ROLE OF STEAROYL-COA DESATURASE- 1 (SCD1) IN THE ACTIVATION OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) IN LUNG CANCER CELLS

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ABSTRACT OF THE THESIS

The Role of Stearoyl-CoA desaturase-1 (SCD1) in the activation of Epidermal Growth Factor Receptor (EGFR) in lung cancer cells

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Cancer cells activate lipogenic enzymes, including StearoylCoA Desaturase-1 (SCD1), the key enzyme that converts saturated fatty acids (SFA) into monounsaturated fatty acids (MUFA). Previously, we established that SCD1 regulates lipogenesis, cell proliferation and invasiveness in lung cancer cells, as well as tumor formation in mice. We recently reported that SCD1 modulates the PI3K/Akt pathway, a central signaling cascade, along with ERK, which are involved in the regulation of lipid biosynthesis, growth and survival of mammalian cells. Growth factor-activated tyrosine kinase receptors, such as epidermal growth factor (EGF) receptors (EGFR), are main activators of Akt and ERK signals, two cascades that are most often deranged in cancer.

A hallmark of cancer is the metabolic shift towards macromolecular synthesis to support cell replication. SCD1 expression increases in cancer cells. The molecular mechanisms by which SCD1 regulates the biological phenotype of cancer cells is still unknown. The poor prognosis and ineffective treatments of some cancers, such as lung cancer, calls for better understanding of their mechanisms and for finding novel targets that, like SCD1, modulate the Akt and ERK pathways. Here we provide evidence that SCD1

activity controls the activation of EGFR and its downstream signaling targets, Akt and ERK. Using H460 human lung cancer cells, we observed that the activating phosphorylation of Tyr1068 and Tyr1086 residues in EGFR upon EGF stimulation was markedly impaired when SCD1 activity was blocked with CVT-11127, a novel small molecule SCD inhibitor. In addition, supplementation with oleic acid, the product of SCD1, restored EGF-induced phosphorylation of EGFR but not the full phosphorylation of Akt. Finally, abrogation of SCD1 dramatically altered distribution of rafts and non-raft domains, suggesting that the regulation of EGFR function by SCD1 may involve the alteration of membrane lipid domains. All results are representative of 3 separate experiments. In conclusion, our data indicate that SCD1 may coordinate the regulation of lipid biosynthesis and the transduction signals that control cancer cell metabolism, proliferation, survival and tumorigenesis by modulating EGFR activation, which subsequently modifies the Akt and ERK signaling platforms. Our findings also suggest SCD1 is a potential target for novel pharmacological interventions in lung cancer.

Abbreviations used: SCD1, Stearoyl-CoA desaturase-1; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PI3K, Phosphatidylinositol 3-Kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; NSCLC, non-small cell lung cancer; NSCLC, non-small cell lung cancer.

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Chapter 1: Introduction

SCD1, a key lipogenic enzyme with critical roles in cancer cell proliferation, survival, and tumorigenesis

Lung cancer is currently one of the least successfully treated cancers. Its survival rate is 5-15% within five years of diagnosis, therefore there is a need for better treatment based on the discovery of new therapeutically useful targets. Of all lung cancers, non-small cell lung cancers (NSCLCs) account for 80% of lung cancer types (1). Lung cancer is the second overall cause of death in the United States and Canada (2). A key issue with the poor prognosis is the late detection, which occurs in 70% of NSCLC cases (2). Moreover, recurrence is frequent and the cancer cells usually become resistant to the original drug treatment (3).

Metabolism in cancer cells is very different than the metabolism in non-cancerous wild-type cells. Due to their demanding need for newly synthesized macromolecules to sustain the formation of daughter cells, cancer cells shift metabolism towards anabolic reactions at the expense of suppression of catabolism. For instance, cancer cells exhibit constitutively active global lipid biosynthesis whereas fatty acid oxidation is mostly suppressed (4). Among the most critical lipid biosynthetic enzymes, Stearoyl-CoA desaturases (SCD) catalyze the biosynthesis of monounsaturated fatty acids (MUFA) in mammalian cells by introducing a double bond between carbons 9 and 10 of a saturated acylCoA, preferentially palmitoyl-CoA and stearoyl-CoA, to yield palmitoleoyl-CoA and oleoyl-CoA, respectively. Up to five SCD isoforms are found in mammalian organisms (5). SCD1 to

SCD4 are found in mice (5). Humans express SCD1, as well as SCD5, which is also found in primates and bovine (6;7). Little is currently known about the function and biological role of SCD5. SCD1, however, has been studied extensively. In humans and rodents SCD1 is found in almost all adult tissues and is modulated by a great number of nutrients, growth factors, and hormones **(**8). Research using mouse models as well as human cells has shown that SCD1 is crucial for the overall lipid balance in cells. In $SCD1^{-/-}$ knockdown mice, lack of SCD1 altered not just MUFA content, but also the levels of free fatty acids, triglycerides, and cholesterol (9). SCD1 mutation also inhibited β-oxidation of the fatty acids in mouse heart (9). Other studies showed that deregulation of SCD1 is involved in numerous diseases and disorders such as diabetes, hypertension, cardiovascular disease, immune disorders, obesity and metabolic syndrome (10). Importantly, SCD1 expression has been implicated in cancer cell proliferation, survival, and tumorigenesis (8). In fact, when a siRNA library was screened for targets of cytotoxicity in cancer cell lines, SCD1 was found to be a main target (11). This indicates that cancer cells rely on SCD1 to potentiate their growth.

Studies done in animal and cell models demonstrated that, besides the contribution of dietary fatty acids, SCD1 is the main regulator of the ratio of MUFA to saturated fatty acids (SFA) (12). This is particularly true in cancer cells, in which MUFA is required for acylating reactions that synthesize structural, signaling and energetic lipids for the formation of daughter cells (8). The functional relevance of SCD1 in cancer cells is highlighted by the observation that when the desaturase is inhibited in these cells, the growth and survival rates of cancer cells drop drastically along with a marked increase in the ratio of SFA to MUFA (12-14). Not only does the lack of MUFA halt lipid synthesis needed for the cancer cells to

proliferate, but the buildup of SFA also contributes to induction apoptosis by a cytotoxic effect known as lipotoxicity (12;14).

Initial studies on SCD1 function in cancer cells were performed in models of inhibition of SCD1 gene expression. More recently, the use of newly discovered specific small molecule inhibitors of SCD activity confirmed the role of SCD1 in cell proliferation. When SCD activity was blocked with one of these novel inhibitors, CVT-11127 (CVT), cancer cell proliferation was acutely reduced (60% inhibition in 48h) (13). This decreased proliferation was fully returned to control levels with 100μM oleate, confirming that MUFAs are required for mitosis (13). CVT successfully slows proliferation of cancer cells but it did not significantly effect cell proliferation of WS-1 fibroblast wild-type cells (13). Thus, effectiveness of CVT seems to be dependent on proliferation rate or possibly levels of SCD1. This may be crucial for the potential use of these inhibitors in the treatment of cancer since they appear to only target rapidly growing cancerous cells.

The reduction of MUFA substrates for lipid biosynthetic reactions may be the main reason why lipogenesis is affected by SCD1 inhibition. However, the excessive levels of SFA may also suppress lipogenesis by subduing fatty acid synthesis by inhibiting acetyl-CoA carboxylase (ACC), a rate-limiting enzyme in the fatty acid biosynthetic pathway (13). Further supporting this notion, when a specific small molecule inhibitor blocked ACC, cell number decreased by 30%. This effect was not attenuated with simultaneous SCD1 blockade, suggesting that SCD1 and ACC work on the same pathway for lipogenesis (13).

Recent work from our lab revealed that SCD1 is necessary for the progression of the cell cycle (11). In this study, when SCD1 activity was blocked, there was a 75% reduction of

cells in S-phase of the cell cycle, a 50% reduction of cells in G_2/M phase and an accumulation of cycling cells in G_1 phase (11). These observations suggest that SCD1 activity is needed for progression into S-phase of the cell cycle. There was also an increase in the number of cells undergoing apoptosis, indicating that cancer cells induce the mechanisms of cell suicide when they cannot complete the cell cycle (11). Supplementation with oleate overcame the inhibition of SCD1 activity and prevented apoptosis, further confirming the role of MUFA synthesis in the enhancement of cell proliferation (11).

SCD1 activity is also crucial for tumor formation and tumor growth. Studies from our lab show that SCD1-deficient human lung cancer cells implanted in immunodeficient mice were not as efficient in producing tumors as control cancer cells (12). Control athymic nude mice took a mere two weeks to form measurable tumors; whereas, mice injected with cancer cells that had reduced SCD1 expression took an average of 80days to form tumors (12). Not only was the onset of tumorigenesis delayed without SCD1, but also when the rate of tumor growth in mice injected with SCD1-depleted cells proceeding the onset of tumorigenesis was significantly lower than in animals injected with control cancer cells (12). Altogether, these data strongly suggest the participation of SCD1 in the tumor-forming process.

Role of EGFR→Akt/ERK signaling pathways in cancer. Implication of MUFA synthesis.

As aforementioned, activation of lipogenesis promoted by SCD1 appears to be crucial to promote and sustain typical traits of malignant behavior, such as high rate of cell proliferation and cell survival. Recent studies indicate that changing levels of SCD activity may have significant implications in the response of cancer cells to mitogenic stimuli that activates survival signaling pathways. Studies done in lung cancer cells and, more recently,

in prostate cancer cells, revealed that SCD1 is a key modulator of the phosphatidylinositol-3 kinase/Akt pathway (12;15), a central signaling cascade involved in the regulation of lipid biosynthesis, growth and survival of mammalian cells (16). Akt, found ubiquitously, can phosphorylate up to 9,000 different proteins (16). Interestingly, phosphorylation of Akt has been detected in NSCLC tumors and has been significantly positively correlated with overall survival (17).

The mechanisms by which SCD1 activity, growth and survival signaling pathways, and their downstream biological targets are integrated remain unknown. However, it is conceivable that SCD1 activity, by controlling the acyl composition of membrane phospholipids, modulates, either sequentially or concurrently, lipogenic and mitogenic pathways by affecting plasma membrane-resident signaling platforms linked to the Akt cascade, such as growth factor-activated tyrosine kinase receptors. A major mechanism for the activation of Akt in cancer cells involves the ligand-mediated stimulation of EGF receptors, also known as ErbB, prototypical tyrosine kinase receptors that are critically involved in the pathogenesis of lung cancer (18). The ErbB family comprises four members: ErbB1 or EGFR, ErbB2 (Neu or HER2), ErbB3 (HER3), and ErbB4 (HER4). Chronic overactivation of ErbB, particularly EGFR, by mutation has been implicated in several types of cancer (19). These mutations are among the most frequent genetic alterations found in lung cancer, particularly in NSCLC (19). In lung cancer, overexpression of EGFR is associated with poor prognosis (2) and is a significant factor in the prediction of response to treatment (3).

ErbB receptors are the first step in multiple signaling pathways that regulate cell division, survival and tumor formation and progression (19). Binding of epidermal growth factor (EGF) to ligand-binding sites of the ErbB receptor induces a conformational change

that promotes receptor homo- and hetero-dimerization, leading to autophosphorylation of multiple *tyr* residues ultimately, for functional activation (19;20) (Figure 1). The phosphorylated receptor then migrates along the plasma membrane in order to activate its downstream targets (21). Phosphorylated Tyr residues are able to couple to effector proteins that will specifically activate several crucial signaling pathways, including that of PI3K/Akt, Ras-MAPK (ERK) and protein kinase C. These cascades transduce signals that activate both lipid synthesis and mitogenesis (22). Activating phosphorylation of *tyr1068* and *tyr1086* initiates the binding to the SH2 domain of p85 subunit of PI3K, an event that will subsequently activate this kinase and its downstream effector, Akt (23). The PI3K/Akt pathway is a central signaling pathway that regulates lipogenesis, cell proliferation and survival and is one of the most deranged signaling cascade in cancer (18). Although mutated EGFR has also been linked to cancer, cancers with wild-type EGFR have been shown to have a shorter survival time (17). ErbB receptos, particularly EGFR, are among the most prominent clinical targets for pharmacological interventions in lung cancer and others (24;25).

Previous reports from our lab and others have suggested a functional connection between SCD1 activity and activation of Akt signals (12;15). The mechanisms that connect these two events in cancer cells has not been established. However, it can be hypothesized that an overly active SCD1 could contribute to overactivation of mitogenic signaling cascades triggered by tyrosine kinase receptors, such as EGFR, typically observed in a number of cancer cells (26). Although direct evidence supporting this proposed hypothesis is lacking, *in vitro* studies indicate that phospholipids enriched in MUFA induce activation of EGFR by triggering tyrosine autophosphorylation (27) whereas saturated phosphatidylcholine (PC) causes the opposite effect (28). Changing levels of PUFA are known to affect the activity of

EGFR receptors at the cell membrane (22) but a similar regulation of tyrosine-kinase receptors by SCD1-derived MUFA has yet to be demonstrated.

ERK

EGFR activation is also involved in the development of metastasis by activating the migration of cancer cells via the ERK/MAPK pathway (29). Most tissues have both ERK1 and ERK2 (30). Activation of ERK causes down stream induction of the transcription factor for *slug,* which cause the degradation of the surrounding extracellular matrix and permits the cell to move (29). Since SCD1 has been shown to be involved in invasiveness (*Scaglia and Igal, unpublished data*), it is possible that SCD1 upregulation in cancer affects EGFR-mediated activation of ERK, thereby inducing cell migration. ERK activation triggered by EGFR, was shown promote DNA damage repair, which may help the cells proceed through the cell cycle (18).

Implication of Lipid rafts in the regulation of EGFR activation

Given its impact on fatty acid composition of cell membranes, SCD1 activity could hypothetically modify the activation of tyrosine-kinase receptor signaling platforms, such as EGFR, by changing the lipid profile of the plasma membrane where these signaling effectors reside or interact. Activation and function of EGFR, a plasma membrane-resident protein, is known to be modulated by its surrounding lipid microenvironment (31). Thus, a change in membrane MUFA content may modify the signal transduction that is initiated at the transmembrane receptors. It has been postulated that specific lipid domains in plasma membranes, raft and non-raft structures, constitute functional microdomains for signaling mechanisms induced by tyrosine kinase receptors, including EGFR (31). Although these

microdomains have been controversial in the past because of differing results from various procedures, it is now established that lipid rafts not only exist, but display critical functions in the cell (32;33). The lipid domains are segregated into lipid rafts (enriched in cholesterol, gangliosides, and saturated PC) and non-raft domains (enriched in more fluid phospholipids) (30). These raft domains attract membrane-embedded receptors, such as EGFR, due to their hydrophobic interactions (22;34). The cholesterol and SFAs in lipid rafts decrease the membranes fluidity (35). Receptors are dependent on the raft and non-raft domains to induce their activation or inactivation (22;32). They also may help co-localize receptors with their signaling proteins.

Although there is conflicting data, it has been shows that alterations in the content and metabolism of raft-forming lipids modifies the structure and biological functions of these microdomains. It has been reported that polyunsaturated fatty acids (PUFAs) in lipid rafts alter cell signaling mechanisms and induce apoptosis in breast cancer cells (33). Moreover, PUFA supplementation reduced sphingomyelin and cholesterol, two main components of lipid rafts, in the plasma suggesting a connection between these lipid alterations and the observed lower rate of apoptosis (33). On the other hand, treatment of cancerous neuronal tissue with stearic acid, a SFA, significantly reduced cancer cell survival in vivo (10;36). An association of higher SFA and perturbations of raft structure has not yet been demonstrated, but it could have potential implications in the anti-cancer effect of these fatty acids. Researchers have recently discovered that low levels of cholesterol, an important modulator of membrane fluidity, also correlate with low levels of EGFR in lipid rafts (33). The same study also showed that when PUFA was added, EGFRs downstream target, MAPK/ERK, was more active than in control conditions (33). In a similar study by Rogers et al.,

docosahexaenoic acid (DHA), a highly unsaturated long chain fatty acid, changed the activation of Ras proteins that are known for their involvement in cancers (22). They also found that EGFR was less likely to be localized in lipid rafts with excess DHA and that it was more active at the tyr1068 site. Though the localization of Ras did not change, their activation significantly decreased (22).

Summary and Hypothesis

Although it is clear that the lipid composition of plasma membranes is an important modulator of EGFR functionality, the molecular mechanisms whereby lipids regulate EGFR activation are far from understood. By introducing specific changes in the molecular species of plasma membrane phospholipids and, thereby, the composition and abundance of raft and non-raft domains, an overly active SCD1 could contribute to the overactivation of EGFR and its mitogenic signaling cascade in lung cancer cells (Figure 2). The molecular mechanisms by which SCD1 modulates lipogenesis and, consequently, the rate of proliferation and survival in cancer cells is also unknown. H460 cells are an ideal cell line for this study since they are human lung adenocarcinoma cells that resemble the most prevalent lung cancer form, and exhibit wild-type EGFR expression. In this work, we investigated the mechanisms by which SCD1 regulates the activation of EGFR as well as its subsequent mitogenic response to EGF via the Akt and ERK signaling cascades.

Chapter 2: Materials and Methods

Cell Culture

For routine cell culture, H460 human lung cancer cells were grown in DMEM (cellgro, Manassa, VA) with 10% Fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% non-essential amino acids, and 1% vitamins at 37°C in 5% CO_2 and 100% humidity (37). Dilutions of 1:10 were prepared for each passage. Cells were grown in 100mm dishes (Corning Incorporated, Corning, NY).

Immunoblotting

Immunoblotting protocol was modified from Scaglia et al. (37). Briefly, cells at 80% confluency were treated with 1μM CVT-11127 (N-(2-(6-(3,4-dichlorobenzylamino)-2-(4-methoxyphenyl)-3-oxopyrido[2,3-b]pyrazin-4(3H)-yl)ethyl)acetamide (38) or DMSO vehicle in serum-free DMEM with 1% penicillin/ streptomycin, 1%, non-essential amino acids, 1% vitamins for 24h. Under these conditions, SCD1 activity is reduced by 90% (13). Cells were then stimulated with EGF or NRG (neuregulin) in the presence of DMSO vehicle or CVT-11127 (1μM) for 5 minutes. Incubation was stopped by discarding treatment media with vacuum and residual media on cells was discarded by washing the cell monolayers with two additions of ice-cold phosphate buffer saline (PBS). Cells were then collected and homogenized by sonication on ice in hypotonic lysis buffer (20mM Tris-HCl pH 7.5, 10mM

NaF, 1mM EDTA, diH_2O). Aliquots of cell homogenates were stored for total protein determination by Bradford method (13) and the remaining homogenate was resuspended in gel loading Laemmli buffer (0.35M Tris-HCl pH 6.8, 10% SDS, 30% Glycerol, 9.3% DTT pH 6.8, 0.175mM Bromophenol blue), boiled for 5 minutes, and stored at -80°C until use.

Proteins of cell homogenates were separated by 7.5% SDS-PAGE gel electrophoresis and transferred onto a nitrocellulose membrane. Membranes were blocked in Tris-Buffered Saline and Tween 20 (TBS-T) with 5% BSA for 2h and then incubated with the following antibodies: phospho-AKTs473, phosphor-AKT tyr308, flotillin, and caveolin-1, total MAPK, total AKT, total EGFR, EGFR tyr992, EGFR tyr1086, EGFR tyr1068, and EGFR tyr1045, pERK, SCD1, and SCD5. Dilutions are shown in Table 1. Anti-human SCD1 antibody was a gift of J-B Demoulin, Belgium. All other antibodies were from Cell Signaling Technology (Danvers, MA), BD Biosciences (San Diego, CA), Santa Cruz Biotechnology (Santa Cruz, CA), or Sigma Chemical Co. (St. Louis, MO). After the incubation with primary antibodies, the blotting membranes were washed with TBS-T three times and incubated with either goat-anti-rabbit or donkey-anti-mouse complexed with horse radish peroxidase. Membranes were incubated with chemiluminiscence reagents (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) and protein bands were detected using BioRad Universal Hood II ChemiDoc with QuantityOne software. In cases in which detection was below the sensitivity range of Chemidoc, protein bands were developed on Kodak film by Mini medical 9 X-ray developer. Images shown in this work correspond to a representative Western Blot from 3 separate determinations.

Determination of cell proliferation

Cell proliferation rate in H460 cells was assessed by Crystal violet assay modified from Jeng and Watson (39). Briefly, cells were seeded in 24-well plates allowed to proliferate until they reached 30% confluency. Triplicate wells were then treated with 1 ml/dish of 2% FBS DMEM containing 1μM CVT-11127 (CVT), 100ng/ml EGF, 100μM sodium oleate, a combination of these treatments, or DMSO as a control. All media contained 50μM BSA, which was used to produce fatty acid:BSA complex in a 2:1 ratio. Cells were subjected to these treatments for 48 hours. At the end of the incubation, cell monolayers were washed twice with ice-cold PBS. Cells were then fixed with pure methanol. After 10 min, methanol was discarded with vacuum, cells were dried out and incubated with 1ml 0.1% Crystal violet solution for 10min. At the end of the incubation, staining solution was removed, cells were washed with deionized water and 200μl 10%MeOH, 5% acetic acid were added to each well. The Optical density (OD) reading of the destained solution in the well was determined in a Versamax turntable microplate reader at 585nm using SoftmaxPro 3.1.2 software. OD values were expressed as a percentage change compared to controls (100%).

Cell transfection of SCD1 siRNA

In order to silence the expression of SCD1 in H460 cells, a transient transfection with siRNAs (ON-TARGETplus SMARTpool Dharmacon) was performed (40). To do so, a 24-well plate was seeded with 1:25 diluted H460cells in penicillin/streptomycin -free DMEM and

allowed to grow to ~85% confluency. Cells were then transfected with 20nM control or SCD1 siRNAs. After 48 hours, transfected cells were stimulated with EGF (1mg/ml) for 5 minutes. Samples were washed with ice-cold PBS, 30µL Laemli gel loading buffer was added to the cells and left at 4°C. After an overnight incubation, cell suspensions were harvested and proteins were resolved by immunoblotting for SCD1, Total Akt, pAkt, and EGFR tyr1086.

Isolation of raft and non-raft plasma membrane fractions

In order to separate raft and non-raft membrane fractions, cell homogenates were subjected to a sucrose gradient protocol modified from MacDonald and Pike (41). Briefly, a 5%-40% sucrose gradient was prepared with Rabbit Peristaltic Pump minipuls2 and Hoefer SG50 gradient maker. The sucrose buffer contained 10% Tris-HCl (20mM) and 10% NaCl (150mM) pH 7.6. Cells were starved of FBS for 24 h when they reached 80% confluency, and treated with either DMSO vehicle or CVT-11127 (1µM). Cells were stimulated for 5 minutes with EGF (100ng/ml) and then washed with washing buffer (10% NaCl, 10% $MgCl_2$, 10% $CaCl_2$ (1mM) and 70% TrisCl (20mM) pH 7.8). Next, cells were harvested in lysis buffer and homogenized by passing the cells through a 22G needle. Next, homogenates were placed at the bottom of the centrifuge tube with 80% sucrose solution for a final concentration of 40% and they were centrifuged in Beckman Coulter Optima LE-80K Ultracentrifuge at 39,000rpm at 4°C for 14 hours. One-milliliter aliquots were removed from the top down to separate fractions by density; with 500µl removed from each fraction for lipid analysis. 100µl of each sample resolved in a 7.5% SDS PAGE gel with 100µl loaded from each fraction. The gel was then transferred to a nitrocellulose membrane for 16 hours at 0.3milliamps and

immunoblotted as described above. A representative Western blot image of 3 experiment is shown in this work.

Statistical Analysis

Cell proliferation data are represented with means ± S.D. Statistical significance was measured by Students t-test with values of at least $P \leq 0.05$ being considered significant.

Chapter 3: Results

Previous research from our lab and others showed that SCD1 is a crucial enzyme in the regulation of fatty acid balance and overall lipid homeostasis in mammalian cells. This is especially true for cancer cells, which have a high demand for lipids with an appropriate fatty acid composition, particularly, membrane-forming lipids (8). Typical biological features of cancer, such as unremitting cell proliferation and survival, invasiveness and tumorigenesis rely on the constant activation of signaling cascades that transduce the oncogenic stimuli (26). Examples of these critical pathways are PI3K/Akt and ERK signaling cascades. It is shown that SCD1 depletion leads to decreased cell proliferation, invasiveness, and tumor formation and that these effects were connected to a reduction in the activation of Akt phosphorylation (8;13). Since Akt activation depends on the activation of tyrosine-kinase receptors in plasma membranes, such as EGFR, we hypothesized that changes in the species of phospholipids in the plasma membrane induced by changing levels of SCD1 may likely affect the ability of EGFR to be activated by its ligand, EGF, and subsequently activate downstream effectors.

We first analyzed the activation of EGFR upon stimulation with EGF in relation to SCD1 activity by assessing the levels of phosphorylation of *tyr* residues 1086, 1068, and 1045, which are critically related to the receptors activation rate. In cells undergoing SCD1 inhibition with CVT or vehicle, DMSO, EGFR was not phosphorylated in the non-stimulation conditions (Figure 3), indicating that the receptor remained inactivated in the absence of its ligand. Upon stimulation with EGF, a strong phosphorylation signal was detected in all *tyr* residues in controls cells. This effect was specifically and directly caused by the binding of

EGF to EGFR receptors since cells treated with neuregulin (NRG), a cytokine that binds to other ErbB receptors but not to EGFR, did not induce phosphorylation. In EGF-stimulated cells, the decrease in the levels of total EGFR was likely due to the conversion into a phosphorylated receptor. Interestingly, in cells undergoing SCD1 inhibition the levels of phosphorylation, although strong, was markedly decreased relative to controls in all residues, suggesting that SCD activity modulates the overall activation of EGFR in these cells.

Because the phosphorylation of EGFR tyrosine residues was reduced when SCD1 was inhibited, we supplemented cells with the product of SCD1, oleic acid, to discern its ability to recover phosphorylation to control conditions by bypassing the blockade of SCD1. In non-stimulated conditions, *tyr* residues 1068 and 1086 were again found unphosphorylated (Figure 4). Upon EGF stimulation, both *tyr* residues were markedly phosphorylated. Supplementation of control cells with oleate further increased the phosphorylation of *tyr* residues. Remarkably, in cells undergoing inhibition of SCD1 with CVT, oleate recovered *tyr* phosphorylation induced by EGF indicating that MUFA are a prerequisite for EGF mediated activation of EGFR. In summary, these results show that EGFR activation in cancer cells is dependent on the activity of SCD1 for MUFA production.

We next determined whether these effects on EGFR activation translate to the downstream components of the EGFR signaling cascade, Akt and ERK. In unstimulated conditions, cells undergoing SCD1 inhibition and controls showed only slight phosphorylation of both Akt and ERK (Figure 5). However, upon EGF stimulation, the signal for Akt and ERK stimulation substantially increased, an effect that was amplified by oleate supplementation. The levels of phosphorylation of both signaling proteins were decreased when SCD1 was abrogated and reduced to unstimulated control levels. SCD1 abrogation showed reduced

phosphorylation levels relative to controls. However, EGF stimulation and oleate supplementation did partially recover the phosphorylation state. Altogether, these data suggest that the full induction of both Akt and ERK require EGFR activation by an SCD1-regulated mechanism.

All of these data suggest that SCD1 activity provides suitable conditions for EGFR activation and subsequent cell proliferation since main downstream components of the pathway, like Akt and ERK, are active. To determine if the reduction in the activity of the EGFR pathway in SCD1 deficient cancer cells promote alterations in cell proliferation, an analysis of cell growth by Crystal violet assay was performed. DMSO-treated cells without EGF stimulation was the control set to 100%. A ~35% increase in cell proliferation was observed in cell stimulated with EGF with respect to unstimulated controls (Figure 6), suggesting that EGF alone can induce cell proliferation in these cancer cells. However, when SCD1 is inhibited, the proliferation in non-stimulated cells significantly dropped to below 50% of controls. Cell proliferation in SCD1-ablated cells remains below 50% despite stimulation with EGF. This is strong evidence that the mechanisms of cell replication by EGFR-mediated pathways rely on the presence of active SCD1 in order to be fully activated.

Although CVT-11127 is a potent and specific inhibitor of SCD activity (8), H460 cells contain measureable amounts of SCD5, whose potential blockade by the inhibitor may contribute to its effects on EGFR-activated mechanisms. It is unknown how SCD5 is affected by CVT treatment; however, SCD5 levels did not change when SCD1 was knocked down (*Igal, unpublished data)*. We silenced the SCD1 gene via siRNA to confirm the data pointing to the necessity of SCD1 for EGFR activation and subsequent induction of Akt and ERK activation leading to cell proliferation. As shown in Figure 7, SCD1 was successfully silenced

as only traces of SCD1 protein were detected with the specific anti-human SCD1 antibody. In cells with ablated SCD1, Akt could not be phosphorylated in spite of being stimulated by EGF, confirming that EGFR-dependent activation of Akt phosphorylation is dependent on phosphorylation of EGFR, which requires SCD1 activity. It is unknown why control siRNA with EGF stimulation inhibited Akt phosphorylation. Perhaps an inherent toxic effect was induced by the siRNA. These data also confirm that the previous findings were not an unspecific effect of the small molecule SCD inhibitors, but indeed, the abrogation of SCD1 that is responsible for changes in EGFR phosphorylation, subsequent activation of its pathways, and cell proliferation.

To elucidate the mechanism by which the abrogation of SCD1 modulates EGFR activation, we postulated that the product of SCD1 is necessary to regulate the structure and activity of lipid rafts in the plasma membrane. Since EGFR must traverse the plasma membrane raft domains to a non-raft domain in order activate its downstream targets, the lipid content of phospholipids may alter EGFRs ability to migrate (21). To assess a change in lipid domain distribution and the localization of EGFR in these domains upon EGF stimulation, the presence of both total and phosphorylated EGFR was analyzed in membrane lipid domains based in a continuous sucrose gradient was prepared (Figure 8). Flotillin was used as a marker for lipid rafts because the majority of flotillin proteins localize to raft domains (31). Although the exact function of flotillin remains to be elucidated, it has been shown to affect trafficking within the membrane and is associated with EGFR signaling pathways (42). Under DMSO treatment conditions, flotillin was found in the least dense gradient fraction predicted to contain lipid rafts. However, the presence of flotillin, in heavier gradient fractions and in the sediment of the preparations may indicate the presence of more dense rafts structures or a

certain degree of contamination of this fraction with cell debris and non-homogenized subcellular fractions (Figure 8A). Both EGFR total (unphosphorylated) and EGFRtyr1068 appear to co-localize with lipid rafts as shown by their co-localization with flotillin. When SCD1 activity was blocked, flotillin was detected in more dense fractions as if the lipid domain distribution shifted to have contained more dense raft domains, probably due to accumulation of SFA in their lipids. Both phosphorylated and total EGFR were found in the more dense portions of the gradient where flotillin was present (Figure 8B) suggesting that this alteration in the structure and/or lipid composition of rafts may in turn affect the ability of phosphorylated EGFR to activate the Akt and ERK signaling cascades in their plasma membrane compartments.

Chapter 4: Discussion

In the present study we demonstrate that the key enzyme of MUFA synthesis, SCD1, regulates the rate of cancer cell proliferation by impairing the activation of the EGFR signaling platform by its ligand, EGF. EGFR's ability to autophosphorylate upon stimulation by its ligand, EGF, then migrate across the plasma membrane initiates two critical cascades, Akt and ERK, involved in cell proliferation, survival and tumorigenesis.

SCD1 is responsible for maintaining the balance of MUFA and SFA in cell lipids, particularly in membrane forming phospholipids (8). The present work also suggests that MUFA, likely from endogenously synthesized pools, is an essential regulator of cell signaling in cancer cells. However, the observation that exogenous oleate only partially relieved the effect of SCD1 inhibition on the phosphorylation of Akt implies the requirement of molecules other than MUFA to sustain a full activation of this signaling cascade. The finding that EGF-mediated induction of cell proliferation was suppressed in conditions of SCD1 ablation confirms that the alteration of EGFR signaling affects cancer cell phenotype and is influenced by SCD1.

The main issue arises as EGFR attempts to phosphorylate its downstream effectors, Akt and ERK. Our results reveal that Akt and ERK are not within the same pathway but different cascades leading to similar results: increased cell proliferation, survival, and tumor formation. These data coincide with previous studies, which show an overlap in the pathways of ERK and Akt but they are not solely dependent on each other. Meng et al. found that treatment with AZD6244, an ERK inhibitor, and MK2206, an Akt inhibitor, apoptosis was significantly higher when combined than when each is used individually in NSCLCs in vitro

and in vivo (25). Furthermore, the combination of drugs exhibited significantly smaller tumors in mouse xenografts than each alone. The same study showed inhibition of ERK resulted in significantly smaller tumors and significantly longer survival time in mice than when Akt alone was blocked (25). The combination of drugs had the greatest effect; again, suggesting Akt and ERK are critical in cancer cell survival and tumorigenesis.

Besides EGFR, in which phosphorylation was restored to control levels by oleate, Akt has been shown to be also sensitive to changing levels of FAS, SCD1, and glucose (13) implying that this signaling pathway depends on lipogenesis for its full activity. The observation that addition of oleate does not completely restore the low levels of Akt activation in SCD1-depleted cells is somehow surprising, but previous research showed that activation of Akt by oleate may require other signals (13). EGFR is not the sole activator of Akt; thus, despite EGFR phosphorylation recovery by oleate, Akt may not present a similar restoration due to its many other influences.

Although the present work does not provide an answer to how SCD1 activity affects the components of the EGFR→Akt signaling system, changes in plasma membrane lipid domains caused by SCD1 may be responsible, at least in part, for those effects. Alteration of lipid raft formation has previously been shown to modulate the activity of receptors in the plasma membrane, including EGFR (22). We observed a dramatic alteration in the distribution of raft domains. Although in cells with blocked SCD1, EGFR could become phosphorylated, the activated receptor may not be in the appropriate compartment to proceed with activation of downstream signaling targets. EGFR requires a shift from a lipid raft to a non-raft domain (43) for it to be capable to phosphorylate Akt and ERK (22).

Finally, our findings may provide relevant information that can be used for novel treatments of NSCLCs since EGFR has been hypothesized to be the likely culprit in cell proliferation and tumorigenesis. It is now possible to conclude that SCD1 is a vital component in the mechanism of EGFR activation and downstream Akt and ERK effects. Future investigation will be required to reveal the identity of the mechanistic components of these intertwined pathways of MUFA synthesis and signaling.

Table 1. Antibodies used for Western Blots. All antibodies were diluted in TBS-T with 5% BSA.

	Antibody	Dilution	Source
Mouse	pAkts473	1:1000	Cell Signaling
	pAkt308		
	Flotillin		BD Biosciences
	Caveolin		
	β-actin	1:5000	Sigma Aldrich
Rabbit	MAPK total	1:1000	Cell Signaling
	Akt total		
	EGFR total		
	EGFR tyr992		
	EGFR tyr1086		
	EGFR tyr1068		
	EGFR tyr1045		
	pERK	1:600	
	SCD1	1:5000	Santa Cruz
Goat	Anti-rabbit	1:1000	Cell Signaling
Donkey	Anti-mouse	1:2000	Santa Cruz

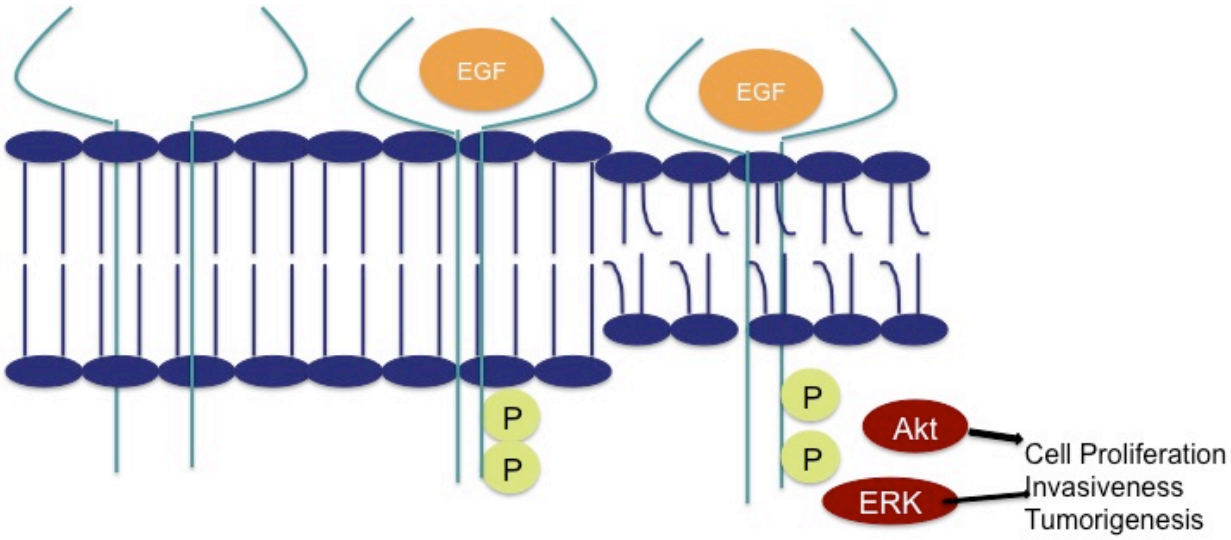

Figure 1. EGFR activation and migration. EGF, epidermal growth factor.

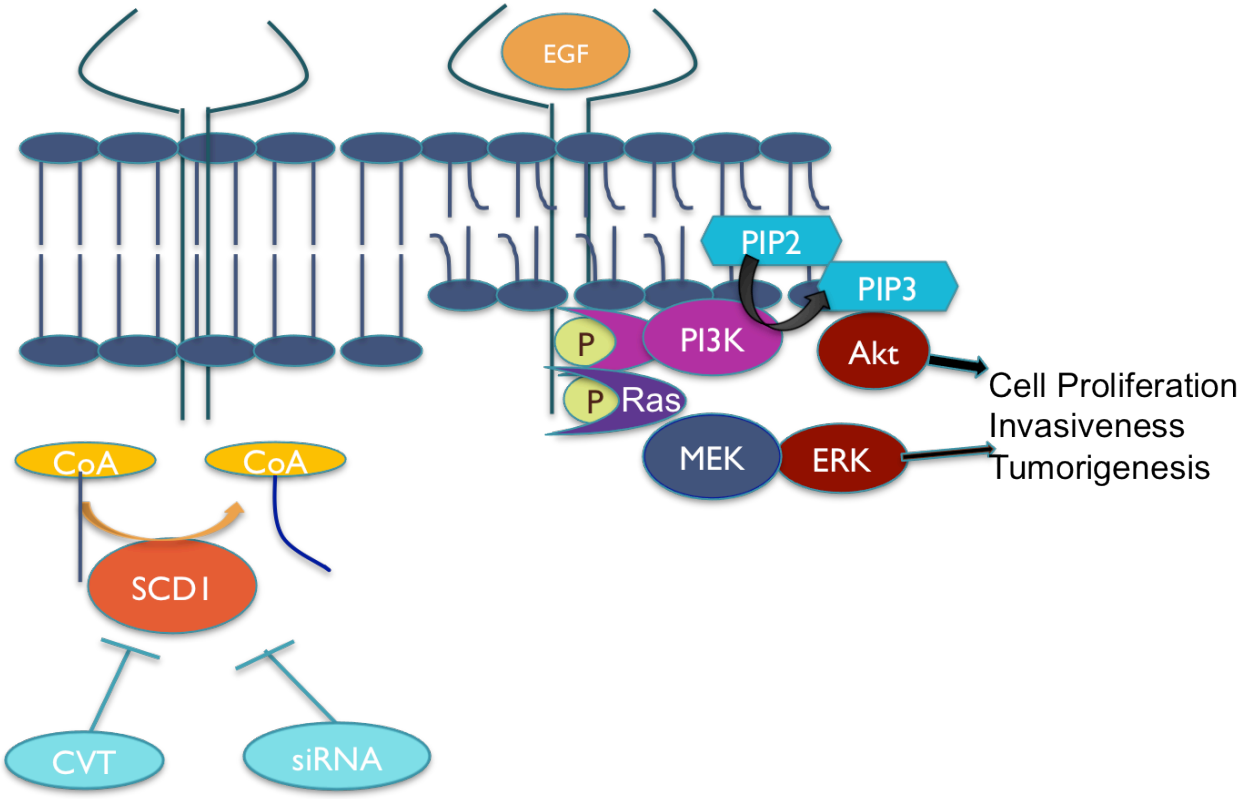

Figure 2. Hypothetical mechanism of regulation of EGFR activation by SCD1 and subsequent activation of Akt and ERK. EGF, epidermal growth factor; SCD1, Stearoyl-CoA desaturase 1; CVT, CVT-11127.

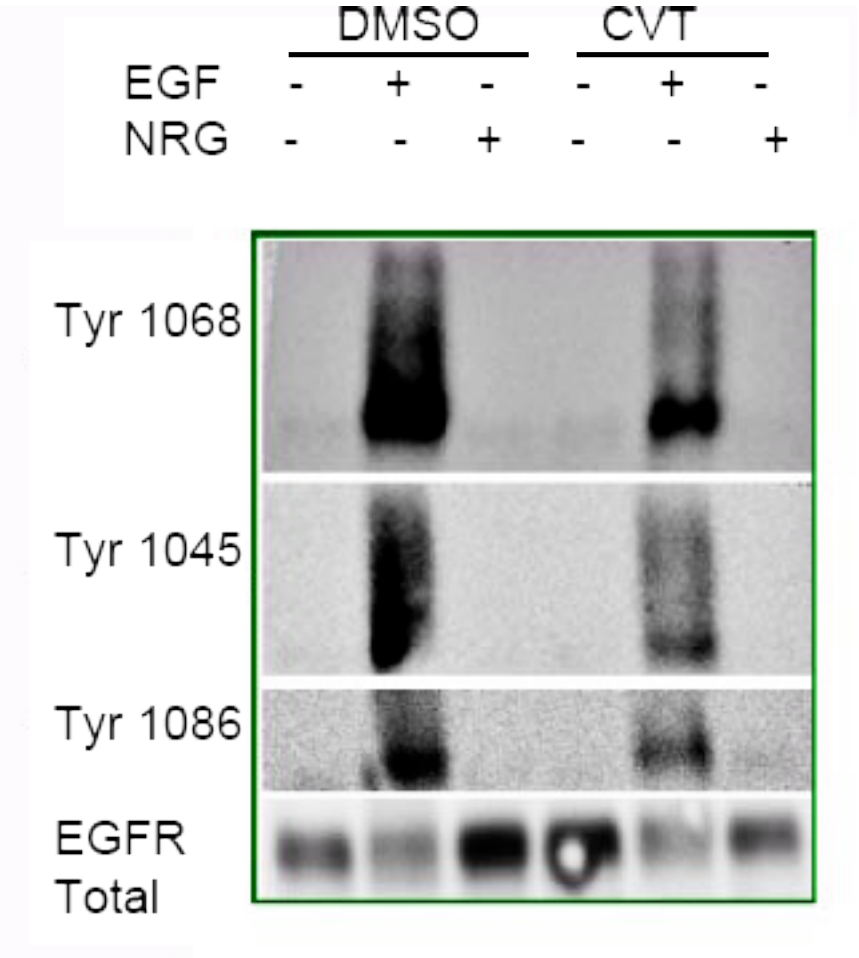

Figure 3. SCD1 inhibition decreases activation of EGFR by EGF. H460 cells were treated with DMSO or CVT-11127 (CVT) for 24h. DMSO, EGF, or NRG (1μM) were used to stimulated cells for 5 min. Levels of phosphorylated EGFR at tyrosine sites 1068, 1045, and 1086 were determined by Western Blot on 7.5% SDS-PAGE gel. Membranes were incubated with antibodies for EGFRtyr1068, EGFRtyr1045, and EGFRtyr1086. EGFR total detects only unphosphorylated protein. Results representative of 3 separate experiments.

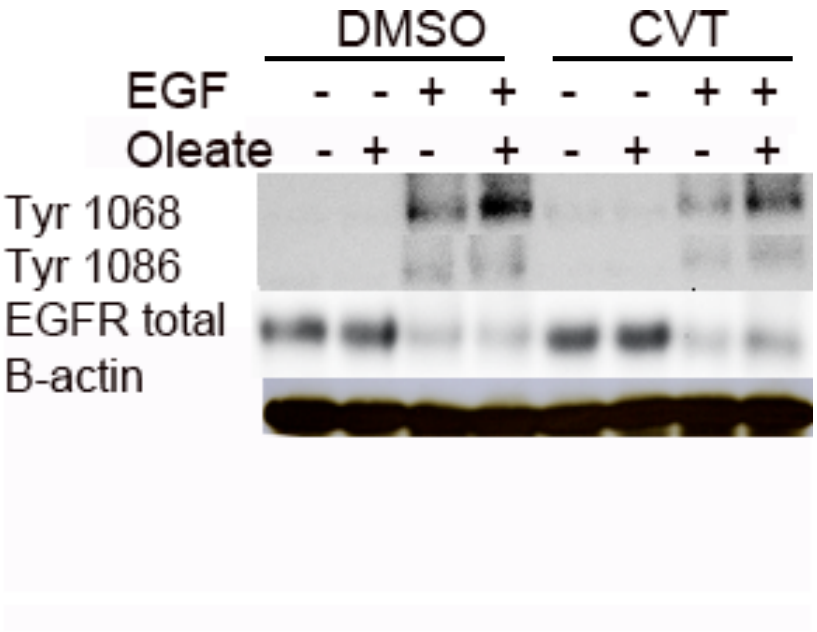

Figure 4. Oleate recovers EGFR phosphorylation when SCD1 is abrogated. H460 cells were treated with DMSO, CVT, or Oleate for 24h. DMSO or EGF (1μM) were used to stimulate cells for 5 min. Levels of phosphorylated EGFR at tyrosine sites 1068 and 1086 were determined by Western Blot on 7.5% SDS-PAGE gel. Membranes were incubated with antibodies for EGFRtyr1068, EGFRtyr1086, Total EGFR (unphosphorylated protein), and β-actin.

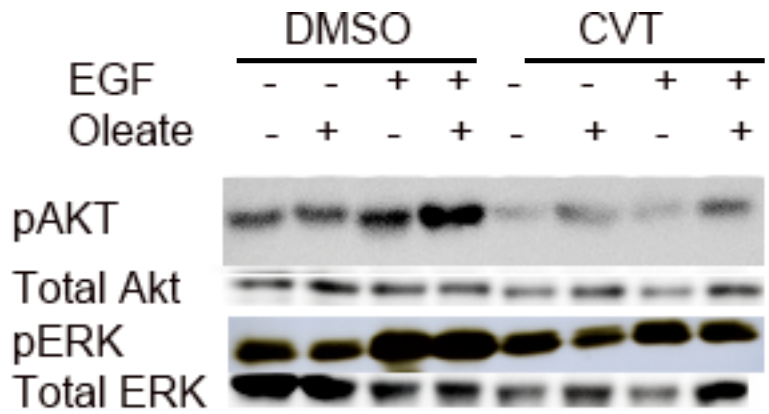

Figure 5. Activation of downstream targets of EGFR, Akt and ERK, requires SCD1 via EGFR activation. H460 cells were treated with DMSO, CVT, or Oleate for 24h. DMSO or EGF (1μM) were used to stimulate cells for 5 min. Levels of pAktser473, Total Akt, pERK, and Total ERK were determined by Western Blot on 7.5% SDS-PAGE gel. Membranes were incubated with antibodies for pAktser473, Total Akt (unphosphorylated protein), pERK, and Total ERK (unphosphorylated protein).

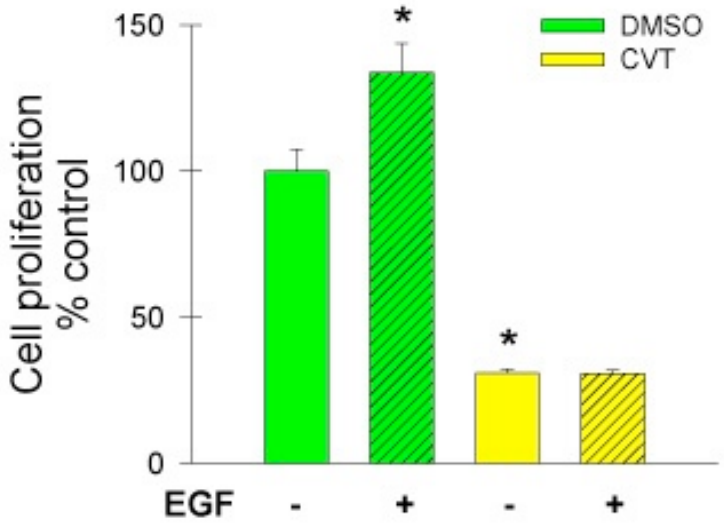

Figure 6. Inhibition of SCD1 prevents EGF induced cell proliferation. H460 lung cancer cells were treated with 1μM CVT-11127 (CVT) or DMSO in 2%FBS DMEM for 48h in presence or absence of 100μM oleate. Cells were stimualted with 100ng/mL EGF. Cell proliferation was determined by Crystal violet assay in a 24-well plate done in triplicates. Results are representative of 3 separate experiments. *$p<0.05$ or less vs. vehicle-treated control cells.

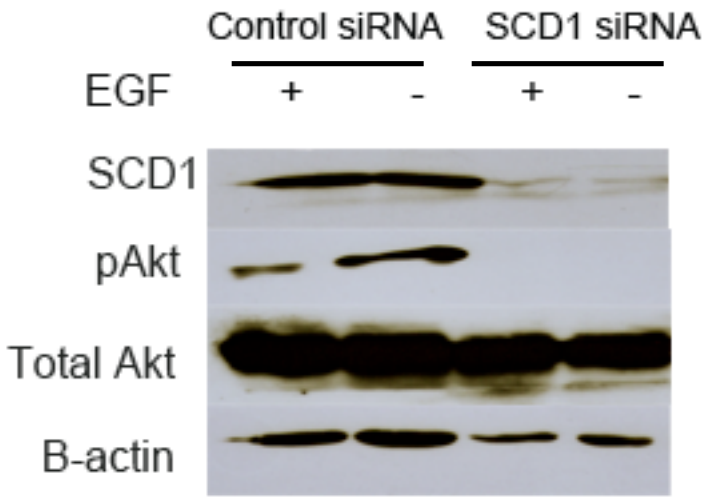

Figure 7. With SCD1 knocked down, Akt cannot become phosphorylated. H460 cells were stably transfected with control or SCD1 siRNA (20μM). DMSO or EGF (1μM) were used to stimulate cells for 5 min. Levels of SCD1, pAktser473, Total Akt, and β-actin were determined by Western Blot on 7.5% SDS-PAGE gel. Membranes were incubated with antibodies for SCD1, pAktser473, Total Akt (unphosphorylated protein), and β-actin.

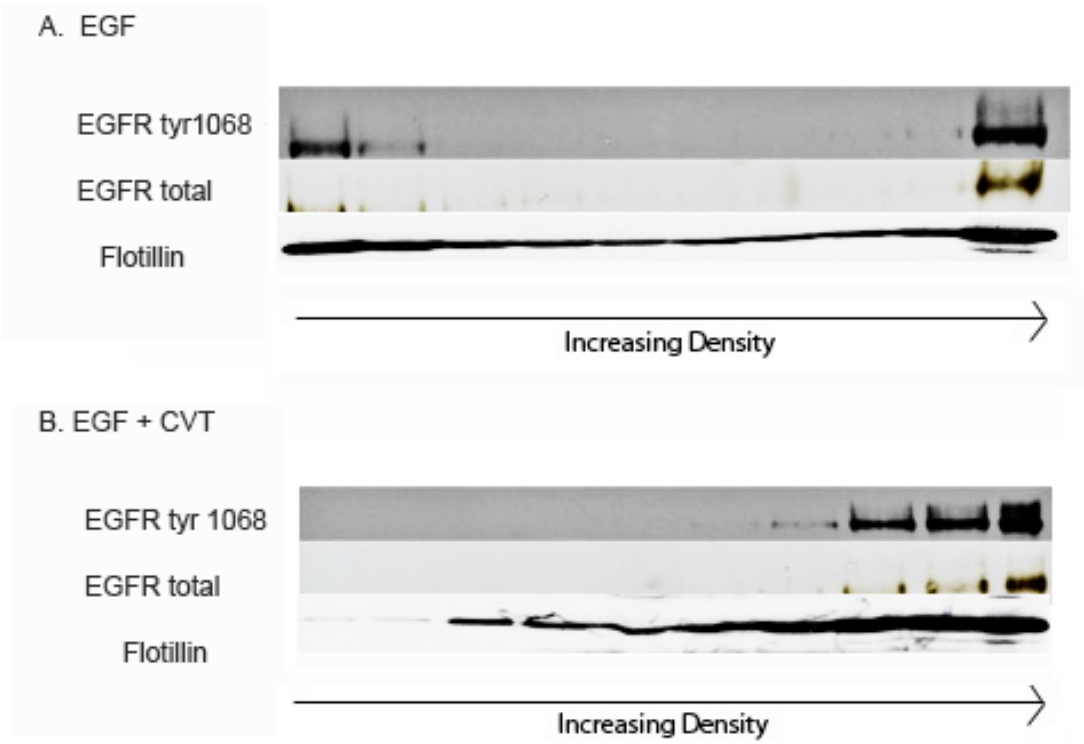

Figure 8. SCD1 blockade alters distribution of EGFR within lipid rafts. H460 cells were treated with (A). DMSO or (B). CVT and stimulated with EGF (1μM) for 5 min. 7.5% SDS-PAGE gel was used to resolve proteins. Flotillin is a marker for lipid rafts. Low density (5% sucrose) corresponds to left side and continuously increases to 40% sucrose. Membranes were incubated with antibodies for EGFRtyr1068, EGFR total, and Flotillin. Results are representative of 3 separate experiments.

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