Biochemical and Biophysical Research Communications xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Clinical and biochemical relevance of monounsaturated fatty acid metabolism targeting strategy for cancer stem cell elimination in colon cancer

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ARTICLE INFO

Article history:
Received 22 August 2019
Accepted 24 August 2019
Available online xxx

Keywords:
Stearoyl-CoA desaturase 1
Lipidomics
Cancer stem cell
Mono-unsaturated fatty acid

ABSTRACT

Lipid metabolism is associated with colon cancer prognosis and incidence. Stearoyl-CoA desaturase 1 (SCD1), which converts fully saturated fatty acids (SFAs) to monounsaturated fatty acids (MUFAs), has been suggested as a vulnerable target for selective elimination of cancer stem cells (CSCs). However, the clinical significance and physiological role of SCD1 in CSCs has not been well demonstrated. Here, we showed the clinical and biochemical relevance of blocking SCD1 to target CSCs by analyzing human colon cancer data from TCGA and through lipidomic profiling of CSCs with or without SCD1 inhibition using mass spectrometry. Positive associations between SCD1 expression and colorectal cancer patient clinical status and the expression of CSC-related genes (WNT and NOTCH signaling) were found based on TCGA data analysis. Lipidomic profiling of CSCs and bulk cancer cells (BCCs) using mass spectrometry revealed that colon CSCs contained a distinctive lipid profile, with higher free MUFA and lower free SFA levels than in BCCs, suggesting that enhanced SCD1 activity generates MUFAs that may support WNT signaling in CSCs. In addition, all identified phosphatidyl-ethanolamine-containing MUFAs were found at higher levels in CSCs. Interestingly, we observed lower phosphatidyl-serine (18:1/18:0), phosphatidyl-choline (PC; p-18:0/18:1)), and sphingomyelin (SM; d18:1/20:0 or d16:1/22:0) levels in CSCs than in BCCs. Of those, SCD1 inhibition, which efficiently diminished free MUFA levels, increased those specific PC and SM and MUFAs in CSCs promptly. These results suggest that these specific lipid composition is critical for CSC stem cell maintenance. In addition, not only free MUFAs, which are known to be required for WNT signaling, but also other phospholipids, such as SM, which are important for lipid raft formation, may mediate other cell signaling pathways that support CSC maintenance. Comparison of the lipidomic profiles of colon cancer cells with those of previously reported for glioma cells further demonstrated the tissue specific characteristics of lipid metabolism in CSCs.

1. Introduction

Excess energy is stored in the body as fat, which plays a crucial role in promoting obesity. Exactly how obesity increases the risk of cancer is not well understood. However, obesity or even overweight increases the risk for 15 cancer types [1,2]. Especially in colorectal cancer, a high-fat diet is classified as a possible risk factor.

On the other hand, most cancer cells are in an environment that provides insufficient nutrition, primarily due to shortage of blood supply. Therefore, cancer cells often have altered their own glucose [3] and lipid metabolism [4]. In most tissues, the fatty acid (FA) demand is satisfied through dietary FAs in the bloodstream. Only the liver, adipose tissue, kidney and lactating breast perform de novo synthesis of FAs [5,6]. However, cancers perform de novo FA synthesis [4,7] probably due to meet the cost of membrane

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https://doi.org/10.1016/j.bbrc.2019.08.137
0006-291X/

generation and signaling for their growth and survival in insufficiently vascularized tumor areas [8].

When lipogenesis is enhanced in cancer cells, more lipid modifying enzymes are also required to generate diverse lipid species. In vertebrates, fatty acid(FA) synthase produces palmitic acid (16:0) as the final product [9], and additional long-chain fatty acids can be extended. The double bond at the 9, 10 position of the acyl chain in palmitic acid is catalyzed by stearoyl-CoA desaturases (SCDs) [10]. Most common FAs, palmitic acid and stearic acid (18:0) [11] are transformed by SCD to mono-unsaturated FAs (MUFAs), palmitoleic acid and oleic acid, respectively. In humans, SCD1 is the main enzyme catalyzes the rate-limiting step in the production of MUFAs [12,13].

In prostate, liver, kidney and breast cancer, SCD1 overexpression has been found to be correlated with reduced survival. Only few studies have shown the influence of SCD1 on lipid metabolism and survival in cancer stem cells (CSCs). Ovarian CSCs contain high levels of MUFAs [14], and SCD1 inhibition synergistically kills CSCs in lung cancer [15]. We reported that MUFA and cholesterol synthesis are essential metabolic processes for GBM CSC maintenance [16].

CSCs are a small subpopulation of cancer cells responsible for tumor initiation, resistance and recurrence. Therefore, targeting strategy to CSCs has received much attention [17]. Several signaling pathways, including WNT and NOTCH, have been intensively investigated as potential therapeutic targets for CSC therapy. Although SCD1 is suggested as a promising target for selective elimination of CSCs by us [16] and others [14,15,18], why the activity of this enzyme is essential in CSCs is not clear. One of the best-supported hypotheses is that a free MUFA (Palmitoleic acid,16:1) is used for modification of WNT ligands, which is essential in stem cells [19], and thus, SCD1 inhibition suppresses WNT signaling [20].

In this study, we demonstrated an association between SCD1 and CSC marker expression in colon cancer tissues from patients and in colorectal cancer cell lines. Further, we demonstrated that lipidomic profiles of colon CSCs are different from those of bulk culture cells (BCCs) or glioblastoma CSCs and only a small number of lipids are modulated by SCD1 inhibition. We also identified a few phospholipids whose composition is modulated by SCD1 inhibition and that may be related to critical CSC signaling other than WNT signaling.

2. Materials and methods

2.1. Bioinformatic analysis

The comprehensive TCGA PanCanAtlas 2018 data set (Colon Adenocarcinoma) was acquired from The Cancer Genome Atlas (TCGA) for gene expression and tumor stage analyses. The gene expression association among human tissues were performed as described from the TCGA dataset [21].

2.2. Statistical analysis of the data

Most statistical analyses and visualizations were conducted using GraphPad Prism version 5 software. Differences between tumor stage were compared using an unpaired *t*-test. For heat map construction, median values were used for calculating scores, and Cluster 3.0 and Tree view were used to visualize the results.

2.3. Cell culture

The human colon cancer cell lines HT29 and SW480 were purchased from American Type Culture Collection (ATCC, USA) and cultured in DMEM and RPMI1640 respectively with 10% fetal bovine serum (FBS; Gibco, USA) in BCC condition. CSCs were enriched in serum-free conditioned DMEM/F-12 (Thermo Fisher, USA) containing 20 ng/ml EGF, 20 ng/ml basic fibroblast growth factor, and B27 supplement as described previously [22]. PluriSIn#1 and MF-438 were purchased from Selleckchem and Merck Millipore, respectively.

2.4. Quantitative reverse transcription PCR (qPCR) and western blotting

Total RNA was extracted from BCCs and CSCs using TRIsure (Bioline, Korea). cDNA was transcribed from the extracted RNA with a SuperScript II kit (Invitrogen). qRT-PCR was carried out a using SensiFAST SYBR Hi-ROX kit (Bioline). Ribosomal RNA L32 and actin mRNA were used as controls. The sequences of primers and siRNAs used are listed in supplementary materials. For western blotting, the antibodies were purchased from Abcam (ab19862, SCD1) and Santa Cruz Biotechnology (sc23948, α-tubulin).

2.5. Lipid metabolite profiling

HT29 CSCs with or without SCD1 inhibitor treatment (MF-438, 10 nM) or cultured in standard way with FBS (BCC) were collected. The experiments were conducted in 4 replicated sets of samples. Heptadecanoic acid (17:0, Sigma) was used as a reference mass compound from the point of lipid extraction step. Lipid metabolomics was conducted using a quadrupole time-of-flight mass spectrometer (Q-TOF MS) as published before [16].

3. Results

3.1. Association between SCD1 expression and cancer progression

To assess the possible existence of an association between SCD1 expression and colorectal cancer patient clinical status, SCD1 expression data sets of colorectal adenocarcinoma in TCGA Pan-Cancer Atlas analyzed. Based on American Joint Committee on Cancer tumor staging, stage III showed significant differences from stage I (P-value = 0.0151) in the expression level of SCD1 (Fig. 1A). The same data set was reorganized with the mRNA level represented by a Z score. Patients with a higher SCD1 mRNA level (Z score of more than 2) were found only in stage II ~ stage IV specimens, and the SCD1 mRNA level was found to increase in accordance with advances in stage (Fig. 1B). These data support the hypothesis that SCD1 is associated with colon cancer progression.

3.2. Association between SCD1 expression and cancer stem cells

Because CSCs are linked to cancer progression [17,23], we further investigated whether CSC signaling molecules are related to SCD1 expression in human colon cancer specimens and in cell lines (Fig. 2). The expression of the canonical WNT signaling genes TCF7 and AXIN2 and the alternative downstream effector YAP [24] was found to be associated with SCD1 expression in human colorectal specimens. Notch1 gene expression was also associated with SCD1 expression. These data show that in vivo cell populations expressing SCD1 may be associated with cells expressing WNT or NOTCH signaling genes (Fig. 2A). Since the NOTCH and WNT signaling pathways are key regulators in colon CSCs [25,26], this result supports that SCD1 is strongly associated with the stem cell population in colon cancer in vivo. In addition, we confirmed that not only mRNA but also the protein of SCD1 expressed more in CSCs than BCC of colon cancer cell lines (Fig. 2B and C).

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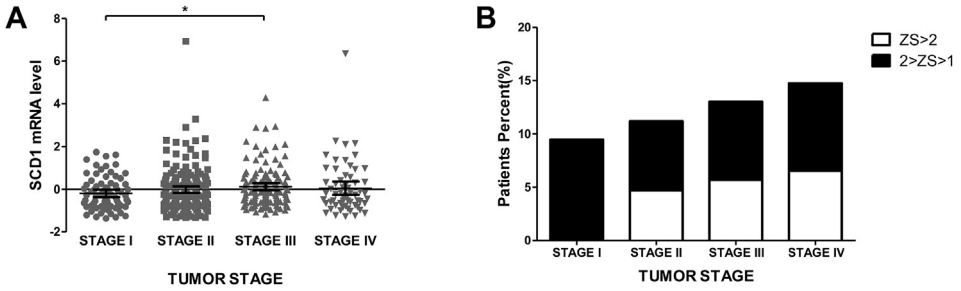

Fig. 1. SCD1 expression is associated with the progression of colon cancers. (A) mRNA level of SCD1 gene in colorectal cancer patients. Tumor stage is based on AJCC staging (STAGE I = 74; STAGE II = 170; STAGE III = 123; STAGE IV = 61; Total(n) = 428). (B) The percentage of patients with each SCD1 mRNA expression level in their stage.*P < 0.05.

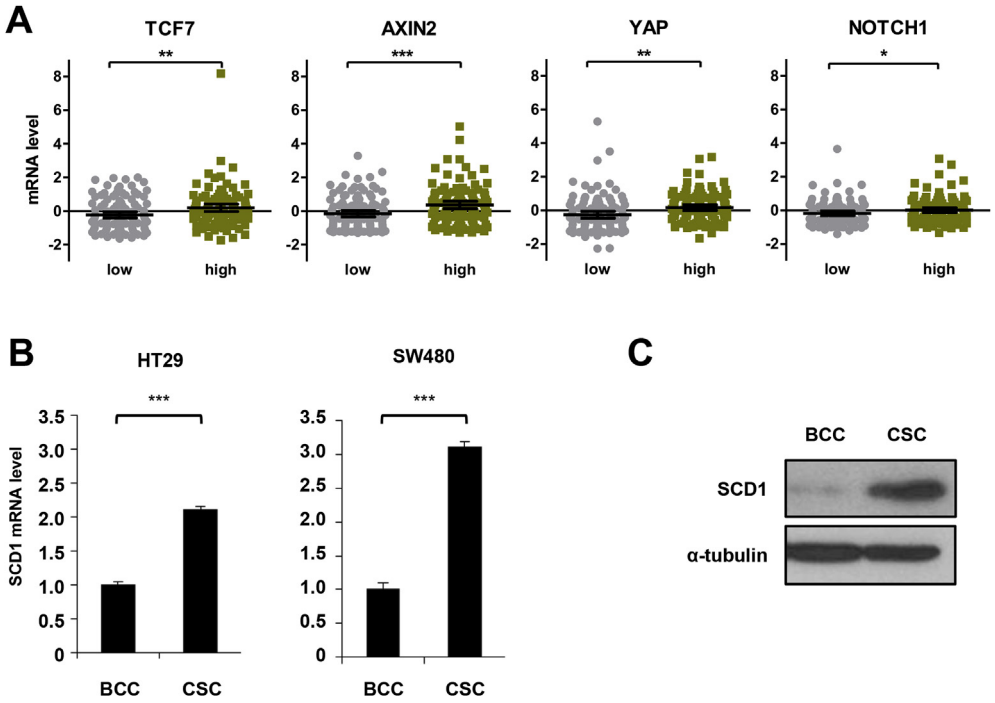

Fig. 2. SCD1 expression is associated with cancer stem cell signaling in vivo and in vitro. (A) Scatter plots showing the each gene's mRNA levels of patients expressing upper 25% of SCD1 mRNA level (high) compared with patients having the lower 25% level (low). (B) RT-qPCR analysis of SCD1 in cell lines cultured in standard way (BCC) and CSC enriched (CSC). (C) Western blot for SCD1 in HT29 cell line. α-tubulin was used as internal control. *P < 0.05, **P < 0.01, ***P < 0.001.

3.3. SCD1-dependent lipid unsaturation is important for CSCs

The enhanced expression of SCD1 in CSCs strongly suggests that the enzymatic activity of SCD1 plays an important role in CSCs. We previously showed that the lipidomic profile of GBM CSCs is quite different from that of BCCs. Especially, we found many phospholipids with MUFAs in CSCs more, suggesting that SCD1 activity is important in GBM CSCs. We confirmed that inhibition of SCD1 activity using two specific inhibitors disrupts CSC maintenance in two colorectal cancer cell lines (Fig. 3A). The siRNA against SCD1 completely disrupts the CSC population showing molecular biological intervention also lead same effect with the pharmacological blocking (Fig. 3B). To determine whether colorectal CSCs have a unique lipid profile related to SCD1 activity and whether treatment with inhibitors changes the unique lipid profile of CSCs, we performed lipidome level profiling of a colorectal cancer cell line using mass spectrometry coupled with ultraperformance liquid chromatography, similar to our previously published study [16] on GBM cells. The PLS-DA score plot of lipidome mass data from BCCs, CSCs and CSCs treated with an SCD1 inhibitor (MF-438, 10 nM) and the 2-way hierarchical clustering analysis showed that the lipidomic profile of CSCs is quite distinguishable from that of BCCs (Fig. 3C and D). Moreover, 24-h treatment with an SCD1 inhibitor only altered a small number of the differential metabolites. Since we previously demonstrated a different free MUFAs and SFAs (saturated FA) composition between CSCs and BCCs in GBM and others have reported similar results in ovarian cancer, we first compared the amount of these free FAs in colon cancer cells. Similar to the GBM data, the SFA, palmitic acid and stearic acid levels were lower in CSCs than in BCCs. We found that more oleic acid (18:1) exists in CSCs and that is decreased by SCD1 inhibition, suggesting that the lipid profile of SFAs and MUFAs in colon CSCs may be similar to that of GBM CSCs. The relative amount of SFA and MUFA in BCC and CSCs also suggest the increased activity of SCD1 in colon CSC (Fig. 3E).

3.4. Lipid metabolites changes in CSCs that may be important for CSC signaling

Because we previously reported higher levels of 15 unsaturated phospholipids in GBM CSCs relative to BCCs, we also investigated

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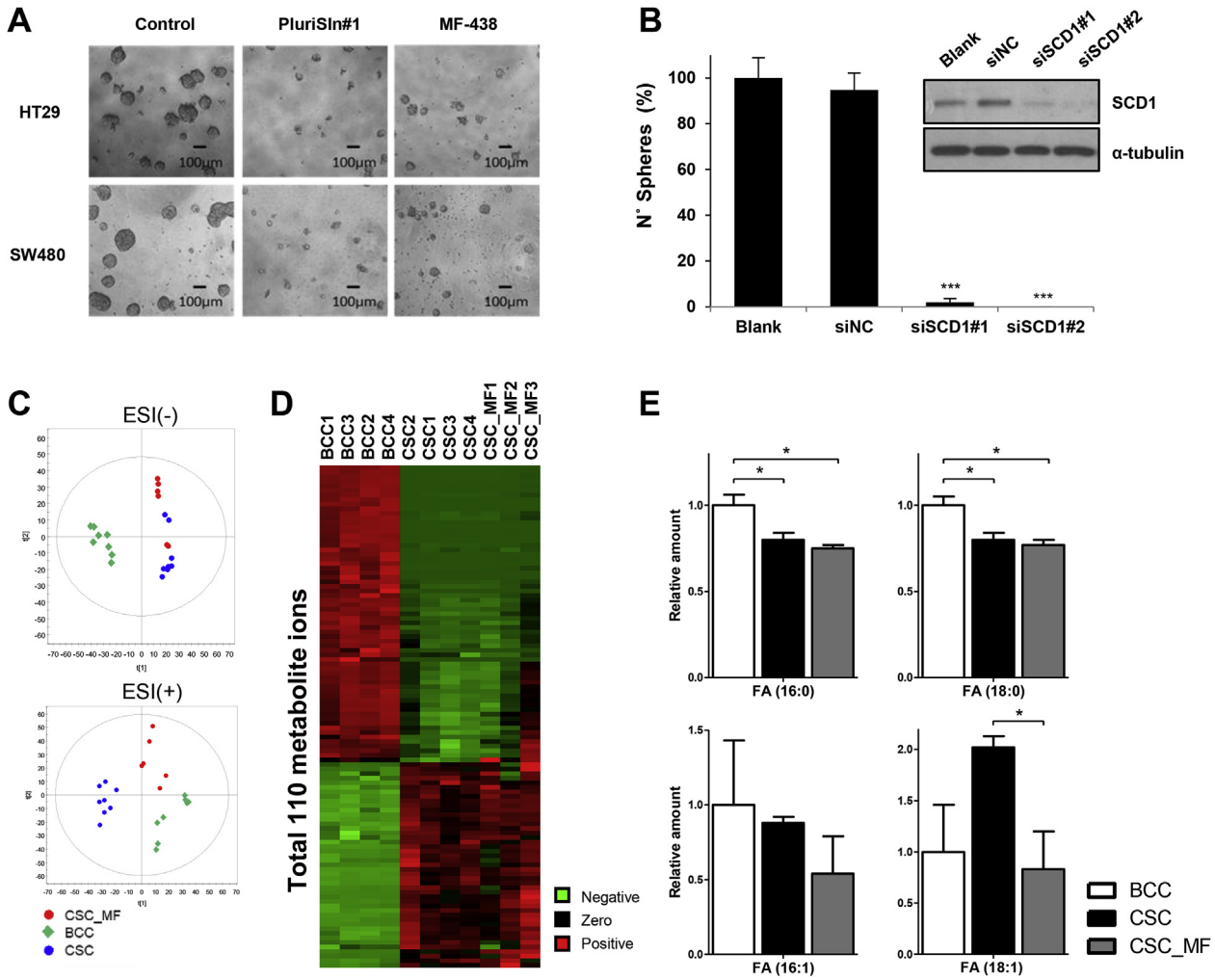

Fig. 3. Colorectal stem cells have distinctive compositions in unsaturated and saturated fatty acids. (A) Representative images of CSC spheroids treated with vehicle, PluriSIn#1 or MF-438 for 72hr. Scale bar 100 μm. (B) The relative amount of CSC spheres after transfection of scrambled siRNA(siNC) or siRNA of SCD1 in HT29. Bars represent the relative mean ± S.D. (C) Metabolite ion profile (PLS-DA score plot) in BCC (bulk cultured cells), CSC (CSC enriched cells) and MF-438 treated CSC. Multivariate statistical analysis of lipid profiles acquired from UPLC-qTOF-MS data. PLS-DA score plots were derived from metabolite ions acquired from ESI- (Left) and ESI+ (Right) modes. (D) Heat map for lipid metabolite mass ions by 2-way hierarchical clustering analysis. Represented 110 metabolites were significantly differentially regulated metabolite mass ions between BCC and CSC (P-value = 0.001). Each colored cell represents normalized intensity of each mass ion. (E) Relative amount of fatty acid (FA) in CSC and MF-438 treated CSC compared to BCC (palmitic acid (16:0), stearic acid (18:0), palmitoleic acid (16:1), oleic acid (18:1)). *P < 0.05, ***P < 0.001.

these phospholipids in colon cancer cells (Fig. 4). The level of most phosphatidylcholines (PC) containing MUFAs (16:1 or 18:1) were similar between the colon CSCs and BCCs, which differs from the GBM results [16]. The more quantity of phosphatidylethanolamines (PE) contains MUFA chains in colon CSCs than in colon BCC was similarly to the results in the GBM [27]. However, lower PC (p-18:0/18:1) and PS (18:1/18:0) lipid levels were observed in colon CSCs than in colon BCC and it is opposite to the GBM results. The level of a newly identified phospholipid, SM (d18:1/20:0 or d16:1/22; 0), was lower in CSCs. Of all the phospholipids we identified, only PC (p-18:0/18:1) and SM (d18:1/20:0 or d16:1/22; 0) levels were increased by SCD1 inhibitor treatment. MF-438 at 10 nM did not kill the colon CSC population cells within 24 h but modulated the expression of many CSC-related signaling genes (AXIN, LEF1, and Notch1; unpublished results). Collectively, these findings suggest that the decrease in free MUFA (18:1) and increase in PC (p-18:0/18:1) or SM (d18:1/20:0 or d16:1/22:0) caused by SCD1 inhibition treatment may be responsible for modulation of CSC signaling.

4. Discussion

Further understanding of the molecular mechanisms underlying cancer stem cell (CSC) maintenance and searching for selective CSC targets are urgent issues for better cancer treatment outcomes. Although an increasing number of studies have shown that lipid metabolism is important for cancer cells and CSC characteristics, how lipids regulate cancer cell stemness remains largely unknown [28]. However, SCD1 has been identified as a key factor in lung cancer [15], ovarian cancer [14], and GBM [16] CSCs.

Through analysis of TCGA data, we demonstrated that SCD1 expression is associated with disease progression in colorectal cancer patients. We also observed that SCD1 expression is associated with CSCs in human colon tumor tissues and colon cancer cell lines. Furthermore, in the lipidomics study, we found that colon CSCs have high metabolic flux from SFAs to MUFAs through the activity of SCD1. We also demonstrated the characteristic colon CSC lipidome and identified candidate phospholipids that may be responsible for the anti-CSC potential of SCD1 blockage.

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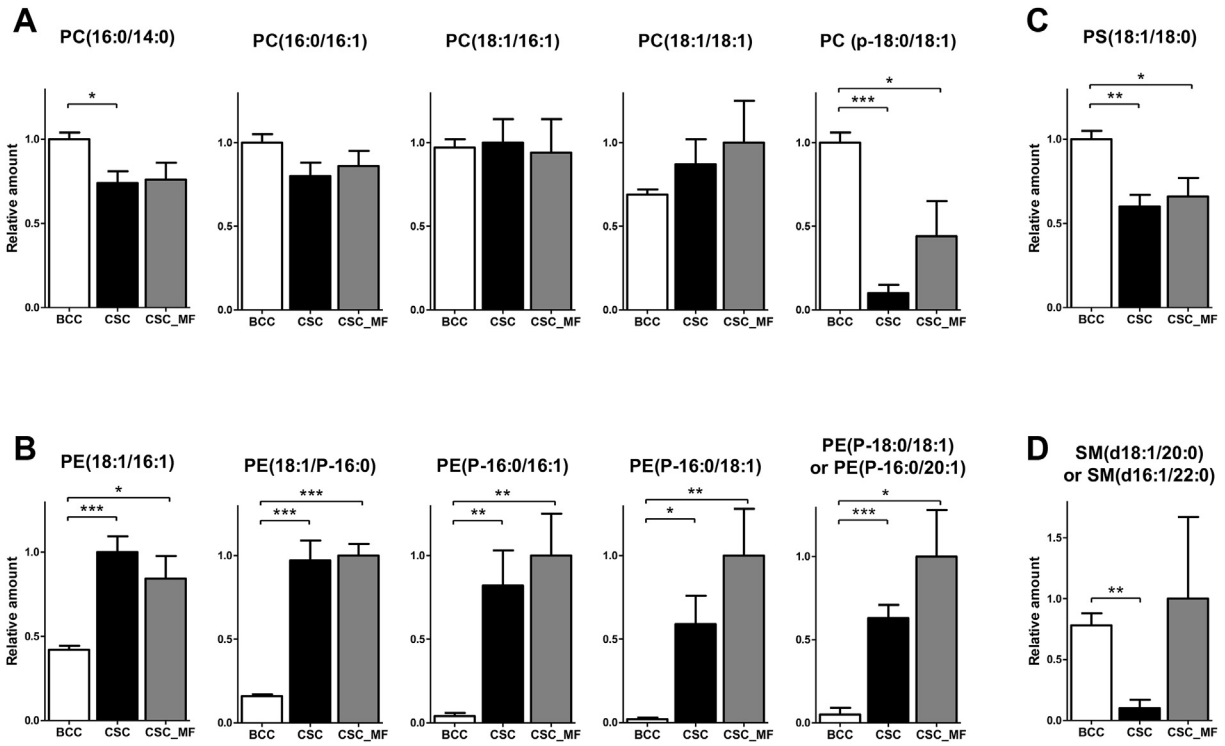

Fig. 4. Differential composition of phospholipids between colon CSCs and BCCs are modulated by SCD1 inhibition. (A, B) Relative amount of phosphatidylcholines (PCs), phosphatidylamine (PEs) in BCC (bulk cultured cells), CSC (CSC enriched cells) and MF-438 treated CSC. (C,D) Relative amount of phosphatidyserinee (PS, 18:1/18:0) and sphingomyelin (SM, d18:1/20:0 or d16:1/22:0). The data are shown as means +SEM,*, $P < 0.05$; **,$P < 0.01$; ***, $P < 0.001$.

Membrane lipid composition and fluidity are important to maintain membrane topology, mobility and membrane bound proteins. The biological roles of lipids in cell signaling are diverse and important [28]. More than one hundred proteins have been identified to be acylated, using free FAs [28]. The signaling required for stem cell maintenance [29] is also associated with this lipid modification. Especially, Wnt3a modification with palmitoleic acid (16:1) is required for WNT secretion [19,30]. Therefore, SCD1 may mediate cancer stem cell maintenance though WNT-mediated β-catenin [28] and YAP/TAZ activation [20]. In current study, a positive correlation between SCD1 and WNT target gene expression found in colorectal cancer patients supports these hypothesis. We also showed that SCD1 inhibition efficiently reduced free MUFA levels in colon CSCs accompanied by CSC elimination. Therefore, current result also supports that the SCD1 inhibitor-induced reduction in MUFAs causes WNT signaling blockade, leading to CSC suppression.

Previously, we showed that many PC, PE, and PS unsaturated lipid chains are enriched in CSC membranes in GBM [16]. However, in the current study, we found that most of the PC lipids with MUFA chains were not accumulated more in colon CSCs than in colon BCCs. Conversely, all identified PEs with MUFAs were enriched in colon CSCs compared with colon BCCs, similar to our findings in GBM. Even more interestingly, PC (p-18:0/18:1), PS (18:1/18:0), and SM (d18:0/20:0 or d16:1/22:0) levels were significantly reduced in CSCs compared with BCCs. In addition, out of 12 phospholipids we identified, only PC (p-18:0/18:1) and SM (d18:0/20:0 or d16:1/22:0) were increased by treatment of MF-438 for 24 h in colon CSCs. PCs are one of the most abundant phospholipids in biological membranes and have long been considered a primary building block of cell membranes. However, PCs can be cleaved to generate the second messenger diacylglycerol, which activates a variety of signaling pathways. Moreover, additional cell signaling functions, such as a role in insulin responses, are being unveiled [31]. However, the roles of each specific PC form are far from fully understood. Interestingly, CSCs have lower saturated PC (16:0/14:0) levels than BCCs, but SCD1 inhibition failed to increase these levels. The only PC found to be lower in CSCs, PC (p-18:0/18:1), was reincreased by MF-438, suggesting that PC (p-18:0/18:1) not only has general and structural roles but also plays a specific signaling role in these drug-treated CSCs.

Another phospholipid found to exhibit lower levels in CSCs but that were recovered by MF-438 was SM (d18:1/20:0 or d16:1/22:0). SM is abundantly found in the plasma membrane and endoplasmic reticulum and can be converted to the second messenger diacylglycerol. Although the specific role of this specific form of SM (d18:1/20:0 or d16:1/22:0) is not currently known, one important role of SM is to constitute membrane rafts along with cholesterol. Membrane rafts are small microdomains that compartmentalize a variety of cellular signaling molecules [32,33]. We previously showed that high cholesterol levels were present in CSCs and blockade of cholesterol synthesis dramatically hampered CSC survival in GBM and in lung, breast and colon cancer [16]. Especially, Notch, one of the pivotal signaling molecules in CSCs, is mediated by gamma γ-secretase present in lipid raft of plasma membrane. We found that Notch1 expression is positively associated with SCD1 expression in vivo and blockade of SCD1 in CSCs failed to maintain the lower amount of SFAs containing SM (d18:1/20:0 or d16:1/22:0). Therefore, it is possible that this SM in CSCs cooperates with cholesterol for CSC signaling at lipid rafts but that SCD1 blockade can somehow reverse this cooperation. It may also be important to note that only two phospholipids were found to be altered by the brief treatment with the SCD1 inhibitor (MF-438, 10 nM, 24 h). Under this condition, we found that NOTCH and WNT signaling were suppressed, ahead of other signaling changes or CSC marker decreases in colon cancer cells(unpublished results). The

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exact role of this specific and perhaps other not yet identified SM in lipid rafts in CSCs may need to investigated.

Previously, we identified and quantified 16 lipid components, primarily phospholipids, and reported differences between BCCs and CSCs in GBM. In the current study, we found that some of the differences observed in GBM are not present in colon CSCs. In addition, here, we report two unique phospholipids that may modulate CSC signaling under SCD1 function. Considering the clinical significance of SCD1 expression and its observed association with CSC signaling in vivo and in vitro, this biochemical analysis provides important insight into how SCD1 blockade can be used to target stem-like cell activity in colon cancer and gives a further rationale for development of an SCD1-targeting colon cancer therapeutic strategy. More in-depth study of other CSC signaling pathways requiring specific lipid components in CSCs may further reveal the precise mechanism by which synthesis of specific lipids controls CSC survival.

Acknowledgements

This work was supported by Korean Government (NRF-2018R1D1A1B07045153 to WYK).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.08.137.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.08.137.

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