Lipid desaturation-associated endoplasmic reticulum stress regulates MYCN gene expression in hepatocellular carcinoma cells

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Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide due to its high rate of recurrence, in part because of cancer stem cell (CSC)-dependent "field cancerization". Recently, we identified that the oncogene v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN) marked CSC-like subpopulations in heterogeneous HCC and served as a therapeutic target and prognostic marker for HCC. In this study, we explored the molecular basis of upregulated MYCN gene expression in HCC cells. Liquid chromatograph time-of-flight mass spectrometry-based metabolome analysis demonstrated that the content of unsaturated fatty acids was increased in MYCN high expression (MYCNhigh) CSC-like HCC cells. Inhibition of lipid desaturation using either the chemical inhibitor or siRNA/shRNA against stearoyl-CoA desaturase-1 (SCD1) suppressed cell proliferation as well as MYCN gene expression in MYCNhigh HCC cells, grown as both monolayer and spheres. Further mechanistic study using RNA-seq based transcriptome analysis revealed that endoplasmic reticulum (ER) stress related signaling networks such as endocannabinoid cancer inhibition pathway were under the control of SCD1 in MYCNhigh HCC cells. Furthermore, the expression of ER stress-inducible transcription suppressor cyclic AMP-dependent transcription factor (ATF3) was downregulated in MYCNhigh CSC-like HCC cells and CSC-rich spheroids, which was upregulated by inhibition of lipid desaturation or treatment with acyclic retinoid (ACR). Lipid profiling using NMR spectroscopy revealed that the ACR dramatically reduced the content of unsaturated fatty acids in HCC cells. The chemical inducer of ER stress inhibited MYCN gene expression, while the chemical inhibitor of ER stress or knockdown of ATF3 gene expression partially rescued the suppression of MYCN gene expression by ACR in MYCNhigh HCC cells. These data suggested that lipid desaturation-mediated ER stress signaling regulates MYCN gene expression in HCC cells and serves as a promising therapeutic target for the treatment and prevention of HCC.

Introduction

Hepatocellular carcinoma (HCC) is a well-characterized inflammation-driven cancer1 and is the second most lethal cancer worldwide due to its poor prognosis2. Persistent infections of hepatitis B virus (HBV) and hepatitis C virus (HCV) are the major risk factors for HCC3. Advances in antiviral therapy have reduced the risk of developing both HBV4 and HCV-related HCC5. However, obesity-associated inflammation is responsible for increased death rates for all the cancers6. Non-alcoholic steatohepatitis (NASH), which is characterized by continuous hepatocyte death and compensatory proliferation7, has attracted much attention and is believed that it will soon be the leading etiology of HCC8. Lipogenesis is known to be high in individuals with NASH and its upregulation is associated with high risk and poor prognosis of NASH-related HCC9. Lipid metabolic reprogramming allows the cells to adapt to the...
tumor microenvironments. For example, lipogenesis may promote the cell growth and proliferation of MYC-driven cancers, probably by maintaining the rebalanced nutrient supply and demand for cellular hyperproliferation. In addition, lipid accumulation in hepatocytes selectively induces lipotoxicity in intrahepatic CD4+ T cells and promotes HCC development by evading immune surveillance. Furthermore, there is growing evidence about the role of unsaturated fatty acids in tumor initiation by regulating the generation and maintenance of cancer stem cells (CSCs) or tumor-initiating cells (TICs).

Tissue regeneration and tumorigenesis share common molecular pathways in the regulation of cell growth and death. In response to resection or injury, healthy liver has the unique ability to grow back or regenerate by a process of compensatory hyperplasia. In contrast, under chronic inflammation such as lipid-rich environment, repeated liver injury and compensatory proliferation might lead to aberrant stabilization and chronic activation of the oncogenes, which can be considered as one of the leading causes of HCC. The MYC family members are critically involved in the regulation of multiple biological processes, including cell growth, proliferation, apoptosis, energy metabolism, and differentiation, and play dual roles in regulating both hepatocellular proliferation and hepatocarcinogenesis. avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN), is a well-recognized oncogene associated with the progression and prognosis of neuroblastoma. A transcriptome analysis performed by our group in primary hepatocytes during the process of mouse liver regeneration revealed that the expression of Mycn was low in normal hepatocytes, but increased along with hepatocyte proliferation after partial hepatectomy. We also reported that MYCN expression was seen in epithelial cell adhesion molecule (EpCAM)+ liver CSC-like cells and was positively correlated with the recurrence of HCC. However, the mechanism underlying the overexpression of MYCN during chronic liver injury and hepatic tumorigenesis is still unclear.

Acyclic retinoid (ACR) is a synthetic vitamin A-like compound capable of preventing the recurrence of HCC in patients after curative removal of the primary tumors. Recently, we identified that the MYCN high expression (MYCNhigh) liver CSC-like cells are selectively depleted by ACR, suggesting MYCN as a therapeutic target for the prevention and treatment of HCC. Further proteome analysis showed enrichment in MYCNhighEpCAM+ CSC-like HCC cells for lipogenic enzymes such as stearoyl-Coenzyme A desaturase-1 (SCD1), an enzyme that creates double bonds at specific locations in long chain fatty acids. Therefore, in this study, we examined the link between lipid desaturation and MYCN gene expression in HCC cells.

Results

Metabolome characterization of MYCNhigh CSC-like HCC cells

To determine the metabolic characteristics of MYCNhigh CSC-like HCC cells, metabolite analysis was performed on the EpCAM+/- JHH7 cell sorted using fluorescence activated cell sorting (FACS). Peaks of a total of 65 lipophilic metabolites were detected using liquid chromatograph time-of-flight mass spectrometry (LC-TOFMS) (Table S1). Hierarchical cluster analysis (HCA) demonstrated a clear distinction in the abundance of lipophilic metabolites between EpCAM+/- cells (Fig. 1a). The pathway impact analysis of the differentially expressed metabolites with a threshold of fold change of more than 2 using MetaboAnalyst showed that linoleic acid (LA; C18:2) metabolism was the most significantly perturbed pathway between EpCAM+/- cells (Fig. 1b). Furthermore, in accordance with the proteome analysis, the content of unsaturated fatty acids was strikingly increased in the EpCAM+ cells compared with that in the EpCAM− cells. Palmitoleic acid (PA, C16:1; 6.8-fold) and oleic acid (OA, C18:1; 5.6-fold), which are the main monounsaturated fatty acids generated via SCD1, were the two most dramatically enhanced lipophilic metabolites in EpCAM+ cells. In contrast, there were modest increases in the content of saturated fatty acids such as stearic acid (SA, C18:0; 1.6-fold) and palmitic acid (C16:0; 2.1-fold), and almost no changes in cholesterol (0.79-fold) and cholesterol sulfate (1.1-fold) in the EpCAM+ cells compared with those in the EpCAM− cells (Fig. 1c).

MYCN co-expression genes in HCC tumor tissues and cell lines

Next, we undertook a clinical investigation of MYCN co-expression genes in human HCC tumor tissues. The genes were selected using The Cancer Genome Atlas (TCGA) database, which contains a RNA-seq dataset of 372 HCC tumor samples (PanCancer Atlas). With a threshold of Spearman’s correlation coefficient of more than 0.3, 109 genes were selected as MYCN co-expression genes and then subjected to pathway analysis (Fig. 2a). Ingenuity Pathway Analysis (IPA) revealed that the top canonical pathways such as the fatty acid activation and γ-linolenate biosynthesis were correlated with MYCN in HCC (Fig. 2b). Then, we examined the correlation between the MYCN gene expression and well-established hepatic stem/progenitor markers and genes associated with cancer metabolism and metastasis by data mining in the Cancer Cell Line Encyclopedia (CCLE) database. With a threshold expression level of MYCN at 1 read per kilobase of exon per million mapped reads (RPKM), 8 out of a total of 25 HCC cell lines were selected as MYCNhigh cells (≥1 RPKM), while the other 17 HCC cell lines were MYCN low expression (MYCNlow) cells (<1 RPKM).
Hierarchical clustering demonstrated enrichment in MYCN\textsuperscript{high} HCC cells for genes related with liver CSC markers (EpCAM and AFP), stemness regulation (DLK1, NANOG, and ALDH1A1), and fatty acid metabolism (ACACA, FASN, FADS1, SCD1, and FADS2) (Fig. 2c). In contrast, MYCN\textsuperscript{low} HCC cells were characterized by the expression of liver CSC marker CD90 (THY1) and genes involved in glucose metabolism (PKM2 and LDHA) and epithelial-to-mesenchymal transition (c-KIT and TGF\textbeta{}1) (Fig. 2c).

For further functional analysis, HLF and JHH7 cells were used as MYCN\textsuperscript{low} and MYCN\textsuperscript{high} HCC cells, respectively. Morphological analysis showed that the HLF cells had features of mesenchymal-like cells, while the
JHH7 cells had those of epithelial cells (Fig. 2d). Immunofluorescence staining (Fig. 2e) and flow cytometric analysis (Fig. 2f) revealed that the HLF cells were negative for EpCAM and positive for CD90, while in contrast JHH7 cells were positive for EpCAM and negative for CD90. PCR analysis confirmed that the gene expression levels of MYCN, EpCAM, and AFP were dramatically higher, while the expression levels of THY1, TGFβ1, and c-KIT were lower in the JHH7 cells than that in the HLF cells (Fig. 2g). Then, we examined the MYCN expression in a three-dimensional (3D) multicellular tumor spheroid system, which could mimic in vivo 3D tumor growth and promote stemness maintenance. 3D culture increased the MYCN gene expression in JHH7 cells, but not in HLF cells (Fig. 2h). Furthermore, the JHH7 cells showed greater sphere growth ability than the HLF cells (Fig. 2i).

**Loss of function analysis of SCD1 in MYCNhigh HCC cells**

The above data suggested that MYCN was highly expressed in epithelial-like HCC cells with preferentially
high tumorigenic potential and CSC characteristics, which was probably related with the acceleration of unsaturated fatty acid synthesis. To examine this hypothesis, we examined whether the inhibition of lipid desaturation using a chemical inhibitor (CAY10566) or shRNA against SCD1 might play a functional role in the proliferation of MYCN\textsuperscript{high} HCC cells in monolayer and sphere cultures. CAY10566 inhibited the spheroid proliferation in the JHH7 cell 3D culture system (Fig. 3a). To provide functional evidence supporting the lipid desaturation-mediated effects of CAY10566, we measured sphere proliferation with exogenously added lipid supplementation. The combined addition of monounsaturated fatty acids, OA and PA, and polyunsaturated fatty acid LA, but not the saturated fatty acid SA, restored the suppression of sphere proliferation by CAY10566 (Fig. 3b). Furthermore, we compared the effect of CAY10566 on the proliferation of MYCN\textsuperscript{low} HLF cells and MYCN\textsuperscript{high} JHH7 cells (Figure S1). Notably, a significantly greater growth suppressive effect of CAY10566 was observed in JHH7 cells than in HLF cells when grown as both monolayer and spheres. These data supported SCD1-mediated lipid desaturation as a major player in maintaining the sphere growth of MYCN\textsuperscript{high} HCC cells. Similarly, knockdown of SCD1 using its shRNA inhibited the proliferation of JHH7 cells in monolayer culture (Fig. 3c) and there was an increased formation of apoptotic bodies (Fig. 3d). Furthermore, flow cytometric analysis demonstrated that the proportion of Annexin V positive apoptotic cells was increased among these cells (Fig. 3e). PCR analysis also revealed that the expression of pro-apoptotic gene Bcl-2-like protein 11 (BCL2L11; commonly called Bim) was increased following SCD1 knockdown in JHH7 cells in both monolayer and sphere cultures (Fig. 3f). Moreover, knockdown of SCD1 led to the decrease in MYCN gene expression in JHH7 cells, suggesting that SCD1-mediated signaling pathway might act as an upstream regulator of MYCN gene expression in HCC cells.

**RNA-seq based transcriptome analysis identifies molecular targets of SCD1 in MYCN\textsuperscript{high} HCC cells**

To explore the mechanism underlying the regulation of MYCN gene expression by SCD1, RNA-sequencing (RNA-seq) based transcriptome analysis was performed to identify the molecular targets of SCD1 in JHH7 cells with transient transfection of a control siRNA (siCtl) or siRNAs against SCD1 (siSCD1) or MYCN (siMYCN) (Fig. 4a), and FACS-sorted CD90\textsuperscript{+/−} HLF cells and EpCAM\textsuperscript{+/−} JHH7 cells (Fig. 4b). Hierarchical clustering of the RNA-seq based transcriptome profiles with 16,735 genes demonstrated diverse expression profiles between HLF and JHH7 cells (Fig. 4c and Table S2). In accordance with the above PCR analysis, the MYCN gene expression was not detected in CD90\textsuperscript{+/−} HLF cells, while it was highly expressed in the EpCAM\textsuperscript{+} JHH7 cells as compared with that in the EpCAM\textsuperscript{−} cells and was decreased following knockdown of SCD1 or MYCN in JHH7 cells (Fig. 4d). Furthermore, MYCN gene expression was not correlated with that of c-MYC, which is a major member of the MYC family contributing to the genesis of human cancers\textsuperscript{18} (Fig. 4d). With a threshold fold change of more than 4, differentially expressed genes between CD90\textsuperscript{+/−} HLF cells (383 genes), EpCAM\textsuperscript{+/−} JHH7 cells (524 genes), and siSCD1/siCtl (284 genes) and siMYCN/siCtl (228 genes) JHH7 cells were selected for pathway analysis to gain greater insights into the biological function (Fig. 4e). Top diseases or functional annotation (Fig. 4f) and upstream regulator analysis (Fig. 4g) using IPA platform revealed that EpCAM\textsuperscript{+} JHH7 cells and CD90\textsuperscript{+} HLF cells shared similar activation patterns, such as activation of DNA metabolism and nuclear protein 1 (Nupr1)-regulated signaling pathways, and inhibition in cell death program and estrogen-related signaling pathways. This data was in agreement with the previous reports that the stemness could be considered as a state acquired but not defined by a distinct cell type or a cell biomarker\textsuperscript{29}.

Notably, the CSC-related signaling pathways were reversibly affected by knockdown of either SCD1 or MYCN, suggesting that SCD1 and/or MYCN might serve as potential CSC therapeutic targets. To identify the upstream regulators of MYCN gene expression under the control of SCD1, a total of 152 genes were further selected with a threshold fold change of more than 2 according to the following strategy: (1) differentially expressed by siSCD1 transfection in JHH7 cells; (2) differentially expressed between EpCAM\textsuperscript{+/−} JHH7 cells and reversibly changed in comparison to the siSCD1 transfected JHH7 cells; (3) no changes between the CD90\textsuperscript{+/−} HLF cells and by siMYCN transfection in JHH7 cells. Canonical pathways analysis of these genes revealed that endocannabinoid signaling pathways were under the control of SCD1, which contains genes that play critical roles in controlling endoplasmic reticulum (ER) stress such as cyclic AMP response element-binding protein (CREB), DNA damage inducible transcript 3 (DDIT3), and cyclic AMP-dependent transcription factor (ATF3) (Fig. 4i). These data suggested that the SCD1 knockdown-induced ER stress might play a functional role in regulation of MYCN gene expression in the HCC cells.

**ER stress regulates MYCN gene expression in MYCN\textsuperscript{high} HCC cells**

The gene expression of ATF3 was examined using PCR. Knockdown of SCD1 induced the significant expression of ATF3 in JHH7 cells (Fig. 5a). In contrast, the gene expression of ATF3 was decreased in the EpCAM\textsuperscript{+} CSC-
Fig. 3 (See legend on next page.)
like JHH7 cells than that in the EpCAM− JHH7 cells (Fig. 5b). The gene expression of ATF3 was also decreased in JHH7 cells grown in sphere cultures than that in monolayer cultures (Fig. 5c). Data mining in the TCGA database revealed a significant negative correlation between the gene expression of MYCN and ATF3 in human HCC tissues (Fig. 5d). Since ATF3 is an ER stress-inducible transcriptional repressor, we then examined whether ER stress might functionally regulate MYCN gene expression. Tunicamycin, a chemical inducer of ER stress, strongly stimulated the gene expression of ATF3 and inhibited the gene expression of MYCN in the JHH7 cells (Fig. 5e). Finally, we examined whether ER stress is associated with the suppression of MYCN gene expression by ACR. Lipid profiling using 1H nuclear magnetic resonance (NMR) spectroscopy revealed that the treatment with ACR for 4 h dramatically reduced the content of unsaturated fatty acids in JHH7 cells (Fig. 5f). To further explore the molecular basis of the suppressive effect of ACR on lipid desaturation, the effect of ACR on gene expression of lipid desaturases was examined in JHH7 cells (Figure S2). Treatment with ACR for 4 h significantly suppressed the gene expression of SCD1 as well as fatty acid desaturase-1 (FADS1) in JHH7 cells. ACR significantly enhanced the gene expression of ATF3 in JHH7 cells, while co-addition of tauroursodeoxycholic acid (TUDCA), a chemical inhibitor of ER stress (Fig. 5g), as well as knockdown of ATF3 using siRNA (Fig. 5h) significantly diminished the effects of ACR on ATF3 and MYCN gene expression in JHH7 cells. These data suggested ACR suppressed MYCN gene expression in JHH7 cells, at least in part through ER stress induced ATF3 signaling pathways.

**Discussion**

We have previously reported that (1) inhibition of biosynthesis of unsaturated fatty acids, but not glucose metabolism plays a crucial role in the prevention of hepatocellular carcinogen diethylNitrosamine (DEN)-induced hepatic tumorigenesis by ACR in the obesity mice model; (2) ACR can inhibit MYCN gene expression and selectively kill MYCNhigh EpCAM+ CSC-like HCCs, thus, providing a molecular basis by which ACR prevents HCC recurrence; (3) proteome analysis has shown that MYCNhigh EpCAM+ CSC-like HCCs are enriched in enzymes involved in lipid desaturation such as SCD1, FADS1 and FADS2. It should be noted that it is the upregulation of MYCN mRNA expression, but not its amplification status that is correlated with the prognosis of HCC. Therefore, it seems that MYCN gene expression might represent a dynamic stem state of the liver CSC-like cells and an understanding of how MYCN transcription is activated would provide new insights on liver tumorigenesis. In this study, we further provided evidence that (1) the content of unsaturated fatty acids was increased in MYCNhigh EpCAM+ CSC-like HCC cells; (2) inhibition of lipid desaturation using either the chemical inhibitor or siRNA/shRNA against SCD1 suppressed cell proliferation as well as MYCN gene expression in the MYCNhigh HCC cells grown as both monolayer and spheres. These data bridge the gap in the knowledge about the link between lipid desaturation and MYCN gene expression in HCC cells.

It is known that MYC family oncogenes activate the expression of the enzymes involved in fatty acid biosynthesis and promote lipid metabolic reprogramming in support of reprogrammed cell growth and survival in tumorigenesis. Blocking unsaturated fatty acid synthesis with chemical inhibitors had been shown to be toxic to Myc-overexpressing cells. In contrast, our study showed a regulatory role of SCD1 on the MYCN gene expression in HCC cells, suggesting that, in addition to its potential as the major source of energy and building blocks of membranes for cell proliferation, the lipid-rich microenvironment might also contribute to tumorigenesis via mediating oncogenic signaling. Obesity might cause chronic inflammation via promoting the production of tumor-promoting cytokines such as IL-6 and activation of oncogenes such as STAT3, which has a critical role in regulating cell fate, inflammation, and immunity. A new study has demonstrated that the fatty acids could directly activate STAT3 by enhancing its palmitoylation. In addition, there is increasing focus on the emerging roles of lipid metabolism, especially SCD1-mediated lipid desaturation, in the generation and maintenance of CSCs, through the activation of oncogenic signaling pathways such as NF-kB, Wnt/β-catenin and Hippo/YAP.
Fig. 4 (See legend on next page.)
signaling. For example, monounsaturated fatty acids such as OA might support mRNA stabilization of the Wnt receptor LRP5/6 and contribute to the activation of Wnt/β-catenin signaling, which is critical for maintaining stem cell pluripotency. Therefore, targeting lipid desaturation should be a promising therapeutic strategy for HCC treatment via the inhibition of energy production and membrane building blocks, as well as for HCC prevention through the suppression of oncogenic signaling and elimination of CSCs.

We further showed through transcriptome analysis that the ER stress related signaling networks such as endocannabinoid cancer inhibition pathway were under the control of SCD1 in MYCNhigh HCC cells. Furthermore, the chemical inducer of ER stress inhibited MYCN gene expression, while the chemical inhibitor of ER stress partially rescued the suppression of MYCN gene expression by ACR in MYCNhigh HCC cells. Lipid messengers, such as sphingolipids, lysolipids, and endocannabinoids, play critical roles in multiple cellular functions from cell proliferation to apoptosis and are emerging as promising therapeutic targets in cancer. Endocannabinoids are endogenous lipid-based retrograde neurotransmitters and their G-protein-coupled receptors CB1 and CB2 are expressed throughout the nervous system. CB1 activation causes the generation of the lipid second messenger ceramide, which plays central roles in cannabinoid induced apoptosis, partly through triggering of ER stress pathways. The MYCN oncogene is notably amplified in peripheral and central nervous system tumors especially neuroblastoma. In addition, retinoic acids have been shown to transcriptionally induce CB1 expression in hepatocytes via retinoic acid receptor γ-dependent pathways. Therefore, it is possible that ACR regulates MYCN gene expression through endocannabinoid system-mediated ER stress signaling pathways.

It has been reported that decrease in membrane phospholipid unsaturation and increase in cellular saturated fatty acids induced ER stress-dependent cell death. Indeed, activation of ER stress signaling is instrumental in the development of NASH-related HCC by triggering chronic liver damage and inflammation. In contrast, tissue stem cells, as well as CSCs, are featured with their adaptive plasticity to allow them to survive when exposed to a wide range of stresses including ER stress. Reduction in ER stress enables maintenance of functional hematopoietic stem cells, while induction of ER stress causes loss of intestinal epithelial stemness. Importantly, rescue of ER stress in CSC induces differentiation and sensitizes them to chemotherapy in colon cancer and HCC. In agreement with this, we found that the expression of ER stress-inducible gene ATF3 was down-regulated in MYCN-high EpCAM+ CSC-like HCC cells and CSC-rich spheroids, which was upregulated by ACR treatment or lipid desaturation inhibition. Therefore, escaping from the ER stress-mediated programmed cell death in obesity-caused inflammatory microenvironment should play a critical role in the maintenance of liver CSCs and hepatic tumorigenesis and serve as a promising therapeutic target in the prevention of HCC.

In summary, multi-omics analyses revealed that lipid desaturation-mediated ER stress signaling reversibly regulates the MYCN gene expression in HCC cells and might be a promising therapeutic target for the treatment and prevention of HCC.

**Materials and methods**

**Cell culture**

The HCC cell line HLF (JCRB0405) was obtained from the Japanese Collection of Research Bioresources Cell Bank. The HCC cell line JHH7 was kindly provided by Prof. T. Matsuura of the Jikei University School of Medicine, Tokyo, Japan. The cells were maintained at 37°C and 5% CO2, in Dulbecco’s modified Eagle medium (DMEM, Wako Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS; Mediatech, Herndon, VA, USA), 100 U/mL penicillin/streptomycin, and 2 mM L-glutamine (Mediatech).
Chemicals

A SCD1 inhibitor, CAY10566 (ab144421), was purchased from Abcam (Cambridge, MA, USA). Tunicamycin (11445) and TUDCA (20277) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Fatty acids SA (S4751), OA (O1008), PA (P0500), and LA (L1376) were obtained from Sigma (Louis, MO, USA). ACR was supplied by Kowa Co. Ltd (Tokyo, Japan). Dimethyl sulfoxide
(DMSO, Sigma) was used as the primary solvent for CAY10566, tunicamycin, and TUDCA. Isopropanol (Wako Industries) was used as the primary solvent for fatty acids. Ethanol (EtOH, Wako Industries) was used as the primary solvent for ACR.

**FACS sorting**
Flow cytometric analysis was performed as previously described. The cells were incubated with Alexa Fluor 647-conjugated mouse anti-EpCAM (1:20; 324212; BioLegend, San Diego, CA, USA) and/or FITC-conjugated mouse anti-CD90 (328108; BioLegend) at 4 °C for 30 min. The labeled cells were analyzed and sorted on a BD FACSAria (BD Biosciences, San Diego, CA, USA). The data were analyzed in FlowJo software (Tree Star, Inc., Ashland, OR, USA).

**LC-TOFMS metabolome analysis**
The cells were washed with 5% (w/w) mannitol solution twice and harvested in EtOH containing 10 µM internal standards (Human Metabolome Technologies Inc., Tsuruoka, Japan) with scrapers. The cell pellet was diluted in 1000 µL Milli-Q water and supernatant was collected by centrifugation (4400 x g, 4 °C, 5 min). The supernatant was dried and resuspended in 200 µL of isopropanol/Milli-Q water solution (1:1) for LC-TOFMS analysis using an Agilent 1200 series RRLC system SL equipped with an Agilent LC/MSD TOF system (Agilent Technologies, Palo Alto, CA, USA). Primer sequences were as follows: glyceroldehyde 3-phosphate dehydrogenase (GAPDH) forward (5′-CAATGACCCCTTCATGTACG-3′) and reverse (5′-GACAAACGTTCCCGTGTCAGG-3′), SCD1 forward (5′-GTACCCCGGTGCACATCAACTT-3′) and reverse (5′-TTGGAGACTTTCTCAGGGTCAT-3′), FADS1 forward (5′-CTACTCCCAG CGCTACTCCAC-3′) and reverse (5′-CGGTGATCTGACTAGCCACC-3′), MYCN forward (5′-GCGATAGGACCAGCAGTGT-3′) and reverse (5′-AACCGTACCAACGTTTACG-3′), BCL2L11 forward (5′-GGCCCTACTCTCCCTACA-3′) and reverse (5′-GGGT GTGTTGTTGATTGTTCAT-3′), and ATF3 forward (5′-TTTGATACCTGACGCCGCTTT-3′) and reverse (5′-TGA CACTGATTCACGCGAGAAG-3′). PCR reactions were performed using a Roche LightCycler 96 Real-Time PCR System (Roche Diagnostic Co., Ltd., Tokyo, Japan) and the SYBR Premix ExTaq II (TaKaRa Bio). The cells were plated in 24-well plates for 24 h, and then transfected with 100 nM siRNAs using lipofectamine 2000 transfection reagent (Life Technologies, Gaithersburg, MD, USA). On the following day, cells were collected for RNA isolation or treated with chemicals for further analysis.

**Transduction of shRNA lentiviral particles**
SCD1 (sc-36464-V) and control (sc-108080) short hairpin RNA (shRNA) lentiviral particles were obtained from Santa Cruz Biotechnology. The cells were transduced with lentiviral vectors expressing the shRNAs at ~0.5 multiplicity of infection (MOI) using 5 µg/mL Polybrene (Santa Cruz Biotechnology) and then selected with 2 µg/mL puromycin-containing culture medium.

**Immunofluorescence analysis**
The cells were seeded in 96-well plates at 10⁵ cells/mL. After 24 h, the cells were stained with Alexa Fluor 647-conjugated mouse anti-EpCAM (1:20; 324212; BioLegend) and/or FITC-conjugated mouse anti-CD90 (1:20; 328108; BioLegend) at room temperature for 30 min. Images were captured using an ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices, Sunnyvale, CA, USA).

**RNA isolation and real-time (RT)-PCR**
Total RNA was isolated using a FastGene RNA Basic Kit (FG-80250, NIPPON Genetics, Tokyo, Japan) and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized using a PrimeScript RT Master Mix Kit (TaKaRa Bio, USA). Primer sequences were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward (5′-CAATGACCCCTTCATGTACG-3′) and reverse (5′-GACAAACGTTCCCGTGTCAGG-3′), SCD1 forward (5′-GTACCCCGGTGCACATCAACTT-3′) and reverse (5′-TTGGAGACTTTCTCAGGGTCAT-3′), FADS1 forward (5′-CTACTCCCAG CGCTACTCCAC-3′) and reverse (5′-CGGTGATCTGACTAGCCACC-3′), MYCN forward (5′-GCGATAGGACCAGCAGTGT-3′) and reverse (5′-AACCGTACCAACGTTTACG-3′), BCL2L11 forward (5′-GGCCCTACTCTCCCTACA-3′) and reverse (5′-GGGT GTGTTGTTGATTGTTCAT-3′), and ATF3 forward (5′-TTTGATACCTGACGCCGCTTT-3′) and reverse (5′-TGA CACTGATTCACGCGAGAAG-3′). PCR reactions were performed using a Roche LightCycler 96 Real-Time PCR System (Roche Diagnostic Co., Ltd., Tokyo, Japan) and the SYBR Premix ExTaq II (TaKaRa Bio).

**Data mining**
RNA-seq transcriptome profiles of 372 HCC tumor tissues (PanCancer Atlas) were obtained from the TCGA database using cBioPortal software (www.cbioportal.org/).
RNA-seq transcriptome profiles of 25 HCC cell lines were obtained from the CCLE database (https://portals.broadinstitute.org/ccle)27.

Cell viability assay
Cell viability was measured using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Tokyo, Japan). The absorbance was measured using a plate reader (ARVO MX, Perkin Elmer Inc.) at 450 nm.

Annexin V staining assay
Apoptotic cells were detected using the FITC Annexin V Apoptosis Detection Kit with Propidium iodide (PI) (640914, BioLegend). Briefly, the cells were washed thoroughly with cell staining buffer (420201, BioLegend) and resuspended in Annexin binding buffer. The cells were then labeled with Annexin V-FITC and PI at room temperature for 15 min in the dark. The fluorescence of stained cells was measured using a BD LSR flow cytometer and CellQuest Pro software (BD Biosciences). The data were further analyzed in FlowJo software (Tree Star, Inc.).

RNA-seq based transcriptome analysis
RNA-Seq libraries were prepared using TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA), followed by paired-end sequencing on a NovaSeq 6000 instrument (Illumina). STAR (2.5.1b) was used to align reads in fastq files to the UCSC human hg38 reference genome. Stringtie (1.3.5) was used to assemble the transcriptome based on the hg38 reference annotation with Gencode.v30. The quantification of relative abundance of each transcript was reported as fragments per kilobase of exon model per million reads mapped (FPKM). Data downloads are available (Table S2).

Knowledge-based pathway analysis
For the metabolome analysis, the pathway impact was calculated as the sum of the importance measures of the matched metabolites normalized by the sum of the importance measures of all metabolites in each pathway using MetaboAnalyst 4.050. For the transcriptome analysis, the knowledge-based functional analysis was performed using the IPA platform (Ingenuity Systems, Mountain View, CA, USA) as previously described19. The generated biological networks were ranked by score, which is the likelihood of a set of genes being found in the network owing to random chance, identified by a Fisher’s exact test. The generated diseases or functional annotations or upstream regulators were ranked by the activation z score, which can be used to find likely regulating molecules based on a statistically significant pattern match of up- and downregulation, and also to predict the activation state (either activated or inhibited) of a putative regulator. An absolute z score of more than 2 was considered as significant.

Lipid profiling using 1H NMR spectroscopy
The JHH7 cells treated with EtOH or 15 μM ACR for 4 h were harvested and lipid was extracted using the chloroform/methanol/water extraction method as previously described.31 The 1H NMR spectra were measured at 700 MHz on a Bruker AVANCE II 700 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). All NMR spectra were processed using the MestReNova program (Version 12.0.1, MestReNova, Santiago de Compostela, Spain). Tetramethylsilane was used as the internal standard. The double bond regions of unsaturated fatty acids appear mainly from 5.20 to 5.45 ppm.

Statistical analysis
Quantitative data are expressed as the mean ± SD of at least three replicates. The significance of differences between values was assessed using Student’s t-test. A p-value < 0.05 was considered significant.

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Conflict of interest
The authors declare that they have no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the paper; in the decision to publish the results.

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