농학석사 학위논문

Inhibition of Stearoyl-CoA Desaturase 1 (SCD1) induces ER stress-mediated apoptosis in ovarian cancer cells

난소암 세포주에서 SCD1 억제에 의해 유도된 소포체 스트레스 매개 세포사멸 기전 연구

2017년 8월

서울대학교 대학원

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Abstract

Cancer cells are strongly dependent on alterations in specific metabolic pathways. Together with the Warburg effect, enhanced lipid biosynthesis plays a crucial role in the production of energy and metabolic intermediates, leading to uncontrolled proliferation of cancer cells. Stearoyl-CoA desaturase 1 (SCD1) is a rate-limiting enzyme of *de novo* lipid synthesis that catalyzes the biosynthesis of mono-unsaturated fatty acids (MUFAs) by introducing a double bond in the *cis*-Δ9 position of saturated fatty acids (SFAs). MUFAs, such as palmitoleic acid and oleic acid, are indispensable for the synthesis of cell membranes, the production of signaling compounds, and the generation of energy. In this study, we identified that SCD1 is highly expressed in epithelial ovarian cancer (EOC) cells compared to normal ovarian surface epithelial (NOSE) cells. Inhibition of SCD1 reduced cell proliferation and induced apoptotic cell death in ovarian cancer cells. However, it had no significant effect on the viability of NOSE cells and peripheral blood mononuclear cells (PBMCs), indicating the selective cytotoxicity against ovarian cancer cells. Furthermore, suppression of SCD1 induced endoplasmic reticulum (ER) stress by activating PERK, IRE1α, GRP78, ATF4, and CHOP in ovarian cancer cells. Addition of exogenous oleic acid rescued ER stress-mediated apoptosis caused by SCD1 suppression,

highlighting the importance of lipid desaturation for cancer cell survival. Taken together, our findings suggest that SCD1 could be a potential biomarker as well as a novel therapeutic target for ovarian cancer.

Keywords: Ovarian cancer, Lipid metabolism, SCD1, MUFA, ER stress

Student Number: 2015-22694

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List of Abbreviations

GEO: Gene expression omnibus

SCD1: Stearoyl-CoA desaturase 1

SFA: Saturated fatty acid

MUFA: Mono-unsaturated fatty acid

EOC: Epithelial ovarian cancer

NOSE: Normal ovarian surface epithelial

PBMC: Peripheral blood mononuclear cell

ER stress: Endoplasmic reticulum stress

UPR: Unfolded protein response

PARP: Poly ADP-ribose polymerase

PERK: Protein kinase RNA-like endoplasmic reticulum kinase

IRE1α: Inositol-requiring enzyme 1α

GRP78: 78 kDa glucose-regulated protein

ATF4: Activating transcription factor 4

CHOP: C/EBP (CCAAT-enhancer-binding protein) homologous protein

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

OA-BSA: Oleic acid-bovine serum albumin conjugate

FAS: Fatty acid synthase

Introduction

Cancer cells primarily exhibit alterations in several metabolic pathways. They require increased production of energy and cellular building blocks such as proteins, nucleic acids, and lipids for their rapid proliferation [1]. The best-known metabolic phenotype in cancer cells is aerobic glycolysis or the Warburg effect, characterized by excessive glucose uptake and increased lactate production under aerobic conditions [2]. Although the Warburg effect is much less energy-efficient than mitochondrial oxidative phosphorylation, cancer cells rely on this glycolysis to obtain metabolic substrates and maintain redox balance for their survival and rapid growth [3]. Another cancer-specific metabolic alteration is glutamine addiction. Glutamine, one of the major energy source of mammalian cell proliferation, provides carbon and nitrogen for amino acid and nucleotide biosynthesis [4, 5]. The majority of cancer studies have focused on alterations in glucose and glutamine catabolism over the past decades, but more recently, increasing attention has been given to dysregulated lipid metabolism in cancer cells [6].

Fatty acids play crucial roles in cancer cell development, progression, and survival [6-8]. They are the building blocks of phospholipids and other lipids such as sterols and sphingolipids for membrane biosynthesis. Fatty acids also act as secondary messengers in signal transduction, and

they are stored as triacylglycerides. Interestingly, most normal cells use fatty acids obtained from exogenous sources, whereas cancer cells utilize fatty acids generated from *de novo* synthesis [9]. Unlike normal cells, up to 95% of fatty acids in cancer cells are derived from *de novo* fatty acid synthesis regardless of dietary lipid levels [10, 11]. This exacerbated lipogenesis in cancer cells involves the upregulated expression and activity of lipogenic enzymes, such as ATP citrate lyase, acetyl-CoA carboxylase, and fatty acid synthase. Many previous studies have shown that inhibition of different enzymes in the lipogenic pathway can suppress cancer cell proliferation [6, 7, 11], suggesting that enhanced lipogenesis is essential for cancer cell survival.

Stearoyl-CoA desaturase 1 (SCD1), a critical regulator of *de novo* fatty acid synthesis, is a rate-limiting enzyme catalyzing the conversion of saturated fatty acids (SFAs) into mono-unsaturated fatty acids (MUFAs), primarily palmitoyl-CoA and stearoyl-CoA into palmitoleoyl-CoA and oleoyl-CoA, respectively [12]. SCD1 is abundantly expressed in a variety of human cancers, including breast, lung, liver, and ovarian cancers, compared to the corresponding normal tissues [12-14]. The elevated SCD1 levels have been associated with poor prognosis in liver and lung cancers [13, 14]. Moreover, inhibition of SCD1 activity or expression significantly decreased cell proliferation and induced apoptosis in lung and colon cancer cells [12, 15, 16]. However, the

functional role of SCD1 in ovarian cancer and the molecular mechanism underlying cancer cell death induced by SCD1 suppression have not yet been fully elucidated.

In this study, we found that ovarian cancer cells exhibit high levels of SCD1 mRNA and protein expressions relative to normal ovarian cells. Furthermore, genetic and pharmacological inhibition of SCD1 reduced cell proliferation and induced ER stress-mediated apoptosis in ovarian cancer cells without cytotoxic effects on normal ovarian cells and PBMCs. Finally, supplementation with exogenous oleic acid, the main product of SCD1 activity, reversed the suppression of cancer cell proliferation and the induction of ER stress-mediated apoptosis triggered by SCD1 inhibition. Altogether, our results strongly suggest that SCD1 may serve as a promising biomarker and a therapeutic target for ovarian cancer.

Materials and Methods

1. Cell culture

Human ovarian cancer cell lines PA-1, OVCAR-3, TOV112D, and SKOV-3 were purchased from the American Type Culture Collection (Rockville, MD), and SNU840 was obtained from the Korean Cell Line Bank (Seoul, Korea). A2780 was kindly gifted from Prof. Benjamin K. Tsang (University of Ottawa, Canada). Induced ovarian surface epithelial cell lines IOSE385 and SNU3236 were generously gifted from Prof. Young Kee Shin (Seoul National University, Korea) and Prof. Ja-Lok Ku (Seoul National University, Korea), respectively. PA-1 was cultured in MEM (WelGENE, Seoul, Korea), and other cancer cell lines were cultured in RPMI1640 (WelGENE). TOV112D and normal ovarian cell lines were grown in DMEM/F12 (Gibco-BRL, Gaithersberg, MD). All media were supplemented with 10% fetal bovine serum (WelGENE), 100 Units/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL). All cells were cultivated at 37°C in humidified conditions with 5% CO_2. For assays, all cell lines were treated with CAY10566 or SCD1 siRNA for 24-48 hours in 1% serum-containing medium.

2. Reagents and antibodies

CAY10566 was purchased from Cayman Chemical (Ann Arbor, MI). Oleic acid-BSA conjugate and fatty acid-free BSA were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies used for western blotting were as follows: p-PERK (Thr981), PARP, GRP78, and cleaved caspase-3 from Santa Cruz Biotechnology (Santa Cruz, CA), IRE1α and ATF4 from Cell Signaling Technology (Danvers, MA), SCD1 from Abcam (Cambridge, UK), CHOP (GADD153) from Thermo Fisher Scientific (Waltham, MA), and GAPDH from AbFrontier (Seoul, Korea).

3. qRT-PCR (quantitative real-time PCR)

Total RNA was extracted using RNAiso Plus (TaKaRa, Tokyo, Japan), and the concentration of RNA was determined by NanoDrop2000 (Thermo Fisher Scientific). Complementary DNAs were synthesized from 1 μg of total RNA with oligo-dT primers and PrimeScript Reverse Transcriptase (TaKaRa). PCR was performed using QuantiSpeed SYBR No-ROX Kit (PhileKorea, Seoul, Korea) and the following specific primers: SCD1 sense 5'-CGA CGT GGC TTT TTC TTC TC-3', antisense 5'-GGG GGC TAA TGT TCT TGT CA-3' and GAPDH sense 5'-GAG TCA ACG GAT TTG GTC GT-3', antisense 5'-TTG ATT TTG

GAG GGA TCT CG-3'. The amplification conditions were as follows: an initial denaturation step at 95°C for 3 minutes, followed by 45 cycles of denaturation at 94°C for 5 seconds, annealing at 60°C for 15 seconds, extension at 72°C for 10 seconds, and a final extension step at 72°C for 10 minutes. The relative gene expression levels were calculated using the comparative Ct method, and GAPDH was used as a reference gene.

4. Cell proliferation assay

Cell viability was examined by MTT assay. For CAY10566 treatment, cells were seeded into 96-well plates at a density of 4,000-10,000 cells per well. The cells were treated with various concentrations of CAY10566 or DMSO (solvent control) for 24-48 hours. For siRNA transfection, cells were seeded into 6-well plates to be 60-80% confluent at transfection. After 24 hours, the cells were transfected with SCD1 siRNA or scrambled siRNA (negative control), incubated overnight, and then cultured into 96-well plates for 24-48 hours. At the end of the treatment, cells were incubated with 50 μl of MTT solution (2 mg/ml) for 3 hours at 37°C in humidified conditions with 5% CO_2 and subsequently solubilized in 100 μl of DMSO for 30 minutes. The optical density was measured at 540 nm using a Multiskan Ascent plate reader (Thermo LabSystems, Helsinki, Finland).

5. Gas chromatography

Desaturase activity of SCD1 was measured by gas chromatography. CAY10566-treated cells were harvested by centrifugation and freeze-dried using a freeze dryer (LABCONCO, Kansas City, MO). The extraction and methylation of fatty acids were conducted as previously described [17]. For GC analysis, fatty acids were converted to their methyl esters (FAMEs). Gas chromatography was performed using an Agilent 7890A GC system (Agilent Technologies, Wilmington, DE) equipped with a DB-23 capillary column (60 mm x 0.25 mm x 0.25 μm; Agilent Technologies). The GC conditions were as follows: the initial temperature was 50°C for 1 minute, then raised to 130°C at 15°C/min, to 170°C at 8°C/min, to 215°C at 2°C/min, and held for 10 minutes. The injector temperatures was set at 250°C, and the detector temperature was set at 280°C. Pentadecanoic acid (C15:0) was used as an internal standard for quantification. The ratios of palmitoleic acid (C16:1) to palmitic acid (C16:0) and oleic acid (C18:1n9c) to stearic acid (C18:0) were determined for this study.

6. Small-interfering RNA transfection

The SCD1 siRNA target sequence was 5'-GAG AUA AGU UGG AGA

CGA UUU-3'. Scrambled siRNA was used as a negative control (Genolution, Seoul, Korea). Cells were transfected with the siRNA oligonucleotides (100 nM) using Lipofectamine RNAiMAX (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocols. At 24 hours post-transfection, the cells were used for cell proliferation assay, flow cytometry analysis, and western blot analysis.

7. Isolation of peripheral blood mononuclear cells (PBMCs)

To determine the cytotoxic effect of CAY10566 on normal cells, buffy coats from healthy donors were collected under the approval of Seoul National University Hospital Institutional Review Board (C-1307-008-502). Human peripheral blood mononuclear cells were isolated from buffy coats by density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Marlborough, MA) according to the manufacturer's instructions. In brief, buffy coats were diluted in PBS, carefully layered on Ficoll-Paque PLUS, and centrifuged at 400 x *g* for 30 minutes at 20°C. The PBMC layer was transferred to a clean centrifuge tube, washed twice with PBS, centrifuged at 200 x *g* for 15 minutes at 20°C, and suspended in RPMI1640 supplemented with 10% FBS, 100 Units/ml penicillin, and 100 μg/ml streptomycin.

8. Flow cytometry analysis

To analyze both floating and adherent cells, culture media containing floating cells were collected into a round-bottom tube (BD Falcon, San Jose, CA), and adherent cells were trypsinized, washed with cold PBS, and collected by centrifugation at 4°C. All cells were stained with Annexin V-FITC and PI using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. The stained cells were analyzed using a BD FACS Canto II flow cytometer (BD Biosciences) with BD FACS Diva software (BD Biosciences).

9. Western blot analysis

Cells were lysed with the lysis buffer containing the premade 2X lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1 mM EGTA), 1% Triton X-100, 1 mM PMSF, 0.1% DCA, and 1X EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The concentration of protein was determined using BCA Protein Assay Kit (Thermo Fisher Scientific). 10-20 μg of proteins were loaded onto 6-15% SDS-PAGE gels for separation and transferred to nitrocellulose membranes (GE Healthcare Life Sciences) for detection by

immunoblotting. The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20, incubated with primary antibodies overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies for 2 hours at room temperature. Signals were visualized with the enhanced chemiluminescence detection kits, WESTSAVE up (AbFrontier) and ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences).

10. Statistical analysis

All data were expressed as mean ± SEM of three independent experiments. The statistical significance of differences was determined using Student's *t*-test and One-way ANOVA with Bonferroni's post hoc test. All statistical analyses were performed using IBM SPSS Statistics 22 software (SPSS Inc., Chicago, IL). For all analyses, differences with p-values < 0.05 were considered statistically significant.

Results

SCD1 expression is significantly elevated in EOC cell lines compared to NOSE cell lines

SCD1, the enzyme responsible for the production of mono-unsaturated fatty acids, has emerged as a novel therapeutic target for various cancer types, including breast, lung, thyroid, kidney, and colon cancers [13-16, 18-21]. To explore the role of SCD1 in ovarian cancer, we downloaded the microarray data from the NCBI GEO database (accession number GSE14407) and examined the expression levels of SCD1 in epithelial ovarian cancer (EOC) cells and normal ovarian surface epithelial (NOSE) cells. The microarray data containing 12 EOC cells and 12 NOSE cells showed that SCD1 gene expression was highly upregulated in EOC cells compared to NOSE cells (Figure 1A). We further investigated whether SCD1 expression is universally elevated in ovarian cancer cell lines. The qRT-PCR analysis revealed that the expression level of SCD1 mRNA in EOC cells was much higher than that in NOSE cells (Figure 1B). Consistent with the mRNA expression level, western blot analysis confirmed the overexpression of SCD1 protein only in EOC cells (Figure 1C). These results demonstrate that SCD1 is highly expressed at both mRNA and protein levels in EOC cells,

but not in NOSE cells. Based on Figure 1B and 1C results, we chose two cancer cell lines displaying the highest expression of SCD1, PA-1 cells, and the lowest expression of SCD1, SKOV-3 cells, for the next experiments.

Inhibition of SCD1 decreases cancer cell proliferation with no significant effect on normal cell growth

To elucidate the biological function of SCD1 in ovarian cancer, we examined the effect of SCD1 on cancer cell proliferation by using a small-molecule inhibitor of SCD1, CAY10566. CAY10566 significantly reduced cell proliferation in a dose-dependent manner in PA-1 cells (Figure 2A-1). In contrast, no apparent effect of CAY10566 on cell viability was observed in SKOV-3 cells (Figure 2A-1). CAY10566-mediated inhibition of SCD1 activity was confirmed by gas chromatography, followed by calculating the ratios of palmitoleic acid to palmitic acid (C16:1n7/C16:0) and oleic acid to stearic acid (C18:1n9c/C18:0) (Figure 2A-2). Additionally, we used SCD1 siRNA to ablate SCD1 expression in PA-1 and SKOV-3 cells. Although SCD1 levels were decreased by the siRNA in both two cell lines (Figure 2B-1), MTT assays showed that knockdown of SCD1 inhibited cell growth in PA-1 cells, but not in SKOV-3 cells (Figure 2B-2).

Previous studies have reported that *de novo* lipid synthesis is generally limited to specific tissue types, including liver, adipose, and lactating breast tissues [6, 22]. Accordingly, the expression of lipogenesis-related enzymes is present at low levels in most adult tissues, even in normally high proliferative cells such as hematopoietic cells [22]. It also has been known that SCD1 expression is downregulated in normal tissues relative to the adjacent cancer tissues [12]. Consistently, we have demonstrated that normal cell lines had low expression levels of SCD1 mRNA and protein compared to cancer cell lines (Figure 1B and 1C). To evaluate the effect of SCD1 inhibition on normal cell growth, NOSE cell lines (IOSE385, SNU3236) and peripheral blood mononuclear cells (PBMCs) were treated with various concentrations of CAY10566, and cell viabilities were measured by MTT assays. Contrary to the results of cancer cells, CAY10566 did not affect cell proliferation in all normal cells (Figure 2C), suggesting that SCD1 inhibition has a cancer-specific cytotoxicity. Thus, PA-1 cells were used for further experiments elucidating the cell death mechanism involved in SCD1 suppression in ovarian cancer.

SCD1 suppression induces ER stress-mediated apoptosis in ovarian cancer cells

Different fatty acids with various chain lengths and degrees of unsaturation represent the huge structural diversity of membrane lipids [23]. Changes in the degree of fatty acid unsaturation in cell membrane dramatically affect membrane fluidity and protein dynamics [4]. Recent studies have shown that inhibition of SCD1, subsequent accumulation of SFAs, and depletion of MUFAs resulted in the induction of ER stress and activation of the UPR, finally leading to apoptotic cell death [19, 23-25]. To determine whether inhibition of SCD1 triggers apoptotic cell death in ovarian cancer cells, we performed flow cytometry analysis using Annexin V-FITC and PI staining. As shown in Figure 3A, CAY10566 dramatically induced apoptosis in PA-1 cells. Western blot analysis also revealed that the expression levels of apoptosis marker proteins, cleaved PARP and caspase-3, were elevated by CAY10566 (Figure 3B). Consistent with the results of CAY10566 treatment, knockdown of SCD1 by siRNA enhanced apoptosis (Figure 3C), confirmed by western blot analysis (Figure 3D). These results suggest that both genetic ablation and pharmacological inhibition of SCD1 lead to apoptotic cell death.

To further investigate whether SCD1 suppression induces ER stress in ovarian cancer cells, we analyzed the expression levels of ER stress marker proteins, two master regulators (p-PERK, IRE1α) and their downstream effectors (GRP78, ATF4, CHOP). Western blot analysis showed that blockade of SCD1 activity by CAY10566 strongly

upregulated the ER stress-related proteins, suggesting the induction of ER stress and activation of the UPR (Figure 4A). In addition, genetic depletion of SCD1 by siRNA triggered ER stress by increasing the expression levels of ER stress marker proteins (Figure 4B). These findings demonstrate that ER stress can be induced by inhibiting desaturase activity of SCD1 or suppressing gene expression of SCD1.

Supplementation with oleic acid reverses the proliferative defect and ER stress-mediated apoptosis triggered by SCD1 inhibition

It has been reported that the reduction of cell viability and the induction of ER stress-mediated apoptosis triggered by inhibiting SCD1 desaturase activity are abrogated by exogenous application of MUFAs, such as oleic acid and palmitoleic acid [12, 16, 21]. To verify this in ovarian cancer, PA-1 cells were treated with CAY10566 with or without oleic acid conjugated to BSA (OA-BSA) for 48 hours. The CAY10566-induced proliferative defect was completely rescued by oleic acid (Figure 5A). Additionally, we assessed the effect of oleic acid on apoptotic cell death caused by SCD1 suppression. CAY10566-induced apoptosis was eliminated by addition of oleic acid (Figure 5B). Western

blot analysis also confirmed that the cleavage of PARP and caspase-3 was blocked by oleic acid (Figure 5C). Furthermore, we determined whether exogenous oleic acid prevents ER stress triggered by CAY10566. As demonstrated in Figure 5D, the CAY10566-induced upregulation of ER stress marker proteins was reversed by supplementation with oleic acid. These findings suggest that addition of exogenous oleic acid rescues the suppression of cell proliferation and the induction of ER stress-mediated apoptosis generated by SCD1 inhibition.

Figures

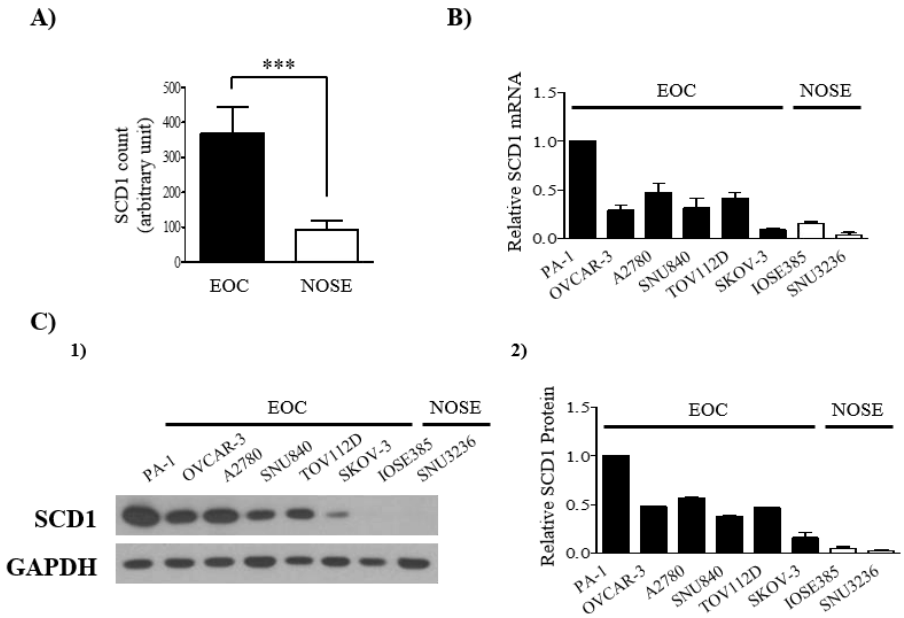
A)
B)
***
SCD1 count (arbitrary unit)
500
400
300
200
100
0
EOC
NOSE
Relative SCD1 mRNA
1.5
1.0
0.5
0.0
EOC
NOSE
PA-1
OVCAR-3
A2780
SNU840
TOV112D
SKOV-3
IOSE385
SNU3236
C)
1)
2)
EOC
NOSE
PA-1
OVCAR-3
A2780
SNU840
TOV112D
SKOV-3
IOSE385
SNU3236
SCD1
GAPDH
Relative SCD1 Protein
1.5
1.0
0.5
0.0
EOC
NOSE
PA-1
OVCAR-3
A2780
SNU840
TOV112D
SKOV-3
IOSE385
SNU3236

Figure 1. SCD1 is highly expressed in EOC cells compared to NOSE cells.

A) The microarray data for SCD1 expression in 12 ovarian cancer epithelial (EOC) cells and 12 normal ovarian surface epithelial (NOSE) cells obtained from the NCBI Gene Expression Omnibus (GEO) database (accession number GSE14407). Data were expressed as arbitrary units.

B) The mRNA expression levels of SCD1 in EOC cell lines and NOSE cell lines. SCD1 mRNA levels were analyzed by qRT-PCR analysis and normalized to GAPDH mRNA levels.

C) The protein expression levels of SCD1 in EOC cell lines and NOSE cell lines. (1) SCD1 protein levels were examined by western blot analysis. (2) The levels were quantified by densitometry using ImageJ software (NIH, Bethesda, MD), followed by normalization to GAPDH protein levels. All data were presented as mean ± SEM of three independent experiments. *** indicates $P < 0.001$.

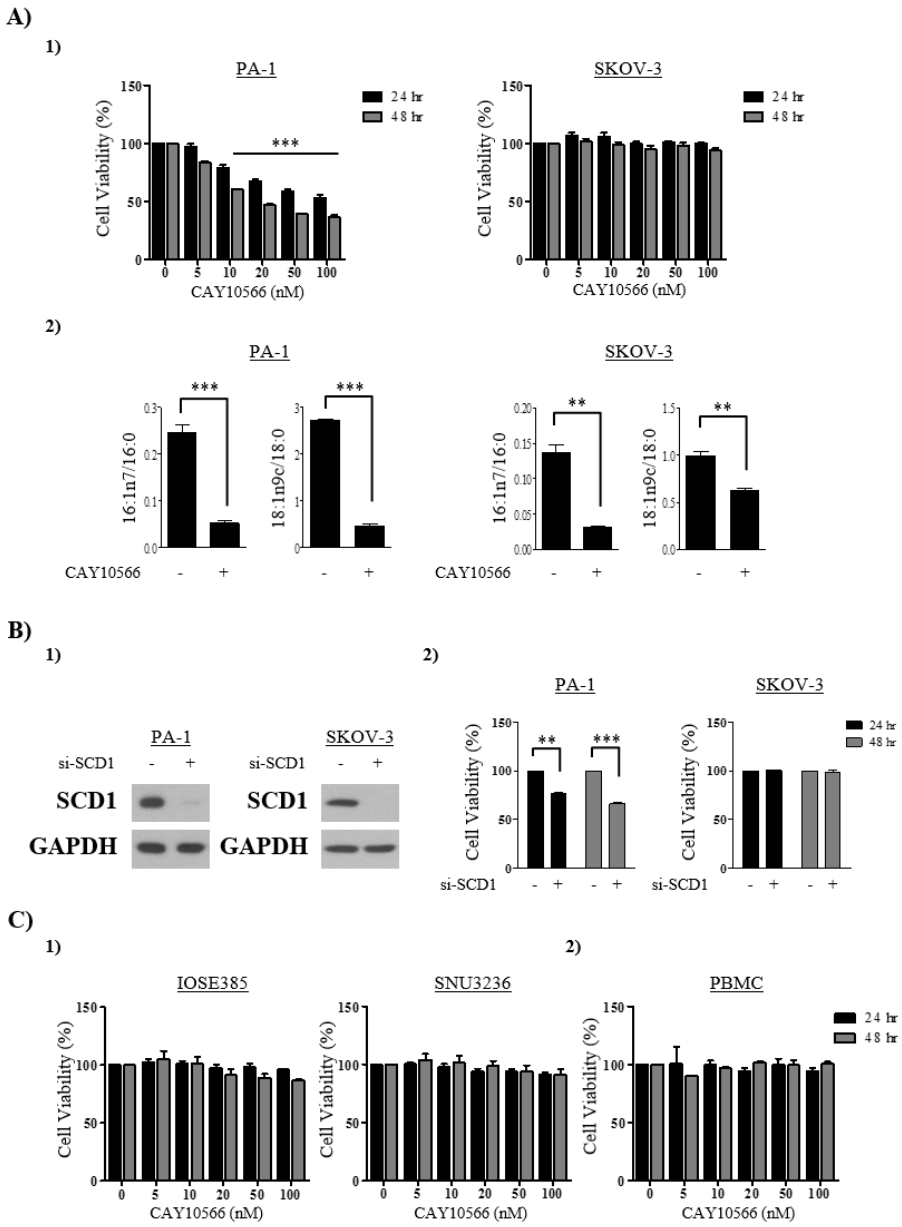
A)
1)
PA-1
SKOV-3
Cell Viability (%)
CAY10566 (nM)
24 hr
48 hr
***
2)
PA-1
SKOV-3
16:1n7/16:0
18:1n9c/18:0
CAY10566
**
B)
1)
PA-1
SKOV-3
si-SCD1
SCD1
GAPDH
2)
PA-1
SKOV-3
Cell Viability (%)
si-SCD1
24 hr
48 hr
C)
1)
2)
IOSE385
SNU3236
PBMC
Cell Viability (%)
CAY10566 (nM)
24 hr
48 hr

Figure 2. SCD1 inhibition reduces cancer cell proliferation with no cytotoxic effect on normal cells.

A) (1) Cytotoxic effect of CAY10566 on ovarian cancer cells. PA-1 and SKOV-3 cells were treated with various concentrations (0, 5, 10, 20, 50, and 100 nM) of CAY10566 or DMSO (solvent control) for 24-48 hours. Cell viability was measured by MTT assay. Results were shown as the percentage of total cell number compared to DMSO-treated control.

(2) Inhibition of SCD1 desaturase activity by CAY10566. PA-1 and SKOV-3 cells were treated with the IC_{50} value of CAY10566 for PA-1 cells (20 nM) for 48 hours. Cells were harvested and freeze-dried using a freeze dryer. Desaturase activity of SCD1 was estimated as the ratios of palmitoleic acid (C16:1n7) to palmitic acid (C16:0) and oleic acid (C18:1n9c) to stearic acid (C18:0) using gas chromatography.

B) (1) Silencing of SCD1 protein expression by siRNA. PA-1 and SKOV-3 cells were transfected with SCD1 siRNA (100 nM) or scrambled siRNA (100 nM) as a negative control. After 72 hours, ablation of SCD1 protein was determined by western blot analysis. GAPDH was used as a loading control.

(2) Cytotoxic effect of SCD1 siRNA in ovarian cancer cells. PA-1 and SKOV-3 cells were transfected with SCD1 siRNA (100 nM). At 72 hours post-transfection, cell viability was analyzed using MTT assay. Results were presented as the percentage of total cell number compared to scrambled siRNA-transfected control.

C) (1) No cytotoxic effect of CAY10566 in normal cells. IOSE385 and SNU3236 cells were treated with different concentrations of CAY10566. After 24-48 hours, cell viability was examined by MTT assay. Results were expressed as the percentage of total cell number compared to DMSO-treated control.

(2) No cytotoxic effect of CAY10566 in PBMCs. PBMCs were treated with increasing concentrations of CAY10566 for 24-48 hours. Cell viability was analyzed using MTT assay. Results were displayed as the percentage of total cell number compared to DMSO-treated control. All data were described as mean ± SEM of three independent experiments. ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively.

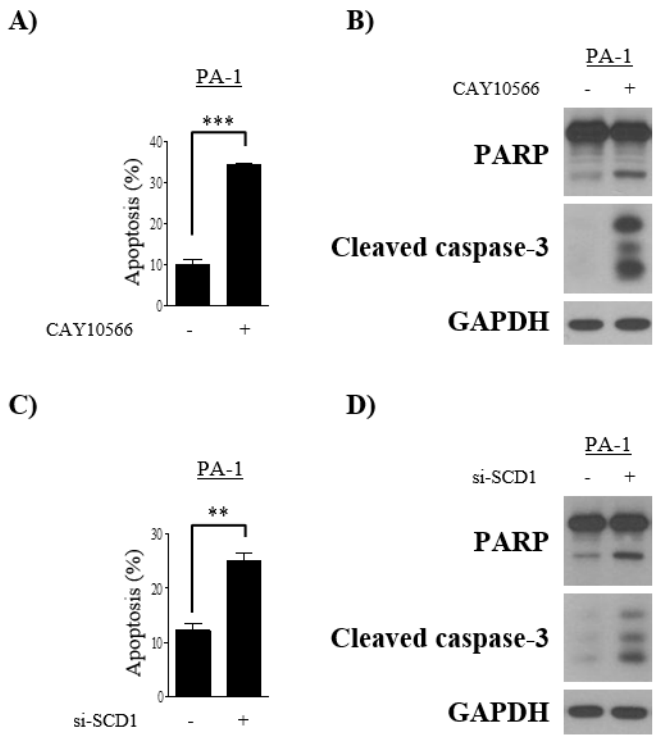
A)
PA-1
***
Apoptosis (%)
40
30
20
10
0
CAY10566 - +
B)
PA-1
CAY10566 - +
PARP
Cleaved caspase-3
GAPDH
C)
PA-1
**
Apoptosis (%)
30
20
10
0
si-SCD1 - +
D)
PA-1
si-SCD1 - +
PARP
Cleaved caspase-3
GAPDH

Figure 3. SCD1 suppression promotes apoptotic cell death in ovarian cancer cells.

A) Induction of apoptosis by CAY10566. PA-1 cells were treated with CAY10566 (20 nM) for 48 hours. The percentage of apoptotic cells was measured by flow cytometry analysis using Annexin V-FITC and PI staining.

B) Cleavage of PARP and caspase-3 by CAY10566. PA-1 cells were treated with CAY10566 (20 nM) for 48 hours. The expression levels of apoptosis marker proteins, cleaved PARP and caspase-3, were detected by western blot analysis. GAPDH was used as a loading control.

C) Induction of apoptosis by SCD1 siRNA. PA-1 cells were transfected with SCD1 siRNA (100 nM). After 72 hours, the percentage of apoptotic cells was determined by flow cytometry analysis using Annexin V-FITC and PI staining.

D) Cleavage of PARP and caspase-3 by SCD1 siRNA. PA-1 cells were transfected with SCD1 siRNA (100 nM). At 72 hours post-transfection, the expression levels of cleaved PARP and caspase-3 were observed by western blot analysis. GAPDH was used as a loading control. All data were presented as mean ± SEM of three

independent experiments. ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively.

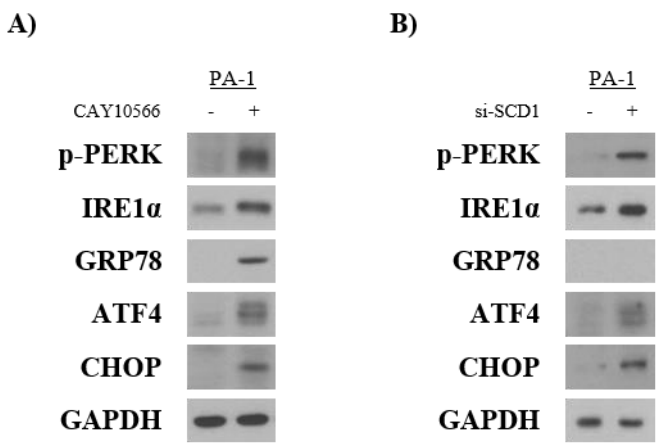
A)
PA-1
CAY10566 - +
p-PERK
IRE1α
GRP78
ATF4
CHOP
GAPDH
B)
PA-1
si-SCD1 - +
p-PERK
IRE1α
GRP78
ATF4
CHOP
GAPDH

Figure 4. Blockade of SCD1 induces ER stress in ovarian cancer cells.

A) Induction of ER stress by CAY10566. PA-1 cells were treated with CAY10566 (20 nM) for 48 hours. The expression levels of ER stress marker proteins, p-PERK, IRE1α, GRP78, ATF4, and CHOP, were examined by western blot analysis. GAPDH was used as a loading control.

B) Induction of ER stress by SCD1 siRNA. PA-1 cells were transfected with SCD1 siRNA (100 nM). At 72 hours post-transfection, the expression levels of p-PERK, IRE1α, GRP78, ATF4, and CHOP were determined by western blot analysis. GAPDH was used as a loading control.

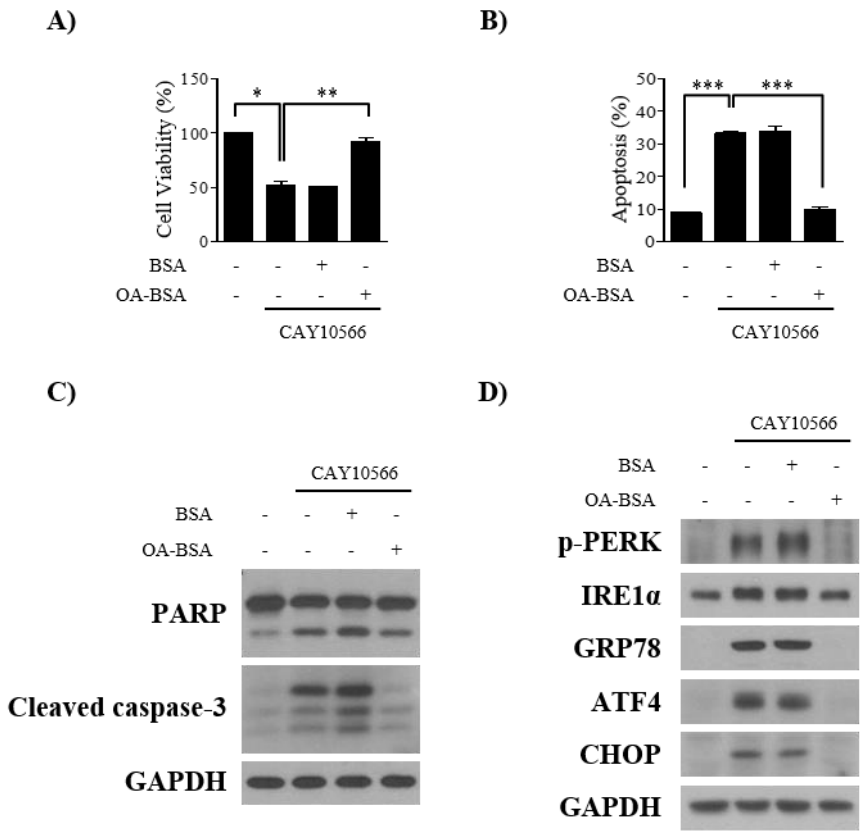
A)
Cell Viability (%)
150
100
50
0
*
**
BSA - - + -
OA-BSA - - - +
CAY10566
B)
Apoptosis (%)
50
40
30
20
10
0
***
***
BSA - - + -
OA-BSA - - - +
CAY10566
C)
CAY10566
BSA - - + -
OA-BSA - - - +
PARP
Cleaved caspase-3
GAPDH
D)
CAY10566
BSA - - + -
OA-BSA - - - +
p-PERK
IRE1α
GRP78
ATF4
CHOP
GAPDH

Figure 5. Addition of exogenous oleic acid rescued ER stress-mediated apoptosis triggered by SCD1 inhibition.

A) Abrogation of CAY10566-induced growth defect by addition of exogenous oleic acid. PA-1 cells were treated with CAY10566 (20 nM) with oleic acid-BSA (10 μM) or fatty acid-free BSA (10 μM) as a vehicle control for 48 hours. Cell viability was analyzed by MTT assay. Results were presented as the percentage of total cell number compared to DMSO-treated control.

B) The reverse effect of oleic acid on CAY10566-triggered apoptosis. PA-1 cells were treated with CAY10566 (20 nM) with or without oleic acid-BSA (10 μM) for 48 hours. The percentage of apoptotic cells was calculated by flow cytometry analysis using Annexin V-FITC and PI staining.

C) Inhibition of CAY10566-mediated PARP and caspase-3 cleavage by supplementation with oleic acid. PA-1 cells were treated with CAY10566 (20 nM) with or without oleic acid-BSA (10 μM) for 48 hours. The expression levels of cleaved PARP and caspase-3 were assessed by western blot analysis. GAPDH was used as a loading control.

D) The rescue effect of oleic acid on CAY10566-induced ER stress.

PA-1 cells were treated with CAY10566 (20 nM) with or without oleic acid-BSA (10 μM) for 48 hours. The expression levels of p-PERK, IRE1α, GRP78, ATF4, and CHOP were determined by western blot analysis. GAPDH was used as a loading control. All data were expressed as mean ± SEM of three independent experiments. *, **, and *** represent $P < 0.05$, $P < 0.01$, and $P<0.001$, respectively.

Discussion

Reprogramming of energy metabolism is one of the main features of cancer [26]. Rapidly proliferating cancer cells exhibit elevated glycolytic metabolism in the presence of oxygen. However, the importance of dysregulated lipid metabolism in cancer is increasingly recognized [4, 6, 27]. Lipids are stored as triacylglycerides, composed of cell membranes as phospholipids, and utilized for the generation of signaling molecules [28]. Surprisingly, cancer cells are highly dependent on *de novo* lipogenesis to obtain sufficient lipids required for their survival and proliferation. Numerous studies have revealed that the lipogenic enzymes are upregulated in various cancers including ovarian cancer, and inhibition of the enzymes causes cancer cell death [7, 11, 29]. So far, most efforts for drug development to target *de novo* lipogenesis have focused on inhibition of fatty acid synthase (FAS) responsible for the production of long-chain fatty acids from acetyl-CoA and malonyl-CoA [30, 31]. However, small-molecule inhibitors of FAS have shown the pharmacological limitations and considerable side effects in pre-clinical models, and only one has recently entered clinical trials [30-32]. To identify novel promising targets, further studies are needed to elucidate the precise contribution of lipogenic enzymes to cancer cell proliferation and survival.

SCDs reside exclusively in the endoplasmic reticulum of the cell and catalyze the introduction of a double bond in the *cis*-Δ9 position of SFAs to produce MUFAs [33]. While mammalian organisms contain up to four SCD isoforms, human tissues have two SCD isoforms, SCD1 and SCD5 [34]. SCD1 is ubiquitously present in all mammalian cells, with high expressions especially in lipogenic tissues, including brain, liver, heart, and lung [28]. However, SCD5 is highly expressed only in human brain, pancreas, and embryo tissues, and its physiological roles remain still unknown [34]. Recently, previous studies have found that cancer cells exhibiting elevated lipid metabolism have a strong dependence on the activity of desaturases. For example, SCD1 expression has been shown to be upregulated in a large number of human cancers [12-14, 20, 21]. Pharmacological inhibition of SCD1 reduced cancer cell growth *in vitro* [13, 18, 20, 21] and suppressed tumor growth in pre-clinical cancer models with no significant change in body weight [12, 16, 35]. Collectively, these studies suggest that targeting desaturase activity could be a novel therapeutic approach for cancers.

Together with these previous findings, our results provide further evidence for the critical role of SCD1 in ovarian cancer survival. We confirmed that SCD1 is highly expressed in ovarian cancer cells compared to normal ovarian cells (Figure 1). We also examined the anti-tumor effect of SCD1 on cancer cells using an inhibitor of SCD1

desaturase activity, CAY10566, and SCD1 siRNA. Consistent with the previous reports, inhibition of SCD1 decreased cancer cell proliferation, but did not affect normal cell viability (Figure 2), suggesting a cancer-specific cytotoxicity of SCD1 suppression. However, Mason *et al*. [15] reported that SCD1 inhibition had limited impact on SKOV-3 cell viability, although it reduced the desaturase activity of SCD1 in SKOV-3 cells. The observation is consistent with our data that SCD1 inhibition or knockdown had no significant effect on SKOV-3 cell viability (Figure 2A and 2B). Based on these results, we speculate that SKOV-3 cells may have less dependency on *de novo* lipogenesis relative to other cancer cells.

We next detected the percentage of apoptotic cells using flow cytometry analysis and determined the cleaved PARP and caspase-3 levels using western blot analysis. Downregulation of SCD1 by CAY10566 and SCD1 siRNA induced apoptosis in ovarian cancer cells (Figure 3). These data are in agreement with previous studies [13, 16, 19]. We further identified that suppression of SCD1 induced ER stress in ovarian cancer cells (Figure 4). The endoplasmic reticulum (ER) is responsible for protein folding, maturation, and trafficking. Numerous physiological and pathological stress conditions including lipotoxic stress can disturb ER homeostasis, leading to defects in protein folding capacity and accumulation of misfolded or unfolded proteins in the ER

[36, 37]. This condition is referred to as ER stress, which triggers the unfolded protein response (UPR) for adaptation to the stress. Activation of the UPR is initiated by three ER transmembrane sensors, IRE-1 (inositol-requiring enzyme 1), PERK (protein kinase RNA-like endoplasmic reticulum kinase), and ATF6 (activating transcription factor 6). They regulates their downstream effectors such as GRP78 (78 kDa glucose-regulated protein), ATF4 (activating transcription factor 4), and CHOP (C/EBP homologous protein) in three distinct pathways to resolve ER stress, resulting in suppression of protein translation, improvement of protein folding capacity, and ER-associated degradation (ERAD) of misfolded proteins [12, 37]. If the stress conditions are not resolved and ER stress is prolonged, programmed cell death can occur through caspase activation [37]. Previous reports have shown that ablation of SCD1 increased Xbp1 mRNA splicing, eIF2α phosphorylation, and CHOP expression, demonstrating the activation of the UPR and the induction of apoptotic cell death [19, 23]. Genetic and pharmacological inhibition of SCD1 induced ER stress and apoptosis in clear cell renal cell carcinoma (ccRCC) cells by increasing the expression levels of GRP78, CHOP, and cleaved PARP [20]. Moreover, Volmer *et al*. [24] confirmed that SCD1 inhibition activated IRE-1α and PERK, but not ATF6. These results were in accordance with our data obtained by western blot analysis.

Interestingly, we found that supplementation with exogenous oleic acid prevented the reduction of cell proliferation and the induction of ER stress-mediated apoptosis caused by SCD1 inhibition (Figure 5). In line with these observations, many previous studies have reported that the proliferative defect and apoptosis induction triggered by SCD1 inhibition were rescued by addition of oleic acid [15, 16, 38]. Furthermore, other recent studies have identified that genetic and pharmacological inhibition of SCD1 triggered ER stress and activated the UPR, which was reversible by exogenous application of oleic acid [20, 21].

In summary, SCD1 expression is significantly elevated in EOC cells compared to NOSE cells. Genetic ablation or pharmacological inhibition of SCD1 triggered the cancer-specific reduction of cell proliferation and the induction of ER stress-mediated apoptosis. Moreover, addition of oleic acid markedly reversed the effects of SCD1 suppression on cell growth inhibition and apoptosis induction mediated by ER stress, suggesting the importance of lipid desaturation catalyzed by SCD1 for ovarian cancer survival. These results implicate that SCD1 may serve as a novel biomarker and a potential therapeutic target for ovarian cancer.

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국문 초록

최근 암 세포의 독특한 대사적 특징 중 항진되어 있는 지방산 생합성 대사에 대한 중요성이 강조되고 있다. SCD1은 지방산 생합성 과정에서 중요한 속도조절 효소로 작용하며, 포화지방산에 이중결합을 도입함으로써 단불포화지방산을 합성하는 역할을 한다. Palmitoleic acid와 oleic acid 같은 단불포화지방산은 세포막을 구성하고 신호전달물질을 생산하며 에너지를 비축하는 데 사용되므로, 암 세포의 생존과 증식에 있어 필수적이다. 본 연구에서는 SCD1의 억제가 난소암 세포주에서 소포체 스트레스 매개 세포사멸을 유도한다는 것을 증명하였다. 먼저, 정상 난소 상피 세포보다 상피성 난소암 세포에서 SCD1 mRNA와 단백질의 발현이 더 증가되어 있는 것을 확인하였다. SCD1을 억제하였을 때 난소암 세포의 증식이 감소되었고 세포사멸이 유도되었지만, 정상 난소 상피 세포와 말초혈 단핵세포에서는 효과가 나타나지 않았다. 이를 통해, SCD1의 억제가 난소암 세포에 특이적으로 세포독성을 나타낸다는 것을 확인하였다. 또한, SCD1을 억제하였을 때 난소암 세포에서 PERK, IRE1α, GRP78, ATF4, CHOP이 활성화되어 소포체 스트레스가 유도되었다. 그리고 SCD1의 산물인 oleic acid를

추가로 넣어주었을 때 SCD1의 억제에 의해 유도된 소포체 스트레스 매개 세포사멸이 회복되었다. 이를 통해, 난소암 세포의 생존에 지방산 불포화가 필수적이라는 것을 밝혔다. 이상의 결과들을 종합하여 볼 때, 본 연구에서는 SCD1이 난소암의 종양표지자 및 난소암 치료의 표적이 될 수 있다는 것을 시사한다.