*Original Research*

# Stearoyl-CoA desaturase activity modulates the activation of epidermal growth factor receptor in human lung cancer cells

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## Abstract

**Stearoyl-CoA desaturase-1 (SCD1), the main enzyme that converts saturated fatty acids into monounsaturated fatty acids, is a key factor in the mechanisms of cancer cell proliferation, survival and tumorigenesis. Evidence indicates that SCD1 activity regulates these events in part by targeting the phosphatidylinositol-3 phosphate kinase/Akt and Ras/extracellular signal-regulated kinase (ERK) pathways, but the molecular mechanisms remain unknown. We now show that in H460 lung cancer cells, the suppression of SCD activity with CVT-11127, a specific small molecule SCD inhibitor, impairs the ligand-induced phosphorylation of epidermal growth factor (EGF) receptor, causing the inactivation of its downstream targets Akt, ERK and mammalian target of rapamycin. Importantly, the mitogenic response to EGF was markedly defective in SCD-depleted cancer cells. The inactivation of EGF receptor (EGFR) promoted by SCD inhibition may be caused by perturbations in the lipid microenvironment surrounding the receptor, since we detected significant alterations in the lateral mobility of plasma lipid microdomains. Finally, incubation of lung cancer cells with SCD blockers potentiated the antigrowth effect of gefitinib, an EGFR inhibitor employed in cancer treatment. Altogether, our data indicate that SCD activity may control cancer cell metabolism, proliferation and survival by modulating the EGFR→Akt/ERK signaling platforms. Our studies also suggest a value for SCD inhibitors as novel pharmacological agents in lung cancer, one of the most common and lethal forms of cancer for which therapeutic options remain very limited.**

**Keywords:** MUFA synthesis, lung cancer, Akt, ERK, mTOR, cancer cell proliferation, EGFR inhibitor

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## Introduction

A signature alteration in cancer cells and tumors is the conspicuous presence of aberrant metabolic activity, especially high rates of glycolysis and lipid biosynthesis.[1] In the search for novel therapeutic approaches to cancer, enzymes of lipid biosynthesis are receiving increasing attention as valuable targets for pharmacological intervention in cancer. The process of metabolic transformation requires the activation of lipogenic enzymes, particularly those in the fatty acid biosynthetic pathway, for the production of new structural, signaling and energetic lipids needed for the survival and propagation of cancer cells. A concerted increase in the expression of ATP-citrate lyase, acetylCoA carboxylase, fatty acid synthase (FAS) and stearoyl-CoA desaturases (SCD) allows for the unlimited production of saturated fatty acid (SFA) and monounsaturated fatty acids (MUFA), the preferred building blocks for lipid biosynthesis in cancer cells.[2,3] The activity of every enzyme in the tandem is essential for cancer cell mitogenesis and tumorigenesis since inhibition of each fatty acid biosynthetic enzyme caused attenuation or suppression of cancer cell and tumor growth.[4–9] Recently, more attention has been focused on the novel role of SCD1 as a key regulator of the program of oncogenic transformation. Abnormally high levels of SCD1 have been detected in several types of experimental and human cancers, including colon, liver, breast and lung cancers.[10–12] Studies in cancer cells have unambiguously showed that SCD1 stimulates cell proliferation by facilitating the progression of the cell cycle,[13] and by inhibiting the mechanisms of programmed cell death and SFA-induced cytotoxicity.[14–16]

SCD1 may contribute to the onset and progression of cancer by promoting metabolic and signaling conditions that induce and sustain the biological traits of cancer. For instance, SCD1 activity has been implicated in a shift in lipid metabolism towards anabolism in cancer cells by



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activating lipogenesis and inhibiting the activity of catabolic activators, such as AMP-activated protein kinase.[15] Moreover, recent data demonstrate that the activation of phosphatidylinositol-3 phosphate kinase (PI3K)/Akt pathway, a central signaling cascade involved in the regulation of lipid biosynthesis, growth and survival of mammalian cells,[17] is modulated by SCD1 activity in cancer cells.[9,18] The mechanisms by which the balance of MUFA/SFA determined by SCD activty modulates Akt activation are not fully understood.

A major mechanism for the activation of Akt in cancer cells involves the ligand-mediated stimulation of epidermal growth factor (EGF) receptors (also known as ErbB), prototypical tyrosine kinase receptors that are activated by autophosphorylation upon ligand binding.[19] Chronic overactivation of ErbB, particularly EGF receptor (EGFR), by mutation or deletion has been implicated in the pathogenesis of cancer.[19,20] These mutations are among the most frequent genetic alterations found in lung cancer, particularly in non-small-cell lung cancer (NSCLC), the most common and lethal type of lung cancer.[21] In lung cancer, overexpression of EGFR is associated with a negative prognosis,[22] and is a significant factor in the prediction of response to treatment.[23] Moreover, recurrence is frequent and cancer cells usually become resistant to the original drug treatment;[24] hence, the discovery of new pharmacological targets is critical for improving treatment outcomes.

As a plasma membrane-resident protein, EGFR activation and function is modulated by its surrounding lipid microenvironment. Specific lipid domains in plasma membranes, raft and non-raft structures, constitute functional platforms for signaling mechanisms associated with a number of tyrosine kinase receptors, including EGFR.[25,26] Lipid domains are segregated into more structurally ordered lipid rafts (enriched in cholesterol, gangliosides and saturated phosphatidylcholine) and less ordered non-raft domains (enriched in more unsaturated phospholipids). It has been postulated that localization of EGFR to raft and non-raft lipid domains modulates both the ligand-binding and tyrosine kinase activity[26] and that changes in membrane lipid composition have dramatic repercussions on the partition and activation of EGFR.[27] SCD1 has been shown to be the main factor in the regulation of the abundance of SFA and MUFA in cellular membranes of cancer cells;[3] therefore, changing levels of SCD activity might be able to modulate the activation of EGFR signaling platform by regulating the composition and mobility of lipid fluid (non-raft) and rigid (raft) domains in plasma membranes in which this signaling platform is embedded. This potential SCD-mediated mechanism of EGFR regulation has not been investigated.

In the present study, we show that the activation of EGFR is markedly impaired in lung cancer cells in which SCD activity was blocked by specific small molecule inhibitors. We also found that the EGFR inactivation promoted by the blockade in MUFA synthesis produced a consequent inhibition of Akt, extracellular signal-regulated kinase (ERK)1/2 and mammalian target of rapamycin (mTOR), the main downstream signaling effectors of the receptor. Importantly, we found that the mitogenic response of cancer cells to EGF was suppressed by the blockade in SCD activity. In addition, we observed that SCD inhibition led to an alteration in the mobility of plasma membrane lipid domains, a perturbation that may be critical for the activation of plasma membrane-resident signaling platforms, such as EGFR→PI3K/Akt and EGFR→Ras/ERK signaling pathways. Finally, we report that the SCD inhibitors potentiated the suppressing effect on cancer cell growth of gefitinib, an EGFR inhibitor employed in cancer treatment. These results suggest that a combined pharmacological approach that blocks both signaling and metabolic targets may enhance the therapeutic effect of cell signaling inhibitors like gefitinib.

## Material and methods

### Materials

H460, A549 and H1299 human lung adenocarcinoma cells, A431 human mucoepidermoid carcinoma cells, WI38 normal human lung fibroblasts and SV40-WI38 human lung fibroblasts were from ATCC (Manassas, VA, USA). Human lung normal and tumor homogenate samples were from ProSci Inc. (Poway, CA, USA). Cell culture media and other culture reagents were from Invitrogen Life Technologies (Carlsbad, CA, USA). Ultrafiltered fetal bovine serum (FBS), fatty acid-free bovine serum albumin, phosphatase and protease inhibitor cocktail were purchased from Sigma (St Louis, MO, USA). Cell culture supplies, silica gel 60 chromatography plates, and analytical-grade solvents were from Fisher Scientific (Morris Plains, NJ, USA). All antibodies were from Cell Signaling Technology (Danvers, MA, USA), except human SCD1 antibody,[28] which was kindly donated by Jean-Baptiste Demoulin, Université Catholique de Louvain, Belgium, and SCD5 antibody,[12] which was a gift from Brent Rupnow, Bristol Myers-Squibb (Princeton, NJ, USA), respectively. The EGFR inhibitor gefitinib was a generous gift from AstraZeneca (Wilmington, DE, USA).

### Cell culture

Unless otherwise stated, cells were grown in Dulbecco's modified eagle's media ( DMEM) supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (10 $\mu$g/mL), 1% non-essential amino acids and 1% minimum essential medium vitamin solution (growing medium), at 37°C, 5% $CO_2$ and 100% humidity.

### Extraction of cell lipids and determination of total cellular fatty acid composition

Preconfluent cell monolayers were treated with 1 $\mu$mol/L CVT-11127, a specific SCD small molecule inhibitor,[29] or dimethyl sulfoxide (DMSO) (vehicle) for 1, 12 and 24 h in 10% FBS DMEM. At the end of incubations, cells were harvested in ice-cold methanol and total lipids were extracted as previously described.[14] Heptadecanoic acid (C17:0) was added as an internal standard at the beginning of the lipid extraction process. Fatty acids from total lipids were transesterified and methylated according to the method described by Lepage and Roy.[30] The composition of fatty

acid methyl esters was determined by gas chromatography using a Varian 3800 GC (Varian Inc., Palo Alto, CA, USA), equipped with a DB-23 column (J&W Scientific Inc., Folsom, CA, USA) and flame ionization detector. Fatty acid methyl ester identification and response factors were assessed by using mixtures of pure fatty acid standards (NuChek Prep Inc., Elysian, MN, USA). Chromatographic peaks were identified by comparison of their retention times with those of standards and percent distribution was calculated.

### Cell proliferation assay

To determine the effect of SCD inhibition on EGF-induced cell proliferation, preconfluent H460 human lung adenocarcinoma cells were treated with CVT-11127 or DMSO for 48 h, in the presence or absence of 100 nmol/L EGF, in 2% FBS DMEM for 24 h. At the end of incubation, cell monolayers were fixed and cell density was determined by crystal violet assay as described.[15] Results were expressed as percentage change in cell proliferation with respect to optical density values of vehicle-treated control (100%). Similar cell proliferation assays were conducted in H460 cells that were treated for 48 h with CVT-11127 or CVT-12012, an orally bioavailable SCD inhibitor[31] or vehicle in 10% FBS DMEM, in the presence or absence of 20 $\mu$mol/L gefitinib, an inhibitor of EGFR-tyrosine kinase activity. Additionally, groups of cells were treated with CVT-11127 or DMSO plus or minus any of the following compounds: Ly294002 (PI3K inhibitor, 20 $\mu$mol/L final concentration), U0126 (MEK inhibitor, 20 $\mu$mol/L) or rapamycin (mTOR inhibitor, 100 nmol/L).

### Fluorescence recovery after photobleaching analysis

Lateral mobility of plasma membrane lipid domains was assessed by fluorescence recovery after photobleaching (FRAP), as described by Lambert *et al.*[32] Cells were cultured on 35 mm chamber slides and kept in serum-free media for 24 h previous to FRAP analysis. Cells were stained with fluorescent markers of raft-like and non-raft-like lipid domains cholera toxin B-Alexa488 and DiIC18, respectively, for 10 min at 4°C. FRAP determinations were performed at 37 °C with a Leica TCS SP5 confocal microscope equipped with an argon laser (Leica Microsystems, Inc., Buffalo Grove, IL, USA). Fluorescence recovery was calculated as a percentage of postbleach fluorescence as previously described.[32]

## Results

### SCD1 protein levels are increased in human lung tumors

Cancer cells and tumor tissues cells exhibit a great abundance of MUFA in their major acyl-containing lipids.[3,33] Although it is now well documented that high SCD activity is largely the main factor that contributes to the enrichment of lipid with MUFA in cancer cells,[3] it was not clear whether this effect was produced by changing levels of one or both human SCD isozymes, SCD1 and SCD5. In Figure 1a, we

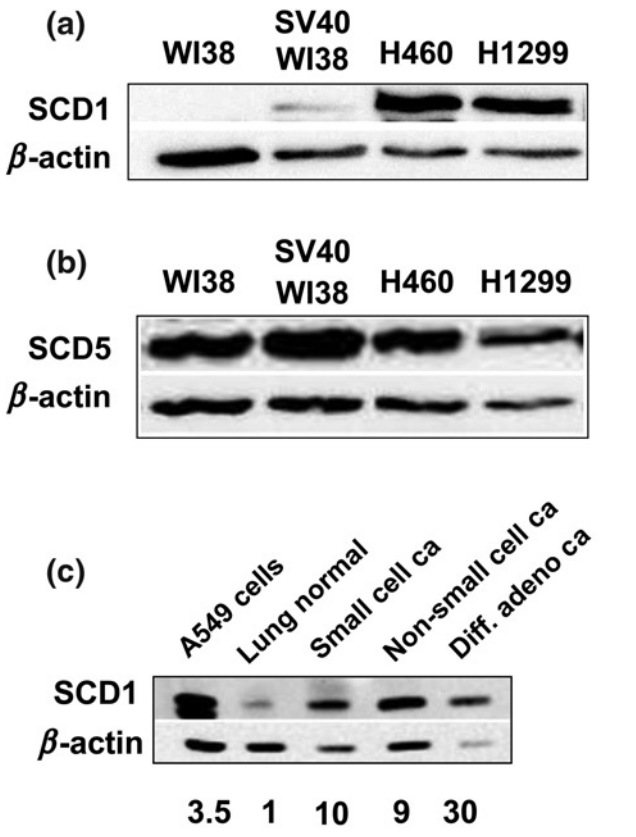


**Figure 1** Increased stearoyl-CoA desaturase 1 (SCD1) expression in human lung cancer cells and tumor tissues. Western blot analysis of SCD1 (a), SCD5 (b) and $\beta$-actin in human lung normal (WI38), SV40-transformed WI38 cells and H460 and H1299 lung cancer cells. SCD1 levels were also determined in homogenates of A549 cells, normal lung tissue, small-cell carcinoma, non-small-cell carcinoma and differentiated adenocarcinoma tissues ((c), fold-change versus normal lung). $\beta$-Actin was employed as loading control to normalize SCD1 levels

show that in *sv40*-oncogene transformed human lung fibroblasts, as well as in the tumor-forming lung cancer cell lines A549, H460 and H1299 human lung adenocarcinoma cells, the content of SCD1 protein was drastically increased compared with WI38 normal human lung fibroblasts, demonstrating that the increased Δ9-desaturase activity observed in cancer cells is the result of greater abundance of SCD1 protein. We detected the presence of SCD5 protein in both normal and lung cancer cells, but its levels were virtually similar among cell strains (Figure 1b), which may imply a less influential role for this SCD isoform in the biochemical transformation of cancer cells than SCD1. Importantly, SCD1 expression was also increased in homogenate samples of human lung cancer tissues compared with normal lung tissue preparations (Figure 1c), suggesting that the up-regulation of SCD1 is a rather ubiquitous feature of the cancer cell phenotype, at least in lung cancer.

### Pharmacological inhibition of SCD activity acutely reduces MUFA/SFA ratios in cancer cell lipids

Previous studies demonstrated that SCD1 is the main regulator of MUFA/SFA balance in cancer cells.[9,12,14,15,18] In a recent study, we observed that a 24-h blockade of SCD1

with CVT-11127, a specific small molecule inhibitor of the enzyme, promoted a marked alteration in the MUFA/SFA ratio in lipids from H460 human lung adenocarcinoma cells.[15] Here, again using H460 cells in which SCD activity was blocked with CVT-11127 for 1, 12 and 24 h, we detected that the main MUFA-to-SFA ratios exhibited a time-dependent decrease in MUFA-to-SFA (Figure 2). We observed that the oleic acid (18:1n-9)-to-stearic acid (18:0) ratio was reduced by 16%, 45% and 60% after 1, 12 and 24 h of treatment with the SCD blocker (Figure 2a). The inhibition of SCD activity also evoked a progressive decrease in the palmitoleic acid (16:1n-7)-to-palmitic acid (16:0) ratio (Figure 2b). Interestingly, cancer cells exhibited low levels of polyunsaturated fatty acids, with arachidonic acid (20:4 n-6) and docosahexenoic acid (22:6 n-3) as the most abundant members of this fatty acid group. These fatty acids were slightly increased in cells with suppressed SCD activity (20:4 n-6: Control, $5.77 \pm 0.16$, CVT, $6.11 \pm 0.16$; 22:6 n-3: Control, $1.33 \pm 0.04$, CVT, $1.53 \pm 0.04$). Taken together, these results reveal that the pharmacological inhibition of SCD activity globally impairs the synthesis of MUFA species, similarly to what we reported in cells with genetic ablation of SCD1 expression.[14] More importantly, our data show that the ablation of Δ9-desaturase activity has an immediate and profound impact on the fatty acid content of cell lipids and unambiguously indicates that SCD activity is the most critical determinant of the MUFA/SFA balance in cancer cells.

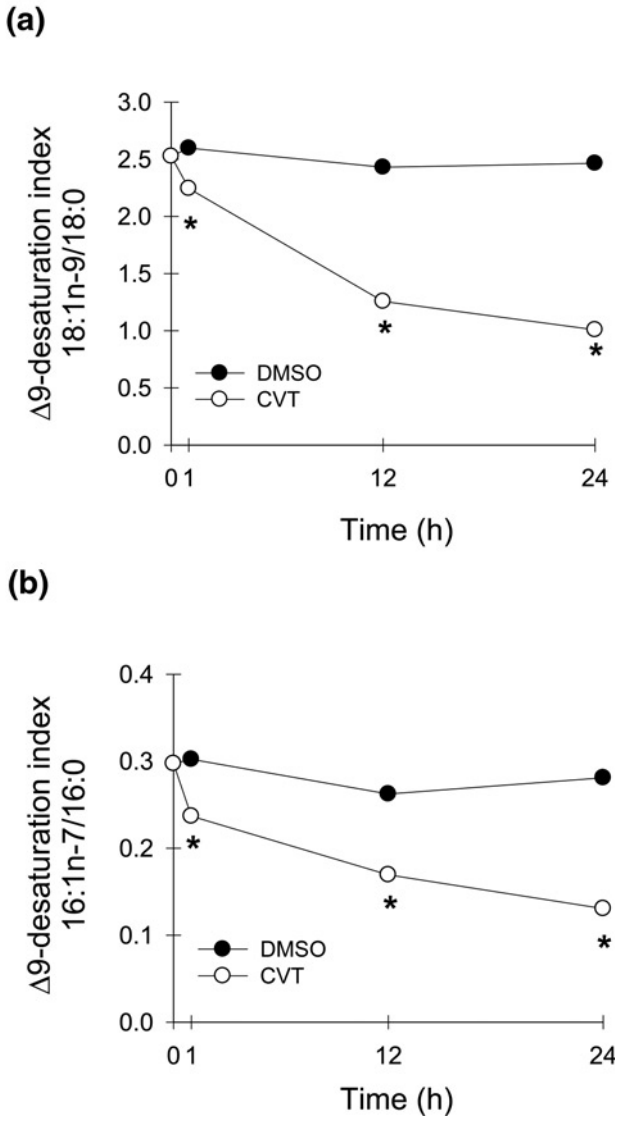


**Figure 2** Effect of stearoyl-CoA desaturase inhibition on monounsaturated fatty acid/saturated fatty acid ratios in total cell lipids. H460 cells were incubated with 1 μmol/L CVT-11127 (CVT) or dimethyl sulfoxide (DMSO) for up to 24 h. Total cell lipids were extracted and fatty acid composition was assessed by gas chromatography. Values express the ratio of oleic/stearic acids (a) and palmitoleic/palmitic acids (b) and represent the mean ± SD of 4–5 samples. $^{*}P < 0.01$ or less, by Student's *t*-test

## Inhibition of SCD1 alters the EGF-dependent signaling mechanism

Recent data indicate that SCD1 controls the oncogenic program by the coordinated regulation of lipid biosynthetic routes and signaling pathways critical for cell survival such as PI3K/Akt and Ras/MAPK (ERK) pathways.[9,18] In these studies, it was observed that inhibition of SCD1 leads to lower basal levels of phosphorylated (catalytically active) Akt and ERK. The mechanisms by which SCD1 modulates these signaling cascades have not yet been explored; hence, we investigated the role of MUFA biosynthesis in the functionality of EGFR, a tyrosine kinase receptor that activates Akt and ERK cascades and is critical for lung cancer onset.[20] To evaluate the role of SCD activity in activation of EGFR, H460 cells, after a 24 h-treatment with CVT-11127 or DMSO (vehicle), were stimulated with EGF for 5 min and the phosphorylation of tyrosine (Tyr) residues in EGFR was examined by immunoblotting. EGFR contains 12 Tyr sites that are targeted for phosphorylation upon ligand binding, each one eliciting specific responses related to activation of several signaling pathways, including PI3K/Akt, Ras and protein kinase C,[34,35] such as Tyr1068 and Tyr1086. Figure 3a shows that in control cells, EGF produced a robust phosphorylation of these two Tyr residues whereas stimulation with neuregulins, a ligand that activates ErbB3/ErbB4 receptors[36] and functions as a negative control for EGFR activation, failed to induce Tyr phosphorylation. When cells undergoing a blockade of SCD were stimulated with EGF, the phosphorylation of EGFR Tyr1068 and Tyr1086 by EGF was markedly reduced, indicating for the first time a direct relationship between MUFA synthesis and activation of EGFR in cancer cells. The EGF-induced phosphorylation of Tyr1045, an EGFR site that is relevant for the internalization and degradation of the receptor,[37] was also impaired by the inhibition of SCD by CVT-11127 (Figure 3a), suggesting that the overall activation of EGFR may be affected by changing levels of MUFA synthesis. In addition, it was observed that the blocking effect of SCD inhibition was restricted to the EGFR, at least in H460 cells, since phosphorylation of Tyr877 and Tyr1212 of ErbB2 (Her2) by EGF was not affected by CVT-11127 (data not shown).

In order to further validate the concept that MUFA are essential molecules in the regulation of the EGFR signaling pathway, we determined if addition of exogenous oleate

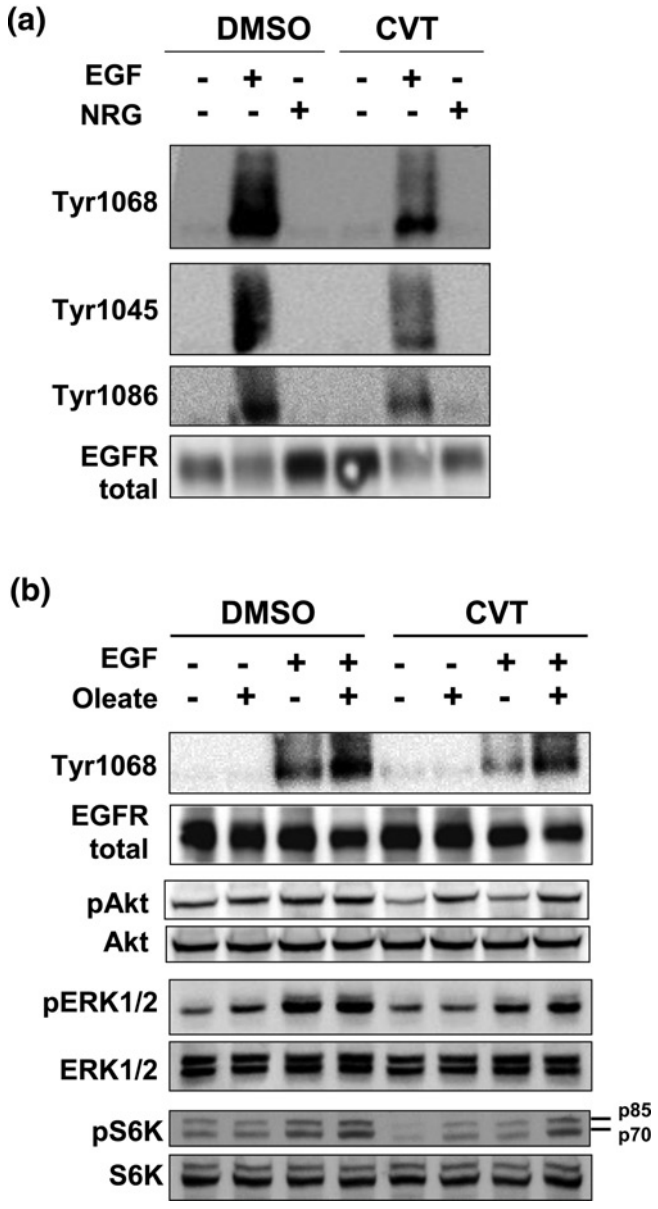


**Figure 3** The inhibition of stearoyl-CoA desaturase activity impairs the ligand-induced Tyr phosphorylation of epidermal growth factor receptor (EGFR). H460 lung cancer cells were treated with 1 $\mu$mol/L CVT-11127 (CVT) or dimethyl sulfoxide (DMSO) for 24 h and stimulated with 100 ng/mL EGF or NRG (a). Cells were also treated with CVT or DMSO for 24 h in the presence or absence of 100 $\mu$mol/L oleate and phosphorylation of tyrosine residues in EGFR was determined by Western blot (b)

would restore the low phosphorylation levels of EGFR in cells with ablated SCD activity. Thus, H460 cells treated with the SCD inhibitor or DMSO vehicle, both in the presence or absence of 100 $\mu$mol/L sodium oleate, were stimulated with EGF for 5 min and phosphorylation of Tyr1068 was determined (Figure 3b). In control cells, oleic acid alone did not have any effect on Tyr1068 phosphorylation but significantly enhanced the EGF-mediated phosphorylation of this Tyr residue, demonstrating that membrane MUFA do not stimulate EGFR activation *per se* but are essential for the ligand-induced stimulation of the receptor. Moreover, in cells in which SCD activity was ablated, addition of oleic acid restored the EGF-induced Tyr phosphorylation only to a level that was similar to control cells stimulated with the ligand, suggesting that exogenous MUFA can only partially replace their endogenously synthesized counterparts.

Phosphorylation of Tyr1068 and Tyr1086 in EGFR are functionally linked to PI3K→Akt activation; thereby, we next analyzed the effect of SCD1 blockade on EGF-mediated activation of Akt in H460 cells. In agreement with previously reported observations,[9,18] the levels of phosphorylated Akt in unstimulated conditions were lower in cancer cells where SCD was inhibited than in the respective controls (Figure 3b). Treatment of non-stimulated cells with oleate increased the content of phosphorylated Akt, an effect that implies a role for MUFA as sole activators of Akt in cancer cells. Incubation with EGF for 5 min evoked an activation of Akt in vehicle-treated controls, but this response was notably impaired in cells with a blockade in SCD activity. The presence of oleic acid restored basal Akt phosphorylation in SCD inhibitor-treated cells both in the presence and absence of EGF to levels detected in control cells, indicating that MUFA are required for full activation of EGFR→PI3K/Akt cascade.

Interestingly, we also found that SCD activity modulates the activation of the mTOR pathway, a known downstream target for PI3K/Akt.[38] mTOR controls multiple cellular functions by triggering the phosphorylation of p70-S6 kinase, one of its main substrates and signaling surrogates.[39] In control cells subjected to stimulation with EGF, the phosphorylation of p70-S6 kinase was markedly increased, whereas oleic acid alone did not modify the phosphorylation level in this protein (Figure 3b). The presence of oleic acid in control cells enhanced the phosphorylation of p70-S6 kinase upon stimulation with EGF. In unstimulated cells, the blockade of SCD activity led to a drastic reduction in the levels of phospho-p70-S6K which was reversed by addition of oleic acid (Figure 3b). Stimulation of p70-S6K phosphorylation by EGF was similar to oleic acid in CVT-treated cells; however, there was an additive effect on p70-S6K when CVT-treated cells were treated with both oleic acid and EGF.

Akt is also modulated by the rate of dephosphorylation. Akt is inactivated by the phosphatase and tensin homolog (PTEN), a phosphatase that removes the 3′-phosphate from PIP3 introduced by PI3K.[40] Therefore, a potential increase of PTEN levels in SCD1-deficient cells could contribute to downregulating the activity of the Akt pathway since it is possible that an accumulation of SFA in cells with reduced SCD1 expression could activate PTEN.[41] However, no changes in the expression of PTEN were detected in cells with a blockade in SCD activity (data not shown). Moreover, protein phosphatase 2A (PP2A), a major protein serine/threonine phosphatase that regulates many signaling pathways in mammalian cells, is upregulated by palmitic acid, leading to dephosphorylation of Akt.[42] We have observed an accumulation of palmitic acid in cancer cells with reduced SCD1 expression; therefore, it may be possible that the low levels of phosphorylated Akt in these cells could be attributed to an SFA-mediated induction of PP2A. However, we did not detect a significant change in the levels of PP2A in cells undergoing inhibition

of SCD1 for 24 h (data not shown), confirming that SCD1-mediated regulation of Akt occurs mainly through inhibition of Akt phosphorylation.

EGFR is also known to activate the Ras→ERK cascade, which is a critical effector of cancer cell proliferation.[19,20] Unlike its effect on Akt phosphorylation, treatment of unstimulated cells with oleic acid did not increase the content of phosphorylated ERK in either SCD-deficient or control cells (Figure 3b). As expected, stimulation of H460 cells with EGF increased the phosphorylation of ERK, but this effect was markedly blunted by the inhibition of SCD1 activity with CVT-11127 (Figure 3b). In EGF-stimulated cells, the low levels of phosphorylated ERK in SCD1-depleted cells were restored, albeit partially, by the presence of exogenous oleic acid, demonstrating the critical role of MUFA in the activation of Akt and ERK pathways.

### Inhibition of SCD activity decreases the mobility of fluid domains in plasma membranes

The activation of plasma membrane-resident signaling platforms, like EGFR-associated signaling cascades, is sensitive to the composition of the surrounding lipid microenvironment.[25,26] It has been reported that the membrane lipid microenvironment in cancer cells and tumor tissues is significantly more fluid than in their normal counterparts,[43–45] likely due to their greater MUFA/SFA ratio in phospholipids promoted by high SCD activity.[14,15,43] Having observed that ablation of SCD activity substantially reduces the MUFA/SFA ratio in cell lipids, we inferred that these marked compositional changes may affect the mobility of plasma membrane lipid domains, particularly the MUFA-enriched non-raft structures. Thus, in living H460 cells with reduced SCD activity and in controls, we determined the rate of lateral diffusion of lipids by FRAP. We used the fluorescent-labeled probes cholera toxin B-Alexa, which specifically binds to ganglioside GM3 in liquid-ordered raft domains, and DiIC18, which specifically partitions in liquid-disorder (non-raft) domains. It was observed that blockade in SCD1 activity clearly decreased lateral mobility of fluid domains in the plasma membrane of cells (Figure 4a), suggesting that MUFA modulates the fluidity of these lipid domains. SCD appears to specifically affect the dynamics of non-raft domains since the mobility of lipid rafts was virtually unmodified by ablation of SCD activity (Figure 4b).

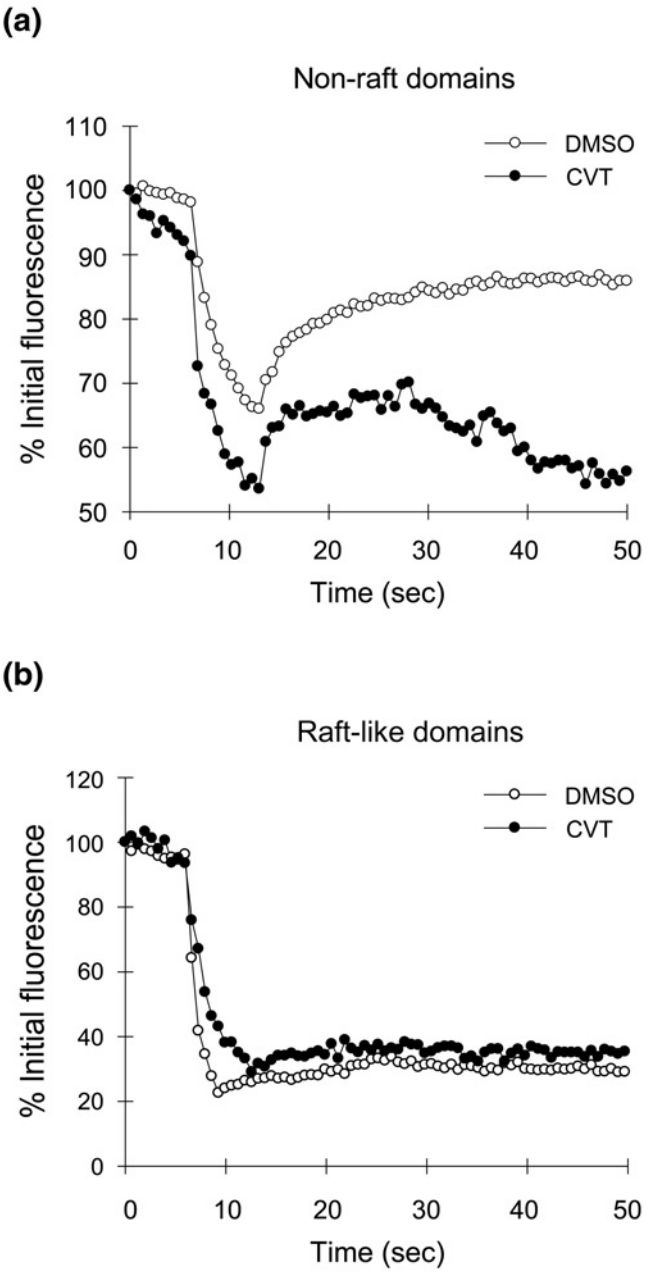


**Figure 4** Reduced lateral mobility of non-raft lipid domains in stearoyl-CoA desaturase 1-deficient cells. In H460 cells treated with either 1 $\mu$mol/L CVT-11127 (CVT) or dimethyl sulfoxide (DMSO) for 24 h, lateral mobility of non-raft (a) and raft (b) lipid domains was determined by fluorescence recovery after photobleaching with DiIC18 and cholera toxin B-Alexa488, respectively

### Abrogation of SCD activity reduces EGF-induced proliferation in lung cancer cells

The functionality of the EGFR pathway is critical for cancer cell mitogenesis.[19] Having observed a significant deactivation of EGF-stimulated Akt, ERK and mTOR signals, the main mitogenic downstream effectors of EGFR, we evaluated if induction of cell proliferation by EGF could be impaired in SCD-depleted cells. We observed that addition of EGF for 48 h increased the population of H460 cells with respect to unstimulated cells (Figure 5a). Notably, the EGF-induced mitogenesis was fully blocked in SCD-inactivated cells, implying that EGFR-mediated enhancement of cell proliferation was indeed compromised in cells with impaired MUFA synthesis.

### Sensitivity to the EGFR inhibitor gefitinib in cells with a blockade in SCD activity

Selective small molecule inhibitors of the tyrosine-kinase domain in EGFR have been tested as monotherapy in patients with relapsed NSCLC, but patients become resistant to anti-EGFR therapies.[24] Having observed that SCD blockade markedly impairs the functionality of the EGFR signaling platform, we aimed to investigate if SCD inhibitors could

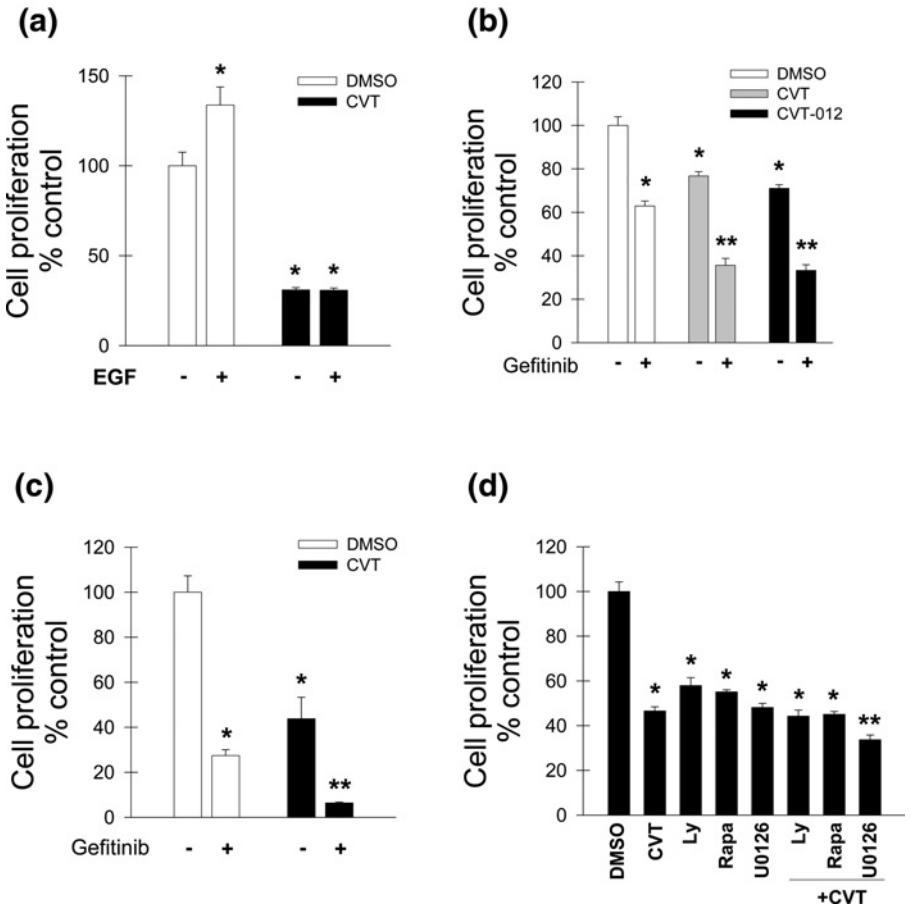


**Figure 5** The inhibition of stearoyl-CoA desaturase (SCD) activity impairs epidermal growth factor (EGF)-mediated proliferation. Effect of SCD and EGF receptor inhibitors. (a) Cell proliferation was determined in H460 lung cancer cells that were treated with 1 $\mu$mol/L CVT-11127 (CVT) or dimethyl sulfoxide (DMSO) in 2% fetal bovine serum Dulbecco's modified eagle's media for 48 h in the presence or absence of 100 ng/mL EGF. Additionally, cell proliferation was assessed in H460 cells (b) and A431 cells (c), treated with either 1 $\mu$mol/L CVT-11127, an orally bioavailable SCD inhibitor CVT-01012, or DMSO for 48 h in the presence or absence of 10 $\mu$mol/L gefitinib. A similar cell growth determination was performed in H460 cells incubated with CVT plus or minus Ly294002 (phosphatidylinositol-3 phosphate kinase inhibitor, 20 $\mu$mol/L final concentration), U0126 (MEK inhibitor, 20 $\mu$mol/L) or rapamycin (mammalian target of rapamycin inhibitor, 100 nmol/L) (d). In all experiments, density of cell populations was determined by crystal violet assay. $^{*}P < 0.05$ or less versus vehicle-treated control cells

synergize the cytotoxic effect of gefitinib on cancer cells. Gefitinib-resistant H460 lung cancer cells were incubated with 10 $\mu$mol/L gefitibib in the presence or absence of 1 $\mu$mol/L CVT-11127 or CVT-12012, an orally bioavailable SCD inhibitor from a distinct chemical scaffold, for 24 h and cell proliferation was analyzed (Figure 5b). It was observed that the EGFR inhibitor alone led to an ~40% decrease in the cell proliferation rate. Remarkably, the combination of gefitinib and either SCD inhibitor had a significant additive inhibitory effect on cell growth. A more profound inhibitory response was observed in A431 carcinoma cells, a gefitinib-sensitive cell line, when these cells were treated with CVT, gefitinib or a combination of these two inhibitors (Figure 5c). Furthermore, the antigrowth effect of the combined treatment was not cell strain-specific since a similar reduction in cell number was observed in two additional gefitinib-resistant lung cancer cell lines, H1299 and A549 cells (data not shown).

Inhibitors of signaling proteins belonging to the EGFR signaling cascade, such as PI3K, ERK and mTOR, have profound antigrowth activity in cancer cells.[46] In order to evaluate if suppressing the activity of SCD would potentiate the cytostatic activity of these signaling inhibitors, H460 cells were treated with LY29004, U0126 and rapamycin, which are inhibitors of PI3K, ERK and mTOR, respectively, in the presence of CVT-11127 or vehicle and cell proliferation was determined. As expected, a significant suppression of cell growth was observed when cells were incubated with either LY29004, U0126 or rapamycin (Figure 5c). With the exception of the ERK inhibitor, which exhibited a modest additive effect, the association of these cell growth signaling blockers with the SCD inhibitor did not amplify the antigrowth impact of these compounds, suggesting that these inhibitors may suppress common metabolic and signaling routes. Altogether, our results indicate that treatment of lung cancer cells with a combination of an EGFR signaling pathway inhibitor and an SCD inhibitor may be an improved therapeutic strategy in lung cancer intervention.

## Discussion

Increasing evidence suggests that the constitutive activation of MUFA synthesis, propelled by elevated SCD1 expression,

plays a critical role in the process of malignant transformation.[3,33] As a key contribution to the metabolic shift toward lipogenic conditions observed in cancer cells, SCD1 promotes the activation of the fatty acid biosynthetic pathway while suppressing lipid oxidation signals.[14,15] Recently published research also show that the activity of signaling pathways that induce cell proliferation and promote cell survival, such as Akt and ERK, are positively associated with the expression and activity levels of SCD1.[9,18] However, the molecular mechanisms by which SCD activity affects signaling regulation have not been revealed. Using EGFR, a paradigmatic tyrosine kinase receptor involved in lung cancer onset as a signaling platform model, we here show that SCD regulates the activity of growth and survival signaling pathways by modulating the ligand binding-induced phosphorylation activation of the tyrosine-kinase receptor. Our finding that MUFA biosynthesis is required for the full activation of EGFR is consistent with *in vitro* studies showing that phospholipids enriched in MUFA induce activation of EGFR by triggering tyrosine autophosphorylation whereas saturated phosphatidylcholine causes the opposite effect.[47] In studies done in fibroblasts, oleic acid added to culture media was shown to activate EGFR phosphorylation.[48] Unsaturated fatty acids other than MUFA appear to have a positive effect on the activating autophosphorylation of EGFR in cancer cells since docosahexenoic acid (22:6n-3) was shown to increase phosphorylation of its Tyr1068 residue when offered to A549 lung cancer cells.[49]

Importantly, our studies show that unlike FAS inhibition, which decreases the levels of both total and phosphorylated EGFR,[50] pharmacological suppression of SCD activity appears to specifically affect the mechanism of EGFR autophosphorylation. Furthermore, our finding that abrogation of SCD activity impaired the phosphorylation of all four Tyr residues in EGFR tested in our experiments implies the notion that SCD1 modulates the overall process of ligand-mediated receptor activation. This global alteration in EGFR phosphorylation produced by abrogation of SCD activity carried functional consequences for the cancer cell, since the EGF-mediated activation of its main downstream effectors Akt, mTOR and MAPK was observed to be markedly impaired. Among the tyrosine residues in EGFR analyzed in the present study, phosphorylation of Tyr1068 is critical for Akt activation whereas Tyr1086 and Tyr1173 residues signal PI3K through activation of Grb2-Ras and PLC-γ.[51,52] Therefore, it is conceivable to postulate that the reduced EGF-induced phosphorylation of Akt and ERK in SCD1-ablated cells may have been directly caused by a global impairment of the ligand-mediated phosphorylation of EGFR residues in these cells.

The EGFR-activated signaling cascades transduce signals that globally stimulate lipid synthesis,[53] and activate mitogenesis and the oncogenic program in cancer cells.[20] Thus, the observation of a suppressed mitogenic response in cells with blocked SCD activity may be the result of an impaired activation of the EGFR signaling complex exhibited by these cells. Furthermore, the impact of the deregulation of EGFR signaling caused by the inhibition of SCD activity may also affect lipid metabolism. EGF is a known activator of lipogenesis in normal cells;[20] hence, it is possible that the inactivation of the EGFR→Akt/ERK/mTOR signaling network by SCD1 ablation may be responsible for the low lipogenesis rate previously reported in SCD1-deficient neoplastic cells.[14,15] Moreover, since EGF stimulates the biosynthesis of MUFA by inducing the expression of SCD1,[28] it can be hypothesized that by maintaining this positive feedback loop involving the activation of EGFR and SCD1, cancer cells are able to promote metabolic (lipogenic) and signal transduction (progrowth and prosurvival) conditions that are optimal for the full expression of the malignant phenotype.

Although the mechanistic connection between the SCD and the activation of EGFR and downstream signals remain unknown, it is plausible that greater levels of endogenously produced MUFA may modify the architecture and dynamics of plasma membrane lipid domains, therefore altering the functionality of membrane-resident signaling platforms such as EGFR. It was reported that cancer cells and tumor tissues exhibit a more fluid membrane microenvironment,[43–45] likely due to dramatic enrichment of major structural phospholipids in cell membranes with MUFA.[9,14,15] In CHO cells, elevated SCD1 expression increases the abundance of less ordered lipid domains (non-raft) in plasma membranes.[54] We found that the profound reduction in the MUFA-to-SFA ratio in lipids of SCD-depleted cells was accompanied by a significant decrease in the lateral mobility of non-raft domains in plasma membrane, reinforcing the notion of a critical role for SCD activity in the regulation of plasma membrane homeostasis. As a plasma membrane-resident protein, EGFR activation and function is modulated by its surrounding lipid microenvironment[40] and a change in membrane lipid composition, particularly its MUFA content, may modify the signal transduction that is initiated at the transmembrane receptors. Elegant studies by Pike's lab indicated that in basal conditions, EGFR is found in equal proportions between raft and surrounding non-raft domains, whereas upon stimulation with EGF, the great majority of EGFR (~90%) migrates to more fluid non-raft structures.[25] This is consistent with data from other reports suggesting that migration of raft-resident EGFR to the surrounding non-raft compartments may be required for its functional activation.[32,55] Moreover, exogenous polyunsaturated fatty acids were shown to alter the partition of EGFR in rafts and increase the phosphorylation of the receptor upon stimulation.[49] We observed that mobility of non-raft fractions in the plasma membrane is severely constrained by the abrogation of SCD activity, suggesting that the physicochemical properties of these lipid domains may not be optimal for EGFR activation. Downstream signaling effectors of EGFR such as Akt and Ras oncogenes have been shown to also partition between rafts and non-raft structures in plasma membrane during their ligand-triggered activation;[56,57] thereby, an alteration of the structural and functional homeostasis of these lipid microdomains may directly affect the activation rate of these signaling proteins.

Our findings that SCD activity is a major regulator of the EGFR pathway may be clinically relevant. Drugs that block the tyrosine-kinase activity of EGFR in lung cancer cells are

currently used in conventional chemotherapy; however, some lung cancer types, such as NSCLC, display resistance to this pharmacotherapy.[24] The fact that that proliferation of H460 cells, a NSCLC cell type that is resistant to EGFR blockers, was more drastically reduced by co-treatment with SCD inhibitors and gefitinib, a well-studied EGFR tyrosine kinase antagonist, suggests a potential for SCD blockers in the treatment of chemo-resistant types of lung cancer. Interestingly, the SCD inhibitor potentiated the anti-growth action of the MAPK inhibitor U0126, but not PI3K and mTOR inhibitors, implying that these latter agents may target similar biological mechanisms than the SCD blocker in the cancer cell. Finally, the finding that blocking SCD activity synergizes the cytotoxic effect of both EGFR and MAPK inhibitors further suggests a therapeutic value for SCD inhibitors, alone or in combination with other anti-cancer agents, in lung cancer treatment.

In conclusion, our studies provide the first evidence showing that the rate of MUFA biosynthesis, which in cancer cells appears to be the main controller of membrane fatty acid composition, directly modifies the functionality of EGFR in cancer cells. We provide information supporting a novel mechanism in which SCD1 modulates cancer cell growth by controlling the activation of EGFR and, consequently, their downstream effectors in the Akt, ERK and mTOR pathways. A key aspect of our hypothetical mechanism is that the constitutive activation of SCD (predominantly SCD1 activity) in cancer cells dramatically elevates the MUFA-to-SFA ratio in membrane lipids, which promotes an increase in the mobility of non-raft lipid domains of plasma membranes. This alteration in the lipid microenvironment may favor the partition of EGFR out of rafts and into more fluid non-raft lipid domains of plasma membrane that facilitates autophosphorylation and activation of the receptor signaling cascade (Figure 6). The enhanced activity of the EGFR-induced signaling pathways driven by chronically elevated SCD activity may ultimately provide the appropriate intracellular signals that trigger and sustain the metabolic and biological traits of cancer cells. However, the temporal sequence of events and topographical interaction of the signaling and enzymatic components that control cancer life that are modulated by SCD1 are presently unknown and will require additional research for their elucidation.

**Author contributions:** MN, JWC and RAI conceived, designed and performed the experiments, analyzed the data and wrote the paper.

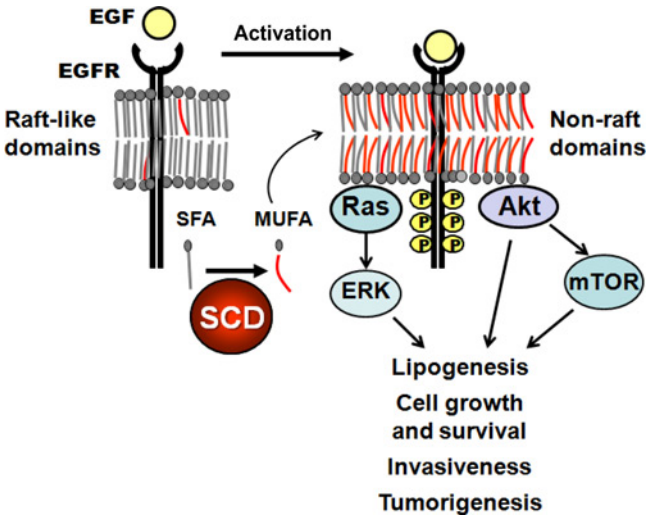


**Figure 6** Hypothetical mechanism of regulation of epidermal growth factor receptor (EGFR) signaling activation by stearoyl-CoA desaturase 1 (SCD1) in lung cancer cells. Constitutive activation of SCD1 in cancer cells leads to a greater enrichment of phospholipids lipids with monounsaturated fatty acids, which increases the fluidity of non-raft microdomains in plasma membrane. These changes in the dynamics of non-raft structures may favor the partition and consequent activation of plasma membrane-resident EGFR and its downstream signaling effectors Akt and Ras that interact with this membrane

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