

Article

# Inhibition of Stearoyl-CoA Desaturase-1 Activity Suppressed SREBP Signaling in Colon Cancer Cells and Their Spheroid Growth

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**Abstract:** Unsaturated fatty acids are critical in promoting colon tumorigenesis and its stemness. Stearoyl-CoA desaturase-1 (SCD1) is a rate-limiting lipid desaturase associated with colon cancer cell proliferation and metastasis control. This study aims to evaluate the effects of SCD1 inhibition on colon cancer spheroid growth in a three-dimensional cell culture system. An analysis of clinical data showed that increased *SCD1* gene expression in colon tumors was negatively correlated with the prognosis. A chemical inhibitor of SCD1, CAY10566, inhibited the growth of colon cancer cells in both monolayer and sphere cultures. In addition, oleic acid administration—a monounsaturated fatty acid generated by the action of SCD1—prevented the suppression of sphere formation by CAY10566. RNA-sequencing data from 382 colon tumor patient samples obtained from the Cancer Genome Atlas database showed that 806 genes were SCD1-associated genes in human colon cancer. Correlation analysis identified the master regulator of lipid homeostasis sterol regulatory element-binding protein 2 (SREBP2) as a prominent transcription factor, whose expression was positively correlated with *SCD1* in human colon cancer. *SCD1* knockdown using siRNA in colon cancer samples, suppressed *SREBP2* gene expression, providing direct evidence that SREBP signaling is under the control of SCD1 in these cells. Pathway analysis in the Ingenuity Pathways Analysis platform showed that *SCD1* expression positively correlated with genes involved in multiple pathways, including lipid synthesis and incorporation, cell proliferation, and tissue tumorigenesis. Further network analysis revealed a central role for Myc in the network hierarchy of the *SCD1*-correlated genes. These findings suggested that SCD1 inhibition would be an effective strategy for suppressing colon cancer spheroid growth, partly through downregulating SREBP-mediated lipid and cholesterol metabolism and Myc signaling.

**Keywords:** stearoyl-CoA desaturase-1; colon cancer; spheroid culture; fatty acid; sterol regulatory element-binding protein 2

## 1. Introduction

Colon cancer, a well-characterized inflammation-driven disease, is the third leading cause of cancer-related deaths worldwide, with more than one million new cases reported each year [1]. These outcomes are mainly due to its poor prognosis, where long term treatment leads to patients acquiring resistance to therapy. In China, according to the data of a population-based cancer registry, the age-standardized 5-year relative survival rate for colon cancer is less than 50% [2]. The major cause of colon cancer therapy failure is the emergence of tumor-driving cells or cancer stem cells (CSCs), which possess high plasticity and a mesenchymal-like phenotype [3]. Among these contributors, leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5)<sup>+</sup> colon stem cells, existing near the

base of intestinal crypts that normally contribute to all cell lineages in the intestinal epithelium, are the most rapidly self-renewing epithelial tissues in mammals [4] and are known to serve as a cell of origin for colon cancer due to their inherent plasticity and longevity [5]. Therefore, new therapies based on understanding the molecular mechanisms underlying colon CSC regulation are needed.

There is growing evidence associating colon cancer development with environmental causes, such as a high-fat diet [6]. Pro-obesity diets enhance the numbers and function of the Lgr5<sup>+</sup> stem cells and/or colon tumorigenesis by activating multiple pathways, including peroxisome proliferator-activated receptor delta (PPAR- $\delta$ ) and Wnt/ $\beta$ -catenin signatures [7], retinol binding protein 4 (RBP4)-stimulated by the retinoic acid 6 (STRA6) pathway [8], and fatty acid oxidation [9]. Similar changes were observed when dietary constituents, such as palmitic acid and oleic acid (OA), were administered, suggesting a critical role for fatty acid in the maintenance of colon CSCs.

Fatty acids function as critical constituents of membrane structure, large energy sources, and serve as signal mediators for signal transduction [10]. Stearoyl-CoA desaturase-1 (SCD1), a transmembrane protein predominantly located at the endoplasmic reticulum, is a rate-limiting lipid desaturase responsible for generating monounsaturated fatty acids, mainly palmitoleic acid and OA [11]. We recently showed that SCD1 is upregulated in CSC-like cell populations compared with non-CSC populations in human liver cancer cell lines [12,13]. Other studies also shed light on the roles of unsaturated fatty acids in maintaining CSC stemness by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) [14] and Wnt- $\beta$ -catenin [15] signaling pathways. These studies indicate that lipid desaturation is a metabolic signature, and SCD1 may be an important therapeutic target in CSCs.

SCD1 has been reported to promote the metastasis of colon cancer by inducing epithelial–mesenchymal transition [16]. Furthermore, the inhibition of SCD1 suppresses colon cancer cell proliferation [17]. Although the underlying molecular mechanism has not been fully understood, possible pathways include either induced ceramide biosynthesis [17] or the depletion of monounsaturated fatty acid [18]. However, a limitation of this study is that in vitro monolayer cell cultures did not mimic in vivo three-dimensional (3D) tumor growth [19]. Therefore, here we focus on the effects of SCD1 inhibition on the spheroid growth potential of a human colon cancer cell line. We employed a simple 3D multicellular tumor spheroid system known to be significantly more resistant to chemotherapeutic agents compared with monolayer cultures [20] and found that a chemical inhibitor of SCD1, CAY10566, efficiently prevented the 3D spheroid formation of colon cancer cells.

## 2. Results

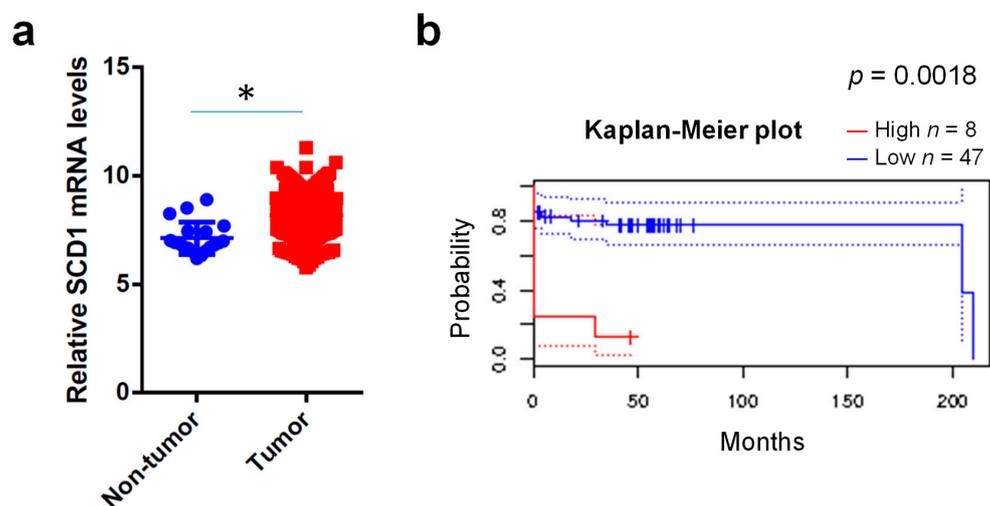
### 2.1. Clinical Expression Levels of SCD1

First, we examined the clinical and prognostic implications of SCD1 expression in human colon cancer. The mining of microarray data from the Gene Expression Omnibus (GEO, accession no. GSE40967) [21] demonstrated that SCD1 gene expression was significantly increased in colon tumors compared with the adjacent non-tumor colon tissues (Figure 1a). Using the PrognosScan database [22], Kaplan–Meier estimates of the proportion of colon cancer patients who survived over 15 years, showed that patients with high SCD1 gene expression in tumors had significantly lower survival rates (Figure 1b). This is in accordance with a recent report, relying on the Cancer Browser database, that colon cancer patients with high SCD1 levels had a poor overall survival rate [16]. These data suggested that SCD1 and lipid desaturation play important tumor-promoting roles in colon cancer.

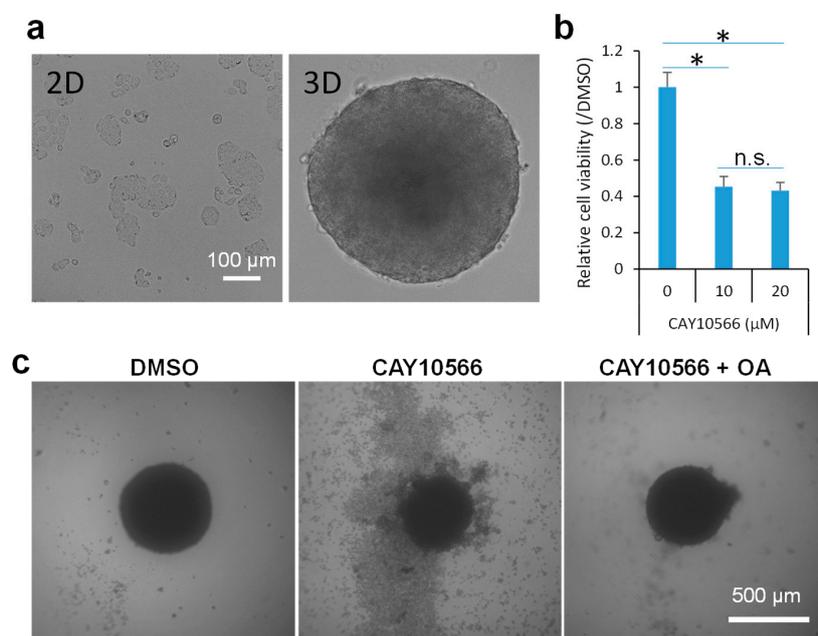
### 2.2. Inhibition of SCD1 Suppresses Sphere Growth of Colon Cancer Cells

We then examined whether inhibiting lipid desaturation using a chemical inhibitor of SCD1, CAY10566 [14], can suppress the growth of a colon cancer cell line—WiDr cells grown as both monolayers and spheres (Figure 2a). In WiDr monolayers, CAY10566 significantly decreased cell viability (Figure 2b). Similarly, CAY10566 inhibited the spheroid formation in the WiDr 3D culture

system (Figure 2c). To provide functional evidence supporting the lipid desaturation-mediated effects of CAY10566, we measured sphere formation with exogenously added lipid supplementation. The combined addition of OA, which is a monounsaturated fatty acid synthesized by SCD1 [23], restored the suppression of sphere formation by CAY10566 (Figure 2c). These data supported SCD1 and lipid desaturation as major players in maintaining colon cancer spheroids.



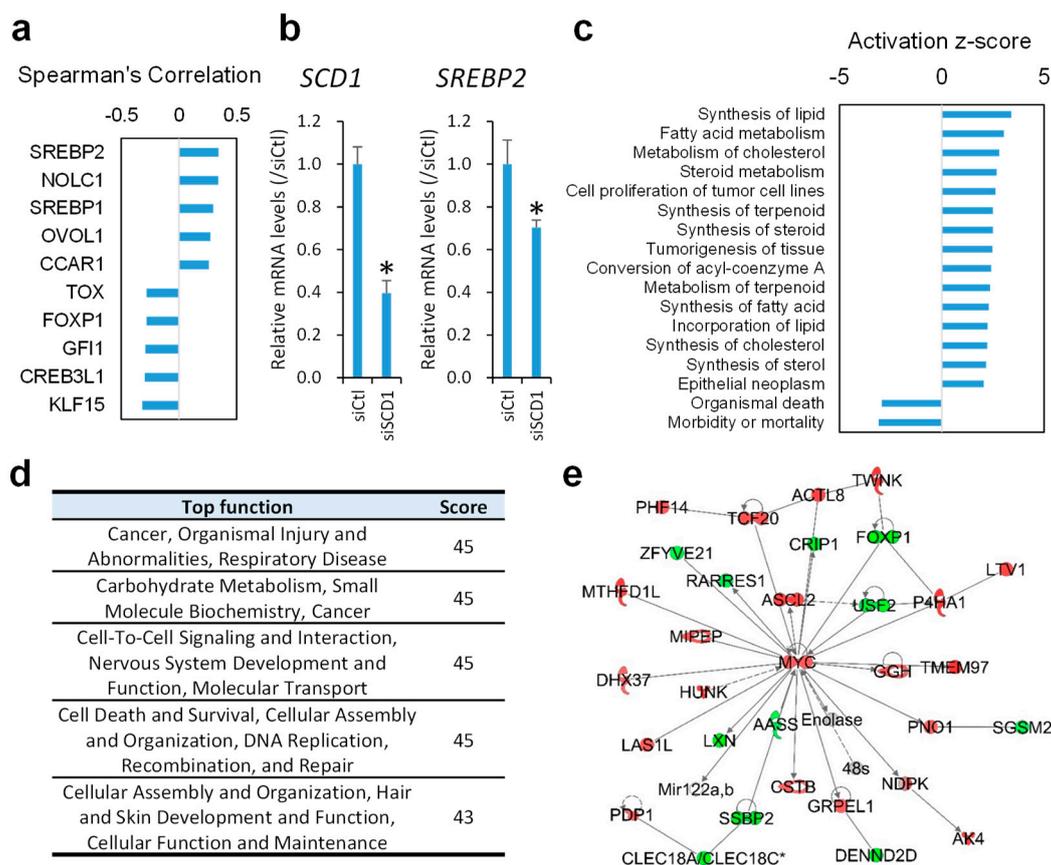
**Figure 1.** Clinical expression levels of stearoyl-CoA desaturase-1 (SCD1). (a) The expression of *SCD1* mRNA in human colon cancer (right,  $n = 566$ ) and adjacent non-tumor tissues (left,  $n = 19$ ) (Gene Expression Omnibus (GEO) dataset GSE40967). Statistical significance was evaluated using the Mann–Whitney U test. \*  $p < 0.05$ . (b) The clinical significance of *SCD1* expression in predicting the prognosis of colon cancer evaluated using the PrognosScan database analysis.



**Figure 2.** Inhibition of SCD1 suppresses colon cancer spheroid growth. (a) Representative microscopic images of WiDr cells grown as monolayers (left) and spheres (right). Scale bar, 100 μm. (b) Dose-dependent decrease in relative cell viability of WiDr cells grown as monolayers treated with increasing concentrations of a SCD1 inhibitor, CAY10566, for 48 h. The data are presented as means ( $n = 3$  replicates)  $\pm$  SD; \*  $p < 0.05$ , Student's *t*-test. n.s., not significant. (c) Representative microscopic images of WiDr cells grown as spheres treated with 20 μM CAY10566 in the absence or presence of 100 μM oleic acid (OA) for 3 days. Scale bar, 500 μm.

### 2.3. Inhibition of SCD1 Suppresses Myc Signaling in Colon Cancer Cells

Finally, to gain more insight into the molecular basis for the role of SCD1 in human colon cancer, we identified the SCD1-correlated genes from the Cancer Genome Atlas (TCGA) database, which contains RNA-sequencing data from 382 colon tumor samples. With a threshold of Spearman's correlation coefficient of  $>0.2$ , 806 genes were selected as SCD1-associated genes in human colon cancer (Figure 3a). To obtain direct and functional evidence regarding the correlation between SCD1 and its potential downstream targets in colon cancer cells, the effect of SCD1 knockdown on the expression of SCD1-correlated transcriptional regulators was examined using polymerase chain reaction (PCR) analysis. Transfection of WiDr cells with a pool of three target-specific siRNAs towards SCD1 significantly suppressed the expression of sterol regulatory element-binding protein 2 (SREBP2; Figure 3b). The SCD1-correlated genes were imported into the Ingenuity Pathways Analysis (IPA) platform to investigate possible biological interactions. The diseases or functions annotation analysis showed that the genes involved in controlling lipid synthesis and incorporation, cell proliferation, and tissue tumorigenesis positively correlated with SCD1, while the genes regulating organismal death negatively correlated with SCD1 in human colon cancer (Figure 3c). Further network analysis of the biological functions of the top five IPA-generated networks are summarized in Figure 3d. The most highly populated biological networks entitled "Cancer, Organismal Injury and Abnormalities, Respiratory Disease" (Score = 45) revealed a central role for Myc in the network hierarchy of the SCD1-correlated genes (Figure 3e).

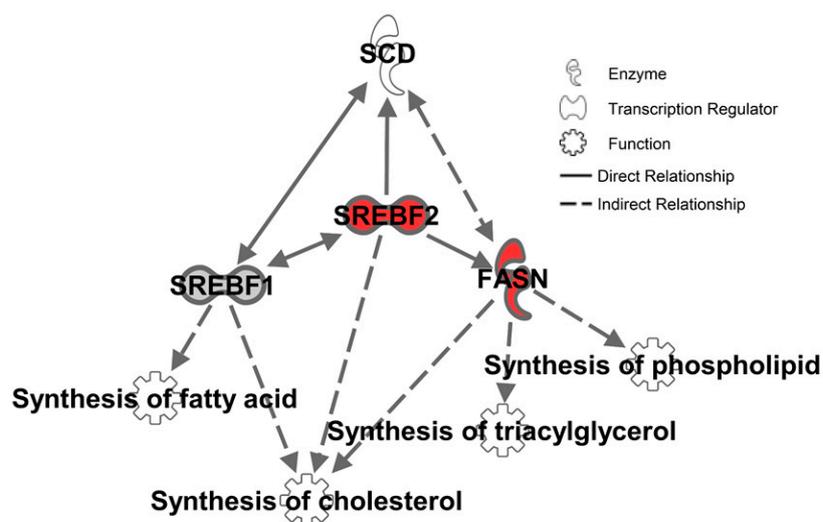


**Figure 3.** Inhibition of SCD1 suppresses sterol regulatory element-binding protein 2 (SREBP) signaling. (a) The top transcription regulators correlated with SCD1 gene expression in human colon cancer selected through an analysis of the Cancer Genome Atlas (TCGA) database. The genes were ranked by Spearman's correlation coefficient. (b) The decrease in SCD1 (left) and SREBP2 (right) gene expression in WiDr cells grown as monolayers treated with 100 nM siSCD1 for 24 h. The data are presented as means

( $n = 3$  replicates)  $\pm$  SD. \*  $p < 0.05$ , Student's  $t$ -test. (c) The top diseases or functions annotation associated with the 806 genes correlated with *SCD1* in 382 human colon tumors (Spearman's correlation coefficient of more than 0.2) performed in the Ingenuity Pathways Analysis (IPA) platform. The pathways were ranked according to their activation  $z$  score, which can be used to predict either the activated or inhibited state based on a statistically significant pattern match of up- and downregulation, respectively. (d) The top five associated network functions generated by the IPA platform. (e) A representative network related with Myc signaling entitled "Cancer, Organismal Injury and Abnormalities, Respiratory Disease".

### 3. Discussion

Our data show that *SCD1* inhibition is an effective strategy for suppressing the spheroid growth of colon cancer cells. Mechanically, data mining in the TCGA database revealed that the master regulator of lipid homeostasis *SREBP2* acted as a master transcription factor, whose gene expression positively correlated with *SCD1* in human colon cancer. Importantly, siRNA knockdown of *SCD1* significantly inhibited *SREBP2* expression, suggesting that the anti-tumor effects of *SCD1* inhibition are mediated, at least in part, through the downregulation of the *SREBP* signaling pathway. *SREBP2* belongs to a family of transcription factors that regulate the expression of genes required for the synthesis of fatty acids, cholesterol, triacylglycerol, and phospholipid (Figure 4) [24]. Recently, the inhibition of *SREBP* has been shown to suppress tumor growth and initiation in colon cancer through the downregulation of cellular lipid biosynthesis [25]. Our data provide direct evidence that *SREBP* signaling is under the control of *SCD1* in colon cancer cells.



**Figure 4.** A schematic model of the *SCD*-dependent regulatory network in the control of lipid metabolism generated using the IPA platform. Red, genes whose expression were positively correlated with *SCD1* gene expression in human colon cancer selected through an analysis of Cancer Genome Atlas (TCGA) database.

The pathway and network analyses suggested that the pro-tumorigenic effects of lipid desaturases, such as *SCD1*, in a human colon cancer cell lines are related to Myc signaling. Myc (*c-Myc*), a basic helix–loop–helix–zipper transcription factor, is reported as one of the Yamanaka factors [26] and plays a critical role in maintaining stem cell pluripotency. Myc also functions as an oncogene, contributing to both tumor initiation and maintenance by controlling cell growth [27]. A recent study has shown that *c-Myc* activation positively regulates cell self-renewing and is indispensable for crypt regeneration and colon tumorigenesis [28]. Of interest, a domain-based interaction resource suggested a binding between human Myc and *SCD1* proteins [29]. It has been reported that aberrant stabilization and dysregulated activation of Myc reprograms cancer cell metabolism to enhance precursor provisions for

phospholipids [30]. In addition, OA administration partially rescues cell death-induced Myc signaling suppression, highlighting a critical role for lipid biosynthesis in Myc-driven cancer survival [30]. It seems plausible that there is a positive feedback loop between Myc activation and lipid metabolism reprogramming, which is probably regulated by the phosphatase and tensin homolog (PTEN)/the mechanistic target of rapamycin (mTOR) signaling pathways [31,32].

In this study, we observed an increase in *SCD1* gene expression in human colon cancer, which was significantly correlated with a poor prognosis. It has been reported that Toll-like receptor 4 (TLR4) deficiency can lead to the reduction of mRNA levels of genes involved in de novo lipogenesis, such as SCD1, but not genes involved in gluconeogenesis and fatty acid oxidation [33]. These data suggest that SCD1 expression might be regulated by the lipopolysaccharide (LPS)/TLR4 signaling pathways. Studies from the past few years demonstrate a strong impact of environmental factors, such as high-fat diet, on microbial composition and colon cancer risk [34]. Enhanced TLR4 expression in the intestinal epithelium has been shown to cause the expansion and proliferation of Lgr5<sup>+</sup> colon stem cells [35]. Enhanced TLR4 expression in colon cancer is related to increased metastasis [36] and poor prognosis [37]. Therefore, it seems reasonable to hypothesize that increased *SCD1* gene expression in colon cancer is controlled by microbial dysbiosis and the activation of TLR4 signaling.

## 4. Materials and Methods

### 4.1. Cell Culture

WiDr cell, derived from human colon adenocarcinoma cell line HT-29 [38], was maintained at 37 °C and 5% CO<sub>2</sub>, in Dulbecco's modified Eagle medium (DMEM, Wako Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS; Mediatech, Herndon, VA, USA), 100 U/mL penicillin/streptomycin, and 2 mM L-glutamine (Mediatech).

### 4.2. 3D Spheroid Culture and Chemical Treatment

The cells were seeded onto non-adherent 96-well round-bottomed Sumilon PrimeSurface™ plates (MS-9096U, Sumitomo Bakelite, Tokyo, Japan) [20], in 100 µL of media (DMEM plus 10% FBS), at a concentration of 5000 cells per well. The spheroids were grown for 4 days, and then, 50 µL of media were exchanged with 50 µL fresh serum-free media containing 40 µM CAY10566 (ab144421, Abcam, Cambridge, MA, USA), a SCD1 inhibitor, and/or 200 µM OA (O1008, Sigma, Louis, MO, USA). The spheroids were further cultured for 3 days, and photos were taken using an optical microscope (DS-Fi1, NIKON, Tokyo, Japan). Dimethyl sulfoxide (DMSO, Sigma) was used as the primary solvent for all chemicals.

### 4.3. Cell Viability Assay

Cell viability was measured using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan). The absorbance was measured using a plate reader (ARVO MX, Perkin Elmer Inc., Waltham, MA, USA) at 450 nm.

### 4.4. In vitro RNA Interference

A pool of 3 target-specific siRNAs targeting human SCD1 (sc-36464; siSCD1) and a control siRNA (sc-37007; siCtl) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). WiDr cells were plated in 48-well plates for 24 h and then transfected with 100 nM siRNAs using Lipofectamine 2000 transfection reagent (Life Technologies, Gaithersburg, MD, USA). On the following day, cells were collected for RNA isolation and further analysis.

### 4.5. RNA Isolation and Real-time (RT)-PCR

Total RNA was isolated using a FastGene RNA Basic Kit (FG-80250, NIPPON Genetics, Tokyo, Japan) and quantified using a NanoDrop spectrophotometer (NanoDrop products, Wilmington,

DE, USA). cDNA was synthesized using a PrimeScript RT Master Mix Kit (TaKaRa Bio, Otsu, Japan). Primer sequences were as follows (5' to 3'): glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) forward (CAATGACCCCTTCATTGACC) and reverse (GACAAGCTTCCCGTTCTCAG), *SCD1* forward (GTACCGCTGGCACATCAACTT) and reverse (TTGGAGACTTCTTCCGGTCAT), and *SREBP2* forward (ATCGACCTAGGCAGTCTGGT) and reverse (ATAGAGGGCTTCTGGCTCA). PCR reactions were performed using a Roche LightCycler 96 Real-Time PCR System (Roche Diagnostic Co., Ltd., Mannheim, Germany) and the SYBR Premix ExTaq II (TaKaRa Bio).

#### 4.6. Data Mining

Normalized *SCD1* expression from a clinical dataset, which contains transcriptome profiling of 566 colon cancer samples and 19 adjacent non-tumor samples, was downloaded from the GEO database ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo), accession no. GSE40967) [21]. A Prognoscan database (<http://www.abren.net/Prognoscan/>) analysis, which contains a large collection of publicly available cancer microarray datasets with clinical annotations, was conducted to investigate the clinical significance of *SCD1* expression in predicting cancer prognosis [22].

#### 4.7. Knowledge-Based Pathway Analysis

Genes correlated with *SCD1* gene expression in colon cancer were selected through an analysis of the TCGA database using cBioPortal software [39]. With a threshold of Spearman's correlation coefficient of >0.2, 806 genes were selected and input into the knowledge-based functional analysis software IPA (Ingenuity Systems) as previously described [40]. The generated biological networks were ranked by score, which is the likelihood of a set of genes being found in the networks owing to random chance, identified by a Fisher's exact test. The generated diseases or functions annotations were ranked by the activation z-score, which can be used to find likely regulating molecules based on a statistically significant pattern match of up- and downregulation and also to predict the activation state (either activated or inhibited) of a putative regulator. An absolute z-score of more than 2 was considered to be significant.

#### 4.8. Statistical Analysis

Quantitative data were expressed as the mean  $\pm$  SD of at least three replicates. The significance of differences between the values was assessed using Student's *t*-test or Mann-Whitney *U* test. *p*-values of <0.05 were considered significant.

## 5. Conclusions

Our current findings suggest a potential role for unsaturated fatty acid-associated metabolic changes during colon tumorigenesis. Furthermore, lipid desaturases such as *SCD1* might serve as therapeutic targets for colon cancer via the inhibition of *SREBP*-mediated lipid and cholesterol metabolism.

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