FOX1 Promotes HCC Proliferation and Metastasis by Upregulating DNMT3B to Induce DNA Hypermethylation of CTH Promoter

Zhuoying Lin
Huazhong University of Science and Technology

Wenjie Huang
Huazhong University of Science and Technology

Qin He
Huazhong University of Science and Technology

Dongxiao Li
Huazhong University of Science and Technology

Zhihui Wang
Huazhong University of Science and Technology

Yangyang Feng
Huazhong University of Science and Technology

Danfei Liu
Huazhong University of Science and Technology

Tongyue Zhang
Huazhong University of Science and Technology

Yijun Wang
Huazhong University of Science and Technology

Meng Xie
Huazhong University of Science and Technology

Xiaoyu Ji
Huazhong University of Science and Technology

Mengyu Sun
Huazhong university of science and technology

Dean Tian
Huazhong University of Science and Technology

Limin Xia (xialimin@tjh.tjmu.edu.cn)
Huazhong University of Science and Technology  https://orcid.org/0000-0002-6327-6034

Research

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Abstract

**Background:** Forkhead box C1 (FOXC1), as a member of the FOX family, is important for promote HCC invasion and metastasis. FOX family protein lays a pivotal role in metabolism. ROS is involved in tumor progression and is associated with the expression of lots of transcription factors. We next explored the mechanism underlying FOXC1 modulating the metabolism and ROS hemostasis in HCC.

**Methods:** We used amino acids arrays to verify which metabolism is involved in FOXC1-induced HCC. The kits were used to detect the ROS levels in HCC cells with over-expression or down-expression of FOXC1. After identified the downstream target genes and candidate pathway which regulated by FOXC1 during HCC progression *in vitro* and *in vivo*, we used western blot, immunohistochemistry, bisulfite genomic sequencing, methylation-specific PCR, chromatin immunoprecipitation analysis and luciferase reporter assays to explore the relationship of FOXC1 and downstream genes. Moreover, the correlation between FOXC1 and target genes and the correlation between target genes and the recurrence and overall survival were analyzed in two independent human HCC cohorts.

**Results:** Here, we reported that FOXC1 could inhibit the cysteine metabolism and increase reactive oxygen species (ROS) levels by regulating cysteine metabolism-related genes, cystathionine γ-lyase (CTH). Overexpression of CTH significantly suppressed FOXC1-induced HCC proliferation, invasion and metastasis, while the reduction in cell proliferation, invasion and metastasis caused by the inhibition of FOXC1 could be reversed by knockdown of CTH. Meanwhile, FOXC1 upregulated de novo DNA methylase 3B (DNMT3B) expression to induce DNA hypermethylation of CTH promoter, which resulted in low expression of CTH in HCC cells. Moreover, low levels of ROS induced by N-acetylcysteine (NAC) which is an antioxidant inhibited the cell proliferation, migration, and invasion abilities mediated by FOXC1 overexpression, whereas high levels of ROS induced by L-Buthionine-sulfoximine (BSO) rescued the suppression results mediated by FOXC1 knockdown. Our study demonstrated that the overexpression of FOXC1 that was induced by the ROS dependent on the extracellular regulated protein kinases 1 and 2 (ERK1/2)-phospho-ETS Transcription Factor 1 (p-ELK1) pathway. In human HCC tissues, FOXC1 expression was positively correlated with oxidative damage marker 8-hydroxy-2'-deoxyguanosine (8-OHdG), p-ELK1 and DNMT3B expression, but negatively correlated with CTH expression. HCC patients with positive co-expression of 8-OHdG/FOXC1 or p-ELK1/FOXC1 or FOXC1/DNMT3B had the worst prognosis, whereas HCC patients who had positive FOXC1 and negative CTH expression exhibited the worst prognosis.

**Conclusion:** In a word, we clarify that the positive feedback loop of ROS-FOXC1-cysteine metabolism-ROS is important for promoting liver cancer proliferation and metastasis, and this pathway may provide a prospective clinical treatment approach for HCC.

**Background**

Metabolic reprogramming is one of the essential features of tumors(1). Specific metabolic processes can be directly involved in the transformation process or biological processes that support tumor growth(2). Amino acids play a number of roles in tumor cell growth and survival, including providing carbons to the tricarboxylic acid cycle (TCA cycle), nitrogen to nucleobase synthesis, in maintaining redox homeostasis and other metabolic activities(3). Meanwhile, amino acids can regulate the development of tumor cells by activating some oncogenes(4). The liver is the key organ for coordinating metabolic activities, including nitrogen metabolism, detoxification and energy metabolism. In physiological and pathological conditions, the liver provides the energy necessary to maintain the function of different organs. Abnormalities in circulating amino acid metabolite levels were observed in hepatocellular carcinoma (HCC). Recently, clinical studies showed that circulating levels of some biogenic amines and branched-chain, aromatic and glucogenic amino acids were closely associated with the risk of HCC(5). Therefore, further studies of molecular mechanism underlying amino acid metabolism with HCC which are able to assist with developing novel therapeutic strategies are urgently needed.
Moreover, another risk factor associated with tumorigenesis and tumor progression is an increase in reactive oxygen species (ROS) abundance, which is caused by the production and elimination of an imbalance in the composition of reactive oxygen species(6). An increase in ROS has been detected in various cancers and has been shown to have multiple roles in activating pro-tumorigenesis signals, driving DNA damage and genetic instability, and enhancing cell survival and proliferation. Counterintuitively ROS can also promote anti-tumorigenic signaling, and trigger tumor cell death induced by oxidative stress. Furthermore, as the important messenger, ROS is associated with the expression of lots of transcription factors(7).

The forkhead box (FOX) protein family consists of a group of evolutionarily conserved transcription factors characterized by a common DNA-binding domain known as the forkhead box domain(8). FOX family proteins involve in cell growth, differentiation and other biological processes(9). The deregulation of Fox family transcription factors is important for the development and progression of tumors(8). Some researchers have shown that abnormal expression of FOX family protein plays a pivotal role in metabolism. The forkhead box O (FOXO) family participates in the regulation of a large number of biological activities from development, cell signal transduction, tumorigenesis to cell metabolism (10). The balance of FOXO and FOXM1 transcription factors integrates Adenosine 5’-monophosphate (AMP)-activated protein kinase (AMPK)-mediated metabolic status and cell cycle regulation through competitive regulation of target genes (including Insulin-like growth factor-1 (IGF1)) in neonatal cardiomyocytes(11). FOXK1 and FOXK2 are important in the regulation of mitochondrial function, metabolism and apoptosis (12). FOXO family facilitated the cellular antioxidant defense, and on the other hand, ROS may regulate FOXO activity at many levels(13). Therefore, FOX family proteins involve in the development of diseases by altering the activities of cell metabolism and the accumulation of ROS.

As a member of FOX family proteins, FOXC1 was first found to be associated with the ocular dominant genetic disease Axenfeld-Rieger syndrome (ARS)(14). FOXC1 regulated normal embryonic development and is involved in the development and function of multiple organs(15). Some research have shown that FOXC1 is positively correlation with poor prognosis of a variety of tumors, including pancreatic ductal adenocarcinoma, acute myeloid leukemia, basal-like breast cancer, gastric cancer and colon cancer(16–20). Our previous research indicated that FOXC1 is important for promoting HCC metastasis(21, 22). Nevertheless, whether FOXC1 promotes HCC progression through amino acid metabolism is unclear. Using amino acid metabolism RT² Profiler PCR array (Supplementary Table S1), we found that FOXC1 downregulated cystathionine γ-lyase (CTH) expression, which is associated with cysteine metabolism. Cysteine metabolism is involved in redox balance by regulating the ROS level.

In this study, we demonstrated that FOXC1 upregulated DNA methylases 3B (DNMT3B) to induce DNA hypermethylation of CTH promoter and CTH gene silencing, which resulted in the decrease of cysteine levels and increases of ROS levels.

Moreover, the high level of ROS increased the expression of FOXC1 through extracellular regulated protein kinases 1 and 2 (ERK1/2)- phospho-ETS Transcription Factor 1 (p-ELK1) pathway, which formed a ROS-FOXC1-cysteine metabolism-ROS positive feedback loop to promote HCC proliferation and metastasis.

Materials And Methods

ROS levels measurement

The 2',7'- dichlorofluorescein diacetate (DCFH-DA) (Beyotime, China) was used to detect the ROS levels. Firstly added DCFH-DA to DMEM(without FBS) in a ratio of 1:1000, and then a volume of 2mL mixed solution was added to the HCC cells, which were washed 2 times by PBS. Then we put the cells into incubator for 30 min at 37 °C in dark. Then HCC cells were washed again with DMEM (without FBS) for three times, we measured the ROS levels by fluorescence microscope.

GSH/GSSG, GSH and cysteine levels measurement
The GSH/GSSG ratio, GSH levels and cysteine levels were detected by Reduced glutathione (GSH) assay kit (Nanjing jiancheng, China), Total glutathione / Oxidized glutathione assay kit (Nanjing jiancheng, China) and Cysteine content test kit (Nanjing jiancheng, China) on the basis of the manufacturer's instructions. The transfected cells were lysed in culture dishes containing a lysis buffer, and 0.5ml supernatant was taken from the resulting lysates which were centrifuged. 2ml of the application solution was added and mixed evenly, and then centrifuged at 3500rpm for 10min. Lastly 1ml of the supernatant was taken for color reaction. The measurements were conducted with a UV–visible spectrophotometer.

A detailed description of the materials and methods used in this study can be found in the online supplementary material.

Results

Overexpression of FOXC1 downregulates CTH expression and increases ROS levels.

To explore whether FOXC1 participates in the regulation of amino acid metabolism in HCC cells, we performed an amino acid metabolism RT2 Profiler PCR array to examine transcriptome changes mediated by FOXC1 overexpression in Huh7 cells (Supplementary TableS1) and FOXC1 down-expression in MHCC97H cells (Supplementary TableS2). Using twofold as a cut-off to designate differentially expressed genes, 21 out of the 158 amino acid metabolism genes were down-regulated in Huh7 cells which overexpressed FOXC1, while 30 genes were up-regulated in MHCC97H cells which down-expressed FOXC1. Among the overlap of down-regulated genes in Huh7-FOXC1 and up-regulated genes in MHCC97H-shFOXC1 cells (Fig. 1A), CTH attracted our attention, which is strongly inhibited by FOXC1 overexpression. CTH is the key enzyme for cysteine synthesis (Fig. 1B) and cysteine is the precursor of ROS scavenger(23). We found that the levels of ROS increased in HCC cells with FOXC1 overexpression and decreased when FOXC1 is down-regulated (Fig. 1C). Moreover, when FOXC1 overexpressed, the ratio of glutathione (GSH)/ Glutathione (Oxidized) (GSSG) and the level of cysteine and GSH decreased. Correspondingly, the ratio of GSH/GSSG and the level of cysteine and GSH increased when FOXC1 is down-regulated (Fig. 1D).

According to the data from The Cancer Genome Atlas dataset (TCGA), we found that compared with mRNA level of the CTH in normal liver tissues, the mRNA levels of CTH was markedly lessened in HCC specimens. Furthermore, based on TCGA data, the mRNA expression of CTH decreased in cholangiocarcinoma (CHOL), kidney renal clear cell carcinoma (KIRC) and thyroid carcinoma (THCA) tissues (Supplementary Figure S1A). Human Protein Atlas program database showed high or medium CTH staining intensity in normal liver samples, whereas liver cancer samples showed low staining of CTH by immunohistochemistry (IHC) tissue microarray data (Supplementary Figure S1B). Kaplan-Meier analysis based on TCGA data showed that HCC patients who had low CTH mRNA level, their overall survival time of were significantly shorter than that with high CTH mRNA level (Supplementary Figure S1C). These studies suggested that low levels of CTH indicated poor prognosis, and CTH may act as an important tumor suppressor gene (TSG) in human HCC.

FOXC1 facilitates HCC proliferation and metastasis by inhibiting CTH expression.

To explore whether FOXC1 regulates CTH expression, we first detected CTH level in four stable cell lines, Huh7-FOXC1 and MHCC97H-shFOXC1 and their control groups. Up-regulation of FOXC1 expression meaningfully inhibited the expression of CTH, whereas down-regulation of FOXC1 increased the CTH levels (Fig. 1D). We upregulated the expression of CTH in Huh7-FOXC1 cells and knocked down CTH in MHCC97H-shFOXC1 cells with lentivirus transfection (Fig. 1E). Up-regulation of CTH abolished FOXC1-facilitated cell proliferation, migration and invasion, and konckdown of CTH had the opposite results (Fig. 1F-J).

Up-regulation of CTH significantly inhibited tumor growth induced by Huh7-FOXC1 cells, whereas down-regulated CTH rescued the decreased tumor growth mediated by FOXC1 knockdown by in vivo tumorigenicity assays (Fig. 1K-L). Consistently, in vivo metastasis assay indicated that overexpression of CTH reduced the HCC metastasis with Huh7-
FOXC1 cells, which prolonged overall survival time. In contrast, the down-regulation of CTH recused the inhibition of HCC proliferation and metastasis in the MHCC97H-shFOXC1 group (Fig. 1M-Q). These studies showed that FOXC1 facilitated HCC proliferation and metastasis via inhibiting CTH expression.

**FOXC1 upregulates DNMT3B expression, which results in the DNA hypermethylation of CTH promoter and CTH gene silencing.**

Epigenetic modifications such as DNA methylation of TSG promoters contribute to the progression and metastasis of HCC(24). Recent studies indicated that the inhibition of CTH gene transcription resulted from DNA hypermethylation of CpG rich region in the CTH promoter(25–27). To determine whether promoter DNA hypermethylation results in CTH gene silencing in FOXC-overexpressing HCC cells, the methylation status of CpG island in the CTH promoter was analyzed by bisulfite genome sequencing (BGS) analysis, which covered 13 CpG sites from −206 to -20 of the CTH promoter (Fig. 2A). Hypermethylation at CpG sites in the CTH promoter region was detected in Huh7-FOXC1 cells, whereas less methylation was detected in MHCC97H-shFOXC1 (Fig. 2A and 2B). Compared with adjacent nontumor tissues, HCC tissues showed much higher methylation levels at CpG sites in the CTH promoter region (Fig. 2C). In addition, treatment with a demethylation agent, the DNA methyltransferase inhibitor 5-aza-2’-deoxycytidine (5-Aza), restored CTH expression in Huh7-FOXC1 cells, indicating that promoter DNA hypermethylation contributes to the transcriptional silencing of CTH gene (Fig. 2D).

As the important DNA methyltransferases, DNMT3A and DNMT3B are involved in de novo methylation patterns, which are maintained by DNMT1(28). Therefore, the DNA hypermethylation of CTH promoter may be induced by DNMTs. We found that the expression of DNMT3B is increased in Huh7-FOXC1 cells, whereas DNMT3B expression is downregulated in MHCC97H-shFOXC1 cells. However, the expression of DNMT1 and DNMT3A had no significant change in Huh7-FOXC1 and MHCC97H-shFOXC1 cells compared to control cells (Fig. 2E). Moreover, ectopically overexpression of DNMT3B decreased the expression of CTH in MHCC97H-shFOXC1 cells, whereas knockdown of DNMT3B increased CTH expression in Huh7-FOXC1 cells (Fig. 2F). These studies suggested that FOXC1 upregulated DNMT3B expression, which resulted in CTH gene silencing in HCC cells.

**DNMT3B is a direct transcriptional target of FOXC1.**

The mechanism by which FOXC1 upregulated DNMT3B expression is still unclear. We hypothesized that FOXC1 may directly transactivate DNMT3B. A dual-luciferase reporter assay showed that overexpression of FOXC1 enhanced the transcription of the luciferase reporter in the DNMT3B plasmid constructs compared to that of the controls (Fig. 2G). In order to clarify the regulation mechanism of DNMT3B, the promoter sequence of DNMT3B was analyzed and we found 3 putative FOXC1 binding motifs. To further confirm whether FOXC1 targets these potential binding sites to regulate DNMT3B, we designed a series of reporter plasmids containing truncated or mutated DNMT3B promoter sequences and used them in luciferase reporter assay. Huh7 cells were transfected with these plasmids to assess their reaction to FOXC1 overexpression. The results suggested that FOXC1-induced luciferase reporter expression was significantly eliminated by deletion in the −195～+109 bp region compared to that of the controls. Consistently, compared with the control group, mutations at the putative binding sites 2&1 of the DNMT3B promoter significantly reduced the activity of the FOXC1 overexpressed luciferase reporter gene, suggesting that these binding sites were essential for FOXC1 transactivation (Fig. 2H). A chromatin immunoprecipitation (ChIP) assay demonstrated the direct binding of FOXC1 to the putative site in the DNMT3B promoter in Huh7-FOXC1 cells (Fig. 2I upper). Moreover, we investigated whether FOXC1 binds to the same regions in patient samples, and the results revealed that compared to healthy controls, FOXC1 binding sites in the HCC samples were indeed enriched in these regions. (Fig. 2I, lower).

Then we examined the protein levels of CTH and promoter methylation levels of CTH in two independent cohorts of human HCC tissues. Microvascular invasion, poor tumor differentiation, and higher TNM stage were positively associated
with the depletion of CTH (Table 1). Loss of CTH expression was an independent and meaningful risk factor for recurrence and reduced survival according to the multivariate analysis (Table 2). The expression of CTH was inversely correlated with promoter methylation levels of CTH in both cohorts (Fig. 2J). In addition, promoter methylation of CTH gene was positively correlated with aggressive tumor behaviors (Supplementary Table S3). HCC patients with promoter methylation of CTH had higher recurrence rates and shorter overall survival time than HCC patients without promoter methylation of CTH (Fig. 2K).
Table 1
Correlation between CTH expression and clinicopathological characteristics of HCCs in two independent cohorts of human HCC tissues

| Clinicopathological variables | Cohort I |  | Cohort II |  |
|------------------------------|---------|---|-----------|---|
|                              | Tumor CTH expression |  | Tumor CTH expression |  |
|                              | Negative (n = 152) | Positive (n = 128) |  | Negative (n = 109) | Positive (n = 101) |  |
| Age                          | 51.63(9.148) | 52.85(10.966) | 0.283 | 50.95(10.857) | 54.59(9.943) | 0.546 |
| Sex                          |  |  |  |  |
| female                       | 25 | 20 | 0.872 | 20 | 19 | 1.000 |
| male                         | 127 | 108 |  | 89 | 82 |  |
| Serum AFP                    |  |  |  |  |
| ≤ 20 ng/ml                   | 24 | 25 | 0.433 | 19 | 31 | 0.034 |
| > 20 ng/ml                   | 128 | 103 |  | 90 | 70 |  |
| Virus infection              |  |  |  |  |
| HBV                          | 108 | 85 | 0.0807 | 76 | 82 | 0.129 |
| HCV                          | 22 | 22 |  | 11 | 8 |  |
| HBV + HCV                    | 9 | 7 |  | 5 | 5 |  |
| none                         | 13 | 14 |  | 17 | 6 |  |
| Cirrhosis                    |  |  |  |  |
| absent                       | 38 | 41 | 0.230 | 28 | 29 | 0.644 |
| present                      | 114 | 87 |  | 81 | 72 |  |
| Child-pugh score             |  |  |  |  |
| Class A                      | 122 | 114 | 0.049 | 81 | 77 | 0.752 |
| Class B                      | 30 | 14 |  | 28 | 24 |  |
| Tumor number                 |  |  |  |  |
| single                       | 91 | 100 | 0.001 | 56 | 68 | 0.024 |
| multiple                     | 61 | 28 |  | 53 | 33 |  |
| Maximal tumor size           |  |  |  |  |
| ≤ 5 cm                       | 78 | 84 | 0.021 | 45 | 54 | 0.097 |
| > 5 cm                       | 74 | 44 |  | 64 | 47 |  |
| Tumor encapsulation          |  |  |  |  |
| absent                       | 54 | 21 | < 0.001 | 62 | 24 | < 0.001 |
| present                      | 98 | 107 |  | 47 | 77 |  |
| Microvascular invasion       |  |  |  |  |
| absent                       | 81 | 91 | 0.003 | 41 | 75 | < 0.001 |
| present                      | 71 | 37 |  | 68 | 26 |  |
| Tumor differentiation        |  |  |  |  |
| I-II                         | 100 | 107 | 0.001 | 76 | 91 | < 0.001 |
| III                          | 52 | 21 | 33 | 10 |  |  |
| TNM stage | Cohort I | Cohort II | p-value   |
|-----------|----------|-----------|-----------|
| I-II      | 101      | 122       | < 0.001   |
| III       | 51       | 6         | 36        | 4         | < 0.001   |
Table 2
Univariate and Multivariate Analysis of Factors Associated with Time To Recurrence and Overall Survival in Cohort I HCC Patients (n = 280) and Cohort II HCC Patients (n = 210)

| Cohort I (n = 280) | Time To Recurrence | | Overall Survival | |
|--------------------|-------------------|---|------------------|---|
| **Clinical Variables** | HR(95%CI) | P value | HR(95%CI) | P value |
| **Univariate Analysis** | | | | |
| Age | 0.994(0.979–1.009) | 0.427 | 0.989(0.973–1.004) | 0.152 |
| Sex (female versus male) | 0.861(0.574–1.293) | 0.861 | 0.902(0.592–1.373) | 0.630 |
| Serum AFP (≤ 20 versus > 20 ng/ml) | 1.418(0.927–2.170) | 0.108 | 1.301(0.849–1.994) | 0.227 |
| Virus infection (no versus yes) | 0.986 (0.796–1.222) | 0.896 | 1.002(0.808–1.243) | 0.986 |
| Cirrhosis ( absent versus present) | 1.039(0.741–1.456) | 0.825 | 1.132(0.797–1.606) | 0.489 |
| Child-pugh score (A versus B) | 1.254(0.835–1.884) | 0.274 | 1.247(0.824–1.887) | 0.296 |
| Tumor number (single versus multiple) | 2.596(1.903–3.540) | < 0.001 | 0.343(0.250–0.470) | < 0.001 |
| Maximal tumor size (≤ 5 versus > 5 cm) | 0.583(0.482–0.706) | 0.013 | 0.696(0.509–0.951) | 0.023 |
| Tumor encapsulation (absent versus present) | 0.341(0.248–0.469) | < 0.001 | 3.065(2.221–4.230) | < 0.001 |
| Microvascular invasion (absent versus present) | 2.338(1.720–3.179) | < 0.001 | 0.405(0.296–0.554) | < 0.001 |
| Tumor differentiation (I-II versus III-) | 0.295(0.241–0.361) | < 0.001 | 0.317(0.229–0.439) | < 0.001 |
| TNM stage (I-II versus III) | 3.032(2.193–4.191) | < 0.001 | 0.145(0.102–0.205) | < 0.001 |
| CTH (negative versus positive) | 6.289(4.444–8.901) | < 0.001 | 3.733(2.629–5.299) | < 0.001 |
| **Multivariate analysis** | | | | |
| Tumor number (single versus multiple) | 0.707(0.473–1.057) | 0.091 | 0.616(0.413–0.920) | 0.018 |
| Maximal tumor size (≤ 5 versus > 5 cm) | 1.050(0.749–1.471) | 0.778 | 1.085(0.766–1.537) | 0.645 |
| Tumor encapsulation (absent versus present) | 1.448(0.937–2.238) | 0.096 | 1.392(0.889–2.178) | 0.148 |
| Microvascular invasion (absent versus present) | 0.674(0.469–0.968) | 0.033 | 0.631(0.436–0.912) | 0.014 |
| Tumor differentiation (I-II versus III-) | 0.858(0.514–1.435) | 0.560 | 0.933(0.548–1.587) | 0.798 |
### Cohort I (n = 280)

| Clinical Variables                          | Time To Recurrence | Overall Survival |
|---------------------------------------------|--------------------|------------------|
| TNM stage (I-II versus III)                 | 0.357(0.197–0.645) | 0.335(0.184–0.611) | < 0.001 |
| CTH (negative versus positive)              | 2.759(1.941–3.922) | 2.969(2.054–4.293) | < 0.001 |

### Cohort II (n = 210)

| Clinical Variables                          | Time To Recurrence | Overall Survival |
|---------------------------------------------|--------------------|------------------|
| TNM stage (I-II versus III)                 | 7.507(4.967–11.345) | 0.124(0.082–0.188) | < 0.001 |
| CTH (negative versus positive)              | 2.617(1.783–3.822) | 4.409(2.950–6.588) | < 0.001 |

### Univariate Analysis

| Clinical Variables                          | HR(95%CI) | P value |
|---------------------------------------------|-----------|---------|
| Age                                         | 0.987(0.970–1.004) | 0.122   |
| Sex (female versus male)                    | 0.769(0.499–1.184) | 0.232   |
| Serum AFP (≤ 20 versus > 20 ng/ml)          | 1.199(0.788–1.825) | 0.397   |
| Virus infection (no versus yes)             | 0.959(0.700–1.313) | 0.792   |
| Cirrhosis (absent versus present)           | 0.873(0.595–1.282) | 0.489   |
| Child-pugh score (A versus B)               | 0.973(0.649–1.460) | 0.896   |
| Tumor number (single versus multiple)       | 1.984(1.398–2.817) | < 0.001 |
| Maximal tumor size (≤ 5 versus > 5 cm)      | 1.328(0.934–1.888) | 0.114   |
| Tumor encapsulation (absent versus present) | 0.354(0.249–0.505) | < 0.001 |
| Microvascular invasion (absent versus present) | 2.319(1.629–3.301) | < 0.001 |
| Tumor differentiation (I-II versus III)     | 2.128(1.422–3.184) | < 0.001 |
| TNM stage (I-II versus III)                 | 7.507(4.967–11.345) | < 0.001 |
| CTH (negative versus positive)              | 0.261(0.178–0.382) | < 0.001 |

### Multivariate analysis

| Clinical Variables                          | HR(95%CI) | P value |
|---------------------------------------------|-----------|---------|
| Tumor number (single versus multiple)       | 1.011(0.667–1.534) | 0.958   |
| Maximal tumor size (≤ 5 versus > 5 cm)      | 0.856(0.510–1.436) | 0.556   |
| Tumor encapsulation (absent versus present) | 1.679(1.012–2.786) | 0.045   |
## DNMT3B is critical for FOXC1-induced HCC proliferation and metastasis.

To explore whether DNMT3B was involved in FOXC1-mediated HCC proliferation and metastasis, we knocked down DNMT3B in Huh7-FOXC1 cells and ectopically upregulated DNMT3B expression in MHCC97H-shFOXC1 cells with lentivirus transfection (Fig. 2F). Down-regulation of DNMT3B inhibited FOXC1-facilitated HCC proliferation, migration, and invasion abilities, while up-regulation of DNMT3B had the opposite result (Fig. 3A-E). *In vivo* tumorigenicity assays suggested that down-regulated DNMT3B significantly inhibited tumor growth induced by Huh7-FOXC1 cells (Fig. 3F, left), whereas upregulation of DNMT3B salvaged the suppression tumor growth induced by FOXC1 knockdown (Fig. 3F, right). Representative Ki67-stained images are shown (Fig. 3G). *In vivo* metastatic assay suggested that down-regulated DNMT3B eliminated HCC metastasis with Huh7-FOXC1 cells, which prolonged overall survival time. In contrast, overexpression of DNMT3B rescued the inhibition of HCC metastasis in the MHCC97H-shFOXC1 groups (Fig. 3H-K). Representative H&E-stained images are shown (Fig. 3L). These results identified that FOXC1 facilitated HCC proliferation and metastasis through upregulating DNMT3B expression.

## FOXC1 expression is positively correlated with DNMT3B expression and negatively correlated with CTH expression in human HCC tissues.

Immunohistochemical (IHC) analysis was used to verify the clinical relevance of FOXC1 and DNMT3B or CTH in human HCC specimens from two independent cohorts. The representative images of FOXC1, DNMT3B, and CTH expression were showed by IHC staining (Fig. 4A). Compared to adjacent nontumor tissues, DNMT3B expression was observably increased in HCC tissues, whereas CTH expression was observably decreased in HCC tissues. FOXC1 expression was positively associated with DNMT3B expression but negatively associated with CTH expression in both cohorts (Fig. 4B). Up-expression of DNMT3B was positively correlation with poor prognosis (Fig. 4C and 4E, upper panel) and aggressive tumor behavior (Supplementary TableS4). Both TCGA database and Human Protein Atlas program database showed that compared to the normal liver tissues, the mRNA and protein levels of DNMT3B in liver cancer tissues were much higher (Supplementary Figure S1D-E). Kaplan–Meier analysis based on TCGA data displayed that compared to HCC patients with low DNMT3B mRNA levels, patients who have high DNMT3B mRNA levels had shorter overall survival time and disease-free survival time (Supplementary Figure S1F). In addition, the reduced expression of CTH indicated poor prognosis (Fig. 4D and 4F, upper panel). Patients who had both of high expression of FOXC1 and DNMT3B endured the highest recurrence rates and lowest overall survival time (Fig. 4C and 4E, lower panel). Consistently, patients with the FOXC1(+)/CTH (-) expression pattern had the highest recurrence rates and lowest overall survival time (Fig. 4D and 4F, lower panel).

## High levels of ROS are critical for FOXC1-mediated HCC cell proliferation, migration and invasion.

To explore whether ROS regulate FOXC1-mediated HCC proliferation, migration and invasion, N-acetylcysteine (NAC) which is an antioxidant(29) and L-Buthionine-sulfoximine (BSO) which decreases GSH levels(30), were used to treat Huh7

| Cohort I (n = 280) | Time To Recurrence | Overall Survival |
|--------------------|--------------------|------------------|
| Microvascular invasion (absent versus present) | 1.111(0.660–1.871) | 0.693 | 1.000(0.591–1.694) | 1.000 |
| Tumor differentiation (I-II versus III-IV) | 0.851(0.547–1.324) | 0.475 | 0.778(0.499–1.212) | 0.267 |
| TNM stage (I-II versus III) | 0.173(0.094–0.321) | 0.001 | 0.172(0.091–0.323) | < 0.001 |
| CTH (negative versus positive) | 3.403(2.243–5.164) | < 0.001 | 3.816(2.467–5.903) | < 0.001 |
-FOXC1 cells and MHCC97H-shFOXC1 cells, respectively. NAC (0.2 mM) treatment abolished high levels of ROS induced by FOXC1 overexpression, whereas BSO (30 µM) treatment rescued the decreased ROS levels induced by FOXC1 knockdown (Fig. 5A). Meanwhile, low levels of ROS induced by NAC inhibited the cell proliferation, migration, and invasion abilities mediated by FOXC1 overexpression and high levels of ROS induced by BSO rescued the suppression results mediated by FOXC1 knockdown (Fig. 5B-E).

High Levels of ROS upregulates FOXC1 expression through ERK1/2-pELK1 pathway.

We have elucidated that overexpression of FOXC1 increased ROS levels by inhibiting cysteine metabolism and ROS promotes HCC progression and metastasis. Meanwhile, we previously reported that FOXC1 is over-expressed in human HCC tissues and promoted HCC progression and metastasis(21, 22). Therefore, we determined whether high levels of ROS regulate FOXC1 expression in HCC cells. Treatment with BSO and NAC could increase and decrease ROS levels respectively (Fig. 5F). BSO treatment significantly increased FOXC1 expression and NAC treatment decreased FOXC1 expression at protein level in HCC cells (Fig. 5G). Notably, with the stimulation of BSO, FOXC1 promoter activity was significantly increased, indicating that high levels ROS transactivated FOXC1 promoter to elevated the expression of FOXC1 (Fig. 5H).

To verify the accurate location of cis-regulatory elements in the FOXC1 promoter sequence that reacted to ROS, we generated a series of truncated mutants of the FOXC1 promoter (Fig. 5I). Dramatic suppression in FOXC1 promoter activity were found in mutants with two deletions from nt-1058 to nt-422 and nt-422 to nt-66, suggesting that these sequences are important in allowing ROS-enhanced FOXC1 activation. The prior region contains one ELK1 binding site, one specificity protein 1 (SP1) binding site and one ATF2 binding site. And the later region contains one SP1 binding site and ELK1 binding site. Notably, site-directed mutagenesis at the ELK1 binding sites inhibited the ROS-promoted FOXC1 activity, while no effect was found for mutations at the SP1 binding site and ATF2 binding site (Fig. 5I). Meanwhile, knockdown of ELK1 abolished FOXC1 promoter activity which is increased by BSO (Fig. 5J). We found that BSO didn’t increase the expression of ELK1 but activate phosphorylation of ELK1 when activated FOXC1 expression (Fig. 5K).

ROS activates Nuclear factor kB (NF-kB), c-Jun N-terminal kinase (JNK), ERK1/2, p38 kinases and Phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) pathways to promote cancer progression(31). To verify that ROS regulate FOXC1 expression via which pathway, we treated cells with PI3K, p38 kinases, JNK, NF-κB and ERK1/2 inhibitors. Preconditioning cells with ERK1/2 inhibitor decreased ROS-induced FOXC1 expression (Fig. 5L). Nevertheless, there was no effect on ROS regulating FOXC1 expression when cells were pretreated with other inhibitors. Further, ChIP assays showed that the ERK1/2 inhibitor dramatically weakened ELK1 binding to the FOXC1 promoter, while there were on significant changes on the binding of ELK1 to the FOXC1 promoter by other inhibitors (Fig. 5M). These results demonstrated that ROS induced FOXC1 over-expression via the ERK1/2-pELK1 signaling pathway.

8-OHdG is the oxidative damage marker(32). IHC was used to detect 8-OHdG, phospho-ELK1 (activated ELK1) expression in two independent cohorts of human HCC tissue arrays. Compared to adjacent nontumor tissues, both 8-OHdG and p-ELK1 expression was markedly elevated in HCC tissues. 8-OHdG and p-ELK1 expression were localized in the nucleus (Fig. 6A). FOXC1 expression was positively associated with both 8-OHdG and p-ELK1 expression in two cohorts (Fig. 6B). patients with high expression of 8-OHdG (Fig. 6C and 6E, upper panel) and p-ELK1 (Fig. 6D and 6F, upper panel), compared to patients with low expression of 8-OHdG and p-ELK1, had shorter overall survival and higher recurrence rates. Elevated expression of both 8-OHdG and p-ELK1 were positively correlated with loss of tumor encapsulation, microvascular invasion, poor tumor differentiation, and a higher TNM stage (Supplementary TableS5-S6). Furthermore, patients with positive co-expression of 8-OHdG (Fig. 6C and 6E, lower panel) and FOXC1 or co-expression of p-ELK1 ((Fig. 6D and 6F, upper panel)) and FOXC1 had the highest recurrence rates and lowest overall survival times.

Discussion
Hepatocellular carcinoma has a high rate of cancer-related death (33). Early recurrence and metastasis often occur after radical resection of HCC, which leads to poor prognosis of HCC patients (34). Thus, the molecular mechanism of HCC metastasis needs to be further elucidated to develop novel therapeutic strategies. As we all know, dysregulated metabolism is a hallmark of cancer, manifested through alterations in metabolites (1). Studies reported that the progression of HCC is associated with the aberrant levels of amino acids (5). However, how deregulation of amino acid metabolism affect HCC proliferation and metastasis remains unclear. Cysteine is a semi-essential amino acid, which can be acquired from the diet or synthesized from methionine through the reverse transsulfuration pathway by cystathionine γ-lyase (CTH) (23). Depletion of CTH results in oxidative stress, vascular defects, abnormal stress responses, and hyperhomocysteinemia (35, 36). Cysteine produced the ROS scavenger glutathione, which decrease ROS levels, by the enzymes GCLC, GCLM (37). Cysteine levels determines the GSH levels and influence the ROS levels. Uncontrolled increase in ROS production result in damage to large molecules such as DNA, proteins and lipids, leading to genomic instability and changes in cell growth (38). As a messenger, ROS also affect several transcription factors, such as HIF1-α, NF-κB, AP-1, NRF2, which are important for cancer development (6). Our previous studies showed that FOXC1 is important for promoting HCC metastasis (21, 22). In this study, our amino acid metabolism RT² Profiler PCR array indicated that FOXC1 inhibited CTH expression, which is involved in cysteine pathways. Overexpression of FOXC1 decreased the cysteine level and increased the ROS level in HCC cells. Overexpression of CTH significantly decreased FOXC1-mediated HCC proliferation and metastasis, while knockdown of CTH recused the suppression of cell proliferation and metastasis that was induced by the down-regulation of FOXC1. In human HCC tissues, FOXC1 expression was negatively associated with CTH expression, and the patients with high expression of FOXC1 and low expression of CTH exhibited the worst prognosis. These results indicated that FOXC1 facilitated HCC proliferation and metastasis through inhibiting CTH expression and increasing ROS levels.

Although we found that overexpression of FOXC1 inhibited CTH expression, its underlying mechanism remains unclear. Some researchers reported that the attenuation of CTH gene transcription is resulted from DNA hypermethylation of CpG rich region in CTH promoter (25, 27). DNA methylation, driven by DNMT1, DNMT3A and DNMT3B, can inhibit gene expression. DNMT1 has high affinity for hemi methylated DNA and maintain the constitutive methylation status of DNA (39). DNMT3A and DNMT3B act primarily as de novo methyltransferases to constitute DNA methylation (40). Our study indicated that the CpG island of CTH promoter was highly methylated and the expression of DNMT3B was markedly increased in FOXC1-overexpressing HCC cells (Huh7-FOX1) than the control groups. In contrast, the expression of DNMT3B was downregulated and the DNA hypermethylation of CTH promoter was inhibited in FOXC1-knockdown cells (MHCC97H-shFOX1) as compared to control cells. Interestingly, FOXC1 upregulated DNMT3B expression through directly binding to its promoter and transactivated its promoter activities. Moreover, downregulation of DNMT3B decreased FOXC1-mediated HCC proliferation and metastasis, whereas upregulation of DNMT3B reversed the inhibition of HCC proliferation and metastasis caused by FOXC1 down-regulated. In human HCC tissues, FOXC1 expression was positively associated with DNMT3B expression, and the patients with positive co-expression of FOXC1 and DNMT3B had the worst prognosis. These studies indicated that overexpression of FOXC1 induced the DNA hypermethylation of CTH promoter and CTH gene silencing through upregulating DNMT3B expression, which resulted in HCC proliferation and metastasis.

Although we identified FOXC1 altered the cysteine metabolism and ROS levels, and FOXC1-mediated high level of ROS promoted HCC proliferation and metastasis, the mechanism underlying FOXC1 overexpression in HCC needs to be clarified. In this study, we observed that high level ROS upregulated FOXC1 expression via the ERK1/2-pELK1 pathway in HCC cells. Overexpression of FOXC1 increased ROS levels through regulating cysteine metabolism, which formed a positive feedback loop to facilitate HCC progression. Our in vitro study showed that the antioxidant NAC inhibited ROS-mediated FOXC1 upregulation, thereby inhibiting ROS-FOXC1-cysteine-ROS signaling-mediated HCC proliferation and invasion. Furthermore, FOXC1 expression was positively correlated with 8-OHdG (oxidative damage marker) and p-ELK1 (activated ELK1) expression in human HCC tissues. HCC patients with positive coexpression of 8-OHdG/FOX1 or p-
ELK1/FOXC1 exhibited the worst prognosis. These studies indicated that ROS-ERK1/2-p-ELK1 signaling mediated FOXC1 overexpression promoted HCC progression.

Conclusions

In conclusion, we found the ROS-ERK1/2-p-ELK1 signaling axis upregulated FOXC1 expression in HCC cells. Overexpression of FOXC1 induced the DNA hypermethylation of CTH promoter and CTH gene silencing through upregulating DNMT3B expression, which resulted in the decrease of cysteine levels and increases of ROS levels. Thus, we demonstrated that the positive feedback loop of OS-FOXC1-cysteine metabolism-ROS is important for promoting liver cancer proliferation and metastasis, and this pathway may provide a prospective clinical treatment approach for HCC.

Abbreviations

BGS, bisulfite genomic sequencing; BSO, L-Buthionine-sulfoximine; ChIP, chromatin immunoprecipitation analysis; CTH, cystathionine γ-lyase; DNMTs, DNA methylases; FOXC1, forkhead box C1; HCC, hepatocellular carcinoma; MSP, methylation-specific PCR; NAC, N-acetylcysteine; pELK1, phospho-ETS Transcription Factor 1; ROS, reactive oxygen species; TNM, tumor-node-metastasis; 5-Aza, 5-aza-2'-deoxycytidine; 8-OhdG,8-hydroxy-2'-deoxyguanosine.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology. All patients provided full consent for the study. All animal experiments are conducted in accordance with the principles and procedures approved by the Ethics Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology for animal experiments.

Consent for publication

All authors have agreed to publish this manuscript.

Availability of data and materials

The data supporting our conclusion were obtained from the TCGA database (https://cancergenome.nih.gov) and Human Protein Atlas online database (https://www.proteinatlas.org).

Competing interests

The authors declare no conflict of interests.

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Authors' contributions

Zhuoying Lin performed experiments, analyzed data and wrote manuscript. Wenjie Huang, Qin He, and Dongxiao Li assisted in plasmid construction and luciferase reporter assay. Zhihui Wang performed the statistical analysis. Yangyang Feng, Danfei Liu, Tongyue Zhang, and Yijun Wang gave assistance in immunohistochemistry staining, IHC staining and
animal experiments. Meng Xie, Xiaoyu Ji and Mengyu Sun assisted in collecting tissues samples. Dean Tian participated in conceiving experiments and analyzing data. Limin Xia and Zhuoying Lin designed studies and wrote the paper. All authors read and approved the final manuscript.

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Authors' information

1Department of Gastroenterology, Institute of Liver and Gastrointestinal Diseases, Hubei Key Laboratory of Hepato-Pancreato-Biliary Diseases, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

2Hepatic Surgery Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology; Clinical Medicine Research Center for Hepatic Surgery of Hubei Province; Key Laboratory of Organ Transplantation, Ministry of Education and Ministry of Public Health, Wuhan, Hubei, 430030, China

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