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Antidiabetic drug metformin suppresses tumorigenesis through inhibition of mevalonate pathway enzyme HMGCS1

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Yiyan Chen[1,‡], **Min Li**[1,2,‡], **Yanying Yang**[1], **Yan Lu**[1,3,*], **and Xiaoying Li**[1,4,*]

From the [1]*Ministry of Education Key Laboratory of Metabolism and Molecular Medicine, Department of Endocrinology and Metabolism, Zhongshan Hospital, Fudan University, Shanghai, China;* [2]*The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong, China;* [3]*Institute of Metabolism and Regenerative Medicine, Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China;* [4]*Shanghai Key Laboratory of Metabolic Remodeling and Health, Institute of Metabolism and Integrative Biology, Fudan University, Shanghai, China*

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Metformin, an antidiabetic drug, shows some potent antitumor effects. However, the molecular mechanism of metformin in tumor suppression has not been clarified. Here, we provided evidence using *in vitro* and *in vivo* data that metformin inhibited mevalonate pathway by downregulation of 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1), a key enzyme in this pathway. Our results further demonstrated that metformin downregulated HMGCS1 expression through inhibition of transcription factor nuclear factor E2–related factor 2. In addition, we determined that HMGCS1 was highly expressed in human liver and lung cancer tissues and associated with lower survival rates. In summary, our study indicated that metformin suppresses tumorigenesis through inhibition of the nuclear factor E2–related factor 2–HMGCS1 axis, which might be a potential target in cancer prevention and treatment.

Tumor growth and metastases need a constant and excessive supply of nutrients as building bricks, fuels, and signals so that altered nutrient metabolism is widely accepted to be added to the list of hallmarks of cancer (1). Among those nutrient sources, mevalonate pathway intermediates have been reported significantly accumulated in multiple tumors (2). Recent studies reported that mevalonate pathway and its key enzyme 3-hydroxy-3-methylglutaryl-CoA receptor (HMGCR) had additional potent tumor-supportive effects besides sterol biosynthesis. Driven by oncogenes, such as mutant p53 (3, 4) and MYC (5), the mevalonate flux plays a favorable role in several types of cancers. Several studies focus on the mevalonate flux's role in the misfolded state and stability of tumor-suppressor gene p53 (6, 7), thus forming a positive feedback loop in tumor formation. The mevalonate pathway provides several intermediate products that are important to tumor growth, for example, ubiquinone to adjust redox control and mitochondrial activity during nutrient restriction, and squalene for prevention of oxidative cell death, bile acid (8) signaling, and its crosstalk with microbiota, let alone cholesterol, which is important for cell membrane formation and signal transduction in cancer cells (9).

Metformin, an oral hypoglycemic agent mainly prescribed to patients with type 2 diabetes, is known for its pleiotropic functions and safety. Besides the antidiabetic effects, metformin also shows the latent capacity to prevent and improve the prognosis of multiple cancer such as breast cancer (10) and prostate cancer (11). According to ClinicalTrials.gov (https://clinicaltrials.gov/), 393 clinical trials are registered using metformin in the treatment of cancer till February 2022. Diabetes Prevention Program Outcomes Study demonstrated that metformin was associated with a 12% lower risk of cancer in a 22-year follow-up (12). After adjusting for age, gender, viral hepatitis, and other variables, metformin still reduced the incidence of hepatocellular carcinoma (HCC) (13). Since liver is a key organ for cholesterol homeostasis and bile production, it would be of interest to explore the effect of metformin on mevalonate pathway and HCC development. HCC accounts for nearly 90% of all liver cancers, casting heavy burden on public health and global economy (14, 15). Therefore, there is an urgent need to identify the critical signaling pathways and molecular targets in the HCC for the prevention of progression and metastasis.

To date, there is no report about metformin-induced suppression of tumorigenesis through inhibition of mevalonate pathway. Nuclear factor E2–related factor 2 (NRF2) is a transcription factor in oxidative stress response. NRF2 activation is reported to be associated with poor survival and tumor invasion of HCC (16). In this study, we investigated the role of mevalonate pathway enzyme 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) in tumorigenesis and hypothesized that metformin functioned through inhibition of the NRF2–HMGCS1 axis to diminish tumor proliferation.

Results

Metformin inhibits mevalonate pathway enzyme HMGCS1 expression in cancer cells

Some pieces of research showed that metformin improved survival rate in patients with HCC (17, 18) through pleiotropic effects including the influence of cholesterol biosynthesis in

‡ These authors contributed equally to this work.
* For correspondence: Xiaoying Li, li.xiaoying@zs-hospital.sh.cn; Yan Lu, rjluyan@126.com.

cancer cells. We postulated that metformin may achieve this effect through the regulation of mevalonate pathway.

We first checked the enzymes in the mevalonate pathway for their mRNA levels in HepG2 cells treated with metformin and verified that HMGCS1, rather than ACAT, HMGCR, HMGCS2, was the mere enzyme that underwent an expression change (Fig. 1, *A* and *D*). HepG2 cells treated with 20 mmol/l metformin for 48 h showed a time-dependent reduction (Fig. 1*A*) in mRNA level of HMGCS1 and was significantly reduced from 5 mmol/l to 20 mmol/l of metformin (Fig. 1*B*). There was a sharp decrease in the protein level of HMGCS1 while treated with 20 mmol/l metformin for 48 h (Fig. 1*C*). We used the ultraperformance liquid chromatography (UPLC)–MS to measure the contents of metabolites in the mevalonate pathway in hepG2 cell after metformin treatment (Fig. 1*E*). The concentrations of β-hydroxy β-methylglutaryl-CoA (HMG-CoA) and mevalonic acid showed a significant decrease, whereas there was no significance change in acetyl-CoA and acetoacetyl-CoA concentrations. We then put emphasis on HMGCS1, the enzyme for HMG-CoA production. The inhibition of HMGCS1 expression by metformin was also observed in lung cancer cells A549 (Fig. S1*A*) and H1299 (Fig. S1*B*). These results show that metformin inhibits HMGCS1 expression in both mRNA and protein levels in liver cancer and lung cancer cell lines.

Metformin regulates HMGCS1 expression through NRF2

To determine whether metformin regulates the expression of HMGCS1 through transcription factors, we conducted luciferase promoter assays in hepG2 cells and human embryonic kidney 293T (HEK293T) cells under the treatment of metformin (Fig. 2, *A* and *B*). We shortened the promoter activity region of HMGCS1 and narrowed down the binding sites of metformin on HMGCS1 to -267 to -838 (Fig. 2*C*). With the help of the PROMO website (19), we found out several transcription factors that may work on

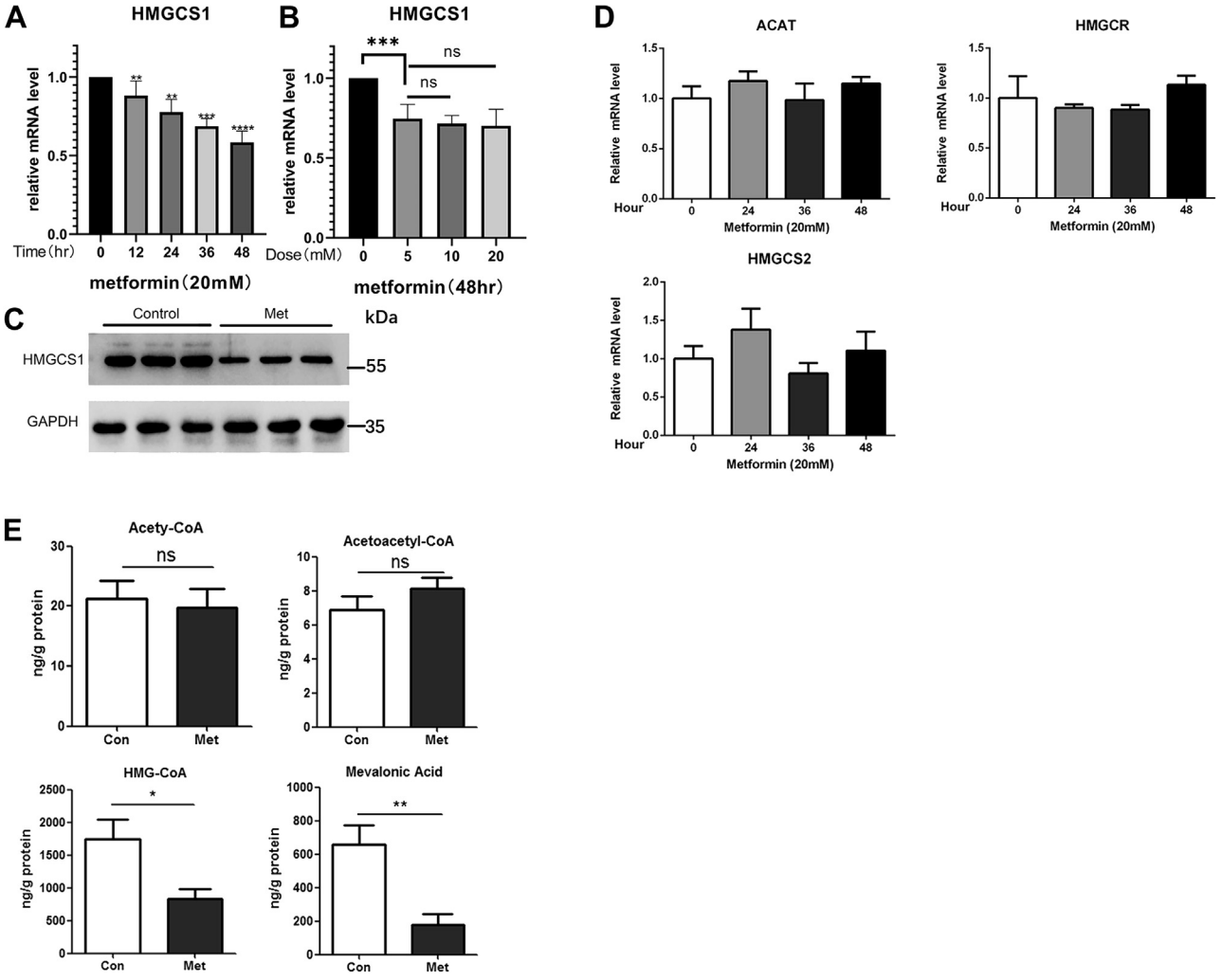

Figure 1. Metformin downregulates mevalonate pathway enzyme HMGCS1 in liver cancer cells. *A*, time-dependent mRNA expression of HMGCS1 in metformin-treated HepG2 cells. Shown are mean ± SD (n = 3). Cells were treated by 20 mmol/l metformin for 0, 12, 24, 36, and 48 h. *B*, dose-independent mRNA expression of HMGCS1 in metformin-treated HepG2 cells. Shown are mean ± SD (n = 3). Cells were treated for 48 h by 0, 5, 10, and 20 mmol/l metformin. *C*, the levels of expression of HMGCS1 proteins in HepG2 cells, after 20 mmol/l metformin treatment for 48 h, normalized with GAPDH. *D*, real-time PCR comparing expression of ACAT1, HMGCR, and HMGCS2 in metformin-treated HepG2 cells. Shown are mean ± SD (n = 3). *E*, UPLC/MS analysis of metabolites in the mevalonate pathway. Cells were treated by 20 mmol/l metformin for 48 h. HMGCR, 3-hydroxy-3-methylglutaryl-CoA receptor; HMGCS, 3-hydroxy-3-methylglutaryl-CoA synthase; UPLC, ultraperformance liquid chromatography.

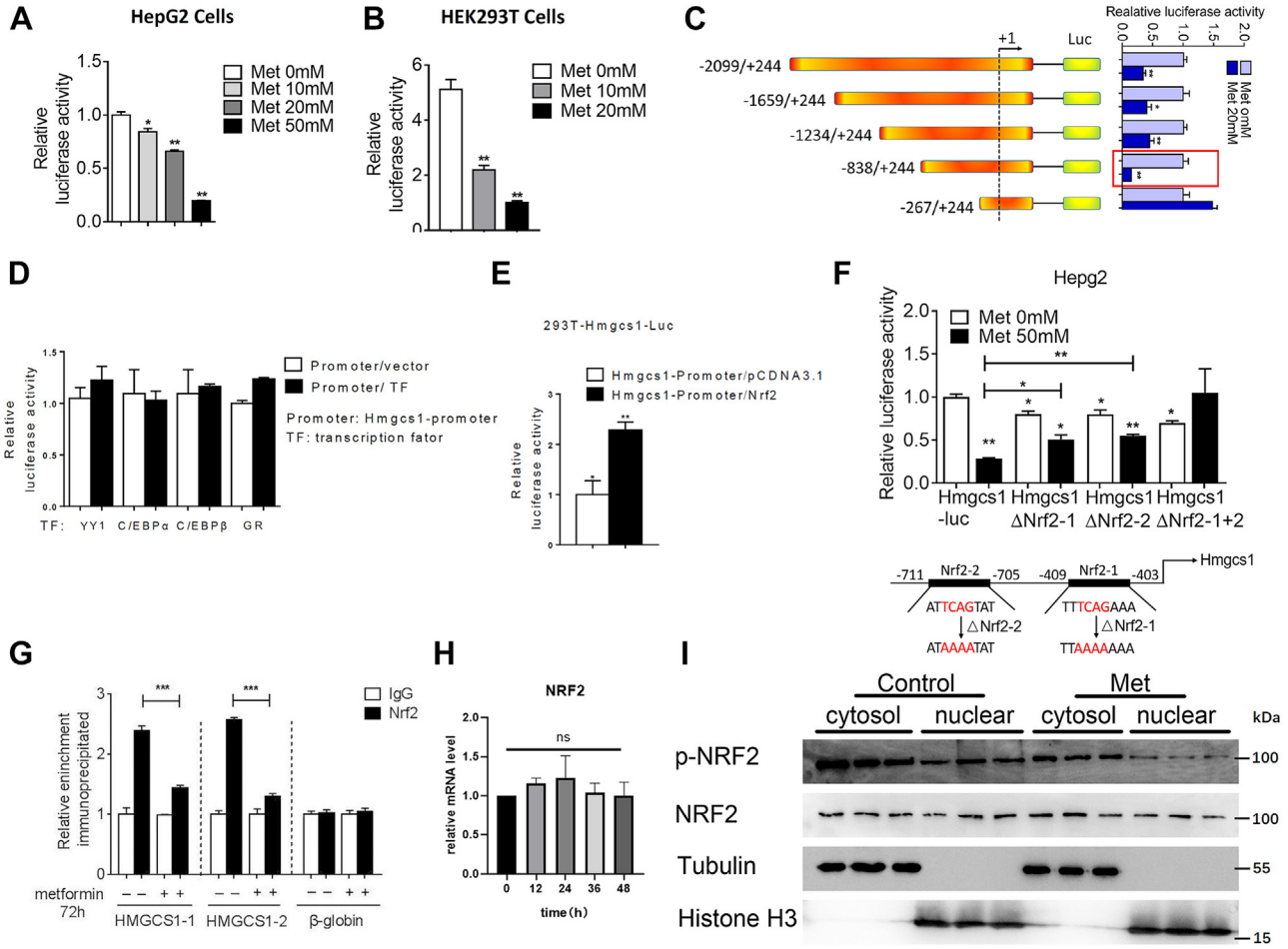

Figure 2. Metformin regulates HMGCS1 through NRF2. *A*, luciferase results for HMGCS1 promoter activity in HepG2 cells when treated with different concentrations of metformin for 48 h. Shown are mean ± SD (n = 3). *B*, luciferase results for HMGCS1 promoter activity in HEK293T cells treated with different concentrations of metformin for 48 h. Shown are mean ± SD (n = 3). *C*, luciferase results for HMGCS1 promoter activity in HEK293T cells in promoter deletion analysis of HMGCS1 when transfected with pcDH-NRF2 plasmid or its control plasmid for 48 h, with or without 20 mmol/l metformin. Shown are mean ± SD (n = 3). *D*, luciferase results for HMGCS1 promoter activity in HEK293T cells overexpressing each of these transcription factors (YY1, C/EBPalpha, C/EBPbeta, and GR) predicted by PROMO. Shown are mean ± SD (n = 3). *E*, luciferase results for HMGCS1 promoter activity in HEK293T cells overexpressing NRF2. Shown are mean ± SD (n = 3). *F*, luciferase results for HMGCS1 promoter activity in HEK293T cells with mutations in one, two binding sites, and control. Shown are mean ± SD (n = 3) (above). A schematic graph about the mutation strategy of the NRF2 core binding site (TCAG) on Hmgcs1 promoter (below). *G*, relative enrichment of ChIP obtained with NRF2 antibody over the IgG control in HEK293T cells with 20 mmol/l metformin treatment for 72 h. *H*, real-time PCR comparing expression of NRF2 in 20 mmol/l metformin-treated HepG2 cells. Shown are mean ± SD (n = 3). *I*, the levels of expression of phospho-NRF2 and NRF2 proteins in the cytosol and nuclear of SNU182 cells, after 20 mmol/l metformin treatment, normalized with tubulin and histone H3. $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$. ChIP, chromatin immunoprecipitation; HEK293T, human embryonic kidney 293T cell line; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; IgG, immunoglobulin G; NRF2, nuclear factor E2–related factor 2.

the binding sites, including YY1, C/EBPalpha, C/EBPbeta, GR, and NRF2. By overexpressing each of these transcription factors in HEK293T cells, we found out only NRF2 upregulated the luciferase promoter assay of HMGCS1 (Fig. 2, *D* and *E*). Mutations of the two binding sites of NRF2 ceased its inhibition of HMGCS1 promoter by metformin (Fig. 2*F*). Chomatin immunoprecipitation (ChIP) provided further evidence between metformin treatment and the reduction in NRF2 and HMGCS1 combination (Fig. 2*G*). Since metformin did not change the mRNA expression of NRF2 (Fig. 2*H*), we examined the phosphorylation level of NRF2 to investigate the mechanism through which metformin inhibits NRF2. After treatment of metformin, phosphorylation levels of NRF2 were reduced in nuclear and cytosolic extracts of HepG2 cells, especially in the nucleus (Fig. 2*I*).

We established NRF2-overexpressed A549 cells (Fig. S2*A*) and witnessed an increase in HMGCS1 mRNA levels (Fig. S2*B*). To elucidate whether NRF2 regulates the expression of endogenous HMGCS1, we used NRF2 activator oltipraz or NRF2 inhibitor ML385 (Fig. S2, *C*–*E*) in cancer cell lines. Oltipraz upregulated the expression of NRF2 downstream genes Npnt, Bmpr1a, and Igf1 as well as HMGCS1 (Fig. S2*C*), whereas ML385 led to the opposite trend, causing downregulation of Npnt and Igf1 together with HMGCS1 (Fig. S2*D*). Protein levels of endogenous HMGCS1 expression also changed synchronously with NRF2 (Fig. S2*E*). These results are consistent with the former data that oltipraz (20) and ML385 (21), although known as activator and inhibitor of NRF2, which could also change the protein expression of NRF2.

These results suggest that endogenous HMGCS1 can be transcriptionally regulated by NRF2 in response to metformin treatment.

HMGCS1 overexpression reverses tumor-suppressive effect of metformin

To determine whether the suppression of tumorigenesis by metformin depends on the inhibition of HMGCS1, we constructed overexpression plasmid of HMGCS1. Cell viability assessed by Cell Counting Kit-8 (Dojindo) was markedly restored after overexpressing HMGCS1 in HepG2 cells (Fig. 3*A*) and A549 cells (Fig. S3*A*). The percentage of apoptotic cells was soared with the treatment of metformin and returned to the baseline level by add-on of overexpression of HMGCS1 (Fig. S3, *B* and *C*). We further explored the antitumor activity of metformin *in vivo*. Transplanted tumor models in nude mice were established, using HepG2 cells and A549 cells transfected with HMGCS1 and control vectors, and treated with metformin (200 mg/kg) or solvent control by gastric perfusion and sacrificed to measure the volume and weight of tumors and relative gene expression. The first day of cell injection was recorded as day 0, and metformin was introduced from day 9, with a total observation period of 15 days. Tumor growth was suppressed by metformin in liver cancer (Fig. 3, *B*, *D*, and *E*) and lung cancer (Fig. S3, *D*–*F*), and the tumor-suppressing effect of metformin was reversed by HMGCS1 overexpression in nude mice xenograft models of both liver cancer (Fig. 3, *C*, *D*, and *E*) and lung cancer (Fig. S3, *D*, *E*, and *G*) without changing the weight of the mice (Figs. 3*F* and S3*H*).

Based on these facts, we verify that the tumor-suppressive effect of metformin is mediated by mevalonate pathway key enzyme HMGCS1.

RNA interference of HMGCS1 exhibits tumor-suppressive effect

To test the oncogenic role of HMGCS1, we used RNA interference of HMGCS1 in tumorigenicity assays. HepG2 cells (Fig. 4*A*) and A549 cells (Fig. S4*A*) were transfected with three different siRNA to knock down HMGCS1 for 4 days, respectively. RNA interference of HMGCS1 in both cell lines using potent KD2 siRNA demonstrated a marked tumor-suppressive effect, as shown in cell viability (Figs. 4*B* and S4*B*), bromodeoxyuridine proliferation assay (Figs. 4*C* and S4*C*), and cell cycle (Fig. 4*D* and S4*D*). RNA interference also led to tumor cell apoptosis (Figs. 4*E* and S4*E*). These findings collectively indicate that RNA interference of HMGCS1 imitates the effect of metformin.

Upregulation of HMGCS1 is associated with poor survival of liver and lung cancer patients

We have investigated the oncogenic role of HMGCS1 in liver cancer cells and animals. We further explored the role of HMGCS1 in human liver cancer. In human HCC samples, expression of HMGCS1 was markedly increased in mRNA levels (Fig. 5*A*) and protein levels by immunohistochemistry

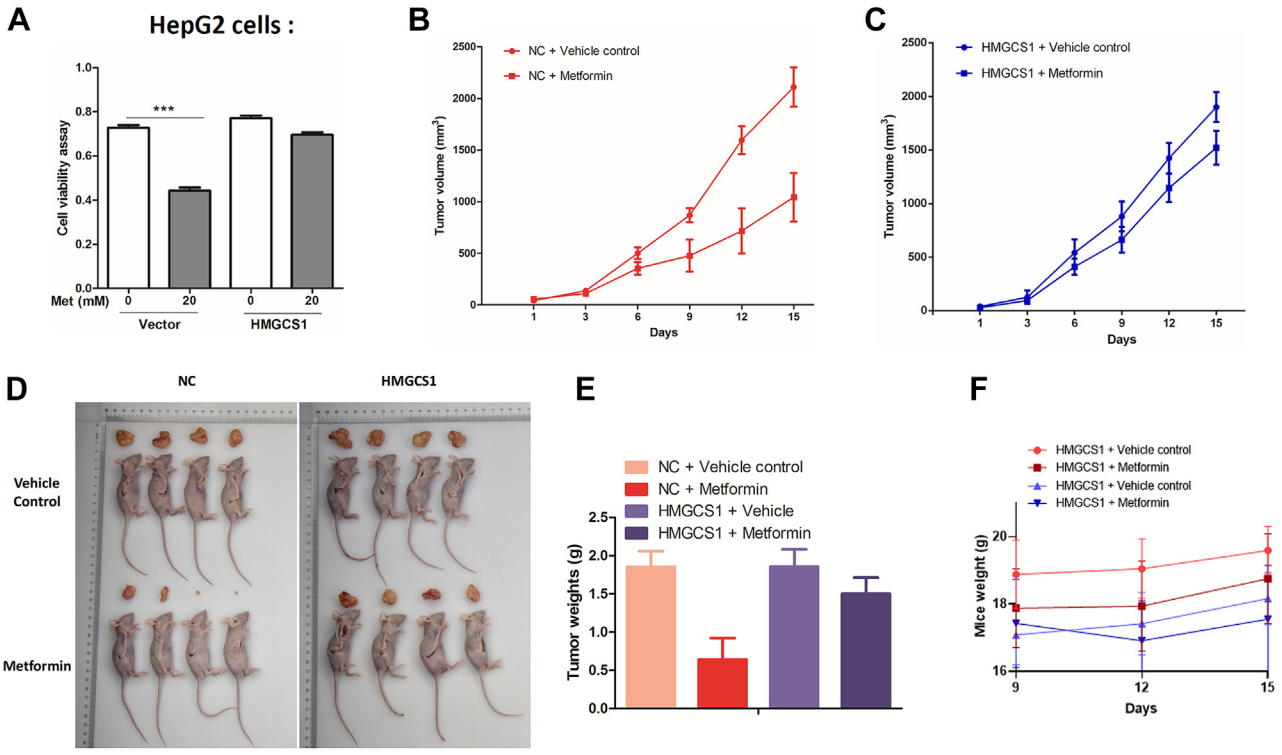

Figure 3. HMGCS1 overexpression partially reversed tumor-suppressive effect of metformin. *A*, effects of HMGCS1 overexpression on cell viability by CCK-8 in HepG2 cells with or without metformin at 20 mmol/l for 48 h. Shown are mean ± SD (n = 6). *B*, tumor volume (mm^3) over time (days) in nude mice subcutaneously inoculated with HepG2 cells, treated with metformin or vehicle control (n = 4). *C*, tumor volume (mm^3) over time (days) in nude mice subcutaneously inoculated with HepG2 cells overexpressed HMGCS1, treated with metformin or vehicle control (n = 4). *D*, effect of HMGCS1 overexpression on the growth of HepG2 cells inoculated into nude mice, with or without metformin treatment (n = 4). *E*, effect of HMGCS1 overexpression on the tumor weights of tumor-forming experiment in nude mice using HepG2 cells, with or without metformin treatment. Shown are mean ± SD (n = 4). *F*, mice weight (g) of the four groups inoculated with HepG2 cells, presented as the mean ± SEM (n = 4). *$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$. CCK-8, Cell Counting Kit-8; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1.

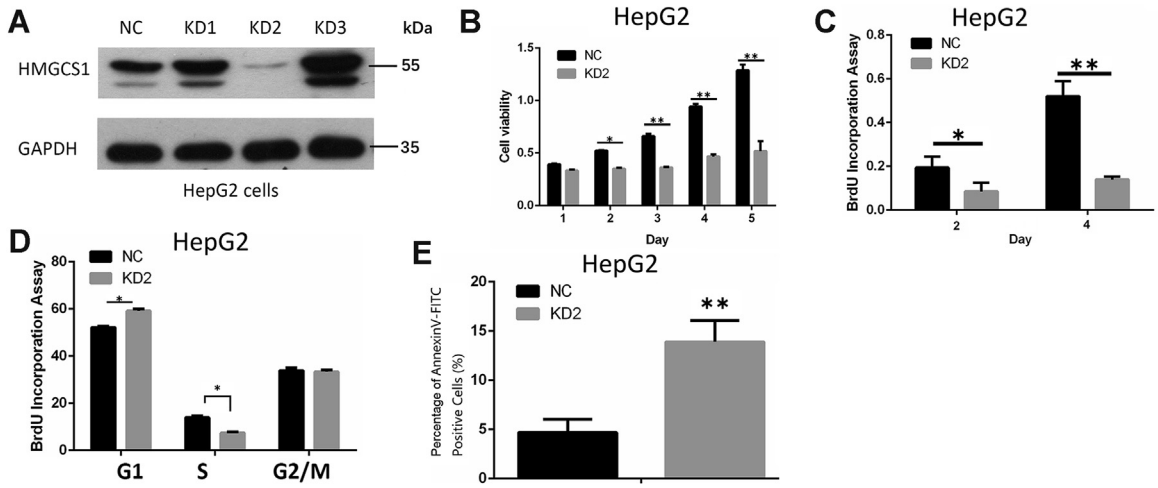

Figure 4. RNA interference of HMGCS1 imitated tumor-suppressive effect of metformin. *A*, the levels of HMGCS1 protein in HepG2 cells transfected with siRNA for HMGCS1 for 4 days, normalized with GAPDH. *B*, effects of different incubation time of siRNA for HMGCS1 (1–5 days) on the cell viability by CCK-8 of HepG2 cells. Shown are mean ± SD (n = 6). *C*, bromodeoxyuridine (BrdU) incorporation assay in HepG2 cells, 2 and 4 days after transfected with siRNA for HMGCS1. Shown are mean ± SD (n = 3). *D*, cell cycle distribution measured by flow cytometry using a BrdU Flow Kit in HepG2 cells transfected with siRNA for HMGCS1 for 4 days. *E*, percentage of apoptotic HepG2 cells transfected with siRNA for HMGCS1 for 4 days. Apoptotic cells are labeled with annexin V–FITC and measured by flow cytometry. Shown are mean ± SD (n = 3). *$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$. CCK-8, Cell Counting Kit-8; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1.

staining compared with the paired adjacent noncancerous tissues (Fig. 5*B*). Higher HMGCS1 expression in HCC tissues leads to a poor overall survival rate in HCC patients (Fig. 5*C*). Similar results were observed in the patients with lung cancer (Fig. S5, *A*–*C*).

Discussion

Several previous studies revealed the relationship between mevalonate pathway and tumor formation. In this study, we shed light on the tumor-suppressive role of metformin mediated by inhibition of mevalonate pathway enzyme HMGCS1. The mevalonate pathway exists in almost all cell types, so its oncogenic role and possibility as a drug target are worth further investigation. HMGCS1, the first key enzyme in mevalonate pathway, turns acetoacetyl-CoA into HMG-CoA, which further undergoes the catalysis of HMGCR to generate mevalonic acid. Jiang *et al.* (22) reported that HMGCS1 expression could be suppressed by metformin. Our results verified that HMGCS1 is an important target to mediate the antitumor activities of metformin. The expression levels of HMGCS1 and its downstream metabolite mevalonate decrease after treatment with metformin (Fig. 1), and this decrease is synchronizing with the cell viability and tumor growth (Fig. 3). We also confirm that HMGCS1 is highly expressed in human liver cancer and lung cancer tissues and related to poor prognosis and overall survival rate (Fig. 5). It is possible that increased HMGCS1 expression in tumor tissues might contribute to cell division, proliferation, and signal transduction.

The mevalonate pathway contributes to the progression of several types of cancers through its key enzymes and metabolites. Some researches were consistent with our point of view by pointing out that HMGCS1 is highly expressed in most cancer types, and it is also related to the poor prognosis and drug resistance (23). Other studies found out that HMGCR-driven MYC activation (24) and cholesterol production (25) supports the growth of liver cancer. Lipid reprogramming including regulation of HMGCS1 and HMGCR contributes to epithelial-to-mesenchymal transition in HCC and invasive phenotype like invadopodia formation. In a mouse xenograft model of HT29 human colon cancer cell line, mevalonate replenish experiment reversed the tumor-suppressing effect of

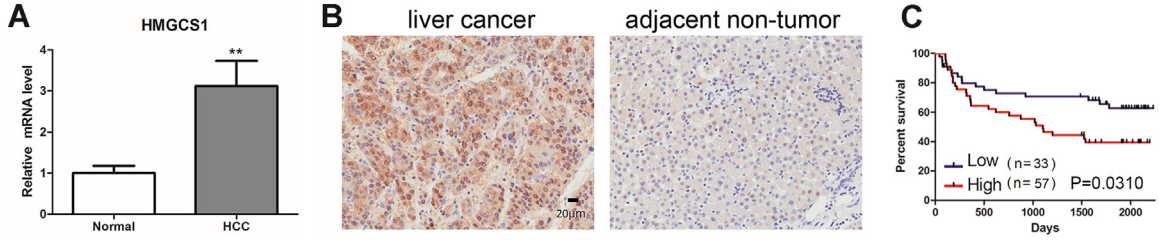

Figure 5. Upregulation of HMGCS1 is associated with poor prognoses of liver cancer. *A*, real-time PCR analysis of the HMGCS1 expression in hepatocellular carcinoma (HCC) samples and the paired noncancerous adjacent tissues. Shown are mean ± SD (n = 14). *B*, HMGCS1 immunostaining in HCC and adjacent noncancerous tissues. *C*, Kaplan–Meier curve showing the percent survival in HCC patients with *high* (n = 57) or *low* (n = 33) HMGCS1 expression ($p = 0.031$). *$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$. HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1.

metformin (26). This result is consistent with our HPLC/MS results (Fig. 1*E*) and verified our speculation that the presence of mevalonate is essential in tumor proliferation. Cell viability restored by mevalonic acid replenishment could be inhibited by metformin (Fig. S6). All these facts provide reasons why the inhibitors of the mevalonate pathway are under heated investigation for their antitumor activities (27, 28).

Studies over the past decade revealed that metformin functions through inhibition of mammalian target of rapamycin (mTOR) to a great extent (29). It happened that an observation gave a clue for the metabolite mevalonate's role in activation of mTOR (30). Chances are that there is a convergence of the antitumor role of metformin and the mevalonate pathway, and mTOR is their shared downstream target. A study on renal cell carcinoma showed the additive effect of mevalonate pathway inhibitor statins and mTOR inhibitor (31).

Our study uncovers that the mevalonate pathway dependence is a common feature in liver cancer and lung cancer. Between the two lung cancer cell lines we used, A549 cells have more epithelial characteristics, whereas H1299 cells are derived from mesenchymal connective tissues. We also used HCC cell line SNU182 and hepatoblastoma cell line HepG2. These four tumor cell lines are from different tissues and origins, all demonstrating the same trend that mevalonate pathway together with cell activity can be inhibited by metformin. These results validate our hypothesis that the oncogenic role of mevalonate pathway and HMGCS1 is universal in multiple tumors. We speculate that the antitumor effect of metformin could be reproduced in other tumor cell lines and even tumors *in vivo*.

Here, our results showed that HMGCS1 could be transcriptionally upregulated by NRF2. Previous studies reported NRF2 mutations and NRF2-Keap1 pathway activation throughout the hepatocarcinogenesis (32). Constitutive NRF2 activation was observed in human cancer tissues (33). The elevated expression of NRF2 may serve as a compensatory mechanism to resist ischemia and subsequent hypoxia (34). We discovered that a large dose of metformin weakened the transcription activity of NRF2 by reducing its protein phosphorylation, leading to a decrease of HMGCS1 expression and tumor arrest. These observations are consistent with the established theory that NRF2 inhibitors are effective in treatment of aggressive tumors that grow rapidly and are resistant to treatment, whereas NRF2 inducers are used to protect normal cells from carcinogens through antioxidative effects (35).

A piece of previous study showed that *hmgcs1* gene was negatively regulated by Nrf2 in mice (36). To our knowledge, it is the first time to demonstrate that NRF2 regulates HMGCS1 through transcriptional activity in controlling cancer progression. We postulate that the change of phosphor-NRF2 under the treatment of metformin is probably induced by PKC λ/ι (37, 38) because we used an antibody specific for Nrf2 phosphorylated at S40. In previous studies, metformin not only inhibits the phosphorylation levels of Nrf2 (39) but also mediates Nrf2 degradation (40).

To sum up, this present study revealed the key role of the NRF2–HMGCS1 axis in the antitumor activity of metformin. Consistent with this notion, we assume that HMGCS1 might serve as a novel therapeutic target for liver cancer, which adds another piece to the puzzle of the relationship between metformin, cancer, and metabolism.

Limitations of the study

In this research, mice that underwent a high dose of metformin remained the same weight, indicating that metformin suppresses the tumor progression at a safe dose without disturbance of energy metabolism or aggravation of cachexia. However, it should be noted that we used metformin with a dose beyond the physiological amount (41). In this regard, more investigations are needed to show the potential of metformin within the regular dosage for patients. Targeting the delivery system of metformin to improve its therapeutic efficacy is worth equal attention. The targets we predicted using the PROMO website may not include all possible scenarios. Based on the transcriptional regulation role of GATA 1 on HMGCS1 in the process of erythropoiesis, further investigations can be made to determine the role of GATA 1 in regulation of HMGCS1 in the development of solid tumors (42). Noteworthy, the downstream mechanism of HMGCS1-mediated tumor promotion function needs further investigation.

Conclusions

In summary, we unveil that metformin suppresses tumor growth and progression through inhibition of mevalonate pathway enzyme HMGCS1 (Fig. 6). HMGCS1 also serves as a predictive biomarker of poor prognosis in patients with liver cancer and lung cancer.

Experimental procedures

Reagents

Metformin was purchased from Sigma–Aldrich. Nrf2 pathway activator oltipraz and inhibitor ML385 were obtained from MedChemExpress. Antibody for HMGCS1 was purchased from Abcam (catalog no.: ab194971). The anti-NRF2 antibody was purchased from Proteintech (catalog no.: 16396-1-AP) and Abcam (catalog no.: ab62352). The anti-phosphor-NRF2 antibody was purchased from Abcam (catalog no.: ab76026). The antibodies against GAPDH (catalog no.: 3683; Cell Signaling Technology), tubulin (catalog no.: 5346; Cell Signaling Technology), and histone H3 (catalog no.: AH433; Beyotime) served as internal references for Western blots.

Tumor xenograft model

All animal protocols were reviewed and approved by the Animal Care Committee of Zhongshan Hospital, Fudan University. All mice were housed in a temperature-controlled environment (20–22 °C) with 12:12-h light/dark cycles with free access to food and drinking water. HepG2

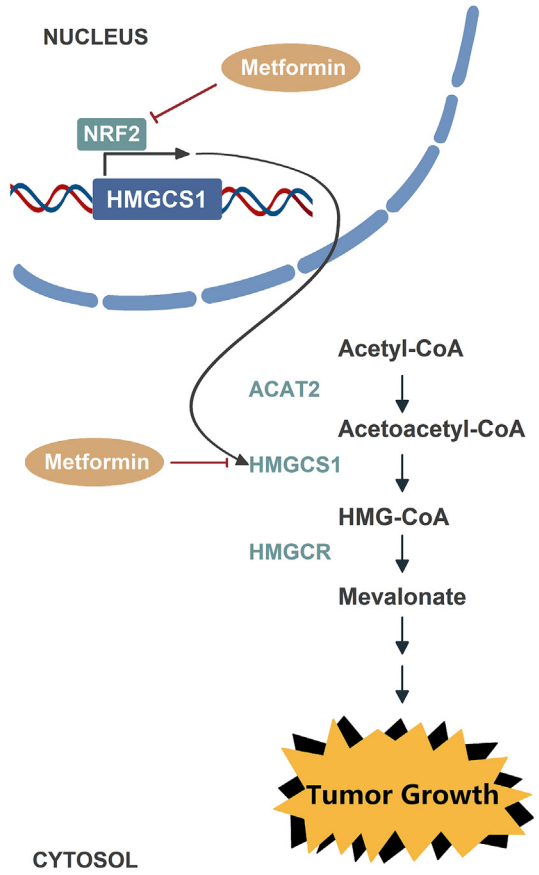

Figure 6. Schematic depiction of metformin's role in tumor suppression through mevalonate pathway.

and A549 cells with HMGCS1 overexpression for 48 h or vehicle control were transplanted to nude mice. Each mouse was subcutaneously inoculated with HepG2 or A549 tumor cells at a volume of 5 × 10^5/200 μl. In 2 weeks, tumor-bearing nude mice were allocated to metformin and PBS control groups, respectively. A 15-day treatment with metformin was applied then. Metformin (200 mg/kg) or PBS control was administrated by gastric perfusion. Four mice were used in each group. Tumor volume was measured every 3 days according to the diameters. Body weight was assessed every 3 days.

Cell lines and culture conditions

Cell lines and the origins are listed as follows: HepG2 (HCC), SNU182 (HCC), A549 (lung cancer), H1299 (lung cancer), and HEK293T.

Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% penicillin and streptomycin at 37 °C and 5% CO_2.

Transfection and agents

siRNA targeted to HMGCS1 and negative control were purchased from Jima Biotechnology Co, Ltd. Overexpressed plasmids (HMGCS1, NRF2, YY1, C/EBPα, C/EBPβ, and GR) were purchased from JiKai Gene Co, Ltd. Cell transfection was performed on HEK293T cells, HepG2, and A549 using the Lipofectamine 3000 reagent (Thermo Fisher Scientific) following the manufacturer's instructions.

Cell growth and viability assays

Cells were seeded at 5000 per well in 96-well plates and incubated overnight before drug treatment. About 10 μl Cell Counting Kit-8 was added to the 90 μl cell culture system, and cells were incubated for 1 to 4 h at 37 °C and 5% CO_2 before measurement.

Cell cycle analysis

Flow cytometry (Accuri C6 FACS) was used to analyze the cell cycle through Cell Cycle Analysis kit (BD Biosciences). The FlowJo software (BD FACSCalibur) was used for data analysis in flow cytometry.

Reporter gene assays

293T cells were seeded in 24-well plates, cotransfected with siRNA targeting different active regions of HMGCS1, pcDH-NRF2 plasmids, or its control plasmids. The luciferase activity was assessed 24 h after transfection.

Quantitative real-time PCR analysis

Total RNA was extracted using the TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Takara PrimeScript RT reagent kit was used to obtain complementary DNA (Takara). Primers for quantitative PCR (qPCR) were as follows: HMGCS1 forward: 5′-TGTACACATCTTCAGTATATGGTTCCC-3′, reverse: 5′-AAGAAAACACTCCAATTCTCTTCCCT-3′, HMGCR forward: 5′-TGATTGACCTTTCCAGAGCAAG-3′, reverse: 5′-CTAAAATTGCCATTCCACGAGC-3′, ACAT1 forward: 5′-AAGGCAGGCAGTATTGGGTG-3′, reverse: 5′-ACATCAGTTAGCCCGTCTTTTAC-3′, HMGCS2 forward: 5′-GCCCAATATGTGGACCAAACT-3′, reverse: 5′-GAAGCCCATACGGGTCTGG-3′, NRF2 forward: 5′-TCAGCGACGGAAAGAGTATGA-3′, reverse: 5′-CCACTGGTTTCTGACTGGATGT-3′, NPNT forward: 5′-GTAAGCACAGGTGCATGAACA-3′, reverse: 5′-GAACCATCCGGCATGAGCATA-3′, BMPR1A forward: 5′-TGAAATCAGACTCCGACCAGA-3′, reverse: 5′-TGGCAAAGCAATGTCCATTAGTT-3′, AND IGF1 forward: 5′-GCTCTTCAGTTCGTGTGTGGA-3′, reverse: 5′-GCCTCCTTAGATCACAGCTCC-3′. All data were normalized to 36B4, and the expression levels were calculated by the 2–DDCT method.

Western blot analysis

Total proteins were extracted, separated by 10% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore). The membrane was blocked for 1 h with 5% bovine serum albumin, washed with Tris-buffered saline with Tween, and incubated overnight at 4 °C with a primary antibody listed as before. Then the membrane was incubated with secondary antibodies for 1 h at room temperature and detected using ECL reagents.

ChIP–real-time qPCR

Control immunoglobulin G- and NRF2-ChIPed DNA libraries were prepared using MAGnify Chromatin immunoprecipitation system (Thermo Fisher Scientific). qPCR was performed using the same procedure described previously in the qRT–PCR methods.

UPLC–MS/MS for metabolite detection

Cells are seeded in 6-well plates and cultured for 48 h until the confluence of 80 to 90%. Cells in each well were washed with 1 ml PBS buffer (precooled at 4 °C) twice and added with 0.4 ml HPLC grade methanol (precooled at −80 °C) containing 1 μg/ml tridecanoic acid (catalog no.: 91988-5G; Sigma–Aldrich). Cells were harvested by scraping and gently transferred to Eppendorf tubes. Samples were stored at −80 °C for late assessment by UPLC–MS/MS.

Histological analysis

Liver cancer and lung cancer specimen and the corresponding adjacent tissues were obtained by surgery. For histological analysis, the tissues were fixed in 4% neutral-buffered formalin overnight. Paraffin embedding, sectioning, blocking, incubation, and antigen retrieval were performed according to the standard protocols. The human study was approved by the Human Research Ethics Committees of Zhongshan Hospital, Fudan University. Written informed consent was obtained from each subject.

Human studies

A total of 90 subjects with liver cancers and lung cancers, respectively, were recruited for survival analysis. The human study was approved by the Human Research Ethics Committees of Zhongshan Hospital, Fudan University. Written informed consent was obtained from each subject.

Statistical analysis

Data are presented as the mean ± SD. Differences among groups were analyzed by one-way ANOVA followed by a *t* test. A two-sided $p < 0.05$ was considered significantly different.

Data availability

All data discussed here are presented in the article and supporting information.

Supporting information—This article contains supporting information.

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Abbreviations—The abbreviations used are: ChIP, chomatin immunoprecipitation; HCC, hepatocellular carcinoma; HEK293T, human embryonic kidney 293T cell line; HMG-CoA, β-hydroxy β-methylglutaryl-CoA; HMGCR, 3-hydroxy-3-methylglutaryl-CoA receptor; HMGCS, 3-hydroxy-3-methylglutaryl-CoA synthase; mTOR, mammalian target of rapamycin; NRF2, nuclear factor E2–related factor 2; qPCR, quantitative PCR; UPLC, ultraperformance liquid chromatography.

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