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Stearoyl-CoA Desaturase regulates sorafenib resistance via modulation of ER stress induced differentiation

Mark Kin Fai Ma[1,2*], Eunice Yuen Ting Lau [1,7,8 *], Doris Hoi Wing Leung[1,7], Jessica Lo[1,7], Nicole Pui Yu Ho[1,7], Lily Kwan Wai Cheng[3], Stephanie Ma[1,3], Chi Ho Lin[4], John A Copland[5], Jin Ding[6], Regina Cheuk Lam Lo[1,2], Irene Oi Lin Ng[1,2 #], Terence Kin Wah Lee[1,7,9 #]

[1]State Key Laboratory for Liver Research, The University of Hong Kong, [2]Department of Pathology, [3] School of Biomedical Sciences, [4] Centre for Genomic Science, Li Ka Shing Faculty of Medicine, The University of Hong Kong, [5]Department of Cancer Biology, Mayo Clinic Florida, Jacksonville, [6]Eastern Hepatobiliary Surgery Hospital, The International Cooperation Laboratory on Signal Transduction,[7]Department of Applied Biology and Chemical Technology,[8]Department of Clinical Oncology, Queen Elizabeth Hospital, [9]State Key Laboratory Chirosciences, The Hong Kong Polytechnic University

*contributed equally

[#]Corresponding authors:

Dr. Terence K.W. Lee is Room 805, Block Y, Department of Applied Biology and Chemical Technology, Lee Shau Kee Building, The Hong Kong Polytechnic University, Hong Kong. Tel: (852) 3400-8799; Fax: (852) 2364-9932; Email: terence.kw.lee@polyu.edu.hk or Prof. Irene O.L. Ng, Room 127B, University Pathology Building, Department of Pathology, The University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong. Tel: (852) 2255-3967; Fax: (852) 2872-5197; Email: iolng@hku.hk

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BACKGROUND & AIMS: We investigated the functional role and clinical significance of Stearoyl CoA desaturase-1 (SCD1) mediated endoplasmic reticulum (ER) stress in regulation of liver tumor-initiating cells (T-ICs) and sorafenib resistance, aiming to develop a novel therapeutic strategy against hepatocellular carcinomas (HCCs)

METHODS: We evaluated the clinic-pathological relevance of SCD1 and its correlation with sorafenib resistance in large cohorts of HCC clinical samples by qPCR and immunohistochemical analyses. Lentiviral-based overexpression and knockdown approaches were performed to characterize functional roles of SCD1 in regulation of liver T-ICs and sorafenib resistance. Molecular pathways mediating the phenotypic alterations was identified through RNA sequencing analysis and functional rescue experiments. The combinatorial effect of SCD1 inhibitor and sorafenib was tested using our patient-derived tumour xenograft (PDTX) model.

RESULTS: SCD1 overexpression was found in HCC which was associated with shorter disease free survival. SCD1 was found to regulate the populations of liver T-ICs; while its suppression by SCD1 inhibitor suppressed liver T-ICs and sorafenib resistance. Interestingly, SCD1 was markedly upregulated in our established sorafenib-resistant PDTXs, and its overexpression predicts the clinical response of HCC patients to sorafenib treatment. Suppression of SCD1 forces liver T-ICs to differentiate via ER stress induced unfolded protein response (UPR), resulting in their enhanced sensitivity to sorefenib. Using a patient-derived xenograft model (PDTX#1), we found that a novel SCD1 inhibitor (SSI-4) demonstrated maximal growth suppressive effect when combined with sorafenib treatment.

CONCLUSIONS: SCD1 mediated ER stress regulates liver T-ICs and sorafenib sensitivity. Targeting SCD1 alone or in combination with sorafenib might be a novel personalized medicine against HCC.

LAY SUMMARY:

In this study, we found that SCD1 plays a critical role in regulation of liver tumor initiating cells and sorafenib resistance through regulation of ER stress mediated differentiation. Targeting SCD1 in combination of sorafenib may be a novel therapeutic strategy against liver cancer.

Liver cancer (hepatocellular carcinoma, HCC) is one of the deadliest diseases, being the fifth most commonly diagnosed cancer and second leading cause of cancer mortality in the world (1). The frontline treatment for this disease is liver transplantation and surgical resection. Despite intensive research into better therapeutic options, the only success we have so far is with the multi-kinase inhibitor sorafenib. It is the only drug currently approved by the FDA and serves as the standard treatment for HCC patients in advanced stages. However, sorafenib is reported to only increase the median survival of HCC patients by 3 months (2), partly due to drug resistance (3,4). Thus, it is clear that there is an urgent need to seek novel therapeutic targets that have high efficacy and specificity to HCC cells.

Recently, solid evidence has emerged in support of a cancer stem cell (CSC)/tumor-initiating cell (T-IC) model in leukemia and a wide range of solid tumors, including HCC (5-8). T-ICs are now regarded as the source of tumor origins and are implicated in recurrence and therapeutic resistance. Recently, we have established sorafenib-resistant cell lines and found that these cells are endowed with enhanced T-IC properties and exhibit an increase in liver T-IC markers, including CD47 (9). Our data, together with other studies showing that $EpCAM^{+}$ and label-retaining HCC cells are more resistant to sorafenib treatment, strongly support the hypothesis that targeting the signaling pathways that mediate the functions of liver T-ICs may be a possible way to reverse sorafenib resistance in cancers. As part of the continuous pursuit of novel signaling pathways in liver T-ICs, we used our previously established chemoresistant hepatospheres (10) and analyzed the genes/pathways that were upregulated in this enriched T-IC population. Interestingly, we found activation of a lipogenesis pathway, in which stearoyl-CoA desaturase-1 (SCD1) was found to be the most upregulated enzyme. SCD1 is a key enzyme located in the endoplasmic reticulum that catalyzes desaturation of lipid (11). In lipogenesis pathways, SCD1

works synergistically with other major lipogenic enzymes such as fatty acid synthase, acetyl Co-A carboxylase and ATP citrate lyase. However, the role of SCD1 in liver T-ICs remains unknown, and its role in HCC has not been clearly addressed.

In this study, we examined whether SCD1 plays a crucial role in sorafenib resistance through the regulation of liver T-IC function. First, we found that over-expression of SCD1 in a cohort of HCC samples was associated with shorter disease-free survival. SCD1 was found to regulate the traits of CSCs. The results were also validated by the employment of SCD1 inhibitor A939572. Furthermore, SCD1 was markedly upregulated in our established sorafenib-resistant cell lines as well as in patient-derived xenografts (PDTXs), and its suppression sensitized HCC cells to the effects of sorafenib. Clinically, SCD1 serves as a good predictive marker for patient responses to sorafenib treatment. Using a patient-derived xenograft model (PDTX#1), we found that a novel SCD1 inhibitor, SSI-4, demonstrated maximal growth suppressive effects when combined with sorafenib treatment. Mechanistically, we found that the activity of the endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR) was upregulated in SCD1-knockdown cells. Suppression of SCD1 forces liver CSCs to differentiate via the induction of ER stress, which results in their enhanced sensitivity to sorafenib both *in vitro* and *in vivo*.

Methodology

Human tissue specimens. All the human tissue specimens were obtained from the Department of Surgery, Queen Mary Hospital, Hong Kong. Specimens were collected with informed consent, and the study was approved by the Institutional Review Board of the University of Hong Kong and Hong Kong Hospital Authority. Liver tumor specimens of two HCC patients who had undergone hepatectomy between 2011 and 2012 were chosen for the xenograft experiment (PDTX#1 and PDTX#5).

In vivo therapeutic targeting and its combined effect with sorafenib. The study protocol was approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at Hong Kong Polytechnic University. The xenograft was established in 4- to 6-week-old male NOD/SCID mice using the PDTX#1. Treatment was started once the size of the xenograft reached approximately 5×5 mm (length × width). The mice were then randomly assigned into four groups, each consisting of 4 mice. To investigate the effect of SCD1 inhibitor alone and in combination with sorafenib, the four groups of mice were assigned as follows: control (solvent only), sorafenib (10 mg/kg), SSI-4 (10 mg/kg) and sorafenib with SSI-4 (both 10 mg/kg). Sorafenib was dissolved in DMSO, while SSI-4 was dissolved in PEG 400 (Affymetrix) and Tween 80 (Anatrace) (PEG 400:Tween 80 ratio is 4:1). Sorafenib was dissolved in 100% dimethyl sulfoxide (DMSO) to prepare a stock solution of 200 mg/mL. Each mouse received sorafenib diluted in H2O at 10mg/kg (final concentration, 1% v/v) or control vehicle (DMSO 1%, v/v). The mice were fed with SCD1 inhibitor or sorafenib orally on a daily basis. The size of the tumors was recorded every other day continuously for 26 days. Harvested tumors were fixed with formalin and subjected to immunohistochemical (IHC) analysis.

Statistical analysis. The statistical significance of the results obtained from qPCR, spheroid formation assays, flow cytometry analysis, invasion and migration assays was determined by Student's t-test using Microsoft Office Excel software (Microsoft Corporation, Redmond, WA, USA). The results are shown as the means and standard deviations, and p-values less than 0.05 were considered statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Kaplan-Meier survival analysis was used to analyze disease-free survival and overall survival, and the statistical significance was calculated by log-rank test; these analyses were carried out using SPSS 20. Additional experimental procedures are provided in the supplementary information.

RESULTS

SCD1 was upregulated in enriched liver T-IC populations and sporadically expressed in human HCC specimens

In our previous study, we established drug-resistant hepatospheres derived from PLC/PRF/5 by the administration of chemotherapeutic drugs (cisplatin and doxorubicin), through 16 serial passages (10). Successful enrichment of liver T-ICs was evidenced by increases in the abilities of self-renewal and tumor initiation. The genetic profiles between the drug-resistant hepatospheres and their differentiated counterparts were analyzed by cDNA microarray. From the upregulated genes, we found that genes related to the lipogenesis pathway were highly upregulated, among which stearoyl-CoA desaturase isoform 1 (SCD1) was found to be the most upregulated gene (Figure 1A). To further validate the results from the microarray data, the expression of SCD1 was confirmed by qPCR. Consistently, the drug-resistant hepatospheres showed more than a 28-fold upregulation of SCD1 expression when compared to their differentiated progenies (Figure 1B). To examine whether SCD1 expression is clinically relevant, we examined the expression of SCD1 at

both the mRNA and protein levels in a cohort of HCC clinical specimens. First, we randomly selected 70 HCC patient specimens and analyzed the expression of SCD1 in tumor and non-tumor counterparts by qPCR analysis (Figure 1C). Approximately 60% of the patients showed an upregulation of SCD1 mRNA in their tumor tissue, when compared with the non-tumor counterpart. A cutoff value of T/N > 2 was used to classify SCD1 expression in HCC patients. Immunohistochemical staining was also employed to examine the protein expression level of SCD1 in patient samples (Figure 1D). A quick score method was employed to quantitate the expression of SCD1 in 44 cases of HCC specimens (details can be found in the supplemental information). Patients with a high expression of SCD1 were correlated with a shorter disease-free survival (p = 0.008, log rank test) (Figure 1E). Using an Oncomine analysis, we found that overexpression of SCD1 was also observed in a variety of other cancers, including renal, colon, breast, bladder, pancreas and stomach (Supplementary Figure 1).

SCD1 regulates self-renewal, tumorigenicity, differentiation, and expression of liver T-IC markers

To further examine whether SCD1 functionally contributes to the traits of stem/progenitor cells, we performed a SCD1-knockdown experiment using a lentiviral-based approach. By qPCR and western blot analyses, we found a high expression of SCD1 in Huh7 and PLC/PRF/5, among others, in a panel of HCC cell lines (Figure 2A&B); therefore, these two cell lines were chosen for the knockdown experiment. After confirmation of successful SCD1 knockdown (shSCD1) (Figure 2B), we examined the tumorigenicity of PLC/PRF/5 cells upon SCD1 knockdown. As few as 500 cells derived from PLC/PRF/5 was sufficient to initiate tumor formation (Figure 2C). However, we found that both the number and size of the tumors formed in NOD/SCID mice were lower in

the shSCD1 clones compared with those in the non-target control group (Figure 2C, Supplementary Table 1A). A similar observation was found in Huh7 cells upon SCD1 knockdown (Figure 2C, Supplementary Table 1B). To determine whether SCD1 could regulate the self-renewal of HCC cells, control cells and shSCD1 cells were subjected to a spheroid formation assay. When compared to the control cells, significantly less and smaller spheroids were observed in shSCD1 cells from both PLC/PRF/5 and Huh7 cell lines (Figure 2D). To further investigate whether SCD1 regulates the T-IC population in HCC cells, we measured and compared the expression of two liver T-IC markers, CD24 and CD47, in shSCD1 cells and their control counterparts by flow cytometry. We found that shSCD1 cells exhibited a decrease in the percentages of these markers in HCC cells (Figure 2E). Next, we examined whether SCD1 regulates the liver T-IC population through intervening in the differentiation process. To examine this, we evaluated the expression of two differentiation markers (HNF4α and UGT2B7) in shSCD1 cells by qPCR analysis. Compared to the non-target control, we consistently found upregulation of these two differentiation markers in shSCD1 cells, suggesting that SCD1 maintains liver T-ICs by the suppression of differentiation (Figure 2F). To further confirm the role of SCD1 in the regulation of liver T-ICs, we employed the lentiviral-based overexpression approach to ectopically transfect SCD1 ORF into SCD1-low expressing Bel7402 cells. The successful establishment of a stable SCD1 overexpression clone was confirmed by western blot analysis (Supplementary Figure 2A). Opposite to the effects of SCD1 suppression, SCD1-overexpressing Bel7402 cells exhibited the enhanced abilities of tumorigenicity, self-renewal and expression of liver T-IC markers (Supplementary Figure 2B-D, Supplementary Table 1C).

SCD1 regulates invasion, migration and chemoresistance of HCC cells

Several reports have suggested that T-ICs possess a high metastatic potential (12,13). Consistent with the above findings, Matrigel-coated (for invasion) or Matrigel–uncoated (for migration) transwell assays showed that shSCD1 cells displayed significantly lower migration and invasive efficiencies in both PLC/PRF/5 and Huh7 cells (Figure 3A and B). Conversely, SCD1-overexpressing Bel7402 cells demonstrated a significant increase in migration and invasion capabilities (Figure 3C). Furthermore, knockdown of SCD1 also resulted in the sensitization of HCC cells to the commonly used chemotherapeutic drug doxorubicin (Figure 3D). Flow cytometry analysis of the percentage of dead cells demonstrated a decreased survival ability and lower resistance to doxorubicin following SCD1 knockdown, while the opposite effect was observed in Bel7402 cells upon SCD1 overexpression (Figure 3E).

SCD1 is upregulated in sorafenib-resistant cells and its expression predicts the clinical response of patients receiving sorafenib

In our previous study, we found that sorafenib-resistant HCC cells demonstrated enhanced T-IC properties, including tumorigenicity, self-renewal and expression of liver T-IC markers (9). This data, together with the data showing a regulatory role for SCD1 in liver T-ICs, led us to investigate whether SCD1 sensitizes the effect of sorafenib through the regulation of liver T-ICs. To test this hypothesis, we first examined the expression of SCD1 in our established sorafenib-resistant Bel7402 and Huh7 cells. Using western blot analysis, we found an upregulation of SCD1 protein in sorafenib-resistant HCC cells compared with their corresponding mock controls (Figure 4A). To further confirm this observation, we employed two sorafenib-resistant, patient-derived HCC tumor xenografts (PDTX#1 and PDTX#5) after treating with sorafenib for several rounds *in vivo*. By IHC staining, we consistently found the upregulation of SCD1 in the sorafenib-resistant

PDTXs compared with mock-treated controls (Figure 4B). Next, we wanted to confirm the role of SCD1 in the regulation of sorafenib resistance by examining the effect of SCD1 knockdown on the sensitivity of sorafenib in HCC cells. By annexin V staining, we found that shSCD1 cells in both Huh7 and PLC/PRF/5 cell lines showed decreased survival ability and lower resistance to sorafenib (Figure 4C). In addition, the effect of sorafenib on apoptosis in shSCD1 and control cells was evidenced by differential expression of apoptosis cascade proteins including Bcl2, Bax, cleaved caspase-9 and PARP (Supplementary Figure 3). To further investigate whether the role of SCD1 in sorafenib resistance is clinically relevant, we utilized a tissue microarray consisting of 90 HCC samples from patients who had been treated with sorafenib. The possible role of SCD1 in sorafenib resistance was examined by correlating SCD1 expression with the recurrence status as well as the patient survival by IHC staining (Figure 4D). A quick score method was employed to quantitate the expression of SCD1 in these samples as described above (Figure 4E). By IHC staining, we found that patients with high SCD1 expression were correlated with a higher chance of HCC recurrence after sorafenib treatment ($p<0.001$, χ^2 test) (Figure 4F). In addition, patients with high SCD1 expression were correlated with shorter disease-free survival ($p=0.022$, log-rank test) (Figure 4G). This result showed that SCD1 expression is a good predictive marker for the sorafenib response in HCC patients.

Pharmacological inhibition of SCD1 by A939572 suppresses self-renewal, migration, invasion and sorafenib resistance

To further explore the potential of therapeutic targeting of SCD1 in HCC, we wanted to examine whether the pharmacological inhibition of SCD1 by A939572 (BioVision) exerted a similar effect to SCD1 knockdown using the lentiviral-based approach. To address this question,

we first determined the dose of A939572 at which cell proliferation is minimally affected. By direct cell counting up to day 6, we found that doses of A939572 ranging from 1 μM to 5 μM exerted minimal effects on cell growth in both SCD1-high expressing Huh7 and PLC/PRF/5 cells (data not shown). Based on this result, we then determined whether A939572 at doses ranging from 1 μM to 5 μM exerted a suppressive effect on self-renewal ability. By the sphere formation assay, we found that A939572 suppressed the number and size of spheres formed in a dose-dependent manner (Figure 5A). In the migration and invasion assays, 5 μM A939572 significantly suppressed both the migration and invasion of Huh7 and PLC/PRF/5 (Figure 5B and C). To further confirm whether SCD1 suppression sensitized the cells to the effects of sorafenib, Huh7 and PLC/PRF/5 cells were treated with A939572 alone and in combination with sorafenib. By annexin V staining, we found that suppression of SCD1 by A939572 in both Huh7 and PLC/PRF/5 cells showed decreased survival ability and lower resistance to sorafenib (Figure 5D). To demonstrate the role of sorafenib resistance is specific for SCD1 among other genes in lipogenesis pathway, we have also examined the effect of targeting another lipogenic gene, FASN, on sorafenib sensitization. We found that Cerlenin, a potent FASN inhibitor, only exerted significant effect on sorafenib sensitization at the dosage of 20 μM, but not at lower doses that we used for A939572 (5 μM and 10 μM) (Supplementary Figure 4). This result showed that SCD1 plays a prominent role in regulation of sorafenib resistance among other genes in lipogenesis pathway.

SCD1 regulates the traits of liver T-ICs and sorafenib resistance through regulation of ER stress

To determine the major downstream mediator of SCD1, we employed RNA sequencing analysis to compare the gene expression profiles of shSCD1 (#13) and non-target control cells derived from both Huh7 and PLC/PRF/5. Using a fold-change cutoff of 1.2-fold and a p value ≤ 0.05, 406 genes

were found to be commonly de-regulated (Figure 6A). A pathway enrichment analysis identified critically over-represented pathways related to the biosynthesis of unsaturated fatty acids, the metabolism of xenobiotics by cytochrome P450, MAPK signaling pathway, TGF-beta signaling pathway and protein processing in the endoplasmic reticulum (Supplementary Figure 5). After subsequent qPCR confirmation of these pathways, we found upregulation of genes related to ER stress induced UPR activation in protein processing in the endoplasmic reticulum. As shown in Figure 6B, preferential overexpression of ER stress-related UPR genes in shSCD1 cells in both PLC/PRF/5 and Huh7 cells was observed. To determine whether SCD1 regulates the traits of liver T-ICs and sorafenib resistance through the suppression of ER stress, we suppressed ER stress by the administration of a potent inhibitor of ER stress, tauroursodeoxycholic acid (TUDCA), in the presence of A939572 to investigate whether the effects of SCD1 suppression could be eliminated upon addition of TUDCA. By western blot analysis, we found that TUDCA successfully inhibited the ER stress created by administration of A939572, as evidenced by the decrease in BIP expression upon administration of 100 μM or 200 μM TUDCA (Figure 6C). Next, we examined whether administration of TUDCA at 100 μM or 200 μM could reverse the suppressive effect of A939572 in self-renewal. By the sphere formation assay, we found that the suppressive effect of A939572 on sphere formation was offset by administration of TUDCA in a dose-dependent manner (Figure 6D). Consistently, we found that suppression of SCD1 by A939572 forced the differentiation of liver T-ICs, while this effect was reversed upon the application of TUDCA, as evidenced by the decrease in expression of differentiation markers (HNF4α and UGT2B7) (Figure 6E). Lastly, we examined whether A939572 sensitized HCC to sorafenib treatment via the activation of UPR. By annexin V staining, we found that the sensitization effect of SCD1 suppression by A939572 was reversed in both PLC/PRF/5 and Huh7 cells upon co-treatment with

TUDCA, and this offsetting effect was found to be dose-dependent (Figure 6F). In parallel with this finding, shSCD1 cells demonstrated increased ER stress, as evidenced by increased expression of CHOP and BIP when compared with control cells (Supplementary Figure 3). Given that monounsaturated fatty acid oleate is one of the enzymatic products of SCD1, we would like to further examine whether SCD1 regulates sphere formation, sorafenib sensitivity and ER stress due to production of oleate. For this purpose, we added exogenous oleic acid (100 μg/mL and 500 μg/mL) in HCC cells treated with A939572 to examine whether oleic acid rescues the effect of SCD1 suppression on the above phenotypes. We found that exogenous oleic acid rescued the effect of A939572 on sphere formation, sorafenib sensitization and ER stress (Supplementary Figure 6).

SCD1 inhibitor in combination with sorafenib results in maximal tumor suppression in a PDTX model

Given the crucial role of SCD1 in regulating sorafenib resistance, we examined the therapeutic role of targeting SCD1 alone and its combined effect with sorafenib *in vivo* using the PDTX#1 sample. In this study, we employed a novel SCD1 inhibitor (SSI-4) developed by Dr. Copland from the Mayo clinic to target SCD1 expression in HCC cells *in vivo*. Prior to evaluating its combinatorial effect with sorafenib, we first determined the toxicity and potency of SSI-4 in the suppression of tumor growth in the PDTX#1 sample which showed high SCD1 expression. Three different doses of SSI-4 ranging from 10, 50 to 100 mg/kg were used for this initial analysis. We found that 10 mg/kg of SSI-4 showed a similar potency as 50 and 100 mg/kg without a loss in body weight after treatment for 14 days (Figure 7A and B). Based on this study, 10 mg/kg of SSI-4 was selected for the subsequent *in vivo* experiments. The following therapeutic regimens were used: 1) SSI-4 (10 mg/kg), 2) 10 mg/kg sorafenib, 3) 10 mg/kg sorafenib plus 10 mg/kg of SSI-4,

or 4) vehicle (DMSO, PEG400/Tween 80). Treatment was initiated once the size of the xenografts had reached approximately 5×5 mm. The efficacy of the combined drug treatment was evaluated by the tumor size and compared to single drug treatment groups. During the experiment, no signs of toxicity (infection, diarrhea, or loss of body weight) were observed in the animals undergoing SSI-4 treatment. The tumors and their corresponding volumes are shown in Figures 7C and 7D after treatment for 26 days. SSI-4 suppressed the tumor volumes in a manner similar to the effect of sorafenib. In addition, SSI-4 combined with sorafenib exerted a synergistic effect, resulting in maximal suppression of the tumors, compared with the control group. To further examine whether sorafenib sensitization was due to an increase in ER stress, we performed IHC staining to examine the expression of BIP in the tumors of the different treatment groups. As shown in Figure 7E, the percentage of cytoplasmic Bip expression was dramatically increased in the SSI-4 treated tumors compared with the control group. Furthermore, SSI-4 combined with sorafenib treatment showed further increases in Bip expression compared to SSI-4 alone (Figure 7E). This *in vivo* result further supports that the sensitization effect of the SCD1 inhibitor to sorafenib treatment is due to the activation of the ER stress pathway.

DISCUSSION

In this study, we adopted serial passages of hepatospheres combined with anticancer chemotherapeutic drugs as a strategy to establish chemoresistant hepatospheres with enhanced self-renewal capacity and tumorigenicity. Interestingly, lipogenesis pathways were highly upregulated in the drug-resistant hepatospheres. Lipogenesis is important to maintain the stemness properties of glioma stem cells (14) and is also crucial for stem cell self-renewal and proliferation (15,16). When we examined the lipogenesis pathways in drug-resistant hepatospheres, SCD1 demonstrated the highest expression. SCD1 has been studied for many years in metabolic diseases, such diabetes and obesity (17-18), and has been suggested as a target for the treatment of metabolic syndrome (19). The role of SCD1 in cancer was not explored until recently, as SCD1 was found to be upregulated in various cancers, including prostate cancer (20), clear cell renal cell carcinoma (21), lung cancer (22), and colorectal cancer (23). Specifically, SCD1 was found to be overexpressed in HCC (24). Overexpression of SCD1 was induced by AKT-mTORC1-RPS6 signaling leading to HCC development (25). More recently, SCD1 was found to play crucial role in fibrosis-induced HCC via wnt positive-signaling loop by stabilization of low-density lipoprotein-receptor-related protein 5 and 6 (26). Although evidence suggests SCD1 is important in cancer progression (27), the role of SCD1 in liver T-ICs remains to be investigated.

By qPCR analysis, we found 60% of patient tumor samples exhibited SCD1 upregulation, which is consistent with the previous study showing upregulation of this protein in HCC (24). Upregulated SCD1 protein expression in HCC patients was significantly correlated with disease-free survival. Because SCD1 is upregulated in liver CSCs, both *in vitro* and *in vivo* assays were employed to study the functional roles of SCD1 using overexpression and knockdown approaches. First, SCD1 expression levels positively correlated with the expression levels of liver CSC

markers, including CD24 (13) and CD47 (10). Consistently, SCD1 was found to play a regulatory role in lung-initiating cells (22). In this study, we found that suppression of SCD1 not only suppressed the sphere-forming ability of HCC cells but also inhibited their tumor forming ability and chemoresistance. In addition, we confirmed the role of SCD1 in the regulation of HCC invasion and migration. Interestingly, we found that SCD1 regulates the T-IC population by guiding the differentiation process, as evidenced by the increase in expression of differentiation markers upon SCD1 knockdown. This result echoed with recent study demonstrating the role of SCD1 in the differentiation process of hepatocytes from hiPS cells (28).

Here, we found that SCD1 was highly upregulated in both sorafenib-resistant cell lines and PDTXs when compared to controls. The upregulation of SCD1 in sorafenib-resistant cell lines and PDTX mice suggests a critical role for SCD1 in sorafenib resistance and the survival of liver CSCs. Although SCD1 partially mediates chemoresistance in breast cancer (29), the role of SCD1 in sorafenib resistance has not yet been studied. We consistently found SCD1 inhibition sensitized the effect of sorafenib treatment. To further determine whether SCD1 is a potential biomarker for the prediction of the sorafenib response in HCC patients, immunohistochemical staining was performed to analyze 90 cases of post-sorafenib treatment patient tumor specimens. IHC staining revealed a correlation between high SCD1 expression after sorafenib treatment and poor sorafenib response in patients, both with regard to of HCC recurrence and disease-free survival. SCD1 expression levels may represent a novel prognosis marker to predict patient responses to sorafenib treatment.

A939572 suppressed the self-renewal, migration and invasion of HCC cells. These results are in line with the findings of Noto A et al. in lung CSCs (22). In addition, when A939572 was combined with sorafenib, cell apoptosis was significantly enhanced. The sensitization of HCC to

sorafenib treatment induced via the knockdown of SCD1 and administration of A939572, making SCD1 an attractive therapeutic target and leading us to further investigate whether the application of an SCD1 inhibitor augments the efficacy of sorafenib *in vivo*. To do so, the novel SCD1 inhibitor SSI-4 was tested in PDTX tumor-bearing nude mice. We found that SSI-4 at the dose of 10 mg/kg suppressed tumor growth but did not cause serious side effects in tumor-bearing nude mice during treatment. Notably, the dose of SSI-4 used in this study is much lower than in the previous study using A939572 (100 mg/kg) for pre-clinical cancer therapy (30), indicating the high potency of SSI-4 in tumor suppression. Next, we examined the therapeutic efficacy of SSI-4 in combination with sorafenib by oral gavage. After 28 days, the effects of SSI-4 and sorafenib treatment alone were relatively modest compared to the co-treatment group. Interestingly, the co-treatment group exhibited a significant reduction in tumor volume, suggesting that SSI-4 may be used as a novel inhibitor to augment the efficacy of sorafenib and target HCC and liver CSCs.

Next, we elucidated the downstream mechanism of SCD1 by performing RNA sequencing of the SCD1-knockdown clones and their control cells. In comparing their gene expression profiles, ER stress-induced activation of the unfolded protein response (UPR) was identified as a potential downstream pathway of SCD1 to regulate the traits of liver T-ICs. The unfolded protein response (UPR) is triggered when the amount of newly synthesized polypeptides overloads the folding capacity of the ER. After the identification of the UPR as a downstream pathway of SCD1, we confirmed the upregulation of several ER stress genes upon SCD1 knockdown by qPCR analysis. Although the correlation between SCD1 and ER stress has been reported by several studies (23,31), how SCD1 regulates T-ICs and drug resistance via ER stress has not yet been addressed. To further confirm the UPR-induced ER stress as the downstream effector of SCD1, the chemical chaperone TUDCA was employed to reverse the phenotypic effects induced by

A939572. TUDCA reversed the suppressive effects of A939572 during hepatosphere formation and desensitized HCC to sorafenib treatment, further supporting the UPR as a potential downstream pathway of SCD1 that regulates the traits of liver CSCs and sorafenib resistance. In addition, we found that TUDCA decreased the expression of differentiation induced by A939572, indicating that suppression of SCD1 forces liver CSCs to differentiate via the induction of ER stress. Increase accumulation of saturated fatty acid (SFA) in cell membrane alters the integrity as well as the functionality of endoplasmic reticulum, leading to ER stress response (32). Desaturation of fatty acids by SCD1 is thought to counter these effects, and is protective against SFA-mediated stress (33). In addition, oleic acid, one of the major products of SCD1, was found to alleviate SFA mediated ER stress (34). This, together with the data showing the rescue effect of oleic acid in sphere formation, sorafenib sensitization and expression of ER marker upon SCD1 suppression (Supplementary Figure 6), we believe that SCD1 might regulate ER stress and related phenotypes through desaturation of fatty acid. To support this *in vitro* data, an increase in ER stress was also evidenced by the increased expression of Bip protein in SSI-4-treated PDTX compared with mock-treated controls, which results in enhanced sensitivity to sorafenib both *in vitro* and *in vivo*. These results are aligned with recent studies showing the role of ER stress in the induction of differentiation and resulting in the sensitization of colon cancer stem cells to chemotherapy (35).

In conclusion, we have demonstrated that SCD1 regulates liver T-ICs and sorafenib resistance through the regulation of ER stress (Supplementary Figure 7). Targeting T-ICs with an SCD1 inhibitor in combination with sorafenib seems to be a promising, novel therapeutic strategy for improving the efficacy of sorafenib.

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Figure 1. Up-regulation of SCD1 of self-renewing liver cancer cells in chemoresistant hepatospheres. (A) SCD1 was found to be the most upregulated gene in lipogenesis pathway in chemoresistant hepatospheres. (B) Up-regulation of SCD1 expression in chemoresistant hepatospheres was confirmed by qPCR (*P<0.05, t test). (C) Over 60% (42/70) of patients has T/N > 2-fold upregulation of SCD1 in their tumor samples when compared to the non-tumor counterpart. (D) Representative IHC images showing two HCC cases graded with low SCD1 expression and high SCD1 expression. A representative photo showing the boundary between non-tumor and tumor region (M indicates the margin). (E) The disease-free survival rate of HCC patients with high SCD1 over-expression was significantly lower than those patients with low SCD1 expression (P=0.008, log-rank test). Error bars represent the standard deviation (SD) from at least three independent experiments.

Figure 2. **SCD1 knockdown reduced stem/progenitor characteristics of HCC cells.** (A) By qPCR analysis, SCD1 was found to be highly expressed in Huh7 and PLC/PRF/5 among other cell lines. (B) Similar observation was found by western blot analysis. Two different shSCD1 sequences were used (#13 and #15). Western blotting showed the successful knockdown of SCD1 in PLC/PRF/5 and Huh7 cells. (C) Knockdown of SCD1 in Huh7 and PLC/PRF/5 cell lines suppressed the tumorigenicity when compared with NTC cells. Representative photos showing injection of 500 and 10000 cells derived from PLC/PRF/5 and Huh7 were shown. (D) Knockdown of SCD1 also reduced the size and number of hepatospheres formed by PLC/PRF/5 and Huh7 cells (**P<0.01 and ***P<0.001, respectively, t test). (E) Knockdown of SCD1 decreased expression of CD24 and CD47 in both PLC/PRF/5 and Huh7 cells, when compared with NTC cells (*p<0.05, ** p<0.01, t test). (F) Knockdown of SCD1 increased expression of differentiation markers

including HNF-4a and UGT2B7(*p<0.05, ** p<0.01, *t* test). Error bars represent the standard deviation (SD) from at least three independent experiments.

Figure 3. SCD1 regulates the abilities of migration, invasion and chemoresistance of HCC cells. (A&B) Transwell migration and invasion assays demonstrated the decreased migratory (**P<0.01 and ***P<0.001, respectively, *t* test) and invasive abilities (**P<0.01 and ***P<0.001, respectively, *t* test) of shSCD1 transfected PLC/PRF/5 and Huh7 cells *in vitro*. (C) SCD1 OE demonstrated increased migratory (** p<0.01, *t* test). and invasive abilities (*p<0.05, *t* test) in Bel7402, when compared with EV control cells. (D) shSCD1 cells showed a higher percentage of Annexin V-positive cells in response to doxorubicin treatment at 1μg/mL for 24 hours, when compared with the NTC control cells (**P<0.01 and ***P<0.001, respectively, *t* test). (E) SCD1 OE cells showed lower percentage of Annexin V-positive cells in response to doxorubicin treatment at 1μg/mL for 24 hours, when compared with the EV control cells (**P<0.01, respectively, *t* test). Error bars represent the standard deviation (SD) from at least three independent experiments.

Figure 4. The regulatory role of *SCD1* in sorafenib resistance. (A) By western blot analysis, SCD1 was overexpressed in sorafenib-resistant cell lines Bel7402 and Huh7. **(**B) IHC staining also confirmed overexpression of SCD1 in sorafenib-resistant PDTX#1 and PDTX#5. (C) SCD1 knockdown PLC/PRF/5 and Huh7 clones showed increased sensitivity to sorafenib, as indicated by a greater percentage of Annexin V-positive cells (*P<0.05, **P<0.01 and ***P<0.001 respectively, *t* test). (D) A TMA consisting of 90 tumor tissues with post-treatment of sorafenib was subjected to IHC analysis. Tissues were stained with anti-SCD1 antibody and brownish

staining indicated the overexpression of SCD1 in the patient specimens. (E) Representative IHC image showing four HCC cases graded with low SCD1 expression (case 40 and 65) and high SCD1 expression (case 10 and case 73). (F) Patients with high SCD1 expression after sorafenib treatment were significantly correlated with HCC recurrence. (G) Patients with high SCD1 expression after sorafenib treatment would have shorter disease-free survival than those with lower SCD1 expression ($p = 0.022$). Error bars represent the standard deviation (SD) from at least three independent experiments.

Figure 5. Pharmacological inhibition of SCD1 by A939572 on T-IC properties. (A) Inhibition of SCD1 by A939572 significantly suppressed sphere formation of PLC/PRF/5 and Huh7 cells at dose of 3μM and 5μM (*$p<0.05$, ** $p<0.01$, *t* test). (B &C) A939572 at the dose of 5μM suppressed the migratory and invasive abilities of PLC/PRF/5 and Huh7 cells (*** $p<0.001$, *t* test). (D) Inhibition of SCD1 by A939572 significantly sensitized the effect of sorafenib to both PLC/PRF/5 and Huh7 cells (*$P<0.05$, **$P<0.01$ and ***$P<0.001$ respectively, *t* test). Error bars represent the standard deviation (SD) from at least three independent experiments.

Figure 6. SCD1 regulates liver T-IC properties via modulation of ER stress. (A) Commonly deregulated genes in PLC/PRF/5 and Huh7 cells upon SCD1 suppression were shown. (B) By qPCR analysis, a list of genes related to ER mediated UPR were activated in shSCD1 cells from PLC/PRF/5 and Huh7 cells (*$P<0.05$, **$P<0.01$ and ***$P<0.001$ respectively, *t* test). (C) By western blot analysis, BIP was found to be upregulated in shSCD1 cells, while this effect was offset by administration of TUDCA at 100 μM and 200 μM. (D) TUDCA at 100 μM and 200 μM reversed the suppressive effect of A939572 on sphere formation (*$p<0.05$, ** $p<0.01$, *t* test). (E)

TUDCA at 100μM and 200μM reversed the effect of A939572 on expression of differentiation markers (*p<0.05, ** p<0.01, *t* test). (F) TUDCA at 100 μM and 200 μM conferred sorafenib resistance in A939572-treated PLC/PRF/5 and Huh7 cells (*P<0.05, **P<0.01 and ***P<0.001 respectively, *t* test). Error bars represent the standard deviation (SD) from at least three independent experiments.

Figure 7. SSI-4 antibody in combination with sorafenib for HCC cancer therapy. (A) Cells from a PDTX (PDTX#1) were subcutaneously injected into nude mice. Three different dosages of SSI-4 were examined in PDTX#1 bearing nude mice. (B) The toxicity of SSI-4 was evaluated by the percentage of the weight loss in the mice. (C&D) SSI-4 and sorafenib was given orally daily at 10 mg/kg. The subcutaneous tumor volume was measured and recorded every 2-3 days from the initial treatment to tumor harvest (Day 26). (E) By IHC staining, BIP expression was found to be drastic increase in the tumor treated with both sorafenib and SSI-4 when compared mock control.