Received: 4 March 2022 | Revised: 21 May 2022 | Accepted: 26 May 2022

DOI: 10.1111/cas.15449

ORIGINAL ARTICLE

Cancer Science WILEY

SREBF2–STARD4 axis confers sorafenib resistance in hepatocellular carcinoma by regulating mitochondrial cholesterol homeostasis

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Funding information
National Science Foundation of China, Grant/Award Number: 81972819; Natural Science Foundation of Shandong Province, Grant/Award Number: ZR2020YQ57; Young Innovation Team Foundation of Shandong University.

Abstract

Sorafenib resistance limits its survival benefit for treatment of hepatocellular carcinoma (HCC). Cholesterol metabolism is dysregulated in HCC, and its role in sorafenib resistance of HCC has not been fully elucidated. Aiming to elucidate this, in vitro and in vivo sorafenib resistant models were established. Sterol regulatory element binding transcription factor 2 (SREBF2), the key regulator of cholesterol metabolism, was activated in sorafenib resistant HepG2 and Huh7 cells. Knockdown of SREBF2 resensitized sorafenib resistant cells and xenografts tumors to sorafenib. Further study showed that SREBF2 positively correlated with StAR related lipid transfer domain containing 4 (STARD4) in our sorafenib resistant models and publicly available datasets. STARD4, mediating cholesterol trafficking, not only promoted proliferation and migration of HepG2 and Huh7 cells, but also increased sorafenib resistance in liver cancer. Mechanically, SREBF2 promoted expression of STARD4 by directly binding to its promoter region, leading to increased mitochondrial cholesterol levels and inhibition of mitochondrial cytochrome *c* release. Importantly, knockdown of SREBF2 or STARD4 decreased mitochondrial cholesterol levels and increased mitochondrial cytochrome *c* release, respectively. Moreover, overexpression of STARD4 reversed the effect of SREBF2 knockdown on mitochondrial cytochrome *c* release and sorafenib resistance. In conclusion, SREBF2 promotes STARD4 transcription, which in turn contributes to mitochondrial cholesterol transport and sorafenib resistance in HCC. Therefore, targeting the SREBF2–STARD4 axis would be beneficial to a subset of HCC patients with sorafenib resistance.

KEYWORDS
cholesterol transport, mitochondrial membrane permeability, sorafenib resistance, SREBF2, StAR

Abbreviations: Dox, doxycycline; HCC, hepatocellular carcinoma; LPDS, lipoprotein-deficient serum; MMP, mitochondrial membrane permeabilization; SREBF2, sterol regulatory element binding transcription factor 2; STARD4, StAR related lipid transfer domain containing 4; StAR, steroidogenic acute regulatory protein; T-IC, tumor-initiating cell; X-XO, xanthine plus xanthine oxidase..

1 | INTRODUCTION

Hepatocellular carcinoma is the most common form of primary liver cancer, and is currently the major malignancy worldwide.[1] Typically, patients are diagnosed when the advanced stages are already present. For these cases, sorafenib is the first FDA-approved drug for treatment.[2] Two large-scaled phase III clinical trials showed that sorafenib treatment expanded the median survival of patients with advanced stage HCC.[2,3] Recently, lenvatinib has been approved by the FDA for unresectable HCC, but sorafenib is still the most effective single-drug therapy.[4] Despite initial response, the survival benefits of sorafenib treatment are limited, partly due to sorafenib resistance.[3,5] As sorafenib, a tyrosine kinase inhibitor, could target multiple growth factors, including Raf, c-Kit, VEGF, and PDGF,[6–8] sorafenib resistance could include many complicated mechanisms, which are still unclear. In order to overcome sorafenib resistance in HCC, it is urgent to further elucidate the precise underlying molecular mechanism.

Metabolic reprogramming is a hallmark of cancer.[9] Among them, reprogramming of lipid metabolism plays a critical role in the pathogenesis of tumors.[10] Cholesterol is a major type of lipid, which accounts for more than 20% of lipids in the mammalian cell membrane.[11,12] In HCC, reprograming of cholesterol metabolism includes increased cholesterol esterification, enhanced cholesterol synthesis and uptake, and activated cholesterol trafficking.[13–16] All of these contribute to progression of HCC in many aspects, such as cell proliferation, tumor-associated immune microenvironments, and migration and invasion.[15,17,18] Sterol regulatory element binding transcription factor 2, a master regulator of cholesterol metabolism, mainly activates transcription of cholesterol synthesis genes, such as *HMGCR*, *HMGCS1*, and *SQLE*. A previous study reported that SREBF2 activity was significantly elevated in HCC patients with poorer prognosis.[19] Emodin, fluvastatin, and simvastatin, the cholesterol-lowering drugs, have been reported to sensitize different type of tumors to sorafenib,[20–22] suggesting that cholesterol metabolism might play a certain role in sorafenib resistance. In 2008, Montero et al. reported that mitochondrial cholesterol contributes to chemoresistance in HCC by regulating mitochondrial membrane permeability and mitochondrial cytochrome *c* release.[16] Until now, the role of SREBF2 in sorafenib resistance of HCC has not been fully elucidated. Therefore, understanding the role of SREBF2 in sorafenib resistance of HCC would benefit the search to overcome it.

Besides cholesterol synthesis, uptake, and efflux, intracellular cholesterol distribution involves in cholesterol homeostasis. Steroidogenic acute regulatory proteins, containing an internal hydrophobic cavity for lipid binding, play important roles in the trafficking of cholesterol between diverse intracellular membranes.[23,24] Among them, STAR, a member of StARs, contributes to the mitochondrial intermembrane trafficking of cholesterol, which in turn mediates resistance of HCC to chemotherapy,[16] indicating that the other StARs might also be involved in chemoresistance. A previous report showed that STARD4, belonging to the family of StARs, is highly expressed in mouse liver tissue.[25] Further studies indicated that STARD4 associates with many types of cancers, including breast cancer, head and neck squamous cell carcinoma, and prostate cancer.[26–28] However, the role of STARD4 in HCC progression and sorafenib resistance has not been reported. Many studies showed that STARD4 is capable of delivering cholesterol to isolated mitochondria,[29–31] which could affect mitochondrial membrane permeability and mitochondrial cytochrome *c* release, involving in sorafenib-induced cell death. This hypothesis needs deep investigation.

Here, our study reveals that SREBF2 directly binds to STARD4 promoter for its transactivation, which in turn increases mitochondrial cholesterol levels and inhibits mitochondrial cytochrome *c* release, contributing to sorafenib resistance in HCC. This study provides potential therapeutic targets for HCC patients with sorafenib resistance.

2 | MATERIALS AND METHODS

2.1 | Cells and reagents

HepG2, Huh7, and HEK293 cells were purchased from Beyotime Biotechnology and National Collection of Authenticated Cell Cultures, which were cultured in DMEM with 10% FBS at 37°C. All experiments were carried out with mycoplasma-free cells. The specific reagents used in this study Dox and sorafenib (Sigma).

2.2 | Vectors and oligos

The Dox-induced SREBF2-knockdown vector was purchased from Vigene Biosciences, and the shRNA targeted sequence is shown in Table S1. The SREBF2 and STARD4 shRNAs (Table S1) were synthesized by Sangon Biotech and cloned in pLVX-Puro vector. Polymerase chain reaction-amplified mature human SREBF2 (1–468 bp) and STARD4 were each cloned into Flag-tagged pCMV vector. Full-length STARD4 promoter and truncates were amplified using the specific primers and cloned into pGL3-basic vector. The specific primers for cloning and gene expression and siRNA oligos were synthesized by Sangon Biotech (Table S1).

2.3 | Mouse models

Female nude mice (6–8 weeks) were purchased from Vital River and maintained under specific pathogen-free conditions. All animal experiments were carried out under the approval of the Institutional Animal Care and Use Committee at School of Basic Medical Sciences, Shandong University and conformed to the provisions of the Declaration of Helsinki.

Sorafenib-resistant xenograft models were established as previously described.[32] HepG2 cells (1×10^7) were injected into the flank of each mouse to form xenograft tumors. The mice were then intraperitoneally treated with sorafenib (50 mg/kg/every 2 days) or vehicle. Xenograft tumors showing increased growth (responding to

sorafenib more than 15 days and increasing more than 30% volume in 3 days) and relapsed (eliminated in the first 2–3 weeks and regrowing) were considered sorafenib resistant.[32]

To evaluate the function of SREBF2 or STARD4 in sorafenib resistance, established sorafenib-resistant xenografts were randomly separated into three groups. One group was continuously treated with sorafenib (50 mg/kg/every 2 days). The second group of mice were intratumorally injected with viruses (shSREBF2 or shSTARD4 viruses) (1×10^8 pfu/tumor three times). The third group of mice were treated with a combination of sorafenib with virus injection.

HepG2 cells were transduced with SREBF2 knockdown and/or STARD4 overexpression viruses to establish the stable cells. The stable cells (1×10^7) were subcutaneously implanted into the flank of female nude mice (6–8 weeks old) to form xenograft tumors. The mice were randomly separated into four groups according SREBF2 and STARD4 expression. The mice were then treated with sorafenib (30 mg/kg/every 2 days) and/or Dox (2 mg/ml) in drinking water.

Tumor volumes (V) were monitored and calculated using the following formula: V (mm^3) = $L\times W^2/2$, with L and W representing the longest and shortest diameters, respectively. The mice were killed and the tumors were used to make paraffin sections for TUNEL assay and Ki-67 staining.

2.4 | Mitochondrial cytochrome *c* release

Isolated mitochondria were incubated with xanthine (0.1 mM) plus xanthine oxidase (40 mU/ml; X-XO) (Sigma) for 5 min at room temperature. The protein in the supernatants was collected. The level of cytochrome *c* was determined using anti-cytochrome *c* (4272; Cell Signaling Technology) by western blot analysis.

2.5 | Statistical analysis

Statistical significance of the results obtained by Student's *t*-test or two-way ANOVA. Data are presented as mean and SD, and $p<0.05$ was considered statistically significant. $^*p\le0.05$, $^{**}p\le0.01$, $^{***}p\le0.001$.

3 | RESULTS

3.1 | SREBF2 is activated in sorafenib resistant HCC

SREBF2 is the master regulator of cholesterol metabolism.[19] Here, the cell viability of SREBF2-knockdown HepG2 and Huh7 was decreased more clearly than that of control siRNAs transfected cells after sorafenib treatment (Figure 1A,B), suggesting SREBF2 plays a role in sorafenib resistance of HCC. To further evaluate it, sorafenib resistant cell lines were established and were defined as HepG2R and Huh7R. Indeed, these cells were less sensitive to sorafenib than parental cells (HepG2P and Huh7P) (Figure 1C). Mounting evidence has shown that sorafenib resistance was driven by T-ICs.[32,33] Consistently, the properties of T-ICs, cell migration and sphere formation, were increased in sorafenib resistant cells, displaying as more migrated cells and larger and more spheres were observed in sorafenib resistant cells than in parental cells (Figure 1D,E). Furthermore, the mRNA levels of *CD133*, *EpCAM*, *SOX2*, and *NANOG*, the markers of T-ICs, were higher in sorafenib resistant cells than in parental cells (Figure 1F). Interestingly, the elevation of mature SREBF2 protein was observed in sorafenib resistant HepG2 and Huh7 cells (Figure 1G). The expression of SREBF2 target genes, such as *HMGCR*, *MVD*, and *IDI1*, also showed the trend of upregulation in specimens from sorafenib resistant HCC patients (Figure 1H), but the trend was not statistically significant due to limited samples. All of these data suggest activation of SREBF2 involves in sorafenib resistance of HCC.

3.2 | Knockdown of SREBF2 resensitizes sorafenib resistant HCC to sorafenib treatment

To further explore the role of SREBF2 in sorafenib resistance, sorafenib resistant properties were examined in HepG2R and Huh7R with SREBF2 knockdown. SREBF2 was knocked down in HepG2R and Huh7R cells by two different siRNAs (Figure S1A). As shown in Figure 2A, knockdown of SREBF2 resensitized HepG2R and Huh7R cells to sorafenib at different concentrations. To confirm these results, HepG2R and Huh7R cells were transduced with Dox-induced shSREBF2 viruses (Figure S1B). The ability of cell migration was decreased in HepG2R and Huh7R with Dox treatment. Notably, the number of migrated cells were dramatically decreased in HepG2R and Huh7R cells with sorafenib and Dox combined treatment compared to each individual treatment (Figure 2B). Similarly, smaller and fewer spheres were observed in combined treated cells than each individual treatment (Figure 2C).

To further verify the role of SREBF2 in sorafenib resistance in vivo, a sorafenib resistant xenograft tumor mouse model was established (Figure S1C).[32] Endogenous SREBF2 was knocked down by intratumoral injection of viruses armed with shSREBF2 (Figure S1D). As shown in Figure 2D, shSREBF2 virus injection inhibited tumor growth compared to the sorafenib continuous treatment group. Importantly, the tumor growth rate was dramatically decreased in xenografts tumors with sorafenib and shSREBF2 virus combined treatment. Consequently, more TUNEL-positive cells, and fewer Ki-67-positive cells were observed in xenograft tumors with sorafenib and shSREBF2 combined treatment than in those with each individual treatment (Figure 2E). Above all, SREBF2 confers sorafenib resistance in HCC.

3.3 | SREBF2 positively associates with STARD4 in sorafenib resistant HCC

As SREBF2 is the master regulator of cholesterol synthesis, total cholesterol levels were measured in parental and resistant liver

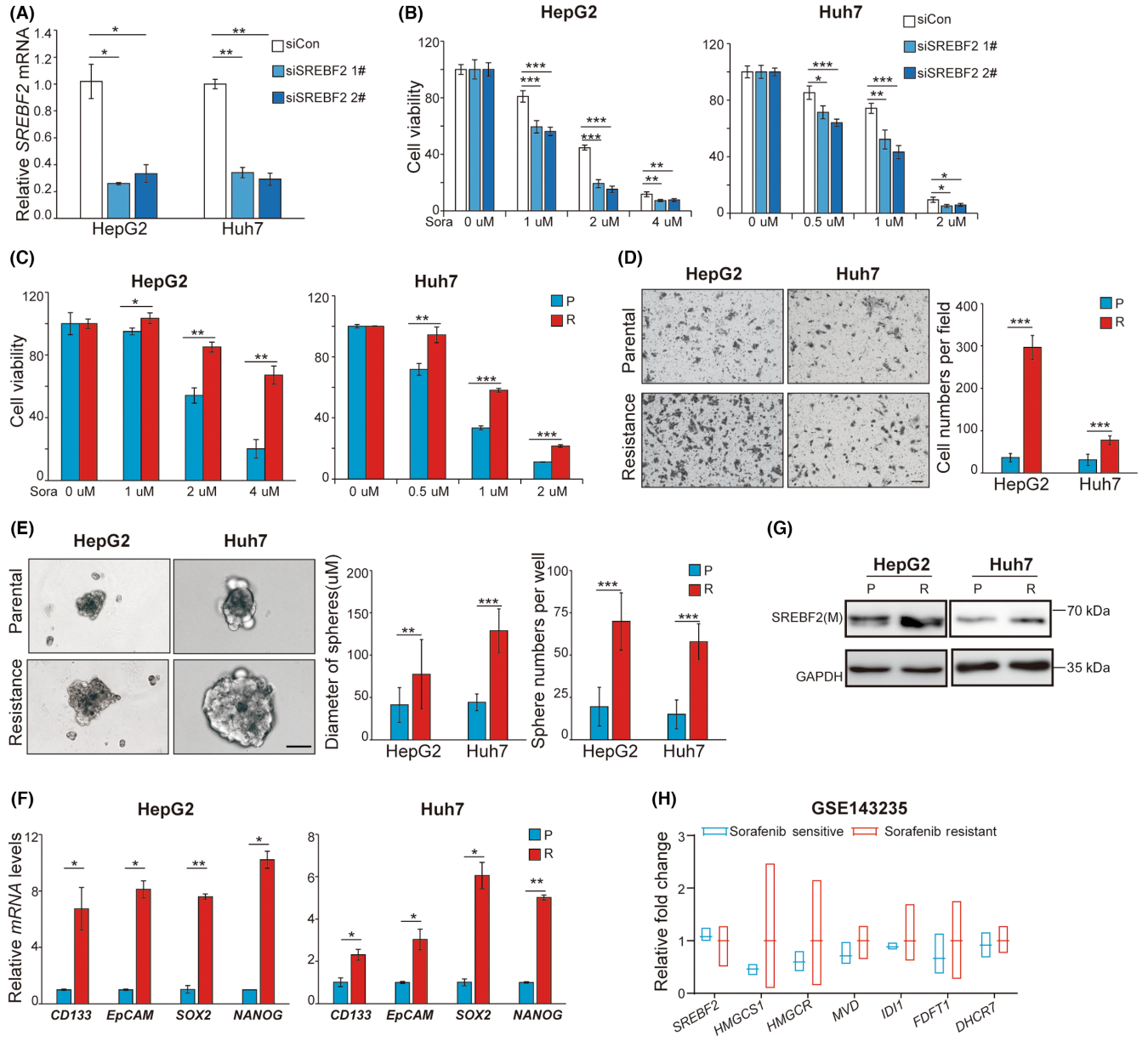

FIGURE 1 SREBF2 expression is elevated in sorafenib resistant cells and specimens. (A) Endogenous SREBF2 was examined in HepG2 and Huh7 cells with siSREBF2 transfection by quantitative RT-PCR (RT-qPCR). (B) Cell viability was detected in SREBF2-knockdown HepG2 and Huh7 cells after sorafenib treatment. (C) Cell viability was determined in sorafenib resistant (R) and parental (P) HepG2 and Huh7 cells with sorafenib treatment. (D) Cell migration assays were carried out with sorafenib resistant and parental HepG2 and Huh7 cells. Left: representative images; right: statistical analysis. (E) Sorafenib resistant and parental HepG2 and Huh7 cells were used to evaluate sphere formation ability. Left: representative images; middle and right: statistical analysis. (F) mRNA levels of tumor-initiating cell markers were detected in parental and resistant HepG2 and Huh7 cells by RT-qPCR. (G) Protein levels of SREBF2 were examined in parental and resistant HepG2 and Huh7 cells by western blot analysis. (H) Cholesterol synthesis enzymes were analyzed in a sorafenib resistant HCC cohort (GSE143235). $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$

cancer cells by using flow cytometry. As shown in Figure S2A, total cholesterol levels had no significant difference between parental and resistant cells, determined by Amplex Red Cholesterol Assay Kit (Invitogen, USA). Inflorescence microscopy showed that the distribution of cholesterol in parental HepG2 and Huh7 cells is different than that in sorafenib resistant cells. Especially, localization of cholesterol in mitochondria of sorafenib resistant cells were increased (Figure 3A). StARs regulate cholesterol distribution in mammary cells.[24] Here, STARD3 and STARD4 were highly expressed in liver tissues (Figure S2B, https://www.proteinatlas.org). However, only STARD4 was clearly upregulated in our established sorafenib resistant HepG2 and Huh7 cells (Figure 3B). Consistent to our results, the expression of STARD4 is also dramatically increased in two publicly available sorafenib resistant datasets[32,34] (Figure 3C). Moreover, the expression of well-known SREBF2 targets *HMGCR* and *SQLE* were upregulated in LPDS cultured HepG2 and Huh7 cells. Importantly,

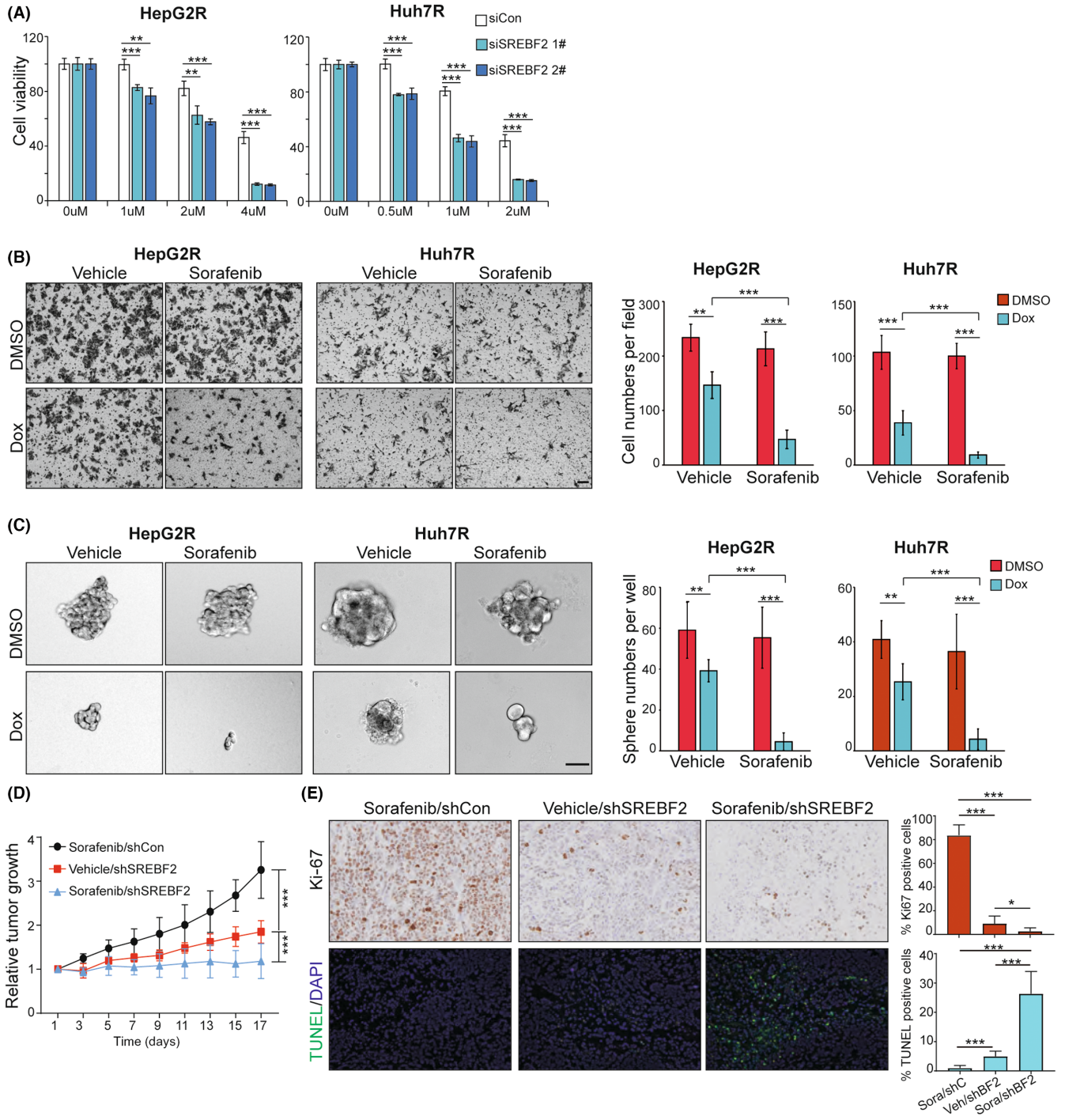

FIGURE 2 Knockdown of SREBF2 reverses the sorafenib resistant properties of hepatocellular carcinoma. (A) HepG2R and Huh7R cells were transfected with siSREBF2s or control (siCon) for cell viability after 24 h of treatment with sorafenib. (B, C) HepG2R and Huh7R cells were transduced with doxycycline (Dox) inducible SREBF2-knockdown viruses for cell migration (B) and sphere formation (C). (D) Growth curves of sorafenib resistant xenograft tumors with sorafenib and/or shSREBF2 were displayed. (E) Paraffin xenograft tumor tissues were used to carry out TUNEL assays and Ki-67 staining. Representative images and statistical data are presented. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$. shBF2, shSREBF2; shC, shControl; Sora, sorafenib; Veh, vehicle

the mRNA levels of *STARD4* were also clearly increased in these LPDS cultured cells (Figure 3D). Furthermore, overexpression of mature SREBF2 increased the mRNA levels of *STARD4* (Figure 3E). Reciprocally, knockdown of SREBF2 decreased the expression of STARD4 (Figures 3F and S2C). Interestingly, the mRNA levels of *SREBF2* and *STARD4* were positively correlated in two independent patients cohorts (Figure 3G).[35] Above all, SREBF2 upregulates STARD4 at transcriptional level.

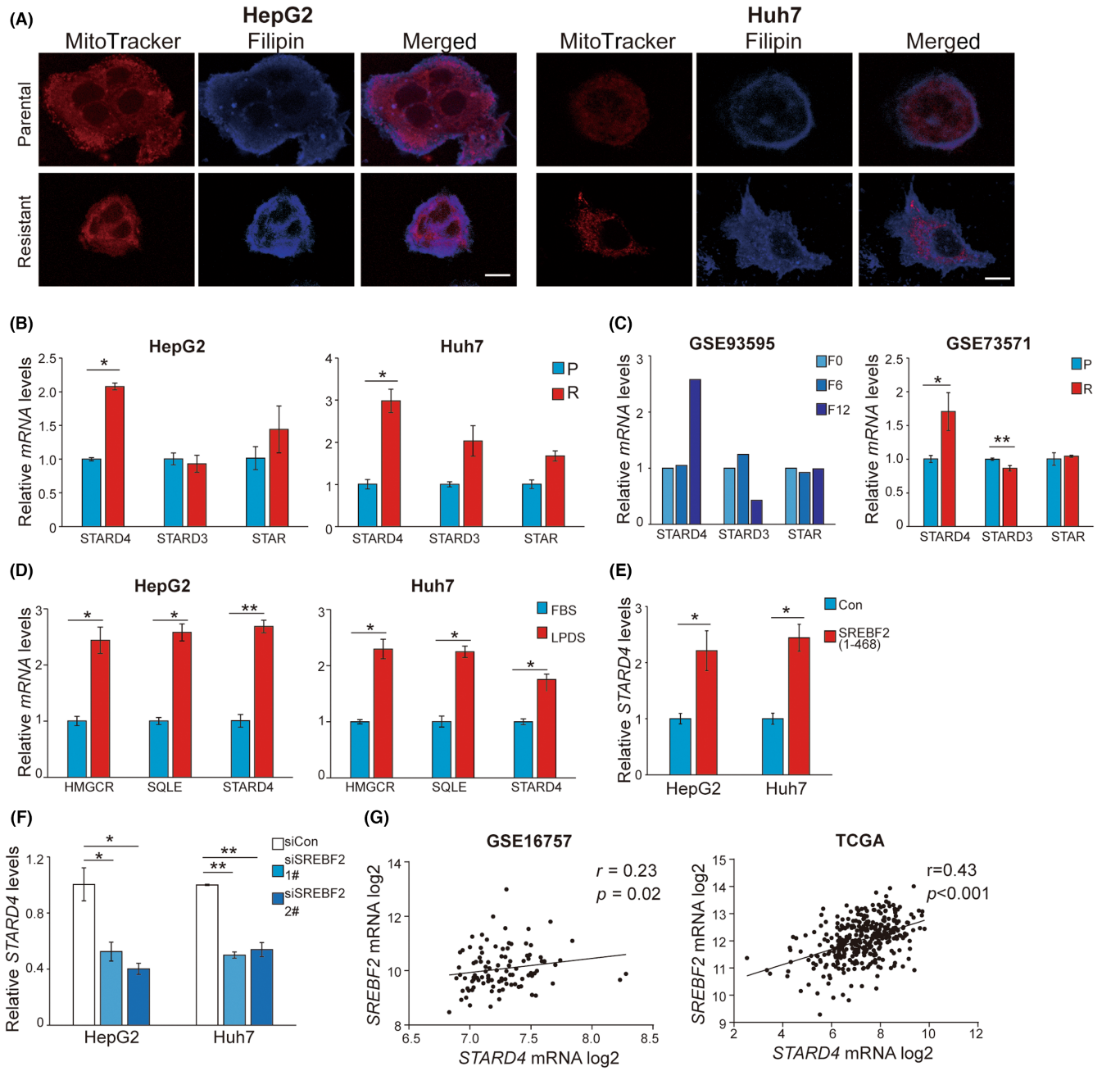

FIGURE 3 SREBF2 promotes STARD4 expression at transcriptional level. (A) Sorafenib resistant (R) and parental (P) Huh7 and HepG2 cells were stained with filipin (cholesterol) and MitoTracker (mitochondria). (B) Expression of StARs were examined in parental and resistant cells by quantitative RT-PCR (RT-qPCR). (C) mRNA levels of StARs were evaluated in two sorafenib resistant HCC models (GSE93595 and GSE73571). F0, parental Huh7 cells; F6 and F12, sorafenib resistant Huh7 cells. (D) mRNA levels of *HMGCR*, *SQLE*, and *STARD4* were evaluated in HepG2 and Huh7 cells with indicated treatments. (E, F) *STARD4* mRNA levels were determined in HepG2 and Huh7 cells with SREBF2 manipulation by RT-qPCR. (G) Positive correlation of SREBF2 and STARD4 expression was observed in two publicly available datasets (GSE16757 and The Cancer Genome Atlas [TCGA]). $^{*}p \le 0.05$, $^{**}p \le 0.01$

3.4 | SREBF2 directly binds to the promoter of STARD4 for transactivation

To decipher the molecular mechanism of SREBF2 in promoting STARD4 transcription, the dual luciferase reporter plasmid of STARD4 promoter (−1942 to +90 bp) was successfully constructed (Figure 4A). Both mature SREBF2 and LPDS cells increased luciferase activity of STARD4 promoter reporter compared to that of the control vector and FBS, respectively (Figure 4B). The promoter region of STARD4 was analyzed by using JASPAR (http://jaspar.genereg.net/), an online database to predict transcriptional factor binding sites. As shown in Figure 4C, five putative SREBF2-bound sites

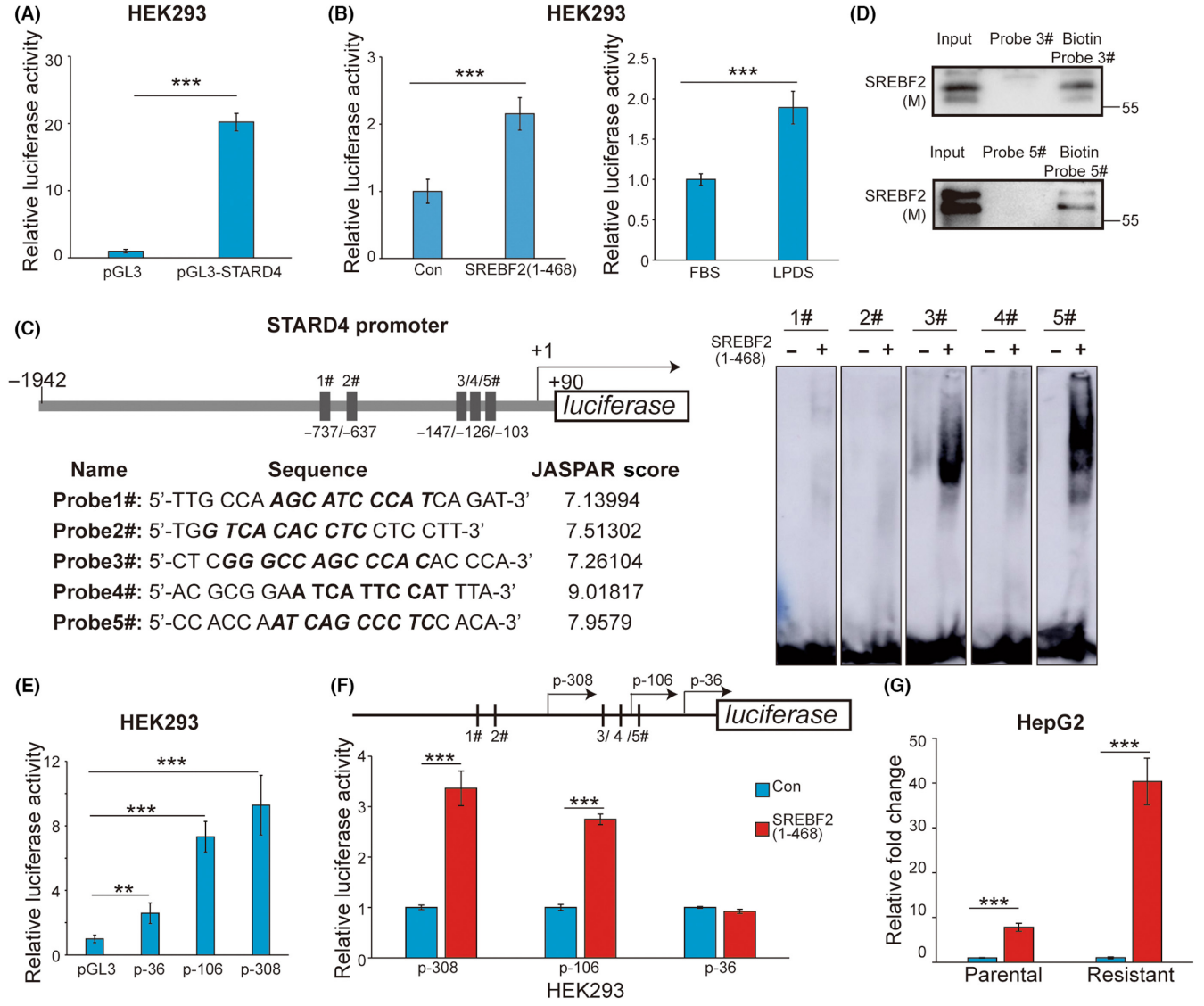

FIGURE 4 SREBF2 binds to STARD4 promoter region for transactivation. (A) Full-length STARD4 promoter reporter or basic reporter were transfected in HEK293 to examine luciferase activity. (B) Luciferase activity of STARD4 promoter was determined in HEK293 cells with indicated manipulation. Con, control; LPDS, lipoprotein-deficient serum. (C) Left: location of five putative SREBF2-bound sites on STARD4 promoter; right: representative gel-shift images of EMSA assays. (D) Biotin-labeled probes pull-down protein was examined by western blot. M, mature. (E) Luciferase activity of STARD4 promoter truncates were determined in HEK293 cells. (F) Luciferase activity of STARD4 promoter truncates were determined in HEK293 cells with mature SREBF2 or control vector transfection. (G) Genomic DNA fragments were pulled down from sorafenib parental and resistant HepG2 cells using IgG and SREBF2 Ab to undertake quantitative PCR. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Con, control

were identified on STARD4 promoter region. Moreover, the EMSA results showed that mature SREBF2 directly bound to the sites of 3# and 5# on STARD4 promoter (Figure 4C, right panel). Consistently, the biotin-labeled 3# and 5# probes directly pulled down mature SREBF2 (Figure 4D). The series of STARD4 promoter truncated reporters were then constructed (Figure 4E). When both sites of 3# and 5# were deleted, the mature SREBF2-induced increase of luciferase activity was eliminated (Figure 4F). Moreover, ChIP assay showed that recruitment of SREBF2 on STARD4 promoter is clearer in sorafenib resistant HepG2 cells than in parental cells (Figure 4G). Therefore, SREBF2 directly binds to STARD4 promoter region for its transactivation.

3.5 | STARD4 promotes HCC development and sorafenib resistance

To the best of our knowledge, there are limited reports about the effect of STARD4 in HCC. The expression of STARD4 was evaluated in HCC tumor and para-tumor tissues. As shown in Figure S3A, STARD4 mainly localized in cell cytoplasm, and STARD4 protein levels were higher in tumors than in paratumors (Figure S3B), suggesting STARD4 correlates with HCC progression. Endogenous STARD4 levels were knocked down by two different shSTARD4 in HepG2 and Huh7 (Figure S3C). Moreover, knockdown of STARD4 inhibited cell proliferation and cell migration in HepG2 and Huh7

cell lines (Figure S3D,E). Analysis of publicly available datasets (The Cancer Genome Atlas) revealed that patients with high expression of STARD4 were correlated to poorer prognosis than those with low STARD4 expression (http://kmplot.com/analysis/) (Figure S3F). Taken together, STARD4 is positively associated with HCC progression.

To evaluate the role of STARD4 in HCC sorafenib resistance, STARD4 was knocked down in HepG2R and Huh7R cells by a previously proven siRNA (Figure 5A).[30] The cell viability of STARD4-knockdown sorafenib resistant HepG2 and Huh7 cells was lower than those of siCon transfected cells (Figure 5B). Concurrently, fewer migrated cells and smaller and fewer spheres were observed in sorafenib resistant cells with siSTARD4 and sorafenib combined treatment than in those with each individual treatment (Figure 5C,D). Sorafenib resistant xenograft tumor models were then treated with sorafenib and/or shSTARD4. As shown in Figure 5E, shSTARD4 inhibited xenograft tumor growth. Notably, the combination of sorafenib and shSTARD4 inhibited tumor growth more dramatically than sorafenib or shSTARD4 individual treatment. Consistently, more TUNEL-positive cells and fewer Ki-67-positive cells were observed in combined sorafenib and shSTARD4 treated xenograft tumors than in those treated with sorafenib or shSTARD4 individually (Figure 5F). All of these data demonstrate that STARD4 promotes sorafenib resistance in HCC.

3.6 | SREBF2 regulates mitochondrial membrane permeability and mitochondrial cytochrome *c* release in HCC through STARD4

As STARD4 affects mitochondrial cholesterol metabolism,[26–28] the mitochondrial cholesterol levels of parental and sorafenib resistant HepG2 and Huh7 cells were measured. The results showed that mitochondria were successfully isolated (Figure 6A). Notably, the cholesterol levels were higher in mitochondria isolated from sorafenib resistant HepG2 and Huh7 cells than from parental cells (Figure 6A). Mitochondrial cholesterol regulates MMP and mitochondrial cytochrome *c* release.[16] To evaluate MMP, mitochondrial permeability transition was triggered by using the superoxide anion-generating system, X-XO, to induce the release of mitochondrial cytochrome *c*.[36] As shown in Figure 6B, isolated mitochondria from HepG2R and Huh7R resisted X-XO-induced cytochrome *c* release. Additionally, mitochondrial ROS levels were decreased in sorafenib resistant cells compared to parental cells (Figure S4A). Importantly, sorafenib treatment induced diffusion of cytochrome *c* is more obvious in parental Huh7 cells than that in sorafenib resistant Huh7 cells (Figure S4B).

To clarify the relationship between SREBF2 and STARD4 in MMP, mitochondrial cholesterol levels and mitochondrial cytochrome *c* release were analyzed. As shown in Figure 6C,E, knockdown of SREBF2 or STARD4 reduced mitochondrial cholesterol in sorafenib resistant HepG2 and Huh7 cells, respectively. Importantly, HepG2R and Huh7R cells resensitized to X-XO-induced cytochrome *c* release after knockdown of SREBF2 or STARD4 (Figure 6D,F). STARD4 was also overexpressed in SREBF2-knockdown HepG2 and Huh7 cells (Figure S4C). The results revealed that the reduction of mitochondrial cholesterol levels in SREBF2-knockdown HepG2 and Huh7 cells were recovered after overexpression of STARD4 (Figure 6G). Consequently, overexpression of STARD4 reversed the increase of X-XO-induced mitochondrial cytochrome *c* release in SREBF2-knockdown HepG2 and Huh7 cells (Figure 6H). Therefore, STARD4 is the mediator of SREBF2 regulating mitochondrial MMP.

3.7 | SREBF2 contributes to sorafenib resistance in HCC through STARD4

To verify the role of the SREBF2–STARD4 axis in sorafenib resistance of HCC, cell viability was measured in HepG2 and Huh7 cells with siSREBF2 and/or Flag-tagged STARD4 transfection. Consistently, knockdown of SREBF2 increased sensitivity of HepG2 and Huh7 cells to sorafenib. Meanwhile, ectopic expression of STARD4 partially reversed the siSREBF2-induced increase of sorafenib sensitivity in HepG2 and Huh7 cells (Figure 7A). Flag-tagged STARD4 and/or Dox-induced shSREBF2 viruses were used to transduce HepG2 cells (Figure S4D). Similarly, knockdown of SREBF2 increased the sorafenib inhibitory effect on tumor growth. Importantly, ectopic expression of STARD4 partially reversed the effect of SREBF2 knockdown on sorafenib sensitivity (Figure 7B). Moreover, TUNEL assays and Ki-67 staining showed the same trends (Figure 7C). The analysis of survival rate revealed that patients with high SREBF2/STARD4 expression had a poorer prognosis than those with low SREBF2/STARD4 expression (http://kmplot.com/analysis/) (Figure 7D). These data further indicate that STARD4 is the downstream target of SREBF2, and the SREBF2–STARD4 axis contributes to sorafenib resistance in HCC.

4 | DISCUSSION

Cholesterol metabolism is dysregulated in HCC, including enhancement of cholesterol esterification, synthesis, and uptake, and activation of cholesterol trafficking.[13–16] However, the key regulator of cholesterol metabolism, SREBF2, in sorafenib resistance of HCC has not been clearly elucidated. Here, our data showed that knockdown of SREBF2 not only increased sensitivity of HCC to sorafenib, but also resensitized sorafenib resistant HCC to sorafenib treatment both in vitro and in vivo, indicating that SREBF2 plays a key role in HCC sorafenib resistance. Further results indicated that SREBF2 positively correlated with STARD4 and bound to the STARD4 promoter region for transactivation. Importantly, STARD4 promoted mitochondrial cholesterol trafficking and decreased MMP, leading to reducing of mitochondrial cytochrome *c* release. SREBF2 contributes to sorafenib resistance in HCC through STARD4-mediated mitochondrial cholesterol transport (Figure 7E). Therefore, understanding the role of the SREBF2–STARD4 axis in HCC sorafenib

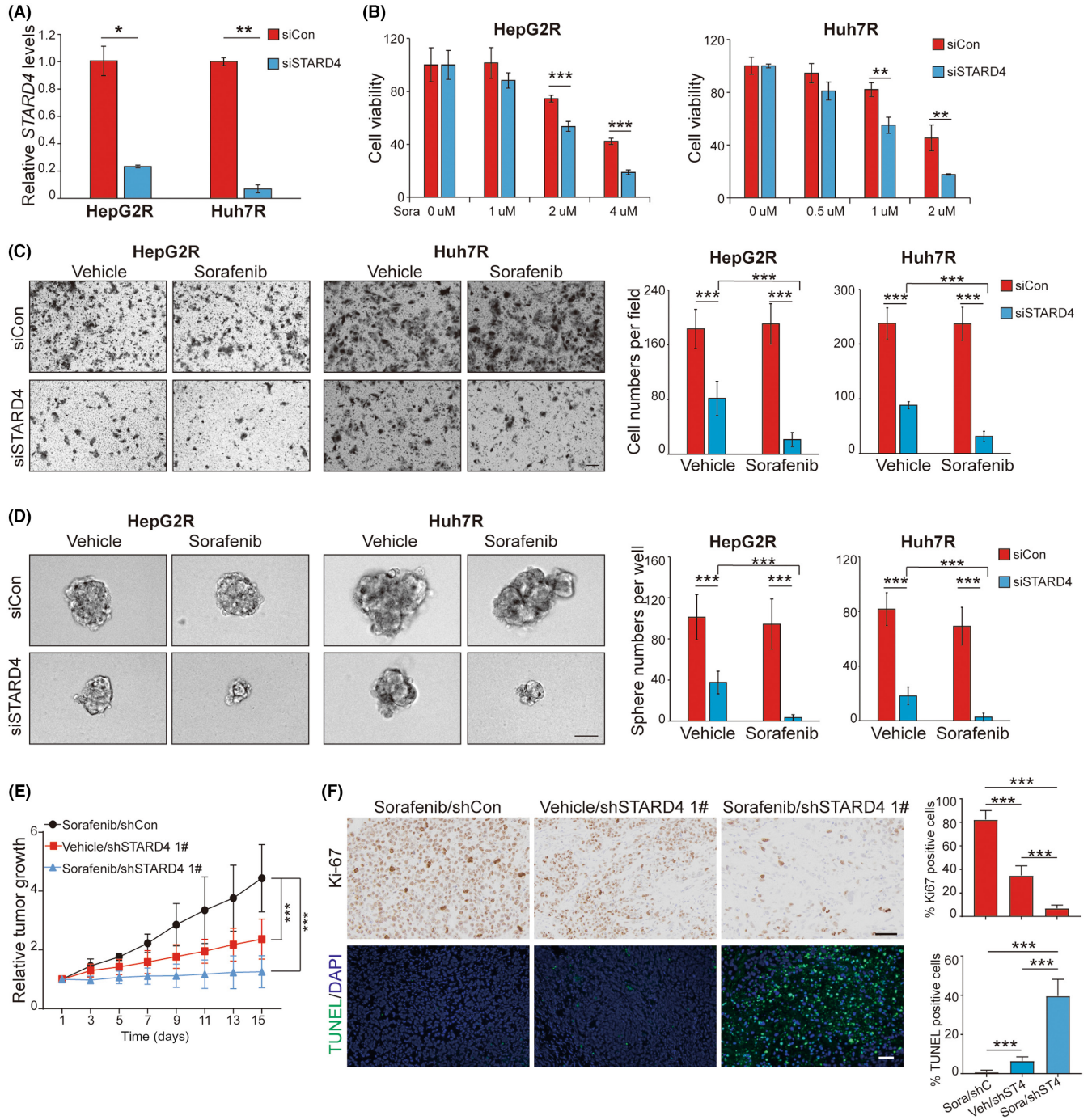

FIGURE 5 Knockdown of STARD4 resensitizes sorafenib resistant hepatocellular carcinoma to sorafenib. (A) *STARD4* mRNA level was determined in siSTARD4 transfected resistant HepG2 and Huh7 cells. (B) HepG2R and Huh7R cells were transfected with siSTARD4 and control (siCon) to detect cell viability after treatment of sorafenib. (C, D) HepG2R and Huh7R cells were transfected with siSTARD4 or control (siCon) to undertake cell migration (C) and sphere formation (D) assays, respectively. (E) Growth curves of xenograft tumors with shSTARD4 virus and/or sorafenib treatment. (F) TUNEL and Ki-67 staining were determined in paraffin xenograft tumor tissues. Representative images and statistical data are presented. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$

resistance will provide potential therapeutic strategies for HCC patients with sorafenib resistance.

As the master regulator of cholesterol metabolism, SREBF2 mainly activates expression of cholesterol synthesis key enzymes to promote de novo cholesterol synthesis.[17,19] Much evidence has illustrated that the cholesterol-lowering drugs, targeting the SREBF2 target gene *HMGCR*, sensitized different types of tumors to sorafenib.[20–22] Our data showed that direct knockdown of SREBF2 increased sorafenib sensitivity of HCC and resensitized sorafenib resistant HCC to sorafenib both in vitro and in vivo. The extent of SREBF2 activation differs between sorafenib resistant Huh7 (mutant p53 cell) and HepG2 (WT p53 cell). Previous reports have

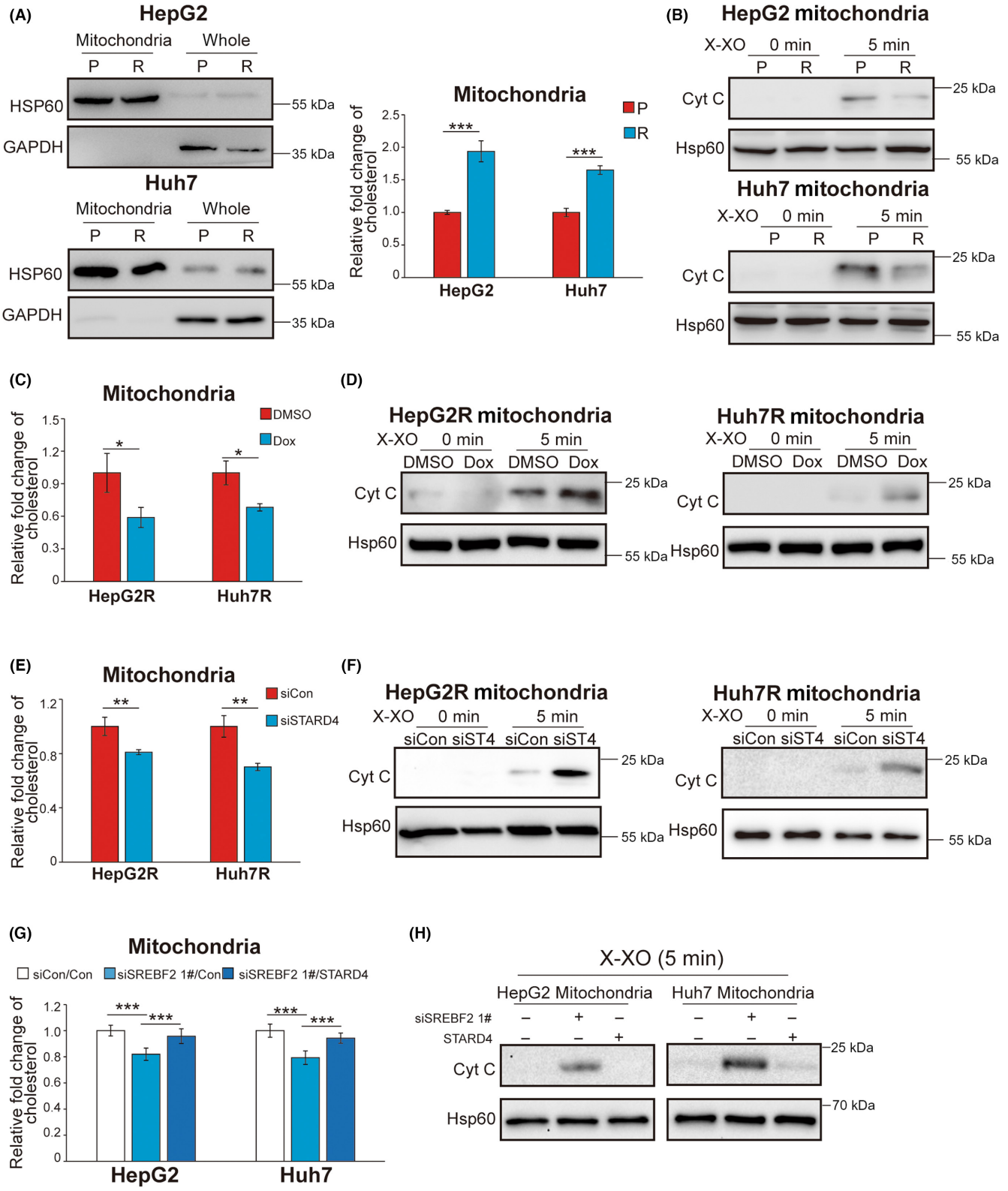

FIGURE 6 SREBF2 regulates mitochondrial membrane permeability through STARD4 in hepatocellular carcinoma. (A) Mitochondria of resistant and parental cells were isolated for cholesterol measurement. Left: purity of isolated mitochondria; right: statistical data of mitochondrial cholesterol. (B) Released mitochondrial cytochrome *c* (Cyt c) was determined by western blot analysis, induced by xanthine plus xanthine oxidase (X-XO). P, parental; R, resistant. (C, E) Mitochondria were isolated from SREBF2-knockdown (C) or STARD4-knockdown (E) sorafenib resistant HepG2 and Huh7 cells for measuring cholesterol levels, respectively. (D, F) Isolated mitochondria from SREBF2-knockdown (D) or STARD4-knockdown (F) sorafenib resistant HepG2 and Huh7 cells were used to induce mitochondrial cytochrome *c* release. (G) Mitochondrial cholesterol levels were determined in HepG2 and Huh7 cells with siSREBF2 and/or STARD4 transfection. (H) X-XO induced mitochondrial cytochrome *c* release in HepG2 and Huh7 cells with siSREBF2 and/or STARD4 transfection. Dox, doxycycline; HSP60, heat shock protein 60. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$

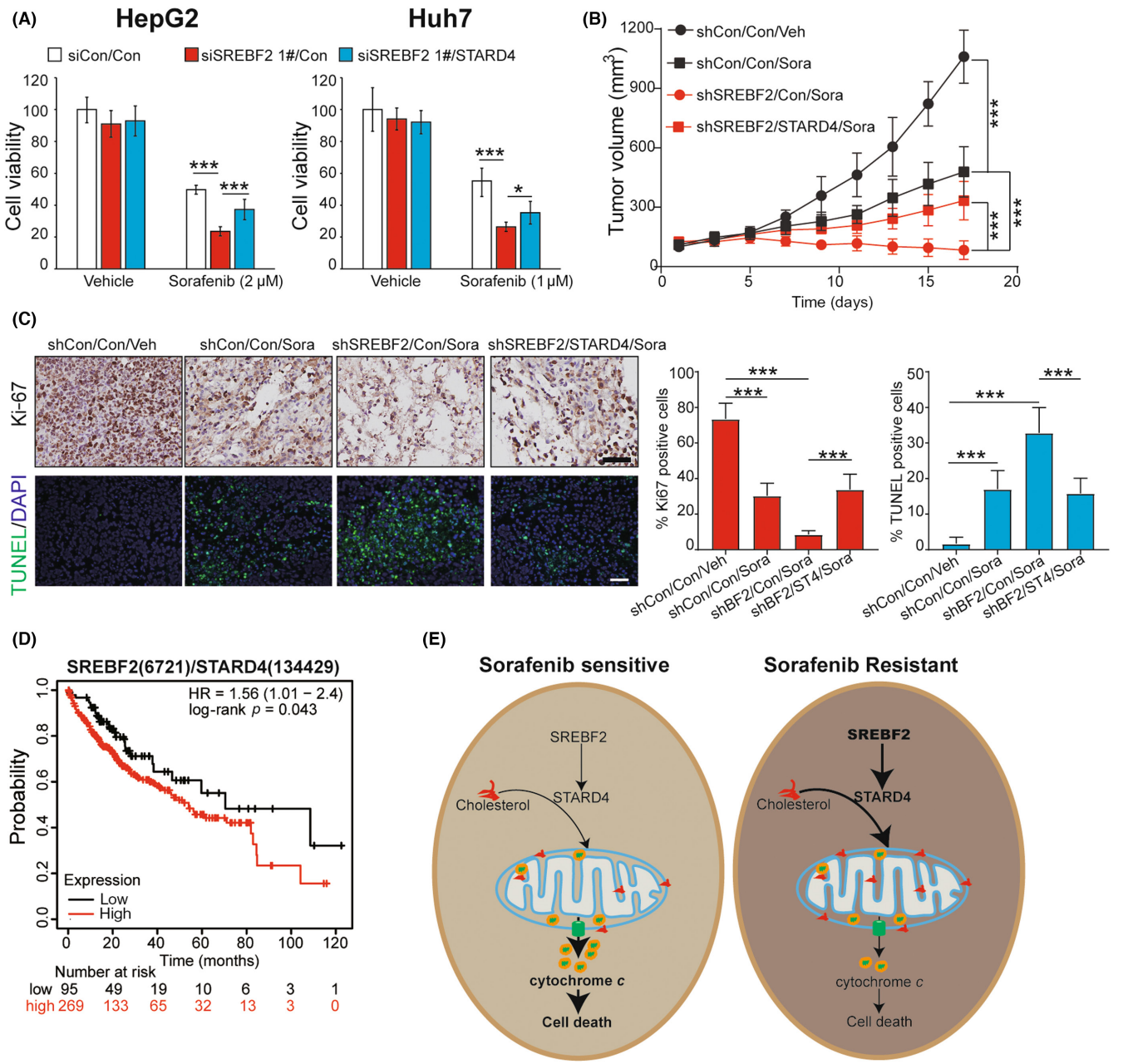

FIGURE 7 SREBF2 confers sorafenib resistance through STARD4 in hepatocellular carcinoma (HCC). (A) HepG2 and Huh7 cells were transfected with siSREBF2 and/or STARD4 to determine cell viability after sorafenib treatment. (B) Doxycycline (Dox)-induced shSREBF2 and/or Flag-tagged STARD4 viruses transduced HepG2 cells were used to establish xenograft tumors. Growth curves of xenograft tumors were presented. (C) Tumor tissues were used to undertake TUNEL assays and Ki-67 staining; representative images and statistical data are presented. (D) Kaplan–Meier curves indicate the overall survival curves of HCC patients (The Cancer Genome Atlas [TCGA]) with high SREBF2/STARD4 and low SREBF2/STARD4 expression. (E) Working model for this study. $^{*}p \le 0.05$, $^{***}p \le 0.001$

shown that WT p53 controls SREBF2 maturation through ABCA1.[37] Therefore, the p53 status might affect SREBF2 maturation and sorafenib resistance of HCC. Intriguingly, we found that total cholesterol levels were not changed in sorafenib resistant cells, in which SREBF2 was activated. Rohrl et al.'s study found that thapsigargin induced SREBF2 maturation could not lead to increase of cholesterol levels in HepG2 cells.[38] The reason of this phenomenon needs to be investigated in a future study.

An important finding of this study is that SREBF2 positively associates with STARD4 in liver cancer cell lines and patient samples. STARD4 belongs to the StARs family, which plays a major role in intracellular cholesterol transport.[24] However, the research about its function in HCC is limited. In this study, our data showed that STARD4 was highly expressed in the patient samples with HCC. Knockdown of STARD4 inhibited proliferation and migration of HepG2 and Huh7 cells. Clinically, high expression of STARD4

is associated with poor prognosis of HCC patients. The underlying mechanism of STARD4 in HCC development is still not clear. STARD4 could rapidly equilibrate sterols between membranes, especially membranes with anionic lipid headgroups.[39] Whether STARD4 is involved in HCC through regulation of membrane sterols needs further investigation.

Until now, how SREBF2 regulates STARD4 is not fully understood. Based on bioinformatics analysis, we found five putative SREBF2 binding sites upstream of the transcription start point of STARD4 promoter. Using the dual luciferase reporter, EMSA, ChIP, and pull-down assays, we clarified that SREBF2 directly bound to two specific motifs on the promoter region of STARD4. In addition, SREBF2 target genes and STARD4 were elevated in a sorafenib resistant cohort,[40] further indicating that SREBF2 contributes to sorafenib resistance in HCC through upregulation of STARD4. STAR and STARD3 had no significant difference in sorafenib resistant HCC samples. How SREBF2 specifically activates STARD4 in sorafenib resistant HCC is still unclear.

Importantly, our data revealed that STARD4-mediated mitochondrial cholesterol transport played an important role in SREBF2 promoting sorafenib resistance of HCC. Although many studies reported cholesterol enrichment in mitochondria, which could disrupt specific mitochondrial component and impair mitochondrial function in cancer cells,[41–43] its effect on sorafenib-induced cell death susceptibility has not been well investigated. Here, our results indicated that STARD4-mediated mitochondrial cholesterol trafficking decreased MMP to inhibit release of mitochondrial cytochrome *c*. By using the superoxide anion-generating system,[36] our data showed that mitochondria from sorafenib resistant HepG2 and Huh7 cells resisted to X-XO-induced cytochrome *c* release, and knockdown of SREBF2 or STARD4 restored X-XO-induced cytochrome *c* release. More importantly, overexpression of STARD4 reversed the decrease in mitochondrial cholesterol levels and increase of mitochondrial cytochrome *c* release in SREBF2-knockdown HepG2 and Huh7 cells. Therefore, strategies of targeting MMP through modulation of the SREBF2–STARD4 axis could contribute to resensitizing sorafenib resistant HCC to sorafenib.

In conclusion, we have characterized that HCC sorafenib resistance is driven by the SREBF2–STARD4 axis in this study. These findings suggest that targeting SREBF2–STARD4 axis seems to be a promising novel therapeutic strategy for improving the efficacy of sorafenib therapy.

AUTHOR CONTRIBUTIONS

Xuetian Yue designed and performed the experiment, analyzed the data, wrote and reviewed the manuscript; Youzi Kong, Yankun Zhang, and Shuyue Liu performed the experiment and analyzed the data; Zhuanchang Wu, Lifen Gao, Xiaohong Liang, and Chunhong Ma wrote and reviewed the manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation of China (no. 81972819), Natural Science Foundation of Shandong Province (no. ZR2020YQ57), and Young Innovation Team Foundation of Shandong University.

DISCLOSURE

The authors have no conflict interest.

ETHICS STATEMENT

Approval of research protocol by an Institutional Review Board at the School of Basic Medical Sciences, Shandong University.

ANIMAL STUDIES

The animal studies were approved by the Ethics Committee of School of Basic Medical Sciences, Shandong University.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Yue X, Kong Y, Zhang Y, et al. SREBF2–STARD4 axis confers sorafenib resistance in hepatocellular carcinoma by regulating mitochondrial cholesterol homeostasis. *Cancer Sci*. 2023;114:477-489. doi: 10.1111/cas.15449