stage of the cell cycle) and after two additional days in G_1, treating the cells with an induction "cocktail" consisting of cAMP (or methylisobutylxanthine), dexamethasone, and a high level of insulin (or a low level of IGF1). Following withdrawal of cAMP and dexamethasone the induced cells synchronously reenter the cell cycle, undergo ~2 rounds of division (mitotic clonal expansion) (Tang et al. 2003), and return to the state of G_1 growth arrest. At this point expression of the lipogenic proteins/enzymes of the adipocyte differentiation program occurs synchronously (Tang et al. 2003; Tang and Lane 2012). The use of such established preadipocyte lines have been indispensable for the identification and characterization of the key steps in the differentiation program. A large body of evidence (Tang and Lane 2012) shows that these preadipocyte models faithfully recapitulate differentiation of mouse embryonic fibroblasts (MEFs) in cell culture (Tang et al. 2003; Zhang et al. 2003). Likewise, the authenticity of model systems has been validated in vivo by demonstrating that these preadipocyte lines give rise to normal adipose tissue when implanted subcutaneously into athymic mice without exogenous inducers (Mandrup et al. 1997).

Gene Expression During Adipocyte Differentiation

The utilization of the 3T3-L1 cell line in culture represents a useful model system for investigating the mechanisms of cellular differentiation (Tang and Lane 2012). Under appropriate conditions, 3T3-L1 preadipocytes, which initially resemble fibroblast, differentiate readily into adipocytes in cell culture (Green and Kehinde 1975; Mackall et al. 1976). The differentiation process is accompanied by a dramatic increase in the activities of the enzymes associated with de novo lipogenesis (Mackall et al. 1976; Mackall and Lane 1977; Coleman et al. 1978; Student et al. 1980) and triglyceride mobilization (Hirsch and Rosen 1984), as well as an increased sensitivity to lipogenic and lipolytic hormones (Kohanski et al. 1986; Hirsch and Rosen 1984; Watkins et al. 1982). The cells accumulate triglyceride (Green and Kehinde 1975; Mackall et al. 1976) and acquire the morphological characteristics of adipocytes (Novikoff et al. 1980) isolated from normal adipose tissue. Strong evidence indicated that for some of the lipogenic enzymes (e.g., FAS), the increases in

activity during differentiation arise from increased rates of enzyme synthesis (Student et al. 1980) suggesting increased mRNA abundance. Moreover, it was subsequently shown that changes in mRNA expression closely correlated with the increased activity and abundance of the proteins that were differentially expressed (Mandrup and Lane 1997; MacDougald and Lane 1995; Bernlohr et al. 1985a, b; Angus et al. 1981). Therefore, it became evident that to understand the genetic mechanisms responsible for these changes in phenotype, it would be necessary to identify genes whose expression is activated during differentiation of 3T3-L1 preadipocytes into adipocytes. This ultimately led to the discovery that one of the key regulatory genes in this process was stearoyl-CoA desaturase (Ntambi et al. 1988).

Identification of mRNAs Whose Expression Is Induced During Differentiation of 3T3-L1 Preadipocytes into Adipocytes

An initial approach towards determining the mechanism of increased expression of key lipogenic and lipolytic enzymes was in vitro mRNA translation using ^{35}S-methionine followed by immunoprecipitation (Bernlohr et al. 1985a). In the early 1980s antibodies to several lipogenic enzymes were available (ACC, FAS, malic enzyme, pyruvate carboxylase, etc.) and by using in vitro translation of mRNA isolated from either 3T3-L1 preadipocytes or adipocytes followed by immunoprecipitation, one could assess the relative expression of certain mRNAs. William Angus, a PhD student at the time, utilized such an approach and demonstrated increased mRNA levels for pyruvate carboxylase (Angus et al. 1981) and acetyl-CoA carboxylase (MacDougald and Lane 1995). These findings suggested that differentiation of 3T3-L1 preadipocytes in culture likely involved the activation of the entire adipogenic gene expression program. While technically simple, the method was limited by the availability of mono-specific antibodies and was time-consuming and relatively inefficient. This consideration drove the race towards cloning differentially expressed mRNAs (MacDougald and Lane 1995; Cornelius et al. 1994).

A second approach towards analysis of gene expression during differentiation involved the isolation and characterization of cDNAs corresponding to genes that are activated during differentiation. Indeed, during the 1980s the identification of cDNA clones corresponding to differentially expressed genes occupied the focus of a number of laboratories. Toward that end, we constructed and characterized a cDNA library from mRNAs expressed by differentiated 3T3-L1 adipocytes (Bernlohr et al. 1984). A similar approach was taken by Spiegelman and colleagues (Ross et al. 1990) with 3T3-F442A cells, a related mouse preadipocyte cell line. Once a cDNA library was developed a dual-label analysis method was utilized to identify such clones that corresponded to mRNAs that were adipose-specific (Bernlohr et al. 1984). In this method, ^{3}H-labeled cDNA was synthesized using mRNA from preadipocytes and mixed with ^{32}P-labled cDNA synthesized from differentiated adipocytes. The mixed cDNA population was used in colony hybridization studies to identify cDNAs that contained differentially expressed mRNA

(Bernlohr et al. 1984). Using such a dual-label screening method we identified several cDNA clones that correspond to mRNAs that increase in abundance upon differentiation. Many of these clones (e.g., pAL122, pAL421, pAL422) represent mRNAs that are detectable primarily in differentiated cells. From such a screen came the identification of a cDNA, pAL122, corresponding to stearoyl-CoA desaturase (Ntambi et al. 1988).

Identification of Stearoyl-CoA Desaturase as a Differentiation-Induced Enzyme During Adipocyte Differentiation

Differential screening led to the identification of a family of adipocyte mRNAs (Ntambi et al. 1988) that included: (1) The pAL422 mRNA (later identified as encoding FABP4, the adipocyte fatty acid-binding protein also known as aP2) of which numerous clones were obtained owing to its abundant expression in differentiated 3T3-L1 adipocytes (Bernlohr et al. 1984, 1985b) and (2) The pAL122 mRNA was later identified as encoding the stearoyl-CoA desaturase one mRNA (Ntambi et al. 1988).

It should be noted that at the time of cloning these cDNAs their identities were unknown. The utilization of databases and searchable sequence repositories was in its infancy and even DNA sequencing was time-consuming and laborious. Indeed, since the original cDNA cloning was accomplished using the GC-tailing method, sequencing of such cloned inserts often proved problematic and required many sequencing runs and overlapping replicates to develop reproducible trustworthy results. The pAL422 cDNA was identified from a protein sequence data base as a putative fatty acid-binding protein, aP2, that exhibited ~60 % amino acid sequence identity to mP2, a myelin fatty acid-binding protein (Bernlohr et al. 1985b). James Ntambi undertook the identification of pAL122. It should be noted that Ntambi had little to go on as the pAL122 cDNA nucleotide sequence contained largely 3′-untranslated sequence and only a small amount of coding information upstream of the translational stop site (Ntambi et al. 1988). Northern blotting had revealed that the pAL122 mRNA sequence was ~5 kb, suggesting that the pAL 122 cDNA clone corresponded to less than 30 % of the complete cDNA.

In an act of heroic proportion James Ntambi began a manual “nucleotide-by-nucleotide” sequence comparison of the pAL122 mRNA with any new sequences that appeared in the literature. Remarkably, in 1986 Ntambi found a match with the nearly identical nucleotide sequence of the rat liver SCD1 from Philip Strittmater’s laboratory that appeared in the Journal of Biological Chemistry in 1986 (Thiede et al. 1986). Thus, James’ persistence paid off with the identification of pAL122 as murine stearoyl-CoA desaturase1 (Ntambi et al. 1988). Armed with the knowledge that pAL122 corresponded to SCD1, Ntambi utilized the pAL122 1.4-kb cDNA to isolate a 4.9-kb full-length cDNA clone and determined the complete mouse SCD1 sequence (Ntambi et al. 1988).

Isolation and Structure of the SCD1 Gene

Stearoyl-CoA desaturase is the key enzyme in the synthesis of unsaturated fatty acids and is under complex transcriptional and posttranscriptional regulation. To develop tools for the molecular analysis of SCD1, Ntambi, along with Susan Buhrow, Klaus Kaestner, Eric Sibley, and Robert Christy in our lab, was able to isolate genomic clones corresponding to murine SCD1 and to identify binding sites in the 5′-flanking regions of the gene that are involved in the coordinate differentiation-induced expression in 3T3-L1 preadipocytes (Ntambi et al. 1988; Christy et al. 1989). From these studies emerged the structural organization of the murine SCD1 gene and comparison to the murine 3T3-L1 SCD1 cDNA (Fig. 1.1). At the same time, genomic clones corresponding to pAL422 (Kaestner et al. 1989) had been obtained and armed with sequence information in the 5′ upstream region from both genes, control elements that may provide clues towards coordinate regulation during differentiation were identified.

Identification and Cloning of a Second Stearoyl-CoA Desaturase, SCD2

Southern blotting of mouse DNA with the SCD1 cDNA suggested that there was a second similar sequence in the genome, and therefore, potentially a second SCD gene. Armed with the SCD1 cDNA, Klaus Kaestner and James Ntambi screened the 3T3-L1 cDNA library and identified a cDNA clone that encoded a second stearoyl-CoA desaturase, SCD2, from 3T3-Ll adipocytes. This second SCD gene was

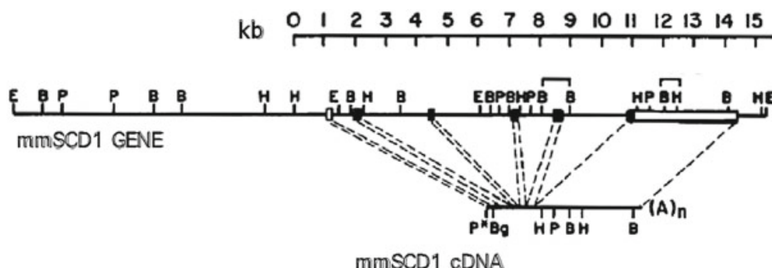

Fig. 1.1 Structural organization of the Mus musculus SCD1 gene and cDNA. The *top scale* indicates nucleotides in kilobases. The *middle scale* indicates the organization of the murine SCD1 gene where *boxes* indicate the exonic regions with open regions representing noncoding and filled regions coding sequence. The *lower scale* indicates the organization of the SCD1 cDNA isolated from 3T3-L1 adipocytes. *Single letters* represent restriction sites; *B* BamH1, *E* EcoR1, *H* HindIII, *P* Pst1, *B* BglII, *kb* kilobase pair. Adapted from (Ntambi et al. 1988)

expressed at tenfold higher levels in 3T3-L1 adipocytes compared to the preadipocytes. The full-length SCD2 cDNA predicted a protein of 358 amino acids with 87 % amino acid sequence identity to murine SCDl (Christy et al. 1989). Utilizing the SCD2 cDNA the murine SCD2 gene was cloned and analyzed to assess its regulation and expression patterns (Kaestner et al. 1989).

SCD2 mRNA is expressed constitutively at a high level in brain and its expression in kidney, adipose, and lung tissue is increased greatly by shifting mice from a diet containing unsaturated fatty acids to a diet devoid of fat (Landschulz et al. 1994). Presumably, the major function of SCD2 in brain is to supply unsaturated fatty acids constitutively for the synthesis of membrane phospholipids, particularly for myelination. In high carbohydrate, fat-free diets, fatty acids are synthesized de novo and are desaturated for membrane phospholipid synthesis and stored as triacylglycerol in the monounsaturated form. Strikingly, SCD2 mRNA is absent from liver regardless of the dietary treatment (Christy et al. 1991). The difference in tissue-specific expression and dietary control of SCD1 and SCD2 expression suggests distinct control mechanisms for the expression of each of the two genes. This in turn led to an analysis of the structure and regulation of SCD2.

The SCD2 gene spans approximately 15 kb pairs and consists of six exons and five introns, with intron–exon junctions similar to those of SCDl (Kaestner et al. 1989; Christy et al. 1989). As determined by primer extension analysis the start site of transcription maps 300 nucleotides upstream of the initiator methionine codon. Unlike the SCDl gene, SCD2 lacks a typical "TATA" box in the 5′ flanking region but has two "CCAAT" boxes at positions −90 and −135 relative to the transcription initiation site. The SCD2 promoter contains a 140-base pair sequence (located between nucleotides −54 and −201) that possesses 77 % sequence identity to a region (located between nucleotides −472 and −325) in the SCD1 promoter. There is a GC-rich sequence in the SCD2 promoter (at nucleotide −175) similar to the binding site for the nuclear transcription factor Sp1 as well as an element with homology to the core consensus sequence of the glucocorticoid receptor positioned a −500 and a potential CCAAT box/enhancer binding protein sequence at position −540. Although the promoter of the two genes differs markedly, there is one region with high sequence identity. A nucleotide sequence comparison of the 5′ flanking regions of the SCD2 and SCDl genes revealed a stretch of 146 bp of striking sequence homology. The sequence between −201 and −54 bp in the SCD2 gene is 77 % identical to the sequence between −472 and −325 in the SCDl gene, including conservation of the two CCAAT boxes. This marked sequence homology suggests that duplication occurred during evolution of the SCD gene family. Presumably after gene duplication, either a sequence insertion or deletion occurred to yield the different promoters for SCDl and SCD2. The SCD2 promoter lacks the potential cAMP core regulatory sequence present in the SCDl promoter, but contains a potential binding site for the CCAAT/enhancer binding protein (Kaestner et al. 1989).

C/EBPα as Transcriptional Inducer of Gene Expression During Preadipocyte Differentiation

In view of the coordinate nature of the increase in expression of the 422/aP2 and SCD1 genes (Bernlohr et al. 1985a; Kaestner et al. 1989), the possibility was considered that a common transcription factor might activate expression of this group of adipocyte genes. Thus, we employed both DNase I footprinting and gel retardation analysis to show that nuclear factors present in differentiated—but not undifferentiated—3T3-L1 adipocytes bind to regions within the 5′-flanking regions of the promoters of the 422/aP2 and SCD1 genes (Christy et al. 1989). Subsequently we searched for and found that a factor present in nuclear extracts of differentiated—but not undifferentiated—3T3-L1 cells "foot-printed" the similar sites in the 5′-flanking regions of several differentiate-induced genes, notably the 422/aP2 and SCD1 genes (Christy et al. 1991). It was also shown that the differentiation-induced nuclear factor was CCAAT/enhancer binding protein alpha (C/EBPα) (Christy et al. 1991), a DNA-binding protein first isolated from rat liver (Landschulz et al. 1988). Bacterially expressed recombinant C/EBP binds to the same site at which the differentiation-specific nuclear factor interacts within the promoter of each gene. Northern analysis with RNA from 3T3-L1 cells shows that C/EBPα mRNA abundance increases markedly during differentiation. Co-transfection of 422(aP2)-chloramphenicol acetyl transferase or SCD1-chloramphenicol acetyl transferase constructs with a C/EBPα expression vector into 3T3-L1 preadipocytes revealed that C/EBPα trans-activated both the 422(aP2) and SCDl gene promoters, thus implicating C/EBPα in differentiation-specific gene expression. Finally, transient co-transfection studies with a C/EBPα expression vector showed that C/EBPα could serve as trans-activator of both the 422(aP2) and SCDl gene promoters (Fig. 1.2). Later we found that C/EBPα also served as a transcriptional activator of the leptin (Hwang et al. 1996, 1997; MacDougald et al. 1995) and GLUT4 genes (Kaestner et al. 1990) during the differentiation process (Fig. 1.2).

As C/EBPα appeared to serve as a pleiotropic activator of gene expression during preadipocyte differentiation (Kaestner et al. 1989, 1990; Hwang et al. 1996, 1997; MacDougald et al. 1995; Lin and Lane 1992), it was of interest to determine how the C/EBPα gene itself is controlled during differentiation. Consistent with its suspected role as a pleiotropic activator, we found that the increase in the C/EBPα message level during differentiation was due to an increase in the rate of transcription of the gene (Christy et al. 1991). We then set out to determine the structure of the C/EBPα gene and the nucleotide sequence of its 5′ flanking region. DNase I footprinting of the 5′ flanking region by nuclear factors from differentiated and undifferentiated 3T3-L1 cells revealed two sites of differential binding (Christy et al. 1991). One site in the gene promoter between nucleotides −252 and −239 binds a nuclear factor(s) present in preadipocytes that is lost or modified upon

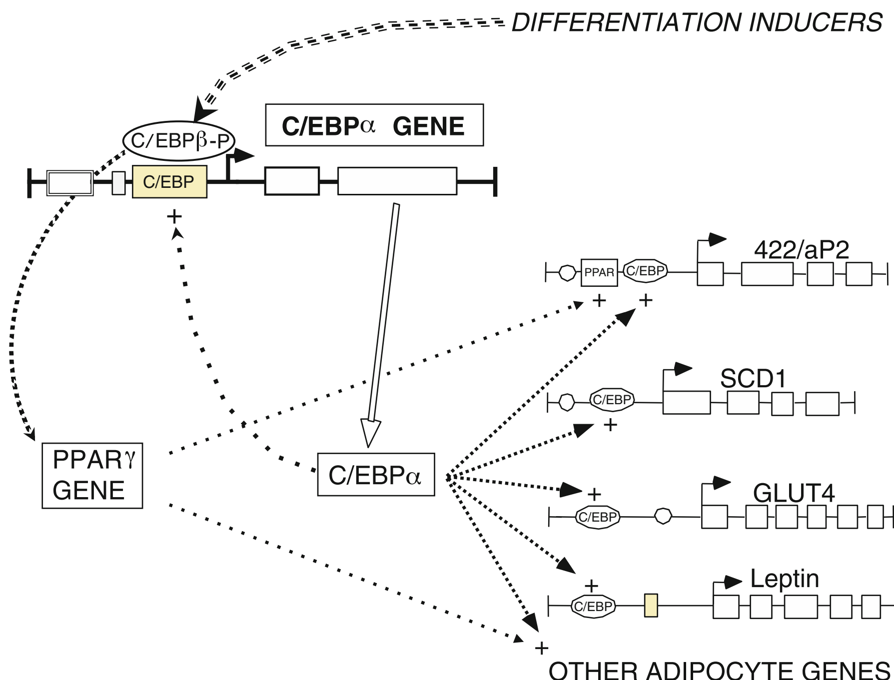

Fig. 1.2 Roles of C/EBPα, C/EBPβ, and PPARγ in the transcriptional activation of adipocyte genes during differentiation of 3T3-L1 preadipocytes. Following induction of differentiation, C/EBPβ is expressed that in turn activates the expression of C/EBPα and PPARγ. C/EBPα and PPARγ act as pleiotropic activators of many adipocyte genes including those encoding 422/aP2 (aP2 or FABP4), a fatty acid-binding protein; *SCD1* stearoyl-CoA desaturase-1, GLUT4 the insulin-responsive glucose transporter; and the leptin gene. C/EBPα also activates its own gene thereby maintaining the differentiated state. C/EBP refers to CCAAT/enhancer binding protein and PPAR to peroxisome proliferator-activated receptor

differentiation. The second site, located between nucleotides −203 and −176, exhibited different—but overlapping—footprints by nuclear factors present in differentiated and undifferentiated cells. Gel shift analysis with oligonucleotides corresponding to these sites revealed protein-oligonucleotide complexes containing these differentially expressed nuclear factors. The factor present in differentiated cells that binds at this site was identified as C/EBP presumably C/EBPα (Kaestner et al. 1989; Christy et al. 1991). These findings suggested that C/EBPα may regulate expression of its own gene (Fig. 1.2). It appears that the continued expression of C/EBPα allowed the maintenance of the differentiated state through interaction of the gene product (e.g., C/EBPα) with the promoter of its own gene. It was later found that C/EBPβ, a related C/EBP family member, also binds at this site and is responsible for activating expression of the C/EBPα gene following induction much earlier in the differentiation program (Fig. 1.2) (Tang and Lane 2012).

Conclusions

From the simple beginning using the differentiating 3T3-L1 model cell system has come a broad and comprehensive analysis of the SCD gene family in many different organisms. Work over the last 20+ years has focused on identifying and analyzing numerous SCD isoforms in a variety of species that are in turn controlled by multiple signaling systems. The genes for SCD have been cloned from human, mice, rats, sheep, hamster, *Drosophila*, and *C. elegans* (reviewed by Paton and Ntambi 2009). In the mouse, four isoforms (SCD1, 2, 3 and 4) have been identified while in the rat only two isoforms have been characterized. In humans, two genes have been identified; a ubiquitously expressed SCD1 and a brain, pancreas form termed SCD5. Given the diverse role of monounsaturated fatty acids in metabolic processes such as the synthesis of phospholipids, triglycerides, cholesteryl esters, wax esters, and alkyldiacylglycerols, it is not surprising that multiple genes exist and that multiple regulatory elements control isoform expression. In addition, tissue-specific dietary control of isoform expression occurs via a series of complex signal transduction schemes making SCD one of the most highly studied gene families. Conditional alleles and corresponding tissue-specific knockout models for many of the murine SCD genes have provided a wealth of information on not only tissue-specific fatty acid metabolism, but also the key transcription factors that regulate SCD expression under a variety of metabolic and genetic backgrounds. Whereas today many genes and gene families are defined and analyzed bioinformatically, early events using the 3T3-L1 system provided the foothold that enabled our current understanding of one of the most interesting and important loci in cellular metabolism.

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Chapter 2
Skin Stearoyl-CoA Desaturase Genes

Satish Parimoo and Pappas Apostolos

Introduction

The hair follicle and its associated sebaceous gland constitute the skin pilosebaceous unit, the biogenesis of which occurs during embryogenesis. In certain skin areas such as eyelids, nipples, prepuce, and labia minora, sebaceous glands may form and function in the absence of hair follicles, but all hair follicles form and function normally in association with sebaceous glands. Although significant advances have been made in our understanding of hair biology during the last decade, cellular and molecular differences of sebaceous glands between various species and skin sites within an organism are poorly understood. Furthermore, it is currently unknown whether differences in sebaceous glands are related to the heterogeneity of hair follicles. It has been hypothesized that during evolution sebaceous glands arose first in the animal kingdom before hair follicles came into existence (Stenn et al. 2008). If this hypothesis is true, it can be logically concluded that sebaceous glands must have played some crucial role in the evolution of hair follicles. In fact, in vitro and in vivo studies suggest that sebaceous glands are important for normal formation or functioning of hair follicle (Williams and Stenn 1994; Allen et al. 2003). Given the relationship between sebaceous glands and hair follicles, an obvious interesting question that arises is what happens to skin and or hair follicles if sebaceous glands have defects in differentiation and as a result do not function properly. Such questions may be addressed with appropriate animal models (Porter et al. 2002; Lunny et al. 2005; Yager et al. 2012). A spontaneous mutant mouse, asebia (*ab*), is known to exist that serves as an excellent model for sebaceous gland deficiency.

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The recessive asebia mutation originated spontaneously in a colony of BALB/cCrglGa mice (Gates and Karasek 1965). The name asebia (*ab*) was initially given to mutant mice as it was believed that they lacked sebaceous glands; however, subsequent closer examination revealed that in fact sebaceous glands were not absent but hypoplastic, and other related glands (meibomian, perianal glands, and ceruminous glands) are affected to various extents in *ab* mice (Josefowicz and Hardy 1978; Pennycuik et al. 1986; Compton et al. 1989; Sundberg et al. 2000). Studies of dermal-epidermal recombination fetal grafts suggested that the functional defect in mutant mice was in the epidermis and not the dermis (Pennycuik et al. 1986). Other prominent features of *ab* mutant mice are their scant subcutaneous fat and corneal opacities.

Identification of *Scd1* Gene Defect in the *Asebia* Mouse by Positional Cloning

An allelic spontaneous mutation, ab^{J}, was identified at the Jackson Laboratories and was mapped to mouse Chromosome 19 (Sweet and Lane 1977). Later another allelic mutation, ab^{2J}, arose spontaneously at the Jackson Laboratories on the inbred mouse strain DBA/1LacJ and its characterization was reported by Sundberg et al. 2000. Differences in appearance of mutant and normal mice and their skin histology are shown in Fig. 2.1. Whereas well-differentiated sebaceous glands are obvious in normal mouse skin section, they are not apparent in ab^{2J} skin section. The gene

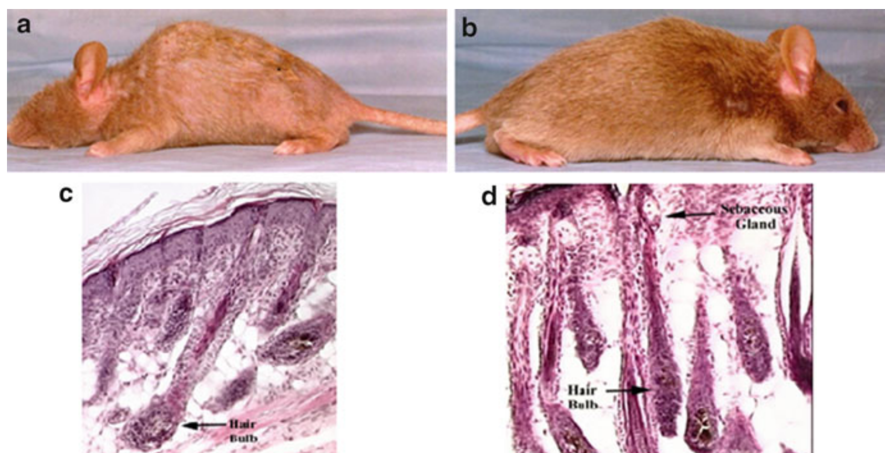

Fig. 2.1 Scaly skin with alopecia in an adult ab^{2J} mutant mouse (**a**) and normal fur of nonmutant littermate (**b**). Histology (H&E) skin sections of ab^{2J} and nonmutant mouse are shown in (**c**) and (**d**), respectively. While well-developed sebaceous glands, as indicated, are visible in (**d**), they are hypoplastic and not obvious in (**c**)

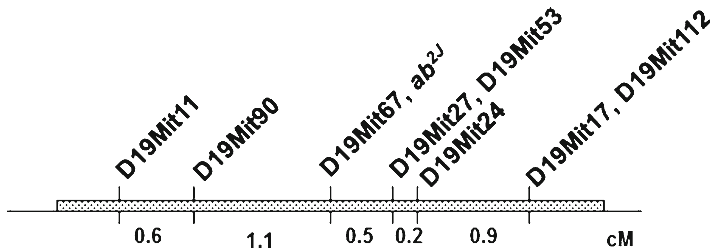

Fig. 2.2 Genetic linkage map of the mouse chromosome 19 region surrounding *ab*^{2J} locus and the associated microsatellite polymorphic markers that were used to obtain the map from backcross progeny as described by Eilertsen et al. (2000). The marker D19Mit67 is tightly linked to *ab*^{2J} as indicated. Inter-marker genetic distances are shown in centimorgans (cM). The *ab*^{2J} mutation is within the 1.6 cM genetic interval defined by D19Mit90 and 19Mit27/D19Mit53

defect in asebia mouse was identified by positional cloning involving two major steps. The first step was obtaining a high resolution genetic and physical map of the locus as reported by Eilertsen et al. (2000), followed by detailed identification and characterization of the genetic lesion in the mutant mouse using a variety of molecular techniques including nucleic acid sequencing (Zheng et al. 1999). These studies mapped the *ab*^{2J} mutation to mouse chromosome 19 that co-segregated with the polymorphic microsatellite marker D19Mit67 when DNA from 644 backcross mouse progeny were examined as reported by Eilertsen et al. 2000. The critical genetic interval in *ab*^{2J} mouse was identified to a fairly narrow region of DNA (1.6 cM) as shown in Fig. 2.2. Further detailed molecular analysis ultimately identified underlying genetic defects in both *ab*^{J} and *ab*^{2J}mice.

After probing skin RNA blots from *ab*^{J} and *ab*^{2J} mutant and normal mice with DNA probes derived from genes and expressed sequence tags (ESTs) from mouse chromosome 19 critical region, one gene probe derived from *Scd1* showed a lack of *Scd1* RNA expression on skin RNA blots from *ab*^{J} and *ab*^{2J} mice in contrast to normal mice (Zheng et al. 1999). Although this was good suggestive evidence that the *Scd1* was most likely the gene that was mutated in *ab* mice, more in-depth analysis of Southern and Northern blots, RT-PCR and DNA sequence analysis from mutant and normal mice, subsequently revealed that the *Scd1* gene expression was indeed disrupted due to mutations in *Scd1* gene in both *ab*^{J} and *ab*^{2J} mice although the nature of genetic lesions was different. Whereas a large deletion of DNA (exon 1–4) is responsible for gene inactivity in *ab*^{J} mice, a more subtle deletion of 18 nucleotides at exon 2 and intron 2 boundary was identified in *ab*^{2J} mice (Zheng et al. 1999). The gel electrophoresis of RT-PCR products from skin and liver RNA samples indicated the expected band of 787 base-pairs derived from *Scd1* RNA of the normal mouse, but mutant samples had either multiple bands due to aberrant RNA splicing in *ab*^{2J} or no bands at all due to larger genomic deletions in *ab*^{J} (Fig. 2.3a). DNA sequencing of RT-PCR products established that all three aberrantly spliced transcripts had in-frame stop codons in *ab*^{2J} (Fig. 2.3b). Deletion of 18 nucleotides in *ab*^{2J} causes loss of the 5′ splice site (GT) at the exon 2–intron 2 boundary used for *Scd1* RNA splicing. As a consequence of this 5′ splice site deletion, genomic DNA

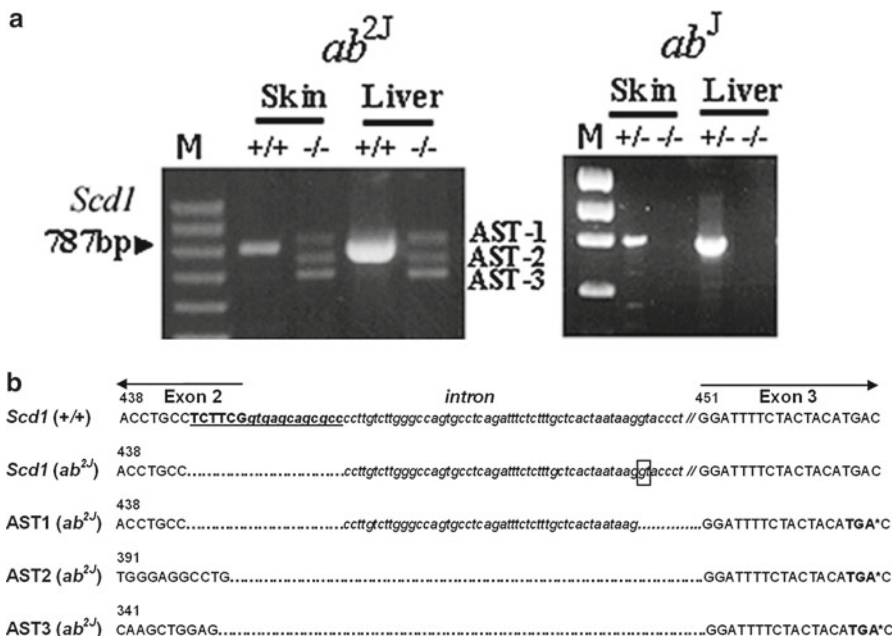

Fig. 2.3 (**a**) Characterization of RT-PCR products from mutants ab^J, $ab^{2,J}$ and nonmutant tissues on agarose gel. *M* marker ladder. AST-1, AST-2, and AST-3 are three aberrantly spliced transcripts from ab^{2J} tissues. *Arrowheads* indicate *scd-1*-specific transcript. +/+ = nonmutant; −/− = mutant; +/− = heterozygote that is phenotypically normal. Reproduced from Zheng et al. (1999) *Nat Genet* 23:268–270. (**b**) Comparison of DNA sequences from genomic DNA of nonmutant (+/+), ab^{2J} mouse and cDNA derived from aberrantly spliced transcripts (AST-1, AST-2, and AST-3) from ab^{2J} mouse. *Asterisk* indicates in-frame stop codons. The GT dinucleotide used as cryptic 5′ splice for the generation of AST-1 is shown as boxed within the intron 2 of genomic sequence of *Scd1* (ab^{2J}). The exon sequences are in the *upper case* and intronic sequences are in the *italics* and *lower case*. The eighteen-nucleotides, encompassing exon 2 and intron 2 junction, that are deleted in ab^{2J} mutant are shown in *bold* and *underlined* in *Scd1* genomic sequence (+/+). *Dots* reflect deletions and // indicates intronic sequence present in the genomic DNA but not shown in the figure. The sequence numbering is same as that of the published cDNA (Ntambi et al. 1988. *J Biol Chem* 263:17291–17300)

codes for the aberrantly spliced transcript (AST-1) in ab^{2J} by utilizing a 5′ cryptic splice site (GT) within the intron as shown in Fig. 2.3b. The AST-1 from ab^{2J} has an insertion of 47 nucleotides from intron 2 as reflected by its larger sized RT-PCR product in comparison to that derived from the nonmutant mouse samples and confirmed by DNA sequencing (Fig. 2.3). Smaller sized RT-PCR products derived from aberrantly spliced transcripts, AST-2 and AST-3, apparently use other upstream 5′ cryptic sites and as a result generate smaller sized RT-PCR products (Fig. 2.3). None of the aberrantly spliced transcripts in ab^{2J} can code for Scd protein since all of them have in-frame stop codons (Fig. 2.3b). The fact that the *Scd1* gene mutation is responsible for the asebia phenotype was also supported by *Scd1* gene knockout studies as reported by Miyazaki et al. (2001). Details of how the *Scd1* gene defect

in asebia impacts sebum synthesis or controls sebaceous gland morphogenesis and function are still. However, the role of various members of fatty acid desaturase gene family in skin has been reviewed recently (Sampath and Ntambi 2011).

Impact of *Scd1* Gene Defect in Mouse

Scd1 mutation causes several distinct changes in mouse when compared to heterozygote or normal mice. Adult homozygous asebia mice develop generalized alopecia, scaly skin, and have a hunched back. The hair shafts formation occurs normally, but they are sparse and matted (Josefowicz and Hardy 1978; Compton et al. 1989; Sundberg 1994). In adult asebia mice, hair follicles protrude deep in subcutis and hair cycles (anagen, catagen, and telogen) last longer than those in the controls (Josefowicz and Hardy 1978; Pennycuik et al. 1986). A subtle abnormality in mutant mice hair shaft includes retention of inner root sheath (IRS) above sebaceous gland that causes the plugging of the hair canal (pilary canal). The abnormally long hair follicles deep in dermis in mutant mice are believed to exist as a consequence of the retention of this cornified cell plug in the pilary canal that impedes the normal exit of hair shaft (Sundberg et al. 2000). The protrusion of hair shaft deep within the skin causes foreign body reaction with the resultant dermal scarring and eventual destruction of the hair follicle itself resulting in progressive alopecia with age in mutant mice. These observations underscore the importance of the sebaceous gland in controlling normal hair shaft growth and hair shaft-sheath interactions. Other studies also suggest that the separation of the hair shaft from the sheath is dependent on an intact mid-follicle/sebaceous gland (Williams and Stenn 1994; Gemmell and Chapman 1971; Williams et al. 1996). Given the similarity between hair follicle destruction observed in ab^{2J} mouse and human scarring alopecia, the asebia mouse has been postulated to be a good model for the pathogenesis of some forms of human scarring alopecia (Sundberg et al. 2000). Besides hair follicle abnormalities, asebia mice have thickened epidermis and dermis which is marked by increased vascularity and cellular infiltrate of mast cells and macrophages (Brown and Hardy 1988, 1989). Dermal inflammation and epidermal hyperplasia in mutant mice are believed to be due to macrophages containing lipid crystals (Brown and Hardy 1988). Skin surface lipid analysis of the asebia mutant mouse showed a marked reduction in sterol esters and cholesterol with apparently total loss of diol diesters compared to littermate controls (Wilkinson and Karasek 1966; Sundberg et al. 2000).

Although adult mutant mice are easy to distinguish visibly from normal mice, they are very difficult to distinguish phenotypically from littermate controls at the time of birth until approximately 1 week of age when hair fibers emerge from the follicles. Histological examination of skin reveals the presence of very small sebaceous glands in mutant skin as compared to controls (Sundberg et al. 2000). Ultrastructural studies of the glands indicate fewer lipid droplets, intracellular abnormalities such as impaired mitochondrial development and dilated smooth endoplasmic reticulum (Josefowicz and Hardy 1978).

The fact that disruption of *Scd1* expression in asebia mice causes dramatic phenotypic changes highlights the importance of this enzyme for normal skin function including sebaceous gland differentiation. How exactly the enzyme or its product is able to control the fate of sebaceous gland morphogenesis is unknown at present. Stearoyl-CoA desaturase (Scd) is a key iron-containing membrane-bound enzyme that catalyzes the rate-limiting step of oxidative conversion of saturated fatty acids to monounsaturated fatty acids, involving the insertion of a cis-double bond between the C9 and C10 positions in the acyl-CoA derivatives of saturated fatty acids such as palmitic and stearic acids to produce palmitoleoyl and oleoyl-CoA, respectively (Tocher et al. 1998; Paton and Ntambi 2009). This process is dependent upon electron flow from NAD(P)H through NADH-cytochrome b5 reductase to cytochrome b5, to Scd and finally to molecular oxygen that is reduced to water. Unsaturated fatty acids are not only involved in energy metabolism, but also in lipid-activated signal transduction (Shinomura et al. 1991; Kasai et al. 1998). Hence, one can conclude that monounsaturated fatty acids or their metabolite products, through an unknown mechanism, affect some pathways that are crucial for the normal differentiation of sebaceous gland and other related glands.

Cloning of *Scd3*, a Gene with Restricted Expression in Skin

Until late 1990s, only two members of *Scd* gene family were known to express in mouse tissues. A novel member of the *Scd* gene family was subsequently discovered while work on DNA sequencing of cDNA clones derived from asebia and normal skin was in progress. This novel member was named *Scd3* and characterization of this gene was reported by Zheng et al. 2001. Nucleotide sequence analysis of cDNA clones indicated that the *Scd3* transcript has 91 and 88 % identity with *Scd1* and *Scd2* coding sequences, respectively, with very limited homology in noncoding regions, suggesting independent control of gene expression in these genes. The amino acid identity of the Scd3-deduced protein sequence, compared to Scd1 and Scd2, is 88.7 % and 84.9 %, respectively. The Scd3 protein sequence of 359 amino acids, like the other two family members (Sdc1, Scd2), possesses four transmembrane domains and all the conserved histidine residues typically found in the desaturases and hydroxylases of several species. *Scd3* gene exists in close proximity to *Scd1* and *Scd2* genes on chromosome 19 as revealed by in situ hybridization and PCR analysis of BAC clones (Zheng et al. 2001).

Expression of Mouse *Scd* Gene Family in Sebaceous Glands and During Hair Cycle

RNA in situ hybridization of frozen mouse skin sections using a *Scd-1* specific probe demonstrated that *Scd-1* expression could be observed in the sebaceous gland but not in the hair follicle or any other cutaneous structure of wild-type mouse skin

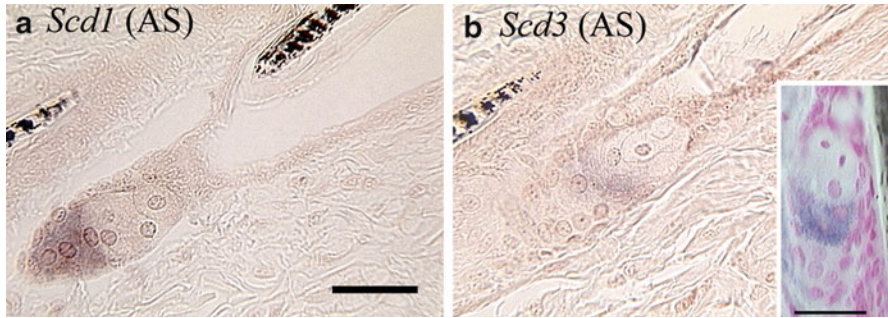

Fig. 2.4 RNA in situ hybridization of mouse skin sections comparing expression of *Scd1* and *Scd3* in the sebaceous gland. The same sebaceous gland is shown on both (**a**) and (**b**) as pictures were derived from two consecutive sections of mouse skin hybridized separately with the *Scd1* and *Scd3* probes. The inset shows an independent section where the slide was stained with hematoxylin and eosin (H&E) after obtaining *Scd3*-specific signal. *AS* antisense probe. The bar is 30 μM. Reproduced from Zheng et al. (2001) *Genomics* 71:182–91 with permission from the publisher (Elsevier)

(Zheng et al. 1999, 2001). *Scd-1* RNA expression was observed in sebaceous glands of skin irrespective of hair cycle stage as reported by Zheng et al. 1999. Similar to *Scd1* expression, *Scd3* expression by in situ RNA hybridization was observed to be restricted to sebaceous glands only (Fig. 2.4). However, the interesting feature of expression of the two genes (*Scd1* and *Scd3*) was that they did not co-express in the same region of sebaceous gland as observed from their expression in two consecutive tissue sections by in situ hybridization. This difference in gene expression may be attributed to the sebocytes of different stages of differentiation and also highlight the independent roles of *Scd1* and *Scd3* in different sebocytes within the sebaceous gland.

It is a well-known fact that hair follicle undergoes cyclic changes of growth (anagen), regression (catagen), and quiescence (telogen). The duration of each stage of the cycle is characteristic of both the hair type and species. Given the close association of sebaceous gland and hair follicle as mentioned earlier, it is of interest to know if hair cycle-induced changes in skin also impact expression of the *Scd* gene family. Such changes can be observed in natural or induced (plucked hair) hair cycles. As shown in Fig. 2.5a, steady-state levels of *Scd-1* transcript change over the natural hair cycle. The level of *Scd1* transcript was very low in skin at day 1 post-birth, but its expression increases considerably in the skin derived from mouse in anagen of the first hair cycle (day 8) and then decreases in regression/resting phases of hair cycle followed by increase in the next growth phase of the second hair cycle (days 28 and 35). *Scd-1* expression is much lower in the telogen hair cycle (days 21 and 42). In contrast to *Scd1*, *Scd2* did not show hair cycle-dependent changes in expression in the skin. This phenomenon of enhanced *Scd1*gene expression in skin with hair follicles in the anagen phase followed by decreased expression in skin containing telogen hair follicles was also observed in induced hair cycles (Fig. 2.5b). These results as reported by Zheng et al. (1999, 2001) do not answer the question if

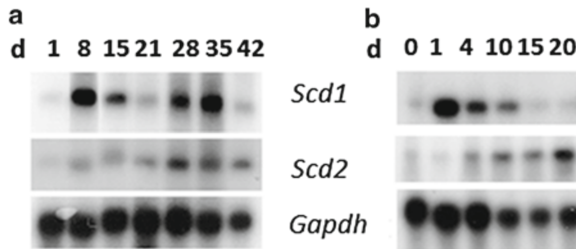

Fig. 2.5 Changes in *Scd-1* expression during spontaneous and induced hair cycles. (**a**) RNA blots of the samples from spontaneous hair cycle obtained from dorsal skin RNA of mice normal mice (DBA/1LacJ) at the age of mice in days (d) as indicated were probed with *Scd1*, *Scd2,* or Gapdh (control)-specific probes. (**b**) RNA blots of the samples from induced hair cycle14 procured from dorsal skin of normal mice (C57BL/6) probed with the same probes as in (**a**). Day 0 (d 0) and day 1 (d 1) represent telogen (quiescent) and early anagen (growth), respectively. The anagen phase ends at about day 18. Adapted from Zheng et al. (1999) *Nat Genet* 23:268–270

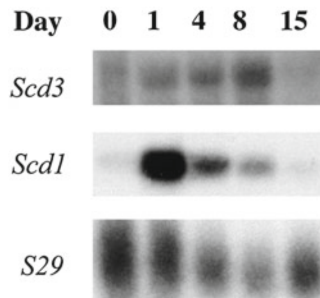

Fig. 2.6 Mouse hair cycle-dependent expression of *Scd3* in comparison to *Scd1*. RNA samples from the skin of 8-week-old mice with hair follicles in quiescent phase (telogen—day 0) and at days 1, 4, 8, and 15 after induction of follicular growth (anagen) by hair depilation were analyzed by RNA blots and probed with *Scd1*- and *Scd3*-derived probes. S29 is a ribosomal protein control probe. Reproduced from Zheng et al. (2001) *Genomics* 71:182–91 with permission from the publisher (Elsevier)

the changes in expression of skin *Scd-1* over the hair cycle are because of changes in *Scd1* expression in the sebaceous gland alone, or whether other tissues such as subcutaneous fat cells also contribute to these changes. Interestingly, in comparison to *Scd1*, *Scd3* transcript level increases gradually during induced hair cycle reaching the highest level at day 8 of anagen (late anagen), and then decreases at catagen (regression phase), which is day 15 of the induced hair cycle (Fig. 2.6). *Scd1* expression is very high in early anagen but relatively low in late anagen. These data also highlight the fact that *Scd1* gene expression is much higher compared to *Scd3* in mouse skin, particularly in early anagen phase (day1) of the induced hair cycle.

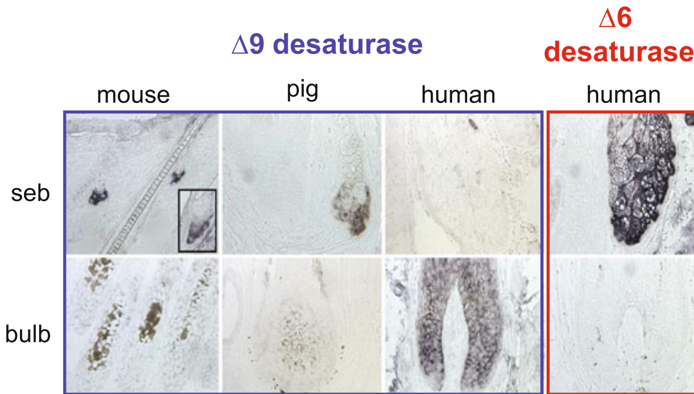

Fig. 2.7 Mouse SCD-1 expression is restricted to the sebaceous gland. Human SCD is weakly expressed in the sebaceous gland, but is highly expressed in the hair follicle bulb and lower hair shaft

Human Skin Desaturases

The human SCD1 gene was cloned by screening a human keratinocyte cDNA library and analysis of 3 h-RACE (rapid amplification of cDNA ends) products from various tissues yielded a 5.2 kb cDNA encoding a 359 amino acid protein with a calculated molecular mass of 41.5 kDa. Analysis of 3 h-RACE products suggested that alternative usage of polyadenylation sites generates two transcripts of 3.9 and 5.2 kb, a result consistent with Northern analysis. Southern analysis demonstrated the existence of two *SCD* loci in the human genome. Chromosomal mapping localized one locus to chromosome 10, and the second locus to chromosome 17. The locus on chromosome turned out to be transcriptionally inactive, fully processed pseudogene. Although the primary sequence and intron–exon structure of *SCD* is phylogenetically conserved, divergence between rodent and human is seen in the number of *SCD* genes and in the generation of alternative transcripts, suggesting a species-specific component of *SCD* regulation of unsaturated fatty acid metabolism.

In skin, mouse SCD-1 is restricted to and abundantly expressed in mouse sebaceous gland, whereas human SCD is weakly expressed in human sebaceous gland (Fig. 2.7). In skin, human SCD is highly expressed in the hair follicle bulb, lower hair shaft, and in the eccrine sweat gland, suggesting a role in the production of hair and sweat. Pig SCD is similar to mouse with sebaceous gland expression, and similar to human with sweat gland (apocrine) expression. However, pig differs from human in that the hair follicle lacks SCD expression. The differences between species in pilosebaceous expression of SCD do not seem to correlate with the number of SCD genes. The difference in gene number, polyadenylation, and expression

pattern indicate that the SCD genes underwent species-specific selection during evolution, and that species-specific function of the pilosebaceous unit may contribute to this diversification. Among human, pig, mouse, and sheep, humans are the only species to express the delta 6 desaturase in sebaceous glands and if we consider that they are the only species that develop acne, that makes it a valid target for that human-specific disease. As described above, the essential role of the SG in hair follicle growth comes from the asebia mouse, which demonstrated hypoplastic SGs and alopecia, and due to a loss-of-function mutation in the Scd-1 gene; highly and specifically expressed in murine sebocytes (Gates and Karasek 1965; Zheng et al. 1999). From the expression of FAD2 in the human sebaceous gland we could conclude that the delta 6 desaturase may participate towards the differentiation process of sebocytes in adult sebaceous glands since its expression is mainly in suprabasal sebocytes.

Human sebaceous glands (SG's) have unique anatomical and biochemical properties since the sebaceous follicle is characterized by large, multi-lobular SGs that are attached to a hair follicle bearing a small hair shaft (Kligman 1974). But the most striking exceptionality is that the human sebum contains an abundant fatty acid that is unique among the animal kingdom. This fatty acid is named sapienic acid, thus "sapien" is the root of its name, and is a 16 carbon monounsaturated fatty bearing the double bond on the *n*10 or delta 6 position (Nicolaides 1974; Stewart et al. 1986). This fatty acid is implicated in the pathogenesis of acne, a disease that is mainly manifested in humans (Downing et al. 1986; Stewart et al. 1986). The levels of sapienic acid are multiple folds higher than any of its derivatives or isomers or other monounsaturated fatty acids found in sebum, which combined with the lack of SCD expression in human SG suggested that other members of the desaturase superfamily, particularly Δ-6 desaturase (also known as *f*atty *a*cid *d*e*s*aturase-2, FADS2), may be expressed in human SG (Ge et al. 2003; Pappas et al. 2002; Pappas 2009).

Concluding Remarks

Based on the dramatic cutaneous pathology observed in asebia mouse, it has become clear that *Scd-1* gene product plays an important role for maintaining normal skin and eye phenotype. Mechanistic understanding of how the *Scd-1* gene product contributes to normal differentiation and function of sebaceous glands or Meibomian glands is unclear at present. Histological studies of normal and asebia mice suggest that the sebaceous gland also plays an important role in dissociating the hair follicle sheath from the outgrowing shaft during hair growth cycle, but molecular basis for this process is again unclear. It is possible that monounsaturated fatty acids or their derivatives may play an important role in sebocyte membrane synthesis, in sebum production, or in sebocyte signaling pathways (Shinomura et al. 1991; Kasai, et al. 1998). Monounsaturated fatty acids have been implicated in maintaining retinol homeostasis based on the observation that skin retinol/retinoic acid and the retinoic acid-induced genes were elevated in *Scd1* gene knockout mice (Flowers et al. 2011).

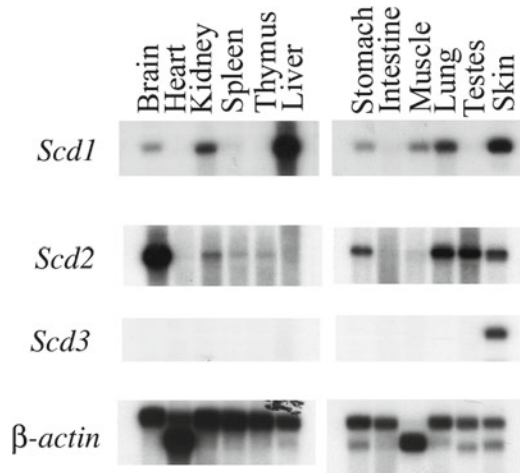

Fig. 2.8 Tissue-specific expression pattern of *Scd3* from RNA blots. The membrane blots were probed with cDNA probes from the noncoding regions specific for each *Scd* gene. The β-actin probe was used as a control. The exposure times for *Scd3* blot was adjusted more than fivefold in order to achieve comparable signal intensity to that of *Scd1* probed blot. Reproduced from Zheng et al. (2001) *Genomics* 71:182–91 with permission from the publisher (Elsevier)

Expression of multiple *Scd* genes has been reported in rodent skin (Zheng et al. 1999, 2001). Although the predicted Scd1 and Scd3 protein sequences have high sequence homologies, it is unknown if there are any differences in the activity of the two gene products. However, differences in the gene promoter sequences suggest different gene control mechanisms that may be responsible for observed tissue specificity with respect to their expression (Fig. 2.8). It is noteworthy that the lack of expression of *Scd1* in asebia mouse does not alter the expression of *Scd3* gene, at least in young mice (Zheng et al. 2001). Decrease of *Scd3* expression with advanced age in mutant mice is most likely related to the destruction of follicles and sebaceous glands due to the scarring of the pilosebaceous units (Sundberg et al. 2000). However, it is obvious that in asebia mutant mice with rudimentary sebocytes, *Scd3* gene expression in young mice cannot compensate for the lack of *Scd1* expression. The spatial expression of *Scd1* and *Scd3* in the mouse sebaceous gland underscores their roles in distinct cellular populations of sebaceous gland. Interestingly *Scd3* gene expression is slightly higher in male skin than in female skin (Zheng et al. 2001). Whether this differential regulation is achieved by androgen-mediated up-regulation in male or estrogen-mediated down-regulation in female mouse is unknown, although an estrogen receptor recognition site was observed in the *Scd3* distal promoter (Zheng et al. 2001).

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Chapter 3
Stearoyl-CoA Desaturase-1 Is a Biological Regulator of Energy Homeostasis

James M. Ntambi

Role of Hepatic SCD1 in Regulation of Lipogenesis and Fatty Acid Oxidation

Stearoyl-CoA desaturase-1 (SCD1) has since been found to be very important for the synthesis of oleate despite the fact that mammalian diets supply abundant dietary oleate. If this is true, then why is SCD1 a highly regulated enzyme? Using the asebia mouse strains (ab^{j} and ab^{2j}) that have a naturally occurring mutation in SCD1 isoform (Zheng et al. 1999) as well as a mouse model with a targeted disruption (SCD1−/−) (Miyazaki et al. 2001a, b), our laboratory showed that SCD1−/− mice are deficient in triglycerides, cholesterol esters, wax esters, and alkyldiacylglycerols (Miyazaki et al. 2001a, b; Attie et al. 2002). The levels of palmitoleate (16:1n7) and oleate (18:1n9) are reduced in the plasma and tissue lipid fractions of SCD1−/− mice while palmitate and stearate are increased. These changes are correlated with a decrease in desaturation index (18:1/18:0 or 16:1/16:0 ratio) in liver tissue and plasma (Attie et al. 2002).

Normally, a high carbohydrate diet fed to mice or rats induces the expression of the hepatic SCD1 gene and other lipogenic genes through insulin-mediated sterol regulatory element binding protein-1 (SREBP-1)-dependent mechanism. This mechanism results in an increase of monounsaturated fatty acids (MUFAs) and hepatic triglycerides (Shimomura et al. 1999a, b). One of our observations is that SCD1−/− mice on a high carbohydrate diet increase plasma insulin levels but they fail to accumulate hepatic triglycerides and cholesterol esters (Miyazaki et al. 2001a, b). Supplementation of the high carbohydrate diet with high levels of triolein or tripalmitolein can normalize cholesterol ester levels but the triglyceride levels cannot be returned to the levels found in the wild type mouse (Miyazaki et al. 2001a, b). In

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addition, the SCD1−/− mice have very low levels of triglycerides in the very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions compared to their wild type counterparts. Furthermore, the rate of VLDL-triglyceride secretion, as measured by inhibition of VLDL clearance using Triton-1339, was dramatically reduced in the SCD1−/− mice (Cohen et al. 2002). Transient transfections of an SCD1 expression vector into Chinese hamster ovary (CHO) cells result in increased SCD1 activity and esterification of cholesterol to cholesterol esters (Miyazaki et al. 2000). These observations reveal that endogenously synthesized MUFAs by SCD most likely serve as the main substrates for the synthesis of hepatic triglycerides and cholesterol esters. The enzymes involved in the de novo synthesis of triglycerides and cholesterol ester including SCD, acyl-CoA:cholesterol acyl transferase (ACAT), diacylglycerol acyl transferase (DGAT), and microsomal glycerol phosphate acyl transferase (GPAT) are located in the endoplasmic reticulum (ER) membrane. A possible physiological explanation for the requirement of SCD expression in the synthesis of the triglycerides and cholesterol esters is the production of more easily accessible MUFAs within the vicinity of ACAT, DGAT, and microsomal GPAT. These enzymes probably exist in a complex promoting substrate channeling and fuel partitioning into various metabolic pathways. It was demonstrated previously that SCD1 and DGAT-2 colocalize in ER of liver (Man et al. 2006).

It became interesting to determine whether the impairment in acyl-CoA desaturation in the SCD1−/− mice would alter the whole-animal energy homeostasis, or whether dietary MUFAs would ameliorate the deficiency. Although the growth curves of male SCD1−/− mice were similar to that of the wild type sibs on chow diet, a high fat diet revealed large differences in weight gain in both males and females. On average, the SCD1−/− mice consumed 10–15 % more food than wild type mice even though they were leaner and accumulated less fat in their adipose tissue. The epididymal fat pad mass was markedly reduced in male SCD1−/− vs. wild type mice on a chow diet and a high fat diet. Decreases in fat pads' weights were observed in subcutaneous, mesenteric, and retroperitoneal both on chow and high fat diets. The livers of the wild type and SCD1−/− mice were grossly normal and of similar mass on a chow diet. However, on a high fat diet the livers of the wild type were lighter in color than knockout mice suggesting steatosis. Masses of white adipose depots in SCD1−/− mice were universally decreased compared with wild type, regardless of diet. Thus, SCD1−/− mice were resistant to diet-induced weight gain and fat accumulation, despite increased food intake (Ntambi et al. 2002).

What then is the fate of the dietary fat in excess? Indirect calorimetry was carried out to investigate whether the resistance to weight gain was due to increased energy expenditure. The SCD1−/− mice exhibited consistently higher rates of oxygen consumption (higher metabolic rates) than their wild type littermates throughout day and night. It was hypothesized that the increased energy expenditure in the SCD1−/− mice was due to increased lipid catabolism. Although ketone bodies were undetectable in plasma from either strain during postprandial conditions, β-hydroxybutyrate levels were consistently much higher in knockout mice following a 4-h fast indicating a higher rate of β-oxidation in SCD1−/− mice.

DNA micro arrays were employed to identify genes whose expression was altered in the liver of SCD1−/− mice. Two hundred mRNAs that were significantly different between the livers of SCD1−/− and wild type mice were identified. The most striking pattern was the genes involved in lipogenesis and fatty acid β-oxidation. Lipid oxidation genes such as acyl-CoA oxidase (ACO), very long chain acyl-CoA dehydrogenase (VLCAD), carnitine palmitoyltransferase-1 (CPT-1), and fasting-induced adipocyte factor (FIAF) were up regulated while lipid synthesis genes such as SREBP-1, FAS, and mitochondrial glycerol phosphate acyl-CoA transferase (GPAT) were down regulated in the SCD1−/− mice (Ntambi et al. 2002). SREBP-1c is the main SREBP-1 isoform expressed in liver and regulates the expression of lipogenic genes (Shimomura et al. 1999a, b). Insulin levels, dietary carbohydrate, fatty acids, and cholesterol regulate the SREBP-1 gene expression and protein maturation. Thus, the down regulation of SREBP-1 gene expression in the SCD1−/− could have numerous effects on various metabolic pathways regulated by SREBP-1. For instance, the induction of SREBP-1 by insulin and cholesterol greatly enhances the synthesis and secretion of triglycerides by the liver (Shimomura et al. 1999a, b). However, in the SCD1−/− mice, as well as in the liver-specific knockout of SCD1 mice, carbohydrate feeding fails to induce SREBP-1 and lipogenic gene expression to the same level found in the wild type mice (Miyazaki et al. 2001a, b; Miyazaki et al. 2007).

CPT, ACO, VLCAD, and FIAF are known targets of peroxisome proliferator-activated receptor-α (PPARα) and contain PPARα response regions in their promoters (Kersten et al. 2000). Since PPARα mRNA level is unchanged (Miyazaki et al. 2004), the up regulation of enzymes of fatty acid β-oxidation in the SCD1−/− mice must be downstream of PPARα transcription. Thus, the characteristics exhibited by the SCD1−/− mice are consistent with presence of a PPARα activator with reduced activity in wild type mice but this was not found to be the case (Miyazaki et al. 2004). The SCD1−/− mice exhibit increase in the contents of saturated fatty acids (C16:0 and C18:0) while the contents of the polyunsaturated fatty acids (PUFAs) of the n-6 and n-3 are not changed. Changes in the levels of saturated fats may indirectly stimulate the burning of fat by mitochondria.

If SCD1 is blocked, cells may no longer generate normal levels of monounsaturated fats, which are required for normal synthesis of VLDL and triglycerides. If that happens, saturated fatty acids might build up. Scientists reported in the 1970s that fatty acids inhibit acetyl-CoA carboxylase-1 and 2 (ACC1 and ACC2) which produce malonyl-CoA. Such a buildup of saturated fatty acids might act to decrease malonyl-CoA levels and stimulate fatty acid import into mitochondria and in turn the burning of fat. Malonyl-CoA is known to inhibit the catabolism of fats and one possibility is that somehow SCD1 controls malonyl-CoA levels. In this mechanism the saturated fatty acyl-CoAs would allosterically inhibit ACC, thus reducing cellular levels of malonyl-CoA (Lunzer et al. 1977). Malonyl-CoA is required for fatty acid biosynthesis and also inhibits the mitochondrial carnityl palmitoyl transferase shuttle system, the rate-limiting step in the import and oxidation of fatty acids in mitochondria (McGarry et al. 1978). Thus, reduced levels of SCD1 would lead to a decrease in the cellular levels of malonyl-CoA and derepress fatty acid oxidation. The findings in the SCD1−/− mice are therefore similar to those observed in mice lacking

ACC2, which also have increased fatty acid oxidation in skeletal muscle and possess a lean phenotype (Abu-Elheiga et al. 2001).

Another mechanism that could account for the increased energy expenditure and protection against diet-induced adiposity and insulin resistance in the SCD1-deficient mice is associated with increased activity of AMPK (Dobrzyn et al. 2004; Hulver et al. 2005), an enzyme that has been shown to stimulate fatty acid oxidation following leptin administration. SCD1 deficiency could also be associated with direct or indirect effects on uncoupling proteins that are involved in thermogenesis (Lee et al. 2004).

Finally the fur and lipid abnormalities associated with defects in SCD1-deficient mice could be associated with increased energy dissipation. The SCD1−/− mice as well as the asebia mutant mice show cutaneous abnormalities with atrophic sebaceous glands and narrow eye fissure with atrophic meibomian glands, suggesting an important role of MUFAs in skin homeostasis (Zheng et al. 1999; Sundberg et al. 2000). Studies on the role of lipids in cutaneous biology have mainly focused on PUFAs (alpha-linoleic acid) and their role in skin barrier function (Marcelo et al. 1992). The role of MUFA in skin homeostasis is unknown. However, it is possible that these fatty acids, which are the products of SCD, play an important role in differentiation of the sebaceous gland and a parallel can be drawn to the activation of SCD and production of MUFA during 3T3-L1 preadipocyte differentiation (Casimir and Ntambi 1996; Christianson et al. 2008). Recent studies have attributed the failure of SCD1−/− mice to regulate adaptive thermogenesis and to protect against diet-induced obesity was caused by defective skin lipid barrier due to deficiency in ceramides and transepidermal water loss (TEWL) (Binczek et al. 2007). However, earlier studies had showed that the SCD1-deficient mice have a normal skin barrier but had reduced hydration due to deficiency in glycerol. In addition, the defective skin lipid barrier is due to deficiency of SCD2 isoform in the epidermis of neonates (Miyazaki et al. 2005). Further, there are studies showing that obese humans have increased TEWL (Loffler et al. 2002) supporting our contention that the hypermetabolism in SCD1-deficient mice may not be due to TEWL and an impaired epidermal lipid barrier.

We have recently found that mice with a specific deletion of SCD1 in the skin (SKO) recapitulate some of the phenotypes observed in GKO mice including increased whole-body energy expenditure, protection against dietary-induced adiposity, hepatic steatosis, and glucose intolerance (Sampath et al. 2009). The increased energy expenditure in SKO mice does not result simply from heat loss through the skin. Rather, thermogenesis appears to be constitutively activated in these mice regardless of changes in the ambient temperature (Flowers et al. 2011). Genes of fat oxidation, lipolysis, and thermogenesis, including uncoupling proteins and PPARγ co-activator-1α, are up regulated in peripheral tissues of SKO mice. However, unlike mice globally deficient in SCD1, SKO mice have an intact hepatic lipogenic response to acute high carbohydrate feeding. The up regulation of thermogenic processes for temperature maintenance at the expense of fuel economy illustrates the cross talk between the skin and peripheral tissues in maintaining energy homeostasis.

The SKO mice exhibit a dramatic decrease in the levels of 18:1n9 and 16:1n7 MUFAs in neutral lipid fractions of the sebaceous glands despite sufficient levels of

these fatty acids in the diets. Furthermore, the levels of retinol and the expression of retinoic acid (RA)-target genes including the gene for the secreted adipokine, lipocalin-2, are increased in the skin of SKO mice. Severe alterations in retinol availability or retinoic acid (RA) levels can affect the regulation of adaptive thermogenesis and adiposity, two processes which are fundamental to energy balance (Bonet et al. 2003). For example, retinaldehyde inhibits adipogenesis and blocking retinaldehyde metabolism to RA reduces diet-induced obesity (Ziouzenkova et al. 2007). The net effect of retinoids on adipogenesis and other physiological processes results from a complex balance between RA metabolism and relative amounts of retinoid receptor subtypes (RXR and RAR) (Villarroya et al. 1999). RA is a potent signaling molecule that is essential for many biological processes, and its levels are tightly regulated by mechanisms that are only partially understood.

The synthesis of RA from its precursor retinol, or vitamin A, is a major mode in the regulation of RA levels in cells and tissues (Napoli 1999). To generate RA, retinol is oxidized in two sequential reactions, catalyzed by retinol and retinal dehydrogenases (Napoli 1999), whose activities regulate RA homeostasis. The balance between retinol and retinyl esters may also regulate the availability of retinol for these reactions. Retinol esterification is carried out by two distinct enzymatic activities. One is mediated by LRAT, which catalyzes the covalent joining of a fatty acyl moiety from lecithin (phosphatidylcholine) to retinol that is bound to cellular retinol-binding protein-1 (CRBP-1) (MacDonald and Ong 1988). Unbound retinol is esterified by acyl-CoA:retinol acyltransferase (ARAT) activity that has been very difficult to purify. However, DGAT-1 has ARAT activity and it has recently been shown that DGAT-1 functions as the major ARAT in murine skin (Yen et al. 2008) where it acts to maintain retinoid homeostasis and prevent retinoid toxicity. When dietary retinol is abundant, DGAT-1 deficiency results in elevated levels of RA in skin and cyclical hair loss; both are prevented by dietary retinol deprivation (Shih et al. 2009). Since the skin and metabolic phenotypes of DGAT-1 (Smith et al. 2000) and SCD1 knockout mice are similar, it is very likely that the endogenous MUFAs produced by SCD1 are not available for esterification of excess unbound retinol to retinyl esters by DGAT-1 leading to accumulation of retinol. Both DGAT-1 and SCD1 are therefore important for handling free retinol that exceeds the capacity of LRAT. Some of the excess retinol is subsequently converted to retinoic acid, which initiates the signaling that leads to the phenotype observed in both the SCD1 and DGAT-1 knockout mice. Interestingly SCD1-gene expression is activated by retinol in liver (Miller et al. 1997) and muscle possibly to enhance esterification of excess retinol. In addition, we demonstrated previously that SCD1 and DGAT-2 colocalize in ER of liver (Man et al. 2006). It is very possible that SCD1 and DGAT-1 colocalize in ER of the sebaceous glands.

Plasma levels of lipocalin-2 are also elevated in the SKO mice. Lipocalin-2 is a known secretory protein produced by the human sebaceous glands and has been implicated in obesity and insulin resistance both in humans and rodents (Yan et al. 2007) but its in vivo role in metabolic regulation is largely unknown. It is hypothesized (Fig. 3.1) that the endogenous 18:1n9 or 16:1n7 made by SCD1 are required for esterification of excess retinol into retinyl esters for storage and detoxification in the sebaceous glands. In the absence of SCD1, the excess free retinol is subsequently

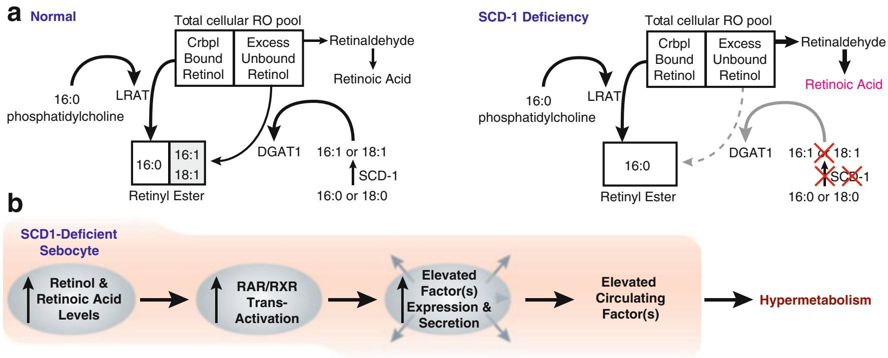

Fig. 3.1 The model proposed by Chad Paton showing how SCD1 deficiency modulates retinoic acid (RA) signaling in the sebaceous gland and leads to increased expression and secretion of a factor(s)

converted to RA, which initiates the RAR–RXR-mediated signaling in the sebaceous gland (Fig. 3.1b). The activation of RA signaling leads to secondary effects via elevations in sebaceous gland-derived lipocalin-2 or other circulating factor(s), that is, a sebokine or an adipokine released into the circulation that interact with peripheral tissues to affect whole-body energy expenditure (Fig. 3.1b). However, potentially TEWL could be the mechanism to hypermetabolism. The hypermetabolism/TEWL could still be due to sebocyte dysfunction but not dependent on a circulating factor. Retinol toxicity (or potentially cholesterol toxicity) may be causing sebocyte atrophy and a disturbed epidermal lipid barrier. This could be the reason for the hypermetabolism, and the effect on a circulating factor is just an associated gene expression change. This should be considered as an alternative hypothesis and should be pursued in case the circulating factor hypothesis does not fully explain the origin of the skin SCD1 deficiency phenotype.

Conclusion

The recent studies using the knockout mouse models have revealed the phenotypes generated as a result of SCD1 gene deficiency. SCD is critical in the biosynthesis of several classes of lipids including phospholipids, triglycerides, cholesterol esters, wax esters, and 1-alkyl-2,3-diacylglycerol. SCD1 deficiency either directly or indirectly induces a signal that partitions fatty acids towards oxidation rather than synthesis. High SCD1 expression favors fat storage while low SCD expression leads to leanness, increased metabolic rate, and insulin sensitivity. Current studies have demonstrated that liver SCD1 deficiency protects mice against high carbohydrate-induced obesity while skin SCD1 deficiency protects mice against high fat diet-induced obesity mediated by a circulating factor derived from the skin. More experiments will be required to provide physiologically relevant information on the role that skin SCD1 gene plays in energy homeostasis.

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Chapter 4
Role for Stearoyl-CoA Desaturase-1 in the Metabolic Effects of Leptin

Paul Cohen

Obesity and Metabolic Disease

Over the last few decades, there has been a worldwide increase in overweight and obesity. This has been associated with a dramatic rise in the incidence of diabetes and other metabolic diseases. The burden of these illnesses threatens to undermine many of the medical advances of the past generation. Obesity is also placing a strain on the healthcare system and economies of many nations, which could have broad societal implications.

In the United States, where obesity has had perhaps the most profound impact, more than one-third adults are obese and approximately two-thirds are overweight (Flegal et al. 2012). Body mass index (BMI), which is the most common metric of overweight and obesity, is associated with increased relative risk of diabetes, hypertension, and coronary heart disease (Kopelman 2000). Obesity is at the center of the metabolic syndrome, which has been defined based on increased visceral adiposity in conjunction with elevated blood pressure, elevated blood glucose, elevated triglycerides, and decreased HDL cholesterol. Obesity is also associated with an increased risk of dyslipidemia, nonalcoholic fatty liver disease, cancer, osteoarthritis, gout, gallstones, and sleep apnea. In addition to these comorbid conditions, obesity, and particularly visceral obesity, has been found in some studies to be linked to an increased risk of death (Pischon et al. 2008).

Obesity also carries a painful social stigma. Individuals who are overweight and obese tend to be viewed as lacking in willpower. Advances in basic science have started to dispel these misconceptions, with the increasing appreciation that body weight is under tight biological regulation (Friedman 2003). A deeper understanding of the mechanisms underlying body homeostasis will be crucial in helping to develop therapies for obesity and comorbid conditions.

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Leptin and the Regulation of Body Weight

The hormone leptin is the central regulator in an endocrine loop regulating appetite and energy homeostasis (Friedman 2009). The existence of such a pathway controlling homeostasis was suggested by parabiosis experiments done on the morbidly obese mouse mutants, *ob/ob* and *db/db*. These studies indicated the existence of a circulating factor regulating body weight, which *ob/ob* mice lack while *db/db* mice lack the ability to respond to it (Coleman 1978).

These predictions were confirmed with the positional cloning of the *ob* gene, which encodes the adipose-derived hormone leptin (Zhang et al. 1994). *Ob/ob* mice are massively obese and hyperphagic. They also develop severe insulin resistance and ectopic fat deposition in the liver and other peripheral tissues. Subsequent studies identified the leptin receptor as product of the gene mutated in *db/db* mice (Chen et al. 1996; Lee et al. 1996).

Leptin is a circulating hormone produced by adipocytes that acts via its receptor in the hypothalamus to coordinate food intake and whole body metabolism. The leptin receptor has five different isoforms (denoted ObRa-ObRe), which have also been detected on some peripheral tissues (Fei et al. 1997). In normal physiology, leptin is secreted from adipose tissue and signals the body's nutritional state to the hypothalamus, which coordinates food intake and energy expenditure to try and maintain energy balance. Treatment of wild-type or *ob/ob* mice with leptin results in voluntary reduction of food intake, increased energy expenditure, and weight loss (Halaas et al. 1995; Campfield et al. 1995; Pelleymounter et al. 1995). Leptin also acts to signal nutritional deprivation. Low leptin levels lead to an adaptive response to conserve energy, with increased food intake and suppression of the reproductive and other neuroendocrine axes (Ahima et al. 1996).

Central administration of leptin can recapitulate the effects of peripheral administration at doses that have no effect when delivered peripherally (Campfield et al. 1995). In addition, neuronal-specific deletion of the leptin receptor results in the same obesity phenotype as that seen in mice with whole body deletion of the receptor (Cohen et al. 2001). Leptin, via its receptor in the hypothalamus, activates anorexigenic pathways and inhibits orexigenic pathways (Saper et al. 2002). These pathways then signal to other brain centers to ultimately coordinate feeding behavior and energy expenditure.

Humans with mutations in leptin or the leptin receptor have also been described. They too have morbid obesity, hyperphagia, and insulin resistance (Strobel et al. 1998; Montague et al. 1997). Leptin replacement in humans with leptin mutations results in a dramatic correction of the obesity phenotype (Farooqi et al. 1999). Unfortunately, leptin mutations are very rare and a rather small percentage of obese humans are leptin-deficient. The great majority of overweight and obese individuals have increased plasma leptin levels, proportional to their fat mass (Maffei et al. 1995). Because most obesity is associated with leptin resistance, leptin, on its own, is not likely to be an effective weight loss drug. In order to develop therapies for obesity and metabolic diseases, it is important to understand the molecular basis for leptin's effects.

Metabolic Effects of Leptin

Several lines of evidence indicate that leptin produces unique metabolic effects distinct from those seen with food restriction alone. *Ob/ob* mice that are food restricted to the level that leptin-treated mice voluntarily consume (pair-fed) lose significantly less weight than leptin-treated counterparts (Levin et al. 1996). Additionally, leptin results in selective loss of fat mass, while food restriction leads to decreased fat and muscle mass. Food restriction is also associated with a compensatory decrease in energy expenditure, whereas leptin-mediated weight loss is associated with increased energy expenditure. Leptin deficiency is also characterized by ectopic lipid deposition in liver, muscle, and other peripheral tissues. This excess lipid is the hallmark of nonalcoholic fatty liver disease. It is also thought to play a pathophysiological role in the development of insulin resistance and diabetes. Leptin, much more so than pair-feeding, is able to potently deplete lipid from these tissues, and thereby results in rapid improvement in insulin resistance and other metabolic defects (Shimabukuro et al. 1997). Unlike with food restriction, leptin treatment is not associated with increased free fatty acids or ketones, supporting a unique mechanism of fatty acid oxidation. These findings indicate that leptin mediates weight loss, at least in part by activating a molecular program with unique effects on metabolism.

Identification of SCD-1 as a Leptin-Repressed Gene in Liver

Leptin-deficient *ob/ob* mice develop severely enlarged livers that are overloaded with lipid. Leptin treatment corrects the hepatic steatosis in these animals far more profoundly than food restriction. Following 12 days of leptin treatment, the liver of an *ob/ob* mouse is indistinguishable from that of a wild-type littermate, at both the gross and histological level. These dramatic effects led us to examine the molecular basis by which leptin depletes hepatic lipid stores. We postulated that this pathway would be important to liver metabolism and whole body energy homeostasis.

To determine the molecular mechanism by which leptin corrects hepatic steatosis we used oligonucleotide microarrays to identify leptin-regulated genes in the liver. *Ob/ob* mice were followed over a time course of either leptin treatment or vehicle treatment with pair-feeding for 2, 4, or 12 days. Free fed *ob/ob* mice treated with saline were also studied. Leptin-treated mice lost significantly more weight and showed a much greater correction of hepatic steatosis than their pair-fed counterparts. Liver RNA was isolated from mice from each group and hybridized to Affymetrix arrays. At the time this experiment was performed, the standard murine arrays contained 6,500 genes. In total, this experiment generated over 50,000 data points.

We developed computational approaches to identify leptin-regulated genes from this large data set (Soukas et al. 2000). Cluster analysis is a well described method

for finding groups of genes with coordinate patterns of expression. In this data set, six clusters of genes were specifically regulated by leptin. However, this list still contained several hundred genes. To prioritize this further, we used a more focused approach to identify genes specifically repressed by leptin. We developed an algorithm to rank genes based on the following criteria: (1) increased expression in *ob/ob* relative to wild-type liver, (2) reduced expression with leptin treatment, and (3) maximally different expression between leptin and pair-feeding. This resulted in a prioritized list of genes with increased expression in *ob/ob* liver, which were specifically repressed by leptin. We hypothesized that downregulation of these genes might mediate some of the novel metabolic actions of leptin.

The gene encoding the enzyme stearoyl-CoA desaturase-1 (SCD-1) was the top-ranked gene with this approach (Cohen et al. 2002). SCD-1 is the rate-limiting enzyme in the biosynthesis of monounsaturated fats. The desaturation reaction requires NADPH, cytochrome b5, and cytochrome b5 reductase. The substrates of SCD-1 are palmitic (16:0) and stearic acid (18:0) which it desaturates to generate palmitoleic (16:1) and oleic acid (18:1). These products are among the most abundant fatty acids found in triglycerides, phospholipids, and cholesterol esters. SCD-1 is localized to the endoplasmic reticulum and undergoes rapid turnover in response to a number of nutritional and hormonal cues (Heinemann and Ozols 2003). SCD-1 is also under transcriptional regulation by SREBP-1, ChREBP, and other factors (Ntambi et al. 2004). SCD-1 is broadly expressed and is one of four characterized SCD genes in mice, which are in a cluster on chromosome 19. Humans have a single SCD gene with high homology to murine SCD-1. SCD homologs have also been described in yeast, flies, and worms.

To confirm that this algorithm properly identified SCD-1 as a leptin-regulated gene, an independent time course was done. Livers were isolated and SCD-1 RNA levels, enzymatic activity, and substrate/product levels were measured (Cohen et al. 2002). SCD-1 RNA levels were markedly elevated in *ob/ob* liver. Upon leptin treatment, SCD-1 RNA levels normalized by day 2, and within 4 days had fallen to levels below that in wild-type mice. Pair-fed mice showed a less robust and delayed decrease in SCD-1 expression. Measures of enzymatic activity reflected these trends in RNA levels. SCD enzymatic activity was more than sevenfold elevated in *ob/ob* livers. Leptin treatment completely normalized enzymatic activity, while pair-feeding had a more modest effect. The levels of 16:1 and 18:1, the products of SCD-1, were elevated in *ob/ob* liver and were preferentially normalized by leptin. Importantly, leptin also repressed SCD-1 in wild-type mice, suggesting that its effects on SCD-1 expression are not simply secondary to its correction of the obesity phenotype.

SCD-1 Repression Mediates the Metabolic Effects of Leptin

To determine whether repression of SCD-1 is important in mediating the metabolic effects of leptin, we studied mouse models of SCD-1 deficiency. Asebia mice (*ab^J/ab^J*) are a spontaneous mutant strain with a genomic deletion of the first four exons of

SCD-1. These mice have no functional SCD-1 mRNA or protein, though the expression of the related gene SCD-2 is unchanged (Zheng et al. 1999). SCD-1 knockout mice have also been generated by standard gene targeting ($SCD1^{-/-}$) (Ntambi et al. 2002). The phenotype of these two mutant strains is largely indistinguishable.

Asebia mice have severe ocular and cutaneous abnormalities and have received their name due to an absence of sebaceous glands. These mice also have decreased synthesis of palmitoleic and oleic acid and reduced levels of hepatic triglycerides, cholesterol esters, and VLDL production (Miyazaki et al. 2000). 16:1 and 18:1 are among the most abundant lipid species found in the diet. However, dietary supplementation is unable to correct the decrease in monounsaturated fats seen in mutant animals (Miyazaki et al. 2001). This indicates that there is a requirement for endogenous synthesis of these lipids.

Upon evaluating asebia mice, we appreciated that they were clearly leaner than their control littermates (Cohen et al. 2002). Despite normal body weight, asebia mice have reduced fat mass and plasma leptin levels. We next examined whether SCD-1 deficiency might replicate any of the effects of leptin. We predicted that *ob/ob* mice lacking SCD-1 would resemble leptin-treated *ob/ob* animals. Double mutant ab^J/ab^J;*ob/ob* mice were generated and found to have a dramatic reduction in body weight relative to *ob/ob* littermates. By 16 weeks of age, double mutants weighed 30 % less than *ob/ob* mice, a weight comparable to that seen in *ob/ob* mice after 12 days of leptin treatment. Body composition analysis confirmed a significant reduction in adiposity in double mutant mice, with an increase in lean body mass. Additionally, $SCD\text{-}1^{-/-}$ mice were protected from diet-induced obesity. The weight of every white adipose depot was significantly lower in knockout mice relative to controls (Ntambi et al. 2002).

To understand the mechanism whereby SCD-1-deficient mice were protected from obesity we measured food intake and energy expenditure (Cohen et al. 2002). Surprisingly, we found that mice lacking SCD-1 ate significantly more food than controls. Food intake in asebia mice was equivalent to that of *ob/ob* mice, and double mutant ab^J/ab^J;*ob/ob* mice consumed even more. We therefore predicted that mice lacking SCD-1 must have increased energy expenditure. We performed indirect calorimetry and found that asebia mice had significantly greater total and resting oxygen consumption than littermate controls. *Ob/ob* mice are known to have markedly reduced energy expenditure, since the absence of leptin signals a state of perceived starvation. However, *ob/ob* mice lacking SCD-1 had a complete correction of this hypometabolic state, with oxygen consumption equivalent to or greater than wild-type littermates.

SCD-1 and Fatty Liver Disease

Since leptin treatment potently depletes hepatic lipid while repressing SCD-1, we examined whether SCD-1 deficiency could protect *ob/ob* mice from hepatic steatosis. Livers from double mutant ab^J/ab^J;*ob/ob* mice were grossly and histologically

indistinguishable from wild-type (Cohen et al. 2002). Triglyceride content was reduced more than threefold in double mutant relative to *ob/ob* livers, with levels comparable to those found in wild-type livers. Triglyceride levels in ab^J/ab^J mice were reduced even further than those of wild-type mice.

This protection from hepatic steatosis prompted us to evaluate whether SCD-1 deficiency could protect from lipid accumulation in other models. Lipodystrophy is defined by partial or complete absence of adipose tissue and is associated with leptin deficiency, insulin resistance, diabetes, and hepatic steatosis (Reitman et al. 2000). Human lipodystrophy can occur due to congenital mutations or due to acquired conditions such as with anti-retroviral therapy for HIV. Leptin replacement in a mouse model of lipodystrophy and in humans with the condition can markedly improve insulin resistance, diabetes, and fatty liver (Shimomura et al. 1999; Oral et al. 2002).

We examined whether hepatic SCD-1 is dysregulated in a mouse model of lipodystrophy. aP2-nSREBP1c mice have enlarged fatty livers with increased SCD-1 expression and enzymatic activity. Central administration of leptin (at doses that have no effect when delivered peripherally) completely normalized SCD activity in this lipodystrophic model (Asilmaz et al. 2004). This indicates that the effects of leptin on hepatic SCD-1 are centrally mediated. To assess the role of SCD-1 in the liver pathology in these mice, double mutant ab^J/ab^J*;aP2-nSREBP1c tg* mice were generated. Double mutant mice demonstrated a significant improvement in hepatic lipid overload, though it was not completely corrected.

Mechanism for the Metabolic Effects of SCD-1 Deficiency

Our data indicated that SCD-1 was likely to be an important metabolic control. Mice deficient in this enzyme were resistant to obesity and hepatic steatosis. This is due to markedly increased energy expenditure. To understand the mechanism underlying this phenomenon, we further examined hepatic metabolism in the setting of SCD-1 deficiency. Fatty acids in the liver are typically esterified to glycerol or cholesterol and can then (1) accumulate leading to hepatic steatosis, (2) be packaged into VLDL for export to other tissues, or (3) be oxidized (Cohen and Friedman 2004). Monounsaturated acids, which are the products of SCD-1, are required for triglyceride and cholesterol ester synthesis and for VLDL production. We predicted then that in the absence of SCD-1 there is a defect in lipid storage and VLDL synthesis, and that as a default, there is increased fatty acid oxidation. Histological analysis and triglyceride quantitation confirmed that lipid storage was impaired in SCD-1-deficient animals. We next measured VLDL production by treating mice with tyloxapol, an inhibitor of VLDL hydrolysis. Following injection, we measured plasma triglyceride levels over time. Since VLDL hydrolysis is blocked, the levels rise in a linear fashion in relation to the rate of VLDL production. We found that VLDL synthesis is significantly increased in *ob/ob* mice and reduced in ab^J/ab^J;*ob/ob* mice to levels similar to that in wild-type controls (Cohen et al. 2002).

We therefore assumed that SCD-1 ablation must result in increased fatty acid oxidation. This is consistent with the finding of decreased adiposity and increased

energy expenditure, in the setting of increased food intake. Moreover, mice lacking SCD-1 have increased plasma ketones, a marker of fatty acid oxidation. Finally, gene expression profiling in SCD-1$^{-/-}$ mice demonstrated increased expression of genes involved in fatty acid oxidation (Ntambi et al. 2002).

We therefore considered a model to explain the mechanism by which SCD-1 deficiency might result in increased fatty acid oxidation. When SCD-1 is repressed, saturated fatty acyl CoAs cannot be desaturated and would therefore accumulate. Saturated fatty acyl CoAs potently allosterically inhibit acetyl CoA carboxylase (ACC), the enzyme that converts acetyl CoA into malonyl CoA, an intermediate in fatty acid biosynthesis (Lunzer et al. 1977). Malonyl CoA is known to inhibit carnityl palmitoyltransferase-1 (CPT-1), the rate-limiting enzyme in the transport of fatty acids from the cytosol, where they are synthesized, into the mitochondria, where they are oxidized (McGarry et al. 1977). When malonyl CoA levels fall, as would be expected in SCD-1 deficiency, CPT-1 is derepressed and fatty acids are shuttled into the mitochondria for oxidation. Decreased malonyl CoA would also result in reduced fatty acid biosynthesis. SCD-1 deficiency could also result in increased activity of AMP-activated protein kinase (AMPK), an intracellular control point for energy metabolism, that is activated by leptin (Minokoshi et al. 2002; Dobrzyn et al. 2004). In fact, unifying these two models, we found that SCD-1 deficiency is associated with increased AMPK activity along with reduced malonyl CoA levels, increased CPT activity, and increased fatty acid oxidation (Dobrzyn et al. 2004).

We also considered other mechanisms that could account for the increased energy expenditure in animals deficient in SCD-1. For example, inhibition of SCD-1 could increase the levels of a ligand for peroxisome proliferator-activated receptor α (PPARα) or another nuclear receptor. Interestingly, the PPARα target gene and rate-limiting enzyme in peroxisomal fatty acid oxidation, acyl CoA oxidase, is increased in livers of SCD-1$^{-/-}$ mice (Ntambi et al. 2002). SCD-1 inhibition could also modulate non-shivering thermogenesis, via actions on uncoupling proteins. Finally, SCD-1 null mice have defects in fur and skin lipids, which could result in increased energy dissipation.

In work done by other groups, it has become clear that inhibition of other genes in the same metabolic pathway as SCD-1 is also linked to increased energy expenditure. Mice with a knockout of ACC-2 demonstrate decreased malonyl CoA levels, increased fatty acid oxidation, and protection from obesity (Abu-Elheiga et al. 2001). The drug C75 causes weight loss by inhibiting the enzyme fatty acid synthase and by derepressing CPT-1 (Loftus et al. 2000). Additionally, deletion of acyl CoA:diacylglycerol acyltransferase (DGAT), which catalyzes the final step in fatty acid biosynthesis, results in some of the same cutaneous and metabolic findings as that seen in SCD-1 deficiency (Smith et al. 2000; Chen et al. 2002).

One of the roles of the triglyceride biosynthetic pathway in the liver is to generate products that can be packaged into VLDL for export to adipose and other tissues. Genetic mutations inhibiting VLDL delivery and hydrolysis are also associated with protection from obesity. Mice lacking the VLDL receptor are lean and protected from dietary and genetic obesity (Frykman et al. 1995; Weinstock et al. 1997). *Ob/ob* mice deficient in adipose tissue lipoprotein lipase, the enzyme required

for triglyceride hydrolysis, also show reduced adiposity (Weinstock et al. 1997). Lastly, *ob/ob* mice overexpressing apolipoprotein C1, which inhibits VLDL hydrolysis, have a nearly complete correction of obesity (Jong et al. 2001).

SCD-1 and Other Components of the Metabolic Syndrome

Given that SCD-1 deficiency is associated with increased energy expenditure and resistance to obesity, we also evaluated whether it protects against other metabolic phenotypes, such as diabetes and hyperlipidemia. SCD-1$^{-/-}$ mice have improved glucose and insulin tolerance, suggesting that inhibition of SCD-1 might protect against diabetes (Ntambi et al. 2002). However, SCD-1 deficiency was not able to improve the severe diabetic phenotype seen in lipodystrophic mice (Asilmaz et al. 2004).

Mice lacking SCD-1 have significantly reduced synthesis of triglycerides, cholesterol esters, and VLDL (Miyazaki et al. 2000). Oleic acid, one of the products of SCD-1, is the preferred substrates for acyl CoA:cholesterol acyltransferase (ACAT), the rate-limiting enzyme in cholesterol esterification (Buhman et al. 2000). Mice deficient in SCD-1 have decreased cholesterol ester synthesis, despite normal ACAT activity. Additionally, in the *hyplip* murine model, hypertriglyceridemia is associated with SCD-1 activity (Attie et al. 2002). In a human cohort, SCD-1 activity was able to explain 44 % of the variance in triglyceride levels. These data suggest that inhibition of SCD-1 might protect against atherosclerosis.

SCD-1 as a Potential Therapeutic Target

Since our reports on the regulation of hepatic SCD-1 by leptin, additional studies have further clarified our original conclusions. Antisense oligonucleotides (ASOs) to SCD-1 reduced SCD-1 expression, decreased fatty acid synthesis, and increased fatty acid oxidation in primary hepatocytes. Administration of these ASOs to mice was able to prevent diet-induced obesity (Jiang et al. 2005). A separate study used ASOs to SCD-1 in mouse and rat models of insulin resistance. Insulin clamps showed that these ASOs were able to markedly improve hepatic insulin resistance (Gutierrez-Juarez et al. 2006).

Additional animal models have also further illuminated the role that SCD-1 in liver and other tissues contributes to body weight and metabolism. Mice with a liver-specific deletion of SCD-1 have been generated. These animals were protected from high carbohydrate, but not from high fat-induced obesity and hepatic steatosis (Miyazaki et al. 2007). This model suggests that SCD-1 activity in tissues other than liver plays a role in the phenotype seen with whole body deficiency. This was confirmed with the generation of mice with a skin-specific deletion of SCD-1. These mice were protected from high fat diet-induced obesity (Sampath et al. 2009).

These mice appear to have increased thermogenesis to compensate for defective insulating capacity by the skin.

In the aggregate, abundant evidence exists to support an important role for SCD-1 in whole body metabolism. Hepatic SCD-1 activity is markedly elevated in leptin-deficient animals. Leptin-mediated repression of hepatic SCD-1 appears to be an important component of the metabolic effects of leptin. Subsequent studies have shown that SCD-1 in the skin is important for maintaining insulation, and in its absence, animals are protected from obesity. Liver-specific small molecule inhibitors of SCD-1 have been generated (Oballa et al. 2011). These compounds may help further pinpoint the specific role of SCD-1 in obesity and other metabolic diseases. Further studies will be needed to determine the full spectrum of tissues where SCD-1 and other SCD isoforms impact upon energy homeostasis.

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Chapter 5
Regulation and Metabolic Functions of White Adipose Tissue Stearoyl-CoA Desaturase

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Introduction

White adipose tissue (WAT) is the main site for the storage of excess energy, in the form of fat, in the body. In addition to its energy-storage function, adipose tissue also secretes numerous bioactive peptides collectively known as adipokines. These adipokines have important auto/paracrine as well as endocrine functions in regulation of energy balance, glucose homeostasis, and blood pressure. Therefore, adipose tissue is considered to be a dynamic endocrine organ.

As discussed in previous chapters, stearoyl-CoA desaturase (SCD) is a microsomal enzyme which catalyzes the rate limiting step of monounsaturated fatty acid (MUFA) synthesis from saturated fatty acid (SFA) precursors. Humans have two SCD isoforms, SCD1 and SCD5, of which WAT mainly expresses SCD1. Mice have four SCD isoforms, of which WAT expresses SCD1 and SCD2 encoded by *Scd1* and *Scd2,* respectively. The preferential substrates for SCD are palmitoyl-CoA and stearoyl-CoA, which are converted to palmitoleoyl-CoA and oleoyl-CoA, respectively. The latter acyl-CoAs are incorporated into triglycerides, with oleic acid being the predominant fatty acid in human adipose tissue triacylglycerols (Mauvoisin and Mounier 2011). Therefore SCD plays an important role in triglyceride storage in adipose tissue.

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In obese individuals, there is excessive triglyceride storage in adipocytes, which leads to adipocyte hypertrophy. This is also associated with development of a chronic low-grade inflammation in WAT, which leads to a dysregulation of adipokine secretory patterns. These changes lead to metabolic derangements and result in diseases such as type-2 diabetes and hypertension. Since SCD plays a key role in triglyceride storage in adipose tissue, it is likely that SCD plays an important role in modulating adipose tissue function as well as whole body metabolism. In this context, it is important to understand the factors which regulate SCD expression in WAT. This chapter provides an overview of adipose tissue structure and function, factors affecting WAT SCD expression and how adipose tissue SCD expression affects whole body metabolism.

Structure and Functions of WAT

Adipose tissue is considered as an endocrine organ, which consists of ~50 % fat cells (adipocytes) and ~50 % stromal vascular fraction that contains blood cells, endothelial cells, adipose tissue precursors, and macrophages in normal weight people (Wang and Nakayama 2010). These cells along with vascular and nervous tissue in the adipose tissue are supported by an extra-cellular matrix (Kalupahana et al. 2011). Adipose tissue macrophages (ATM) are classified according to the receptors they express as well as by their cytokine profile. Lean adipose tissue mainly contains M2 or alternatively activated macrophages, which secrete anti-inflammatory cytokines such as interleukin (IL)-4 and IL-10 (Kalupahana et al. 2012b). In contrast, adipose tissue from obese individuals contains predominantly M1 or classically activated macrophages, which secrete pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1 and IL-6. In terms of lymphocytes, lean adipose tissue contains predominantly FoxP3^{+} regulatory T cells (Tregs). This, however, changes with obesity so that the Tregs are reduced and other T cell subsets such as T helper 1 (Th1) and CD8^{+} effector T cells are increased (Feuerer et al. 2009; Nishimura et al. 2009).

A major function of adipose tissue is to store excess energy in the form of triglycerides. Excess fatty acids from exogenous (dietary) and endogenous (mainly liver) sources are directed to the adipose tissue, while excess carbohydrates are converted to fatty acids (mainly SFA) by the liver (de novo lipogenesis) and then transported to the adipose tissue. De novo lipogenesis occurs to a certain extent in the adipose tissue as well. Fatty acids from both these sources are incorporated into triglycerides and stored in adipocytes. As described previously, SFA-induced lipogenesis is dependent upon SCD1. For example, in global SCD1 knockout mice, stearate does not induce lipogenesis (Sampath et al. 2007). Therefore, SCD is crucial in regulating triglyceride storage in adipocytes. Insulin is the main hormone which promotes lipid storage. In fasting stage, due to low insulin levels, lipolysis is stimulated and fatty acids are released from the adipose tissue to the blood. These fatty acids are taken up by tissues such as skeletal muscle, stored and/or oxidized to yield energy.

Increased lipid accumulation in skeletal muscle partially contributes to muscle insulin resistance. Defects in lipid storage by the adipose tissue have deleterious effects on body metabolism. For example, in insulin-resistant states and in conditions such as lipodystrophy, there is ectopic fatty acid deposition in organs such as liver, skeletal muscle, and pancreas, which leads to lipotoxicity and the development of diabetes (Hegele 2001). Therefore a normal lipid storage capacity of the adipose tissue is essential for homeostasis.

Adipokines, Obesity, and Adipose Tissue Inflammation

Adipose tissue secretes bioactive peptides termed adipokines. Some of the major adipokines secreted by the adipose tissue are leptin, adiponectin, resistin, angiotensinogen, and pro-inflammatory cytokines such as IL-6, monocyte chemotactic protein (MCP)-1, plasminogen activator inhibitor (PAI)-1, and TNF-α. Leptin functions as a major regulator of energy balance by reducing energy intake and increasing energy expenditure. Mice with genetic mutations of the leptin gene (Ob/Ob mice) or the leptin receptor (db/db mice) are severely obese, highlighting the critical role of this adipokine in energy homeostasis. Adiponectin is an insulin sensitizer, while resistin induces insulin resistance. Angiotensinogen (Agt), the precursor peptide of the hypertensive hormone angiotensin (Ang) II, is an important regulator of blood pressure and fluid balance. Recent evidence has identified Agt as an important regulator of glucose homeostasis as well (Kalupahana and Moustaid-Moussa 2012b). We and others have shown that Agt and Ang II derived from adipose tissue have important endocrine functions (Kalupahana et al. 2012a; Kalupahana and Moustaid-Moussa 2012a).

In states of chronic energy excess, adipose tissue expands by two mechanisms. First, new adipocytes are formed from adipocyte precursors such as preadipocytes and stem cells. This process is known as adipocyte hyperplasia. Second, excessive triglyceride storage within adipocytes leads to an increase in adipocyte size, which is known as adipocyte hypertrophy. Enlarged adipocytes change adipokine secretory patters, characterized by increased production of leptin, Agt, and pro-inflammatory cytokines such as MCP-1 and IL-6 and a reduced production of anti-inflammatory adipokines such as adiponectin (Kalupahana et al. 2011). Adipocyte hypertrophy also leads to adipocyte death. Endoplasmic reticulum stress and adipose tissue hypoxia are two mechanisms responsible for these changes (Boden et al. 2008; Pasarica et al. 2010). These changes in adipocytes are accompanied by changes in adipose tissue immune cell populations as described earlier, leading to a state of chronic low-grade inflammation in the adipose tissue. These changes in adipo/cytokine secretory patterns coupled with excessive lipolysis leading to lipotoxicity are considered to be causative factors for the generation of insulin resistance in the liver and skeletal muscle. Long-standing insulin resistance coupled with pancreatic dysfunction leads to the development of type-2 diabetes (Kalupahana et al. 2012b).

Regulation of Adipose Tissue SCD Expression

Hormonal Control

Human adipose tissue expresses SCD1, while murine adipose tissue expresses both SCD1 and SCD2 isoforms (Kaestner et al. 1989). In 3T3-L1 murine preadipocytes, SCD2 is expressed at higher levels than SCD1 (Kim et al. 2000). When differentiated by adding dexamethasone, methylisobutylxanthine, and insulin (DMI), SCD1 expression dramatically increases, while the increase in SCD2 is modest. Peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor which is important in adipogenesis as well as maintaining normal systemic insulin sensitivity. PPARγ agonists have insulin-sensitizing effects. PPARγ agonist troglitazone increases 3T3-L1 preadipocyte differentiation. However, it suppresses SCD1 expression. Similar effects have been reported for pioglitazone, another PPARγ agonist (Kurebayashi et al. 1997). Expression of SCD2 is independent of both these pro-adipogenic mediators (DMI and troglitazone). In humans, WAT PPARγ expression is positively associated with SCD1 expression. Unlike the effect of troglitazone on SCD1 expression in murine adipocytes, patients treated with PPARγ agonist pioglitazone have a twofold increase in subcutaneous adipose tissue (SAT) SCD1 mRNA and protein expression (Yao-Borengasser et al. 2008). Further, pioglitazone increases SCD1 expression in cultured adipocytes (Yao-Borengasser et al. 2008). Therefore, while SCD expression increases with adipogenesis in humans and rodents, the effects of different PPARγ agonists on SCD expression is inconsistent. There appear to be response elements in the SCD promoter which respond to adipogenic agents (Ntambi et al. 1988).

WAT SCD expression is sensitive to both hormonal and nutritional signals, which are summarized in Fig. 5.1 and Table 5.1. Leptin is a major regulator of adipose tissue SCD expression. Similar to its action in the liver (Cohen et al. 2002), systemic administration of leptin decreases SCD1 expression in WAT (Macek Jilkova et al. 2010). Intracerebroventricular leptin administration also reduces WAT SCD1 expression, indicating that the central action of leptin on the brain has an effect on adipose tissue SCD expression (Gallardo et al. 2007; Lin et al. 2003). Since this effect of leptin is absent in the presence of a melanocortin antagonist, leptin-mediated suppression of WAT SCD1 appears to be mediated via melanocortin receptors (Lin et al. 2003). Conversely, low-leptinemic states such as caloric restriction are characterized by high adipose SCD1 levels (Macek Jilkova et al. 2010). While the anorexigenic hormone leptin decreases WAT SCD1 expression, central administration of the orexigenic hormone ghrelin increases WAT SCD1 expression (Theander-Carrillo et al. 2006). Considering the important function of SCD1 in mediating energy storage, this illustrates the opposite effects of leptin and ghrelin on energy balance—the former promoting energy expenditure and reducing energy storage, with the latter promoting energy storage.

Effects of other hormones involved in metabolism on WAT SCD expression are less clear. For example, WAT SCD1 is increased in mice lacking beta adrenergic

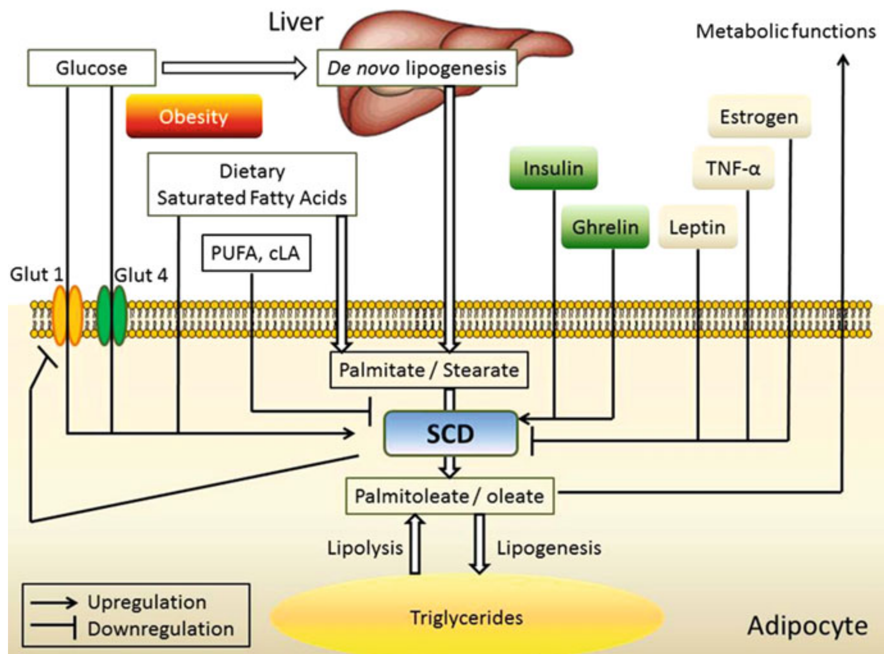

Fig. 5.1 Regulation and functions of white adipose tissue SCD. SCD catalyzes the desaturation of saturated fatty acids (palmitate and stearate) to from MUFAs (palmitoleate and oleate). Since these latter fatty acids are the preferred forms for triglyceride formation, SCD is crucial in regulating adipose tissue lipogenesis. Palmitoleate and oleate also have important regulatory functions in metabolism. In obesity, there is an increase in dietary intake of carbohydrates and saturated fats. Excess glucose from carbohydrates is converted into saturated fats by the liver (de novo lipogenesis). Both de novo-synthesized and dietary saturated fats upregulate SCD expression in adipose tissue, while PUFA and cLA downregulate its expression. Glucose enters adipocytes via Glut-4 and Glut-1 transporters and also upregulate SCD expression. SCD in turn has an inhibitory effect on Glut-1 expression. Hormonal control of SCD expression includes inhibitory effects from leptin, estrogen, and TNF-α and stimulatory effects from ghrelin and insulin. *PUFA* polyunsaturated fatty acids, *cLA* conjugated linoleic acid, *TNF* tumor necrosis factor

receptors. However, SCD1 expression is not suppressed by systemic administration of epinephrine (Mainieri et al. 2007). The effect of insulin on SCD1 expression is also inconsistent (Table 5.1). While we found that insulin did not have an independent effect on SCD1 gene transcription in 3T3-L1 adipocytes (Jones et al. 1998), others have reported that insulin increases SCD1 expression in the same adipocyte cell line (Weiner et al. 1991). TNF-α, a pro-inflammatory cytokine mediating insulin resistance in obese individuals, inhibits SCD1 expression in murine adipocytes in vitro (Weiner et al. 1991). Estrogen also appears to have an inhibitory effect on WAT SCD1 expression in humans (Lundholm et al. 2008) as well as mice (Bryzgalova et al. 2008). There are other hormones which influence SCD1 expression in a tissue-specific manner. For example, while growth hormone increases

Table 5.1 Factors which regulate white adipose tissue SCD1 expression

Factor	Species/cell line	Effect on expression	References
Troglitazone, pioglitazone (PPARγ agonists)	Mouse/3T3-L1	Decrease	Kurebayashi et al. (1997)
Pioglitazone	Human	Increase	Yao-Borengasser et al. (2008)
Leptin	Mouse	Decrease	Macek Jilkova et al. (2010), Gallardo et al. (2007), Lin et al. (2003)
Ghrelin	Mouse	Increase	Theander-Carrillo et al. (2006)
Insulin	Mouse/3T3-L1	Increase/no change	Weiner et al. (1991), Jones et al. (1998)
TNF-α	Mouse/3T3-L1	Decrease	Weiner et al. (1991)
Estrogen	Human	Decrease	Lundholm et al. (2008)
Estrogen	Mouse	Decrease	Bryzgalova et al. (2008)
KLF15	Mouse	Decrease	Nagare et al. (2011)
Hypercaloric diet	Human	Increase	Shea et al. (2009)
Caloric restriction	Human	Decrease	Franck et al. (2011), Johansson et al. (2012)
Caloric restriction	Mouse	Increase	Macek Jilkova et al. (2010)
PUFA	Rat	Decrease	Jones et al. (1996)
cLA	Mouse, 3T3-L1	Decrease	Jaudszus et al. (2010), Choi et al. (2000)
SFA	Mouse	Increase	Nikonova et al. (2008)
Glucose	Mouse/3T3-L1	Increase	Jones et al. (1998)
High-carbohydrate diet	Human	Increase/no change	Mangravite et al. (2007), Chong et al. (2008)

PPAR peroxisome proliferator-activated receptor, *TNF* tumor necrosis factor, *KLF* Kruppel-like factor, *PUFA* polyunsaturated fatty acid, *cLA* conjugated linoleic acid, *SFA* saturated fatty acid

SCD1 expression in the rodent liver, it has no effect on WAT SCD1 expression (Frick et al. 2002).

Certain transcription factors also regulate adipose SCD1 expression. For example, Kruppel-like factor 15 (KLF15) is a member of the Kruppel-like factor family of transcription factors, which is downregulated in WAT of obese animals. Transgenic overexpression of KLF15 downregulates adipose SCD1 expression (Nagare et al. 2011), possibly indicating that KLF15 is a negative regulator of SCD1 in WAT.

Nutritional Control

WAT SCD expression is regulated by both energy content as well as type of macronutrient in the diet. For example, consuming a 40 % hypercaloric diet for 7 days increases abdominal subcutaneous WAT SCD expression by fivefold in lean individuals (Shea et al. 2009). Conversely, caloric restriction leads to suppression of WAT SCD expression in humans (Franck et al. 2011; Johansson et al. 2012). WAT of both obese humans as well as animal models exhibit higher SCD expression, indicating a positive correlation of SCD expression with chronic energy excess.

We have shown that WAT SCD1 expression is increased in obese Zucker rats (Jones et al. 1996). Chronic food restriction/re-feeding also results in an increase in WAT SCD1 expression (Turyn et al. 2010).

WAT SCD1 exhibits a differential response to high-fat and high-carbohydrate diets. A high-polyunsaturated fatty acid (PUFA) diet can reduce the SCD1 mRNA expression by about 75 %, both in vivo as well as in vitro, suggesting that PUFA negatively regulates SCD1 expression in adipocytes (Jones et al. 1996). The *trans*-10, *cis*-12 isomer of conjugated linoleic acid (CLA) also suppresses SCD1 expression in rodents (Jaudszus et al. 2010; Choi et al. 2000). In contrast, SFA-rich diets increase SCD1 expression in murine WAT (Nikonova et al. 2008). Unlike in other tissues, the regulation of adipose SCD1 gene expression by PUFA appears to be at the posttranscriptional level. For example, arachidonic acid decreases the SCD1 mRNA half-life, while oleic and stearic acids do not have an effect on it (Sessler et al. 1996). Transcriptional or posttranslational regulation does not seem to be of major importance in the PUFA-mediated suppression of SCD1 expression in murine adipocytes. The reduction in SCD1 enzyme activity by PUFA can be accounted by the decrease in mRNA levels. The mRNA stability of SCD1 in adipocytes is possibly regulated via mRNA sequences in the 3′-untranslated region (UTR). The unusually long 3′-UTR in rodent and human SCD1 cDNA contains several structural motifs characteristic of mRNA destabilization sequences. Whether these sequences are possible targets of PUFA merits further investigation.

While WAT SCD1 expression is positively regulated by glucose in murine adipocytes (Jones et al. 1998), the effect of high-carbohydrate diets on WAT SCD1 expression in vivo is inconsistent (Table 5.1). For example, while a 3-day high-carbohydrate diet increases the desaturation index in the venous blood draining SAT, WAT SCD1 expression remains unchanged (Chong et al. 2008). Others have reported positive associations between carbohydrate content in the diet and WAT SCD1 expression in humans (Mangravite et al. 2007). Low-fat high-carbohydrate diets also increase SCD2 expression in adipose tissue of mice (Kaestner et al. 1989).

Functions of WAT SCD

Regulation of Adipogenesis and Lipogenesis

In 3T3-L1 adipocytes, SCD1 desaturates palmitic (16:0) and stearic (18:0) acids to form palmitoleic (16:1 *n*-7) and oleic (18:1 *n*-9) acids, respectively. SCD2 appears to be more specific for the conversion of stearate to oleic acid (Kim et al. 2000).

SCD2 has an important function in preadipocyte differentiation. Knockdown of SCD2, but not SCD1, by small interfering RNA reduces adipogenesis in 3T3-L1 preadipocytes (Christianson et al. 2008). This could be possibly mediated via PPARγ, because SCD2 knockdown leads to reduced PPARγ transcription as well as translation (Christianson et al. 2008).

SCD and Obesity

SCD mRNA expression in WAT is greatly increased in genetic rodent models of obesity, such as the Zucker rat (Jones et al. 1996). In humans, BMI positively correlates with the adipose tissue desaturation index, suggesting an increased desaturase activity in obesity (Yee et al. 2012; Gong et al. 2011a; Okada et al. 2005). WAT SCD1 activity also closely correlates with insulin resistance (Sjogren et al. 2008). Further, large insulin-resistant adipocytes express higher levels of SCD1 compared to small insulin-sensitive ones (Matsubara et al. 2009). In conditions of chronic energy excess, there is a higher availability of SFAs to the WAT (either via dietary intake or due to de novo lipogenesis). Since MUFA are the preferred form of fatty acids to be stored in triglycerides via the action of SCD, it is plausible that the increase in SCD expression in obesity is a physiological adaptation to increased SFAs availability. This assertion is supported by the fact that intake of SFAs is associated with higher WAT SCD1 expression in rodents (Nikonova et al. 2008). Moreover, SCD1 knockout mice have low adiposity and reduced MUFA in adipocyte triglycerides (Liu et al. 2010).

However, other evidence suggests that SCD might have a regulatory role in energy balance and adiposity. For example, genetic polymorphisms in the SCD1 gene are associated with fat distribution and insulin sensitivity (Gong et al. 2011b). Moreover, products of reactions catalyzed by SCD such as palmitoleate (Cao et al. 2008) and oleate (Liu et al. 2010) are mediators of inflammation. Palmitoleate is considered to be an insulin-sensitizing lipokine in animal models (Cao et al. 2008). Interestingly, SAT from the lower body (gluteo-femoral) expresses higher SCD1 and produces more palmitoleate compared to SAT from anterior abdominal wall (Pinnick et al. 2012). Since lower-body adipose tissue expansion is associated with a favorable metabolic profile, it is possible that SCD and palmitoleate are possible mediators of these beneficial effects.

Effects of SCD on Adipose Tissue Inflammation

Obesity and increased adiposity lead to a chronic low-grade inflammation in the adipose tissue (described above). This adipose tissue inflammation is causally linked to the pathogenesis of obesity-associated insulin resistance and metabolic syndrome. Since adipose SCD expression is increased in obesity, it is plausible that SCD is a link between obesity and adipose inflammation. Indeed, loss of SCD in diet-induced and genetic models of obesity protects against adipose inflammation (Liu et al. 2010). Moreover, adipocyte-conditioned medium from SCD1 knockout mice does not induce pro-inflammatory cytokine gene expression in macrophages to the same extent as the ones from wild-type mice (Liu et al. 2010). However, SCD1 knockout mice (Ntambi et al. 2002) as well as mice with knockdown of SCD1 by SCD1-specific antisense oligonucleotide inhibitors (Jiang et al. 2005) have lower body weights, adiposity, and WAT lipogenic markers compared to

wild-type counterparts. Moreover, these mouse models exhibit improved whole body insulin sensitivity. Therefore, reduced adipose inflammation in SCD1 knockout mice could be secondary to improvements in body weight and adiposity. Further, the less inflammatory nature of adipocyte-conditioned medium from SCD1 knockout mice could be due to their smaller adipocyte size.

To test a direct role of WAT SCD in modulating adipose inflammation, adipose tissue-specific SCD knockout has been employed. These mice were generated using the cre-loxp technology. Unlike the global SCD1 knockout mice, these adipose-specific SCD1 knockout mice (ASCD-KO) are not protected against high-fat diet-induced adiposity. Moreover, the ASCD-KO mice have comparable glucose tolerance to their wild-type counterparts. Taken together, this suggests that SCD expression in WAT per se does not regulate lipogenesis or adipose tissue inflammation and has a less important role in the regulation of energy balance. Interestingly, adipocytes from these ASCD-KO mice show increased GLUT1 expression with reduced adiponectin expression. Moreover, SCD inhibition in 3T3-L1 adipocytes leads to increased basal, but not insulin-stimulated glucose uptake. In contrast, global SCD1 KO mice exhibit increased GLUT4 and adiponectin expression (Hyun et al. 2010). This further supports the assertion that improvements of adipose tissue inflammation in global SCD1 knockout mice are secondary to reductions in adiposity.

Conclusion

SCD plays a pivotal role in metabolic function. In the WAT, SCD expression is regulated by dietary and hormonal factors. Hypercaloric diets, high-carbohydrate diets, and SFAs upregulate SCD expression, while PUFA and caloric restriction downregulates it. Of the hormonal factors, leptin is a potent inhibitor of SCD expression. WAT SCD upregulation in obesity appears to be an adaption to increased availability of carbohydrates and SFAs. In addition to facilitating lipogenesis via desaturation of fatty acids, WAT SCD also plays an important role in modulating metabolic functions. In this regard, palmitoleate and oleate, two products of the reaction catalyzed by this enzyme, have important metabolic functions. WAT SCD also appears to be important in modulating adipose tissue inflammation in obesity.

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Chapter 6
Function and Regulation of Macrophage Stearoyl-CoA Desaturase in Metabolic Disorders

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Introduction

Macrophages are key members of the innate immune system, which reside in most tissues of the body. The primary function of macrophages was traditionally considered to be phagocytosis and killing of foreign pathogens. More recently, a preponderance of evidence from cell to human studies suggests that macrophages play an important role in metabolic homeostasis as well (Bhargava and Lee 2012). For example, macrophages in adipose tissue and skeletal muscle regulate local inflammatory responses and insulin sensitivity, while macrophages in arterial walls are involved in atherosclerotic plaque formation. Further, macrophages in the liver, also known as Kupffer cells, are responsible for hepatic inflammation, steatosis, and insulin resistance. Therefore, macrophages play a key role in the pathogenesis of metabolic disorders such as type-2 diabetes and atherosclerosis.

Stearoyl-CoA desaturase (SCD) is a microsomal enzyme which facilitates the formation of monounsaturated fatty acids (MUFA) from saturated fatty acid (SFA) precursors (discussed in previous chapters). More specifically, SCD converts palmitoyl-CoA and stearoyl-CoA to palmitoleoyl-CoA and oleoyl-CoA, respectively (Mauvoisin and Mounier 2011). Of the two human SCD isoforms (SCD1 and SCD5), macrophages mainly express SCD1. While mice have four SCD isoforms, only SCD1 has been widely studied in macrophages, with a few studies reporting the expression of SCD2 as well (Saez et al. 2007; Sun et al. 2003).

Interestingly, macrophages isolated from subjects with type 2 diabetes have a higher linoleic acid content and fatty acid profiles consistent with increased SCD

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activity compared to control subjects (Senanayake et al. 2007). Moreover, incubation of human macrophages in serum isolated from dyslipidemic subjects leads to increased expression of SCD1 (Wong et al. 2011). Macrophages from obese animal models also express higher levels of SCD1 (Leroux et al. 2012). Therefore, it is likely that SCD plays an important role in the pathogenesis of macrophage-mediated metabolic disorders. This chapter discusses the functions of macrophages in relation to metabolic derangements, regulation of macrophage SCD expression, and effects of macrophage SCD expression in metabolic disorders.

Role of Macrophages in Metabolic Disorders

Macrophages and Obesity-Associated Insulin Resistance

Adipose tissue is considered as an endocrine organ (discussed in a separate chapter in this book), which consists of adipocytes and a stromal vascular fraction. The latter contains blood cells, endothelial cells, adipose tissue precursors, and macrophages (Kalupahana et al. 2012). Adipose tissue from a lean, normal-weight person contains macrophages which are of the M2 type. These are adipose tissue-resident macrophages (Rull et al. 2010), which have anti-inflammatory functions (Rull et al. 2010; Mosser and Edwards 2008). Adipose tissue of an obese individual is characterized by increased adipocyte (fat cell) numbers (hyperplasia) and cell size (hypertrophy). Obesity and adipocyte hypertrophy can cause lack of nutrients for cells, hypoxia, and endoplasmic reticulum stress, which can lead to adipocyte death via necrosis and apoptosis. Dead adipocytes are surrounded by macrophages, which can be identified microscopically as a “crown-like structure” (Yudkin 2007). The majority of macrophages in expanded adipose tissue are of the M1 type, which are mainly induced and recruited by proinflammatory cytokines. Expanded adipose tissue has an increased local inflammatory response. Monocyte chemoattractant protein 1 (MCP-1) is a chemokine secreted by adipocytes, macrophages, and activated endothelial cells in expanded adipose tissue. MCP-1 promotes the recruitment of monocytes into adipose tissue. Subsequent exposure of the monocytes to macrophage colony-stimulating factor causes differentiation to macrophages, which can lead to secretion of more MCP-1 (Kanda et al. 2006). M1 type macrophages in expanded adipose tissue secrete not only MCP-1 but also other proinflammatory cytokines including tumor necrosis factor α (TNFα), interleukin (IL)-6, IL-1, and IL-8, which can further amplify the local inflammatory response and decrease adipocyte insulin sensitivity (Coppack 2001; Zeyda and Stulnig 2009).

Obesity and increased fat mass are also accompanied with hepatic steatosis and inflammation. Since hypertrophic, insulin-resistant adipocytes have increased lipolytic rates, expanded adipose tissue continuously releases free fatty acids into the blood stream. These fatty acids can be taken up by the liver, skeletal muscle, and other tissues. Increased uptake of free fatty acids from adipose tissue and triglyceride-rich lipoproteins including chylomicrons and very low-density lipoproteins

(VLDLs) can increase fat accumulation in the liver, resulting in hepatic steatosis (Maher et al. 2008). Excess fat accumulation can recruit more inflammatory cells into the liver. These immune cells and Kupffer cells can secrete more inflammatory factors and amplify this inflammatory response, which can lead to hepatic insulin resistance (Huang et al. 2010; Neyrinck et al. 2009). Kupffer cells in obese rodents also express high levels of lipogenic genes such as SCD1, fatty acid synthase (FAS), and diacylglycerol acyltransferase 2 (DGAT2) (Leroux et al. 2012). As a consequence, these cells have elevated lipid content and recruit more immune cells, producing high levels of inflammatory factors.

Macrophages play an essential role in skeletal muscle repair during tissue damage and exercise (Chazaud et al. 2009). Increased blood free fatty acid concentrations can cause elevated fat content in skeletal muscle, which may recruit more immune cells including macrophages. This fat accumulation and inflammation can decrease insulin sensitivity in the muscle (Trayhurn et al. 2011).

Macrophages and Atherosclerosis

Macrophages play an important role in atherosclerotic lesion progression by facilitating cholesterol accumulation and amplifying the local inflammatory responses in blood vessel walls (Kunjathoor et al. 2002; Ludewig and Laman 2004). As early as in 1958, experimental data in rabbits suggested that the increase in white blood cells adhering to the vessel wall signaled an early stage of atherosclerotic lesion progression (Lusvarghi et al. 1958). It was likewise demonstrated that fatty streaks, the earliest detectable atherosclerotic lesions, contain macrophage-derived foam cells (MDFC) which were differentiated from blood monocytes. Monocytes adhere to the blood vessel wall by inflammatory signals such as vascular cell adhesion molecule-1 (VCAM-1), P-selectin, and E-selectin (Hope and Meredith 2003). Under normal conditions the endothelial monolayer of the vasculature resists adhesion of blood-borne monocytes. The presence of VCAM-1, P-selectin, and E-selectin increases the adhesion of monocytes to the blood vessel wall. Once adherent to the endothelial monolayer, the monocytes migrate between endothelial cells at their junction to penetrate the intimal layer (Parker et al. 2001). Modified lipoprotein particles, especially high levels of low-density lipoprotein (LDL) and low levels of high-density lipoprotein (HDL), have been shown to increase the expression of VCAM-1 (Lopez-Garcia et al. 2005; Baker et al. 1999). MCP-1, a chemoattractant cytokine (chemokine), also plays an important role in the recruitment of monocytes into the arterial intima (Sheikine and Hansson 2004).

After monocyte migration into the arterial intima, these monocytes differentiate into macrophages in response to macrophage colony-stimulating factor (Tojo et al. 1999). Macrophages express many scavenger receptors and membrane proteins, which can regulate cellular cholesterol and fat content. Macrophage scavenger receptor 1 (MSR1) and CD36 are membrane proteins involved in the uptake of cholesterol-rich modified lipoproteins (Kunjathoor et al. 2002; de Winther et al. 2000). Two important macrophage membrane proteins involved in cholesterol

efflux are ATP-binding cassette transporter A1 (ABCA1) and scavenger receptor B class 1 (SR-B1). ABCA1 promotes cholesterol and phospholipid efflux from cells to HDL, while SR-B1 mediates the selective efflux of cellular cholesterol to HDL (Vedhachalam et al. 2004; Baranova et al. 2002). Cholesteryl ester (CE) in HDL is then selectively taken up by hepatocytes and used to synthesize bile acids or excreted into the bile completing a process termed reverse cholesterol transport. When macrophage cholesterol influx is greater than cholesterol efflux, cholesterol homeostasis in the macrophages is disturbed and CE accumulates in cytoplasmic droplets. These lipid-laden macrophages are known as MDFC. Both macrophage- and smooth muscle cell-derived foam cells characterize the early atherosclerotic lesion (Guyton and Klemp 1996). This cholesterol accumulation in the vessel intima leads to the formation of a cholesterol core, characteristic of advanced atherosclerotic lesions. MDFC in the atheroma secrete several inflammatory factors and proteases involved in lesion progression. Secretion of MCP-1 and matrix metalloproteinases promotes the progression of the atherosclerotic lesion formation.

Regulation of Macrophage SCD Expression

Only a few studies have investigated the regulation of SCD1 expression in macrophages. These studies indicate that macrophage SCD1 is regulated mainly by transcription factors and polyunsaturated fatty acids (PUFA).

Several transcription factors regulate SCD1 expression in macrophages. For example, activation of liver X receptor (LXR)/retinoid X receptor (RXR) via 22(R)-OH cholesterol and 9-*cis*-retinoic acid leads to increased expression of SCD1 and SCD2 in peritoneal macrophages (Martin-Fuentes et al. 2009). CCAAT/enhancer-binding protein (CEBP) β is a transcription factor which represses SCD1 expression (Zhang et al. 2012). The peroxisome proliferator-activated receptor (PPAR) γ agonist troglitazone suppresses macrophage SCD1 expression (Wang et al. 2004). Atorvastatin, an inhibitor of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, also suppresses SCD1 expression in THP-1-derived macrophages. β-Amyloid peptide is an important mediator of Alzheimer's disease (AD), which induces inflammation in brain macrophages (microglia). One study has shown that this peptide can increase SCD1 expression in microglial cells, suggesting that it is a positive regulator of SCD1 in macrophages (Uryu et al. 2003).

Similar to its regulation in other tissues, macrophage SCD1 expression is repressed by PUFA. For example, alpha linolenic acid (ALA), an n-3 PUFA, reduces SCD1 expression in THP-1 MDFC (Zhang et al. 2011). The mechanism of ALA-induced SCD1 repression is as follows. ALA activates the nuclear receptor farnesoid-X-receptor (FXR), which increases the expression of small heterodimer partner (SHP), which in turn decreases LXR-dependent sterol regulatory element-binding protein 1c (SREBP1c) transcription, leading to repression of SCD1 expression (Zhang et al. 2012). Conjugated linoleic acid (CLA) also suppresses SCD1 in macrophages (Wang et al. 2004).

SCD and Macrophage Biology in Metabolic Disorders

Role of Macrophage SCD in the Pathogenesis of Obesity-Related Inflammation

Macrophages play a key role in the pathogenesis of adipose tissue inflammation and obesity-associated metabolic disorders such as type-2 diabetes and atherosclerosis. While obesity promotes a proinflammatory macrophage phenotype, the key molecules and the molecular signaling pathway (s) involved in this process are not clearly known. Interestingly, macrophages from subjects with type-2 diabetes exhibit higher accumulation of linoleic acid and fatty acid profiles consistent with increased SCD activity compared to control subjects (Senanayake et al. 2007). Recent studies have found that peritoneal macrophages from global C/EBPβ deletion exhibit significantly higher SCD1 gene expression and attenuated the expression of palmitate-induced inflammatory cytokines including TNFα (Rahman et al. 2012). Thus, it is reasonable to propose that forced expression of macrophage SCD1 might be protective against high-fat diet-induced macrophage lipotoxicity.

The assertion that macrophage SCD1 expression is unlikely to promote an inflammatory phenotype is also supported by findings from SCD1 knockout mice. While SCD1 deficiency in obese mouse models such as Agouti and high-fat diet-fed mice prevents inflammation in white adipose tissue and adipocytes, it does not prevent the inflammatory activation of peritoneal macrophages (Liu et al. 2010). Moreover, adipocytes isolated from SCD1 knockout adipose tissue show a blunted response to lipopolysaccharide (LPS) stimulation. However, peritoneal macrophages isolated from SCD1 knockout and wild-type (WT) mice show similar expression of inflammatory genes when challenged with LPS. These results suggest that the modulation of inflammation by SCD1 is cell type-specific. In order to assess a paracrine connection between adipocytes and macrophages, Liu et al. collected conditioned medium (CM) from WT and SCD1 knockout adipocytes and tested its effects on inflammation in RAW264.7 macrophage cells. The induction of proinflammatory genes TNFα and IL-1β was significantly lower in RAW264.7 macrophages treated with SCD1-deficient conditioned medium (CM), compared with the treatment with WT CM. These data indicate that adipocyte-derived soluble factors are the likely mediators of inflammation in macrophages and that the levels of these factors are lower in SCD1-deficient CM.

Macrophage SCD as a Mediator of Atherosclerosis

Macrophage-mediated inflammation also plays a critical role in the pathogenesis of atherosclerosis, a leading cause of cardiovascular morbidity and mortality in the world. Macrophages play a central role in atherogenesis through regulation of accumulation of cholesterol and inflammation (discussed above). As discussed earlier in

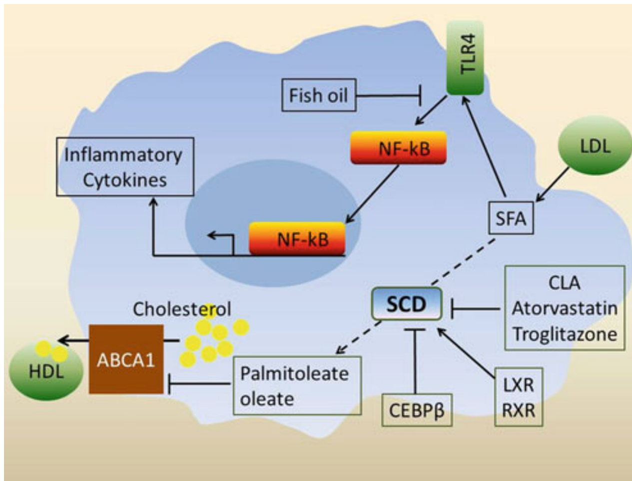

Fig. 6.1 Regulation and functions of macrophage SCD. Macrophage SCD expression is induced by activation of LXR/RXR transcription factors, while repressed by CEBPβ. SCD activity is also inhibited by PUFA, Atorvastatin, and PPARγ agonist troglitazone. SCD catalyzes MUFA formation from SFA, which in turn destabilize ABCA1 leading to reductions in cholesterol efflux. Therefore, SCD inhibition leads to increased cholesterol efflux. However, SCD inhibition leads to accumulation of SFA, which can activate TLR4 and thereby the NF-kB signaling pathway, leading to production of proinflammatory cytokines, which can aggravate atherogenesis. Fish oil can prevent this effect, probably via inhibition of the NF-kB pathway. *SCD* stearoyl-CoA desaturase, *LXR* liver X receptor, *RXR* retinoid X receptor, *CEBP* CCAAT/enhancer-binding protein, *PUFA* polyunsaturated fatty acids, *PPAR* peroxisome proliferator-activated receptor, *MUFA* monounsaturated fatty acids, *SFA* saturated fatty acids, *ABCA1* ATP-binding cassette transporter A1, *TLR4* toll-like receptor-4, *NF-kB* nuclear factor-kB

this chapter, ABCA1 is an important cholesterol efflux receptor, which promotes cholesterol and phospholipid efflux from macrophages to HDL. When macrophage cholesterol influx is greater than cholesterol efflux, cholesterol homeostasis in the macrophage is disturbed and cholesteryl ester accumulates in cytoplasmic droplets. These lipid-laden macrophages are known as MDFC, which characterize the early atherosclerotic lesion (Vedhachalam et al. 2004). Cholesterol efflux from these foam cells provides protection against the progression of the plaque.

Because of the promising role of SCD in the regulation of metabolic functions, much effort has been directed in elucidating the role of SCD in atherosclerosis. A summary is given in Fig. 6.1. Sun et al. have shown that SCD1 and SCD2 are induced in peritoneal macrophages after treatment with LXR/RXR activators, 22(R)-OH cholesterol and 9-*cis*-retinoic acid. This induction is associated with inhibition of ABCA1 (Sun et al. 2003). Other studies also report the LXR-mediated induction of SCD gene expression and subsequent reduction of ABCA1 in macrophages (Wang et al. 2004). Conversely, SCD inhibitors, CLA and troglitazone attenuate the ABCA1 destabilization induced by palmitate and stearate but not by linoleate. These results suggest that LXR/RXR ligands activate SCD, which in turn promote the production

of MUFA from SFA precursors, which destabilize ABCA1. However, there is a body of literature showing that LXR agonists increase both ABCA1 and ATP-binding cassette subfamily G member 1 (ABCG1) gene transcription and cholesterol efflux in macrophages (Larrede et al. 2009; Zhou et al. 2010). These studies, however, do not clarify whether LXR activation-mediated increase of ABCA1 and ABCG1 gene expression and therefore cholesterol efflux in macrophages is dependent on SCD expression (Beyea et al. 2007; Teupser et al. 2008). Thus, the role of SCD in LXR activation-mediated induction of ABCA1 and ABCG1 remains unclear. Further work in this area to determine the exact relationship between LXR agonism, SCD1 expression, and cholesterol efflux is certainly warranted.

Consistent with the effect of SCD inhibitors on attenuating SFA-induced ABCA1 destabilization, SCD inhibition also promotes cholesterol efflux from foam cells. For example, ALA treatment and siRNA-mediated knockdown of SCD1 significantly reduce SCD1 expression in macrophage foam cells with concomitant increases in cholesterol efflux and reductions in intracellular cholesterol storage in these cells (Zhang et al. 2012). SCD1 knockdown also reduces fatty acid content, increases ABCA1 protein levels, and increases cholesterol efflux in cultured human macrophages (Wong et al. 2011). Taken together, this suggests a negative effect of macrophage SCD expression on cholesterol efflux, thereby potentially promoting atherogenesis. Interestingly, the anti-atherogenic pharmacological agent atorvastatin is also known to suppress SCD gene expression in macrophages. In one such study, Martín-Fuentes et al. assessed the effects of atorvastatin on oxidized low-density lipoprotein (oxLDL)-loaded human macrophage cells (THP-1). They found a significant reduction in the percentage of palmitoleic and oleic acids in THP-1 cells incubated with atorvastatin, which was associated with marked reduction of SCD and sterol regulatory element-binding protein 1 (SREBP1) gene expression (Martin-Fuentes et al. 2009).

Since increased macrophage SCD expression is associated with reduced cholesterol efflux, SCD inhibition could possibly have a favorable effect on atherogenesis. Paradoxically, rodent studies have indicated otherwise. Brown et al. used antisense oligonucleotides (ASO) to inhibit SCD1 in a mouse model of hyperlipidemia and atherosclerosis (LDLr−/−Apob100/100) and showed that inhibition of SCD1 attenuated diet-induced obesity, insulin resistance, and hepatic steatosis but promoted aortic atherosclerosis, which was not reversed by dietary oleate (Brown et al. 2008). Furthermore, these mice had increased levels of SFA in plasma and tissues, reduced plasma triglycerides, and normal LDL cholesterol levels. Next, they examined whether increased SFA availability in this model affected toll-like receptor-4 (TLR4) activation. As expected, mice with SCD1 inhibition exhibited hypersensitivity to TLR4 agonists. It is possible that inhibition of SCD1 in the liver leads to secretion of VLDL particles that are enriched in SFA-rich cholesteryl esters (CE), giving rise to SFA-CE-rich LDL particles. Since the macrophages also have reduced SCD1 expression, they are unable to convert the SFA delivered to them via LDLs to MUFA, and therefore are exposed to high levels of SFA. This in turn will activate TLR4 which can further activate the nuclear factor-kB (NF-kB) pathway, leading to transcription of proinflammatory cytokines (Fig. 6.1). This proinflammatory environment promotes atherogenesis, especially in hyperlipidemic conditions.

This model is further supported by the finding that the atherosclerosis in LDLr−/−Apob100/100 mice with SCD1 knockdown can be prevented by the co-treatment with fish oil (Brown et al. 2010). We and others have previously shown that eicosapentaenoic acid (EPA), an n-3 PUFA found in fish oil, can inhibit the NF-kB pathway, possibly via activation of the G protein-coupled receptor 120 (Siriwardhana et al. 2012; Oh et al. 2010). It is plausible that fish oil attenuates the SFA-induced activation of TLR4 and subsequent activation of the NF-kB pathway and transcription of proinflammatory cytokines in the LDLr−/−Apob100/100 mice treated with SCD1 ASO, providing protection against the development of atherosclerosis.

Other studies have also shown that SCD1 deficiency promotes atherosclerosis under hypercholesterolemic conditions. For example, low-density lipoprotein receptor (LDLr)-deficient mice with SCD1 deficiency have increased atherosclerosis when fed a Western-type diet (Macdonald et al. 2009). In addition, SCD1 deletion in bone marrow cells of LDLr−/− mice also does not prevent atherosclerotic lesion formation. However, SCD1 knockdown in the chronic intermittent hypoxia mouse model, a model of obstructive sleep apnea which exhibits atherosclerosis, is protected from atherosclerosis. This discrepancy of the effects of SCD1 knockdown on atherosclerosis could indicate that under hypercholesterolemic conditions, SCD might protect against SFA-mediated inflammation (Liu et al. 2011). Taken together, this indicates that while macrophage SCD deficiency seems to promote cholesterol efflux, the proinflammatory environment induced by SFA-mediated TLR4 activation promotes atherogenesis.

Conclusion

SCD plays a pivotal role in metabolic function and exerts diverse cell-specific patterns of function and regulation. SCD is nutritionally and hormonally regulated and is altered in various metabolic and inflammatory disorders including obesity and diabetes. The role of SCD in macrophages in obesity and atherosclerosis is not fully understood and very limited studies have addressed its regulation in immune cells. Most of the studies were conducted either in RAW macrophages or in peritoneal macrophages isolated from global SCD1−/− mice. Several isoforms of SCD exist (Liu et al. 2011) and their comparative role in macrophage inflammation and lipotoxicity in response to obesity and its associated disorders have not been addressed. Future studies with isoform-specific roles of SCDs in macrophages might shed new insights into SCD's role in obesity-induced macrophage lipotoxicity and metabolic disorders.

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Chapter 7
Stearoyl-CoA Desaturase-1 in the Regulation of Toll-Like Receptor Signaling and Endoplasmic Reticulum Stress Signaling

Tabitha C. Ting and Makoto Miyazaki

SCD1 and Lipid Biosynthesis

Stearoyl-CoA desaturase 1 (SCD1) is an enzyme involved in the de novo lipogenesis of fatty acids (Fig. 7.1). In the first step of this biosynthetic pathway, acetyl-CoA carboxylase converts acetyl-CoA to malonyl-CoA. Next, malonyl-CoA is converted into the saturated fatty acid (SFA) palmitate (C16:0), through a multiple-step process catalyzed by fatty acid synthase (FAS). Elongases convert palmitate into stearate (C18:0), the SFA substrate for SCD. Following elongation, SCD catalyzes the conversion of stearate into the monounsaturated fatty acid (MUFA) oleate (C18:1). MUFAs such as oleate serve as major substrates for the synthesis of complex lipids such as phospholipids, cholesterol esters, and wax esters. Since SCD1 controls the delicate balance between SFAs and MUFAs, SCD1 plays an important role in determining the composition of fatty acids and complex lipids in the cell. An SFA to MUFA ratio that is either excessive or insufficient can lead to severe metabolic consequences, including atherosclerosis, obesity, and type II diabetes (Sampath et al. 2007). To study global and tissue-specific effects of SCD1 on these diseases, researchers have developed several models that have a deletion or decreased expression of SCD1. These include conditional knockout mouse models ($SCD1^{-/-}$) or transient knockdown mouse models using antisense oligonucleotide technology (SCD1 ASO). Inhibition of SCD1 in these models leads to an increase in SFAs such as stearate and a decrease in MUFAs such as oleate (Sampath et al. 2007). These are therefore very useful models to study the physiological role of SFAs and MUFAs.

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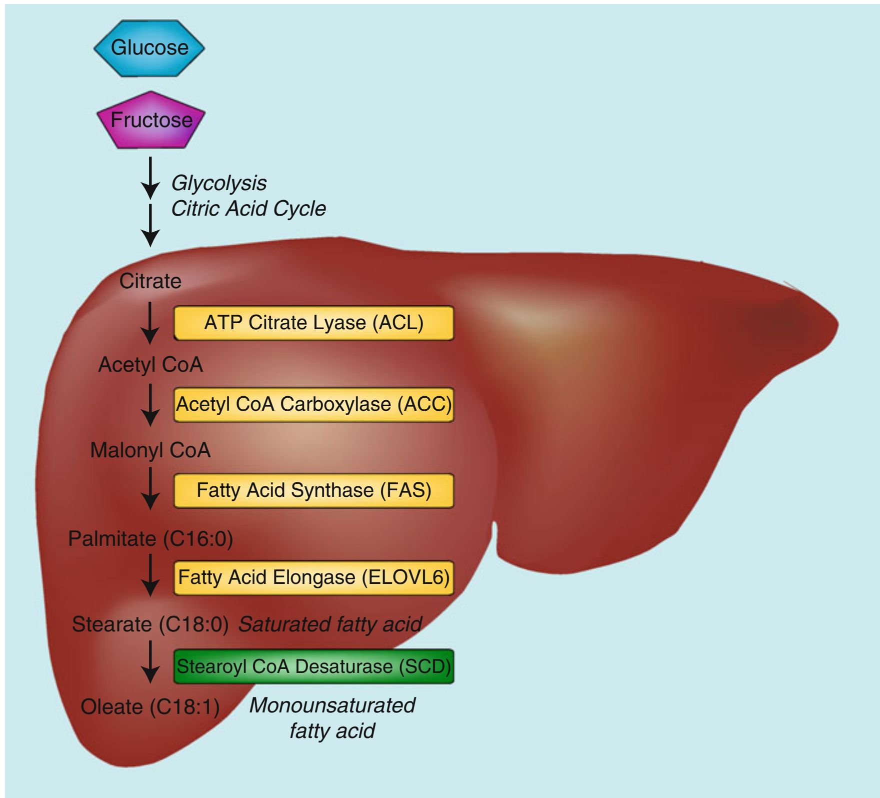

Fig. 7.1 SCD1 in de novo lipogenesis. Dietary carbohydrates such as glucose and fructose are major substrates of hepatic de novo lipogenesis. The carbohydrates are converted to citrate through glycolysis and citric acid cycle. Citrate is converted back to acetyl-CoA in the reaction of ATP citrate lyase (ACL). Acetyl-CoA carboxylase (ACC) catalyzes the irreversible conversion of the 2-carbon acetyl-CoA to the 3-carbon intermediate, malonyl-CoA. Malonyl-CoA serves as the precursor for the endogenous synthesis of fatty acids via the fatty acid synthase (FAS) multienzyme complex. FAS catalyzes seven cycles of sequential condensation, reduction, and dehydration reactions to form the 16-carbon saturated fatty acid (SFA), palmitate (16:0). Further elongation generally occurs through the actions of microsomal elongase (Elovl6) to form stearate (18:0). Cellular levels of stearate are regulated by a lipogenic enzyme, stearoyl-CoA desaturase, which catalyzes the conversion of stearate to oleate. More than 60 % of stearate derived from foods or de novo lipogenesis is converted into oleate. The reaction of stearoyl-CoA desaturase involves the introduction of the first *cis*-double bond in the Δ9 position in a spectrum of saturated fatty acyl-CoAs

SCD1 and Inflammation

Inflammation is a key event in the pathogenesis of many vascular diseases, including atherosclerosis. SCD1 may have an intriguing role in macrophage-mediated inflammation. Studies on β amyloid peptide and toll-like receptor 4 (TLR4)/NF-κB signaling demonstrate that SCD1 inhibition accelerates inflammation, whereas other studies report that SCD1 inhibition exerts no effect on inflammation (Macdonald et al. 2009a, b; Brown et al. 2008; Uryu et al. 2003; Liu et al. 2010,

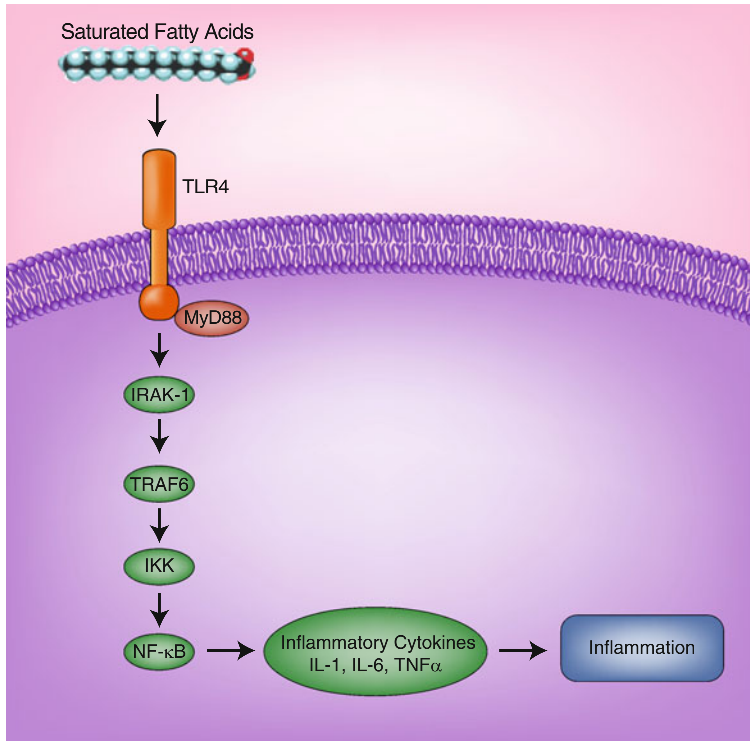

Fig. 7.2 SFAs in TLR4 signaling. SFAs directly bind and activate TLR4, resulting in inflammation. *TLR4* toll-like receptor-4, *MyD88* myeloid differentiation primary response gene-88, *IRAK-1* interleukin-1 receptor-associated kinase 1, *TRAF6* TNF receptor-associated factor-6, *IKK* IκB kinase, *NF-κB* nuclear factor kappa-light-chain-enhancer of activated B cells

2011). In studies supporting the pro-inflammatory role of fatty acids, accumulation of SFAs was shown to directly induce inflammation through TLR4 and NF-κB signaling (Lee et al. 2001, 2003a, b, 2004). In this pathway, SFAs activate TLR4, a pattern-recognition receptor that plays a role in activating innate immunity and inflammation (Fig. 7.2). TLR4 then induces NF-κB, a protein complex involved in cellular responses to harmful stimuli such as stress, free radicals, and toxic lipids. NF-κB activation increases the transcription of most enzymes in the de novo synthesis of ceramides, which are sphingosines covalently linked to a fatty acid. De novo ceramide synthesis uses SFAs, but not unsaturated fatty acids. Therefore, an increase in SFAs due to SCD1 inhibition leads to ceramide accumulation. Ceramides potently inhibit Akt, a serine/threonine kinase that upregulates nutrient storage and inhibits apoptosis. Increased ceramide and the resulting Akt inhibition lead to the activation of pro-apoptotic pathways through enzymes such as caspase-9 (Holland et al. 2007; Summers 2006). This pathway seems to be differentially activated in various tissues, since it has been shown that stearate activates inflammatory genes in macrophages but not in adipose tissue. In addition to TLR4, it has also been shown that SFAs induce macrophage inflammation through TLR2 when the receptor is dimerized

with TLR6 or TLR1. In patients with atherosclerosis, TLR4 signaling is increased particularly in endothelial cells and macrophages, suggesting that the TLR4-NF-κB pathway plays an important role in atherosclerotic lesions.

In addition to the TLR4 studies using diets high in SFAs mentioned above, other studies actually inhibited SCD1 itself to see the effects of the resulting SFA accumulation on inflammation (Brown et al. 2008; Flowers et al. 2008). One of these early studies examining the β amyloid peptide suggested that SCD1 inhibition promotes inflammation in macrophages (Uryu et al. 2003). Using an oligonucleotide microarray analysis, SCD1 was found to be specifically and significantly upregulated by β amyloid peptide (Aβ) during Aβ-induced macrophage activation. However, this study did not propose a mechanism explaining how SCD1 expression was correlated with macrophage inflammation (Uryu et al. 2003).

The pro-inflammatory effects of SCD1 inhibition were also confirmed in related studies on toll-like receptor signaling (Brown et al. 2008). Using ASO-mediated knockdown of SCD1, a study reported that SCD1-knockdown mice demonstrated increases in SFA-enriched plasma lipoproteins and TLR4 hypersensitivity. SCD1 inhibition leads to an accumulation of SFAs, which serve as ligands for TLR4 and consequently mediate atherosclerotic progression by activating TLR4-driven pro-inflammatory responses in macrophages. In addition to activating the innate immune response, transmembrane receptor TLR4 is highly expressed in macrophages present in atherosclerotic plaques and plays an important role in vascular endothelial cell activation, inflammatory cytokine recruitment, and macrophage apoptosis (Brown et al. 2008). In another study, SCD1 inhibition similarly leads to TLR4 activation and increased atherosclerosis, but these effects appear to be reversible by dietary supplementation of ω-3 polyunsaturated fatty acids (PUFAs) from fish oil. Using a hyperlipidemic mouse model, a combination of SCD1 ASO and fish oil treatments decreased both metabolic syndrome and atherosclerosis (Brown et al. 2010).

Although the mechanisms by which SFAs induce TLR4 have yet to be elucidated, one putative explanation is that SFAs change lipid and protein composition of microdomains on raft membranes. Changes in membrane composition will affect TLR signaling since activated TLR4 is thought to translocate to these domains. SFAs will allow for easier TLR4 trafficking to domains, while PUFAs will disturb lipid composition and raft order thus interfering with TLR4 recruitment to rafts. As seen in the study using SCD1 ASO and fish oil-derived ω-3 PUFAs, dual therapy of SCD1 inhibitors and other anti-inflammatory agents may be effective in reducing both metabolic syndrome and atherosclerosis (Brown et al. 2010). Although TLR4 antagonists appear to be a putative treatment for atherosclerotic progression in mice models, the role of TLR4 in human atherosclerosis has been debated. Therefore, further studies will be required to determine whether TLR4 activation is necessary for SFA-induced atherosclerosis and whether these results can be applied clinically.

In contrast to the Aβ and TLR4 studies, others reported that SCD1 inhibition exerts no effect on inflammation. A recent study compared peritoneal macrophages from SCD1-deficient mice and wild-type mice but reported no differences between groups when the macrophages were treated with LPS (Liu et al. 2010). In a similar study using peritoneal macrophages from SCD1-deficient mice, macrophage

inflammation also did not differ from the control, although inflammatory changes in the skin and plasma were observed (MacDonald et al. 2009b). One plausible explanation for this discrepancy may be due to the presence of more than one SCD isoform. SCD2 is more highly expressed in macrophages than SCD1 and therefore might be compensating for the low expression of SCD1 in macrophages. The expression of SCD1 and SCD2 in different cell types may be responsible for differences in inflammatory responses (Liu et al. 2010, 2011; Liu and Ntambi 2009). Although it is well-known that SCD1 deficiency exerts harmful pro-inflammatory effects in several cell types including skin and β-cells (Zheng et al. 1999; Flowers et al. 2007), the role of SCD1 in macrophage inflammation is still ambiguous. Further investigation will be necessary to determine the complex regulation of SCD1 in macrophage inflammation.

SCD and ER Stress

A second method by which SCD1 inhibition and SFAs promote lipid-related diseases is by inducing stress in the endoplasmic reticulum (ER), a central organelle for protein processing and lipid synthesis (Fig. 7.3). ER stress is an accumulation of misfolded or unfolded proteins in the endoplasmic reticulum due to adverse conditions such as ischemia, hypoxia, heat shock, oxidative stress, or depletion of stored ER calcium. ER stress triggers a restorative/corrective pathway known as the unfolded protein response (UPR), which attempts to restore proper ER function. The UPR alleviates ER stress through three main compensatory mechanisms: (1) decreasing the load of proteins that enter the ER by suppressing translation, (2) raising protein-folding capacity by increasing the number of available chaperones, or (3) increasing degradation of misfolded proteins through a ubiquitin-proteasome pathway. If all three compensatory mechanisms fail and homeostasis cannot be reestablished, prolonged ER stress and UPR activation can trigger apoptosis. The UPR comprises three branches mediated by ER-resident transmembrane proteins: PKR-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor-6 (ATF6). These three ER-resident signaling proteins are activated either by binding to a protein chaperone called BiP or by directly sensing the presence of misfolded proteins in the ER lumen. Following activation, these ER-resident proteins induce downstream effectors such as XBP-1 (X-box binding protein 1) and CHOP (C/EBP-homologous protein) (Flowers et al. 2007; Tabas and Ron 2011; Walter and Ron 2011).

Recent studies have shown that abnormal lipid metabolism activates ER stress and prolonged UPR in atherosclerotic plaques. In normal, healthy cells, macrophages transport lipoprotein-cholesterol to the ER, where the cholesterol is esterified (Scull and Tabas 2011). However, in atherosclerotic lesions, this process is interrupted and massive amounts of free cholesterol accumulate. SCD can regulate intracellular free cholesterol levels, since its product oleate is a preferred substrate for cholesteryl ester synthesis. $SCD1^{-/-}$ mice have higher levels of free cholesterol in the skin, liver, and aorta compared to wild-type mice. In addition to this increase in free

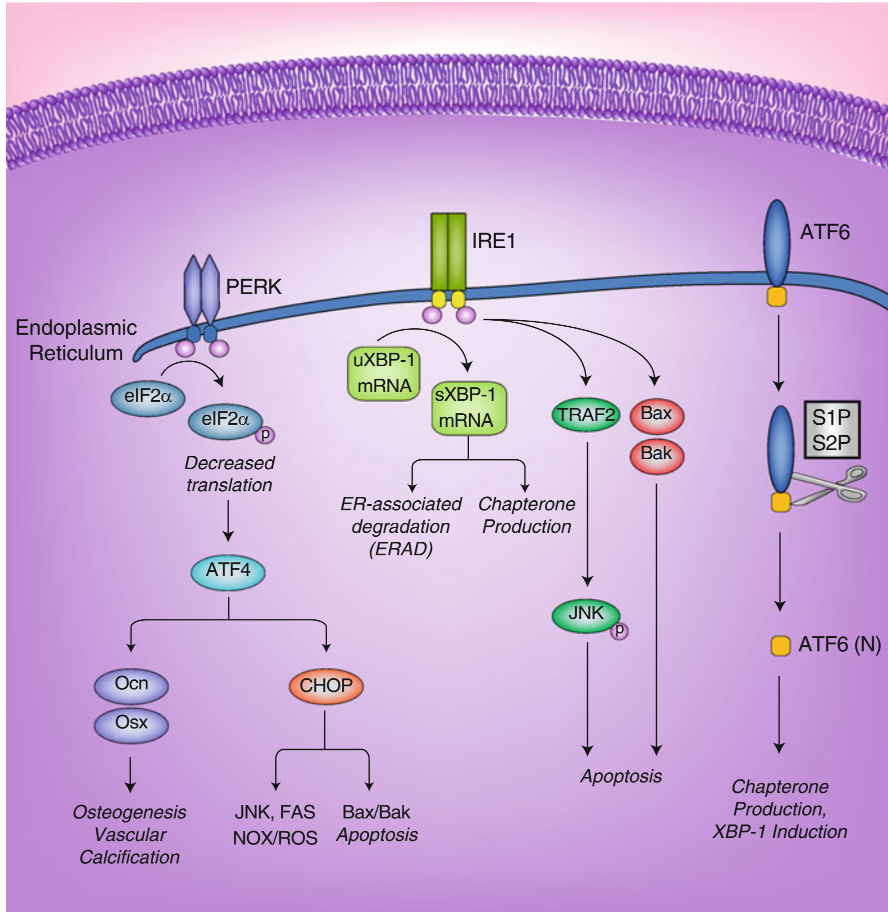

Fig. 7.3 ER stress signaling. Upon accumulation of unfolded proteins and lipids such as SFAs in the ER, three ER stress sensors are activated and initiate signal transduction events that control cell survival or death. *PERK* PKR-like ER kinase, *IRE1* inositol requiring enzyme 1, *ATF6* activating transcription factor-6, *eIF2α* eukaryotic translation initiation factor 2α, *ATF4* activating transcription factor-4, *CHOP* C/EBP-homologous protein, *Ocn* Osteocalcin, *Osx* Osterix, *uXBP-1* unspliced X-box binding protein 1, *sXBP-1* spliced X-box binding protein 1, *TRAF2* TNF receptor-associated factor 2, *JNK* Jun N-terminal kinase, *Bax* Bcl-2-associated X protein, *Bak* Bcl-2 homologous antagonist/killer, *S1P* site-1 protease, *S2P* site-2 protease

cholesterol, lesion exposure to SFAs such as stearic acid induces ER stress, leading to macrophage apoptosis in atherosclerotic plaques (Anderson et al. 2012). Several ER stress transducers and their downstream effectors have been implicated in the development of atherosclerosis. However, better understanding of the molecular mechanisms behind this induction is required. Expanding knowledge of how lipids induce ER stress and atherosclerosis may lead to better treatment of atherosclerosis and associated vascular calcification.

The PERK branch of the UPR is activated by SCD1 inhibition and the resulting accumulation of stearate (Masuda et al. 2012). In this UPR branch, PERK oligomerizes and phosphorylates itself before phosphorylating the α subunit of eukaryotic initiation factor 2 (eIF2). Phosphorylation of this α subunit renders eIF2α inactive, resulting in the inhibition of 80S ribosomal assembly and translation as well as a reduction in the protein load entering the ER. Although most protein translation is reduced when eIF2α is phosphorylated, translation of activating transcription factor-4 (ATF4) increases because ATF4 possesses upstream open reading frames that are bypassed only when eIF2α is phosphorylated. ATF4 activates its targets including the pro-apoptotic CHOP and proteins involved in osteoblast differentiation such as Osteocalcin (Ocn) and Osterix (Osx) (Masuda et al. 2012). During prolonged ER stress, CHOP induces apoptosis through BH3-only proteins, Bcl-2-associated X protein (Bax), and Bcl-2 homologous antagonist/killer (Bak). Using in vitro models, our group has previously shown that SCD1 inhibition increases stearate, leading to increased ATF4 and CHOP expression through the PERK-eIF2α pathway. We found that stearate most potently induces vascular calcification in preference to other fatty acids. ATF4 knockdown inhibits stearate-induced vascular calcification. We therefore concluded that UPR activation of the PERK-eIF2α-ATF4 branch contributes to stearate-mediated vascular calcification (Masuda et al. 2012).

A second branch of the UPR, the IRE1 branch, has also been shown to be activated by SFA accumulation (Wei et al. 2006). In this branch, IRE1 cleaves XBP-1, which then activates the transcription of UPR genes for chaperones, lipid synthesis, and ER-associated degradation of misfolded proteins. IRE1 can also recruit TRAF2, leading to the activation of caspase-12 and Jun N-terminal kinase (JNK). Apoptosis can then be activated either by the TRAF2-JNK pathway or by pro-apoptotic proteins Bax and Bak, which are targets of IRE1 as well as CHOP (Choi et al. 2011). Our study showed that SCD1 inhibition induces the expression of other ER stress targets including spliced XBP-1 in vascular smooth muscle cells (Masuda et al. 2012). In studies on β cells, SFAs such as palmitate were shown to induce the IRE1 pathway, leading to an increase in the IRE1-dependent JNK response (Ron and Walter 2007). Other studies have also confirmed that both stearate and palmitate can induce the IRE1 branch of the UPR, leading to an increase in pro-apoptotic proteins such as caspase-3 (Wei et al. 2006). These changes were accompanied by increases in other UPR-related proteins including ATF4, CHOP, chaperone GRP78, and growth arrest and DNA damage-inducible protein (GADD34). Although β-cell ceramide synthesis has been correlated with fatty acid-induced apoptosis, this study showed that UPR activation by stearate and palmitate could occur independently of de novo ceramide synthesis. Taken together, these studies demonstrate that another UPR branch, the IRE1 pathway, can also mediate the effects of SFAs on apoptosis.

PERK and IRE1 branches of the UPR have been found to link lipotoxic signals to atherosclerosis and other diseases (Scull and Tabas 2011; Erbay et al. 2009). ER stress signaling is significantly activated in animal models of atherosclerosis and other lipid-mediated disorders and human atherosclerotic lesions (Myoishi et al. 2007; Duan et al. 2009). CHOP deficiency decreases atherosclerotic plaque lesions, cell death lesions, and vascular remodeling in hyperlipidemic ApoE$^{-/-}$ and

LDLR$^{-/-}$ mice (Thorp et al. 2009; Gao et al. 2011). Chemical chaperones such as 4-phenyl butyrate and taurine-conjugated chenodeoxycholic acid were shown to reduce atherosclerotic lesion area, accompanied with reduced ER stress (Erbay et al. 2009). In a study examining macrophage ER stress, toxic lipids such as oxidized LDL and palmitate (C16:0) induced ER stress through a macrophage lipid chaperone called fatty acid-binding protein-4 (aP2). Their results suggested that toxic lipids accumulate and upregulate aP2, which then inhibits de novo lipogenesis and leads to activation of UPR-mediated apoptosis (Erbay et al. 2009). Other studies have observed the induction of ER chaperone GRP78, PERK, and CHOP, following lipid accumulation in mouse and rat models on high fat diets (Brookheart et al. 2009). In rat models of nonalcoholic fatty liver disease, high SFA diets and prolonged ER stress have also been shown to activate caspase-3, a crucial death protease that mediates apoptosis. Collectively, these studies suggest that dyslipidemia plays a pivotal role in inducing ER stress and apoptosis. Although links between dyslipidemia and ER stress have been found, mechanisms by which SFAs induce ER stress have not been fully elucidated. Several studies have proposed potential mechanisms. In a study using SCD1$^{-/-}$ mice on very low fat diets, SCD1 loss led to increases in spliced XBP-1, CHOP, and ATF3 (another ER stress-induced transcription factor) in the liver. In this study, Flowers et al. postulated that SCD1 inhibition activates ER stress by altering hepatic fatty acid composition of cellular membranes, leading to impaired function of membrane transport proteins (Flowers et al. 2008). Another explanation attributes the effects of SFAs on ER stress to unsaturated fatty acid depletion, which may contribute to oxidative stress (Flowers et al. 2008). Our studies have shown that the SFA stearate must be converted to its CoA conjugated form (stearoyl-CoA) in order to activate ER stress and vascular calcification (Masuda et al. 2012). We hypothesize that stearoyl-CoA is incorporated into an ER membrane lipid. This altered membrane composition is then sensed by ER stress transducers, which activate the UPR. Although current evidence links SCD1 deficiency to ER stress, the necessity for defining mechanisms behind ER stress induction warrants further investigation.

In addition to SFAs themselves, downstream products of fatty acids such as phospholipids have been shown to regulate ER stress. For example, previous studies have demonstrated that phospholipids play a role in activating ER stress (Testerink et al. 2009; Van Der Sanden et al. 2003; Fu et al. 2011). The two most abundant phospholipids in the ER membrane are phosphatidylcholine (PC) and phosphatidylethanolamine (PE). When lipogenesis or dietary lipid intake is increased, PC is the most common phospholipid component for packaging and storing lipid droplets and lipoproteins. In addition, SCD inhibition changes intracellular PC and PE levels (Dobrzyn et al. 2005). PC synthesis is catalyzed by choline-phosphate cytidylyltransferase A (PCTY1a), while PC to PE conversion is regulated by phosphatidylethanolamine N-methyl transferase (PEMT) (Fu et al. 2011). In ER samples isolated from the obese liver tissue of leptin-deficient mice, the PC/PE ratio was higher than

in the control. Since the PC/PE ratio in lean controls was the same as that found in their diet, the increased PC/PE ratio in obese hepatic ER seemed to result from de novo lipogenesis rather than from dietary sources (Fu et al. 2011). Similar to free cholesterol, higher PC levels in ER membranes were shown to inhibit the activity of sarco/endoplasmic reticulum calcium ATPase (SERCA), a transport protein that maintains calcium homeostasis. Altered calcium levels caused by SERCA dysfunction impaired protein-folding chaperones such as BiP and calnexin, and thus activate ER stress. This upregulation of ER stress was evidenced by IRE1α and eIF2α phosphorylation, accompanied by increased expression of ER chaperones GRP78 and GRP94. Conversely, a decreased PC/PE ratio caused by PEMT suppression also led to IRE1α and eIF2α phosphorylation and the induction of CHOP, homocysteine-inducible ER stress-inducible protein (HERP), and Der1-like domain family member 2 (DERL2). Other studies have also implicated the role of PC in the activation of ER stress. The active spliced form of an IRE1 target, XBP-1, was found to be associated with ER expansion and increased PC synthesis in fibroblasts in vitro. Collectively, these studies seem to indicate that an overabundance of PC is integral to the development of ER stress (Testerink et al. 2009; Van der Sanden et al. 2003). These studies suggest that an alteration of PC/PE ratio in the ER membrane contributes to ER stress mediated by SCD inhibition and SFA overload.

Conclusion

In this chapter, we have discussed specific pathways by which SCD1 and its substrates, SFAs, contribute to inflammation and apoptosis (Fig. 7.4). The SCD1 activity is tightly regulated mostly at the transcription level. However, once this tight regulation is interrupted, the induction of SCD1 expression increases levels of oleate, which is a preferred substrate for triglyceride and cholesteryl ester synthesis. Conversely, substantial reduction of SCD1 increases levels of intracellular SFAs such as stearate and palmitate, leading to TLR4 and ER stress activation. ER stress is induced by abnormal ratios of lipid species such as saturated/unsaturated fatty acids, free/esterified cholesterol, and PC/PE, leading to apoptosis frequently found in atherosclerotic lesions. These ratios can be regulated through the modulation of SCD1 activity. Thus, composition and ratio of lipid species contributing to SFA-mediated ER stress remain to be determined through further investigation. Turning to the broader perspective of disease pathology, we see that the development of atherosclerosis is characterized by all of these factors: abnormal lipid accumulation, TLR4-mediated inflammation, and ER stress-mediated apoptosis. These factors clearly suggest that SCD1, its SFA substrates, and its MUFA products play a multifaceted role in the development of vascular diseases such as atherosclerosis and vascular calcification.

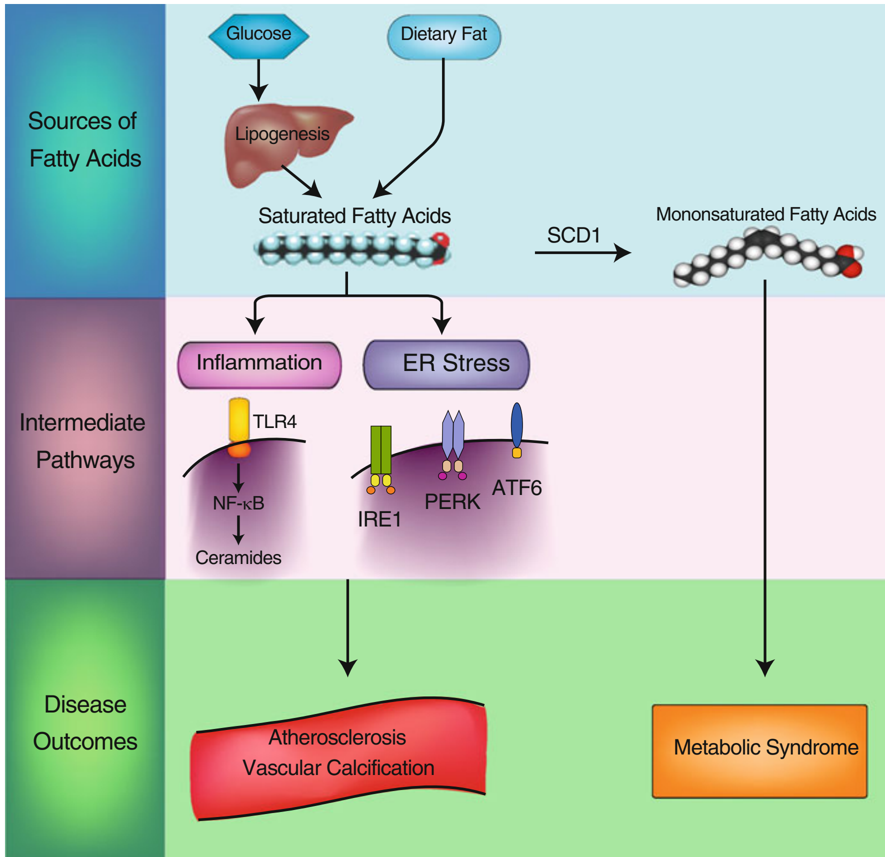

Fig. 7.4 SCD1 in inflammation and ER stress. Loss of SCD1 and the resulting accumulation of SFAs activate TLR4 and ER stress signaling, resulting in inflammation and cell death leading to vascular diseases such as atherosclerosis and vascular calcification. *TLR4* toll-like receptor-4, *NF-κB* nuclear factor kappa-light-chain-enhancer of activated B cells, *PERK* PKR-like ER kinase, *IRE1* inositol requiring enzyme 1, *ATF6* activating transcription factor-6

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Chapter 8
Stearoyl-CoA Desaturase in the Control of Heart Metabolism

Pawel Dobrzyn and Agnieszka Dobrzyn

Introduction

Stearoyl-CoA desaturase (SCD) is a rate-limiting enzyme that catalyzes the synthesis of monounsaturated fatty acids, mainly oleate and palmitoleate, from saturated fatty acyl-CoAs. The preferred substrates for SCD are palmitoyl- and stearoyl-CoAs (Dobrzyn and Ntambi 2004). Four isoforms of SCD have been identified in the mouse (SCD1-4) (Kaestner et al. 1989; Zheng et al. 2001; Miyazaki et al. 2003) and two (SCD-1 and SCD-5) in the human genome (Zhang et al. 1999; Beiraghi et al. 2003). Human SCD1 shows 85 % homology to murine SCD1 (Zhang et al. 1999). In an adult mouse, under normal dietary conditions, SCD1 mRNA is highly expressed in white adipose tissue, brown adipose tissue, meibomian glands, Harderian and preputial glands (Miyazaki et al. 2001). The mRNA is dramatically induced in the liver in response to a high-carbohydrate diet (Ntambi and Miyazaki 2004). SCD2 is expressed predominantly in the brain (Kaestner et al. 1989) but also, to a lesser extent, in the kidney, spleen, skeletal muscles, heart, and lungs, where its expression is induced in response to a high-carbohydrate diet (Kaestner et al. 1989; Zheng et al. 2001). SCD3 is expressed mainly in the differentiated sebocytes (Zheng et al. 2001), and SCD4 is expressed exclusively in the heart (Miyazaki et al. 2003). The physiological role of each SCD isoform and the reason for having multiple SCD gene isoforms that share considerable sequence homology and catalyze the same biochemical reaction are still under investigation.

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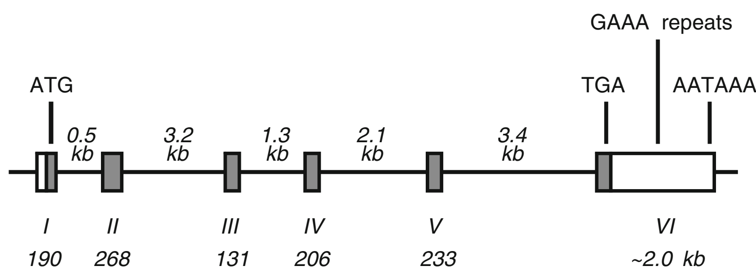

Fig. 8.1 Structure of mouse SCD4 gene. SCD4 is expressed exclusively in the heart. Adopted from Miyazaki et al. (2003)

In the heart, three SCD isoforms (SCD1, SCD2, and SCD4) are expressed. The expression of SCD1 is several times higher in the murine heart compared with that of SCD4 (Miyazaki et al. 2003; Dobrzyn et al. 2010a). However, SCD4 expression is significantly increased after a high-fat diet (Miyazaki et al. 2003), in obese leptin-deficient mice (Miyazaki et al. 2003; Dobrzyn et al. 2010a), and in the state of SCD1 deficiency (Miyazaki et al. 2003; Dobrzyn et al. 2008b). The cardiac SCD4 cDNA encodes a 353-amino acid residue protein with four transmembrane regions that is more than 80 % identical to the other three mouse SCD genes (Miyazaki et al. 2003). SCD4 also contains three conserved histidine-rich motifs that are present in the other SCD isoforms and essential for Δ9-desaturase function (Ntambi et al. 1988; Kaestner et al. 1989; Zheng et al. 2001). This enzyme desaturates both stearate and palmitate to the corresponding monounsaturated fatty acids (Miyazaki et al. 2003). SCD4 maps to mouse chromosome 19 D2, where three other SCD genes are located within a 200-kb region. SCD4 lies between the SCD1 and SCD2 genes, indicating that this region is composed of a cluster of Δ9-desaturases. The 3.1-kb transcript is the predominant species; thus, the SCD4 mRNA is smaller than the 4.9 kb of the other SCD isoforms because of a shorter 3′-untranslated region (Miyazaki et al. 2003) (Fig. 8.1). While in the liver, SCD1 is a major target gene of leptin, in the heart, leptin represses the expression of SCD4 but not that of SCD1 or SCD2 (Miyazaki et al. 2003). It is important to note that the expression of cardiac SCD4 is induced by a high-carbohydrate diet and LXRα agonists; however, unlike SCD1, it is not repressed by polyunsaturated fatty acids (PUFA) (Miyazaki et al. 2003).

The heart has a limited capacity for lipogenesis and de novo lipid synthesis. Why, then, is cardiac SCD such a highly expressed and regulated enzyme? Many studies underline the important role of lipogenic enzymes in proper heart function and suggest that the role of lipogenic genes in the heart may be distinct from other tissues. Cardiac sterol regulatory element-binding protein 1 was shown to activate the G-protein coupled inwardly reflecting K^+ channel, leading to enhanced acetylcholine-sensitive K^+ currents and reduced arrhythmias post-myocardial infraction (Park et al. 2008). It has also been shown that (1) the heart-specific knockdown of peroxisome proliferator-activated receptors γ (PPARγ) (Duan et al. 2005) and acyl-CoA synthase 1 (Ellis et al. 2011) induces cardiac hypertrophy; (2) transgenic overexpression of fatty acid transport protein 1 in the heart causes lipotoxic cardiomyopathy

(Chiu et al. 2005) and (3) fatty acid synthase (FAS), the enzyme that catalyzes de novo fatty acids (FA), is involved in the regulation of the ability of the heart to respond to stress through the activation of Ca^{2+}/calmodulin-dependent protein kinase II (Razani et al. 2011). Studies on mouse strains that have a mutation in the SCD1 gene provided evidence that SCD also plays an important role in the regulation of cardiac metabolism and function (Dobrzyn et al. 2008b, 2010a, b; Paton and Ntambi 2009). The lack of SCD1 expression decreases FA uptake and oxidation and increases glucose transport and oxidation in the heart (Dobrzyn et al. 2008b). Disruption of the SCD1 gene improves cardiac function in obese leptin-deficient ob/ob mice by correcting the systolic and diastolic dysfunction (Dobrzyn et al. 2010a). The improvement is associated with the reduced expression of the genes involved in FA transport and lipid synthesis within the heart along with decreased cardiac free fatty acid (FFA), diacylglycerol, trigliceride (TG), and ceramide levels, and reduced cardiomyocyte apoptosis (Dobrzyn et al. 2010a). Here, we review recent advances made in understanding the role of SCD in the control of heart metabolism and its involvement in the pathogenesis of lipotoxic cardiomyopathy.

Role of SCD in the Regulation of Cardiac Substrate Utilization

The heart has a major energy requirement based on its large work output. Although the cardiomyocytes are capable of using carbohydrates and ketone bodies as energy sources, FA are considered the preferred fuel. Under aerobic conditions, the heart derives 60–90 % of the energy necessary for contractile function from FA oxidation, while the rest is obtained mainly from carbohydrates (glucose and lactate) (Sambandam and Lopaschuk 2003; Stanley et al. 2005). There is evidence to suggest that impaired substrate metabolism contributes to contractile dysfunction and to the progressive left ventricular remodeling that is characteristic of heart failure. In disease states such as ischemia-reperfusion, diabetes, or obesity, cardiac substrate utilization is shifted into an excessive use of FA in place of glucose (Sambandam and Lopaschuk 2003; Chaitman et al. 2004; Stanley et al. 2005). It has been suggested that this shift in metabolism plays a role in the development of cardiomyopathy, which leads to both impaired contractile function and ischemic injury (Stanley et al. 2005; Lopaschuk et al. 2010). Thus, the regulation of substrate metabolism in the heart plays a fundamental role in the control of normal cardiac function.

One of the key control points of FA β-oxidation is the rate of FA transfer into the mitochondria through carnitine palmitoyltransferase 1 (CPT1) (Awan and Saggerson 1993). CPT1 activity and the rate of FA β-oxidation are increased in liver, skeletal muscle, and brown adiposse tissue of SCD1−/− compared with SCD1+/+ mice (Dobrzyn et al. 2004, 2005; Lee et al. 2004a). Interestingly, however, both CPT1 mRNA and protein levels as well as its activity are decreased in the heart of SCD1−/− mice (Fig. 8.2). As a result of lower FA availability and uptake and decreased rate of FA transport into mitochondria by CPT1, the rate of mitochondrial FA oxidation is decreased in the heart of SCD1−/− animals (Fig. 8.2). Remarkably, there was no

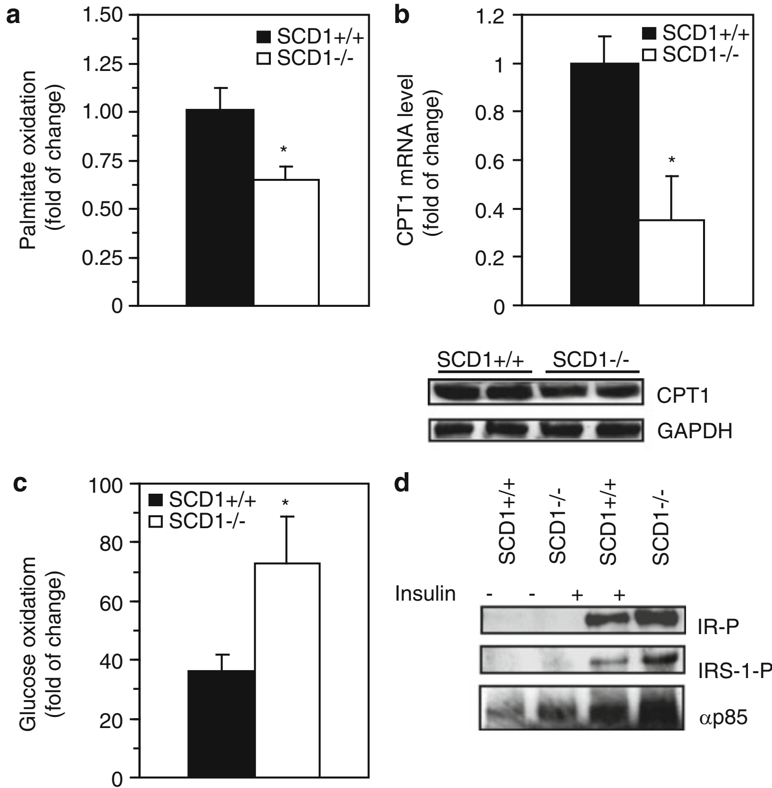

Fig. 8.2 The effect of SCD1 deficiency on the rate of fatty acid and glucose oxidation in the heart. (**a**) The rate of palmitoyl-CoA oxidation in SCD1+/+ and SCD1−/− mice. (**b**) CPT1 mRNA and protein levels. (**c**) The rate of glucose oxidation. (**d**) IR and IRS-1 phosphorylation, and association of IRS-1 with the αp85 subunit of PI3-kinase in basal condition and after insulin administration. *$P<0.05$ vs. SCD1+/+ mice. Adopted from Dobrzyn et al. (2008b)

significant difference in SCD enzyme activity in either group of mice (Dobrzyn et al. 2008b). Furthermore, there were no significant differences in levels of palmitoleic acid, a product of de novo desaturation by SCD, in cardiac lipids between SCD1+/+ and SCD1−/− mice. These results, examined together with increased SCD4 mRNA level in SCD1−/− mice, indicate that the SCD4 compensates for the lack of SCD1 in the heart (Miyazaki et al. 2003; Dobrzyn et al. 2008b). It is possible that this relative lack of a change in cardiac Δ9-desaturase activity may be accountable for the tissue-specific differences in fat oxidation observed in the heart, as opposed to other SCD1 deficient tissues, such as skeletal muscle, liver, or brown adiposse tissue. However, the potential role of SCD4 in the regulation of FA oxidation requires further studies.

The utilization of FA and glucose is tightly coupled in the myocardium (Sambandam and Lopaschuk 2003; Stanley et al. 2005). When FA are unavailable

as a source of ATP, the heart extends its use of carbohydrates as an energy supply. This effect (i.e., increased glucose oxidation) was observed in the heart of SCD1−/− mice to compensate for ATP supply (Fig. 8.2). It was accompanied by increased tyrosine phosphorylation of the insulin receptor (IR) and IR substrate (IRS)-1 and greater IRS association with the αp85 subunit of PI 3-kinase in the heart (Fig. 8.2), despite lower levels of plasma insulin (Dobrzyn et al. 2008b). The increased insulin signaling is responsible for enhanced glucose uptake in the heart of SCD1−/− mice. A markedly increased insulin-stimulated glucose flux in the heart of SCD1−/− mice was also observed by Flowers et al. (2007) in studies using a hyperinsulinemic-euglycemic clamp. The decrease in fat oxidation coupled with increased insulin sensitivity leads to a shift in substrate utilization from FA to glucose in the SCD1-deficient heart (Dobrzyn et al. 2008b). The important role of SCD1 in regulation of cardiac myocyte substrate utilization was also shown in the studies of neonatal cardiomyocytes, where SCD1 overexpression attenuated palmitic acid oxidation and restored palmitate-induced suppression of glucose oxidation in the heart (Matsui et al. 2012).

AMP-activated protein kinase (AMPK) is an important factor that regulates metabolic pathways such as FA oxidation, glucose transporter translocation, glucose uptake, and glycolysis (Towler and Hardie 2007). Thus, activation of AMPK in the heart can potentially increase both fat and glucose metabolism (Coven et al. 2003; Xing et al. 2003; Stanley et al. 2005). SCD1 deficiency is known to activate AMPK in liver and skeletal muscle (Dobrzyn et al. 2004, 2005). However, in the heart, AMPK phosphorylation and protein levels were not affected by SCD1 deficiency, indicating that AMPK is unlikely to play a role in the shift in substrate oxidation in the myocardium of SCD1−/− mice (Dobrzyn et al. 2008b).

The transcription factor PPARα is highly expressed in tissues with a high capacity for FA oxidation, including hepatocytes, cardiomyocytes, the renal cortex, and skeletal muscles. Its activation promotes FA oxidation, ketone body synthesis, and glucose sparing (Ferre 2004). Cardiac-specific overexpression of PPARα has been shown to cause insulin resistance and increased FA oxidation in the heart (Park et al. 2005), whereas ablation of fatty acid translocase/CD36 (FAT/CD36) in the context of PPARα cardiac overexpression largely reverses these effects and promotes glucose uptake and oxidation in the heart (Yang et al. 2007). These studies underscore the role of PPARα in regulating cardiac substrate utilization. The expression of PPARα is decreased significantly in the myocardium of SCD1−/− mice. Therefore, it is possible that the decreased expression of genes encoding proteins involved in FA oxidation such as CPT1 and acyl-CoA oxidase is mediated by decreased PPARα activity in the heart of SCD1−/− mice (Dobrzyn et al. 2008b). PGC1α and nonesterified PUFA are two main regulators of PPARα activity (Sampath and Ntambi 2005; Finck and Kelly 2006). Although cardiac PGC1α expression is not affected by SCD1 deficiency, the intracellular PUFA contents are reduced by 30 % in the heart of SCD1−/− mice, suggesting a viable mechanism for the decreased PPARα activity observed in SCD1-deficient hearts (Dobrzyn et al. 2008b).

SCD1 and Cardiac Function

A shift in cardiac substrate utilization may lead to cardiac structural and/or functional abnormalities (Stanley et al. 2005). Transthoracic echocardiography with Doppler flow analysis was used to assess the structure and function of an SCD1−/− heart (Dobrzyn et al. 2008b). Left ventricle (LV) weights normalized to body weight measured by both wet weights obtained at necropsy and echocardiography were ~15 % higher in SCD1−/− compared with SCD1+/+ mice (Table 8.1). The LV diameter in the heart of SCD1−/− mice was significantly larger; however, the LV posterior and anterior wall thicknesses were similar in SCD1-deficient and wild-type hearts. The structural changes in the heart of SCD1−/− mice were not accompanied by functional abnormalities. The heart rate (beats/min) measured during echocardiography was similar in SCD1−/− and SCD1+/+ mice. LV fractional shortening, which is used as a measure of systolic function, and the isovolumetric relaxation time, which is used as a measure of diastolic function, were not significantly affected by SCD1 deficiency (Table 8.1). Also, the myocardial performance index, a Doppler-based measure of left ventricular function and the velocity of blood flow across the mitral valve in early diastole were unchanged in SCD1−/− compared with SCD1+/+ mice (Dobrzyn et al. 2008b).

Ectopic deposition of lipids in the myocardium may lead to functional impairments as observed in obese leptin-resistant Zucker diabetic rats (ZDF) (Zhou et al. 2000) and db/db mice as well as leptin-deficient ob/ob mice (Barouch et al. 2006). Ob/ob mice develop pathologic LV hypertrophy along with elevated TG content and

Table 8.1 Cardiovascular measurements of heart function and structure of SCD1+/+, SCD1−/−, ob/ob and ob/ob;SCD1−/− mice (Dobrzyn et al. 2010a)

	SCD1+/+	SCD1−/−	ob/ob	ob/ob;SCD1−/−
HR (bpm)	453.13 ± 23.1	473.15 ± 28.4	515.13 ± 36.0	481.33 ± 67.1
AWd (mm)	0.83 ± 0.2	0.85 ± 0.2	0.89 ± 0.1	0.85 ± 0.1
PWd (mm)	0.82 ± 0.1	0.86 ± 0.2	0.92 ± 0.1	0.87 ± 0.1
LVDd (mm)	3.27 ± 0.2	3.56 ± 0.5	3.79 ± 0.3[a]	3.34 ± 0.2[c]
LV mass (mg)	100.01 ± 4.4	110.64 ± 7.2	128.64 ± 3.9[a,b]	107.60 ± 3.4[c]
LV mass/BW (mg/g)	3.51 ± 0.3	3.98 ± 0.6	2.01 ± 0.2[a,b]	2.69 ± 0.4[a,b,c]
% FS	52.33 ± 4.6	55.02 ± 5.7	43.1 ± 3.7[a,b]	55.3 ± 7.8[c]
IVRT (s)	0.014 ± 0.006	0.016 ± 0.004	0.020 ± 0.002	0.020 ± 0.003
MPI	0.44 ± 0.2	0.49 ± 0.1	0.57 ± 0.2	0.50 ± 0.1
Ea/Aa	1.78 ± 0.5	1.64 ± 0.2	1.33 ± 0.2	1.07 ± 0.2[a,b,c]
E/Ea	29.03 ± 4.3	27.36 ± 3.6	17.6 ± 5.1[a,b]	22.3 ± 2.4[a]

HR heart rate in beats per minute, *AWd* anterior wall in diastole, *PWd* posterior wall in diastole, *LVDd* left ventricular diameter in diastole, *LV mass* left ventricular mass, *FS* fractional shortening ((LVDd−LVDs)/LVDd), *IVRT* isovolumic relaxation time in seconds, *MPI* myocardial performance index = the ratio of isovolumic contraction and relaxation to ejection time, MPI = $(a-b)/b$, where a = the time of mitral value closure and b = aortic ejection time, *Ea* early diastolic maximal velocity from tissue Doppler, *Aa* late diastolic maximal velocity from tissue Doppler, *E* transmitral early filling velocity

[a]$P < 0.05$ vs. SCD1+/+ mice

[b]$P < 0.05$ vs. SCD1−/− mice

[c]$P < 0.05$ vs. ob/ob mice

increased myocyte apoptosis (Barouch et al. 2003; Miyazaki et al. 2003). A number of studies suggest an important role of SCD in the pathogenesis of lipid-induced heart disease. SCD1 expression and activity are significantly increased in the myocardium of ob/ob and db/db mice (Ge et al. 2012). The activity of SCD is also significantly induced in the hearts of obese diabetic rats fed a high-sucrose diet, and SCD1 overexpression markedly induces lipid droplet accumulation in isolated neonatal rat cardiac myocytes incubated with palmitate (Matsui et al. 2012). Furthermore, cardiac SCD1 and SCD4 are overexpressed in acyl-CoA synthase transgenic mice, which develop severe lipotoxic cardiomyopathy (Lee et al. 2004b). The expression of SCD1 appears to be more abundant in the hearts of diabetic patients than in those from lean subjects and nondiabetics (Matsui et al. 2012).

It has been proposed that SCD1 repression may be a viable approach for decreasing FA uptake and oxidation in the heart and thereby aid in the prevention and treatment of lipotoxic cardiomyopathy observed in the diabetic and obese states. To test this hypothesis, an ob/ob;SCD1−/− double knockout mouse model was generated (Dobrzyn et al. 2010a). Transthoracic echocardiography analysis showed that ob/ob;SCD1−/− mice are characterized by a smaller LV diameter and significantly reduced LV mass compared to the ob/ob mice (Table 8.1), though LV wall thickness was not affected by SCD1 deficiency (Dobrzyn et al. 2010a). Because the body mass of the ob/ob mice was much larger than that of either the wild type or ob/ob;SCD1−/− counterparts, the LV mass/body weight ratio was significantly increased in ob/ob;SCD1−/− mice. Ob/ob mice appeared to have a significant reduction in systolic function, as demonstrated by the impaired fractional shortening (Dobrzyn et al. 2010a). SCD1 deficiency increased the percent of fractional shortening by 28 % in the heart of ob/ob;SCD1−/− compared with ob/ob mice (Table 8.1). The percent of fractional shortening in ob/ob;SCD1−/− mice was not different from that of wild-type animals (Dobrzyn et al. 2010a). The myocardial performance index, a Doppler-based measure of LV function, was marginally different between ob/ob and ob/ob;SCD1−/− mice. The Doppler flow analysis demonstrated that the E/Ea ratio was reduced by 57 % in ob/ob mice compared with the wild-type mice, indicating a diastolic dysfunction in ob/ob mice (Dobrzyn et al. 2010a). Although the E/Ea ratio in the heart of ob/ob;SCD1−/− mice was still significantly lower than that in the wild-type mice, SCD1 deficiency increased the E/Ea ratio by 27 % in the heart of these mice compared with ob/ob mice (Table 8.1). These results showed that disruption of the SCD1 gene improves cardiac function in obese leptin-deficient ob/ob mice.

Reduction of SCD1 activity was also shown to exert a positive effect on heart function in the animal models of diet-induced obesity. Rats fed a high-carbohydrate, high-fat diet developed eccentric hypertrophy, a characteristic of increased preload, defined by increased left ventricular internal diameter in diastole without any changes to relative wall thickness. Consequently, these rats showed impaired systolic function seen as decreased fractional shortening, increased wall stress, increased diastolic stiffness, decreased developed pressure, and decreased LV contractility. Additionally, diastolic, systolic, stroke volumes, and cardiac output were all elevated in high-carbohydrate, high-fat fed rats without any changes in heart rate (Poudyal et al. 2012a). These negative changes in cardiac function were accompanied by

increased SCD activity. A number of studies showed that increased amounts of α-linoleic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) suppress SCD1 expression (Hofacer et al. 2012; Poudyal and Brown 2011). ALA, DHA, and EPA supplementation in high-carbohydrate, high-fat diet-induced metabolic syndrome rats caused lipid redistribution away from the abdominal area and favorably improved glucose tolerance, insulin sensitivity, dyslipidemia, hypertension and left ventricular dimensions, contractility, volumes, and stiffness. These effects were associated with complete suppression of SCD activity (Poudyal et al. 2013), further emphasizing the importance of SCD for heart function regulation.

SCD1 and Cardiomyocyte Apoptosis

Excessive deposition of intramyocardial TG enlarges the intracellular pool of fatty acyl-CoA, thereby providing a substrate for nonoxidative metabolic pathways, such as ceramide synthesis (Mengi and Dhalla 2004). Increased levels of ceramide cause apoptosis of cardiac myocytes, which can result in LV chamber expansion, contractile dysfunction, and impaired diastolic filling contributing to the cardiomyopathy observed in the settings of obesity and diabetes (Zhou et al. 2000). This phenomenon, broadly referred to as "cardiac lipotoxicity," has been observed in several genetic models in mice and rats. Obese ZDF rats develop cardiac dilatation and reduced contractility at 14 weeks of age, effects that are associated with elevated intramyocardial TG, ceramide levels, and increased apoptosis (Zhou et al. 2000). Troglitazone therapy of ZDF rats decreased cardiac TG and ceramide levels, which was associated with the complete prevention of apoptosis and restoration of cardiac function.

Accumulation of intramyocardial ceramide and TG, LV chamber enlargement, and impaired contractile function were also observed in obese leptin-deficient ob/ob mice (Dobrzyn et al. 2010a). Interestingly, the knockout of SCD1 gene resulted in decreased TG and ceramide contents in the heart of ob/ob;SCD1−/− mice in comparison to control ob/ob mice. Notably, the ceramide level in the hearts of ob/ob;SCD1−/− mice was comparable to values noted in the myocardium of the wild-type counterparts (Dobrzyn et al. 2010a). Ceramide may be formed by hydrolysis of sphingomyelin, de novo synthesis via condensation of palmitoyl-CoA and serine, glycosphingolipid breakdown, or conversion of other sphingolipids (Hanada 2003). The decrease in ceramide content in SCD1-deficient hearts appears to be due to decreased de novo synthesis, as evidenced by decreased SPT activity and gene expression, and reduced incorporation of [^{14}C]palmitate into ceramide (Dobrzyn et al. 2010a). The reduced intracellular palmitate level in the heart of ob/ob;SCD1−/− mice resulting from decreased lipogenesis and reduced FA uptake may also be one rate-limiting factor in de novo ceramide synthesis. Similar effects, including reduced SCD activity and decreased intramuscular palmitoyl-CoA content, concomitant with the downregulation of SPT activity and reduction in ceramide synthesis, were observed in the oxidative skeletal muscles of SCD1−/− and SCD1-deficient ob/ob mice (Dobrzyn et al. 2005).

Because the ceramide pathway is the most important of the lipoapoptotic routes in cardiomyocytes (Shimabukuro et al. 1998), decreased ceramide content due to SCD1 deficiency resulted in a reduced rate of apoptosis in the heart of ob/ob mice (Fig. 8.3). It has been shown that two key markers of ceramide-induced apoptosis, NO production (measured by iNOS activity) and caspase-3 activity, were significantly reduced in the hearts of ob/ob;SCD1−/− double mutant mice (Dobrzyn et al. 2010a). Decreased activities of iNOS and caspase-3 might result from inhibition of de novo ceramide synthesis due to SCD1 deficiency (Fig. 8.3). Bielawska and coworkers (1997) proposed that ceramide upregulates iNOS expression and increases NO production, which causes an increase in peroxynitrite and apoptosis. Additionally, downregulation of caspase-3 was often linked with ceramide action (Ravid et al. 2003; Castillo et al. 2007). Ravid et al. (2003) showed that ceramide-mediated apoptosis is blocked by a general caspase inhibitor, Boc-D-fluoromethylketone. Ruvolo et al. (2002) established that exogenous ceramide can downregulate antiapoptotic factor Bcl-2 expression and phosphorylation, thereby activating caspase-3 and apoptosis. It has also been suggested that the antiapoptotic effect of Bcl-2 may occur via the modulation of ceramide production and the prevention of ceramide-mediated caspase activation (Ruvolo et al. 2002; Sawada et al. 2000). Increased mRNA level of Bcl-2 was observed in the heart of ob/ob;SCD1−/− mice compared with ob/ob controls. Thus, the increased gene expression of Bcl-2 could be another factor contributing to the downregulation of caspase-3 activity and the reduction in the rate of apoptosis in the heart of ob/ob;SCD1−/− double knockout mice (Fig. 8.3).

Furthermore, it has been suggested that increased oxidation of palmitate through CPT1 is involved in the occurrence of apoptosis in cardiomyocytes. Previous studies established that palmitate-induced cell death was enhanced by carnitine, a cofactor needed for palmitate transport into mitochondria via CPT1 (Kong and Rabkin 2002). CPT1 mRNA levels were decreased in the heart of ob/ob;SCD1−/− mice compared with ob/ob mice (Dobrzyn et al. 2010a). Therefore, decreased mitochondrial FA oxidation may account for decreased apoptosis in the heart of SCD1-deficient ob/ob mice. As described above, knockout of the SCD1 gene significantly improves diastolic and systolic LV function in ob/ob mice (Table 8.1).

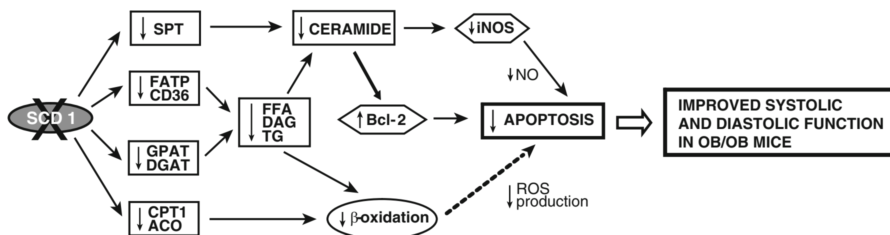

Fig. 8.3 Proposed model for the effect of SCD1 gene deletion on heart lipid metabolism and left ventricle function in leptin deficiency. Reduction in myocardial lipid accumulation and inhibition of lipid-induced apoptosis appears to be the main mechanisms responsible for improved cardiac function in leptin-deficient ob/ob mice caused by lack of SCD1 function. Adopted from Dobrzyn et al. (2010a)

Because reduced apoptosis in cardiomyocytes was shown to improve cardiac function (Barouch et al. 2006), inhibition of lipid-induced apoptosis due to SCD1 deficiency might be directly responsible for the improvement of heart function in ob/ob mice (Fig. 8.3).

Studies performed on isolated cardiomyocytes showed that SCD1 has protective effects against saturated FA-induced apoptosis (Matsui et al. 2012). Palmitic acid or stearic acid increased the activities of caspases 3 and 7 and the number of TUNEL-positive stained cells in neonatal rat cardiomyocytes. In contrast, SCD1 overexpression significantly attenuated saturated FA-induced apoptotic changes. These results suggest that SCD1 suppresses saturated FA-induced apoptosis in cardiac myocytes, as shown first by Listenberger et al. (2003) in CHO cells; however, this effect was observed in studies performed in vitro only.

The Role of Oleate in Regulation of Cardiac Metabolism

Dietary stearic acid intake ranks second among the SFA consumed in the United States, accounting for 25.8 % of SFA intake and 2.9 % of total kcal (Kris-Etherton et al. 2005). Stearate is a precursor to endogenously synthesized oleate by SCD (Sampath and Ntambi 2005). Oleic acid is the most relevant intermediate between saturated FA and polyunsaturated FA, and it is best suited for being stored or incorporated into glycerolipids. It also modulates the basic features of biomembranes (Kamp et al. 2003). Due to its unique features, oleate plays an important metabolic role in the cell (e.g., it directly affects signal transduction by activating the isoenzymes of protein kinase C), regulating the phosphorylation of proteins and modulating the expression of genes (Kennedy et al. 2009; Dziewulska et al. 2012). In the heart, oleate plays an important role in the regulation of substrate metabolism and seems to be involved in the pathogenesis of lipotoxic cardiomyopathy (Posner et al. 1991; Xu et al. 2006; Dobrzyn et al. 2012). It has been shown that the majority of FA undergoing β-oxidation in the heart are not saturated FA but, rather, monounsaturated FA, with oleic acid being the preferred substrate (Lopaschuk et al. 2010). An increased rate of FA β-oxidation was found in the heart of rats fed trioleate- (TO) or tristearate (TS)-supplemented diets, suggesting that both dietary and endogenously synthesized oleate are able to activate oxidative pathways (Dobrzyn et al. 2012). Very little is known about the potential mechanisms involved in the regulation of cardiac FA oxidation by oleate. One of the possible mechanisms that link oleate and FA oxidation may involve the activation of PPARα. Transgenic mice with an overexpression of cardiac PPARα have increased myocardial fatty acid uptake, cardiac TG and ceramide accumulation, cardiac hypertrophy, and left ventricular dilation (Finck et al. 2002, 2003) as well as clear adverse effects on cardiac function and structure (Chiu et al. 2005; Finck et al. 2002). PPARα activation increases fatty acid oxidation and protects the heart from substrate-induced contractile dysfunction when there is an oversupply of FA and TG (Morgan et al. 2006). Gilde et al. (2003) showed that the administration of long-chain saturated and monounsaturated FA

results in moderate increases in the mRNA expression of several PPARα-regulated genes; however, this effect was only seen in isolated cardiomyocytes (Gilde et al. 2003). Recent studies demonstrate that dietary or de novo synthesized oleate increases the expression and activity of PPARα (Dobrzyn et al. 2012). Furthermore, feeding with TS- and TO-supplemented diets results in increased phosphorylation and activity of AMPK in the heart, another important factor for regulating cardiac FA oxidation and glucose transporter 4 (GLUT4) membrane translocation (Lopaschuk et al. 2010; Towler and Hardie 2007). Analyses performed on the perfused heart show that oleic acid activates AMPK in the myocardium (Clark et al. 2004). Thus, it is possible that the activation of both the AMPK and PPARα pathways by oleate is a viable mechanism for increasing mitochondrial FA oxidation in response to increased oleate availability.

As mentioned above, oleate can be easily incorporated into glycerolipids for storage or into phospholipids, leading to changes in plasma membrane properties. As shown using TS- and TO-fed rats, augmentation of cardiac oleate is accompanied by an increased protein level of FAT/CD36 (Dobrzyn et al. 2012), the major protein responsible for membrane FA transport (Carley and Severson 2005). This change could be especially valuable for mitochondrial FA oxidation because overexpression of FAT/CD36 in the skeletal muscle leads to an enhanced level of mitochondrial FA oxidation (Nickerson et al. 2009). An increase in oleate availability also results in increased TG accumulation in the myocardium (Dobrzyn et al. 2012). The increased TG level in the hearts of TO- and TS-fed rats was likely related to the fact that oleate is the preferred substrate for TG esterification. However, both TO and TS feedings resulted in an upregulation of the cardiac lipogenic genes, i.e., FAS, acyl-CoA synthetase, and glycerol-3-phosphate transferase (GPAT) in the heart. The parallel upregulation of SCD1/oleate availability, FAS, and GPAT in conditions of increased cardiac FA oxidation suggests that these proteins may cooperate in maintaining the intracellular oleate level within a narrow range and thus may play a role in the regulation of cardiac energy metabolism.

Increased levels of dietary or endogenously synthesized oleate may be responsible for not only increasing the rate of FA oxidation but also for reducing glucose uptake in the heart (Dobrzyn et al. 2012). Recent studies showed that long-chain FA affect GLUT4 expression and membrane translocation as well as Akt phosphorylation in cardiac tissue. Reporter studies in H9C2 cardiomyotubes have shown that hyperlipidemia in vitro, which is induced by high levels of stearic and oleic acids, repressed transcription of the GLUT4 promoter (Armoni et al. 2005). Hommelberg et al. (2009) observed a reduced rate of GLUT4 translocation and deoxyglucose uptake in L6 skeletal muscle cells incubated with palmitate and stearate. Oleate was shown to reduce Akt phosphorylation in perfused rat livers (Anderwald et al. 2007) and in primary hepatocytes (Liu et al. 2007). Furthermore, prolonged exposure of hepatocytes to oleate decreased the insulin-induced tyrosine phosphorylation of IRS1/2 while slightly increasing the serine phosphorylation of IRS (Liu et al. 2007). Therefore, it is possible that the dietary or de novo synthesized oleate from stearic acid decreases the rate of glucose uptake in the heart by the reduction of Akt phosphorylation and GLUT4 membrane translocation, as was found in TS- and TO-fed

rats (Dobrzyn et al. 2012). These data are in agreement with results obtained in SCD1−/− mice, where increased cardiac glucose transport and oxidation were accompanied by decreased oleic acid content (Dobrzyn et al. 2008b).

Plasma Desaturation Index as Predictor of Cardiac Health

Recent studies establish the important role of SCD activity and plasma fatty acid desaturation on the human heart function and cardiometabolic risks. First, Warensjö et al. (2008a) showed that the plasma SCD desaturation index is elevated by a diet high in saturated fat, compared with a diet rich in unsaturated fat. Next, the same investigators suggested that endogenous fatty acid desaturation, which is partly independent of diet, is associated with and might even contribute to mortality; thus, desaturase indexes may be used to predict cardiovascular mortality risk (Warensjö et al. 2008b). These results were confirmed by Djoussé et al. (2012), who showed that plasma SCD activity (16:1n-7/16:0 ratio) was positively associated with the risk of heart failure, whereas oleic acid and *cis*-vaccenic acid concentrations were not related to heart failure risk. Moreover, the plasma SCD desaturation index was positively associated with heart rate (Ebbesson et al. 2012). Additionally, a study including 93 healthy volunteers showed that SCD desaturation index is closely associated with the features of cardiometabolic risks (i.e., increased body mass index and TG content as well as decreased HDL-cholesterol level) in Koreans (Do et al. 2011). All together, these results suggest that the level of fatty acid desaturation in blood plasma is related to heart rate and function and is possibly related to arrhythmia and sudden death. This would partially explain the observed association between cardiovascular mortality risk and SCD activity.

Conclusion

Research over the past decade has identified SCD as an important regulator of physiological processes and body adiposity. Using knockout mouse models, we have learned that SCD1 is a critical control point of lipid partitioning. While high SCD activity favors fat storage, suppression of the enzyme activates metabolic pathways that promote the burning of fat and decrease lipid synthesis in white adipose tissue and the liver. SCD1 deficiency upregulates insulin-signaling components and affects glycogen metabolism in insulin-sensitive tissues. In the heart, the lack of SCD1 decreases FA uptake and oxidation while increasing glucose transport and oxidation. These metabolic changes cause a decrease in the accumulation of neutral lipids and ceramide thereby inhibiting apoptotic pathways in the LV of leptin-deficient ob/ob mice and improve systolic and diastolic function. The findings on SCD1 point to a potentially novel strategy for the treatment of lipotoxic heart disease. However, the potential use of an SCD inhibitor as a therapeutic agent awaits a more complete

understanding of the mechanism underlying the effects of SCD deficiency and an indication that inhibition of this enzyme is both safe and efficacious.

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Chapter 9
Stearoyl-CoA Desaturase-1 Activity in Skeletal Muscle: Is It Good or Bad?

Joseph Stevens and Matthew W. Hulver

Introduction

Skeletal muscle is known to be an important site for metabolic processes such as glucose disposal and fatty acid oxidation, and, as such, dysregulation of these processes in muscle is associated with and may play a causative role in many disease states and comorbidities including obesity, hypertension, insulin resistance, and hypertriglyceridemia (Lee et al. 2003; McGarry 2002; Petersen and Shulman 2002; Scheuermann-Freestone et al. 2003). Specifically, these disease/co-morbid states are associated with dysregulated glucose and fatty acid metabolism and excess lipid accumulation in skeletal muscle (Flowers and Ntambi 2009; Kahn and Flier 2000). Studies in humans show that SCD1 is associated with insulin resistance, increased intramuscular triacylglycerol (IMTG) content, and reduced fatty acid oxidation (Dubé et al. 2011; Hulver et al. 2005). Studies in rodents clearly show that genetic deletion of SCD1 prevents high-fat diet-induced weight gain and insulin resistance (Ntambi et al. 2002). Conversely, others have suggested that heightened SCD1 activity in skeletal muscle may be protective (Dobrzyn et al. 2010; Schenk and Horowitz 2007). To date, the role of SCD1 in dysregulated metabolism, specifically in skeletal muscle, is not definitively known, as there are conflicting reports. The purpose of this chapter is to highlight the available evidence for both the deleterious and protective roles of SCD1 in the context of metabolic deranged states such as insulin resistance and obesity. The role of SCD1 in the context of inflammatory signaling and adaptation to exercise will also be touched upon.

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A Causative Role for SCD1 in Dysregulated Metabolism

Data from experiments using mouse models with whole-body deletion, a naturally occurring mutation in the gene, or through limited cell culture models with a gain or loss of SCD1 function have all provided insight into this topic; however, there is still much to be learned. Tissue specific deletion of SCD1 has contributed a great deal to the current understanding of the role of SCD1 in the modulation of whole-body metabolism (Liu et al. 2011). Currently, no data from skeletal muscle specific knockout of SCD1 has been published. Cell culture models, where studies in which SCD1 function has been interrupted in myotubes have shown a direct effect on fatty acid metabolism (Hulver et al. 2005; Kim et al. 2011; Peter et al. 2009), suggest a causative role for SCD1 in dysregulated substrate metabolism.

The Role of SCD1 in the Modulation of Substrate Metabolism and Insulin Sensitivity

Obesity is associated with ectopic lipid accumulation and insulin resistance, both of which are risk factors for the development of the metabolic syndrome and type 2 diabetes (Virtue and Vidal-Puig 2010). A majority of research has shown a causative role for SCD1 in the development of obesity, inflammation, and insulin resistance (Kim et al. 2011; Miyazaki et al. 2002; Ntambi et al. 2002; Pinnamaneni et al. 2006; Rahman et al. 2003). However, this causality as it relates to skeletal muscle has been difficult to discern. SCD1 expression is relatively low in skeletal muscle compared to other tissues such as the liver and sebaceous glands of the skin (Paton and Ntambi 2009). However, under high-fat fed conditions, rodent models of SCD1 deletion are protected from insulin resistance and possess reduced levels of intramyocellular lipid intermediates, which are known to disrupt insulin-stimulated glucose uptake (Rahman et al. 2003). Skeletal muscle is an important tissue for postprandial glucose homeostasis; 80 % of insulin-stimulated glucose uptake is accounted for by muscle tissue (Baron et al. 1988). The importance of skeletal muscle in glucose clearance taken along with current research showing a role for SCD1 in contributing to defects in insulin-signaling and glucose uptake suggest a causative role for SCD1 in mediating the alterations in metabolic function. Interestingly, mouse models with liver and/or adipose tissue specific deletion of SCD1 are not protected from the diet-induced obesity observed with the whole-body deletion of SCD1 (Flowers et al. 2012). Because the liver and adipose tissue are paramount to normal whole-body metabolic regulation and substrate homeostasis and the protective effects of high-fat feeding of SCD1 deletion at these sites are not observed, this points to SCD1 function in some other metabolically-important tissue (e.g., skeletal muscle) as the culprit for whole-body metabolic dysregulation.

SCD1 mRNA levels in skeletal muscle are elevated in the obese state and associated with dysregulated lipid metabolism (Hulver et al. 2005). Specifically, a hallmark

feature of obesity is blunted fatty acid oxidation and elevated triacylglycerol accumulation in skeletal muscle, both of which are associated with increased levels of monounsaturated fatty acids (Aguilera et al. 2008). Hulver et al. showed higher SCD1 expression in skeletal muscle of obese humans, relative to non-obese controls, which was associated with decreased AMP-activated kinase (AMPK) phosphorylation and increases in acetyl-CoA carboxylase (ACC) beta levels, both of which occurred in the context of decreased FA oxidation. Consequences of whole-body SCD1 deficiency include increased rates of fatty acid oxidation as well as a reduction in triglyceride synthesis and storage in various tissues including liver, BAT, and skeletal muscle (Flowers et al. 2008; Ntambi et al. 2002; Sampath and Ntambi 2011). SCD1 deficiency appears to increase the rates of FA oxidation in a muscle-fiber type-dependent manner as rates of FA oxidation were increased with SCD1 deficiency in soleus and gastrocnemius, oxidative and oxidative/glycolytic fiber types, respectively (Dobrzyn et al. 2005). Conversely, rates of fatty acid oxidation were not observed in white gastrocnemius or extensor digitorum longus, which are primarily glycolytic fiber types (Dobrzyn and Dobrzyn 2006).

The mechanism by which SCD1 deletion increases FA oxidation is thought to be dependent on AMPK signaling (Dobrzyn et al. 2004a; Kim et al. 2011). It was shown by Dobrzyn et al. that increased AMPK signaling caused a decrease in ACC activity with subsequent reductions in cytosolic malonyl-CoA levels. Malonyl CoA inhibits carnitine palmitoyl transferase 1 (CPT1), which has a primary function of shuttling FA into the mitochondria for oxidation (Hardie and Pan 2002). These findings in SCD1 KO mice are similar to other models in which AMPK signaling is up-regulated (Tomas et al. 2002; Yoon et al. 2006). A similar study in which C2C12 myotubes were treated with the SCD1 inhibitor, 4-(2-cholorophenoxy)-*N*-(3-(methylcarbamoyl)-phenyl) piperidine-1-carboxamide, showed a decrease in ACC and fatty acid synthase (FAS) expression and increase in expression of (CPT1), alternative oxidase (AOX), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) (Kim et al. 2011).

Altered levels of SCD1 expression in skeletal muscle can also have affects on the synthesis sphingolipids molecule, ceramide. Ceramide synthesis occurs through either a de-novo process involving esterification of palmitoyl-CoA and serine by serine palmitoyl transferase (SPT) or through the hydrolysis of sphingomyelin (Menaldino et al. 2003; Perry 2002). Ceramide is known to play a role in apoptosis and insulin resistance through the modulation of intracellular signaling processes (Blázquez et al. 2001; Shimabukuro et al. 1998). In particular, increases in substrates for ceramide synthesis such as FFAs have been shown to contribute to β-cell apoptosis (Shimabukuro et al. 1998). SCD1 KO mice show a 42 % and (Hayashi et al. 1998) 48 % decrease in ceramide content in soleus and gastrocnemius. In addition, sphingomyelin content was significantly reduced (~30 %) in the soleus and gastrocnemius muscles of SCD1 KO compared to their WT counterparts, with no changes in the mRNA levels of the shingomyelinases. This suggests a limited role for SCD1 specifically in sphingomyelin hydrolysis (Dobrzyn and Dobrzyn 2006). Expression of SPT and ceramide are regulated by many factors including intracellular FA content. As such, SCD1 activity may be very important in the modulation

of ceramide-mediated effects on apoptotic processes and the inhibition of signaling events that contribute to insulin resistance.

One factor shown to down-regulate ceramide synthesis is AMPK signaling. This was shown through the use of the ribonucleoside AICAR that stimulates AMPK activity (Hayashi et al. 1998). As stated previously, AMPK signaling has been shown to be responsible for the up-regulation of FA oxidation shown with SCD1 KO mice. Treatment with AICAR resulted in significant decreases in palmitate-induced ceramide synthesis (Blázquez et al. 2001). Another FA and product of SCD1, oleate, has been shown to down-regulate ceramide levels, and this appears to occur through prevention of the generation and/or the scavenging of ceramide molecules (Listenberger et al. 2003). These data suggest that deletion of SCD1 has beneficial effects on cellular signaling processes through increases in AMPK activity and decreases in transcriptional events regulating lipogenic processes.

A consequence of abnormalities in lipid metabolism such as intramyocellular fat accumulation and increased FFA flux in skeletal muscle is insulin resistance (Kahn and Flier 2000; Kelley and Goodpaster 2001; Liu et al. 2007; Roden 2004). Dysregulated lipid metabolism disrupts insulin-signaling and the normal process of glucose transporter 4 (Glut4) translocation to the cellular membrane, and thus, reduces insulin-stimulated glucose uptake (Savage et al. 2007). Lipid metabolites such as ceramide and diacylglycerol can lead to endoplasmic reticulum stress that can cause serine/threonine phosphorylation of the insulin receptor as well as activation of inflammatory pathways (Könner and Brüning 2011; Peter et al. 2009). Decreases in expression of protein tyrosine phosphatase 1-beta (PTP-1b) expression can cause dephosphorylation of the insulin receptor. It can also lead to an inability of the insulin receptor to bind substrates, and through changes in membrane fluidity, it can affect aggregation of the insulin receptor. These effects have been shown to contribute to insulin insensitivity (Dobrzyn and Dobrzyn 2006). Increased SCD1 expression has been shown to induce the changes in FA composition that can affect insulin sensitivity particularly through an increase in the lipid products of SCD1 activity (Attie et al. 2002; Houdali et al. 2003; Warensjo et al. 2009). These lipid products are able to exert deleterious effects on the insulin-signaling machinery that can cause decreases in insulin sensitivity.

As previously stated, models with a disruption in whole-body or skin SCD1 gene expression show improvements in whole-body glucose and insulin tolerance when compared to WT counterparts on the same high-fat diet (Brown and Goldstein 1998; Flowers et al. 2008; Miyazaki et al. 2009; Ntambi et al. 2002). Specifically, KO mice in these studies show improvements in their fasting insulin levels and when put on a high-fat diet, KO mice have an increased ability to clear glucose compared to their WT littermates (Ntambi and Miyazaki 2004). These mice are also resistant high-fat diet induced obesity, which is related to increases in FA oxidation. Increases in FA oxidation lead to decreases in lipid metabolites such as ceramide and fatty acyl CoAs, metabolites that are known to activate protein kinase C (PKC), which has negative effects on the insulin-signaling machinery such as decreased AKT and IRS1 phosphorylation (Geraldes and King 2010; Turban and Hajduch 2011). Along the same line of thought, insulin-resistant, ob/ob mice given the SCD1 inhibitor sterculic acid,

showed improvements in glucose tolerance, which was associated with decreases in both 16:0/16:1 and 18:0/18:1 desaturase indices (Ortinau et al. 2013).

The mechanism by which SCD1 is able to exert these effects on glucose tolerance appears to be through changes in the cell's normal response to glucose. This appears to include, in addition to the above-mentioned mechanisms, a negative influence on the intracellular Glut4 machinery responsible for glucose uptake, particularly under insulin-stimulated conditions (Rahman et al. 2003; Voss et al. 2005). When subjected to over-expression of SCD1, rat myoblasts show a 1.75-fold decrease in total Glut4 levels. These decreases track with suppressed basal and insulin-stimulated glucose uptake measured by 2-deoxyglucose uptake experiments (Voss et al. 2005). Additionally, mice with a deletion of SCD1 show increases in Glut4 levels that occur along with increases in glucose uptake compared to WT mice (Rahman et al. 2003). Whereas SCD1 can negatively influence the insulin receptor and its ability to bind substrate, it can also affect important downstream molecules such as Glut4. Most likely these negative influences on the downstream molecules such as Glut4 are occurring through a mechanism by which upstream molecules such as AKT are inhibited (Dobrzyn et al. 2005).

Another molecule thought to be modulated by SCD1 activity in skeletal muscle is the PTP-1b. This molecule is responsible for the rapid dephosphorylation and subsequent down-regulation of insulin receptor function as well as that of the insulin receptor substrates 1 and 2 (Rahman et al. 2003, 2005). This has been shown through the use of PTP-1b KO mice that have an increase in IR tyrosine phosphorylation in muscle (Elchebly et al. 1999). PTP-1b is down-regulated with SCD1 deletion, and this leads to the autophosphorylation and sustained activity of the insulin receptor. This was associated with increases in insulin-stimulated glucose uptake, and these changes were shown to be independent of circulating insulin levels (Rahman et al. 2003). As of yet, it isn't understood whether the changes in PTP-1b are a direct consequence of decreases in SCD1 activity or a consequence of the alterations in lipid metabolism caused by a lack of SCD1.

There are data showing up-regulation of SCD1 in the presence of certain dietary factors other than fatty acids. Diseases such as type 2 diabetes have been shown to be caused by diets high in carbohydrates (Flowers and Ntambi 2009). High levels of dietary carbohydrates including glucose, sucrose, and fructose significantly up-regulate the expression and activity of SCD1 (Hasty et al. 2000; Matsuzaka et al. 2004; Miyazaki et al. 2004). In the case of a 6-month diet consisting of high sucrose levels, there is a significant increase in levels of oleic acid, the product for SCD1 (Brenner 2003). Excess carbohydrates are metabolized and acetyl-CoA levels rise beyond that needed for ATP production. This results in increased de novo lipid synthesis, which is transcriptionally controlled by lipogenic transcription factors such as SREBP1c and positive modulation SCD1 expression (Hasty et al. 2000; Paton and Ntambi 2009). Rats fed a diet high in glucose show an up-regulation of SCD1 levels (Houdali et al. 2003), and muscle cells treated with high levels of glucose show a very similar effect (Voss et al. 2005).

Another mechanism by which altered SCD1 activity can lead to changes in insulin sensitivity is through its regulation of membrane fluidity; this occurs through the

alteration of the FA composition of the cell membrane (Agatha et al. 2001; Ntambi 1999; Storlien et al. 1996). The ratio of saturated to monounsaturated fatty acids is the major determining factor of membrane fluidity and has been shown to influence insulin signaling. This is related to the amount of polyunsaturated FAs present (Ntambi and Miyazaki 2004). In SCD1 KO models, a decrease in monounsaturated FA content of membranes is met with an increase in polyunsaturated FAs in order to maintain membrane integrity (Ntambi and Miyazaki 2004). This causes increased membrane fluidity, which can lead to aggregation of the IR and subsequent hyperphosphorylation of the IR in response to insulin (Frangioudakis et al. 2010; Lamping et al. 2013) Therefore, data has shown a direct correlation between increases in PUFA content of membranes and the degree of insulin resistance in these same tissues (Dobrzyn and Dobrzyn 2006).

SCD1 and Inflammation

SCD1 has also been shown to play a role in the pro and anti-inflammatory processes that are associated with metabolic disease. Free fatty acids are known to activate pro-inflammatory pathways (Boden et al. 2005; Shi et al. 2006). The substrates as well as the products of SCD1 have been shown to both stimulate and inhibit an inflammatory response (Fessler et al. 2009; Kim and Sears 2010). Specifically, the fatty acid product of SCD1, palmitoleic acid, has been shown to bind to and activate inflammatory signaling pathways (Staiger et al. 2006; Weigert et al. 2004). Palmitoleic acid initiates an inflammatory response through binding of the toll-like receptor 4 and the subsequent activation of the nuclear factor kappa beta (NFκB) transcription factor and stimulation of the machinery involved in initiating inflammatory pathways (Schäffler et al. 2006). NFκB causes the production and activation of inflammatory cytokines, chemokines, and other various inflammatory molecules that can function to inhibit insulin-signaling processes (Hotamisligil 2006; Shoelson et al. 2006; Wellen and Hotamisligil 2005). Free fatty acids such as palmitoleate can also bind to various G-protein coupled receptors that can cause an inflammatory response and down-regulation of the insulin-signaling machinery (Talukdar et al. 2011). In humans, increases in SCD1 activity measured by increases in plasma stearic to oleic acid ratios have been positively correlated with elevated whole-body C-reactive protein, a marker for inflammation (Black et al. 2004; Stryjecki et al. 2012). Research pertaining to an anti-inflammatory role for these substrates and products of SCD1 centers around studies showing that treatment with these molecules can have protective roles against an inflammatory response (Grimble and Tappia 1998; Vassiliou et al. 2009). Similarly, the substrates for SCD1 such as palmitate show a pro-inflammatory role that also appears to function through a TLR4-dependent mechanism (Kim et al. 2007). Mice given diets high in saturated fats such as palmitate show increases in various inflammatory markers including NFκB (Kim et al. 2007; Suganami et al. 2007).

The increases in plasma fatty acids shown with obesity have been shown to cause defects in insulin signaling (Roden et al. 1996). Excess FFAs are able to affect

insulin signaling through their ability to elicit an inflammatory response (Eizirik et al. 2008). Both stearate and palmitate are strong inducers of an inflammatory response and are tightly controlled by SCD1 as they are it's primary substrates (Miyazaki and Ntambi 2003). There appears to be an interesting phenomena relating to the role of SCD1 in modulating inflammation. Evidence exists that SCD1 activity can control the activation of inflammatory pathways that can be causative of problems, especially insulin resistance. The question remains whether this controlling force is by activation or deactivation of these pathways. Mice treated with antisense oligonucleotides (ASO) to inhibit SCD1 show marked increases in saturated fatty acid accumulation in plasma and tissues when fed a westernized high-fat diet. These increases are also demonstrated in macrophages, and these show concomitant increases in TLR4 inflammatory gene expression (Brown et al. 2008). Another model in which mice are deficient in both SCD1 and the low-density lipoprotein receptor shows improvements over control littermates in their metabolic characteristics when given a westernized diet; however, these mice also show a similar increase in inflammation to the Brown et al. study (MacDonald et al. 2009). Saturated FAs have been shown to induce macrophage inflammation through a TLR4-dependent mechanism and ASO treated macrophages show a marked hypersensitivity to TLR4 agonist (Brown et al. 2008; Shi et al. 2006; Suganami et al. 2007). It has been demonstrated previously that macrophage infiltration into adipose and skeletal muscle, and subsequent activation by FFAs can mediate the inflammatory response that can be causative of skeletal muscle insulin resistance (Hevener et al. 2007; Odegaard et al. 2007). This would support a protective role for increases in SCD1 activity. Evidence also exists showing a role for DAG and ceramide in mediating an inflammatory response (Bilan et al. 2009; Holland et al. 2011). Along with this, research using exercise and models with over-expression of SCD1 in skeletal muscle has suggested a role for SCD1 in protecting the body against many of the negative effects of lipid intermediates and alterations in lipid metabolism as they relate to inflammatory pathway activation (Dobrzyn et al. 2010; Schenk and Horowitz 2007). This includes research showing improvements in metabolic outcomes that track with increases in SCD1 levels and activity (Dubé et al. 2008; Peter et al. 2009).

Interestingly, these findings have not been universal as certain models of SCD1 deficiency have shown improvements in inflammatory and stress responses (Liu et al. 2011). Those models that show deleterious effects SCD1 include studies of mice fed a high saturated diet where SCD1 expression and activity correlate with decreased insulin responsiveness (Lee et al. 2006). However, this was shown without studying activation of inflammatory markers. When SCD1 expression was knocked out in two mouse models of obesity, there were marked reductions in adipose tissue-derived inflammatory markers when compared to control mice. This was associated with improvements in basal insulin signaling (Liu et al. 2011). This suggests a possible mechanism by which SCD1 is influencing circulating FA composition and thus contributing to the chronic low-grade inflammation that is characteristic in the obese insulin-resistant state.

With all of this taken into consideration, it is possible that the involvement of SCD1 in these inflammatory processes is cell and model dependent. In models

where the substrates for SCD1 are causing pro-inflammatory responses, SCD1 could have a protective effect by removing these substrates. In the cases where 16:1 or 18:1 fatty acids are causing a similar response, it is possible that SCD1 is contributing to the defect.

A Protective Role for SCD1

While there is considerable evidence for a causative role of SCD1 in the metabolic syndrome and its related comorbidities, there appears to be a collection of research showing SCD1 as a much needed and protective molecule. Both over-expression and exercise models have demonstrated a protective capacity for SCD1 (Dubé et al. 2011; Listenberger et al. 2003; Pinnamaneni et al. 2006). Research in this area has shown, both in rodent and human models, that there are improvements in FA partitioning and oxidation that are dependent on increases in SCD1, and that these lead to positive changes in glucose clearance (Amati et al. 2011; Dobrzyn et al. 2010; Dubé et al. 2008). This leads to the belief that SCD1 may be a double-edged sword capable of both protective and harmful effects. As with the causative mechanisms shown for SCD1 in skeletal muscle, the protective mechanisms seem to differ depending on the specific comorbidity.

Dysregulated Lipid Metabolism and Insulin Resistance

Obese individuals present with increased expression and activity of SCD1 in skeletal muscle that relates to higher rates of IMTG synthesis. Acute and chronic bouts of endurance exercise show a similar phenotype to this in regards to levels of SCD1 (Amati et al. 2011; Dobrzyn et al. 2010; Dubé et al. 2008; Schenk and Horowitz 2007). These alterations appear to be dependent on the muscle-fiber type as these changes have been observed in oxidative, but not glycolytic muscles (Dobrzyn et al. 2010). This follows suit with data showing that when SCD1 is overexpressed in a CHO cell line there is a significant increase in TG synthesis (Listenberger et al. 2003). Over-expression of SCD1 in the L6 muscle cell line exhibits beneficial metabolic effects as they relate to glucose clearance. When treated with palmitate, the cells over-expressing SCD1 showed improvements in triacylglycerol synthesis, which attenuated ceramide and diacylglycerol synthesis that protected against insulin resistance (Pinnamaneni et al. 2006). These opposing data suggest that changes in SCD1 expression can have an important role in glucose clearance, but then it must also be tightly regulated to avoid metabolic consequences. There have also been mouse models supporting this hypothesis (Dobrzyn et al. 2005, 2010). In terms of causation, it is known that SREBP-1 may be central to the up-regulation of SCD1 under exercised conditions (Hodson and Fielding 2013; Sekiya et al. 2003). Data exists showing increases in this transcription factor under exercise conditions with

both mice and humans that is associated with increases in SCD1 and IMTG production (Bergman et al. 2010; Ikeda et al. 2002). Thus it is likely that SREBP1c is functioning in both the deleterious and protective roles for SCD1. There is also data suggesting that FAS may have roles in regulating SCD1 under these conditions. FAS synthesizes palmitic acid de novo, a substrate for SCD1 (Conraads et al. 2002; Mooren et al. 2004). It has been shown that increases in the substrate for SCD1 leads to up-regulation of SCD1 protein levels (Thorn et al. 2010).

In terms of a protective mechanism(s), data suggests a role for SCD1 in alleviating the stressful conditions caused by the overabundance of FA. Exercise studies have shown a role for SCD1 in enhancing the partitioning of FA into IMTG, as well as an increase in the movement of FA into the mitochondria for beta-oxidation (Dobrzyn et al. 2010). Dobryzn et al. showed a significant increase in the expression of proteins involved in the movement of FA in to the mitochondria in mice in response to an endurance exercise protocol. Work by Schenk and Horowitz has demonstrated that an acute bout of exercise (1 h running on a treadmill) prior to being challenged with a lipid infusion was sufficient to increase TAG synthesis. These changes were characterized by increased SCD1 expression and concomitantly reduced DAG and ceramide production (Bergman et al. 2010; Bruce et al. 2006; Dubé et al. 2008). Certain studies have shown increases in DAG with exercise protocols whilst others have shown decreases (Dobrzyn et al. 2010). Schenk et al. was able to show significant increases in DAG content of soleus muscles after a 12-week treadmill running program in mice. Bioactive sphingolipids, such as ceramides, are affected by exercise regimens. Decreases in ceramide content in skeletal muscle have been shown, both in mice and in humans (Amati et al. 2011; Bruce et al. 2006; Dobrzyn et al. 2004b). These changes in lipid intermediates relate to changes seen in the local environment of skeletal muscle under exercised conditions. Adaptation to exercise by skeletal muscle entails both increasing lipogenic activity and oxidative capacity. The increased lipogenic activity serves the purpose of providing ample IMTG to provide a local fuel source of fatty acids to be oxidized in the mitochondria during exercise as well as during the postexercise recovery period. The increased oxidative capacity allows the muscle tissue to predominantly rely on fatty acids as an energy source, as this is the most efficient fuel for ATP production. It has been discussed previously that SCD1 KO mice show increases in AMPK phosphorylation, leading to increases in beta-oxidation. Along these same lines, there are increases in the ability and capacity for oxidizing fatty acids during and after endurance exercise; this appears to be through an AMPK-dependent mechanism (Bruce et al. 2006; Dobrzyn et al. 2010; Russell et al. 2003). Various exercise models using both animals and humans have shown increases in AMPK activity that correlate with increases in FA oxidation (Dobrzyn et al. 2010; Dubé et al. 2008; Schenk and Horowitz 2007). These increases in FA oxidation are shown along with increases in the activity of SCD1. Being that SCD1 is playing a prominent role in the shuttling of monounsaturated FA into TG synthesis (Paton and Ntambi 2009), and these TG are being hydrolyzed to allow influx of FA into the mitochondria, it is possible that SCD1 is playing a role in controlling FA oxidation, particularly the increases seen with endurance exercise.

This data also appears to be supported in humans. Multiple studies in human subjects have shown increases in IMTG that track with increases in SCD1 (Amati et al. 2011; Bergman et al. 2010). Bergman et al. showed that endurance-trained male cyclists have greater skeletal muscle IMTG and SCD1 mRNA expression and protein content than controls. This would suggest that high muscle SCD1 activity is an advantage for these individuals. In addition, Amati et al. showed increases in SCD1 content that was related to increases in IMTG in endurance-trained athletes. These individuals exhibited decreases in both ceramide and diacylglycerol content compared to sedentary counterparts. These data suggest a protective mechanism for SCD1 and a role for SCD1 in mediating the beneficial metabolic changes seen with exercise regimens.

The changes in FA partitioning towards oxidation and away from storage shown with the above models have also shown improvements in glucose tolerance (Amati et al. 2011; Bergman et al. 2010). Whereas increases in IMTG are correlated with insulin resistance in diseased states, in exercised states it has been shown that increases in IMTG are related to improvements in many metabolic characteristics including insulin sensitivity. This implies a certain paradox in which increases in IMTG can either be beneficial or harmful. Supporting this is research that shows individuals who are endurance trained have increased IMTG and increased mitochondria as well as increased insulin sensitivity compared to sedentary individuals. Initially, it was suspected that these changes in insulin sensitivity due to IMTG increases with endurance exercise where related to the removal of harmful lipid intermediates. However, more recent research has shown this may not be true as sedentary and exercised individuals show similar DAG content as well as reduced ceramide content in skeletal muscle (Amati et al. 2011). It is thought that neutral lipid synthesis, instead of concentration of lipid intermediate, may be causing the improvements in insulin sensitivity; that is enhanced clearance of intermediates into synthetic pathways. Research in this area has also shown a role for increases in FA partitioning into IMTG in reducing the inflammatory response seen in insulin-resistant situations (Schenk and Horowitz 2007). This is due to both the removal of pro-inflammatory FFA as they are partitioned into IMTG as well as the removal of lipid intermediates that can lead to activation of inflammatory responses (Schenk and Horowitz 2007).

Conclusions/Future Directions

At this time, knowledge regarding the exact role for SCD1 in mediating metabolic disease in skeletal muscle is lacking. It has been shown that decreases in the expression and activity of SCD1 track with improvements in various metabolic characteristics, including obesity/lipid partitioning as well as insulin resistance and inflammation. While obese and diabetic states both show increases in IMTG and concomitant increases in SCD1, the skeletal muscle of trained individuals also show similar effects. This begs the question, can SCD1 activity be both helpful and harmful.

In skeletal muscle, there appears to be a role for SCD1 in controlling the phosphorylation state of AMPK and subsequently for regulating FA oxidation. However, there are similar effects on AMPK when SCD1 is increased in exercising individuals. Either way, SCD1 appears to have direct affects on the insulin-signaling machinery through its affect of AMPK and the exact mechanism warrants further investigation. There also appears to be a role for SCD1 in controlling the expression of PTP-1b and its role in down-regulation of the insulin receptor and its substrates. PTP-1b appears to decrease with suppression of SCD1 activity and could be causative of the improvements shown in these models. This also warrants further investigation. Thirdly, a mechanism for SCD1 in either causing or suppressing an inflammatory response has been shown. This will become an important area of research as inflammatory processes are involved in many of the chronic diseases related to skeletal muscle metabolism.

Data demonstrates that there is a role for SCD1 in the modulation of skeletal muscle metabolism. However, there is disagreement whether this role is as a protective molecule preventing many of the aberrations caused by dysfunctional lipid metabolism, or whether SCD1 is a contributing factor whose malfunctioning activity is causing disease. It appears as though SCD1 may function on both sides of the argument, and this may be related to the particular metabolic environment by which SCD1 is being regulated. Research using skeletal muscle specific KO would allow for a better understanding of the molecule and its influences on the development and progression of metabolic disease. Of particular interest would be how these mice would adapt to endurance exercise. It is possible that these animals would have a decreased oxidative capacity and therefore a decreased ability to perform aerobic exercise. However, it is also possible that these animals would exhibit compensatory mechanisms that allow them to maintain correct handling and oxidation of FAs. Another research question involving a skeletal muscle SCD1 KO would be how these animals would adapt to a HF diet. It is possible that these animals would exhibit a phenotype similar to SCD1 KO in other tissues such as skin and liver in regards to insulin sensitivity given skeletal muscle's importance in whole-body glucose clearance and thermogenesis.

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Chapter 10
The Cellular Function of Stearoyl-CoA Desaturase-2 in Development and Differentiation

Jennifer L. Cantley, Lucas M. O'Neill, James M. Ntambi, and Michael P. Czech

Introduction

Stearoyl-CoA desaturase-2 (SCD2) is a key member of the murine Δ9-desaturase family and is documented to be the major isoform required for the early development of mouse (Miyazaki et al. 2005). Despite this, little is known about its physiological roles. Historically, SCD2 was labeled as "brain SCD," because at the time, SCD2 was thought to be the only SCD isoform expressed in the brain (Kaestner et al. 1989; DeWillie and Farmer 1992). Despite being highly expressed in the brain, the role of SCD2 in this organ has been largely unexplored. Today, we know SCD2 to be expressed ubiquitously and is the predominate isoform in tissues such as B lymphocytes, epidermis, lung, pancreas, spleen, testis, and ovaries (Sampath and Ntambi 2011a, b; Swick and Lane 1992; Saether et al. 2003). We are also beginning to grasp the temporal expression pattern of SCD2 and its role in adipocytes.

SCD2 is expressed in adult mice, but it is more highly expressed and critical during embryonic development and in newborn mice prior to weaning. Complete loss of SCD2 is embryonic lethal in the C57Bl6 genetic background (B6) (Miyazaki et al. 2005). In the 129SvEv genetic background (129), complete loss of SCD2 is

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      1                                                    50
hSCD1 MPAHLLODDI SSSYTTTTTI TAPPSRVLON GGDKLETMPL YLEDDIRPDI
mSCD2 MPAHILQ.EI SGAYSATTTI TAPPSGGQQN GGEKFEKSSH HWGADVRPEL

      51                                                  100
hSCD1 KDDIYDPTYK DKEGPS.... ..PKVEYVWR NIILMSLLHL GALYGITLIP
mSCD2 KDDLYDPTYQ DDEGPP.... ..PKLEYVWR NIILMALLHL GALYGITLVP

      101                                                 150
hSCD1 TCKFYTWLWG VFYYFVSALG ITAGAHRLWS HRSYKARLPL RLFLIIANTM
mSCD2 SCKLYTCLFA YLYYVISALG ITAGAHRLWS HRTYKARLPL RLFLIIANTM

      151                                                 200
hSCD1 AFONDVYEWA RDHRAHHKFS ETHADPHNSR RGFFFSHVGW LLVRKHPAVK
mSCD2 AFONDVYEWA RDHRAHHKFS ETHADPHNSR RGFFFSHVGW LLVRKHPAVK

      201                                                 250
hSCD1 EKGSTLDLSD LEAEKLVMFO RRYYKPGLLL MCFILPTLVP WYFWGETFQN
mSCD2 EKGGKLDMSD LKAEKLVMFO RRYYKPDLLL MCFVLPTLVP WYCWGETFVN

      251                                                 300
hSCD1 SVFVATFLRY AVVLNATWLV NSAAHLFGYR PYDKNISPRE NILVSLGAVG
mSCD2 SLCVSTFLRY AVVLNATWLV NSAAHLYGYR PYDKNISSRE NILVSMGAVG

      301                                                 350
hSCD1 EGFHNYHHSF PYDYSASEYR WHINFTTFFI DCMAALGLAY DRKKVSKAAI
mSCD2 ERFHNYHHAF PYDYSASEYR WHINFTTFFI DCMALLGLAY DRKRVSRAAV

      351        365
hSCD1 LARIKRTGDG NYKSG
mSCD2 LARIKRTGDG SCKSG
```

Fig. 10.1 Comparison of the amino acid sequences of the mouse SCD2 (mSDC2) and human SDC1 (hSDC1). There is very high sequence similarity between these mouse and human proteins, with highest variability in the N-terminus region. Both the N-terminus and the C-terminus regions of these proteins are thought to be cytoplasmic, while four putative transmembrane regions of these proteins are thought to cross the endoplasmic reticulum membrane. The variable N-terminus region is far removed from the more central segments containing the histidine regions that form the core of the catalytic domain, suggesting they may not be involved in substrate recognition (Zhang et al. 2005)

~70-100 % lethal at birth with few survivors (Miyazaki et al. 2005). In cultured 3T3L1 adipocytes, SCD2 is the only SCD isoform required for adipocyte differentiation (Christianson et al. 2007). The mechanism for this is currently unknown, but it is thought that SCD2 regulates the expression of PPARγ, a key determinant of the adipogenic process. In fact, knockdown of SCD2 is just as powerful at inhibiting adipocyte differentiation as silencing PPARγ itself (Christianson et al. 2007).

There are four known SCD isoforms in mouse (SCD1–4) and two in human (hSCD1 and hSCD5), (Beiragi et al. 2003; Man et al. 2005; Ntambi and Miyazaki 2004; Zhang et al. 1999) but only human SCD1 is considered an orthologue of the mouse SCD isoforms (Evans 2008; Wang et al. 2005). Both mouse SCD1 and SCD2 display approximately 80 % sequence identity to human SCD1 (Fig. 10.1), utilize the same substrates, and share common promoter elements (Fig.10.2) and tissue

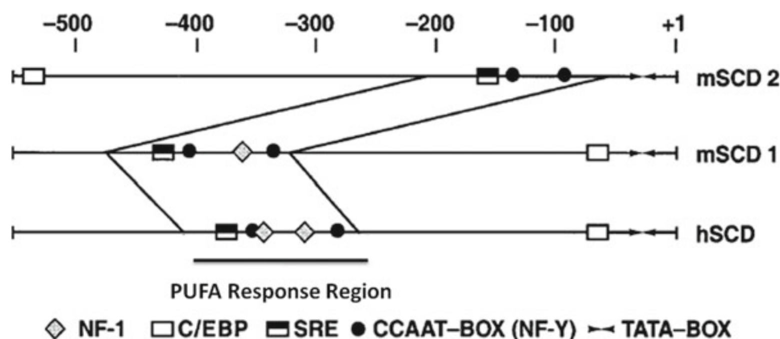

Fig. 10.2 Location of transcription factor recognition sites in the promoter regions of mouse SCD1 (mSCD1), mouse SCD2 (mSCD2), and human SCD1 (hSCD1). The differences in recognition sites and their locations between mSCD1 and mSCD2 likely explain in part their distinct tissue expression profiles, for example that mSCD2 is more highly expressed in embryonic and newborn liver than mSCD1. *NF* nuclear factor, *C/EBP* CCAAT enhancer binding protein, *SRE* serum response factor (Bene et al. 2001)

expression profiles (Hodson and Fielding 2012; Waters et al. 1997; Zhang et al. 2005, 1999). Similar to mouse SCD1 and SCD2, human SCD1 is also ubiquitously expressed with the highest expression in the adipose tissue (Hodson and Fielding 2012; Roongta et al. 2011; Zhang et al. 1999). Human SCD5 is highly expressed in the brain and pancreas, similar to mouse SCD2, despite not sharing functional homology (Wang et al. 2005). Functionally, human SCD1 may encompass the characteristics of both SCD1 and SCD2 in one enzyme: while mouse SCD1 and SCD2 appear to have redundant functions in most tissues, functional differences for these isoforms do appear to exist in adipose tissue, which may be fulfilled solely by SCD1 in humans (Christianson et al. 2007).

The study of SCD2 opens the door to discovering new lipid regulated developmental signaling pathways along with exploring new roles for monounsaturated fatty acids (MUFAs). The legacy of SCD2 is still developing and many questions regarding the physiological roles of this enzyme remain unanswered. Undoubtedly, SCD2 plays a unique and essential role. However, more research is warranted to determine the exact pathways that SCD2 affects and how to use this information in relation to human development.

This chapter will be a cumulative review about SCD2 that will include its regulation and its known physiological roles in different tissues.

Discovery of SCD2

During the initial identification of SCD1, southern blots were performed on 3T3-L1 genomic DNA using a variety of probes against multiple regions of the SCD gene. One probe in particular, containing the entire coding region of SCD1, was hybridized to 3T3-L1 genomic DNA that was collected 5 days after the addition of a

differentiation cocktail consisting of methylisobutylxanthine, dexamethasone, and insulin, and digested with EcoR1. Surprisingly, in addition to the three EcoR1 fragments that comprised the complete gene of SCD1, this probe hybridized to a 14- and 2.2-kb genomic DNA EcoRI fragment (Kaestner et al. 1989). The additional hybridizations could be explained by two possibilities; the 14- and 2.2-kb fragments could be a part of an SCD pseudogene, or secondly, the fragments could be representative of another SCD isoform (Ntambi et al. 1988).

It is at this point, in 1986, that Klaus Kaestner, a graduate student working with James Ntambi in M. Daniel Lane's laboratory at The Johns Hopkins School of Medicine, decided to further explore these unidentified fragments for the possibility that they represented a second SCD gene. Kaestner began his investigation with a clone, designated λE14, which was created during the initial cloning of SCD1 (Kaestner et al. 1989). It contained five exons that were very similar to those in the SCD1 gene, but they were not identical. In order to determine if this clone was a second SCD, Kaestner set out to find its corresponding cDNA. Using a probe containing part of the first exon (nucleotides 205–324), generated by the digestion of λE14 with *PstI*, he screened a mouse 3T3-L1 cDNA library. The longest clone purified from the cDNA library was 2.7kb fragment called λ32 (Kaestner et al. 1989).

The last 200 bp of λ32 was then used to probe the same mouse 3T3-L1 cDNA library, and from this a clone λ53, containing a sequence containing a poly(A)-track, was isolated (Kaestner et al. 1989). Together λ32 and λ53 represent the complete mRNA of 5 kb. This was subcloned and sequenced using dideoxy chain termination. The sequence encoded for a protein that was >87 % similar to SCD1 and was named SCD2 (Kaestner et al. 1989).

SCD2 *Regulation*

Unlike *Scd1*, which is located on the negative strand of chromosome 19, *Scd2* and the other two SCD family members, *Scd3* and *Scd4*, are found on the positive strand of chromosome 19 (Man et al. 2005). *Scd2* is ~15 kb in length and has six exons, five introns, and a uniquely large 3′ UTR that is indicative of the SCD family (Kaestner et al. 1989). Most often, genes that have a long 3′ UTR, including the other SCD isoforms, have multiple polyadenylation signals, so it is surprising that SCD2 has only one such signal. To date, the function of this long 3′ UTR remains unknown.

Many of the regulatory components of *Scd2* were discovered by the differential tissue expression patterns of SCD2 and SCD1 in response to diet. SCD1 and SCD2 are both induced by diets low in unsaturated fatty acids. However, they are induced in different tissues. For example, mice fed a low-fat diet induce SCD1, but not SCD2, expression in the liver; in contrast, this diet induces SCD2 expression in the kidney to a much higher extent than SCD1 (Kaestner et al. 1989). The differences in tissue expression and dietary induction were thought to be differences in the control of gene expression. Kaestner recognized that the promoters of SCD2 and SCD1 are significantly different and that these differences may have an effect on the

expression and induction differences between the two isoforms (Kaestner et al. 1989). A detailed examination of the promoters would come in the years that followed his initial work, but at the time, Kaestner was able to determine that SCD2 has two conserved CCAAT boxes found in SCD1 and that SCD2 has a missing cAMP core regulatory sequence that is present in the promoter of SCD1 (Fig. 10.2) (Kaestner et al. 1989).

During the differentiation of 3T3L1 preadipocytes into adipocytes, genes involved in fatty acid uptake, synthesis, and storage are activated (Christianson et al. 2007; Hodson and Fielding 2012). In 1992, Andrew Swick and M. Daniel Lane discovered that SCD2 contains a preadipocyte repressor element (PRE) site in the promoter (between −435 and −410) that is targeted by a ~58 kDa PRE binding protein causing transcription repression (Swick and Lane 1992).

In 1998, David Tabor and Peter Edwards determined that SCD2 is regulated by the nuclear levels of the pro-lipogenic transcription factor, sterol regulatory element binding protein (SREBP) (Tabor et al. 1999). They also identified a novel sterol regulatory element (SRE) located within the proximal promoter of SCD2 that does not comprise of the usual C/TCAC/T repeats that are separated by a single nucleotide (Fig. 10.2) (Tabor et al. 1999). Additionally, this group found that either a mutation in the SRE or the CCAAT boxes could attenuate SCD2 expression in response to sterols (Tabor et al. 1999). This demonstrated that that sterol-induced transcription is both NF-Y- and SREBP-dependent.

SCD2 Function in the Skin

SCD2 appears to be critical for lipid synthesis during neonatal development, since the SCD2 null mice have a severe skin permeability barrier defect and decreased triglyceride and FFA (free fatty acid) levels in the liver, skin, and plasma. This lack in lipid synthesis appears to be critical for survival because while the SCD2 null mice are born with the expected frequency, >70 % of them die within 24 h after birth when the genetic background is 129SvEv and 100 % die during embryonic development when the genetic background is C57Bl6. Most likely the lethality displayed in the 129SvEv genetic backgound is due to severe dehydration from the skin permeability dysfunction.

The SCD2 null mice that do survive are significantly smaller than the wild-type mice and have reduced body weights. The newborn SCD2 null mice have dry, cracked skin due to the skin permeability barrier defect, with a tightly packed and thickened epidermal stratum corneum. Examination of the lamellar membrane system in the skin reveals there are normal numbers of lamellar bodies in the knockout mice, but a decrease in their internal contents and a delay in lamellar membrane formation. While the epidermis has increased saturated FFA content, it has significantly decreased levels of cholesterol esters, triglyceride, acylceramide, and glucosylceramide, which are the primary constituents of lamellar bodies. Therefore, there appears to be decreased lipid delivery to lamellar granules, leading to deficient deposition of lipids in the stratum corneum interstices.

Additionally, while the total content of linoleic acid is not altered, there is a 80 % decrease of linoleic acid in the acylceramide fraction and an increase of this fatty acid in the phospholipid fraction. Since acylceramide is a major lipid involved in epidermal barrier function, decreasing its content or altering its composition will affect membrane barrier integrity. The epidermal lipid composition also showed the expected decrease in palmitoleate and oleate levels in total lipids of SCD2 null mice, with corresponding increases in saturated fatty acid levels. These differences occurred despite a several fold increase in SCD1 expression in both the epidermis and dermis of the SCD2 null mice, presumably a response to the loss of SCD2. Depletion of SCD2 also decreased the expression of keratinocyte differentiation genes transglutaminase-1 and involucrin, indicating that SCD2 products may play a role in gene expression programming during development. Nonetheless two genes that function in modulating the skin permeability barrier, glucosidase beta and diacylglycerol acyltransferase-2, were not altered in their mRNA levels. Together, these studies indicate that SCD2 is crucial for proper epidermal lipid synthesis, lipid channeling into different epidermal cellular compartments, and skin permeability barrier formation in neonate mice.

SCD2 Function in the Liver

While SCD1 regulates hepatic triglyceride synthesis in adult mice, SCD2 controls hepatic lipid metabolism in embryonic and newborn mice. This concept is consistent with the finding that hepatic total SCD catalytic activity is decreased by 70 % in newborn SCD2 null mice compared to wild-type mice, but not different between adult SCD2 null mice and age-matched controls. In the absence of SCD2, there is a near 50 % reduction in hepatic and plasma triglyceride levels and a 27 % reduction in total hepatic FFA in newborn mice. The decrease in triglyceride content appears to be due to decreased triglyceride synthesis, since ^{3}H-glycerol incorporation after IP injection is 54 % reduced in the triglyceride fraction[11]. However, there are no differences in the plasma glucose concentration, hepatic glycogen content, or hepatic phospholipid and cholesterol ester content. Also, there are no differences in the hepatic phospholipid synthesis rate or in the phospholipid composition as seen in the epidermis of the knockout mice. In the mice that survive past weaning, the defects in lipid synthesis are normalized due to increased SCD1 expression. Therefore, SCD2 is required for appropriate FFA, triglyceride, and VLDL production in the liver of embryos and neonates, but not in adult mice.

SCD2 Function in the Adipose Tissue

In adult mice, SCD1 is highly expressed in white adipose tissue depots and regulates MUFA production (Miyazaki et al. 2001; Ntambi and Miyazaki 2004). However, SCD2 is also expressed in adipose tissue, despite the abundant expression

of SCD1. Evidence suggests that SCD1 and SCD2 may have disparate cellular functions in the white adipose tissue, as opposed to the liver and skin, where SCD1 appears to compensate for SCD2 function. It was reported that in mice fed a high-fat diet there is a large fold induction of SCD2 mRNA expression, much larger than effects on SCD1. In fact, out of 26 lipid metabolizing enzymes examined, SCD2 mRNA expression increased in per cent more than any other enzyme. One possible explanation for this observation is that SCD2 may be required to metabolize particular fatty acid species under high-fat feeding conditions. However, since increasing obesity on a high-fat diet also promotes adipogenesis, the process whereby preadipocyte fibroblasts differentiate into fully mature adipocytes, a role for SCD2 in controlling adipocyte development in the mouse is also possible.

Consistent with this latter hypothesis, studies in 3T3-L1 adipocytes indicate that significant levels of SCD2, but not of SCD1, are required for adipogenesis and for the maintenance of the adipocyte phenotype in fully differentiated adipocytes. The process of adipogenesis occurs through a transcriptional cascade beginning with the rapid and transient expression of C/EBPβ and C/EBPδ. These transcriptional factors then induce the expression of the ligand-activated receptor, PPARγ, and C/EBPα, which are critical for the completion of adipogenesis as well as the maintenance of adipocyte gene expression in fully differentiated cells (Ishida-Yamamoto and Kishibe 2011; Tang and Lane 2012). Recent experiments showed that siRNA-mediated depletion of SCD2 prevented PPARγ mRNA induction during adipogenesis and blocked the conversion of fibroblasts to adipocytes as indicated by oil red O staining of neutral lipid accumulation. Remarkably, silencing SCD2 was as powerful at inhibiting adipocyte differentiation as silencing PPARγ in this cultured preadipocyte system. In line with this interpretation, GLUT4 expression in 3T3-L1 cells was inhibited by about 90 % when either SCD2 or PPARγ were depleted with siRNA prior to the induction of differentiation. PPARγ protein levels were also reduced by about 90 % upon siRNA-mediated silencing of SCD2 under these same conditions. This defective adipogenesis observed upon SCD2 depletion was not restored by the addition of the PPARγ-specific ligand, rosiglitazone, suggesting SCD2 is not controlling the production of a PPARγ ligand.

In fully differentiated adipocytes, SCD2 regulates PPARγ protein expression, rather than transcript levels, which are unchanged after SCD2 depletion. It appears that PPARγ translation is inhibited in the absence of SCD2, while degradation is not affected. This downregulation of PPARγ translation and protein expression then leads to decreased expression of PPARγ target genes and loss of adipocyte function (Fig. 10.3). However, the effect of SCD2 depletion on protein synthesis is not specific to PPARγ since it appears SCD2 is required to maintain general protein synthesis. In fact, [^{35}S] methionine/cysteine labeling shows a small 15 % decrease in newly synthesized protein in 3T3-L1 adipocytes depleted of SCD2 by siRNA silencing. However, unlike PPARγ, many proteins actually decrease at the transcript level, while others increase. Thus, in the mature adipocytes, lack of SCD2 decreases PPARγ protein, which in turn decreases expression of downstream genes important for maintaining the adipocyte phenotype, such as SCD1, fatty acid synthase, and acetyl CoA carboxylase (Fig. 10.3).

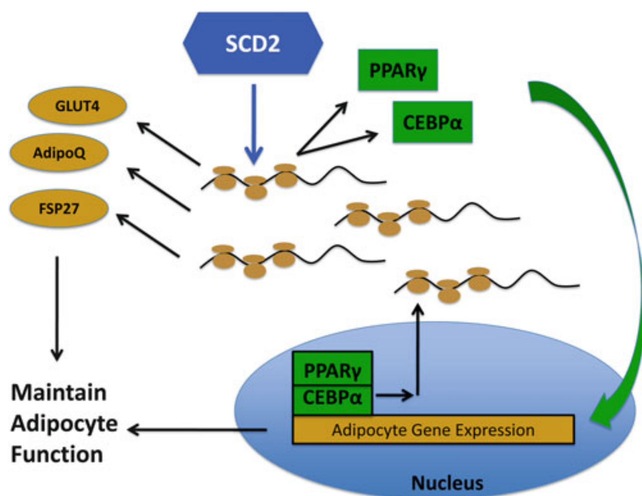

Fig. 10.3 Hypothetical model whereby mSCD2 may function directly or indirectly to selectively stimulate protein translation in cultured adipocytes. Such an action of mSCD2 protein or products that it produces increase PPAR**γ** and CEBPα protein-levels (among other proteins), that in turn function to promote expression of genes [e.g., GLUT4 glucose transporter, Fat-specific protein (FSP) 27, Adiponectin (AdipoQ)] required for adipocyte differentiation. See text and Christianson et al. (2007)

The mechanism by which SCD2 regulates PPARγ mRNA expression in preadipocytes and protein translation in fully differentiated adipocytes remains a mystery. It may be through the production of an SCD2-specific unsaturated fatty acid or the proper shuttling of a fatty acid, as seen in the SCD2 knockout mouse, that regulates the translational machinery. Another possibility is that SCD2 is necessary for a protein–protein interaction that regulates translation. Regardless of the mechanism, these studies have described a distinct function for SCD2, apparently divergent from SCD1, in 3T3-L1 adipocytes. This may also be the case in other mouse tissues, explaining why these two isoforms are often simultaneously expressed. Furthermore, it is possible that human SCD1 also controls adipogenesis and adipocyte function, a hypothesis that deserves testing in future experiments.

SCD2 Expression in Immune Cells

Stearic acid potently inhibits the growth and proliferation of T lymphocytes, whereas B lymphocytes display a high degree of immunity (Pourbohloul et al. 1985; Pourbohloul and Buttke 1990). The reason for the differences in T lymphocyte and B lymphocyte susceptibility can be attributed to their Δ9-desaturase activity. B lymphocytes exclusively express SCD2 to a high level, and in contrast T lymphocytes do not have any detectable SCD transcript (Tebby and Buttke 1992).

Accordingly, when B and T lymphocytes are treated with stearate in vitro, the B cells are able to desaturate up to 25 % of the stearate into oleate, whereas the T cells are not able to desaturate stearate (Buttke et al. 1989). The inability of T cells to desaturate stearate causes the T cell to display 2–4 times more stearate in distearoylphosphatidylcholine than B cells. This level is equivalent to the amount of distearoylphosphatidylcholine that causes hemolysis of erythrocytes (Kuypers et al. 1984). Our knowledge of the role SCD2 in mouse immunology is limited. However, changes in the levels of dietary fat can profoundly alter immune responses in vivo, and it has been suggested that T cells are the predominate sensitive immune cell population (Buttke et al. 1989).

SCD2 Function in Reproduction

Functional testes and ovaries are in part dependent on the proper balance of fatty acids. This balance is critical for maintaining membrane fluidity and motility of sperm, and for membrane fluidity and the proper lipid reserve in the oocytes (Moreau et al. 2005; Saether et al. 2003). Although these alone can be attributed to the abundance and distribution of 22:6(*n*-3), functional sperm also require a balanced composition of *n*-3, *n*-6, and *n*-9 fatty acids. SCD2 is the predominate Δ9 desaturase expressed in the testes and ovaries and is responsible for the Δ9-desaturase activity in these organs (Moreau et al. 2005; Saether et al. 2003).

The roles of palmitoleate and oleate in the testis are not clear. However, the expression pattern of SCD1 and SCD2 within the testis has been established. SCD1 and SCD2 are both highly expressed in the epididymis, whereas only SCD2 is predominate in the testis, germ cells, and Sertoli cells (Saether et al. 2003). Sertoli cells are also known as “nurse cells” for their function of providing nutrients, such as PUFAs, to developing sperm cells. They are thought to be the key provider of PUFAs to the germ cells (Saether et al. 2003). Since SCD2 is highly expressed in the Sertoli cells, it is possible that in addition to providing PUFAs, the Sertoli cells also provide the germ cells with MUFAs (Saether et al. 2003).

The regulation of SCD1 and SCD2 within the testis is very interesting. SCD2 expression is up-regulated by insulin and dexamethasone while SCD1 is down-regulated (Saether et al. 2003). This is surprising, given the opposite effect these hormones have on SCD1 in the liver, where SCD1 is up-regulated by insulin and dexamethasone and SCD2 is down-regulated. One such explanation for this difference is that the regulation may reflect the roles that the testis and liver play in fatty acid metabolism. The primary role of SCD1 in the liver is to provide MUFAs for export, whereas the primary role of SCD2 in the testis is to replenish lost MUFAs (Saether et al. 2003).

Another key regulator of desaturase activity in the testis is follical stimulating hormone (FSH). FSH stimulates both SCD2 and SCD1 to very high levels in the Sertoli cells (Saether et al. 2003). It is reported that in the developing testis, FSH enhances the desaturase expression to levels needed for proper spermatogenesis (Saether et al. 2003).

Female oocytes of a variety of vertebrates have a high abundance of MUFAs, mainly palmitoleate and oleate, in the form of a lipid reserve that is critical for reproduction (Moreau et al. 2005). Much of the work analyzing the expression and regulation of SCD2 in the ovaries was by Céline Moreau and Joëlle Dupont (Moreau et al. 2005). In agreement with the work done on rat testis, by the Haugen group, the level of *Scd2* mRNA was high and predominate in whole ovary compared to *Scd1*, while the opposite is true for liver (Moreau et al. 2005; Saether et al. 2003). Both SCD1 and SCD2 are expressed in the granulosa cells of large follicles, corpus luteum, and cumulus oophorus of the ovary, but SCD2 is predominate in all of these tissues (Moreau et al. 2005). The cumulus cells provide nutrition for the oocyte and given expression of SCD2 is absent in the oocyte (Moreau et al. 2005); it can be speculated that SCD2 is the main depositor of MUFAs within that tissue. Also, SCD2 is highly expressed within the granulosa cell of the cumulus and is virtually absent from the oocytes (Moreau et al. 2005), which supports the hypothesis that lipids are transported into the oocytes from the cumulus. In the ovary *SCD2* is increased during follicular development and is positively regulated by pregnancy hormones that affect follicular development such as eCG and hCG as well as the hormones FSH and IGF1 (Moreau et al. 2005). Using rat granulosa cells to investigate the molecular mechanisms involving IGF1- and FSH-induced SCD2 expression, it was determined that the MAPK3/MAP1 and AKT signaling are involved in inducing expression, respectively (Moreau et al. 2005). This is in agreement with evidence suggesting that MAP3K/MAP1 and AKT pathways are involved in granulosa cell proliferation and steroidgenesis (Moreau et al. 2005).

Human Relevance

The two known human SCD isoforms are hSCD1 and hSCD5. hSCD1 shares high amino acid sequence similarity to SCD2. However hSCD5, also shares a high amino acid sequence similarity to SCD2, but unlike hSCD1, hSCD5 is expressed in a tissue specific manner similar to SCD2 (Beiragi et al. 2003; Dobrzyn and Ntambi 2005; Miyazaki et al. 2006; Wang et al. 2005). In addition, it was discovered that a pericentric inversion of hSCD5 was found in two generations of a family with cheiloschisis or cleft lip (Beiragi et al. 2003). Although defects in SCD2 and hSCD5 produce different tissue morphologies (Beiragi et al. 2003; Miyazaki et al. 2005), there is a strong connection between MUFA synthesis and tissue development.

Conclusions

In recent years it has become evident that SCD2 and the other isoforms of SCD are playing distinct and nonredundant roles. In this chapter we examined the known function of SCD2, both tissue specifically and temporally. Future research on SCD2

will be exciting as there are many unanswered questions about how exactly MUFAs influence early development, tissue morphogenesis, signaling pathways, and adipocyte differentiation.

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Chapter 11
Stearoyl-CoA Desaturase Isoforms 3 and 4: Avenues for Tissue-Specific Δ9 Desaturase Activity

Laura M. Bond and James M. Ntambi

Introduction

Murine stearoyl-CoA desaturase (SCD) isoform 1 was discovered in 1985 by James Ntambi while studying adipocyte differentiation in 3T3-L1 cells (Ntambi et al. 1988). SCD is a critical enzyme in the de novo lipogenesis pathway and catalyzes the Δ9 desaturation of saturated fatty acids to yield monounsaturated fatty acids (MUFAs). As illustrated in the previous chapters of this book, tremendous progress has been made in elucidating the regulation and function of SCD isoform 1. However, in contrast to SCD1, additional isoforms of SCD have been relatively unexplored. Suggestion of multiple SCD isoforms in mice arose just one year after the discovery of *Scd1*, when southern blotting of digested genomic DNA against probes made against *Scd1* revealed DNA bands that did not correspond to the single known *Scd* isoform (Kaestner et al. 1989). After further studies, *Scd2* was isolated and characterized, but a potential third isoform was not investigated until 2001 (Kaestner et al. 1989; Zheng et al. 2001).

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SCD3

Over a decade after the initial discovery of *Scd1*, *Scd3* was reported by scientists at Johnson & Johnson who were studying lipid metabolism in skin to aid developments in pharmaceutics (Zheng et al. 2001). The group was interested in the unique phenotypes of the asebia mouse and was trying to determine the exact location of the mutation that causes alopecia and defects in sebaceous glands (Sundberg et al. 2000). They mapped the asebia mutation to *Scd1*, but in the process of characterizing this mutation, Zheng et al. 2001 identified a previously unreported isoform, which they named *Scd3*.

Tissue Expression

Scd3 is unique to mice and has not been identified in humans, which express h*SCD1* and h*SCD5* (Wang et al. 2005). Zheng and colleagues established with Northern blotting that *Scd3* is expressed in differentiated sebocytes (Zheng et al. 2001). *Scd1*, on the other hand, is expressed in undifferentiated sebocytes, and *Scd2* expression is restricted to the hair follicle (Paton and Ntambi 2009).

The preliminary tissue panel by Zheng et al. surveyed several tissues for expression of *Scd3* by hybridizing tissue blots containing mRNA from 12 tissues with *Scd3* specific probes. Their results suggested that *Scd3* is exclusively expressed in the skin. However, later studies in the mouse harderian gland and preputial gland revealed the expression of *Scd3* in these esoteric tissues (Miyazaki et al. 2001, 2002). Both of these tissues are highly lipogenic; the harderian gland is located within the orbit of the eyeball and is proposed to have a myriad of functions, such as the secretion of hormones and neutral lipids for lubrication (Payne 1994). The preputial glands are anterior to the genitals and are important for reproduction (Thody et al. 1976). *Scd3* expression has also been detected in murine liver, but only in conditions of SREBP-1a overexpression (Horton et al. 2003).

Recent publications report the expression of *Scd3* in subcutaneous white adipose tissue (Bostrom et al. 2012; Yu et al. 2010). Notably, the Speigelman laboratory reported that irisin, a myokine that induces white adipose "beiging", induces expression of *Scd3* (Bostrom et al. 2012).

The detection of *Scd3* expression in white adipose tissue is not entirely surprising, as *Scd3* expression has been reported in 3T3-L1 cultured pre-adipocytes undergoing differentiation (Wang et al. 2008). In fact, *Scd3* exhibits a unique temporal expression pattern. *Scd1* and *Scd2* are expressed in differentiated adipocytes (Kaestner et al. 1989). In contrast, *Scd3* expression is transient, appearing around day 2 and vanishing by day 7 (Wang et al. 2008). Importantly, *Scd3* is not expressed in cultured mature adipocytes. The significance of *Scd3* expression in adipocyte differentiation has not been explored, but it may account for its detection in adipose tissue of adult mice. Alternatively, *Scd3* may be expressed in mature adipocytes in vivo, but cultured mature adipocytes derived from fibroblasts do not express *Scd3* once differentiated, illustrating the limitations of in vitro models.

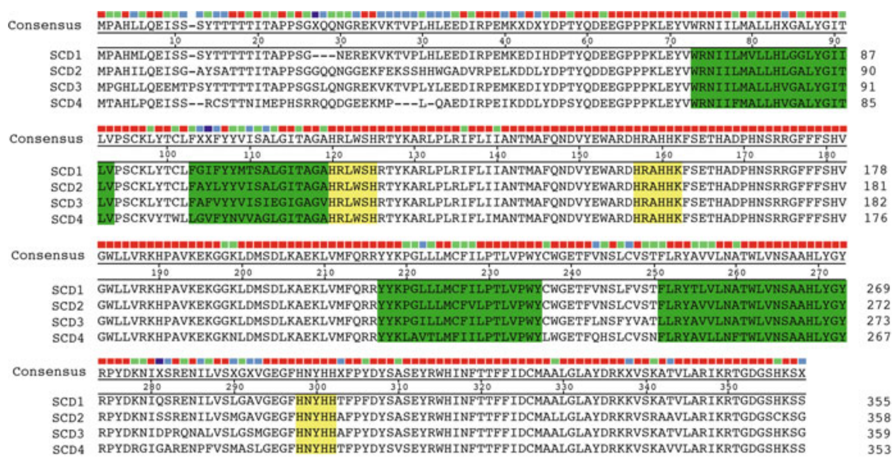

Fig. 11.1 Conservation analysis of murine SCD protein sequences. All murine SCD isoforms contain four transmembrane regions (*green*) and three cytosolic histidine motifs (*yellow*). These histidine residues coordinate elemental iron and are essential for catalytic activity (Shanklin et al. 1994). The histidine motifs are completely conserved in all four isoforms. Degree of conservation is depicted as red (high), green (medium), and blue (low) in the color bar. The consensus sequence is shown below the color bar. Sequence alignment was completed using DNAStar MegAlign ClustalW analysis

Table 11.1 Murine SCD isoforms share strong protein sequence identity

	SCD1	SCD2	SCD3	SCD4
SCD1	–	86	88	79
SCD2	86	–	85	79
SCD3	88	85	–	82
SCD4	79	79	82	–

Percent sequence identity was determined using NCBI BLAST

Protein Structure and Substrate Specificity

SCD3 contains four transmembrane domains and the three histidine motifs that are essential for catalytic activity (Fig. 11.1) (Shanklin et al. 1994). SCD3 shares 88 % and 84 % protein sequence identity with SCD1 and SCD2, respectively (Table 11.1). According to the proposed topology of mouse SCD1, the N and C termini are oriented toward the cytosol (Man et al. 2006).

Despite the strong structural similarities between SCD isoforms 1, 2, and 3, SCD3 exhibits unique substrate specificity. SCD1 and SCD2 introduce a double bond between carbons 9 and 10 of both palmitate and stearate, whereas SCD3 preferentially desaturates palmitate. The first indication that SCD3 desaturates primarily 16:0 came from studies in the mouse harderian gland. SCD1$^{-/-}$ mice show a complete loss

of desaturation toward stearoyl-CoA, but some desaturation toward palmitoyl-CoA remains (Miyazaki et al. 2001). This provides reason to suspect that a harderian gland-specific palmitoyl-CoA Δ9 desaturase must be catalyzing the synthesis of the 16:1(*n*-7) that remains. In 2006, Miyazaki et al. determined the substrate specificity of all four SCD isoforms (Miyazaki et al. 2006). A yeast strain that is deficient in the yeast Δ9 desaturase, OLE1, was transformed with plasmids encoding each mouse SCD gene and then treated with saturated fatty acyl-CoAs of specific lengths. Lipids were extracted and fatty acids were identified with gas chromatography. Yeast given the mouse SCD3 expression vector exhibited no conversion of 18:0 to 18:1(*n*-9), but rather desaturated 16:0 and 14:0. The fatty acid products demonstrated that SCD3 primarily catalyzes the conversion of 16:0 to 16:1(*n*-7). The authors also transfected HeLa cells, which have low basal Δ9 desaturase activity, with the SCD isoforms cloned into a mammalian expression vector. Gas chromatography revealed the identity of the fatty acid products and confirmed that SCD3 primarily catalyzes the conversion of 16:0 to 16:1(*n*-7). The unique substrate specificity of SCD3 is of particular interest in the metabolism field because 16:1(*n*-7) has been proposed to be a lipid-derived hormone, or lipokine, that elicits beneficial metabolic effects. In 2008, this lipokine was reported to improve insulin sensitivity and prevent hepatic steatosis (Cao et al. 2008). However, the effects of 16:1(*n*-7) are controversial and subsequent studies question the original results (Guo et al. 2012; Hodson and Karpe 2013). In vivo and in vitro studies modulating SCD3 activity may elucidate the specific effects of this fatty acid and its participation in tissue cross-talk.

Gene Structure and Regulation

Like other SCD isoforms, *Scd3* maps to chromosome 19 D2 (Fig. 11.2a), has six exons and five introns, and encodes a transcript ~4.9 kb in length (Fig. 11.2b) (Zheng et al. 2001). Scd3 has distinct 5' and 3' noncoding regions and intronic sequences that are significantly longer than those of *Scd1*, *2*, and *4* (Fig. 11.2b).

DNA sequence analysis of the *Scd3* promoter demonstrated that *Scd3* shares a few common regulatory elements with *Scd1* and *Scd2* but lacks several distinct transcription factor binding sites. For example, *Scd3* promoter has two noncanonical TATA boxes, a feature shared with *Scd1*. However, *Scd3* lacks the PUFA response region, SREBP site, and the CHOP/CEBP α-heterodimer binding site, all three of which are present on the promoter of *Scd1* and *Scd2*. Other distinguishing features of *Scd3* include the presence of an estrogen receptor binding site, which is absent in the promoters for *Scd1* and *Scd2*.

Transcription factor binding sites related to sex hormones and determination were identified in the 5' upstream regulatory sequence of *Scd3*, which is consistent with the observation that *Scd3* expression in skin is higher in males than females (Zheng et al. 2001). *Scd3* contains sites for estrogen receptor and sex-determining Y gene product that could facilitate gene repression and activation, respectively, to yield increased expression in males. Consistent with these findings, subcutaneous injection of

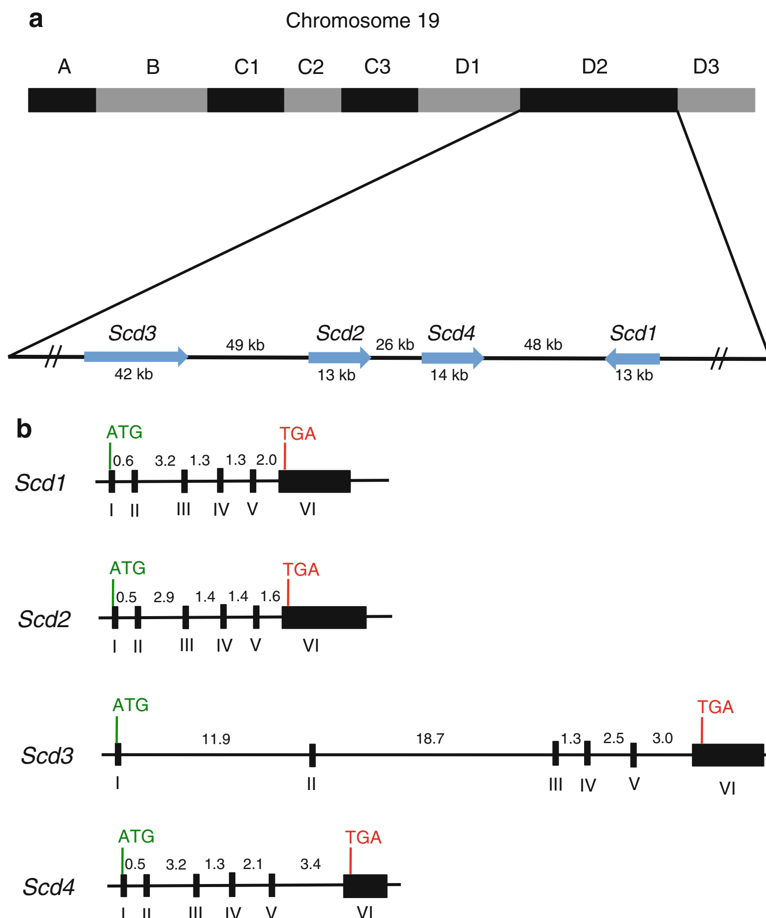

Fig. 11.2 (**a**) All murine SCD isoforms are present on chromosome 19 region D2. *Scd3*, *Scd2*, and *Scd4* are transcribed in the forward direction, while *Scd1* is transcribed in the reverse direction. (**b**) *Scd* isoforms 1-4 contain five introns and six exons. The majority of exon VI is noncoding due to a stop codon that is present towards the 5′ end of the exon

testosterone in mice has been shown to induce *Scd3* expression in the preputial gland; however, the specific transcription factor binding site has not yet been identified (Miyazaki et al. 2002).

Studies in SCD1$^{-/-}$ mice show that loss of SCD1 activity represses *Scd3* expression. The Ntambi lab showed that *Scd3* expression is significantly reduced in the skin, harderian gland, and preputial glands of mice that lack *Scd1* in these tissues (Miyazaki et al. 2001, 2002; Sampath et al. 2009). Zheng et al. also observed a strong reduction of *Scd3* expression in adult *Scd1*-mutant asebia mice, but detected *Scd3* expression in young asebia mice (Zheng et al. 2001). The authors propose that loss of *Scd3* expression may stem from destruction of the sebocytes, when SCD3 activity is not able to

compensate for loss of SCD1 and rescue the sebaceous gland. While this hypothesis is consistent with the requirement for SCD1 activity in sebocyte differentiation, it likely does not account for the observed loss of *Scd3* expression in the harderian and preputial glands of SCD1$^{-/-}$ mice, as these glands maintain their core lipogenic properties and do not exhibit severe phenotypes in the absence of *Scd1*.

SCD products may directly or indirectly regulate transcription, but the mechanism underlying the regulation between isoforms is not well understood. Studies in SCD3$^{-/-}$ knockout mice may reveal if SCD3 activity regulates SCD1 or SCD2 gene expression and forms a positive or negative feedback loop.

SCD4

Discovered in 2003, SCD4 is the most recently identified SCD isoform (Miyazaki et al. 2003). Suggestion of a fourth SCD isoform arose from studies in the *ob/ob* mouse that revealed a paradoxical increase in cardiac MUFA levels despite unaltered expression of *Scd1* and *Scd2* (Miyazaki et al. 2003). The unknown source of Δ9 desaturation was probed and led to the identification of SCD isoform 4. Northern blot analysis of a standard tissue panel, including heart, brain, liver, skeletal muscle, and lung, demonstrated that *Scd4* is expressed in the heart (Miyazaki et al. 2003).

Protein Structure and Function

SCD4 shares 79 % protein sequence identity with SCD1 and SCD2 and 82 % sequence identity with SCD3 (Table 11.1). SCD4 contains the three histidine motifs required for catalytic activity, and hydropathy measurements suggest that all SCD isoforms have four transmembrane domains (Miyazaki et al. 2003) (Fig. 11.1). The conservation of the structural elements in SCD4 that are required by SCD1 suggests that SCD4 possesses similar Δ9 desaturase activity (Shanklin et al. 1994). To demonstrate the activity of SCD4 in vitro, mammalian cells with low endogenous desaturase activity were transfected with a plasmid containing *Scd4*. Microsomal fractions of these cells exhibited Δ9 desaturase activity. Analysis of fatty acid products demonstrated that SCD4 has substrate specificity similar to SCD1 and SCD2 and desaturates both stearate and palmitate (Miyazaki et al. 2003).

Gene Structure and Regulation

Scd4 maps to chromosome 19 region D2 and is located upstream of *Scd2* on the positive strand. *Scd4* is transcribed in the forward direction along with *Scd2* and *Scd3*, but in the reverse direction of *Scd1* (Fig. 11.2a). The predominant *Scd4*

transcript spans 3.1 kb, which is shorter than the other *Scd* isoforms (Fig. 11.2b). Two smaller *Scd4* transcripts, 2.8 and 1.5 kb long, were also detected (Miyazaki et al. 2003). These minor transcripts may result from GAAA repeats in exon 6 that predispose the nascent transcript to premature polyadenylation. It is unknown if these truncated *Scd4* transcripts encode functional desaturase enzymes.

Gene regulation studies constitute the bulk of SCD4 characterization and provide a few clues to the function of this novel isoform. Miyazaki and colleagues selected several key regulatory elements known to modulate murine *Scd1* expression in liver and investigated their effect on *Scd4* expression in the heart (See Hodson and Fielding, 2013 for extensive review of *Scd1* regulation) (Miyazaki et al. 2003). They learned that *Scd4* shares some regulatory patterns with *Scd1* and *Scd2* in the heart but is also subject to unique control mechanisms.

Feeding mice a high-carbohydrate, low-fat diet upregulates both *Scd4* and *Scd1* expression in the heart (Miyazaki et al. 2003; Ntambi 1992). This suggests that *Scd4* is a target of the pro-lipogenic transcription factor, SREBP-1c, which drives *Scd1* expression, but the SREBP binding site, SRE, was not found on the *Scd4* promoter. This element may exist further upstream in an enhancer region not yet explored. Alternatively, the induction of *Scd4* expression after high-carbohydrate feeding could stem from gene activation by ChREBP, another pro-lipogenic transcription factor that was not well-defined at the time of the study (Yamashita et al. 2001). Dietary supplementation of fish oil, a rich source of polyunsaturated fatty acids (PUFAs), decreases *Scd1* and *Scd2* expression, but does not affect expression of *Scd4* (Miyazaki et al. 2003). This dichotomy is consistent with the presence of a PUFA response element on the promoters of *Scd1* and *Scd2*, but not on the *Scd4* promoter. LXR agonists activate expression of *Scd1*, *Scd2*, and *Scd4*, suggesting that all of the isoforms in the heart can be regulated by cholesterol (Miyazaki et al. 2003).

Leptin negatively regulates *Scd4* in the heart, as demonstrated in the *ob*/*ob* mouse studies that led to the discovery of SCD4 (Miyazaki et al. 2003). Leptin is a hormone secreted by adipose tissue that has garnered much attention for its anorectic properties, but its repression of lipogenic genes shows that this adipokine has additional features distinct from its function in satiety (Cohen et al. 2002; Friedman and Halaas 1998). *ob*/*ob* mice have increased cardiac SCD activity and, subsequently, increased MUFA and triglyceride content (Christoffersen et al. 2003; Miyazaki et al. 2003). These phenotypes normalize with intravenous treatment of leptin, and gene expression studies revealed that SCD4—not SCD1 or SCD2—mediates the fatty acid changes observed in the heart (Miyazaki et al. 2003). SCD4 most likely plays a predominant role in the pathogenesis of “fatty heart” that is exhibited in *ob*/*ob* mice. The exact mechanism by which leptin represses *Scd4* expression in the heart has not been determined, but transcriptional reporter assays may reveal the relevant binding region. While *Scd1* and *Scd2* expression are not regulated by leptin in the heart (Miyazaki et al. 2003), the transcriptional repression of *Scd1* and *Scd2* expression by this adipokine has been established in mouse liver (Cohen et al. 2002). Further studies will identify tissue-specific factors that mediate the differential regulation of these isoforms in various organs.

The tissue-specific expression pattern and regulation of *Scd4* expression helps validate the function of having several SCD isoforms. Regulation between the isoforms would further justify having multiple isoforms with high protein sequence identity. Upon the discovery of *Scd4*, Miyazaki and colleagues investigated potential compensatory mechanisms between the predominant heart *Scd* isoforms. $SCD1^{-/-}$ mice exhibit a 3-fold increase in *Scd4* expression (Miyazaki et al. 2003). This compensatory mechanism wherein *Scd4* acts in place of SCD1 explains why $SCD1^{-/-}$ mice have a less dramatic decrease in MUFAs in the heart than in the liver.

Summary

SCD isoforms 3 and 4 have not been fully explored. However, preliminary studies demonstrate that these novel isoforms have unique regulatory properties that manifest in restricted tissue expression. *Scd3* is expressed mainly in the skin, harderian gland, and preputial gland, and *Scd4* is expressed in the heart. Exploring SCD3 and SCD4 will help explain if 16:1(*n*-7) or 18:1(*n*-9) derived from a specific tissue, such as skin or heart, affects the function of these fatty acids as signaling molecules. Since SCD3 desaturates primarily palmitate to yield palmitoleate, a proposed lipokine, this isoform may be a tool for studying increased or decreased levels of this specific MUFA.

These unexplored SCD isoforms are now active sites of investigation. The recent development of SCD3 and SCD4 global knockout mouse models are current tools helping researchers shed light on the nuanced functions of these SCD isoforms. We expect the contents of this chapter to broaden and grow over the next decade.

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Chapter 12
Functional Development of Stearoyl-CoA Desaturase Gene Expression in Livestock Species

Stephen B. Smith

Introduction

Foods high in oleic acid (18:1*n*-9), including oleic acid-enriched beef, reduce risk factors for cardiovascular disease (Kris-Etherton et al. 1999; Adams et al. 2010; Gilmore et al. 2011). Higher concentrations of oleic acid also are positively correlated with overall palatability of beef (Westerling and Hedrick 1979), whereas increased stearic acid (18:0) is the primary determinant of fat hardness (i.e., lipid melting point; Smith et al. 1998; Wood et al. 2004; Chung et al. 2006b). Therefore, increasing the monounsaturated fatty acid (MUFA) to saturated fatty acid (SFA) ratio would increase healthfulness, palatability, and fat softness of animal products. The conversion of SFA to MUFA by the fatty acid Δ^9 desaturase accounts for the majority of MUFA in muscle and adipose tissues of livestock species, i.e., the edible portions of beef, lamb, and pork carcasses (St John et al. 1991; Klingenberg et al. 1995; Lengli and Corl 2007; Jiang et al. 2008; Duckett et al. 2009). The Δ^9 desaturase is encoded by stearoyl-coenzyme A desaturase (SCD), which also is responsible for the conversion of *trans*-vaccenic acid to its corresponding CLA isomer, 18:2 *cis*-9, *trans*-11 CLA (Miyazaki and Ntambi 2003). Several studies have indicated that *SCD* gene expression and/or SCD activity is elevated in adipose tissues containing a high concentration of MUFA (Archibeque et al. 2005; Chung et al. 2007; Duckett et al. 2009; Brooks et al. 2011).

In many species, the concentration of oleic acid in adipose tissue reflects the relative proportion of oleic acid in the diet (St John et al. 1987; Go et al. 2012), but in ruminants such as beef cattle and sheep, dietary oleic acid is hydrogenated largely to stearic acid by ruminal microorganisms before reaching the small intestine (Ekeren et al. 1992). Thus, any accumulation of oleic acid in tissues of ruminants is

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dependent on SCD catalytic activity. In this review we describe the genetic, developmental, and nutritional regulation of *SCD* gene expression and catalytic activity and fatty acid composition in tissues of livestock species.

SCD Tissue Distributions in Livestock Species

In laboratory rodents, *SCD* is expressed in both liver (Ntambi 1992; Waters and Ntambi 1994) and adipose tissue (Jones et al. 1996; Kang et al. 2004), although SCD activity is at least two orders of magnitude higher in mouse liver than in adipose tissue (Enser 1979). Bovine, ovine, and porcine adipose tissues exhibit substantially higher SCD catalytic activity than muscle, liver, or intestinal mucosa (Payne and Masters 1971; St John et al. 1991; Chang et al. 1992; Klingenberg et al. 1995; Archibeque et al. 2005) (Fig. 12.1). *SCD* gene expression is virtually undetectable in bovine liver (Cameron et al. 1994; Brooks et al. 2011), although *SCD* mRNA is readily detectable in porcine liver (Go et al. 2012). Chicken liver exhibits high SCD activity (Legrand and Lemarchal 1991; Legrand and Hermier 1992), although neither SCD activity nor *SCD* gene expression have been reported for

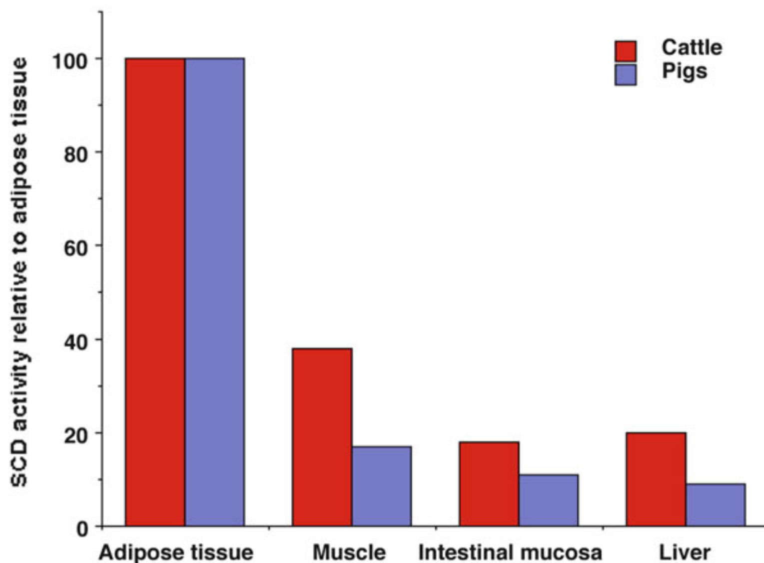

Fig. 12.1 Relative SCD catalytic activities in adipose tissue, longissimus muscle, duodenal intestinal mucosa, and liver of cattle and pigs. Data are expressed relative to adipose tissue activity, and are averages of values reported previously (St John et al. 1991; Chang et al. 1992; Cameron et al. 1994; Klingenberg et al. 1995; Page et al. 1997; Archibeque et al. 2005)

chicken adipose tissue. Consistent with the low hepatic SCD activity in pigs and cattle, the livers of these species are especially high in stearic acid and low in oleic acid (St John et al. 1991; Brooks et al. 2011; Go et al. 2012). Whereas liver lipids of mice contain approximately 12 % stearic acid (Kang et al. 2004), liver lipids of cattle and pigs contain over 25 % stearic acid (St John et al. 1991; Brooks et al. 2011; Go et al. 2012).

The low rates of *SCD* gene expression and SCD activity in bovine, ovine, and porcine liver are consistent with very low rates of de novo fatty acid biosynthesis in the livers of livestock species, relative to adipose tissue depots (Hanson and Ballard 1967; Ingle et al. 1972; Mersmann et al. 1976; Go et al. 2012). Similarly, subcutaneous adipose tissue has approximately twice the SCD activity of marbling adipose tissue (Archibeque et al. 2005), which is consistent with the relative concentrations of MUFA in subcutaneous and marbling adipose tissues (Sturdivant et al. 1992; May et al. 1993; Brooks et al. 2011).

Within an animal, there is a good relationship between *SCD* gene expression and/or SCD activity and tissue fatty acid composition. However, these comparisons do not appear to hold when comparisons are made across breed types. Thus, although Wagyu cattle consistently exhibit higher MUFA concentrations in their muscle and adipose tissues than other breed types (Sturdivant et al. 1992; May et al. 1993; Zembayashi et al. 1995; Siebert et al. 2003), it has not been possible to establish differences in *SCD* gene expression or SCD activity between Wagyu and Angus steers (Cameron et al. 1994; Chung et al. 2007).

SCD Genetic Variation Across Livestock Species

SCD *Chromosomal Locations in Livestock Species*

Although several *SCD* isoforms have been reported for mice, the *SCD*-1 isoform is the most abundant in lipogenic tissues and is common to most species. The sole porcine *SCD* gene, which has been mapped to chromosome 14 (Uemoto et al. 2012), has >80 % homology with other mammalian *SCD* genes (Ren et al. 2004). Similarly, the ovine *SCD* gene, mapped to chromosome 22, is approximately 90 % homologous to bovine and porcine *SCD* genes (Lengli and Corl 2007). The bovine *SCD* gene was mapped to chromosome 26 (BTA26) (Chung et al. 2000; Campbell et al. 2001). Linkage analysis placed the *SCD* gene 3.6 cM distal to microsatellite HEL11 and 2.5 cM proximal to microsatellite BM1314 on chromosome 26 in a comparison of Angus and Brahman populations (Campbell et al. 2001). As in humans, the only other *SCD* isoform identified in livestock species is *SCD-5*, which has 90 % homology with human *SCD-5* (Lengli and Corl 2007; 2008). *SCD-5* gene expression is primarily limited to the brain, although low levels of *SCD-5* mRNA have been detected in liver and muscle.

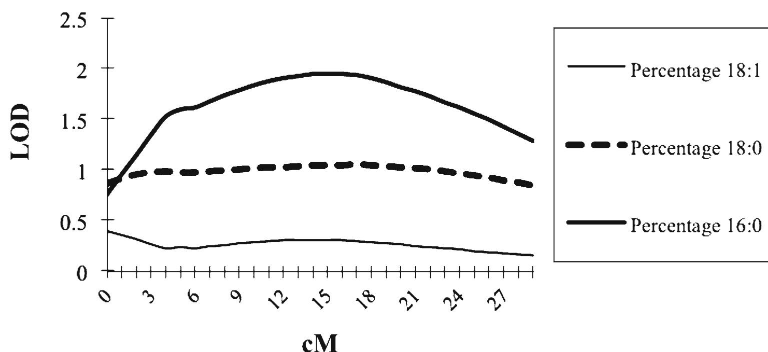

Fig. 12.2 Interval maps for fatty acid composition on BTA26. Palmitic acid approached an LOD score of 2.0, whereas stearic acid and oleic LOD scores were 1.0 and 0.3, indicating no significant quantitative trait loci for these fatty acids on BTA26. *SCD* maps to between 2.5 and 3.6 cm. S. B. Smith and E. M. Campbell, unpublished data

SCD *and Fatty Acid Quantitative Trait Loci*

Interval maps were generated for percentage palmitic acid (16:0), stearic acid, and oleic acid on BTA26 (Fig. 12.2). A quantitative trait locus (QTL) for palmitic acid on BTA26 approached an LOD score of 2.0, whereas LOD scores for stearic acid and oleic QTL were lower. An LOD score of 2.4 was equivalent to an overall *P*-value of 0.05 (Lander and Botstein 1989), so none of the QTL for fatty acids were significant in the region where we mapped the bovine *SCD* gene.

Earlier, we reported significant QTL for stearic acid and oleic acid 18 cM from the centromere on bovine chromosome BTA19 (Taylor et al. 1998). Individuals homozygous for Angus alleles possessed 2.4 % less stearic acid and 3.7 % more oleic acid in their subcutaneous adipose tissue than individuals homozygous for Brahman alleles. However, subcutaneous adipose tissue stearic acid and oleic acid composition did not differ between ¾ Angus:¼ Brahman progeny and ¼ Angus:¾ Brahman progeny from the same cattle population (Smith et al. 2009a, b). Thus, only small differences exist in fatty acid composition between *Bos indicus* and *Bos taurus* breed types.

SCD *Gene Polymorphisms in Asian Cattle*

Large variation in fatty acid composition has been reported between Japanese Black, Korean Hanwoo cattle and the US domestic cattle (Sturdivant et al. 1992; May et al. 1993; Smith et al. 2001, 2009a, b; Chung et al. 2006b). Japanese Black (Wagyu) cattle and Korean Hanwoo cattle share a common ancestry, and they both exhibit high MUFA:SFA ratios in their muscle and adipose tissues (Smith et al. 2009a, b). Although *SCD* gene expression and SCD catalytic activity do not differ between

Wagyu and Angus steers (Cameron et al. 1994; Chung et al. 2007), total MUFA proportions in Wagyu adipose tissue typically are 10 percentage points higher than in the US domestic cattle (reviewed in Smith et al. 2009a, b). Early research with Wagyu cattle reported the existence of *SCD* restriction site polymorphisms (Wilson et al. 1993), and we subsequently reported that significant microsatellite variation exists that is associated with adipose tissue fatty acid composition across geographically isolated populations of Japanese Black cattle (Smith et al. 2001).

Taniguchi et al. (2004) demonstrated single-nucleotide polymorphisms (SNPs) in the open-reading frame of an *SCD* cDNA generated from Japanese Black cattle, in which valine was replaced with alanine. The VA and AA genotypes contributed to higher MUFA and lower melting points in intramuscular adipose tissue than the more infrequent VV phenotype. Matsuhashi et al. (2011) and Yokota et al. (2012) subsequently demonstrated that *SCD* polymorphisms contributed to variations in fatty acid composition and varied across populations of Japanese Black cattle, confirming our earlier observations (Smith et al. 2001). Mannen (2011) confirmed that genetic polymorphisms of the *SCD* and fatty acid binding-protein genes are in part responsible for differences in fatty acid composition within the Japanese Black breed type. Similarly, *SCD* polymorphisms affect fatty acid composition in Japanese Holstein (dairy) cattle (Narukami et al. 2011) and Korean Hanwoo steers (Lee et al. 2008; Oh et al. 2011; Maharani et al. 2012).

SCD *Gene Polymorphisms in Pigs*

Similar associations of *SCD* polymorphisms with fatty acid composition have been reported for pigs (Bartz et al. 2013; Uemoto et al. 2012; Wang et al. 2013). Two groups (Bartz et al. 2013; Uemoto et al. 2012) have identified SNPs in the *SCD* promoter region in pigs that were associated with fatty acid composition, and Uemoto et al. (2012) identified QTL for 14:0, 18:0, and 18:1*n*-9 located near the porcine *SCD* gene.

Adipose Tissue Differentiation and *SCD* Gene Expression and Activity

Bovine Preadipocyte Culture Studies

Casimir and Ntambi (1996) first demonstrated that *SCD* gene expression increased immediately preceding lipid filling in murine 3T3-L1 preadipocytes, and we have demonstrated essentially identical results for bovine preadipocytes (Chung et al. 2006a; Fig. 12.3). Stromal-vascular cells were obtained by collagenase treatment of perirenal adipose tissue from mature Angus steers, were plated at a density of 10^4

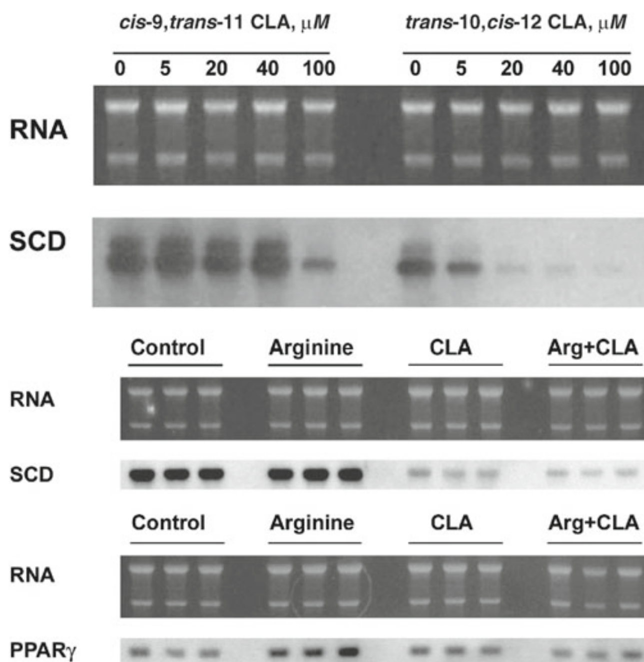

Fig. 12.3 Stearoyl-CoA desaturase gene expression in Angus preadipocytes differentiated in the presence of 5 μM pioglitazone, 10 μg/mL insulin, and DMEM. *Top panel*, preadipocytes were differentiated for 7 days with PI in the presence of *cis*-9, *trans*-11or *trans*-10, *cis*-12 CLA. *Bottom panel*, preadipocytes were differentiated in differentiation medium (Control) or with 5 mM arginine in the absence or presence of 40 μM *trans*-10, *cis*-12 CLA. From Chung et al. (2006a)

cells, and were grown to confluence. At confluence, the medium was supplemented either with insulin plus pioglitazone (a PPARγ agonist) or insulin alone. Prior to confluence, *SCD* mRNA was undetectable, but after 7 days of exposure to insulin plus pioglitazone, *SCD* mRNA and lipid-filled adipocytes were highly abundant.

Ntambi and coworkers demonstrated that the *trans*-10, *cis*-12 isomer of CLA strongly depresses *SCD* gene expression in 3T3-L1 preadipocytes (Choi et al. 2000). In our bovine preadipocyte cell line, *trans*-10, *cis*-12 CLA nearly abolished SCD gene expression, whereas *cis*-9, *trans*-11 CLA was without effect except at the highest concentrations (Fig. 12.3). Although *trans*-10, *cis*-12 CLA also strongly depressed lipid filling, it had no effect on *PPARγ* gene expression in bovine preadipocytes. Conversely, media arginine increased *PPARγ* gene expression, but not *SCD* gene expression, indicating that *PPARγ* and *SCD* gene expression are regulated independently (Chung et al. 2006a).

The depression of *SCD* gene expression by *trans*-10, *cis*-12 CLA is unusual in light of the fact that this CLA isomer is a product of rumen fermentation, and its accumulation would effectively block the conversion of *trans*-vaccenic acid (18:1*trans*-11, a primary product of ruminal fermentation) to *cis*-9, *trans*-11 CLA. Clearly, any production practice that profoundly increases the formation and

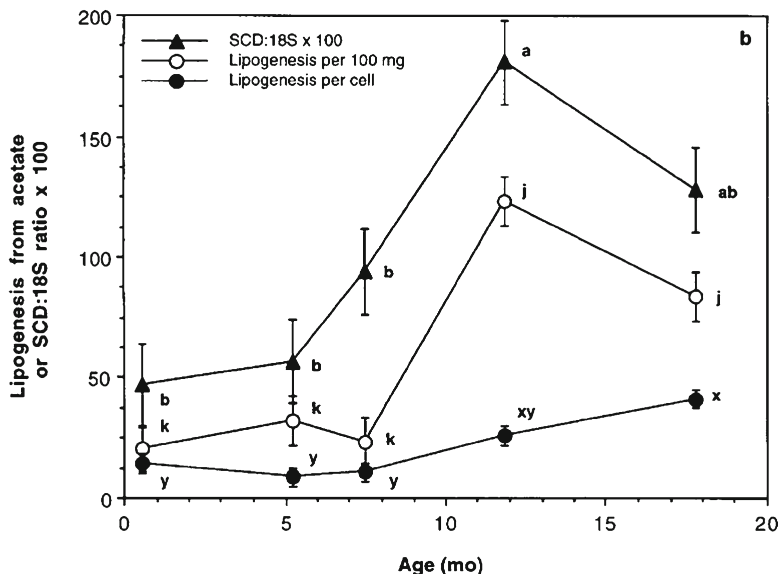

Fig. 12.4 Changes in *SCD* gene expression and lipogenesis during growth in preweaning (2.5 weeks to 7.5 months of age) and postweaning Angus steers. Overall standard errors of the means are affixed to the symbols. [ab,jk,xy]Values within a measurement with the same superscript are not different ($P > 0.05$). Reprinted with permission from Martin et al. (1999)

absorption of *trans*-10, *cis*-12 CLA from the digestive system will depress adipogenesis, and will cause adipose tissue lipids to become more saturated and contain less *cis*-9, *trans*-11 CLA.

Animal Studies

Previous research (Huerta-Leidenz et al. 1996; Chung et al. 2006b) demonstrated that adipose tissue MUFA increase over time. We first demonstrated that *SCD* gene expression attained a plateau at 12 months of age in subcutaneous adipose tissue of steers fed a corn-based finishing diet (Martin et al. 1999) (Fig. 12.4). The rate of de novo fatty acid biosynthesis increased gradually after weaning in adipose tissue of steers, but the rate lagged behind the elevation in *SCD* gene expression (Martin et al. 1999). As in bovine adipose tissue, *SCD* gene expression increased over time in contemporary and obese pigs (Smith et al. 1999) (Fig. 12.5). Adipose tissue *SCD* gene expression was less in contemporary pigs than in obese pigs, and it is less in pigs fed a low-fat, grain-based diet than in pigs fed a high-fat, milk-based diet. Changes in *SCD* gene expression over time were consistent with increases in subcutaneous adipocyte volume, such that there is a highly significant correlation between adipocyte volume and *SCD* gene expression ($R^2 = 0.79$, $P < 0.001$) (Fig. 12.5).

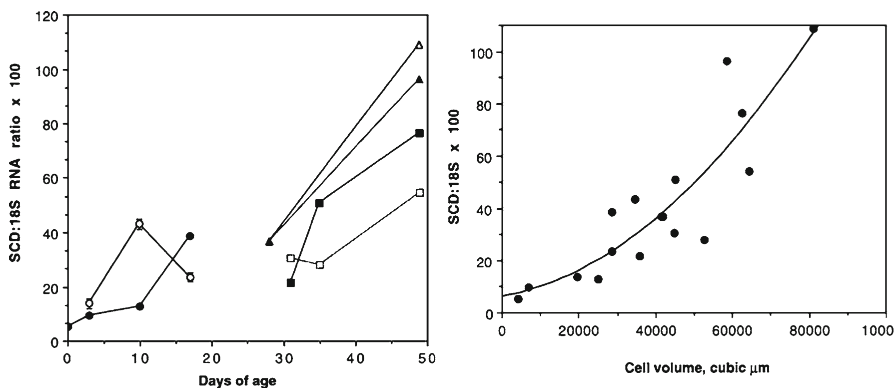

Fig. 12.5 Changes in *SCD* gene expression (*left panel*) and the relationship between *SCD* gene expression and adipocyte volume (*right panel*) in obese (*open symbols*) and contemporary crossbred pigs (*closed symbols*). *Circles*, suckling pigs; *triangles*, pigs fed a high-fat, milk-based diet; *squares*, pigs fed a low-fat, grain-based diet. There was a highly significant correlation between adipocyte volume and *SCD* gene expression ($R^2=0.79$, $P<0.001$). Reprinted with permission from Smith et al. (1999)

The results of these early studies indicated that *SCD* gene expression was coordinated with adipogenesis in cattle and pigs.

A subsequent study (Chung et al. 2007) demonstrated that SCD activity in bovine subcutaneous adipose tissue increased between 16 and 24 months of age in corn-fed steers but not in steers that had been fed a combination of hay and corn (Fig. 12.6). *SCD* gene expression did not change between 16 and 24 months of age in corn-fed steers, but de novo fatty acid biosynthesis from acetate declined sharply (Fig. 12.6). Thus, in this group of animals, fatty acid biosynthesis declined sharply while SCD activity increased. Although feeding the hay/corn depressed overall growth rate as compared to corn-fed steers (Lunt et al. 2005), de novo fatty acid synthesis actually increased between 20 and 28 months of age in the hay/corn-fed steers (Fig. 12.6). Conversely, SCD activity did not increase and *SCD* mRNA decreased. These cattle were raised to larger body weights (625 kg) and much greater adiposity than cattle used in the earlier study (490 kg; Martin et al. 1999), and the data indicates that the regulation of *SCD* gene expression becomes dissociated from de novo fatty acid biosynthesis in overly fattened cattle.

Lee et al. (2008) observed peak *SCD* mRNA at 12 months of age in muscle from Hanwoo steers, and Wang et al. (2013) demonstrated peak *SCD* gene expression at 20 and 25 months of age, respectively, in Piedmontese × Hereford and Wagyu × Hereford crossbred cattle. Smith et al. (2012) demonstrated that *SCD* gene expression attained a peak at 14 months of age and then declined by 16 months of age in brisket (cranial chest) adipose tissue but increased between 9 and 16 months of age in adipose tissue overlying the chuck (shoulder) (Fig. 12.7). *C/EBPβ*, *CPTI*, and *PPARγ* peak mRNA levels were observed at 14 months of age in both adipose tissue depots, and expression of these genes declined profoundly by 16 months of age. Collectively, these data and others (Lee et al. 2008; Brooks et al. 2011;

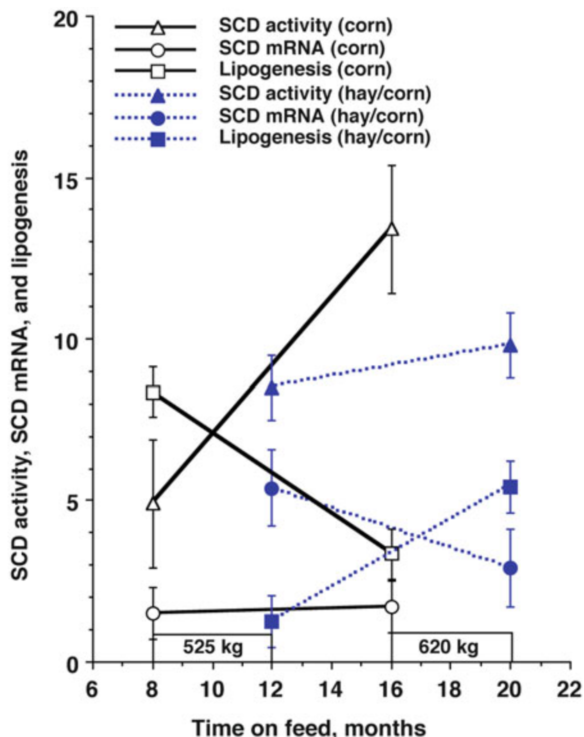

Fig. 12.6 Stearoyl-CoA desaturase catalytic activity (*triangles*), relative mRNA levels (*circles*), and lipogenesis from acetate (*squares*) in subcutaneous adipose tissue of Angus steers fed a corn-based diet (*open symbols*) or hay/corn diet (*filled symbols*). The corn-based diet was designed to yield an average daily gain of 1.36 kg/day and the hay-based diet was supplemented the same corn-based diet to yield 0.9 kg/day. By design, steers fed the corn-based diet for 8 months or the hay/corn-based diet for 12 months (16 and 20 months of age, respectively) weighed 525 kg at sampling. Steers fed the corn-based diet for 16 months or the hay/corn-based diet for 20 months (24 and 28 months of age, respectively) weighed 620 kg at sampling. SCD activity is expressed as nmol/(min·mg protein); *SCD* mRNA is the *SCD*:28S ratio; lipogenesis in nmol/(2 h·100 mg adipose tissue). Pooled standard errors are affixed to the means ($n=4$ for each data point). Adapted from Lunt et al. (2005) and Chung et al. (2007)

Wang et al. 2013) indicate that *SCD* gene expression declines in older, fatter cattle during that period in time in which adipose tissue becomes senescent.

Changes over time in the palmitoleic acid:stearic ratio, an index we have proposed to estimate SCD activity (Chung et al. 2006b; Smith et al. 2009a; Turk and Smith 2009; Smith et al. 2012), were essentially opposite to the pattern observed for *SCD* gene expression in adipose tissues overlying the brisket and chuck (Fig. 12.7). There is a highly significant, negative correlation between adipose tissue palmitoleic and stearic acid (Fig. 12.8), which indicates that the concentrations of palmitoleic acid and stearic acid in adipose tissue are coordinately controlled by SCD activity. Additionally, adipose tissue exhibiting high palmitoleic:stearic acid ratios

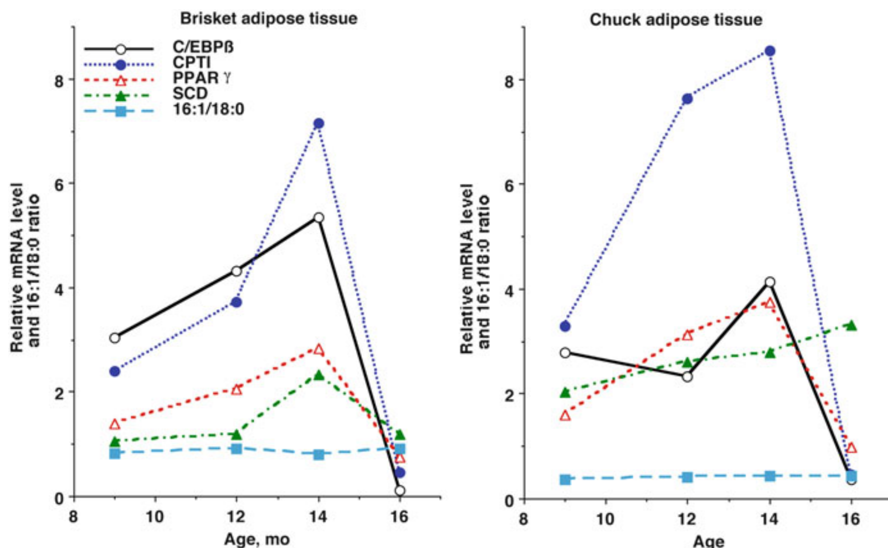

Fig. 12.7 Changes in relative gene expression and the palmitoleic:stearic acid ratio in brisket (*left panel*) and chuck (*right panel*) adipose tissue depots of Angus steers sampled at 9, 12, 14, and 16 months of age. For data pooled over time, *SCD* gene expression was twice as high ($P=0.01$) in chuck as in brisket, whereas the palmitoleic:stearic acid ratio was twice as high ($P=0.01$) in brisket as in chuck. There were no other adipose tissue main effects for relative mRNA amounts. Adapted from Smith et al. (2012)

also are enriched with total MUFA. However, *SCD* gene expression in brisket adipose tissue was only 50 % of the level observed in adipose tissue from the chuck, yet the palmitoleic:stearic acid ratio was twice as high in the brisket as in the chuck. Thus, in adipose tissue overlying the brisket, there is an apparent disconnect between this fatty acid index of SCD activity and *SCD* gene expression. We previously demonstrated that tailhead subcutaneous adipose tissue develops initially as brown adipose tissue (Landis et al. 2002), and brown adipose tissue is unusually concentrated in MUFA (Chen et al. 2007). Because it overlies vital organs, brisket subcutaneous adipose tissue may develop very early as brown adipose tissue, during which time it would have actively converted SFA to MUFA. Thus, the palmitoleic:stearic ratio, and probably other fatty acid indexes of SCD activity (Archibeque et al. 2005), provide at best an index of past *SCD* gene expression and SCD activity.

Regulation of SCD Activity by Fatty Acids

Polyunsaturated fatty acids (PUFAs) decrease *SCD* mRNA in mice and rats (Ntambi 1992; Landschulz et al. 1994). Similarly, feeding fish oil (high in *n*-3 PUFA) depresses *SCD* gene expression in beef cattle (Waters et al. 2009). In cattle and

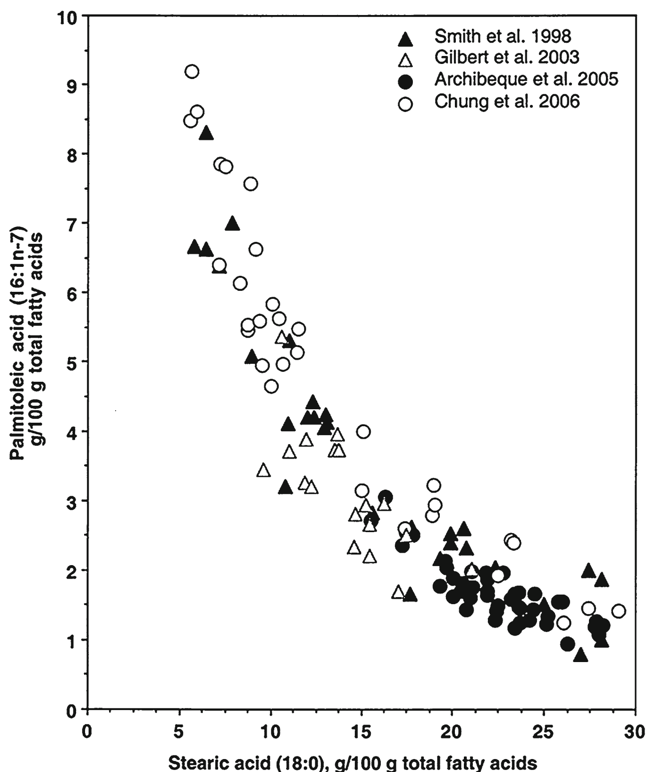

Fig. 12.8 Relationship between stearic acid (18:0) and palmitoleic acid (16:1*n*-7) in subcutaneous adipose tissue lipids of cattle raised in Australia and Japan (Smith et al. 1998) or in the USA (Gilbert et al. 2003; Archibeque et al. 2005; Chung et al. 2006b). Cattle raised in Australia were crossbred Murray Grey, Angus, and Grey Brahman steers fed a grain-based diet for a minimum of 400 days, whereas those raised in Japan were Murray Grey and Japanese Black fed for approximately 570 days. Cattle raised in the USA were Brangus (Gilbert et al. 2003), Angus (Archibeque et al. 2005; Chung et al. 2006b), or American Wagyu (Chung et al. 2006b)

sheep, changes in the ruminal environment in response to grain feeding also may contribute an increase in plasma and adipose tissue PUFA, thereby leading to a decrease in *SCD* gene expression in cattle. Kucuk et al. (2001) reported that increasing dietary forage increased duodenal flow of stearic acid and α-linolenic acid, but decreased the duodenal flow of oleic and linoleic acid. Conversely, feeding a high-corn diet could lower rumen pH sufficiently to decrease *trans*-vaccenic acid and palmitic acid by decreasing protozoal populations (Devillard et al. 2006; Or-Rashid et al. 2007). A prolonged reduction in rumen pH also could cause reductions in the population of bacteria responsible for rumen biohydrogenation (Fukuda et al. 2006; van de Vossenberg and Joblin 2003; Wallace et al. 2006), causing greater flow of linoleic acid (and less stearic acid) to the small intestine.

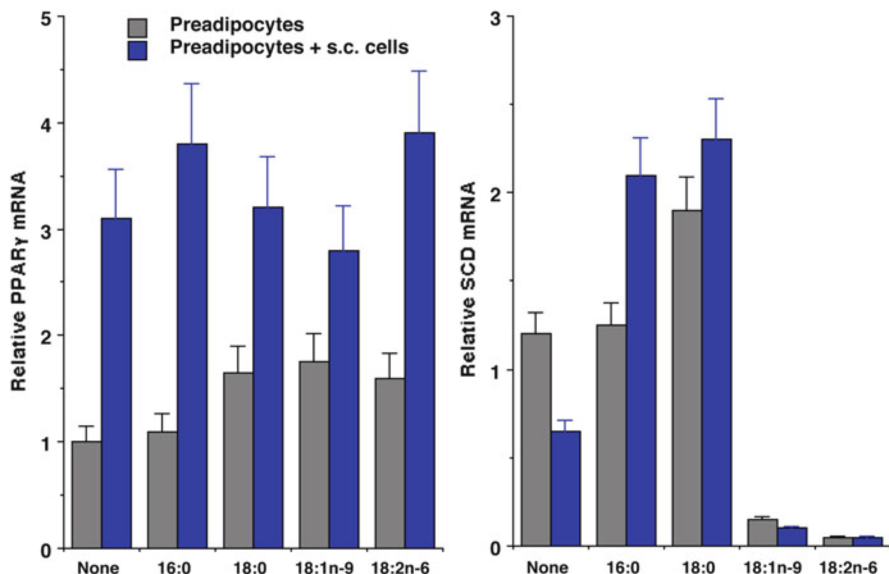

Fig. 12.9 Relative PPARγ (*left*) and SCD gene expression (*right*) in bovine subcutaneous preadipocytes cultured singly or co-cultured with satellite cells (myogenic precursor cells). Single or co-cultured cells were incubated for 7 days medium containing insulin plus ciglitazone or this medium containing 40 μM of the fatty acids indicated. Co-culture with subcutaneous (s.c.) cells increased PPARγ mRNA levels in subcutaneous preadipocytes. In co-cultured preadipocytes, palmitic acid (16:0) and stearic acid (18:0) significantly increased *SCD* mRNA levels, whereas oleic acid (18:1*n*-9) and linoleic acid (18:2*n*-6) strongly depressed *SCD* gene expression in single- and co-cultured preadipocytes (Smith, Johnson, and Choi, unpublished data)

Chung et al. (2006b) demonstrated that the duodenal concentrations of stearic acid and *trans*-vaccenic acid decreased, and linoleic acid and *cis*-9, *trans*-11 CLA increased, with increasing time on a corn-based, finishing diet. Thus, in cattle fed a corn-based, finishing diet, there is an apparent depression in the isomerization of linoleic acid to *cis*-9, *trans*-11 CLA as well as a decrease in the hydrogenation of *cis*-9, *trans*-11 CLA to *trans*-vaccenic acid and hence to stearic acid. These changes in duodenal fatty acid concentrations are reflected in tissue fatty acid compositions (Chung et al. 2006b).

It is possible that linoleic acid contributes to the depression in *SCD* gene expression observed in mature feedlot cattle (Brooks et al. 2011; Smith et al. 2012; Wang et al. 2013). Duodenal linoleic acid essentially doubles, and plasma linoleic acid increases by 50 % between 8 and 16 months of age in cattle fed grain-based diets (Brooks et al. 2011), leading to a significant increase in adipose tissue linoleic acid. Similar results were reported by Chung et al. (2006b). Linoleic acid nearly abolishes *SCD* gene expression in bovine subcutaneous preadipocytes (Fig. 12.9); therefore, linoleic acid could be the primary causative agent in depressing *SCD* gene expression in adipose tissue of mature feedlot cattle.

Regulation of *SCD* Gene Expression by Oleic Acid

It also is possible that circulating oleic acid and/or stearic acid may be responsible for the depression in *SCD* gene expression observed in mature cattle. Oleic acid was nearly as effective as linoleic acid (both 40 μM) in reducing *SCD* gene expression in bovine subcutaneous preadipocytes, although neither oleic nor linoleic acid affected *PPARγ* gene expression at this concentration (Fig. 12.9). In intramuscular preadipocytes, oleic acid as low as 1 μM increased *PPARγ*, whereas only 500 μM oleic acid increased *PPARγ* gene expression subcutaneous preadipocytes (Fig. 12.10). Oleic acid strongly depressed *SCD* gene expression in intramuscular and subcutaneous preadipocytes, as well as bovine muscle satellite cells (myoblasts) at concentrations above 10 μM (Fig. 12.10).

Ovine and porcine *SCD* genes contain completely conserved PUFA response elements (PPRE) (Zulkifli et al. 2010), and it is likely that an identical PPRE exists in the bovine *SCD* promoter region. Also, the PPRE appears to be nearly as sensitive to oleic acid as it is to linoleic acid. This indicates that increasing the adipose tissue concentration of oleic acid, either by endogenous synthesis or uptake from the circulation, would down-regulate *SCD* gene expression in livestock species.

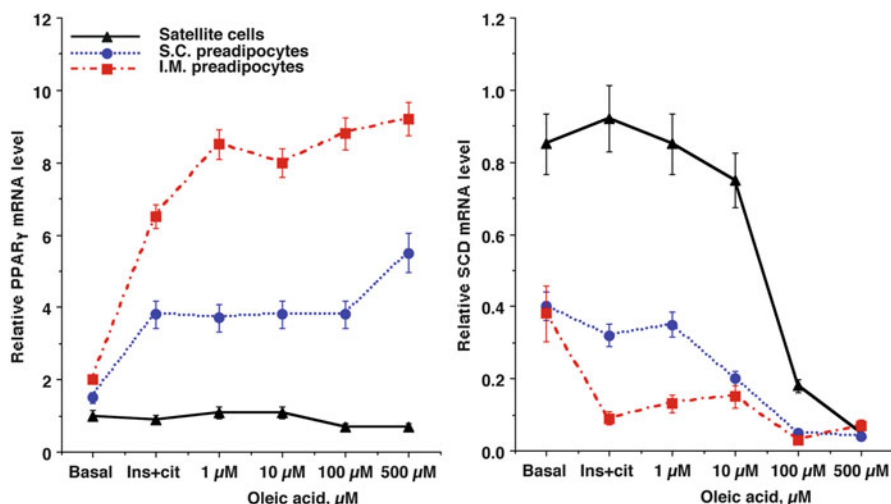

Fig. 12.10 Relative PPARγ (*left*) and SCD (*right*) gene expression in bovine muscle satellite cells (myogenic precursor cells), subcutaneous (SC) preadipocytes, and intramuscular (IM) preadipocytes. Cells were incubated for 7 days in basal medium, medium containing insulin plus ciglitazone (Ins+cit) or medium containing insulin plus ciglitazone and increasing concentrations of oleic. The scale for oleic acid is not linear (Smith, Johnson, and Chung, unpublished data)

Gene Expression in Co-cultured Preadipocytes and Myoblasts

Whereas oleic and linoleic acid depressed *SCD* gene expression, palmitic and stearic acid stimulated *SCD* gene expression in bovine subcutaneous preadipocytes (Fig. 12.9). However, this effect was apparent only in preadipocytes, which were co-cultured with skeletal muscle satellite cells. We demonstrated recently that co-culture of bovine subcutaneous preadipocytes and muscle satellite cells promoted increased *GPR43* gene expression in the preadipocytes and *C/EBPβ* and *PPARγ* gene in bovine myoblasts (Fig. 12.11) (Choi et al. 2013). The mechanism by which myoblasts increase *GPR43* gene expression in co-cultured preadipocytes is not known. We have proposed that oleic acid released from differentiating preadipocytes may promote *C/EBPβ* and *PPARγ* gene expression in the neighboring myoblasts, and this also may occur during growth in livestock species. This remains to be confirmed.

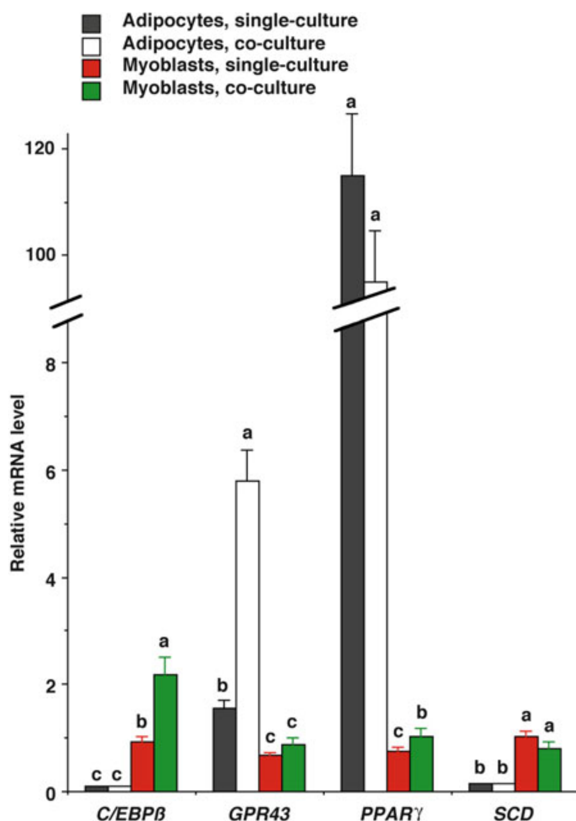

Fig. 12.11 Cell type × culture method interactions for relative mRNA levels in subcutaneous preadipocytes and myoblasts (muscle satellite cells) cultured singly or co-cultured. Co-culture of adipocytes with myoblasts increased *C/EBPβ* and *PPARγ* gene expression in myoblasts. Co-culture of adipocytes with myoblasts increased *GPR43* gene expression in adipocytes. *SCD* gene expression in myoblasts tended to be decreased by co-culture with adipoctyes ($P = 0.15$). Data are means ± SEM ($n = 12$). [abc]Means within a specific gene without common superscripts are different ($P < 0.05$). Adapted from Choi et al. (2013)

Summary

Hepatic *SCD* gene expression is strongly associated with elevated plasma triacylglycerols and obesity in laboratory species and may contribute to adipogenesis in livestock species. Because oleic acid has profound influences on the quality and healthfulness of meat products, strategies have been developed that promote rather than depress *SCD* gene expression and catalytic activity. Endogenously produced oleic acid may actually promote adipogenic gene expression in neighboring preadipocytes and myoblasts.

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Chapter 13
Expression and Nutritional Regulation of Stearoyl-CoA Desaturase Genes in the Ruminant Mammary Gland: Relationship with Milk Fatty Acid Composition

L. Bernard, C. Leroux, and Y. Chilliard

Introduction

In lactating ruminants, stearoyl-CoA desaturase (*SCD*) gene expression in the mammary gland focused particular attention due to its implication in milk technological and nutritional qualities for human consumers. Indeed, the mammary enzyme SCD or Δ9-desaturase by introducing a cis double bond between carbons 9 and 10 of the fatty acids (FA) has a key role in the synthesis of milk monounsaturated FA and specific conjugated linoleic acid (CLA) isomers only found in ruminant products. Then, in milk, about 60 % of oleic (cis-9-18:1; a major milk FA), 50–56 % of palmitoleic (cis-9-16:1), 90 % of myristoleic (cis-9-14:1), and >60 % of the major isomer of CLA (cis-9, trans-11-18:2 or rumenic acid) originate from mammary gland synthesis by the action of SCD enzyme on circulating stearic (18:0), palmitic (16:0), myristic (14:0), and vaccenic acids (trans-11-18:1), respectively. This action is very important due to the impact of milk fat concentration and FA profile in determining milk nutritional quality. Indeed, certain saturated FA (mainly 12:0, 14:0 and 16:0) and trans-FA are considered to exert negative effects when consumed in excess, whereas others (4:0, anteiso-15:0, cis-9-18:1, 18:3*n*-3, and some CLA isomers) have potentially positive effects on human health (Parodi 2005; Shingfield et al. 2008b). For example, cis-9, trans-11-18:2, the major isomer of CLA in ruminant milk, exhibits anticarcinogenic and antiatherogenic (Wahle et al. 2004) properties in animal models. Thus, by contributing to the synthesis of FA that are beneficial from the point of view of human nutrition, e.g., cis-9, trans-11-CLA and to a lesser extent cis-9-18:1, SCD contribute to improve the nutritional quality of milk fat. In addition, by introducing a cis-9 double bond to FA,

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SCD contribute to lower the milk fat melting point (Parodi 1982). Differences in mammary gland level of SCD protein may help in explaining the substantial variation in milk fat content of these fatty acids and thus studies on the regulation of SCD activity and gene expression in the mammary gland have focused major interest in the last decades. Overall, different experiments have been conducted in dairy ruminants for studying the effect of nutrition on milk fat secretion and composition in relation with the regulation of mammary lipogenic gene expression and in particular of *SCD* gene. For this purpose measures were performed at the level of the mammary tissue and milk fat and different variables were determined as follows: (1) mammary *SCD* transcript abundance (*SCD1* and *SCD5* mRNA), (2) mammary SCD activity, (3) the in vivo Δ9-desaturation by a chemical tracer technique, and (4) milk FA Δ-9 desaturation ratios (4 milk ratios of the pairs of FA that represent a product/substrate relationship for SCD: cis-9-14:1/14:0, cis-9-16:1/16:0, cis-9-18:1/18:0, cis-9, trans-11-CLA/trans-11-18:1), which are less invasive indicators commonly used as a proxy for Δ9-desaturase activity due to the difficulty in measuring the activity.

This chapter reviews the present knowledge on *SCD* genes expression in the ruminant mammary gland, in particular, the known effects of nutritional factors in relation with milk FA composition and the effect of specific FA studied using in vitro models. In addition, the known or putative molecular mechanisms underlying these regulations are presented.

Functions of SCD in the Mammary Gland of Ruminant

Milk fat is composed of ca. 98 % of triglycerides (TG) whose FA have a dual origin: (1) either they are de novo synthesized in the mammary gland (Fig. 13.1) from circulating acetate and 3-hydroxybutyrate, produced by ruminal fermentation of carbohydrates and by rumen epithelium from absorbed butyrate, respectively, thus resulting in short and medium chain FA (C4:0 to C16:0) that represent 40–50 % of the FA secreted in milk or (2) they are imported from the plasma, where they are either released by the enzyme lipoprotein lipase (LPL) (Barber et al. 1997) from triglycerides circulating in chylomicra or very low density lipoprotein (VLDL), or derived from the plasma non-esterified fatty acids (NEFA) that circulate bound to albumin, for long-chain FA (≥C18), as well as ca. one half of the 16:0 and very low amount of 14:0 depending on the diet composition. Whatever their origin, these FA may be desaturated by SCD, but not elongated, in the secretory mammary epithelial cells (MECs) (Chilliard et al. 2000). In lactating ruminants, mammary glands have high levels of *SCD* mRNA (Ward et al. 1998; Bernard et al. 2005a) and activity (Bickerstaffe and Annison 1970; Kinsella 1970; McDonald and Kinsella 1973; Bernard et al. 2005b, 2009a, b). The protein SCD is located in the endoplasmic reticulum and catalyses the Δ-9 desaturation, introducing a cis double bond, of a spectrum of fatty acyl-CoA substrates, mainly from C14 to C19. This reaction requires NADH, oxygen, and an electron transport sequence comprising NADH-cytochrome *b*5 reductase, cytochrome *b*5, and SCD. The preferred substrate of SCD

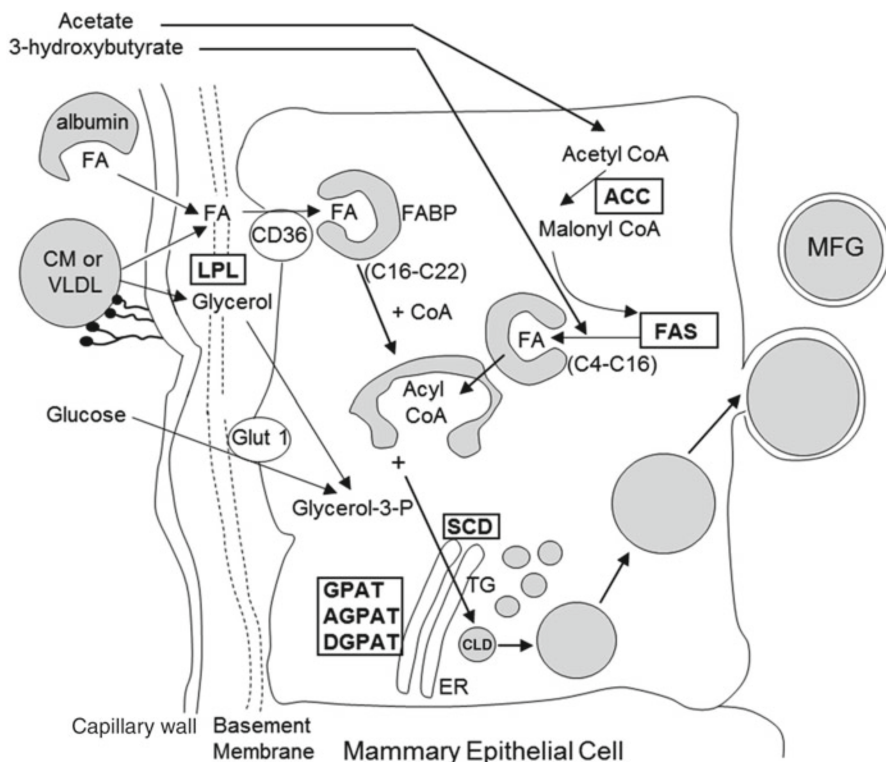

Fig. 13.1 Milk fat synthesis in the ruminant mammary epithelial cell (from Bernard et al. 2008). *ACC* acetyl CoA carboxylase, *AGPAT* acyl glycerol phosphate acyl transferase, *CD36* cluster of differentiation 36, *CLD* cytoplasmic lipid droplet, *CoA* coenzyme A, *CM* chylomicron, *DGAT* diacyl glycerol acyl transferase, *ER* endoplasmic reticulum, *FA* fatty acid, *FABP* fatty acid binding protein, *FAS* fatty acid synthase, *Glut 1* glucose transporter1, *GPAT* glycerol-3 phosphate acyl transferase, *LPL* lipoprotein lipase, *MFG* milk fat globule, *SCD* stearoyl-CoA desaturase, *TG* triglyceride, *VLDL* very low density lipoprotein

is stearic acid leading to the synthesis of oleic acid, the major unsaturated fatty acid found in milk triacylglycerol. In bovine, a number of other saturated acyl-CoA also serve as substrates for SCD including 10:0, 12:0, 14:0, 15:0, and 17:0 (Shingfield et al. 2008b) as well as trans-FA such as trans-7- and trans-12-18:1 (Palmquist et al. 2005; Shingfield et al. 2007). Thus, trans-7, cis-9-18:2 (CLA) in milk originates almost exclusively from the action of Δ9-desaturase on trans-7-18:1 in the mammary gland (Corl et al. 2002). In addition, recent results also suggest that conjugated linolenic acids (CLnA) such as cis-9, trans-11, trans-13-18:3, and cis-9, trans-11, trans-15-18:3 in milk are synthesized endogenously via the action of Δ9-desaturase on trans-11, trans-13-18:2, and trans-11, trans-15-18:2 in the mammary gland, respectively (Lerch et al. 2012). All these trans-18:1 or trans-18:2 FA that may serve as substrate for mammary SCD originate from the ruminal biohydrogenation of the major FA found in the diet delivered to ruminant.

The unsaturation of fatty acid chain is a major determinant of the melting temperature of triglycerides (TG), thus determining the fluidity of milk fat. The fundamental function of mammary SCD in maintaining the physical properties of TG is even increased in ruminants due to the large biohydrogenation processes of dietary PUFA that occur in the rumen which led to the synthesis of saturated FA (mainly 18:0) as well as of numerous intermediates, in particular, trans-FA (Shingfield et al. 2010) that could be further taken up by the mammary gland. Indeed, whereas it is well established that endogenous synthesis of cis-9-18:1 via the action of SCD on 18:0 in the mammary gland is an important point of regulation in milk TG synthesis and maintenance of milk fat fluidity, the presence of a cis- or trans-double bond as well as of short-chain FA and saturated FA also intervene in the maintenance of milk fat fluidity (Chilliard et al. 2000). In this way, a meta-analysis of milk FA yield in relation to duodenal flows (Glasser et al. 2008) showed an interdependence of C18 and C4 to C16 probably due to the esterification step of milk fat synthesis, which involves a putative balance between long-chain and de novo synthesized FA to maintain milk TG fluidity. In the same way it was proposed that, under certain dietary conditions, decreases in the availability of 18:0 for endogenous synthesis of cis-9-18:1 combined with an increase in trans-18:1 at the mammary gland could be a possible mechanism to explain part of the decrease in milk fat secretion (Loor et al. 2005a; Gama et al. 2008). Thus, the ability to maintain milk fat melting point within physiological values could play a role in the regulation of milk fat secretion when milk FA composition varies, for example, in response to feeding factors. In this context, the hypothesis of a contribution of milk fat fluidity to the regulation of milk fat secretion has been proposed (Timmen and Patton 1988; Chilliard et al. 2000) and was confirmed recently in a meta-analysis on cow, goat, and ewe milk FA composition and estimated melting point (Toral et al. 2013a). In addition, studies in goats (Chilliard et al. 2006) on the effect of the CSN1S1 genotype on milk FA profile have reported higher Δ9-desaturation ratios in defective vs. high genotypes suggesting higher expression of SCD in defective genotypes in which the synthesis of short-chain FA is lower. This result led to the hypothesis that higher SCD expression could compensate for the concomitant decrease in short-chain FA and thus to maintain the milk fat melting point in defective genotypes.

Characterization of SCD Genes

Multiple isoforms of *SCD* have been found in most species examined to date. Some rodent genomes (as mice) contain four *SCD* isoforms (*SCD1–4*) with different expression profiles according to tissues. For example, murine *SCD1* is expressed predominantly in lipogenic tissues (Ntambi et al. 1988), while *SCD2* is preferentially expressed in brain and neuronal tissues (Kaestner et al. 1989), *SCD3* is expressed in sebocytes, preputial gland, and Harderian gland (Zheng et al. 2001), and *SCD4* expression is restricted to the heart (Miyazaki et al. 2003). Up to now, the genomes of many other species, including chickens, pigs, cows, and humans, have

been found to contain two *SCD* isoforms. Then, in humans: *SCD1* is highly expressed in adipose tissue and liver while a second *SCD* gene, termed *SCD5*, is highly expressed in brain and pancreas (Wang et al. 2005). In addition to tissue-specific expression profile, there is evidence to suggest that some SCD protein isoforms may differ in their preferred substrate specificity. For example, murine SCD1, SCD2, and SCD4 have been shown to desaturate both palmitoyl-CoA and stearoyl-CoA, while murine SCD3 desaturates palmitoyl-CoA but not stearoyl-CoA (Miyazaki et al. 2006). The tissue and substrate specificity of SCD isoforms suggests that each may have a distinct physiological role.

In ruminants earlier studies reported only one *SCD* gene, generating a transcript of 5 kb in size which was characterized in sheep (Ward et al. 1998), cows (Chung et al. 2000), and goat (Bernard et al. 2001). When compared, the coding region of the caprine *SCD* mRNA shares 98, 95, 90, and 86 % identity with the ovine, bovine, porcine, and human *SCD1* mRNAs, respectively. This *SCD* isoform was highly expressed in lipogenic tissues, adipose tissue, and the lactating mammary gland and was recently renamed *SCD1* since the discovery of the *SCD5* isoform characterized in ruminant species (Lengi and Corl 2007, 2008). In these species *SCD5* is mainly expressed in the brain (Lengi and Corl 2008). In addition to *SCD1* a significant expression of *SCD5* was reported in the lactating mammary gland in cows (Gervais et al. 2009; Jacobs et al. 2011) and goats (Bernard et al. 2010) with probably different regulation for these two isoforms (Gervais et al. 2009; Jacobs et al. 2011). However, both the regulation of the expression and the contribution of *SCD5* isoform to FA Δ-9 desaturation process are still unknown.

SCD1 transcript in goat presents an unusually long (3.8 kb) 3′-UTR sequence which derives from a single exon (Bernard et al. 2001), as observed for human (Zhang et al. 1999), rat (Mihara 1990), and mouse (Ntambi et al. 1988). In the caprine, the 3′-UTR is characterized by the presence of several AU-rich elements as observed in the 3′-UTR region of mouse (Kaestner et al. 1989; Sessler et al. 1996), rat (Thiede and Strittmatter 1985; Mihara 1990), and human (Zhang et al. 1999) *SCD1* transcripts, which could be mRNA destabilization sequences as suggested by Sessler et al. (1996). These AU-rich elements have been shown to play active roles in the mRNA degradation (Vakalopoulou et al. 1991) which is responsible for their turnover. In addition, these motifs could be possible targets of polyunsaturated fatty acids effects on *SCD* mRNA, as proposed by Ntambi (1999) for *SCD1* and *SCD2* mRNA in mouse adipocytes. Otherwise, SCD5 transcript in bovine and ovine was of 2.6 kb in length (Lengi and Corl 2007, 2008) with a 3′UTR corresponding to half the mRNA length.

SCD1 gene was localized on bovine and caprine chromosomes 26q21 and on ovine chromosome 22q21 (Bernard et al. 2001), and *SCD5* was localized on bovine chromosome 6 (Lengi and Corl 2007) and on ovine chromosome 6 (Lengi and Corl 2008). In caprine *SCD1* gene spans about 15 kb and has six exons varying in size from 131 (third exon) to 4,047 (sixth exon) bp, and five introns varying in size from 600 to 3,700 bp (Bernard et al. 2001; Fig. 13.2). This structural organization is similar to *SCD1* gene reported in human (Zhang et al. 1999) and rodents (Ntambi et al. 1988; Kaestner et al. 1989; Mihara 1990). The genomic structure of bovine

Fig. 13.2 Structural organization of the caprine SCD1 transcription unit. Overall structural organization of the caprine SCD1 gene deduced from PCR analysis. *Open bars* represent introns and exons are depicted by *large*, *green* (UTR) or *orange* (TR) *boxes* (from Bernard et al. 2001)

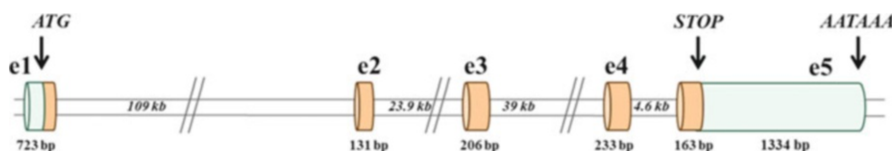

Fig. 13.3 Structural organization of the bovine SCD5 transcription unit. Overall structural organization of the bovine SCD5 gene adapted from Lengi and Corl (2007). *Open bars* represent introns and exons are depicted by *large*, *green* (UTR) or *orange* (TR) *boxes*

SCD5 deduced from known bovine genomic DNA sequence showed that *SCD5* is organized into five exons and four introns in a fragment tenfold longer than *SCD1* spanning more than 175 kb (Lengi and Corl 2007) and is similar to human *SCD5* (Fig. 13.3). Despite the length difference of their genes, the SCD1 and SCD5 encoded proteins are of similar size and are, respectively, composed of 359 and 335 amino acids (AA). Sequence alignment shows 65 % identity between bovine SCD1 and SCD5 at the amino acid level. Both proteins share highly conserved three histidine motifs that are catalytically essential for the desaturase activity. Conversely to SCD1 protein, the N-terminus of the SCD5 protein lacks the conserved PEST (peptide sequence rich in proline, glutamic acid, serine, and threonine). This sequence is present in proteins that have a short intracellular half-time with the hypothesis that PEST sequence acts as a signal peptide for protein degradation (mediated by proteasome or calpaïn). These data suggest that the regulation of SCD1 and SCD5 proteins may differ.

Little is known in ruminant on the molecular mechanisms controlling *SCD* genes expression (see section "Knowledge on the Signalling Pathways Mediating Nutritional Regulation of SCD Gene Expression in Ruminants"). The promoter region of the bovine *SCD1* gene have been studied (Keating et al. 2005) and an area of 36 bp in length was identified as having a critical role in the transcriptional activation and was designated the *SCD* transcriptional enhancer element (STE). Otherwise, a recent study regarding the possible common transcription factors between bovine *SCD5*, expressed in brain and nervous tissue, and rodent *SCD2*, expressed in nervous tissue, demonstrated that early growth response 2 (EGR2) and sterol regulatory element binding protein 1a (SREBP-1a) may bind to the same DNA site in the bovine *SCD5* promoter (Lengi and Corl 2012).

In other respects, in order to explain individual differences in milk fat yield and FA composition, in particular in cis-9 monounsaturated FA, studies in ruminants

were focused on the genetic variability of the region containing *SCD* gene. A lot of programs were dedicated to quantitative trait loci (QTL) detection in dairy bovine breeds on milk parameters. In particular, genome-scan for bovine milk fat composition revealed a QTL for the monounsaturated FA and their unsaturation indices on Bos taurus autosome (BTA) 26 corresponding to the chromosome where *SCD1* gene is located (Stoop et al. 2009). These authors have strongly suggested that this BTA26 QTL was caused by a mutation in the *SCD1* gene. Among the single nucleotide polymorphisms (SNP) detected in *SCD1* gene, a nonsynonymous one is located in exon 5 causing a substitution of valine (V) with alanine (A) at position 293 (A293V; Kgwatalala et al. 2007; Schennink et al. 2008). Several studies in different bovine breeds reported significant association between A293V genotypes and milk FA composition with milk of cows carrying the SCD *AA* genotype having a greater content of cis-9-18:1 and a higher cis-9-14:1/14:0 ratio than did milk of cows carrying the SCD *VV* genotype (Kgwatalala et al. 2009b; Mele et al. 2007; Conte et al. 2010), whereas others (Moioli et al. 2007) could not confirm this association. These latter authors suggested a role of regulatory factors acting on the promoter region of *SCD* gene or through activation of the enzyme. In that way, whereas an earlier study on *SCD1* promoter has not allowed the detection of SNP (Keating et al. 2005), a more recent study highlighted a SNP (133A>C) creating a new consensus site for the transcription factor SP1 binding site, which could be responsible for a difference in the milk FA desaturation ratio (Pauciullo et al. 2012). Moreover, different SNPs were also detected in intronic or exonic regions without AA substitution in the protein. However, their linkage with milk yield was particularly studied in haplotypic configuration suggesting that they could be used as potential genetic markers to improve milk performance (Alim et al. 2012). For example, SNP haplotypes identified in the 3′UTR region were reported to be associated with different 10:0 and 12:0 Δ-9 desaturation ratios in bovine milk (Kgwatalala et al. 2009a).

In small ruminants, although less documented, *SCD1* gene polymorphism has also been studied. The first characterization of the caprine *SCD1* mRNA has revealed a polymorphism involving the deletion of a nucleotide triplet (TGT) in the 3′UTR region (Bernard et al. 2001). The analyses of this mutation in haplotype-based configuration with two other 3′UTR SNP were suggestively associated with lower milk stearic FA content and with higher percentages of PUFA (Zidi et al. 2010). An in silico analysis of the secondary structure of *SCD1* mRNA pointed out the 3nt deletion as being involved in a considerable change in the secondary structure of the *SCD1* mRNA. This change could alter the amount of *SCD1* transcripts in MECs (Zidi et al. 2010). In sheep, a QTL analysis for the FA profile showed a QTL for the ratio of rumenic/vaccenic acid in ovine chromosome 22 which coincided with the position of the *SCD* gene (Carta et al. 2008). However, no association of four SNP previously detected (Garcia-Fernandez et al. 2009) has been observed with the ratio of rumenic/vaccenic acid, whereas a possible effect of the *SCD* polymorphism on milk fat percentage has been suggested (Garcia-Fernandez et al. 2010).

Due to its recent discovery, the polymorphism of *SCD5* gene, located on BTA6, has not been studied to the same extend. A recent study revealed that polymorphisms in the *SCD5* gene were among the most representative markers associated

with unsaturated/saturated FA ratio in milk (Rincon et al. 2012). In this study, two SNP were detected within exon 3 which were, respectively, associated with an increase in saturated FA concentration combined with a decrease in monounsaturated FA, and with a decrease in saturated FA combined with an increase in PUFA. Moreover, an intronic mutation named G allele was shown to be associated with a decrease in saturated FA/unsaturated FA ratio and an increase in monounsaturated FA and Δ-9 desaturation ratios for 14:0 and trans-11-18:1 content in Holstein milk (Rincon et al. 2012). However, the mechanism by which this mutation acts needs to be clarified.

Regulation of Mammary *SCD* Genes Expression by Dietary Factors

Effect of Dietary Factors

In ruminants, milk FA composition is greatly influenced by feeding factors (Jensen 2002; Dewhurst et al. 2006; Chilliard et al. 2007). Thus, modification of milk fat content and FA composition by dietary manipulation has been investigated and few studies were performed to relate the effect of diet on milk FA profile to mammary lipid metabolism considering the major lipogenic genes, in particular, *SCD*. The response of *SCD* gene expression to nutritional changes involves the control of events that could occur at transcriptional (e.g., through transcription factors), post-transcriptional (e.g., such as mRNA stability), translational (e.g., its initiation or through microRNA action), and post-translational (e.g., via turnover or regulation of the activity of the enzymatic protein) levels. However, it is often unclear whether the regulatory factors are the dietary components themselves or their metabolites or hormonal changes produced in response to the nutritional changes. Moreover, in most of the studies, it is difficult to conclude on the level of regulation involved since measurements of *SCD* mRNA of the enzyme protein content and activity are not studied simultaneously. Only few studies have investigated the role of diet on the regulation of *SCD* gene expression and activity in ruminants.

As previously mentioned, prior to the characterization of the bovine *SCD5* gene (Lengi and Corl 2007), as only one *SCD* isoform was thought to be present in this species, the gene was simply referred to as *SCD* for *SCD1*. The in vivo trials reporting the nutritional regulation of mammary *SCD1* and lipogenic genes expression have been carried out in mid-lactation cows and goats and mainly with lipid supplements (Table 13.1). In cows, studies have been primarily realized with diets that induce milk fat depression (MFD). These diets belongs to three types: (1) diets rich in starch without addition of lipid supplements (e.g., high grain/low forage diets), but containing a minimal amount of PUFA in dietary feedstuffs; (2) diets with low level of fiber associated with supplemental PUFA of plant origin; (3) diets associated with dietary supplements of marine oils (fish oils, fish meals, oils from marine

Table 13.1 Effect of dietary lipid supplements on mammary *SCD* and lipogenic genes expression and/or enzyme activity in ruminants

Biochemical pathway[a]	Species	Transcript/protein[b]	Response (%)[c]	Nature of lipid supplement	Inclusion rate (g/kg dry matter)	Response of milk fat yield (%)[c]	Reference
Transcription factors							
SREBF1/S14	Bovine	mRNA	−45/−60	Soyabean oil + fish oil[d]	30 + 15	−38	Harvatine and Bauman (2006)
SREBF1	Bovine	mRNA	−30	Sunflower oil + marine algae[e]	27 + 4	−30	Angulo et al. (2012)
	Bovine	mRNA	−31	Linseed oil + marine algae[e]	27 + 4	−31	Angulo et al. (2012)
SREBF1/PPARG	Bovine	mRNA	NS/NS	Soybean oil + fish oil	25 + 10	−50	Invernizzi et al. (2010)
Desaturation and de novo lipogenesis							
SCD1/ACC	Bovine	mRNA	NS	Rapeseed	33	NS	Delbecchi et al. (2001)
SCD1/ACC/FAS	Bovine	mRNA	(−29)/−46/−64	Fish oil	37	−44	Ahnadi et al. (2002)
SCD1	Caprine	mRNA	−43	Oleic sunflower oil	35	+16	Bernard et al. (2005b)
	Caprine	mRNA	−54	Formaldehyde-linseed	112	+8	Bernard et al. (2005b)
SCD	Caprine	Activity	−30	Sunflower oil	55	+17	Bernard et al. (2009b)
	Caprine	Activity	−22	Oleic sunflower oil	35	+16	Bernard et al. (2005b)
	Caprine	Activity	−27	Linseed oil	55	+15	Bernard et al. (2009b)
	Caprine	Activity	−35	Formaldehyde-linseed	112	+8	Bernard et al. (2005b)
SCD1/FAS	Bovine	mRNA	(−25)/−45	Soyabean oil + fish oil[d]	30 + 15	−38	Harvatine and Bauman (2006)
	Bovine	mRNA	−34.5/−31	Sunflower oil + marine algae[e]	27 + 4	−30	Angulo et al. (2012)
	Bovine	mRNA	−45.5/−33	Linseed oil + marine algae[e]	27 + 4	−31	Angulo et al. (2012)
SCD/ACC/FAS	Caprine	Activity	(−17/+12)/+81	Sunflower oil	61	(−3)	Bernard et al. (2009a)
SCD1/ACC/FAS	Caprine	mRNA	(+15/+19/+12)	Rapeseed	146	(+5)	Ollier et al. (2009)
	Bovine	mRNA	(+39/+38/+36)	Safflower seed	135	NR	Murrieta et al. (2006)
	Caprine	mRNA	+166/+72/+74	Safflower oil	50	+26	Li et al. (2012)
	Bovine	mRNA	(−35)/−28/−41	Sunflower oil[d]	10	−27	Peterson et al. (2003)
	Caprine	mRNA	(−7/−8/+5)	Sunflower oil	61	(+7)	Bernard et al. (2009a)
	Caprine	mRNA	(+10/+15/+6)	Sunflower oil	44	+20	Ollier et al. (2009)
	Caprine	mRNA	(+17/+10/+9)	Sunflower oil	55	+17	Bernard et al. (2009b)

(continued)

Table 13.1 (continued)

Biochemical pathway[a]	Species	Transcript/protein[b]	Response (%)[c]	Nature of lipid supplement	Inclusion rate (g/kg dry matter)	Response of milk fat yield (%)[c]	Reference
	Caprine	mRNA	(+22/+2/+10)	Linseed oil	55	+15	Bernard et al. (2009b)
	Caprine	mRNA	(−16/+4/−4)	Linseed oil	62	+14	Bernard et al. (2009a)
	Caprine	mRNA	+121/−26/+52	Linseed oil	50	+34	Li et al. (2012)
	Caprine	Activity	(+11/−3/+64)	Linseed oil	62	+14	Bernard et al. (2009a)
ACC	Bovine	mRNA	−68	Soyabean oil[d]	50	−43	Piperova et al. (2000)
FAS	Caprine	Activity	+68	Sunflower oil	61	(+7)	Bernard et al. (2009a)
	Bovine	Activity	−61/−44	Soyabean oil[d]	50	−43	Piperova et al. (2000)
	Caprine	mRNA	(−21)	Oleic sunflower oil	35	+16	Bernard et al. (2005a)
	Caprine	mRNA	(−1)	Formaldehyde-linseed	112	+8	Bernard et al. (2005a)
	Caprine	Activity	(+27)	Sunflower oil	55	+17	Bernard et al. (2009b)
	Caprine	Activity	(0)	Oleic sunflower oil	35	+16	Bernard et al. (2005b)
	Caprine	Activity	(+30)	Linseed oil	55	+15	Bernard et al. (2009b)
	Caprine	Activity	(−31)	Formaldehyde-linseed	112	+8	Bernard et al. (2005b)
Fatty acid uptake/processing							
LPL	Caprine	mRNA	(−0.03)	Rapeseed	146	(+5)	Ollier et al. (2009)
	Bovine	mRNA	+50	Safflower seed	135	NR	Murrieta et al. (2006)
	Caprine	mRNA	+43	Safflower oil	50	+26	Li et al. (2012)
	Bovine	mRNA	−61	Soyabean oil + fish oil[d]	30+15	−38	Harvatine and Bauman (2006)
	Bovine	mRNA	−30	Sunflower oil[d]	10	−27	Peterson et al. (2003)
	Caprine	mRNA	(+17)	Sunflower oil	61	(+7)	Bernard et al. (2009a)
	Caprine	mRNA	(+23)	Sunflower oil	44	+20	Ollier et al. (2009)
	Caprine	mRNA	(+1)	Sunflower oil	55	+17	Bernard et al. (2009b)
	Caprine	mRNA	+51	Oleic sunflower oil	35	+16	Bernard et al. (2005b)
	Caprine	mRNA	(−1)	Linseed oil	55	+15	Bernard et al. (2009b)
	Caprine	mRNA	(+26)	Formaldehyde-linseed	112	+8	Bernard et al. (2005b)
	Caprine	mRNA	(+13)	Linseed oil	62	+14	Bernard et al. (2009a)

	Caprine	mRNA	+54	Linseed oil	50	+34	Li et al. (2012)
	Bovine	mRNA	−34	Sunflower oil + marine algae[e]	27 + 4	−30	Angulo et al. (2012)
	Bovine	mRNA	−45	Linseed oil + marine algae[e]	27 + 4	−31	Angulo et al. (2012)
	Caprine	Activity	(+42)	Sunflower oil	61	(+7)	Bernard et al. (2009a)
	Caprine	Activity	(+35)	Sunflower oil	55	+17	Bernard et al. (2009b)
	Caprine	Activity	(+13)	Oleic sunflower oil	35	+16	Bernard et al. (2005b)
	Caprine	Activity	(+21)	Linseed oil	55	+15	Bernard et al. (2009b)
	Caprine	Activity	(−17)	Linseed oil	62	+14	Bernard et al. (2009a)
FACL	Bovine	mRNA	−34	Sunflower oil[d]	10	−27	Peterson et al. (2003)
Esterification							
AGPAT/GPAT	Bovine	mRNA	−27/−52	Sunflower oil[d]	10	−27	Peterson et al. (2003)
GPAM	Bovine	mRNA	−10	Sunflower oil + marine algae[e]	27 + 4	−30	Angulo et al. (2012)
	Bovine	mRNA	−26	Linseed oil + marine algae[e]	27 + 4	−31	Angulo et al. (2012)
FA transport							
FABP	Bovine	mRNA	(−25)	Sunflower oil[d]	10	−27	Peterson et al. (2003)
FABP3/FABP4	Caprine	mRNA	(+11)/+36	Sunflower oil	44	+20	Ollier et al. (2009)
	Caprine	mRNA	(+21)/(+11)	Rapeseed	146	NS	Ollier et al. (2009)

Adapted from Shingfield et al. (2013)

[a]*ACC* acetyl CoA carboxylase, *AGPAT* acylglycerol phosphate acyl transferase, *FAS* fatty acid synthase, *FABP* fatty acid binding protein, *FACL* fatty acyl-CoA ligase, *GPAM* glycerol-3-phosphate acyltransferase 1, *GPAT* glycerol phosphate acyl transferase, *LPL* lipoprotein lipase, *SCD* stearoyl-CoA desaturase, *S14* thyroid hormone responsive spot 14, *SREBF1* sterol response element binding protein

[b]Measurement of tissue transcript abundance (mRNA) or protein activity (Activity)

[c]Response reported when treatment effects were significant ($P < 0.10$) and calculated as [((Treatment − Control)/Control) × 100]. Values in parenthesis are not significant at $P < 0.05$. NS, values not reported because unsignificant

[d]Lipid supplementation also accompanied by decreases in dietary forage:concentrate ratio; NR, not reported

[e]Control treatment, supplement of rumen-stable fractionated palm fat rich in 16:0

mammals, and/or algae) which induce MFD whatever the level of starch or fiber in the diet. In these dietary conditions, variable responses of mammary *SCD1* mRNA abundance have been reported in cows: either no variation with high concentrate diets containing sunflower oil (Peterson et al. 2003) or fish oil and soya bean oil (Harvatine and Bauman 2006); or decreases with diets supplemented with plant oils rich in either *n*-3 or *n*-6 fatty acids (FA) plus docosahexaenoic acid (DHA)-rich algae (Angulo et al. 2012) or with rumen "protected" fish oil (Ahnadi et al. 2002); or surprisingly increases with diets supplemented with fish oil and soya bean oil (Invernizzi et al. 2010). From these studies it is difficult to conclude on the effect of the dietary treatments that induce MFD on *SCD1* mRNA abundance and probably that the forage:concentrate ratio and the nature and the level of the PUFA into the diet may intervene to explain such differences. Otherwise, studies in goats have shown that supplementing diets based on grass hay with sunflower-seed oil rich in oleic acid (Bernard et al. 2005b), sunflower-seed oil (18:2 rich), or linseed oil (18:3 rich) decreased SCD activity (Bernard et al. 2009b) but had no effect on mammary *SCD1* mRNA abundance, whereas supplementing grass hay-based diets with formaldehyde-treated linseed (FLS) decreased mammary *SCD1* mRNA without effect on the SCD activity (Bernard et al. 2005b). In contrast, inclusion of sunflower-seed oil and linseed oil in maize silage-based diets (Bernard et al. 2009a) and of soya beans in lucerne hay-based diets (Bernard et al. 2005a) had no effect on caprine mammary *SCD1* mRNA abundance and/or SCD activity. Lastly, in goat, addition of fish oil and starch to a diet containing sunflower-seed oil has no effect on mammary *SCD1* and *SCD5* mRNA abundances (Bernard et al. 2010).

Overall, these observations (Table 13.1) highlight the importance of interactions between the composition of the basal diet and lipid supplement in the goat with the implication that specific PUFA escaping metabolism in the rumen or specific biohydrogenation intermediates may inhibit SCD activity via transcriptional or post-transcriptional regulatory mechanisms.

In vivo Δ-9 desaturase activity has often been estimated by the milk ratios for the pairs of FA that represent a product/substrate relationship for SCD (Bauman et al. 2001) due to the fact that the in vitro activity assay needs fresh materials and is laborious (Legrand et al. 1997). Moreover, ex vivo activity assay is done in optimal conditions (pH, substrate, cofactors) and may differ from in vivo conditions. From the few studies in goats reporting mammary SCD activity together with milk fatty acid composition, the response to dietary lipids of the four FA pair ratios that represent a proxy for SCD activity was related to the response of the SCD activity itself (Bernard et al. 2008). In some experiments with goats, lipid supplements in the diet have been shown to reduce milk FA desaturation ratios but have no effect on SCD activity (Bernard et al. 2009a, b, c). However, it was shown that the milk ratio of myristoleic acid to myristic acid (cis-9-14:1/14:0) gave the best estimation for the response of mammary SCD activity (Fig. 13.4; from Bernard et al. 2008), probably due to the fact that almost all the myristoleic acid present in the milk is likely to be synthesized in the mammary gland from de novo synthesis of myristic acid followed by desaturation by SCD. Indeed, 14:0 and 14:1 even less are poorly represented in feedstuffs used for ruminants, including lipid supplements (except for 14:0

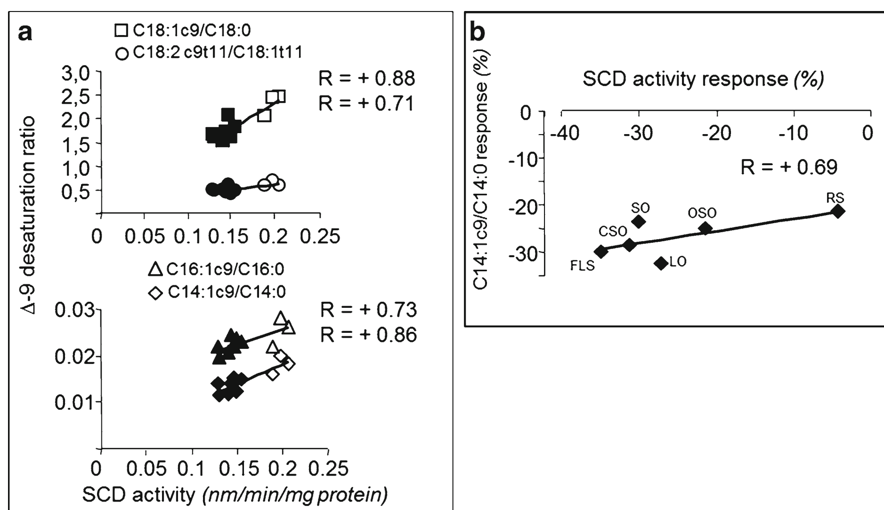

Fig. 13.4 Relationships between milk FA desaturation ratios and stearoyl-CoA desaturase (SCD) activity in 43 goats fed hay-based diet supplemented, or not, with lipids. The lipid supplements were either 3.6 % of lipids from oleic sunflower oil (OSO) or formaldehyde-treated linseed (FLS) (Bernard et al. (2005b)), or 5.8 % lipids from linseed oil (LO) or sunflower oil (SO) (Bernard et al. (2009a)), or 6.5 % whole rapeseed (RS) or 4.5 % of sunflower oil (CSO) (Ollier et al. (2009)) (from Bernard et al. 2008). (**a**) Results are means of the seven lipid-supplemented groups (*black symbols*) and the three control groups (*white symbols*). (**b**) Relationship between the responses (% of the control group) of SCD activity and milk cis-9-14:1/14:0 to the six lipid supplements

in specific lipid supplements such as coconut or krabo oils), and the circulating 14:1 in ruminant is very low (Christie 1981). Otherwise, variable proportions of palmitoleic, oleic, and rumenic acids come from absorption from the digestive tract and/or mobilization of body fat reserves. Consequently, the ratios that involve these latter FA are less indicative of SCD activity even though stearoyl-CoA and palmitoyl-CoA are the preferred substrates for SCD. Indeed, activity of SCD was estimated to contribute to approximately 90 % of cis-9-14:1 compared to 50–56 % of cis-9-16:1, 60 % of cis-9-18:1, and >60 % of cis-9, trans-11-18:2 in bovine milk fat (Enjalbert et al. 1998; Mosley and McGuire 2007; Glasser et al. 2008). Moreover, differential uptake, turnover, and use of different FA of the four pair ratios by the mammary tissue itself may occur.

In other respects, several experiments in goats have provided evidence to support the involvement of post-translational mechanisms in the regulation of SCD activity. Inclusion of cis-9-18:1 enriched sunflower oil (Bernard et al. 2005b), sunflower oil, or linseed oil (Bernard et al. 2009b) in the diet of goats fed grass hay-based diets has been shown to lower mammary SCD activity measured ex vivo without changing mammary *SCD1* mRNA abundance. Otherwise, in goats fed maize silage-based diet, addition of plant oils had no effect on mammary *SCD1* mRNA or SCD activity (Bernard et al. 2009a). However, in four in vivo studies in goats (Bernard et al.

2005b, 2009a, b; Ollier et al. 2009), the relationship between the responses of *SCD1* mRNA and enzyme activity (means per treatment) to the eight lipid supplements (expressed relative to the control) was relatively weak ($r = 0.55$), which may indicate the existence of putative post-traductional regulatory events. In the same way, a recent study in goats using a proteomic analysis suggested an increased protein expression of PSMA5 (proteasome subunit α type 5) and a higher mRNA abundance of *SCD* in the mammary tissue following trans-11-18:1 treatment (by intravenous bolus injection) (Jin et al. 2012). SCD is a short-lived integral membrane protein of the endoplasmic reticulum (Oshino and Sato 1972), and PSMA5 as a part of the ubiquitin proteasome system may be involved in constitutive SCD degradation, and thus could play a role in SCD regulation as further suggested in MAC-T cells (Jin et al. 2012).

Otherwise, in order to determine the in vivo SCD metabolic activity, different methods have been developed and used in ruminants, in particular, to estimate the in vivo conversion of stearic to oleic acid or vaccenic to rumenic acid in the mammary gland. These methods include direct methods, by means of a tracer (for example, [1-^{13}C]trans-11 18:1; Mosley et al. 2006; Bernard et al. 2010), and indirect methods, through quantification of duodenal and milk FA flows (Shingfield et al. 2007; Glasser et al. 2008) or inhibition of the desaturase system using sterculic acid (Griinari et al. 2000; Corl et al. 2001; Kay et al. 2004; Bichi et al. 2012). Studies using these methods provided clear evidence that the endogenous desaturation of stearic and vaccenic acids are the main sources of milk oleic and rumenic acids, respectively. Indeed, about 80 % (Glasser et al. 2008), 80–84 % (Annison et al. 1974), and 63 % (Bichi et al. 2012) of oleic acid comes from stearic acid, respectively, in cows, goats, and ewes. In the same way, 64–97 % (Mosley et al. 2006; Glasser et al. 2008), 63–73 % (Bernard et al. 2010), and 74 % (Bichi et al. 2012) of rumenic acid comes from vaccenic acid, respectively, in cows, goats, and ewes. In addition, few of them provided an estimation of stearic or vaccenic acid desaturation in the mammary gland: for stearic acid it was of 59 % (Mosley and McGuire 2007) and 54 % (Glasser et al. 2008) in cows; for vaccenic acid it was of 25.7 % (Mosley et al. 2006), 28.9 % (Shingfield et al. 2007), 19.8 % (Qiu et al. 2004), 21 % (Glasser et al. 2008) in cows and of 31.6 % in goats (Bernard et al. 2010).

Ruminant Species Specificities

Indirect comparisons from experiments examining in cows and goats the effect of nutrition on milk fatty acid composition outlined several differences in milk cis-9-FA and Δ-9 desaturation ratios responses to lipid supplements among these two species (Chilliard et al. 2007). As a large proportion of the cis-9-FA secreted in milk is synthesized endogenously via the action of mammary SCD, differences in their concentration may suggest specificities in mammary SCD regulation among species. Indeed, in experiments with goats fed maize silage- or grassland hay-based supplemented with plant oils (Chilliard et al. 2007; Bernard et al. 2009c), the

Table 13.2 Effect of inclusion of sunflower oil (rich in cis-9, cis-12-18:2) into a maize silage-based diet on milk fatty acid secretion and composition (including trans-10, cis-12-, trans-10, trans-12-, trans-9, trans-11-CLA) and milk fatty acid desaturation ratio responses in cows and goats

Species	Bovine		Caprine	
Reference	Roy et al. (2006)		Bernard et al. (2009c)	
Major forage of the diet	Maize silage		Maize silage	
Treatment	Control	Sunflower	Control	Sunflower
Oil inclusion rate (g/kg dry matter)	0	52	0	63
Diet composition				
Concentrate (g/kg dry matter)	520	537	612	545
Neutral detergent fiber (g/kg dry matter)	285	266	310	295
Milk fat content and secretion				
Milk fat output (g/d or response)[a]	1,380	(−44.2 %)	107	(+6.5 %)
14:0 (g/d or response)	156.0	(−67.5 %)	12.0	(−28.5 %)
18:0 (g/d or response)	111.1	(−11.9 %)	4.9	(+96.7 %)
trans-10-18:1 (g/d or response)	5.54	(+837 %)	0.44	(+682 %)
Fatty acid (g/100 g fatty acids)				
10:0	3.46	1.2	10.58	6.91
12:0	3.94	1.65	5.72	3.12
14:0	12.12	7.06	12.07	8.10
16:0	32.32	18.93	29.85	18.78
18:0	8.63	13.62	4.88	9.01
trans-10-18:1	0.43	7.22	0.44	3.23
trans-10, cis-12-CLA	0.003	0.024	0.004	0.064
trans-9, trans-11-CLA	0.006	0.008	0.015	0.059
trans-10, trans-12-CLA	0.004	0.015	0.003	0.024
Milk fatty acid desaturation ratios				
cis-9-14:1/14:0	0.094	0.132	0.019	0.012
cis-9-18:1/18:0	1.92	2.08	2.81	1.74
cis-9, trans-11-CLA/trans-11-18:1	0.40	0.53	0.70	0.50

Adapted from Shingfield et al. (2010)

[a]Response calculated as [((Treatment − Control)/Control) × 100]

relative increases in milk cis-9-18:1 concentrations were lower than observed when comparable diets were fed to lactating cows (Roy et al. 2006; Chilliard et al. 2007). These interspecies differences could be related to a greater sensitivity of the SCD enzyme to the inhibitory effects of PUFA in the goat than the cow (Bernard et al. 2008) and/or to larger increases in the enrichment of trans-FA that may act as inhibitor of SCD in the goat compared to the cows receiving similar diets (such as trans-9, trans-11-CLA in goat milk in Bernard et al. (2009c) compared to bovine milk in Roy et al. (2006); Table 13.2; see section "In Vivo Studies with Specific Fatty Acid Infusion or Injection"). Moreover, the balance between (1) the level and nature of unsaturated FA taken up by the mammary gland and (2) the mammary synthesis of short-chain FA and desaturase activity, which play a role in the regulation of milk fat fluidity (Toral et al. 2013a), may intervene in explaining these interspecies differences. Indeed, it may be hypothesized that the goat which has higher

concentrations of milk short-chain FA (Table 13.2) would require lower level of ∆-9 desaturation for maintaining the milk fat melting point. However, in the absence of direct comparisons between bovine and small ruminant species with similar dietary conditions and stage of lactation, it is difficult to draw clear conclusions on species specificities of SCD regulation.

Mechanisms of the Nutritional Regulation of Mammary SCD

In Vivo Studies with Specific Fatty Acid Infusion or Injection

A key role for specific fatty acids on mammary lipogenesis and desaturation was showed from in vivo studies (Shingfield et al. 2010). In particular, studies with specific dietary conditions inducing a dramatic MFD (Bauman and Griinari 2003) underlined the role of specific trans-FA that act as potent inhibitors of mammary lipid synthesis and desaturation process. Indeed, as diet-induced MFD was associated with an increase in milk trans-10-18:1 content (Griinari et al. 1998; Piperova et al. 2000; Loor et al. 2005b) and trans-10, cis-12-CLA (Baumgard et al. 2000), these FA were proposed as candidates for inhibition of mammary lipogenesis (Bauman and Griinari 2001). Studies with post-ruminal infusion of trans-10, cis-12-CLA clearly demonstrated the anti-lipogenic effect of this CLA isomer which was however not always accompanied by a reduction of milk desaturation ratios. Then, post-ruminal infusion studies in lactating cows have established that in amounts above 5 g/day, trans-10, cis-12-CLA typically decreased milk FA desaturation ratios consistent with a decrease in *SCD1* mRNA abundance (Table 13.3). In the bovine, in vivo studies indicated that injection or infusion of trans-10, cis-12-CLA decreased mammary *SCD1* gene transcription (Baumgard et al. 2002; Gervais et al. 2009), whereas no effect was observed on *SCD5* (Gervais et al. 2009). In cows, the responses of mRNA abundances of the lipogenic genes involved in other pathways than desaturation (de novo FA synthesis, FA uptake, transport and esterification) to either diets that induce MFD (Table 13.1) or trans-10, cis-12-CLA infusion (Tables 13.3 and 13.4) showed a large decrease that occurred prior to a decrease in *SCD1* mRNA. It is likely that the responses of lipogenic genes and *SCD1* to these factors reflect a two-step phenomenon which could be related to differences in the response to the transcription factors involved in these regulations (see section "Knowledge on the Signalling Pathways Mediating Nutritional Regulation of SCD Gene Expression in Ruminants"). Otherwise, in the caprine, administration of trans-10, cis-12-CLA directly at the duodenum (Andrade and Schmidely 2006) or fed as calcium salts (Shingfield et al. 2009) was reported to lower milk FA desaturation ratios in the absence (Andrade and Schmidely 2006) or with a small decrease (Shingfield et al. 2009; Table 13.3) in milk fat secretion. Similarly, in the ovine, distribution of trans-10, cis-12-CLA as encapsulated lipids was reported to lower milk fat secretion and FA desaturation ratios (Lock et al. 2006).

Table 13.3 Summary of CLA isomers acting as inhibitors of milk fat synthesis and/or milk fatty acid desaturase ratios in ruminants

Treatment[a]	Species	Dose (g/d)	Duration (h)	Change in milk fat secretion (%)[b]	Milk desaturation ratio response[c] C14/C16/C18/C18:1 t11	Transfer efficiency into milk (%)	Reference
t10c12-CLA							
Post-rumen	Bovine	13.6	5	–48	–32/NS/–17/–24	12	Baumgard et al. (2002)
	Bovine	5	4×24	–28	–10/NS/NS/–10	28	Perfield et al. (2006)
	Bovine	4.2	4×24	–20	–14/NS/–32/–27	19	Sæbø et al. (2005)
	Bovine	5	5×24	–27	–6/–4/–4/–8	22	Perfield et al. (2007)
Intravenous	Bovine	10	5×24	–32	NS		Gervais et al. (2009)
Post-rumen	Caprine	2	3×24	NS	NR/–21/–50/–45	18	Andrade and Schmidely (2006)
Calcium salt	Caprine	7.5	10×24	–20	–70/–53/–57/–17	2.37	Shingfield et al. (2009)
	Caprine	15	10×24	–28	–74/–50/–63/–2	2.38	
	Caprine	22	10×24	–32	–66/–34/–66/–14	2.19	
Lipid encapsulated	Ovine	2.4	10×24	–16	NS/–24/–6/–8	3.8	Lock et al. (2006)
t10t12-CLA							
Post-rumen	Bovine	5	4×24	NS	–19/–18/–10/–15	17	Perfield et al. (2006)
	Bovine	4.7	4×24	NS	–14/NS/–20/–15	23	Sæbø et al. (2005)
t9t11-CLA							
Post-rumen	Bovine	5	5×24	NS	–29/–11/–8/–13	21	Perfield et al. (2007)

[a]Treatment consists of either infusion, abomasal, duodenal, or intravenous of CLA or addition of CLA to the diet as lipid encapsulated or calcium salt
[b]Response reported when treatment effects were significant ($P<0.10$) and calculated as [((Treatment – Control)/Control) × 100]
[c]Response of the milk desaturation ratio (14:1-cis9/14:0) reported when treatment effects were significant ($P<0.10$); NS, values not reported because unsignificant; NR, not reported

Table 13.4 Effect of infusion or injection of trans-10, cis-12-CLA and trans-11-18:1 on mammary mRNA abundances of *SCD1* and lipogenic genes in ruminants

Treatment[a]	Species	Transcript[b]	Dose (g/d)	Duration (h)	Response (%)[c]	Milk desaturation ratio response[d] C9-14:1/14:0	Change in milk fat secretion (%)[c]	Reference
t10c12-CLA								
Post-rumen	Bovine	SCD1	13.6	5	−50	−32	−48	Baumgard et al. (2002)
		FAS/ACC			−40/−39			
		LPL/FABP			−48/−54			
		GPAT/AGPAT			−42/−41			
Intravenous	Bovine	SCD1	10	3×24	NS	NR	−24	Harvatine and Bauman (2006)
		SREBF1/Insig1/S14			−30/−45/−40			
		FAS/LPL			−25/−25			
Intravenous[e]	Bovine	SCD1/SCD5	10	5×24	Tendancy/NS	NS	−32	Gervais et al. (2009)
		ACC/FAS/LPL			−46/−57/tendancy			
		SREBF1			−59			
t11-18:1	Caprine	SCD1	0.3	4×24	+27	+66 (c9t11/t11)	ND	Jin et al. (2012)
Intravenous		ACC/FAS/LPL			−40/−64/NS			

[a]Treatment consists of either post-ruminal infusion and/or intravenous injection of trans-10, cis-12-CLA and trans-11-18:1

[b]Measurement of tissue transcript abundance (mRNA) of *SCD* stearoyl-CoA desaturase 1 and/or 5, *ACC* acetyl CoA carboxylase, *AGPAT* acylglycerol phosphate acyl transferase, *FAS* fatty acid synthase, *FABP* fatty acid binding protein, *GPAT* glycerol phosphate acyl transferase, *Insig1* insulin induced gene 1, *LPL* lipoprotein lipase, *S14* thyroid hormone responsive spot 14, *SREBF1* sterol response element binding protein

[c]Response reported when treatment effects were significant ($P<0.10$) and calculated as [((Treatment − Control)/Control) × 100]

[d]Response of the milk desaturation ratio (14:1-cis9/14:0) reported when treatment effects were significant ($P<0.10$); NS, values not reported because unsignificant; NR, not reported

[e]Control treatment, infusion of cis-9, cis-12-C18:2

From data in ruminant species (Tables 13.1, 13.3, and 13.4), it may be hypothesized that whereas mammary lipogenic genes expression (at the level of the mRNA and protein) in caprine is less sensitive to the anti-lipogenic effect of the trans-10, cis-12-CLA than in cows, for SCD protein the figure could be different with a higher sensitivity in goats. Indeed, indirect comparison from studies in goats (Bernard et al. 2009c) and cows (Roy et al. 2006) with similar dietary conditions (diets based on maize silage and supplemented with 5 % sunflower oil) showed that the four milk FA desaturation ratios decreased in goats, whereas these ratios increased in cows (Table 13.2).

Moreover, post-ruminal infusion studies in cows have shown that in addition to trans-10, cis-12-CLA, trans-10, trans-12- (Sæbø et al. 2005) and trans-9, trans-11-CLA (Perfield et al. 2007) reduced milk FA desaturation ratios (Table 13.3), and therefore, these two latter biohydrogenation intermediates could be specific inhibitors of SCD activity since they were not associated with MFD. In particular, the high response of milk trans-9, trans-11-CLA in goats receiving sunflower oil could play a direct role in the typical decrease of milk FA desaturation ratios in this animal species (Table 13.2). Otherwise, abomasal infusions in cows and ewes (Griinari et al. 2000; Corl et al. 2001; Kay et al. 2004; Bichi et al. 2012) of sterculic oil, known as a strong inhibitor of SCD, and administration of Co-EDTA in the rumen or per os (Shingfield et al. 2006, 2008a; Taugbøl et al. 2008, 2010; Karlengen et al. 2012) decreased the milk FA desaturation ratios without alteration of mammary *SCD1* mRNA abundance (Karlengen et al. 2012) and without decreasing milk fat secretion. It was hypothesized (Shingfield et al. 2008a) that Cobalt interferes with the transfer of electrons from cytochrome b5 to the di-iron protein center of the terminal desaturase protein, thus altering the oxidation–reduction reactions of the conversion of the acyl-CoA substrates. In bovine the concomitant effects of trans-10, cis-12-CLA on mammary *SCD1* mRNA abundance and milk FA desaturation ratios (Table 13.4) suggest an effect at the level of *SCD1* transcription for this CLA isomer. Otherwise, the mechanisms by which trans-10, trans-12 CLA and trans-9, trans-11 CLA decreased milk FA desaturation ratios are not clear and could involve an inhibition of *SCD1* gene transcription or activity.

In Vitro Studies on the Effect of Fatty Acids on Mammary SCD

Few in vitro systems have been developed to investigate the role of specific FA on the regulation of mammary lipogenesis (Table 13.5), including: (1) dispersed bovine mammary epithelial cells (bMECs) (Hansen et al. 1986; Hansen and Knudsen 1987) or primary bMECs (Matitashvili and Bauman 2000); (2) bMEC lines (MAC-T (Jayan and Herbein 2000; Peterson et al. 2004; Kadegowda et al. 2009; Ma and Corl 2012), BME-UV (McFadden et al. 2008)) or the cloned bMECs (Liu et al. 2006; Yonezawa et al. 2008); and (3) mammary slices (Matitashvili et al. 2001; Bernard et al. 2012). Although they are a simplification of physiological conditions, these

Table 13.5 Effect of fatty acid and of agonist or silencer of transcription factor on the regulation of mammary SCD1 and lipogenic gene mRNA abundance and/or enzyme activity in vitro

Biochemical pathway[a]	Transcript/protein[b]	In vitro model	FA supplement/no FA or treatment	Concentration (μM)	Duration treatment (h)	Change in expression or activity (%)[c]	Reference
Nuclear receptors							
SREBF1	mRNA	MAC-T	trans-10, cis-12-CLA	75	48	NS	Peterson et al. (2004)
			cis-9, trans-11-CLA	75	48	NS	
	Precursor protein (125 kDa)		trans-10, cis-12-CLA	75	48	NS	
			cis-9, trans-11-CLA	75	48	−11	
	Mature protein (68 kDa)		trans-10, cis-12-CLA	75	48	−26	
			cis-9, trans-11-CLA	75	48	NS	
SREBF1/PPARG/S14	mRNA	MAC-T	16:0	100	12	NS/NS/NS	Kadegowda et al. (2009)
			18:0	100	12	NS/NS/NS	
			cis-9-18:1	100	12	−139/NS/NS	
			trans-10-18:1	100	12	−48/NS/NS	
			trans-10, cis-12-CLA	100	12	−189/NS/-73	
			20:5	100	12	−125/NS/NS	
			Rosiglitazone	50	12	+100/NS/NS	
SREBF1	mRNA	BME-UV	T0901317 (T09)	2	8	+416	McFadden and Corl (2010)
	Precursor protein		LXR agonist	2	8	+240	
	Mature protein			2	8	+210	
SREBF1	mRNA	Goat MEC	T0901317 (T09)	0.01/0.1/1	24	+132/+269/+381	Wang et al. (2012)
FAS	mRNA		LXR agonist	0.01/0.1/1	24	+127/+157/+223	
LXRA	mRNA			0.01/0.1/1	24	NS/NS/NS	

SREBF1/S14	mRNA	MAC-T	SREBF1-SiRNA[d]	0.1	48	−90/−95	Ma and Corl (2012)
SREBF1	Precursor protein			0.1	48	Undetectable	
	Mature protein			0.1	48	Undetectable	
Desaturation and de novo lipogenesis							
SCD1	mRNA	MAC-T	trans-11-18:1	50	(90)+6	NS	Jin et al. (2012)
PSMA5	mRNA	MAC-T	trans-11-18:1	50	(90)+6	+340	
SCD1	mRNA	bovMEC	trans-11-18:1	200	6 or 72	+200	Matitashvili and Bauman (2000)
			cis-11-18:1			NS	
SCD1	mRNA	MAC-T	trans-10, cis-12-CLA	75	48	−40	Peterson et al. (2004)
			cis-9, trans-11-CLA	75	48	NS	
SCD1	mRNA	MAC-T	16:0	100	12	+95	Kadegowda et al. (2009)
			18:0	100	12	NS	
			cis-9-18:1	100	12	−428	
			trans-10-18:1	100	12	−100	
			trans-10, cis-12-CLA	100	12	−357	
			20:5	100	12	−205	
			Rosiglitazone	50	12	NS	
SCD	Activity	MAC-T	cis-9-18:1/18:0	100	72	−21	Jayan and Herbein (2000)
			trans-11-18:1/18:0	100	72	+72	
ACC/FAS	Activity	MAC-T	cis-9-18:1/18:0	100	72	−27/−35	Jayan and Herbein (2000)
			trans-11-18:1/18:0	100	72	−58/−43	
SCD1	mRNA	MAC-T	SREBF1-SiRNA[d]	0.1	48	−60	Ma and Corl (2012)
ACC/FAS/IDH1	mRNA					−40/−65/−50	
ACSS1/ACSS2	mRNA					NS/−60	

(continued)

Table 13.5 (continued)

Biochemical pathway[a]	Transcript/protein[b]	In vitro model	FA supplement/no FA or treatment	Concentration (μM)	Duration treatment (h)	Change in expression or activity (%)[c]	Reference
SCD1/ACC	Protein					−90/−47	
FAS	mRNA	BME-UV	T0901317 (T09)	2	8	+185	McFadden and Corl (2010)
FAS	mRNA	Goat MEC	T0901317 (T09)	0.01/0.1/1	24	+127/+157/+223	Wang et al. (2012)
SCD	Activity	Goat cell fraction[e]	Oleic (>lin >lnn)[f]	0.8	1	−53	Bickerstaffe and Annison (1970)
Long-chain activation and transport							
ACSL1/FABP3	mRNA	MAC-T	SREBF1-SiRNA[d]	0.1	48	NS/−76	Ma and Corl (2012)
Esterification and protein of milk fat globule							
GPAM/AGPAT6	mRNA	MAC-T	SREBF1-SiRNA[d]	0.1	48	−20/+22	Ma and Corl (2012)
DGAT1						±70	
Protein of the milk fat globule							
PLIN2/BTN1A1/LPIN1	mRNA	MAC-T	SREBF1-SiRNA[d]	0.1	48	−250/NS/−90	Ma and Corl (2012)

[a]*SCD* stearoyl-CoA desaturase, *ACC* acetyl CoA carboxylase, *FAS* fatty acid synthase, ACSS*1* acyl-CoA synthetase short chain family member 1, *ACSS2* acyl-CoA synthetase short chain family member 2, *ACSL1* acyl-CoA synthetase long chain family member 1, *ACSL2* acyl-CoA synthetase long chain family member 2, *IDH1* isocitrate dehydrogenase 1, *S14* thyroid hormone responsive spot 14, *SREBF1* sterol response element binding protein, *PPARG* peroxisome proliferator receptor gamma, *LPIN1* lipin1, *PLIN2* perilipin 2, *BTN1A1* butyrophilin subfamily 1 member A1, *AGPAT6* acylglycerol phosphate acyl transferase 6, *FABP3* fatty acid binding protein 3, *GPAM* glycerol-3-phosphate acyltransferase 1, *DGAT1* diacylglycerol acyl transferase 1

[b]Measurement of tissue transcript abundance (mRNA) or protein activity (Activity)

[c]Response reported when treatment effects were significant ($P<0.10$) and calculated as [((Treatment − Control)/Control) × 100]; NS, values not reported because unsignificant

[d]MACT-T transfected with sterol regulatory element binding protein SREBP-1 small interfering (si) RNA

[e]Goat subcellular fraction of mammary tissue (post-mitochondrial fraction)

[f]lin linoleic acid, lnn linolenic acid

in vitro systems offer an advantage over in vivo nutritional studies because the effects of specific FA in small amount can be evaluated under controlled conditions. Then, several years ago looking at the effects of specific FA on bovine mammary cell line (MAC-T cells), Jayan and Herbein (2000) showed that, compared to stearic acid, oleic acid decreased SCD activity. Similarly, still using MAC-T cell, Kadegowda et al. (2009) demonstrated that oleic acid, among other long-chain FA including 20:5*n*-3, was the most potent inhibitor of *SCD* gene expression, which is suggestive of a feedback-inhibition mechanism. Furthermore, earlier studies (Bickerstaffe and Annison 1970) observed negative effects of oleic, linoleic, and linolenic acids on goat mammary SCD activity measured on the subcellular fraction of goat mammary gland, which have been partly confirmed by in vivo studies on goat (Bernard et al. 2005b, 2009b). Regarding the effect of trans-FA, only two trans-18:1 isomers have been tested in vitro (Table 13.5). Incubation of trans-11-18:1 with bovine MEC increased *SCD1* mRNA abundance (Matitashvili and Bauman 2000) and SCD activity in bovine MAC-T cell line (Jayan and Herbein 2000), whereas trans-10-18:1 reduced *SCD1* mRNA abundance in bovine MAC-T cell line (Kadegowda et al. 2009). Similarly, in goats, intravenous bolus injection of trans-11-18:1 increased both the expression of *SCD* in mammary tissue (Jin et al. 2012) and milk cis-9, trans-11-CLA, suggesting an upregulation of the expression at the protein level. Otherwise, trans-10, cis-12-CLA (and to a lesser extent cis-9, trans-11-CLA) caused a significant reduction in *SCD* transcriptional activity (Keating et al. 2006) in bovine MAC-T cells, due to the *SCD* transcriptional enhancer element region (STE; see section "Characterization of SCD Genes"). The same study also showed that bovine *SCD* promoter was upregulated by insulin and downregulated by oleic acid, whereas linoleic, linolenic, stearic, and vaccenic acids had no effect. Elsewhere, using bovine and caprine mammary slices, Bernard et al. (2012) did not evidenced significant effect of trans-10, cis-12-CLA on *SCD1* mRNA abundance after 20 h incubation time. Conversely, in bovine MAC-T cell, Kadegowda et al. (2009) demonstrated that trans-10, cis-12-CLA reduced expression of *SCD1* together with *SREBF1*. These two latter studies outline differences of response of different in vitro models for studying lipogenic genes expression.

So far, the developed in vitro systems present several limitations: MECs and cultured mammary slices have a limited lifetime, modified cell lines often have abnormal characteristics and low secretory activity. Nevertheless, use of these approaches has allowed to decipher, at least in part, the underlying mechanisms responsible for the inhibitory effects of trans-10, cis-12-CLA on lipogenesis and lipogenic gene expression in primary culture of bMEC (Matitashvili and Bauman 2000), MEC lines (Peterson et al. 2004; McFadden et al. 2008; Kadegowda et al. 2009), and mammary explants (Matitashvili et al. 2001) (see section "Knowledge on the Signalling Pathways Mediating Nutritional Regulation of SCD Gene Expression in Ruminants"). Further research is necessary in ruminants to identify the major regulators of FA desaturation which is hampered by the lack of pure trans-18:1 and 18:2 isomers and by the difficulty to obtain an in vitro functional model for lipid synthesis and secretion (Barber et al. 1997; Bernard et al. 2012).

Knowledge on the Signalling Pathways Mediating Nutritional Regulation of SCD *Gene Expression in Ruminants*

Whereas the signalling pathways involved in the regulation of *SCD* gene expression by dietary factors (e.g., the inhibitory effect of PUFA) have been comprehensively described in rodent tissues (Nakamura and Nara 2004; Ntambi et al. 2004), still little is known about these mechanisms in ruminants. Among the class of transcription factors known as major regulators of lipid synthesis are the sterol response element binding protein (SREBP) family (Eberlé et al. 2004) and peroxisome proliferators-activated receptors (PPARs) (Clarke 2001).

Because different SREBP isoforms have different roles in regulating lipid synthesis, these isoforms have been studied extensively in different species (Brown and Goldstein 1997).

SREBP family is composed of three members: two isoforms of SREBP-1 (a and c) and SREBP-2. Both SREBP-1a and SREBP-1c, which can be differentially expressed in various tissues, are produced from a single gene (*SREBF-1*, located on bovine chromosome 17) presenting alternative splicing. *SREBF1c* and *SREBF1a* use different first exons and both regulate FA synthesis. SREBP-2 is transcribed from a separate gene (*SREBF2*, located on bovine chromosome 5) and regulates the cholesterol biosynthetic pathway (Horton 2002).

Multiple proteins are involved in the SREBP pathway because (1) SREBP are translated as precursor proteins which need maturation steps to become mature that involve several proteins (such as SCAP, INSIG1, 2, S1P, S2P) and (2) the SREBP mature proteins recruit other proteins acting as co-activators to activate the transcription of lipogenic genes to whom belong *LPL*, *ACACA*, *FASN*, and *SCD1* genes.

Regarding PPARs, three different isoforms of PPAR (PPARα, PPARβ/δ, and PPARγ), have been identified and are encoded by three different genes; they exhibit significant differences in tissue distribution and ligands but all function by dimerizing with RXR and binding to the PPAR response element (PPRE) DNA sequence (Sampath and Ntambi 2005).

In lactating cows, in vivo studies have shown the involvement of SREBP-1 and/or PPAR during diet-induced changes in mammary lipogenic gene expression (Angulo et al. 2012; Bionaz and Loor 2008) or in response to 3–5 d intravenous infusions of trans-10, cis-12-CLA (Harvatine and Bauman 2006; Gervais et al. 2009) with the implication that these transcription factors are central elements in the overall regulation of milk fat synthesis.

However, little is known in the ruminant mammary gland on the signalling pathways involved in *SCD* gene expression. Among six in vivo studies (either nutritional or infusion studies) in cows with MFD that measured the mammary mRNA abundances of both lipogenic genes and *SCD1*, only two reported a simultaneous decrease in mRNA abundance of lipogenic genes and *SCD1* (Baumgard et al. 2002; Angulo et al. 2012). Conversely, in the others, either nutritional studies (Ahnadi et al. 2002; Harvatine and Bauman 2006; Peterson et al. 2003) or with intravenous

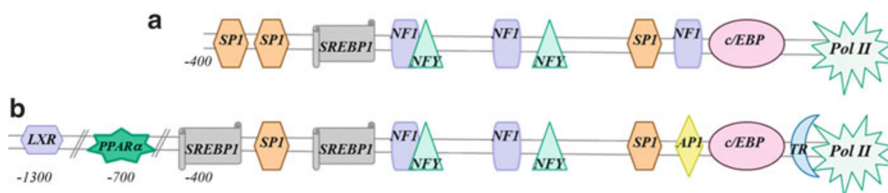

Fig. 13.5 Diagram of the SCD1 gene promoter. The diagram shows the different transcription factors involved in the regulation of SCD1 gene transcription. (**a**) Represents the binding sites of transcription factors deduced by sequence homology between bovine sequence (AY241932) and pig (AY487830) and human (AF 320307). (**b**) Represents elements characterized in human, mouse, and chicken promoters from Mauvoisin and Mounier (2011)

infusion of trans-10, cis-12-CLA (Gervais et al. 2009), a coordinate decrease in the abundance of lipogenic genes was observed with only a tendency to decrease for *SCD1* mRNA suggesting that the transcription of this gene varies less or less rapidly in response to the diet. Probably the other lipogenesis-related transcription factors (e.g., PPARs, SP1, NFY, CEBPs; Fig. 13.5) expressed in the ruminant mammary gland (Bionaz and Loor 2008; Toral et al. 2013b) are involved in the mediation of diet-induced effects on mammary *SCD1* gene expression that may counteract for the effect of *SREBF1*.

Otherwise, in an in vitro study using bovine MAC-T cell line, addition of trans-10, cis-12-CLA and 20:5*n*-3 decreased both *SREBF1* and *SCD1* mRNA abundances with variable effects on the other lipogenic genes and without changing *PPARG* mRNA (Kadegowda et al. 2009). Recently, it was reported that the bovine *SREBF1* gene contained in its promoter responsive elements for both *SREBF1* (SRE) that could allow for a positive feedback regulation by SREBP-1 itself and for liver X receptor (LXR-RE; Lengi and Corl 2010). Therefore, the SREBP-1 responsiveness to LXR activation was demonstrated in BME-UV cell line (McFadden and Corl 2010) using T0901317 (T09), an LXR agonist that resulted in the upregulation of transcription, translation, and proteolytic cleavage of SREBP-1. Because both SRE and LXR-RE have been characterized in *SCD1* gene promoter in human, mouse, or chicken (Mauvoisin and Mounier 2011) and are conserved between species, probably they may also be present in bovine *SCD1* gene promoter. Using bovine MAC-T cell line, Peterson et al. (2004) have demonstrated that the inhibitory effect of trans-10, cis-12-CLA acts through inhibition of the proteolytic activation of SREBP-1, resulting in a reduction in the transcriptional activation of lipogenic genes (*ACACA* and *FASN*) and *SCD1*, without increasing the *SREBF1* expression. The role of SREBP-1 in regulating milk fat synthesis had not been directly evaluated in ruminants until a recent study in MAC-T cells (Ma and Corl 2012) using RNA interference to knock down *SREBF1* (the *SREBF1*-specific siRNA targeted a region of the transcript identical between *SREBF1a* and *SREBF1c* and did not distinguish the two isoforms 1a and 1c) to investigate the specific role of SREBP-1 in affecting the expression of genes encoding key enzymes of milk fat synthesis including *SCD1*. In this study,

FABP3 and *SCD1 mRNAs* were both decreased when SREBP-1 mRNA decreased (and SREBP protein was undetectable), indicating that transport and desaturation of long-chain FA could be regulated transcriptionally by SREBP-1.

Altogether these data underlined the necessity to better characterize the molecular mechanisms involved in the dietary regulation of *SCD* gene expression in ruminant species, and in particular to precise the role of SREBP-1 and PPARs. The observed differences in the response of *SCD* and other lipogenic genes expression to decreased *SREBF1* mRNA abundance (Table 13.1) may suggest a lower threshold response for *SCD* to SREBP-1 level compared to other lipogenic genes which would be in line with the hypothesis of a two-step mechanisms of regulation for these genes, with the implication of other transcription factors in these regulations.

Conclusions

A balance between the endogenously synthesized short-chain FA, the exogenous unsaturated long-chain FA, and the SCD desaturation products must be maintained within the mammary gland in order to preserve the fluidity of milk fat, which emphasizes the importance of knowledge on SCD regulation. Over the last years, the development of studies in ruminants on the nutritional regulation of a few "candidate" genes involved in mammary lipogenesis including Δ9-desaturation (*SCD*) has shown that *SCD* gene regulation differ from other lipogenic genes. Indeed, in cows with dietary conditions that induce a dramatic MFD, a decrease in the mRNA abundance of mammary lipogenic genes was observed which was not always observed for *SCD*. It was hypothesized that the responses of lipogenic genes and *SCD1* to dietary factors that induce MFD in cows occur as a two-step phenomenon, with a first downregulation of lipogenic genes followed by *SCD1*, which could be related to the observed differences in the response to *SREBF1* or to other transcription factors involved in these regulations. In addition, these studies showed that the response of mammary *SCD* mRNA or activity to nutritional factors do not always match with the responses of milk FA Δ-9 desaturation ratios. In goats, *SCD* is regulated at a transcriptional and/or post-transcriptional level, depending on the lipid supplements. Conversely, in cows, data converged to demonstrate that *SCD* mRNA varied little in response to diet except for a decrease when "protected" fish oil or combination of PUFA and DHA-rich algae was fed. Indirect comparison of data from bovine and caprine suggested that SCD in caprine could be more sensitive to the effect of dietary PUFA or their ruminal biohydrogenation intermediates than in cows and that trans-9, trans-11-CLA could play a specific role in the regulation of mammary Δ-9 desaturation in goats receiving PUFA-rich diets. Future experiments examining changes in mammary *SCD* and other lipogenic genes expression in the different ruminant species in response to dietary treatments as well as ruminant *SCD1* and *SCD5* promoter sequencing would provide a more comprehensive insight into the regulation of these genes and their impact in the synthesis of milk fat.

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Chapter 14
Physiological Functions and Regulation of *C. elegans* Stearoyl-CoA Desaturases

Jennifer L. Watts

The *Caenorhabditis elegans* Model for Studies of Lipid Synthesis and Function

The nematode *C. elegans* is an excellent model for genetic studies of complex biological phenomena, and research using this organism has contributed significantly to elucidating the mechanisms of diverse biological processes (Barr 2003). Recent work in *C. elegans* has identified many regulatory proteins and downstream effector genes responsible for lipid homeostasis (Watts 2009; Vrablik and Watts 2012). While *C. elegans* stores lipids in intestinal and hypodermal cells rather than dedicated adipose tissue, other aspects of worm biochemistry and regulation of fat metabolism closely parallel humans. Because of its small size (1.5 mm), rapid life cycle, and ease of laboratory cultivation, *C. elegans* offers great potential for genetic analysis (Riddle et al. 1997). In addition to the traditional forward genetic approaches, reverse genetic studies are popular and powerful because the activity of a target gene can be inactivated in vivo by feeding *C. elegans* bacteria producing double stranded RNA for that gene, a technique referred to as RNA interference, or RNAi (Fire et al. 1998). During feeding the double stranded RNA is absorbed by the intestine and distributed throughout the animal (Timmons and Fire 1998). The construction of RNAi libraries that consist of *E. coli* strains expressing double stranded RNA that correspond to nearly every *C. elegans* gene has allowed for whole-genome screening approaches to identify genes involved in diverse biological processes. These processes include embryogenesis (Sonnichsen et al. 2005), neuronal specification (Poole et al. 2011), and the regulation of longevity (Hamilton et al. 2005).

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C. elegans contains genes encoding enzymes required for fatty acid synthesis, elongation, and desaturation as well as those encoding peroxisomal and mitochondrial β-oxidation enzymes. Fatty acids are esterified to glycerol to form triacylglycerols (TAGs), which are stored in lipid droplets and yolk. In *C. elegans*, TAGs are a vital energy source during embryogenesis, periods of low food availability, and for the specialized non-feeding dauer larval stage. Like other eukaryotes, *C. elegans* obtains fatty acids from its diet and also synthesizes them de novo from acetyl-CoA (Perez and Van Gilst 2008). For de novo synthesis, all of the enzyme activities necessary for the synthesis of palmitic acid (16:0) from acetyl-CoA are encoded by two multifunctional enzymes: acetyl-CoA carboxylase and fatty acid synthase (Wakil 1989; Rappleye et al. 2003; Chirala and Wakil 2004; Wakil and Abu-Elheiga 2008). Palmitic acid can be integrated into TAGs or phospholipids, or it can be modified by fatty acid elongases and desaturases to form a variety of long chain polyunsaturated fatty acids (PUFAs; Wallis et al. 2002; Watts and Browse 2002). Long chain PUFAs are preferentially incorporated into membrane phospholipids, where they affect membrane properties including fluidity, temperature sensitivity, and signaling. In addition, *C. elegans* produces monomethyl branched-chain fatty acids (mmBCFAs), which are essential for growth and embryonic development (Kniazeva et al. 2004, 2012).

C. elegans Encodes Three SCD Orthologs

Due to the abundant and vital roles of lipids, the synthesis and breakdown of fatty acids are subject to many levels of regulation. A critical control point regulating lipid synthesis is the production of monounsaturated fatty acids. *C. elegans* encodes three stearoyl-CoA desaturases (SCDs), also known as Δ9 desaturases, that synthesize monounsaturated fatty acids from saturated fatty acids (Watts and Browse 2000). These enzymes, named FAT-5, FAT-6, and FAT-7, share significant amino acid similarity to rat and mouse SCDs, as well as to the yeast Δ9 desaturase OLE1. By expressing the SCD genes in the yeast *ole1* mutant, the substrate specificities of the three enzymes were determined. The FAT-5 desaturase is specific for palmitic acid (16:0), whereas the FAT-6 and FAT-7 desaturases preferentially introduce a double bond into stearic acid (18:0) (Watts and Browse 2000).

In contrast to mammals, oleic acid produced by the FAT-6 and FAT-7 SCDs is used as a substrate to produce a range of PUFAs. This reaction is mediated by a Δ12 desaturase, an enzyme normally found only in algae and plants (Wallis et al. 2002). Δ12 desaturation of oleic acid produces linoleic acid (18:2), which is further acted on by an omega-3 desaturase FAT-1 and a Δ6 desaturase FAT-3, which, together with elongation and Δ5 desaturation, produces a range of C18 and C20 omega-6 and omega-3 PUFAs (Fig. 14.1) (Watts and Browse 2002).

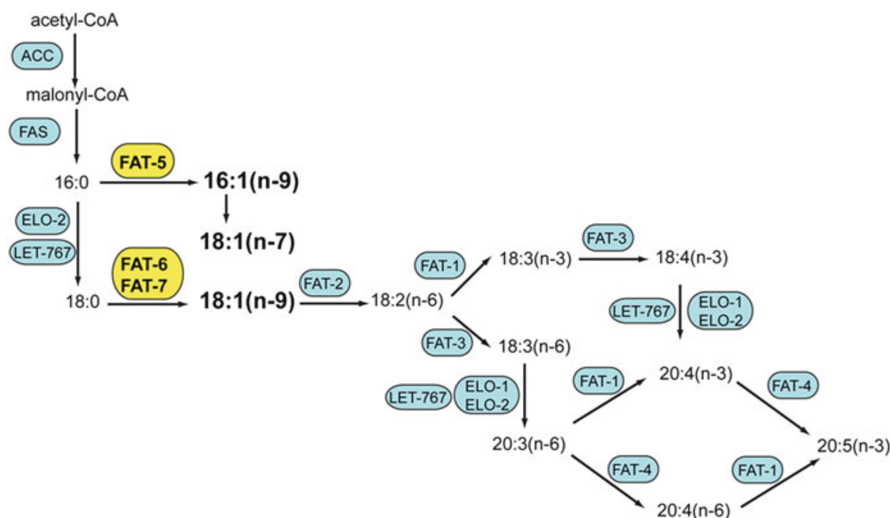

Fig. 14.1 Pathway of synthesis of unsaturated fatty acids in *C. elegans*. A variety of long chain polyunsaturated fatty acids can be synthesized from acetyl-CoA. The SCDs FAT-5, FAT-6, and FAT-7 are highlighted in *yellow*. *ACC* acetyl-CoA carboxylase, *FAS* fatty acid synthase, *ELO* fatty acid elongase, *LET* lethal, *FAT* fatty acid desaturase

Loss of One SCD Isoform Is Compensated for by Up-regulation of the Remaining SCDs

To determine the consequences of depleted SCD activity in *C. elegans*, loss of function mutants in each gene were isolated and analyzed. Gene knockdowns of individual SCD genes using RNAi have also been reported, but due to high sequence similarity between the three SCD genes, which lead to off-target effects, the phenotypes of strains carrying single and double mutations are more easily interpreted. The *fat-5;fat-6;fat-7* triple mutant strain was constructed and was found to be inviable, revealing that endogenous production of monounsaturated fatty acids is essential for survival (Brock et al. 2006). In contrast, the fatty acid composition changes and physiological consequences of the single desaturase mutants are subtle (Brock et al. 2006). The most highly expressed of the three Δ9 desaturases is the FAT-6 SCD, which is expressed in the intestine and hypodermal (skin) tissues (Brock et al. 2006). The *fat-6* mutants display increased stearic acid (18:0) and decreased unsaturated fatty acids, however, the change in fatty acid composition in the *fat-6* mutant worms is modulated by genetic compensation by the other SCDs. Real-time quantitative RT-PCR experiments showed that in the *fat-6* mutants, the *fat-7* and *fat-5* genes are up-regulated three to fivefold. Presumably, this compensation normalizes

the fatty acid composition and allows the strain to grow, reproduce, and behave like wild type (Brock et al. 2006).

Similarly, the *fat-7* mutants have wild type fatty acid composition, fat stores, and reproductive success, even when combined with *klf-3* mutants, which lack a Kruppel-like transcription factor and regulate fat storage in *C. elegans* and in humans (Brock et al. 2006; Zhang et al. 2011). In *fat-7* mutants, both *fat-6* and *fat-5* are up-regulated 1.5–2-fold (Brock et al. 2006). Knockdown of *fat-6* or *fat-7* by RNAi was reported to show decreased resistance to oxidative stress and increased heat tolerance (Horikawa and Sakamoto 2009). However, given that high degree of DNA sequence similarity (86 % identity) between the *fat-6* and *fat-7* cDNA sequences, off-target reduction of the other SCD homologues confounds the interpretation of these results.

In addition to genetic compensation, defects in fatty acid desaturase genes can be compensated for by dietary input. For example, the *fat-5* mutant has only subtle fatty acid composition changes when grown on plate with dietary *E. coli*. This is because palmitoleic acid (16:1) and its elongation product cis-vaccenic acid (18:1*n*-7) are provided by the *E. coli* diet. When *fat-5* mutants are grown axenically in media lacking *E. coli*, the *fat-5* mutants have a more profound fatty acid composition defect, showing greatly increased palmitic acid (16:0) and severely reduced cis-vaccenic acid (18:1*n*-7) (Brock et al. 2006). A recent study examining the relationship between fat stores and dietary restriction in *C. elegans* showed that *fat-5* expression increased two to fourfold in nematodes exposed to dietary restriction during development (Palgunow et al. 2012). Dietary restriction during development resulted in reduced body size, but an increase in the size of lipid droplets and in overall TAG stores. In addition to increases in *fat-5* expression, other lipogenic genes such as those encoding DGAT and acetyl-CoA synthetase were also up-regulated, suggesting that *C. elegans* adapts metabolically to conserve fat stores during periods of suboptimal nutriention (Palgunow et al. 2012).

In spite of compensation to normalize overall fatty acid composition, LC–MS analysis revealed differences in various TAG and PC species among the *fat-5*, *fat-6*, and *fat-7* mutant strains, as well as evidence of increased absorption of dietary fatty acids in the *fat-6* mutant (Castro et al. 2012). Interestingly, metabolomic analysis revealed significant differences in non-lipid metabolites such as alanine and succinate, indicating an increase in catabolism of amino acids and an increase in the activity of the TCA cycle in the *C. elegans* SCD mutants (Castro et al. 2012). Metabolomic studies such as these promise to provide insights as to the complex compensatory mechanisms that occur in animals when lipid homeostasis is altered.

Double Mutant Strains Reveal Additional Requirements for SCDs

Examination of double mutant strains reveals additional roles for monounsaturated fatty acid synthesis in temperature adaptation and in the regulation of fat storage. The *fat-5;fat-7*, *fat-5;fat-6*, and *fat-6;fat-7* double mutant strains display significant

changes in their fatty acid composition. The two strains lacking *fat-5* have increased palmitic acid (16:0) and severely reduced palmitoleic acid (16:1*n*-7) and cis-vaccenic acid (18:1*n*-7). Under standard conditions, these strains grow normally, but they have decreased survival in cold temperature (10 °C), indicating that the higher saturated and lower unsaturated fatty acid composition is detrimental to nematodes at low temperatures (Brock et al. 2007). In addition, the *fat-5;fat-6* double mutant strain was unable to survive starvation stress. After 4 days of starvation of L1 larvae in buffer without nutrients, wild type larvae are viable and maintain a normal fatty acid composition. However, the *fat-5;fat-6* double mutants die and have extremely high saturated fatty acid content in their membranes (Brock et al. 2007). The lack of dietary fatty acids, together with the observation that *fat-7* expression is highly repressed in the absence of food (Van Gilst et al. 2005b), reveals the roles of FAT-5 and FAT-6 in maintaining proper fatty acid composition during starvation.

The *fat-6;fat-7* Double Mutants Have Reduced Fat Stores and Increased Fat Oxidation, Similar to Mouse SCD1 Mutants

The *fat-6;fat-7* double mutant is unable to carry out normal PUFA synthesis, because it cannot synthesize the oleic acid precursor which is the substrate for the remaining PUFAs. However, unusual 18-carbon PUFAs are formed by elongation and desaturation of the FAT-5 product palmitoleic acid (16:1), which apparently partially substitute for vital functions performed by normal PUFAs (Brock et al. 2007). The *fat-6;fat-7* double mutants display slow growth and greatly reduced fertility. Dietary oleic acid partially, but not completely, rescues the growth and fertility deficiencies (Brock et al. 2007).

A striking phenotype of the *fat-6;fat-7* double mutant is greatly reduced fat stores compared to wild type (Brock et al. 2007). Real-time PCR analysis of fatty acid oxidation genes reveals that genes encoding components of mitochondrial fatty acid oxidation are expressed at higher levels in the *fat-6;fat-7* double mutant compared to wild type (Brock et al. 2007). Together with the SCD1 mouse observations, these studies demonstrate that Δ9 desaturase is an important determinant of TAG accumulation and that metabolic changes resulting from decreased SCD signaling elicit protection from obesity. Pharmacological manipulation of SCD activity might therefore benefit treatments of obesity, diabetes, and other diseases of metabolic syndrome. However, a better understanding of the downstream pathways affected by SCD1 signaling will be necessary in order to understand and anticipate potential side effects from such drugs (Dobrzyn and Ntambi 2005).

Nuclear Hormone Receptors Regulate Transcription of SCDs

Nuclear hormone receptors (NHRs) are important regulators of development and metabolism. Upon binding specific ligands, the NHRs dimerize and enter the nucleus where they activate or repress distinct target genes. Such regulation allows

for precise, reversible responses to environmental, developmental, and nutritional signals. *C. elegans* contains a hugely expanded repertoire of NHRs, and sequence analysis reveals that 269 out of 284 *C. elegans* NHRs evolved from an ancient form of the hepatocyte nuclear factor 4 (HNF4) family (Robinson-Rechavi et al. 2005; Taubert et al. 2011). Several NHRs have been implicated in the regulation of fat metabolism in *C. elegans*, and the SCDs are key gene downstream effectors. NHR-80 and NHR-49 are required for expression of the *fat-5, fat-6,* and *fat-7* genes (Van Gilst et al. 2005a; Brock et al. 2006). Both mutant strains have a high stearic acid (18:0) content and reduced oleic acid (18:1*n*-9). The expression of *fat-6* and *fat-7* was also shown to be decreased in *nhr-13* mutants, although the overall fatty acid composition was not affected (Pathare et al. 2012). In contrast, a mutation in the aryl-hydrocarbon receptor, *ahr-1*, results in increased expression of the *fat-7* gene, resulting in small, but statistically significant changes in fatty acid composition (Aarnio et al. 2010).

Both NHR-49 and NHR-80 are required for the up-regulation of *fat-7* needed to compensate for *fat-6* deletion mutants, and the compensation is essential because the *nhr-49;fat-6* and *nhr-80;fat-6* strains are not viable (Brock et al. 2006). Despite the similarities in their effects on Δ9 desaturation, *nhr-49* deletion mutants display more severe growth and reproductive defects than *nhr-80* mutants, probably because NHR-49 also regulates other lipid metabolism genes, including genes involved in the biosynthesis of sphingolipids, genes involved in β-oxidation, and other genes required for the response to nutrient deprivation (Van Gilst et al. 2005b; Pathare et al. 2012).

While mutations in *nhr-49* and *nhr-80* have additive defects in combination with mutations in SCDs, depletion of the *nhr-64* gene results in improved physiology of SCD mutants. The low fat stores, slow growth, and embryonic lethality phenotypes in *fat-6;fat-7* double mutants were improved in combination with *nhr-64*(*RNAi*) (Liang et al. 2010). Likely gene targets of NHR-64 that facilitated these improvements include β-oxidation genes, such as *ech-5* and a thiolase. However, other gene changes in *nhr-64*(*RNAi*) strains were also noted, including reduced expression of several acylCoA synthetase genes and increased expression of *pod-2* (acetyl-CoA carboxylase). These studies illustrate the complexity of compensatory mechanisms regulated by NHRs that balance fat storage, growth, and reproductive efficiency (Liang et al. 2010).

A network of regulators of metabolic genes enriched in NHRs has been experimentally mapped in *C. elegans,* implicating numerous NHRs in control of metabolic processes (Arda et al. 2010). It has been proposed that the expansion of NHRs in *C. elegans* evolved to enable rapid and adaptive responses to environmental clues, including dietary nutrients. It is likely that changes in SCDs are important for the adaptation to diets encountered in nature, which may vary significantly in their lipid content. Thus, the regulation of SCDs by numerous NHRs signifies their important physiological roles.

Regulation of *C. elegans* SCDs by SREBP

The sterol regulatory element binding protein (SREBP) family of basic helix–loop–helix zipper transcription factors are critical regulators of cholesterol and fatty acid homeostasis in mammals (Osborne and Espenshade 2009; Jeon and Osborne 2012). Newly synthesized SREBPs reside in the endoplasmic reticulum (ER) membrane and are inactive. When specific cellular lipid levels are low, the proteins are transported to the Golgi, where they are acted on by site 1 and site 2 proteases released by the N-terminal fragment of SREBP, which then enter the nucleus and activate transcription of lipogenic genes. Mammals express a family of SREBP isoforms (SREBP-1a, -1c, and SREBP-2), in which different isoforms are responsible for the expression of a unique set of lipid metabolism genes. For example, SREBP-2 mainly activates genes responsible for cholesterol uptake and synthesis, while SREBP-1 isoforms regulate genes involved in the biosynthesis of fatty acids, phospholipids, and TAGs. *C. elegans* possesses a single SREBP gene, *sbp-1*. Knockdown of *sbp-1* by RNAi leads to low fat stores, high saturated fatty acid content, impaired growth, and reduced expression of lipogenic genes, including the *fat-5, fat-6,* and *fat-7* SCDs (Kniazeva et al. 2004; Yang et al. 2006). A deletion mutant in the *C. elegans* SREBP homolog, *sbp-1*(*ep79*)*,* displays a similar slow growth and low fat stores as the RNAi treatment (Liang et al. 2010).

The translocation of SREBP from the ER to the nucleus is well understood for the cholesterol-regulated SREBP-2 isoform (Brown and Goldstein 1997). When cholesterol is present, it binds to a cholesterol-sensing protein called SCAP, which promotes association with INSIG (Yang et al. 2002). This complex maintains SREBP in the ER membrane. When cholesterol is absent, INSIG no longer is complexed with SREBP, facilitating trafficking to the Golgi where proteases cleave the transmembrane domains, allowing for translocation to the nucleus (Sun et al. 2007). *C. elegans* does not synthesize cholesterol, and it does not encode an INSIG homolog; however, recent studies have shed light on a conserved mechanism for the nuclear translocation of the SREBP-1 isoform.

The mechanism of nuclear localization of the SREBP-1 isoform depends on membrane phosphatidylcholine (PC) levels. RNAi knockdown of genes encoding s-adenyslyl methionine transferase (*sams-1*) and other genes contributing to the PC synthesis pathway led to increased nuclear localization of SREBP and induction of SREBP target genes (such as *fat-5* and *fat-7*) in *C. elegans* and in mammalian liver cell lines (Walker et al. 2011). When PC is low, the Golgi protease enzymes undergo retrograde transport to the ER, where the proteases cleave the SREBP protein, releasing the transmembrane domains. This liberates the DNA binding portion of SREBP, allowing it to translocate into the nucleus, where it promotes transcription of lipid synthesis genes, including SCDs (Walker et al. 2011). Thus, when PC synthesis is disrupted, SREBP-induced gene expression changes led to increased fat stores in nematodes and hepatic steatosis (fatty liver) in the mouse. An independent study

confirmed that *C. elegans sams-1* and *pmt-1* mutants have high fat stores compared to wild type nematodes, and they express high levels of *fat-7* (Li et al. 2011).

During periods of food deprivation, lipid metabolism is shifted away from synthesis toward breakdown, and oxidation of fatty acids provides energy for the maintenance of the organism. Consistent with this, nuclear SREBP is rapidly depleted during fasting in the mouse liver (Horton et al. 1998) and in *C. elegans* (Walker et al. 2010). This is regulated by the NAD+-dependent deacetylase SIRT1, which directly deacetylates SREBP, leading to ubiquitination, protein degradation, and reduction in target gene expression. The importance of SREBP regulation of the FAT-7 SCDs is apparent, as the expression of the *fat-7* SCD is especially sensitive to fasting, with undetectable transcript levels in the absence of food (Walker et al. 2010).

MDT-15 Interacts with SBP-1 and NHRs to Regulate *C. elegans* SCDs

After binding to target sequences in specific promoters, SREBP and NHRs recruit multi-protein coregulator complexes containing transcriptional cofactors that link the transcriptional activator to the transcription initiation machinery (Blazek et al. 2005). One component, the ARC/mediator subunit MDT-15/MED15, interacts directly and specifically with the activation domain of SREBP (Yang et al. 2006). The *C. elegans* MDT-15 also binds specifically to NHR-49 and NHR-64 (Taubert et al. 2006), which both regulate the expression of genes involved in lipid metabolism. Accordingly, RNAi reduction of *mdt-15* expression results in slow growth, low fat stores, and reduced expression of *fat-7*. Dietary supplementation with oleic acid rescues many of the defects of *sbp-1* and *mdt-15* RNAi animals, indicating that the SCDs are key targets of these regulators (Yang et al. 2006). MDT-15 is also important for broader functions, including induction of detoxification genes in response to certain ingested xenobiotics and heavy metals, suggesting that it interacts with additional transcription factors (Taubert et al. 2008).

FOXO Regulates SCDs to Confer Long Lifespan and Cold Tolerance

Insulin/IGF-1 signaling modulates development, metabolism, stress resistance, and longevity in *C. elegans* (Lin et al. 1997, 2001; Henderson and Johnson 2001). Insulin/IGF-1 signaling during favorable growth conditions negatively regulates the DAF-16/FOXO transcription factor by a phosphorylation cascade that prevents nuclear localization. During unfavorable conditions, such as low nutrients, UV irradiation, certain toxins, and exposure to high temperatures, the phosphorylation cascade is inhibited such that the DAF-16/FOXO transcription factor enters the nucleus,

leading to the expression of many protective genes, such as superoxide dismutase, drug metabolizing enzymes, and molecular chaperones (McElwee et al. 2003; Murphy et al. 2003). This altered gene expression confers long lifespan and stress resistance to the nematodes. SCDs have been identified as targets of DAF-16/FOXO (Murphy et al. 2003; Schuster et al. 2010), and *fat-7*(*RNAi*) leads to a shortened lifespan in the long-lived insulin/IGF-1 receptor mutant *daf-2* (Murphy et al. 2003). A recent study shows that the *age-1* mutant, a long-lived mutant disrupted in the insulin/IGF-1 signaling pathway, is cold-tolerant, and this tolerance depends on DAF-16/FOXO (Savory et al. 2011). While cold stress does not induce nuclear localization of DAF-16/FOXO, cold tolerance in the *age-1* mutants depends on functional SCDs. SCDs have previously been shown to be important for adaptation to cold temperatures in many organisms including plants (Browse and Xin 2001), fish (Tiku et al. 1996), and *C. elegans* (Brock et al. 2007; Murray et al. 2007). Increased SCD expression leads to higher unsaturated fatty acid content, resulting in increased membrane fluidity that is necessary for optimal membrane function in cold environments.

Fatty Acid Desaturation and Other Lipid Modifications in Germ Cells Regulate Lifespan

In *C. elegans*, ablation of germ cells results in a long lifespan. This can be achieved through laser ablation of germ cell precursors or by mutations that block the proliferation of germ cells (Hsin and Kenyon 1999; Arantes-Oliveira et al. 2002). Recent studies reveal that fat metabolism is altered in worms lacking germ cells and that the changes in fat metabolism are required for the increased longevity in these strains (Wang et al. 2008; O'Rourke et al. 2009; Goudeau et al. 2011). Specifically, the lipase *lipl-4* is induced in worms lacking a germline, suggesting that lipase activity might underlie the lifespan extension observed in germline-less animals. In agreement with this, depletion of *lipl-4* shortens the lifespan of worms lacking germ cells (Wang et al. 2008), while overexpression of *lipl-4* is sufficient to extend lifespan in wild type worms (Wang et al. 2008; Lapierre et al. 2011). Autophagy, the process of sequestering and degrading cytosolic components is also increased in germline-less *C. elegans*. The FOXA transcription factor PHA-4 is required for the induction of autophagy-related genes (Lapierre et al. 2011). The process of autophagy mediated through PHA-4 is required for extended lifespan of the germline-less worms, as well as for sustained induction of the *lipl-4* gene (Lapierre et al. 2011).

A recent study demonstrated that SCD activity under the control of NHR-80 is required for the extended lifespan of germline-less worms (Goudeau et al. 2011). The study found that a mutation in *nhr-80* blocks the extended lifespan of a germline-less *glp-1* mutant, while overexpression of *nhr-80* further increases the extended lifespan of *glp-1*. Neither the *nhr-80* mutation nor overexpression of *nhr-80* affected the lifespan of wild type worms (Goudeau et al. 2011). The SCD activity is

required for the extension of lifespan because there was no lifespan extension in the *glp-1;fat-6;fat-7* triple mutants, even in strains where the *nhr-80* gene was ectopically overexpressed. This discovery delineates a link between signals from the reproductive system, SCD activity, and longevity. Further studies will be necessary to determine the key physiological roles of SCDs in longevity, where they may be necessary for optimal membrane fluidity, efficient energy storage, or as precursors of lipid signaling molecules.

Conclusions

SCD activity is essential for proper lipid homeostasis in *C. elegans*. SCDs play important physiological roles in ensuring proper membrane fluidity, synthesizing precursors of PUFAs and other lipid signaling molecules, and enabling efficient fat storage. Whole organism approaches have facilitated studies of the consequences and compensatory mechanisms that occur as a result of reduced SCD activity, including the importance of conserved SCD regulators such as nuclear receptors and SREBP. Future studies promise to clarify lipid-mediated mechanisms contributing to the complex processes of development, reproduction, stress responses, and aging.

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Chapter 15
Remodeling of Membrane Phospholipids by Bacterial Desaturases

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Abbreviations

a-BCFAs	Anteiso-branched-chain fatty acids
ACP	Acyl carrier protein
BCFA	Branched-chain fatty acids
BKDH	Branched-chain keto acid dehydrogenase
CoA	Coenzyme A
Des	Desaturase
DHp	Dimerization and histidine phosphotransfer
Fer	Ferredoxin
Fld	Flavodoxin
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
TM	Transmembrane
TMS	Transmembrane segment
UFA	Unsaturated fatty acid

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Unsaturated Fatty Acids

The physical properties of a biological membrane are highly dependent on the lipid composition, which shows tremendous variation between different membranes and dependence on conditions (Dowhan 1997). This variation is responsible for the plasticity of biomembranes, which plays a role in cellular adaptation to the environment and allows for cellular processes such as energy storage, signaling, transport, and membrane fusion and fission (Dowhan 1997; Aguilar and de Mendoza 2006; Lee 2011). Membrane fluidity is essential for maintaining the membrane barrier and for optimal functioning of membrane proteins (de Mendoza and Cronan 1983; Kaiser et al. 2011). The fluidity of the membrane lipids is in a large extent determined by the ratio of saturated (SFAs) vs. unsaturated fatty acids (UFAs) (Los and Murata 2004). The presence of *cis*-double bonds in membrane lipid acyl chains causes disturbances in the acyl chain packing, which results in much poorer packing of the acyl chains and therefore in a lower gel-to-liquid crystalline phase transition temperature of the membrane. Thus, UFAs are key molecules in the regulation of cellular membrane fluidity (Cronan and Gelmann 1973). In addition to their structural role, UFAs have recently been recognized as signaling molecules involved in several essential cellular processes, such as cell differentiation and DNA replication (for recent reviews see Heird and Lapillonne 2005; Mansilla and de Mendoza 2005).

Furthermore, the UFAs are important in human nutrition (in edible oils) and may be used as reagents of chemical industry (Napier 2007). Fatty acids determine the properties and nutritional value of edible fats. For example, vegetable oils rich in UFAs generally are considered better in cardiovascular health than fats rich in SFAs. Furthermore, the functional groups related to the double bonds such as hydroxy and epoxy groups confer important functional properties to inedible oils, used in the chemical industry (e.g., castor oil). Currently the strategy of choice to produce specific UFAs for the chemical or food industry is the manipulation of the expression of genes encoding desaturases in plants. However, the exploitation of the rich variety of UFAs produced by microorganisms could have a favorable impact in terms of flexibility and speed of production of unusual UFAs (Bonamore et al. 2006).

Unsaturated Fatty Acids Synthesis in Bacteria

Konrad Bloch, a Nobel Laureate chemist, and coworkers determined that UFAs are synthesized in two different ways; one occurs only in aerobic organisms and requires molecular oxygen, while the other pathway is used in anaerobic conditions (Scheuerbrandt et al. 1961; Scheuerbrandt and Bloch 1962).

The anaerobic pathway for UFA synthesis was established in *Escherichia coli*, where it was shown that only two unique biochemical reactions are required to specifically produce UFAs in this bacterium (Cronan et al. 1969; Bloch 1971). Introduction of the double bond involves a dehydration of the 3-hydroxydecanoyl

intermediate of fatty acid synthesis to give a *cis*-3 double bond which would be conserved through subsequent cycles of addition of two carbon atoms to give the membrane lipid UFA moieties (Cronan 2006) (Fig. 15.1a). The double bond is introduced into the growing acyl chain by FabA, an enzyme capable of both the dehydration of β-hydroxydecanoyl-acyl carrier protein (ACP) to *trans*-2-decenoyl-ACP, and the isomerization of *trans*-2 to *cis*-3-decenoyl-ACP. This isomerization is critical to switch the nascent acyl chain into the UFA synthetic pathway, but it is not sufficient. The β-ketoacyl-ACP synthase I (FabB) enzyme is required to elongate the *cis*-3-decenoyl-ACP produced by FabA and its activity is the primary factor in determining cellular UFA content. At this step, the proportion of *cis*-UFA is determined by competition between the use of *cis*-3-decenoyl-ACP by FabB and the use of *trans*-2-decenoyl-ACP by FabI (Zhang and Rock 2008) (Fig. 15.1a). These two proteins, which are involved in type II fatty acid biosynthesis pathway, direct the formation of the two major UFAs that are found in bacteria: 16:1Δ9 and 18:1Δ11 (*cis-vaccenate*). In addition, there is a biochemical mechanism that allows the elongation cycle to respond to the environmental temperature. This mechanism was carefully studied in *E. coli*, and β-ketoacyl-ACP synthase II (FabF) was identified as a key player in the alteration of membrane composition in response to temperature changes (Cronan and Rock 1996; de Mendoza and Cronan 1983). Interestingly, 16:1-ACP is a poor substrate for FabB, and its elongation to 18:1-ACP is controlled by the activity of FabF (Garwin and Cronan 1980; Garwin et al. 1980a, b). It was demonstrated that thermal regulation of membrane fluidity in *E. coli* was due to an intrinsic property of the activity of FabF: the reactivity of FabF toward the palmitoleyl-ACP substrate increases at low temperatures, leading to increased amounts of *cis*-vaccenic acid in membrane phospholipids (Garwin and Cronan 1980). At low temperatures, *E. coli* FabF promotes the production of 18:1Δ11, whereas at higher temperatures the activity of FabF is decreased compared with FabB, the other condensing enzyme, and the formation of 18:1Δ11 is reduced (Garwin et al. 1980a; de Mendoza and Cronan 1983).

Nevertheless, the UFA pathway described in *E. coli* is not widely distributed in anaerobic bacteria that produce UFAs. Genomic analyses indicate that only the alpha and gamma proteobacteria encode the proteins of this pathway (Campbell and Cronan 2001; Heath et al. 2001). However, there are a number of anaerobic bacteria that produce UFAs and do not have a *fabA* homologues in their genomes.

A variation of the FabAB pathway was discovered in *Streptococcus pneumoniae*, where a new enzyme in type II fatty acid synthetase was described (Marrakchi et al. 2002) (Fig. 15.1b). This enzyme, called FabM, carries out the isomerization of *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP, but is not capable of catalyzing the dehydration of a β-hydroxy intermediate. FabM, which lacks homology to FabA, is a member of the hydratase/isomerase superfamily and is present only in streptococcus. *fabM* mutants of *S. pneumoniae* and *Streptococcus mutans* are auxotrophs for UFAs, indicating that FabM is an essential enzyme in streptococci (Altabe et al. 2007). *S. pneumoniae* also possesses a dehydratase that catalyzes the dehydration reaction, FabZ; thus this bacteria uses a combination of FabZ and FabM to introduce the double bond into the growing acyl chain. This pathway utilizes another enzyme, FabK, the enoyl

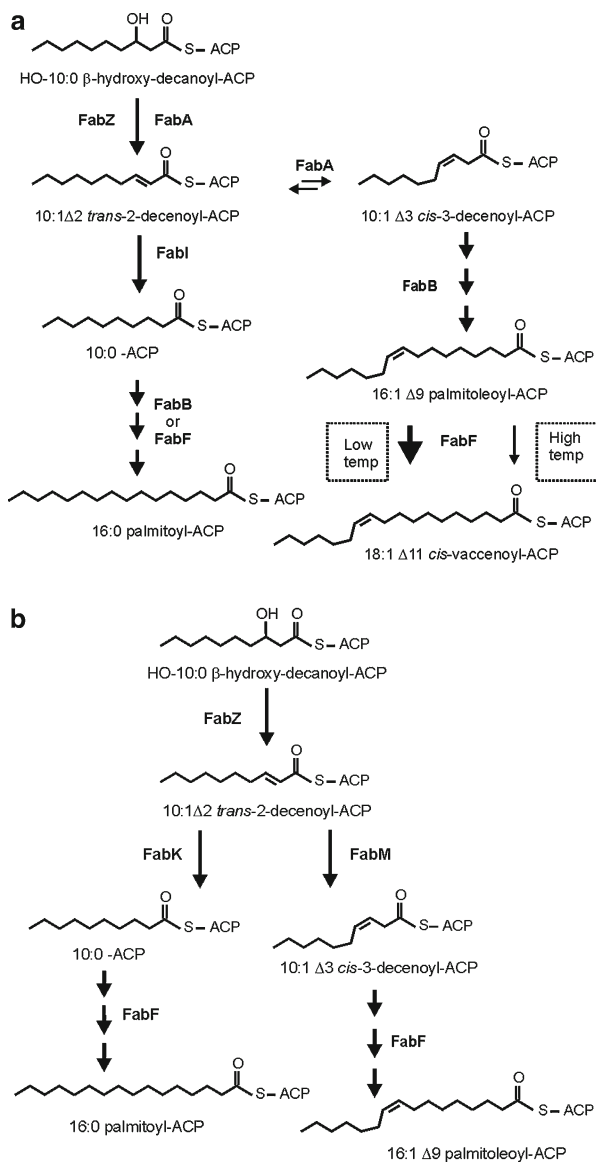

Fig. 15.1 Anaerobic pathways of UFA biosynthesis in *E. coli* and *S. pneumoniae*. (**a**) *E. coli* contains two dehydratases, FabZ and FabA. FabA is a bifunctional enzyme that catalyzes both the removal of water to generate *trans*-2-decenoyl-ACP and the isomerization of this intermediate to *cis*-3-decenoyl-ACP, the key step in UFA production. SFA biosynthesis proceeds by the action of FabI on the *trans*-2 intermediate followed by further elongation cycles initiated by either FabB or FabF condensing enzymes. UFAs require FabB to efficiently utilize the *cis*-3-C10:1-ACP intermediate and initiate the elongation cycles that form 16:1 Δ9 palmitoleoyl-ACP. FabF participates in the elongation of this substrate to 18:1 Δ11 *cis*-vaccenoyl-ACP. The reactivity of this enzyme toward 16:1 Δ9 palmitoleoyl-ACP is increased after a temperature downshift. (**b**) In *S. pneumoniae* there is only a single dehydratase, FabZ, which forms the *trans*-2-decenoyl-ACP intermediate. SFAs are formed by the action of FabK, an enoyl-ACP reductase, followed by further elongation cycles initiated by FabF. UFAs are formed from the isomerization of the *trans*-2 intermediate by FabM, a *trans*-2-*cis*-3-decenoyl-ACP isomerase, followed by further elongation cycles initiated by FabF

reductase that competes with the *cis–trans* isomerase FabM and pulls intermediates to SFA formation. Thus, the branch point in the biosynthesis of UFAs in *S. pneumoniae* occurs following the formation of *trans-2-decenoyl*-ACP, in contrast to *E. coli* where the branch point takes place after the formation of β-hydroxydecanoyl-ACP (Fig. 15.1). The elongation of both saturated and unsaturated acyl-ACPs is carried out by FabF, the only condensing enzyme present in *S. pneumoniae*. The proportion of UFA is controlled by FabT, a transcriptional repressor that regulates the expression of *fabK*, but not *fabM* (Lu and Rock 2006).

Enterococcus faecalis encodes two FabZ homologues and two FabF homologues (FabF is closely related to FabB). It was demonstrated that one of these FabZ homologues, called FabN, has dehydratase/isomerase activity analogous to FabA, whereas the other protein possesses only dehydratase activity (Wang and Cronan 2004). A similar picture was seen for the FabF homologues, one of them, FabO, replaces FabB to perform the synthesis of UFAs, whereas the other functioned only as a FabF enzyme (Wang and Cronan 2004).

On the other hand *Neisseria gonorrhoeae*, a Gram-negative facultative anaerobe, does not encode homologous to any known mechanism of UFA synthesis, either aerobic or anaerobic. Instead, this bacterium possesses a new enzyme, UfaA, that belongs to the 2-nitropropane dioxygenase-like superfamily, which is involved in the synthesis of UFAs when grown anaerobically (Isabella and Clark 2011). Although the mechanism implicated in anaerobic gonococcal UFA synthesis seems to be a distinct form than that of the classical anaerobic UFA synthetic pathway, biochemical understanding of the UfaA reaction mechanism is still fragmentary. Homologues to this enzyme were found in many facultative and obligate anaerobes that produce UFAs but lack *fabA*, suggesting that UfaA is part of a widespread pathway and probably the answer for those organisms that have presented a mystery in their mechanism of UFA synthesis for several years (Isabella and Clark 2011). The aerobic mechanism involved in UFAs synthesis is not yet identified in this bacterium.

De novo fatty acid biosynthesis is finely adjusted by environmental signals to produce acyl chains with the properties that are required to optimize membrane function. However, bacteria are often subjected to abrupt changes, for example in temperature or organic-solvent concentration, that require the immediate modification of existing membrane phospholipid acyl chains to optimize growth under the new conditions. Some prokaryotes such as cyanobacteria, bacilli, mycobacteria, and pseudomonads use a different pathway, which requires molecular oxygen and reduced equivalents, to introduce a *cis* double bond into preexisting fatty acids (Mansilla and de Mendoza 2005; Phetsuksiri et al. 2003; Zhu et al. 2006). This pathway, which is mediated by desaturase enzymes, will be described in the subsequent sections.

Biochemical Characteristics of Desaturation Systems

A desaturase is a special type of oxygenase that can remove two hydrogens from a hydrocarbon chain, especially from a fatty acyl chain, catalyzing the formation of a double bond in the substrate. Unlike normal oxygenases which directly transfer molecular oxygen to a substrate, a desaturase uses activated molecular

oxygen to abstract hydrogens from the substrate creating a carbon/carbon double bond in a fatty acid and a water molecule (Shanklin and Cahoon 1998; Los and Murata 1998; Buist 2004). All these enzymes utilize reducing equivalents obtained from an electron transport chain and are able to introduce the double bond in a chemo-, regio-, and stereoselective manner. Desaturases represent a complex group of enzymes that could be classified in different ways, depending on the solubility of the enzyme, the electron donor, subcellular localization, the regioselectivity of the reaction, and the kind of esterification of the fatty acid used as substrate (Sperling et al. 2003).

According to its solubility these enzymes can be classified into two non-evolutively-related groups: the soluble ACP desaturases, found primarily in the plastids of higher plants (e.g., the castor Δ9 stearoyl-ACP desaturase) (Shanklin and Somerville 1991) and a larger group of membrane-bound desaturases found in a wide range of taxa, typified by the yeast Ole1 Δ9-desaturase (Ole1p) (Stukey et al. 1990) and the *Bacillus subtilis* Δ5-desaturase (Δ5-Des) (Aguilar et al. 1998). The acyl-ACP desaturases catalyze the introduction of *cis* double bonds into fatty acid chains bound to ACP (Shanklin and Cahoon 1998), generally at position 9 relative to the carboxyl end of the fatty acids (Δ9). The analysis of the crystal structure of Δ9 stearoyl-ACP desaturase shows that it has an active di-iron center, and that the amino acid residues involved in attaching the di-iron complex forming two characteristic motifs D/EXXH (Lindqvist et al. 1996).

Membrane desaturases are more widely distributed in nature. These proteins belong to a large family of functionally diverse enzymes which include alkane hydroxylases, xylene monooxygenases, carotene ketolases, and sterol and sphingolipid hydroxylases (Shanklin and Cahoon 1998). Based on the differences in substrate specificity, these enzymes were subdivided into two groups: acyl-CoA desaturase and acyl-lipid desaturases (Shanklin and Cahoon 1998). These integral membrane proteins have a different consensus for the active site with a tripartite motif of eight histidines ($HX_{(3-4)}H$, $HX_{(2-3)}HH$, $HX_{(2-3)}HH$), found in conserved positions of the amino acid sequence. It has been observed by site-directed mutagenesis studies that substitution of any of the conserved histidine residues with another amino acid leads to loss of enzyme activity. This is probably due to the inability to bind iron at the active site of the mutant enzymes (Los and Murata 1998).

The complex process of introducing a double bond into fatty acids requires iron cofactors, molecular oxygen, and two reducing equivalents for catalysis (Shanklin et al. 1994; Sperling et al. 2003). The latter are supplied from NAD(P)H by two different electron transport systems, functionally equivalent, that are specific to the subcellular compartment rather than to the class of the desaturase. In the case of desaturases of plant endoplasmic reticulum and the acyl-CoA or acyl-lipid desaturases of animal and fungi, the donor is cytochrome *b5*, either in the form of a fused domain or in free form. For the soluble acyl-ACP desaturases and the integral membrane acyl-lipid desaturases from plastids and cyanobacteria, electrons are delivered by ferredoxins (Fer) which are ubiquitous soluble iron-sulfur proteins involved in a variety of redox reactions (Sperling et al. 2003).

Three classes of regioselectivity have been observed for fatty acid desaturases that would reflect differences in the position of the active site relative to the features of the substrate binding pocket associated with substrate recognition: the Δx desaturases introduce a double bond x carbons from the carboxyl end; $\omega - x$ desaturases dehydrogenate x carbons from the methyl terminus; while $\nu + x$ desaturases use a preexisting double bond as a reference point and dehydrogenate x carbons from the nearest olefinic carbon (Yadav et al. 1993; Hitz et al. 1994; Uttaro 2006). The Δ9 stearoyl-CoA desaturase locates double bonds counting from the carboxyl terminus of the molecule in a manner similar to that employed by the soluble acyl-ACP desaturases. Acyl-lipid desaturases $\omega 6$ (Δ12) and $\omega 3$ (Δ15) require substrates with a preexisting double bond, most frequently $\omega 6$ desaturase prefers Δ9 UFAs, while $\omega 3$ desaturases prefer dienoic fatty acids Δ9, 12 (Sperling et al. 2003). "Front-end" desaturases introduce the double bond between one preexisting and the carboxyl (front) end of the fatty acid. These enzymes are involved in polyunsaturated fatty acid (PUFA) biosynthesis and all have *citb5* domain fused to the N-terminus (Sayanova et al. 1999; Uttaro 2006) which operates as electron donor during desaturation. The only exceptions among this kind of desaturases are the Δ6 desaturases of cyanobacteria, which use, at least in vitro, Fer as electron donor (Los and Murata 1998).

Bacterial Desaturases

Many bacterial genomes present ORFs with significant similarity to desaturases (Sperling et al. 2003). In this section we will summarize the current knowledge about those desaturases whose function has been experimentally demonstrated.

Desaturases from the Genus Bacillus

Bloch and coworkers were the first to describe the existence of an oxidative pathway for the biosynthesis of long-chain UFAs by microorganisms, thus initiating the study of bacterial desaturases (Scheuerbrandt and Bloch 1962; Fulco et al. 1964). For a number of years Fulco performed detailed in vivo studies with *B. megaterium* demonstrating that the desaturase activity in this bacterium was induced by low growth temperatures (Fulco 1974). Nevertheless, at that time mechanistic insights in desaturation reactions of *Bacillus* could not be established because the studied organisms were not amenable to genetic analysis. This problem was solved when this phenomenon was examined in *B. subtilis*, which is an excellent model because of its general experimental tractability. Genetic and biochemical experiments successfully established that *B. subtilis* contains a single acyl-lipid desaturase, encoded by the *des* gene (Grau and de Mendoza 1993; Aguilar et al. 1998), and that this enzyme catalyzes the introduction of a *cis*-double bond at the Δ5 position of a wide range of SFAs (Altabe et al. 2003). Thus, this protein was named as Δ5-Des.

Δ5-Des exhibits relatively low sequence identity (typically about 23 %) to membrane desaturases from cyanobacteria and plants (Aguilar et al. 1998). It contains the three His clusters found in the known membrane desaturases with the appropriate spacing which might be involved in non-heme iron binding, constituting the active site of the enzyme (Aguilar et al. 1998). An additional, fourth His region not found in other known desaturases is located downstream from the third His cluster of Δ5-Des, but it is not involved in catalysis (Díaz et al. 2002). However, deletion of the C-terminal part of Δ5-Des, downstream of the third His cluster, eliminates enzyme activity, indicating that this region is essential either for desaturase activity or for appropriate protein folding (Díaz et al. 2002). The amino acid sequence of Δ5-Des contains many hydrophobic stretches, consistent with the presence of several membrane-spanning domains. The hydropathy profile of the primary sequence is similar to those of desaturases from cyanobacteria and to almost all known membrane-associated plant desaturases. However, these hydropathy profiles showed some differences compared to AlkB that has provided the only experimental model for this superfamily of enzymes (van Beilen et al. 1992). Therefore, the topology of Δ5-Des was experimentally analyzed in *E. coli* using a set of fusions of N-terminal fragments of the protein to the reporter alkaline phosphatase and surface Lys biotinylation experiments (Díaz et al. 2002). These data, combined with site-directed mutagenesis of His residues conserved in most desaturases, support a model for Δ5-Des as a polytopic membrane protein with six transmembrane segments (TMSs) and one membrane-associated domain, which might be involved in keeping the catalytic site on the cytoplasmic side of the membrane, close to the substrates (Díaz et al. 2002). The proposed topology of Δ5Des, together with the topological features of the mouse stearoyl-CoA desaturase that contains four TMSs (Man et al. 2006), provides two paradigmatics models for the topology of an acyl-lipid desaturases and an acyl-CoA desaturase, respectively, based on experimental grounds.

The complex process of introducing a double bond into fatty acids requires molecular oxygen and two reducing equivalents for catalysis (Shanklin and Cahoon 1998; Sperling et al. 2003). To study this important question in *B. subtilis*, a genetic approach was undertaken to identify the electron donors required to provide the $2e^-$ required for Δ5-Des activity. The *B. subtilis* genome encodes three proteins that can act as potential electron donors of Δ5-Des: Fer and two flavodoxins (Flds), YkuN and YkuP, which belong to the *ykuNOP* operon (Lawson et al. 2004). Thus, mutagenesis of the corresponding genes was performed and the ability of these proteins to support *B. subtilis* desaturase activity was tested in vivo. The result of these experiments demonstrated that Fer, YkuN, and YkuP can act as redox partners for Δ5-Des and suggest that Fer/Flds proteins could function physiologically in the biosynthesis of UFAs in *B. subtilis* (Chazarreta-Cifre et al. 2011). Although Flds have extensively been described as partners in a number of redox processes, this was the first report describing that flavoproteins can transfer electrons to a lipid desaturase and, in conjunction with Fer, support the desaturation of *B. subtilis* lipids.

The *B. subtilis des* gene is essential for cold-shock survival when this bacterium is grown in glucose-minimal medium (Weber et al. 2001). However, this cold-sensitive phenotype is suppressed by addition of the amino acid isoleucine to the growth media. This protective effect is exclusively based on the function of this amino acid in the biosynthesis of anteiso-branched-chain fatty acids (a-BCFA), which have biophysical properties similar to UFAs (Martin et al. 2009b). Thus, Δ5-Des provides a back up mechanism to survive at low temperatures in the absence of an isoleucine-dependent switch for the branching of fatty acids.

Cyanobacterial Desaturases

Cyanobacteria are a versatile group of bacteria capable of carrying out a plant-like oxygenic photosynthesis. Cyanobacteria comprise over 1,600 species with various morphologies and can survive in very hostile environments (Scanlan 2001). It has been well documented that the content of monounsaturated fatty acids (MUFAs) and PUFAs in membrane lipids of cyanobacteria plays essential roles in maintaining the proper function of biological membranes especially under conditions of temperature stress, and energy limitation, growth and photosynthesis (Jeamton et al. 2008; Murata and Wada 1995; Nishida and Murata 1996; Tasaka et al. 1996; Sakamoto and Murata 2002). All known cyanobacterial desaturases are acyl-lipid desaturases (Des) and have rigorous specificity with respect to the position in fatty acid at which double bond is introduced and to the *sn* position of the glycerol to which fatty acids are esterified. Des proteins and their genes have been extensively described in *Synechocystis* sp. PCC 6803 being *desA* the first gene for an acyl-lipid desaturase to be cloned (Wada et al. 1990). Subsequently, it was determined that this bacterium have four specific acyl-lipid desaturases, designated DesA, DesB, DesC, and DesD, that catalyze desaturation at the Δ12, $\omega3$, Δ9, and Δ6 position of C18 acids on the *sn*-1 position, respectively (Wada et al. 1990; Sakamoto et al. 1994a, b, c; Reddy et al. 1993).

The order in which desaturases operates is very strictly determined: the first double bond is introduced by the Δ9 desaturase into stearic acid, and the Δ12 and Δ6 desaturases introduce a double bond into mono or diunsaturated fatty acids that already have a double bond in Δ9 position (Murata et al. 1992). The $\omega3$ desaturase introduces a double bond into fatty acids that have a double bond at the Δ12 position (Murata et al. 1992). Unlike *Synechocystis*, *Synechococcus* has only a single Δ9 desaturase and synthesizes MUFAs exclusively.

The importance of the *des* genes with respect to cold adaptation has been clearly demonstrated by the observation that cyanobacterial mutants defective in these genes are cold-sensitive and grow slower than the wild-type cells (Los and Murata 1999). Disruption of *desA and desD* genes in *Synechocystis* sp. PCC 6803 results in remarkable changes of membrane fatty acids that has deleterious effect on the growth and severe photoinhibition of photosynthesis at low temperatures (Tasaka et al. 1996; Los and Murata 1999).

Pseudomonas aeruginosa *Desaturases*

Pseudomonas aeruginosa is an opportunistic human Gram-negative pathogen that causes nosocomial and life-threatening infections in injured, burned, and immunocompromised patients (Van Delden and Iglewski 1998). The membranes of *P. aeruginosa* contain high quantities of straight-chain UFAs, being *cis*-vaccenate the major species found in the wild-type cells (Zhu et al. 2006). In a recent study it was demonstrated that *P. aeruginosa* has three different pathways to synthesize UFAs, depending on the oxygen availability. In anaerobic conditions, the UFAs synthesis take place exclusively by the actions of the FabAB proteins of the bacterial type II biosynthetic pathway (Fig. 15.1a). In aerobic conditions, besides the FabAB mediated pathway, *P. aeruginosa* employs two oxygen-dependent desaturases, DesA and DesB, which introduce a *cis* double bond into preexisting fatty acids (Zhu et al. 2006). Both desaturases have different substrate specificities and supplement the anaerobic mechanism for UFA synthesis. DesA introduces a double bond at the Δ9 position of fatty acyl chains attached to the *sn*-2 position of existing phospholipids. DesB is also a Δ9 desaturase but introduces double bonds into acyl-CoAs formed from exogenous SFAs, mainly stearate and palmitate, rendering 18:1Δ9 and 16:1Δ9, respectively (Zhu et al. 2006). The *desB* gene is located in an operon with *desC*, a gene encoding an oxidoreductase, which is predicted to function in the electron transport coupled with the DesB desaturation reaction (Zhu et al. 2006). Recently it was demonstrated that DesB is a factor required for full virulence in *P. aeruginosa*, since *desB* mutants are severely deficient in the production of proteolytic enzymes, pyocianin and rhamnolipids, and they show impaired swarming and twitching motilities and reduced virulence in the *Caenorhabditis elegans* infection model (Schweizer and Choi 2011). A *desA* and *desB* double mutant grows well under aerobic conditions, indicating that FabAB is the main pathway to synthesize UFAs (Hoang and Schweizer 1997).

Mycobacterial Desaturases

Mycobacterium tuberculosis is a human pathogen that causes millions of deaths every year. All mycobacteria have a lipid-rich cell envelope containing mycolic acids (Watanabe et al. 2002). Those lipids are complex hydroxylated BCFAs with elevated carbon number (60–90). They may also contain diverse functional groups such as methoxy, keto, epoxy ester groups, cyclopropane rings, and UFAs. Mycolic acids play an important role in reduced cell wall permeability, virulence, and acid fastness characteristic of *M. tuberculosis* (Yuan et al. 1995; Dubnau et al. 2000). Several front-line drugs used for treating tuberculosis inhibit mycolic acid synthesis. Enzymes needed for biosynthesis of mycolic acids are promising drug targets for new anti-TB agents (Tomioka et al. 2008). The mechanisms used to synthesize mycolic acids are not clear at present. Long-chain UFAs containing double bonds in

specific positions are required as building blocks, suggesting that desaturation is probably an essential step in the biosynthesis of mycolic acids (Yuan et al. 1995; Dubnau et al. 2000).

The genome sequence of *M. tuberculosis* and related pathogens has revealed the presence of three genes with similarity to known fatty acid desaturases (Cole et al. 1998). The three putative fatty acid desaturases in the *M. tuberculosis* H37Rv genome, *desA1*, *desA2*, and *desA3*, were cloned and expressed in *Mycobacterium bovis* BCG. DesA3 is a Δ9 membrane-bound desaturase responsible for the synthesis of oleic acid (Phetsuksiri et al. 2003). This enzyme appears to be functionally analogous to mammalian stearoyl-CoA desaturase (Ntambi 1995), which has a number of important physiological roles in mammalian cells (Ntambi 1995; Ntambi et al. 2002). Importantly, DesA3 is one out of the 200 genes required for pathogen survival inside the granuloma enclosing a dormant tuberculosis infection (Sassetti and Rubin 2003). It was demonstrated to be essential for the viability of *M. tuberculosis* and was identified as a target of the anti-tuberculosis drug Isoxyl (Phetsuksiri et al. 2003). DesA1 and DesA2 are annotated as homologues of the soluble acyl-ACP desaturases (Cole et al. 1998; Dyer et al. 2005). Nevertheless the role of these enzymes is unknown and further studies of the properties of these putative desaturases are clearly needed.

Modulation of Membrane Fluidity

When bacteria, which are poikilothermic organisms, are exposed to temperatures below those of their normal conditions, the lipids of their membrane become rigidified, leading to a suboptimal functioning of cellular activities (Phadtare 2004; Mansilla and de Mendoza 2005; Al-Fageeh and Smales 2006). These organisms can acclimate to such new conditions decreasing the transition temperature of the membrane; this is the temperature where membrane lipid bilayers undergo a reversible change of state from a fluid (disordered) to a nonfluid (ordered) array of the fatty acyl chains. In most bacteria, the role of introducing acyl chain disorder is fulfilled by UFAs. As phospholipids containing UFAs have a much lower transition temperature than those lipids made of SFAs (Cronan and Gelmann 1973), the desaturation of the acyl chains of membrane phospholipids results in an increase in fluidity, and membrane lipids returns to their original state, or close to it, with restoration of normal cell activity at the lower temperature. This regulatory mechanism system, called thermal control of fatty acid synthesis, seems to be a universally conserved adaptation response allowing cells to maintain the appropriate fluidity of membrane lipids regardless of the ambient temperature. In this section we will focus on the mechanisms of regulation of bacterial desaturases, developed to adapt the membranes to the new temperatures.

The Des Pathway of **Bacillus subtilis**

B. subtilis cells respond to a decrease in ambient growth temperature by using a dual strategy to restore membrane fluidity. In parallel with a long-term response that leads to an increase in the proportion of saturated a-BCFAs, *B. subtilis* transiently induces the expression of Δ5-Des that desaturates the acyl chain of preexisting membrane phospholipids (Aguilar et al. 1998, 1999, 2001; Altabe et al. 2003). This short-term membrane adaptation requires a canonical two-component regulatory system comprising the histidine kinase DesK and the response regulator DesR (Aguilar et al. 2001). This signal transduction mechanism, termed the Des pathway, enhances the expression of the *des* gene coding for Δ5-Des in response to a decrease in growth temperature (Mansilla et al. 2008). The *B. subtilis* DesK protein is a polytopic protein containing five TMSs that define the sensor domain (Cybulski et al. 2010) and a long cytoplasmic C-terminal tail, which harbors the kinase domain, DesKC. Genetic, physiological, and biochemical evidence proved that DesK is a bifunctional enzyme with both kinase and phosphatase activities that could assume different signaling states in response to changes in membrane fluidity (Aguilar et al. 2001; Albanesi et al. 2004, 2009; Martin et al. 2009a). A kinase-dominant state of DesK predominates upon an increase in the proportion of ordered membrane lipids (Fig. 15.2a). As a consequence of this stimulus perception, DesK undergoes autophosphorylation in the His188 and, subsequently, the phosphoryl group is transferred to the cytoplasmic response regulator DesR. Autophosphorylated DesKC transfers the phosphoryl group to the effector protein DesR that becomes phosphorylated in the predicted Asp 54 residue (DesR-P) (Albanesi et al. 2004). Phosphorylation of the regulatory domain of DesR promotes, in a cooperative fashion, the hierarchical occupation of two adjacent, non-identical, DesR-P DNA-binding sites, so that there is a shift in the equilibrium toward the tetrameric active form of the response regulator (Cybulski et al. 2004). This results in the recruitment of RNA polymerase to the *des* promoter and the activation of *des* transcription, as demonstrated by in vitro experiments (Cybulski et al. 2004) (Fig. 15.2a). Transcriptional activation of *des* is followed by the expression of Δ5-Des and desaturation of the acyl chain of membrane phospholipids (Fig. 15.2b). These newly synthesized UFAs decrease the phase transition temperature of the phospholipids, favoring the phosphatase activity of DesK, resulting in hydrolysis of DesR-P. The unphosphorylated regulator is unable to bind to P*des* and, as a consequence, *des* transcription is turned off (Fig. 15.2b). Using a functional in vitro reconstituted system it was demonstrated that the temperature stimuli comes directly from the membrane lipid bilayer, with no other proteins involved in the sensing or signaling mechanism (Martin et al. 2009a). The opposite activities of DesK, autokinase, and DesR-P phosphatase would be reciprocally regulated by changes in growth temperature that, in turn, adjust the fluidity of membrane phospholipids.

A recent set of crystal structures of DesKC, the catalytic core of DesK, has illuminated the molecular view of how the subdomains of this protein can interact to assemble the three active sites that promote the necessary chemistry for the DesK

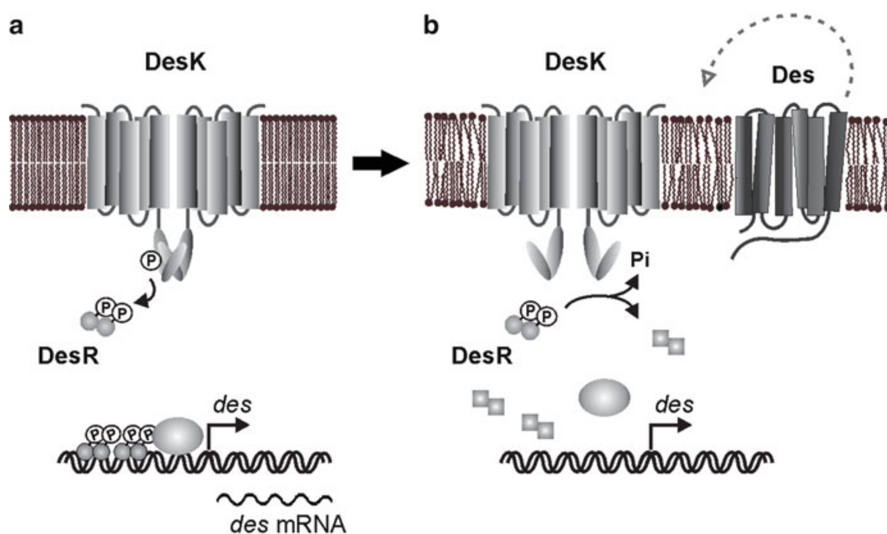

Fig. 15.2 Simplified model of regulation of UFAs synthesis in *B. subtilis*. (**a**) DesK could assume different signaling states in response to changes in membrane fluidity. An increase in the order of the acyl chains of membrane lipids promotes a kinase-dominant state of DesK, which autophosphorylates and transfers the phosphate group to DesR. DesK-mediated phosphorylation of DesR enables interaction of DesR-P dimers with the *des* promoter and RNA polymerase, resulting in transcriptional activation of *des*. (**b**) Δ5-Des is synthesized and desaturates the acyl chains of membrane phospholipids. These newly synthesized UFAs cause a decrease in the order of membrane lipids favoring a phosphatase-dominant state of DesK, leading to dephosphorylation of DesR and thus turning off *des* transcription

regulation cycle and how these interactions might be regulated in response to the cold stimuli (Albanesi et al. 2009). Structural and biochemical approaches showed that DesK is cold-activated through specific interhelical rearrangements in its central four helix bundle domain, known as DHp (for dimerization and histidine phosphotransfer). In a fluid membrane, the transmembrane (TM) domain would stabilize a connecting coiled-coil and the catalytic core into a rigid conformation with the ATP-binding domains attached to the DHp domain. This conformation inhibits autokinase activity and the DHp surface is competent to interact with DesR, resulting in a phosphatase signaling state. Upon cold signal reception, the ensuing structural reorganization would release the ATP-binding domains for histidine phosphorylation (kinase state). Phosphorylation of DesKC induces an as yet different, asymmetric conformation capable of interacting with DesR (phosphotranferase state) (Albanesi et al. 2009). These structural changes of the catalytic domain are promoted by the sensor domain of DesK. One or more of the five TMSs in DesK could undergo a conformational change, in the form of helix rotations and asymmetric helical bends, induced by a modification in the physical state of the membrane lipid bilayer. This information is transmitted to the cytoplasmic domain by the membrane-connecting two-helical coiled-coil, ultimately controlling the alternation between output autokinase and phosphatase activities. Direct support for the

functional importance of the TM helices comes from studies of *B. subtilis* strains producing the soluble DesKC domain. In these strains, the *des* gene is constitutively expressed and its transcription is affected neither by growth temperature nor by the presence of UFAs (Albanesi et al. 2004, 2009; Martin et al. 2009a). Moreover, when DesKC is anchored to the membrane by only one TMS, *des* expression is not induced by cold-shock (Cybulski et al. 2010). Therefore, the TMSs of DesK directly participate in signal transduction recognition and play an essential role in the end-product feedback regulation of *des* transcription.

B. subtilis is also capable of anaerobic growth by nitrate or nitrite respiration. In anaerobiosis *des* is strongly upregulated after a temperature downshift, and this transcriptional induction is dependent on the DesK/DesR system (Beranová et al. 2010). However, there is no fatty acid desaturation after *des* induction, under anaerobic conditions, despite the presence of high levels of Δ5Des. This is due to the strict oxygen requirement of Δ5Des to introduce the double bond into fatty acids. The subsequent transfer of cells exposed to cold temperatures from anaerobic to aerobic conditions rapidly restores the downregulation of *des* transcription. Cold adaptation in *B. subtilis* under anaerobic growth is therefore mediated exclusively by the increased synthesis of a-BCFAs, reaching similar degrees of membrane fluidization that in aerobic conditions (Beranová et al. 2010).

Evidence that membrane fluidity, rather than growth temperature, controls transcription of the *des* gene was obtained by experiments in which the proportion of a-BCFAs of *B. subtilis* membranes was varied controlling the provision of branched-chain acyl-CoA molecules, used as primers (Cybulski et al. 2002). Those primers are obtained by deamination of the amino acids valine, leucine, and isoleucine, and oxidative decarboxylation of the corresponding branched-chain alpha-keto acids, a reaction catalyzed by branched-chain keto acid dehydrogenase (BKDH) (Kaneda 1977). The a-BCFAs, which are synthesized using ketoacids derived from isoleucine as primers (Kaneda 1991), are essential to decrease the transition temperature of *B. subtilis* membrane phospholipids to maintain the appropriate fluidity. Limiting the supply of isoleucine dramatically reduces the amount of a-BCFAs of plasma membrane lipids (Klein et al. 1999), resulting in ordered membrane lipids. Growth of cells in the absence of isoleucine results in activation of *des* transcription under isothermal conditions, using a DesK/DesR-dependent mechanism (Cybulski et al. 2002). Moreover in LipA mutants, which are unable to synthesize BCFA precursors, because of BKDH lack of function in the absence of lipoic acid synthesis (Martin et al. 2009b), transcription of *des* gene reaches to levels fourfold higher than those observed in wild-type strains growing in an isoleucine-free medium at 37 °C. These physiological and transcriptional data give further support to the hypothesis that DesK senses membrane fluidity, since the membrane lipids of *lipA*-deficient cells contain a much higher proportion of high-melting-point SFAs than those of cells expressing LipA (Martin et al. 2009b). Thus, an increase in the order of membrane lipids due to a lower content of membrane isoleucine-derived fatty acids at constant temperature or a decrease in temperature at constant lipid composition can be sensed by DesK, leading to induction of UFAs synthesis.

Two Inducible Aerobics Pathways of UFAs Synthesis in Pseudomonas aeruginosa

P. aeruginosa is able to regulate its aerobic biosynthesis of UFAs in response to changes in the growth conditions. It was suggested that DesA provides a way to quickly adjust the fluidity of the membrane following changes in the growth conditions given that this desaturase introduces double bonds into preexisting membrane lipids. However, rather than being regulated by temperature, like in *B. subtilis*, *P. aeruginosa desA* expression is upregulated when oxygen is limited, which is reminiscent of the induction of OLE1 expression by Mga2p under hypoxic conditions (Jiang et al. 2001; Vasconcelles et al. 2001). However, the mechanism of regulation of *desA* by oxygen availability is not known.

The second aerobic pathway, the DesCB system, is regulated by DesT, a transcriptional repressor that is able to sense the changes in the composition of cellular acyl-CoA pool and modify its binding to DNA accordingly (Zhang et al. 2007). A unique property of DesT is its ability to differentially bind to the *desT–desCB* intergenic region in response to the presence of different acyl-CoA structures (Zhang et al. 2007). DesT binds tightly to DNA in the presence of UFA-CoA ligands. However, the transcriptional repressor is released from its binding site by SFA-CoAs, thus allowing DesB expression. Structural analyses of the DesT/acyl-CoA complexes revealed that the acyl chain shape directly influences the packing of hydrophobic core residues within the DesT ligand-binding domain. These changes are propagated to the paired DNA-binding domains through conformational changes that modulate DesT-DNA affinity (Miller et al. 2010). Thus, under normal growth conditions, DesT exists in equilibrium between the free and DNA-bound forms, and a basal level of *desCB* transcription is maintained. The presence of SFAs in the growth environment leads to an increase in the intracellular saturated acyl-CoA levels. The binding of saturated long-chain acyl-CoAs, such as palmitoyl-CoA, to DesT releases the protein from the *desCB* promoter and derepresses *desCB* transcription. As a result, saturated acyl CoAs are converted to Δ9 UFA-CoAs and incorporated into membrane phospholipids (Zhang et al. 2007). It was proposed that when the intracellular levels of UFA-CoAs augment, either by DesB desaturation or by uptake of free fatty acids that are then converted to UFA-CoAs by the acy-CoA synthase, they could be available to bind DesT. This would increase the affinity of DesT from its binding site followed by transcriptional repression of the *desBC* operon.

It was previously noted that the *fabAB* operon is repressed by exogenous UFAs and that DesT was able to bind to a palindromic sequence located on the promoter region of this operon, suggesting a coordinate expression of anaerobic (*fabAB*) and aerobic (*desCB*) pathways for UFA synthesis (Subramanian et al. 2010). However it was demonstrated that DesT repression seems to play only a modest role in *fabAB* expression, which seems to be controlled by a complex regulatory network composed of a combination of directly acting factors and indirectly acting transcriptional or translational regulators (Schweizer and Choi 2012).

It was suggested that regulation of *desB* expression by DesT could be important when *P. aeruginosa* grows in an environment rich in fatty acids. Thus, the DesT-DesB system could provide a mechanism that allows *Pseudomonas* cells to substitute energy-intensive synthesized fatty acids for environmental fatty acids and at the same time maintain membrane homeostasis (Cronan 2006).

A Multi-Stress Sensor in Cyanobacteria

In cyanobacterial cells, fatty acid desaturation is one of the crucial steps in the acclimation processes during cold-shock. Low temperatures increase the desaturation of fatty acids of membrane lipids in cyanobacterial cells, as a result of upregulation of the expression of the desaturase genes (Los and Murata 1999). Three out of four genes (*desA*, *desB*, and *desD*) coding for desaturases in *Synechocystis* are cold-inducible (Los et al. 1993, 1997).

The systematical inactivation of each of the 43 putative histidine kinases genes (*hik*) in *Synechocystis* led to the identification of Hik33, involved in the sensing of low temperatures (Suzuki et al. 2000). Subsequently, it was demonstrated that the response regulator RpaB along with Hik33 are involved in low temperature signal transduction (Kappell and van Waasbergen 2007). Hik33 has two membrane-spanning domains, a HAMP linker, a leucine zipper, and a PAS domain located at its amino terminus, while the carboxy-terminus contains a highly conserved histidine kinase catalytic domain (Sakamoto and Murata 2002). Hik33 is involved in the cold-inducible expression of *desB* gene, which encodes the $\omega 3$ desaturase of *Synechocystis* (Suzuki et al. 2000). Several lines of evidence support the hypothesis that the transcriptional regulation of *desB* is mediated by Hik33, which in turn is activated by an increase in the order of lipids. It was proposed that the TM domain of Hik33 acts as a sensor of the state of membrane fluidity. If this is the case, the order of membrane lipids could mediate structural rearrangements in the TM domain that would be transmitted to the HAMP domain, which in turn would adjust the signaling state of the Hik33 kinase catalytic domain. Interestingly, the cold induction of *desB* is light-dependent (Mironov et al. 2012). This additional checkpoint in the signal transduction pathway might be related to the function of the PAS domain contained in Hik33.

The cold-regulated induction of gene expression in *Synechocystis* seems to be similar to the pathway controlled by DesK/DesR in *B. subtilis*. Thus, the control of membrane fluidity by cold-sensor histidine kinases could be conserved in several bacteria that use acyl-lipid desaturases to remodel membrane lipids. In contrast to the *B. subtilis* DesK/DesR system which regulates almost exclusively the expression of the *des* gene (Beckering et al. 2002), Hik33 is a global cold sensor involved in the expression of several genes that are induced at low temperature (Murata and Suzuki 2006). In addition, the analysis of the transcriptome of *Synechocystis* indicated that Hik33 is involved in the sensing of hyperosmotic stress (Mikami et al. 2002; Paithoonrangsarid et al. 2004) and salt stress (Shoumskaya et al. 2005), and

is the main sensor of peroxidative stress in this bacterium (Kanesaki et al. 2007). It is remarkable that Hik33 and its cognate regulator(s) controls the expression of distinct respective sets of genes under different kinds of stresses, and this finding cannot be explained by the currently accepted model of two-component systems. A plausible hypothesis is that a non-yet identified factor(s) could determine the specificity of the sensor for a particular stress.

It was shown that a mutation in *hik33* only partially affects the cold induction of *desB* and has not any effect in the temperature regulation of *desA* and *desB* (Suzuki et al. 2001). These findings suggest that a Hik33-independent mechanism operates to regulate these genes. It has early been proposed that temperature-dependent alterations in DNA supercoiling might be involved in the regulation of the expression of the *B. subtilis des* gene (Grau et al. 1994). This observation was extended to cyanobacteria by showing that upon a decrease in ambient temperature the degree of DNA supercoil increases in the promoter region of the *desB* gene (Los 2004). In concert with this finding, it was reported that novobiocin, an inhibitor of the DNA gyrase, prevented the cold-induced expression of several desaturase genes, including *desB* which was the gene more sensitive to inhibition by the antibiotic (Los 2004). DNA microarray-based analysis of gene expression in the presence of novobiocin showed that expression of almost all cold-inducible genes in *Synechocystis* requires DNA gyrase activity (Prakash et al. 2009). Taken together, a plausible model for the perception and transduction of cold-stress signals by cyanobacterial cells would be as follows: the sensory histidine kinase Hik33 perceives the temperature-induced rigidification in the cytoplasmic membrane and acquires a kinase state. Upon autophosphorylation of the Hik33 dimer, it transfers the phosphoryl group to the response regulator RpaB, which binds to promoter region of the genes to induce their transcription. The Hik33-RpaB two-component system regulates 23 out of 48 cold-inducible genes. The cold-inducible genes that are not regulated by Hik33-RpaB could be controlled by a low-temperature induction of negative DNA supercoil. The DNA gyrase-dependent mechanism seems also to be important to control the cold-induced transcription of genes involved in stabilization of mRNA, translation, cell wall metabolism, and regulation of membrane fluidity, which are not dependent of Hik33-RpaB sensing.

Concluding Remarks and Future Perspectives

Much progress has been made over the last several years in the identification and studies of fatty acid desaturases in bacteria. A particularly fundamental question is the mechanistic details of how the acyl-lipid desaturases introduce the double bond into the fatty acid moieties of membrane phospholipids. However, this mechanism has not yet been elucidated because integral membrane proteins are notoriously difficult to study in vitro. The studies of the *B. subtilis* desaturase through the use of genetic techniques coupled with lipid analytics and topological analyses provided important data to understand the structure–function relationship of acyl-lipid

desaturases. Advances in genomics and mass-spectrometry-based shotgun lipidomics promise to facilitate the future biochemical investigations of membrane-associated desaturases present in major human pathogens. Finally, insights into the biochemical genetics of bacterial desaturases will impact other fields since these enzymes are ubiquitous membrane proteins found in all kinds of life.

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