

Original Article

Mechanistic investigation of SOCS2-mediated suppression of hepatocellular carcinoma progression via regulation of ferroptosis through the Nrf2/GPX4 signaling axis

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Abstract: Hepatocellular carcinoma (HCC) is one of the most lethal malignancies worldwide and is associated with a poor prognosis. Ferroptosis, a newly recognized form of programmed cell death, plays a critical role in the initiation and progression of HCC. Suppressor of cytokine signaling 2 (SOCS2) has been reported to exert tumor-suppressive functions in various cancers, however, its specific role in HCC and its relationship with ferroptosis remain to be fully elucidated. Integrated bioinformatic analyses of GEO and TCGA datasets were performed using weighted gene co-expression network analysis (WGCNA) combined with multiple machine learning algorithms to identify key genes associated with hepatocellular carcinoma (HCC), followed by single-cell RNA sequencing analysis to characterize the tumor microenvironment. Functional experiments were conducted in HCC cells with SOCS2 overexpression or knockdown to assess proliferation, migration, invasion, ferroptosis-related phenotypes, and mitochondrial function. In addition, a nude mouse subcutaneous xenograft model was established to evaluate the role of SOCS2 in vivo. Bioinformatics analysis identified 230 core differentially expressed genes (DEGs) in hepatocellular carcinoma (HCC), which were predominantly enriched in pathways related to the cell cycle, ferroptosis, and oxidative phosphorylation. Single-cell analysis revealed that SOCS2, GPX4, and ACSL4 exhibited cell subtype-specific expression patterns across distinct cellular populations. Functional assays demonstrated that SOCS2 overexpression significantly inhibited the proliferation, migration, and invasion of HCC cells, while inducing ferroptosis-related phenotypes characterized by decreased glutathione (GSH) levels, increased malondialdehyde (MDA) and reactive oxygen species (ROS) accumulation, and reduced mitochondrial membrane potential. Notably, these effects were partially reversed by Ferrostatin-1 treatment. Mechanistically, SOCS2 overexpression was accompanied by increased Nrf2 and decreased GPX4 protein levels, and Nrf2 knockdown partially reversed the changes in GPX4 expression and cellular phenotypes. Knockdown of Nrf2 partially restored GPX4 expression and alleviated SOCS2-mediated suppression of cell proliferation as well as oxidative stress. In vivo experiments further confirmed that SOCS2 overexpression markedly inhibited tumor growth, an effect that was partially abrogated by Nrf2 silencing. These results suggest that SOCS2 suppresses HCC cell proliferation and tumor growth in association with ferroptosis-related phenotypes, potentially involving the Nrf2/GPX4 axis. This study provides a preliminary experimental basis for understanding the regulatory network of ferroptosis in HCC and suggests SOCS2 as a candidate molecule for further translational investigation.

Keywords: Hepatocellular carcinoma, SOCS2, ferroptosis, Nrf2, GPX4, oxidative stress

Introduction

The clinical management of hepatocellular carcinoma (HCC), the most common primary malignancy of the liver, remains a formidable challenge, largely reflected in delayed diagnosis, therapeutic resistance, and an unfavorable

prognosis [1, 2]. Because of its insidious onset and the lack of early specific symptoms, most patients are diagnosed at an advanced stage, thereby losing the opportunity for curative treatments such as surgical resection or liver transplantation; moreover, postoperative recurrence rates remain unacceptably high even among

patients who receive curative treatment [3, 4]. In recent years, therapeutic strategies including surgery, chemotherapy, radiotherapy, interventional therapy, targeted therapy, and immunotherapy have expanded substantially; however, targeted agents such as sorafenib and lenvatinib, as well as immune checkpoint inhibitors, confer only limited survival benefits in a subset of patients, and both primary and acquired resistance are difficult to avoid, leaving few effective options after treatment failure [5-7]. In addition, reliable predictive biomarkers are still lacking, making it difficult to identify patients who are most likely to benefit before treatment initiation [8]. Collectively, these factors have precluded a fundamental improvement in the long-term prognosis of patients with HCC [9]. Therefore, the identification of novel molecular biomarkers and the development of effective diagnostic and therapeutic targets are of great significance for the early diagnosis and treatment of HCC.

Ferroptosis is a newly identified form of programmed cell death characterized by the iron-dependent accumulation of lipid peroxides, which is morphologically and mechanistically distinct from apoptosis, necrosis, and autophagy [10, 11]. Increasing evidence indicates that ferroptosis plays a critical role in the initiation and progression of hepatocellular carcinoma (HCC) [12]. The occurrence of ferroptosis is closely associated with elevated lipid peroxidation, disruption of iron homeostasis, depletion of glutathione (GSH), and impaired function of glutathione peroxidase 4 (GPX4) [13].

As a key antioxidant enzyme, GPX4 catalyzes the reduction of lipid peroxides and plays an essential role in maintaining cellular redox homeostasis and regulating reactive oxygen species (ROS) levels [14]. Recent studies have further demonstrated that ferroptosis is critically involved in the regulation of HCC development and progression. For instance, in the context of redox homeostasis associated with ferroptosis, activating transcription factor 4 (ATF4) and ubiquitin-specific protease 8 (USP8) have been shown to enhance the transcriptional activity of SLC7A11 while inhibiting its GlcNAcylation, thereby modulating the Xc-GSH-GPX4 axis and regulating redox balance and ferroptosis [15, 16]. Moreover, the Nrf2/HO-1 signaling pathway suppresses intracellular fer-

roptosis, primarily by reducing ROS generation and modulating iron overload [17, 18]. However, the role of ferroptosis in HCC is complex, and its underlying mechanisms require further in-depth investigation.

The suppressor of cytokine signaling (SOCS) family comprises eight members, including cytokine-inducible SH2-containing protein (CIS) and SOCS1-SOCS7 [19]. SOCS proteins are highly conserved in structure and are characterized by three main functional domains: a central SH2 domain, a C-terminal conserved domain (CTD, containing the SOCS box), and an N-terminal domain (NTD) with variable length and amino acid sequence [20, 21]. Previous studies have demonstrated that downregulation of SOCS2 is closely associated with poor prognosis and accelerated tumor progression in patients with breast cancer, ovarian cancer, prostate cancer, lung cancer, and hematological malignancies [22-26]. In addition, dysregulation of SOCS2 has been implicated in the initiation, progression, metastasis, and prognosis of hepatocellular carcinoma (HCC) [27, 28]. Mechanistic studies suggest that SOCS2 may influence HCC progression by regulating apoptosis or necrosis; however, whether it is involved in other forms of programmed cell death, such as ferroptosis, remains to be further elucidated.

In this study, we investigated the biological role of SOCS2 in hepatocellular carcinoma (HCC) and its association with ferroptosis-related phenotypes, with a particular focus on the potential role of the Nrf2/GPX4 axis. Through a series of functional experiments, we evaluated the effects of SOCS2 on HCC cell proliferation, migration, and invasion. In addition, the effects of SOCS2 on oxidative stress regulation, lipid peroxidation, and mitochondrial function were explored, which may help to clarify the relationship between SOCS2 and ferroptosis-related phenotypes in HCC.

Materials and methods

Data acquisition and preprocessing

This study performed bioinformatics analyses based on publicly available databases. Gene expression datasets related to hepatocellular carcinoma (HCC), namely GSE25097 and GSE76427, were obtained from the Gene

Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI), both of which include tumor tissues and matched adjacent non-tumorous samples. In addition, RNA sequencing data, along with corresponding clinical and survival information for the HCC cohort (TCGA-LIHC), were downloaded from The Cancer Genome Atlas (TCGA) database for validation analyses. All data were pre-processed using R software (version 4.2.0 or higher). For microarray data, background correction, normalization, and log₂ transformation were performed using the Robust Multi-array Average (RMA) algorithm. For RNA-seq data, normalization was conducted using the DESeq2 package (version 1.36.0), followed by variance stabilizing transformation (VST). To minimize systematic bias arising from different data sources, batch effects were corrected using the ComBat method implemented in the sva package (version 3.44.0).

Identification of differentially expressed genes and functional enrichment analysis

Differentially expressed genes (DEGs) were identified using the limma package (version 3.52.0) for microarray data and the DESeq2 package for RNA-seq data. In the GSE25097 and GSE76427 datasets, log₂ fold change (log₂FC) values and adjusted *P* values were calculated using an empirical Bayes approach. The thresholds for DEG selection were set at |log₂FC| > 1 and adjusted *P* < 0.05 (Benjamini-Hochberg correction). The ggplot2 package (version 3.4.0) was used to generate volcano plots for visualizing the overall distribution of DEGs, while the VennDiagram package (version 1.7.3) was employed to perform intersection analysis across different datasets to identify a core set of consistently dysregulated genes. Subsequently, functional annotation and enrichment analyses were conducted on the identified DEGs. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the clusterProfiler package (version 4.4.0) to systematically evaluate their biological significance across biological processes, cellular components, and molecular functions. Enrichment analysis was based on the hypergeometric test, with adjusted *P* < 0.05 considered statistically significant. The enrichplot package (version 1.16.0) was used for visualization of the enrichment results.

Weighted gene co-expression network construction and machine learning-based feature gene selection

The weighted gene co-expression network was constructed using the WGCNA package (version 1.71). Initially, gene expression data were subjected to quality control to remove outlier samples and lowly expressed genes. The optimal soft-thresholding power was determined using the pickSoftThreshold function to ensure that the network satisfied the scale-free topology criterion ($R^2 > 0.85$). Subsequently, network construction and module detection were performed using the blockwiseModules function. Co-expression modules were identified based on a dynamic tree-cutting algorithm and assigned distinct colors for visualization. Pearson correlation analysis was conducted to evaluate the associations between module eigengenes (MEs) and clinical traits, thereby identifying modules significantly related to disease status. In addition, the relationships between module membership (MM) and gene significance (GS) were analyzed and visualized using the ggplot2 package. Based on these results, multiple machine learning approaches were integrated to identify key feature genes, including Random Forest, Support Vector Machine-Recursive Feature Elimination (SVM-RFE), Extreme Gradient Boosting (XGBoost), Least Absolute Shrinkage and Selection Operator (LASSO) regression, and the Boruta algorithm. Candidate genes were selected based on cross-validation or built-in feature importance measures in each method. Finally, the VennDiagram package was used to identify the intersection of genes obtained from different algorithms, thereby yielding a robust and representative set of core feature genes.

Single-cell transcriptomic analysis and cell-cell communication analysis

The scRNA-seq dataset (GEO accession GSE149614) was retrieved from the Gene Expression Omnibus (GEO, NCBI). Libraries were generated using the 10x Genomics Chromium platform with V2 barcoding chemistry, profiling hepatocellular carcinoma (HCC) primary tumor and adjacent non-tumor liver samples from 10 patients (*Homo sapiens*), yielding 21 samples and over 70,000 single-cell transcriptomes. The cohort comprised 9

males and 1 female, with a median age of 64 years (range 48-66 years). Etiologically, 5 patients were positive for hepatitis B virus (HBV), 2 for hepatitis C virus (HCV), and 3 had no viral infection. According to the TNM staging system, clinical stages ranged from I to IV. A total of 10 primary tumor samples, 8 adjacent non-tumor liver samples, 2 portal vein tumor thrombus (PVTT) samples, and 1 metastatic lymph node sample were subjected to scRNA-seq analysis.

Single-cell RNA sequencing (scRNA-seq) data analysis was performed using the Seurat package (version 4.3.0). Raw count matrices were first subjected to rigorous quality control to exclude low-quality cells and potential doublets. The data were then normalized and variance-stabilized using the SCTransform method, with correction for cell cycle effects and mitochondrial gene-related bias. Principal component analysis (PCA) was conducted based on highly variable genes, and an appropriate number of principal components was selected for downstream analysis. Cell populations were subsequently identified using a graph-based clustering approach, and dimensionality reduction for visualization was performed using the Uniform Manifold Approximation and Projection (UMAP) algorithm. Cell type annotation was achieved by integrating automated annotation results from SingleR with known marker genes. The expression patterns of target genes across different cell subpopulations were visualized using FeaturePlot and VlnPlot functions. Furthermore, cell-cell communication was systematically analyzed using CellChat platform. Ligand-receptor interactions were evaluated to characterize intercellular communication among different cell types. Comparative analyses between normal and tumor tissues were conducted to identify alterations in communication patterns. Network visualization and centrality analysis were further employed to identify key cell subpopulations with critical regulatory roles in the intercellular communication network.

Immune infiltration analysis

The infiltration levels of immune cells in the tumor immune microenvironment were evaluated using multiple computational algorithms, including CIBERSORT, TIMER, xCell, MCPcount,

ter, EPIC, and single-sample gene set enrichment analysis (ssGSEA). The immunedeconv package (version 2.1.0) was employed to integrate these deconvolution methods, with normalized gene expression matrices as input to estimate the relative abundance or absolute scores of 22 immune cell subtypes. Spearman rank correlation analysis was performed to assess the relationships between immune cell infiltration and candidate biomarkers. The corplot package (version 0.92) was used to visualize the correlation matrix, where the color intensity represents the strength of the correlation (red indicating positive correlation and blue indicating negative correlation). In addition, dynamic changes in immune infiltration during disease progression were illustrated using line plots generated with the ggplot2 package, providing an intuitive visualization of the trends in different immune cell subpopulations.

Protein structure prediction and analysis

The three-dimensional structures of key proteins were predicted using AlphaFold2 or retrieved from the Protein Data Bank (PDB) if experimentally resolved crystal structures were available. Structural visualization was performed using PyMOL (version 2.5.0) or Chimera (version 1.16). Functional amino acid residues were identified based on published literature, evolutionary conservation analysis using the ConSurf server, and domain annotation from the Pfam and InterPro databases. Interactions between amino acid residues, including hydrogen bonds, salt bridges, and hydrophobic interactions, were systematically analyzed and visualized using LigPlot+ (version 2.2) or RING (version 3.0).

Cell lines and culture conditions

The cell lines used in this study included SK-Hep-1 (ATCC HTB-52; American Type Culture Collection, Manassas, VA, USA), HepG2 (ATCC HB-8065), Huh7 (JCRB0403; Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan), and LO2 (also designated L-02/HL-7702; China Center for Type Culture Collection, Wuhan, China). All cell lines were authenticated by short tandem repeat (STR) profiling and were routinely tested for mycoplasma contamination by PCR-based assays every three months, with negative results. SK-Hep-1, HepG2, and Huh7 cells were cultured

in Dulbecco's Modified Eagle Medium (DMEM), whereas LO2 cells were maintained in RPMI-1640 medium. All culture media were supplemented with 10% fetal bovine serum (FBS; BS-1105, Inner Mongolia Opcel Biotechnology Co., Ltd., China) and 1% penicillin-streptomycin solution. Cells were routinely cultured in a humidified incubator at 37°C with 5% CO₂.

Real-time quantitative PCR (RT-qPCR)

Total RNA from hepatocellular carcinoma cells was extracted using TRIzol reagent (15596026, Invitrogen, Thermo Fisher Scientific, USA), and RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (1011U, NanoDrop, USA). Total RNA was reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (RR047A, Takara, Japan) according to the manufacturer's instructions, with reaction conditions of 37°C for 30-50 min and 85°C for 5 s. RT-qPCR was performed using the Fast SYBR Green PCR Kit (RR820A, Takara, Japan) on an ABI PRISM 7300 system (Applied Biosystems, Thermo Fisher Scientific, USA). The amplification conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. Each sample was analyzed in triplicate, and GAPDH was used as the internal control. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (\text{mean Ct of target gene in the experimental group} - \text{mean Ct of reference gene in the experimental group}) - (\text{mean Ct of target gene in the control group} - \text{mean Ct of reference gene in the control group})$. All experiments were independently repeated three times ($n = 3$ biological replicates). Primers used in this study were synthesized by Sangon Biotech (Shanghai, China) and are listed in [Supplementary Table 1](#).

Western blot (WB) analysis

Proteins from tissues and cells were extracted using RIPA lysis buffer (Beyotime Biotechnology, China) supplemented with 10% protease and phosphatase inhibitor cocktail (NCM Biotech, China). Protein concentrations were determined prior to electrophoresis. Equal amounts of protein (30 µg per sample) were separated by SDS-PAGE under the following conditions: 80 V for 30 min followed by 120 V for 60 min. Subsequently, proteins were transferred onto

PVDF membranes at 250 mA for 100 min. After transfer, membranes were blocked with 5% non-fat milk at room temperature for 60 min and then incubated overnight at 4°C with primary antibodies. The following day, membranes were washed three times with TBST buffer and incubated with appropriate secondary antibodies at room temperature for 60 min. Protein bands were visualized using a ChemiDoc XRS+ imaging system (Bio-Rad, USA). The primary antibodies used in this study included GAPDH (1:3000, AF7021), SOCS2 (1:1000, DF8133), GPX4 (1:1000, DF6701), ACSL4 (1:1000, DF12141), and Nrf2 (1:1000, BF8017), all purchased from Proteintech (China).

Next-generation sequencing (NGS)

SK-Hep-1 cells were transfected with a SOCS2 overexpression plasmid (SOCS2-OV), and total RNA from both the control and overexpression groups was extracted using TRIzol reagent (Accurate Biology, China). RNA sequencing was performed by Allwegene Technologies (Beijing, China). Differentially expressed genes were identified using the edgeR software (version 3.08), and *P* values were adjusted using the Benjamini-Hochberg method. Genes with an adjusted *P* value < 0.05 were considered statistically significant.

Cell counting kit-8 (CCK-8) assay for HCC cell proliferation

Hepatocellular carcinoma cells in the logarithmic growth phase were seeded into 96-well plates at a density of 8×10^3 cells per well and incubated for 24, 48, and 72 h. Subsequently, 10 µL of CCK-8 reagent (96992, Sigma-Aldrich, USA) was added to each well, followed by incubation at 37°C in a humidified incubator for 1 h. The absorbance (optical density, OD) was measured at 450 nm using an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA). Each group included six replicate wells, and all experiments were independently performed three times ($n = 3$ biological replicates).

Colony formation assay

Hepatocellular carcinoma cells were seeded into 6-well plates at a density of 500 cells per well, and the culture medium was replaced every three days. After approximately two weeks of incubation, cells were fixed with 4%

paraformaldehyde at room temperature for 30 min, followed by staining with 0.1% crystal violet for 30 min to assess colony formation. All experiments were independently performed three times ($n = 3$ biological replicates).

Wound healing assay

Hepatocellular carcinoma cells were seeded into 6-well plates at a density of 8×10^5 cells per well. After cell attachment, a straight scratch was created in each well using a 200 μ L pipette tip. Cells were then cultured in RPMI-1640 medium containing 5% FBS for an additional 24 h. Wound images were captured at 0 and 24 h using an inverted microscope (Eclipse Ti2-E, Nikon). Each experiment was independently repeated three times ($n = 3$ biological replicates). Wound width was measured using ImageJ software, and the wound healing rate was calculated as follows: healing rate = (wound width at 0 h - wound width at 24 h)/wound width at 0 h $\times 100\%$.

Transwell invasion assay

A total of 5×10^4 cells were seeded into the upper chamber of Matrigel-coated Transwell inserts in 200 μ L of culture medium containing 1% fetal bovine serum (FBS), while 600 μ L of medium supplemented with 20% FBS was added to the lower chamber as a chemoattractant. After incubation for approximately 24 h, non-invading cells on the upper surface were removed. Invaded cells on the lower surface were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 30 min. Images were captured using an N2-Mi8 inverted microscope, and the invaded cells were subsequently counted and analyzed. All experiments were independently performed three times ($n = 3$ biological replicates).

5-Ethynyl-2'-deoxyuridine (EdU) proliferation assay

Cells were seeded into 96-well plates at a density of 1×10^4 cells per well. On the following day, cells were incubated with EdU solution (1:1000 dilution) for 2 h to label proliferating cells. Subsequently, cells were fixed with 4% paraformaldehyde for 20 min and incubated with Apollo staining solution (RiboBio, China) for 30 min. Cells were then permeabilized with 0.5% Triton X-100 for 10 min and counter-

stained with DAPI for 10 min at room temperature. Images were captured using an N2-Mi8 inverted fluorescence microscope, and cell proliferation was quantified based on EdU-positive cells. All experiments were independently performed three times ($n = 3$ biological replicates). All microscopic images were captured with scale bars added during acquisition or post-processing.

Reactive oxygen species (ROS) detection

Intracellular reactive oxygen species (ROS) levels were measured using a ROS assay kit (Beyotime Biotechnology, China). Cells were seeded into 6-well plates and cultured overnight. On the following day, DCFH-DA was diluted 1:1000 in DMEM and used to incubate the cells for 60 min. After incubation, cells were washed three times with DMEM to remove excess probe and then collected as single-cell suspensions. Intracellular ROS levels were subsequently quantified using flow cytometry. All experiments were independently performed three times ($n = 3$ biological replicates).

Mitochondrial membrane potential assay

Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using rhodamine 123 (Beyotime Biotechnology, China). Cells were seeded into 24-well plates and cultured overnight. On the following day, rhodamine 123 was diluted 1:1000 in the appropriate buffer and used to incubate cells for 60 min. After incubation, cells were washed three times with PBS and collected as single-cell suspensions. Fluorescence images were captured using an N2-Mi8 inverted microscope, and changes in mitochondrial membrane potential were analyzed based on fluorescence intensity. All experiments were independently performed three times ($n = 3$ biological replicates).

Measurement of intracellular GSH and MDA levels

Intracellular glutathione (GSH) and malondialdehyde (MDA), a lipid peroxidation product, were measured using a GSH/GSSG assay kit and an MDA assay kit (Beyotime Biotechnology, China), respectively, according to the manufacturer's instructions. Cells were seeded into 6-well plates and cultured overnight prior to collection. For GSH determination, cells were

resuspended in 30 μ L solution M and subjected to repeated freeze-thaw cycles in liquid nitrogen combined with incubation in a 37°C water bath for lysis. The lysates were then centrifuged at 10,000 rpm at 4°C, and the supernatants were collected. The reaction working solution was prepared according to the kit instructions and incubated at 25°C for 5 min, followed by addition of 50 μ L NADPH. After further incubation for 25 min, absorbance was measured at 412 nm. For MDA measurement, cell lysates were prepared and the supernatants were collected. The samples were mixed with the reaction reagents and heated at boiling temperature for 15 min. After cooling, the mixtures were centrifuged at 1,000 g, and the supernatants were collected. Absorbance was measured at 532 nm to evaluate intracellular lipid peroxidation levels. All experiments were independently performed three times ($n = 3$ biological replicates).

Ferrostatin-1 (Fer-1) treatment

Ferrostatin-1 (Fer-1; #S7243, Selleck Chemicals, Houston, TX, USA) was dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mM stock solution and diluted to 10 μ M in complete culture medium immediately before use. For rescue experiments, SOCS2 overexpressing cells were pretreated with 10 μ M Fer-1 for 2 h before functional assays, and an equal volume of DMSO (final concentration < 0.1%) was used as the vehicle control.

Construction of gene knockdown and overexpression and cell transfection

Gene expression in hepatocellular carcinoma cells was modulated using lentiviral and plasmid-based transfection systems. shRNAs targeting SOCS2 and Nrf2, along with corresponding negative controls (sh-NC), as well as SOCS2 overexpression plasmids and empty vector controls (pcDNA3.1 backbone), were constructed by Shanghai GenePharma Co., Ltd. (Shanghai, China). The target sequences (5'→3') of the shRNAs were as follows: sh-SOCS2-1, GAAGCCAAAGAGAAATTAA; sh-SOCS2-2, GCTAAGACAAGAATATCA; sh-Nrf2, GGAGGAGCTATTATCCATT; and a non-targeting scrambled sequence (sh-NC), TTCTCCGAACGTGTACGTT. For lentiviral transfection, HCC cells were seeded into 6-well plates at a density of 2×10^5 cells

per well and cultured for 24 h prior to infection. After 72 h of lentiviral infection, cells were selected using medium containing 4 μ g/mL puromycin for at least 14 days. Subsequently, cells were maintained in medium containing 2 μ g/mL puromycin for approximately 9 days to establish stable knockdown cell lines, which were then cultured in standard medium for subsequent experiments. For overexpression experiments, exponentially growing HCC cells were seeded into 24-well plates at a density of 8×10^3 cells per well. Upon reaching appropriate confluence, cells were transfected using Lipofectamine 3000 reagent according to the manufacturer's instructions. After 6-8 h, the transfection medium was replaced with complete medium, and cells were further cultured for 48 h prior to collection for subsequent RNA and protein analyses.

In vivo xenograft model

Male BALB/c nude mice (4 weeks old, ~16 g, SPF grade) were purchased from SPF (Beijing) Biotechnology Co., Ltd. (Beijing, China) (license No. SCXK (Jing) 2024-001) and housed under specific pathogen-free (SPF) conditions at $22 \pm 2^\circ\text{C}$ with 50-60% relative humidity and a 12-h light/12-h dark cycle, with free access to sterilized food and water. All animal procedures were approved by the Medical Ethics Committee of the Affiliated Hospital of Hebei University (approval No. HBU2026M024) and were performed in accordance with institutional guidelines for the care and use of laboratory animals. Mice were randomly assigned to four groups using a random number table ($n = 6$ per group; this sample size meets the minimal statistical requirements for the planned comparisons). For tumor implantation, 5×10^6 SK-Hep-1 cells suspended in 100 μ L PBS were injected subcutaneously into the right axillary region of each mouse under anesthesia with isoflurane (2% in oxygen). Tumor size was measured every six days using digital calipers, and tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$; tumor measurement and weighing were performed by an investigator blinded to group allocation. On day 28 after inoculation, mice were euthanized by cervical dislocation under deep anesthesia (sodium pentobarbital, 100 mg/kg, i.p.), and tumors were excised, weighed, and processed for subsequent analyses.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (v 10.0) and R (4.2.0). Continuous variables were tested for normality (Shapiro-Wilk) and homogeneity of variance (Levene's); parametric tests were used only when both assumptions held, otherwise non-parametric tests (Mann-Whitney U or Kruskal-Wallis) were used. Two-group comparisons used Student's t-test, and multiple-group comparisons used one-way ANOVA followed by Tukey's or Bonferroni post hoc correction; time-course data were analyzed by repeated-measures ANOVA with time as a within-subject factor, followed by Bonferroni correction. In vitro data are presented as mean $\pm$ SD from at least three independent biological replicates ($n = 3$), and in vivo data as mean $\pm$ SEM ($n = 6$ per group). Machine-learning model stability was evaluated by ten-fold cross-validation, survival by Kaplan-Meier with the log-rank test, and correlations by Pearson or Spearman coefficients. A two-sided $P < 0.05$ was considered significant.

Results

Integrated WGCNA and machine learning identify key prognostic features in HCC

Based on integrated multi-dataset analyses, this study systematically identified differentially expressed genes (DEGs) associated with hepatocellular carcinoma (HCC) and their potential clinical significance. First, differential expression analysis of the GSE25097 and GSE76427 datasets revealed a set of genes significantly upregulated or downregulated in HCC tissues (**Figure 1A**). By intersecting these DEGs with results from weighted gene co-expression network analysis (WGCNA), a total of 230 consistently altered core genes across multiple datasets were identified, suggesting their potential key roles in HCC initiation and progression (**Figure 1B**). Functional enrichment analysis indicated that these genes were primarily involved in biological processes such as cell cycle regulation, mitosis, chromosome segregation, and DNA replication. They were also significantly enriched in tumor-related pathways, including the cell cycle, DNA replication, ferroptosis, oxidative phosphorylation, and the p53 signaling pathway, suggesting abnormal prolifer-

ation and metabolic reprogramming in HCC cells (**Figure 1C**).

Further WGCNA analysis identified a module (the green module) that was strongly associated with the HCC phenotype. Genes within this module showed a strong correlation between module membership and gene significance, while network topology analysis revealed that multiple hub genes were centrally located within the co-expression network (**Figure 1D-F**). To improve the robustness of feature gene selection, multiple machine learning approaches - including Random Forest, SVM-RFE, LASSO regression, and the Boruta algorithm - were integrated for cross-validation, ultimately identifying six consistently selected core feature genes (**Figure 1G-J**). Correlation analyses demonstrated that SOCS2 was significantly negatively correlated with ferroptosis-related gene GPX4 and lipid metabolism-related gene ACSL4 (**Figure 1K**). A risk score model based on SOCS2 exhibited strong prognostic stratification performance in overall survival analysis, with significantly shorter survival observed in the high-risk group. These findings further support SOCS2 as a potential biomarker, providing a molecular basis for precision diagnosis and personalized treatment of HCC, and highlighting its promising clinical value (**Figure 1L**).

scRNA-seq reveals cellular heterogeneity and cell-cell communication in HCC

This study employed single-cell RNA sequencing (scRNA-seq) to systematically analyze hepatocellular carcinoma (HCC) tissues and matched adjacent normal tissues, providing a transcriptome-wide landscape at single-cell resolution. The analysis delineated cellular composition, intercellular communication patterns, and expression profiles of ferroptosis-related genes within the tumor microenvironment (TME). Cell annotation and communication network analyses identified major cell types, including macrophages, hepatocytes, endothelial cells, monocytes, B cells, T cells, natural killer (NK) cells, and tissue stem cells. Notably, interactions among macrophages, hepatocytes, and endothelial cells were the most prominent, suggesting a central regulatory role for these cell populations in TME remodeling and signaling integration (**Figure 2A**). Compared with normal tissues, tumor tissues exhibited significantly

SOCS2 and ferroptosis-related phenotypes in HCC

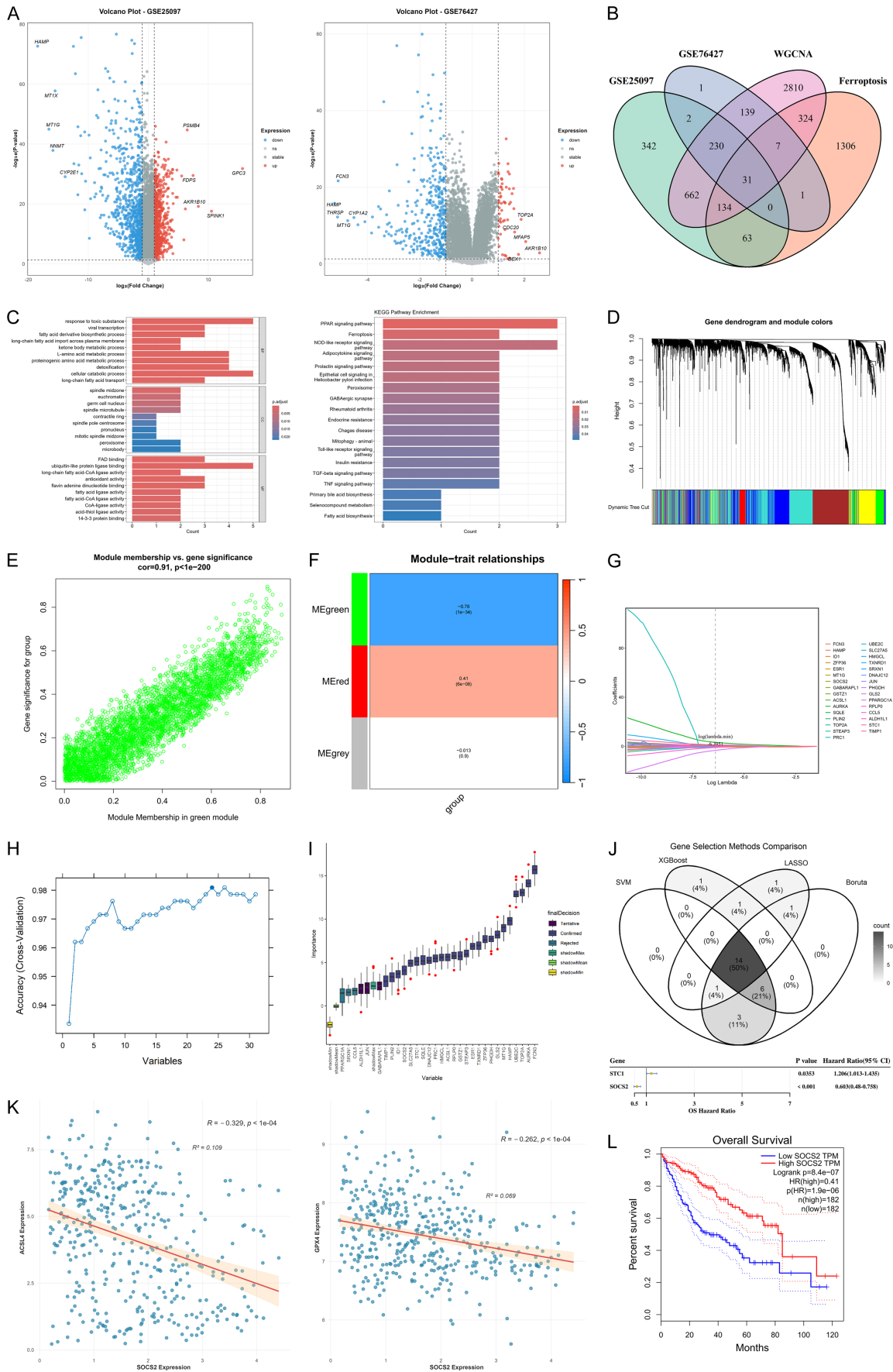


Figure 1. Identification of key genes in hepatocellular carcinoma through integrated WGCNA and machine learning analyses across multiple datasets. A. Volcano plots of differentially expressed genes (DEGs) between hepatocellular carcinoma (HCC) and normal tissues in the GSE25097 and GSE76427 datasets. B. Venn diagram of DEGs identified from GSE25097, GSE76427, and the TCGA-LIHC cohort. C. GO and KEGG pathway enrichment analyses of the intersecting DEGs. D. Correlation analysis between WGCNA modules and HCC phenotype, showing that the green module (MEgreen) was significantly positively correlated with HCC phenotype ($r = 0.81$, $P < 1e-200$). E. Scatter plot of module membership versus gene significance, demonstrating a strong positive correlation in the green module ($cor = 0.91$, $P < 1e-200$). F. Heatmap of correlations between WGCNA modules and clinical traits, indicating that the green module showed the strongest association with HCC phenotype. G. Classification performance of machine learning-based feature gene selection, showing that model accuracy increased with the number of features and stabilized at approximately 30 variables (accuracy ≈ 0.98). H. Intersection of feature genes identified by multiple machine learning algorithms (Random Forest, SVM-RFE, LASSO, XGBoost, and Boruta). I. Comparison of diagnostic performance of classification models (SVM, Random Forest, KNN, and Logistic Regression) constructed based on selected feature genes. J. Venn diagram of genes selected by multiple feature selection methods (XGBoost, LASSO, Boruta, and SVM), identifying core intersecting genes. K. Correlation analysis between SOCS2 and ferroptosis-related genes, showing significant associations with GPX4 ($r = -0.328$, $P < 1e-04$) and ACSL4 ($r = -0.202$, $P < 1e-04$). L. Overall survival analysis based on SOCS2 expression, demonstrating significantly poorer survival in the high-expression group (log-rank $P = 0.001$).

increased immune cell infiltration, particularly elevated proportions of macrophages, T cells, and B cells, accompanied by a marked reduction in hepatocyte abundance. These findings indicate a profound reprogramming of both the immune composition and functional state of the TME in HCC (**Figure 2B**). Ferroptosis-related analyses revealed heterogeneous expression patterns of key ferroptosis-associated genes, including MCL1 and multiple regulatory genes, across distinct cell subpopulations. These genes displayed systematic alterations in tumor-associated cells, suggesting that they may regulate cellular susceptibility to ferroptosis by modulating lipid peroxidation, iron homeostasis, and glutathione metabolism (**Figure 2C, 2G**).

Cell-cell communication analysis further demonstrated that, compared with normal tissues, the interaction network within the tumor microenvironment was markedly more complex, with significantly increased communication number and intensity. This suggests sustained activation of multiple tumor-associated signaling pathways that may contribute to tumor progression, angiogenesis, and immunosuppression (**Figure 2D, 2E, 2J**). UMAP and principal component analysis (PCA) revealed clear segregation of distinct cell populations in reduced dimensional space, with all cells reliably classified into 19 transcriptionally distinct subclusters, highlighting pronounced intratumoral heterogeneity at the single-cell level (**Figure 2F, 2H, 2I**).

Furthermore, key ferroptosis-related genes, including ACSL4, GPX4, and SOCS2, exhibited distinct spatial distribution patterns and expres-

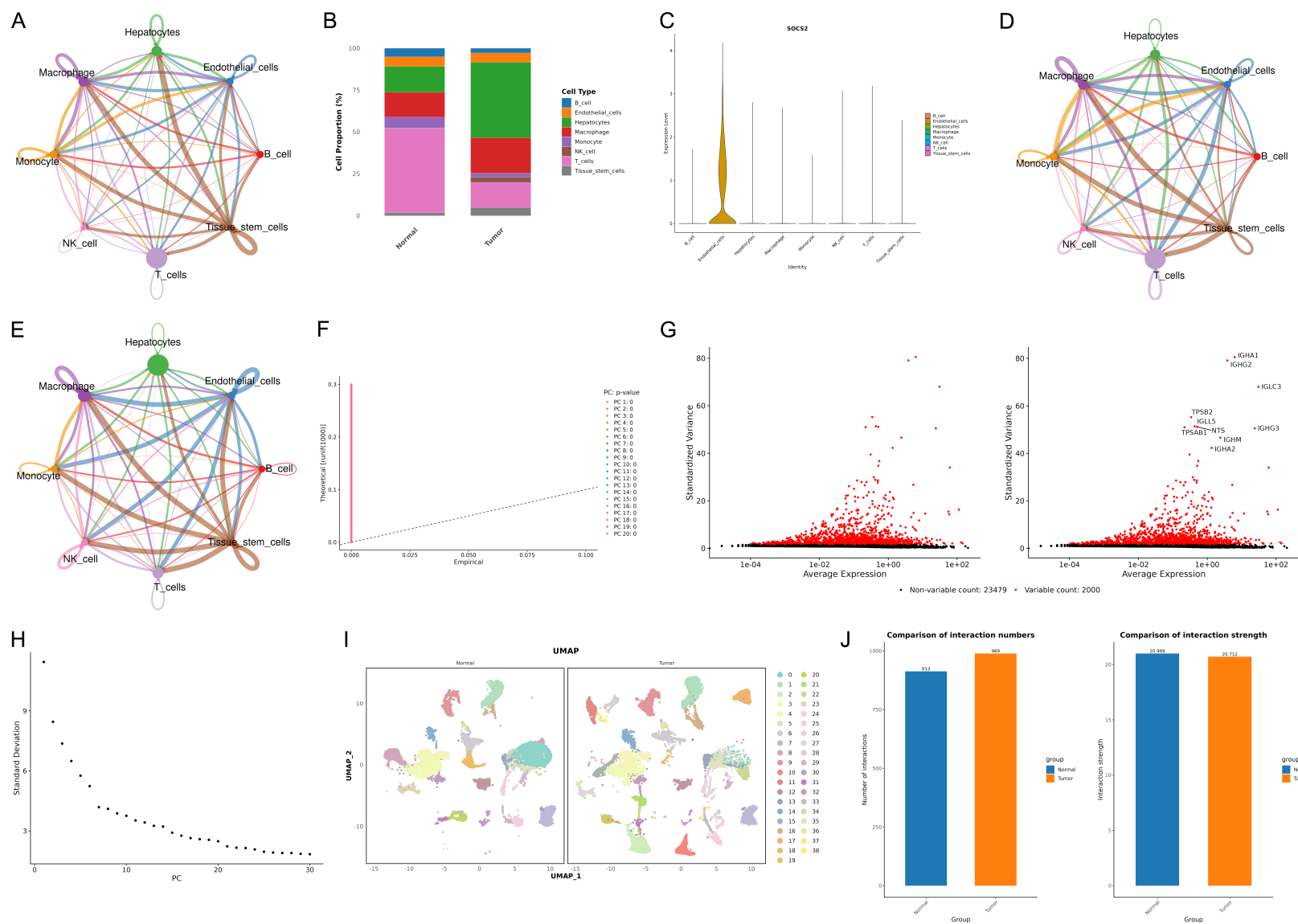
sion gradients across cell subpopulations. GPX4 and ACSL4 were enriched in specific cell clusters, whereas SOCS2 displayed a more complex and heterogeneous expression profile, suggesting a multifaceted regulatory role in ferroptosis modulation and tumor microenvironment remodeling (**Figure 2K-M**). A multi-gene expression heatmap further illustrated the dynamic regulatory landscape of ferroptosis-associated networks in the HCC microenvironment (**Figure 2N**).

Integrated analysis reveals ferroptosis-related signatures and immune features in HCC

To systematically identify ferroptosis-related key genes in hepatocellular carcinoma (HCC), weighted gene co-expression network analysis (WGCNA) was first performed to construct gene co-expression modules. The results showed that several modules were significantly associated with the HCC phenotype, suggesting that genes within these modules may be involved in tumor initiation and progression (**Figure 3A**). Candidate genes extracted from the key module exhibited significant differential expression between tumor and normal tissues, with both upregulated and downregulated genes observed, indicating a complex regulatory landscape of ferroptosis-related pathways in HCC (**Figure 3B**). Although inter-patient variability existed, the overall expression trends remained relatively consistent across samples (**Figure 3C**).

Correlation analysis further revealed that the ferroptosis-related signature score was significantly associated with multiple immune cell infiltration levels, including CD8⁺ T cells, macro-

SOCS2 and ferroptosis-related phenotypes in HCC



SOCS2 and ferroptosis-related phenotypes in HCC

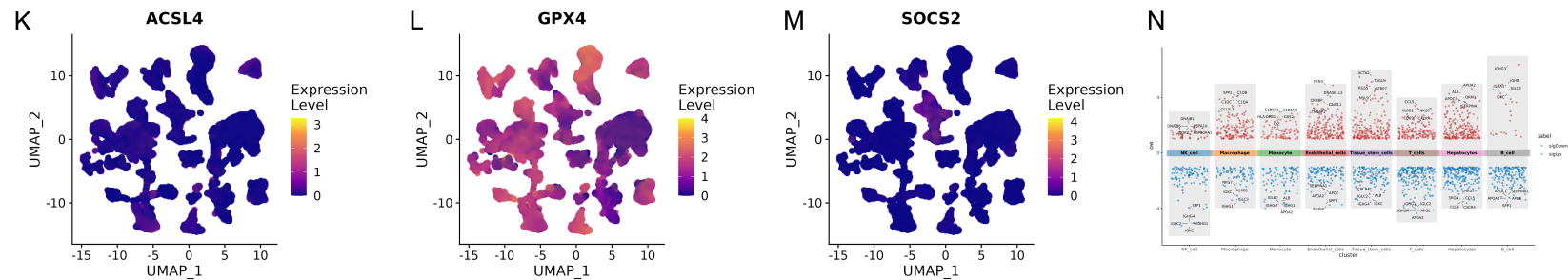


Figure 2. Single-cell transcriptomic analysis reveals cellular composition, intercellular communication features, and key gene expression profiles in the hepatocellular carcinoma tumor microenvironment. A. Cell-cell communication network in normal liver tissue, illustrating ligand-receptor interaction patterns among different cell types. B. Comparison of cellular composition between normal and tumor tissues, showing increased immune cell proportion and decreased hepatocyte proportion in tumor tissues. C. Distribution of SOCS2 expression across different cell types, demonstrating cell type-specific expression patterns. D. Cell-cell communication network in tumor tissues, indicating a more complex intercellular interaction landscape compared with normal tissues. E. Heatmap of intercellular communication strength, revealing globally enhanced cell-cell communication in tumor tissues, with particularly prominent macrophage-associated interactions. F. UMAP dimensionality reduction analysis showing single-cell clustering results, identifying 19 distinct cellular subpopulations. G. Differential gene expression analysis and violin plots demonstrating heterogeneous expression of SOCS2, GPX4, and other genes across cell types. H. UMAP visualization of single-cell samples and cell distribution, showing relatively balanced cell representation across samples. I. UMAP plot illustrating the spatial distribution of 19 identified cellular subpopulations. J. Comparison of intercellular communication number and strength, indicating an overall increase in communication activity in tumor tissues. K-M. Expression patterns of ACSL4, GPX4, and SOCS2 across different cellular subpopulations. N. Differentially expressed genes in each cell type.

SOCS2 and ferroptosis-related phenotypes in HCC

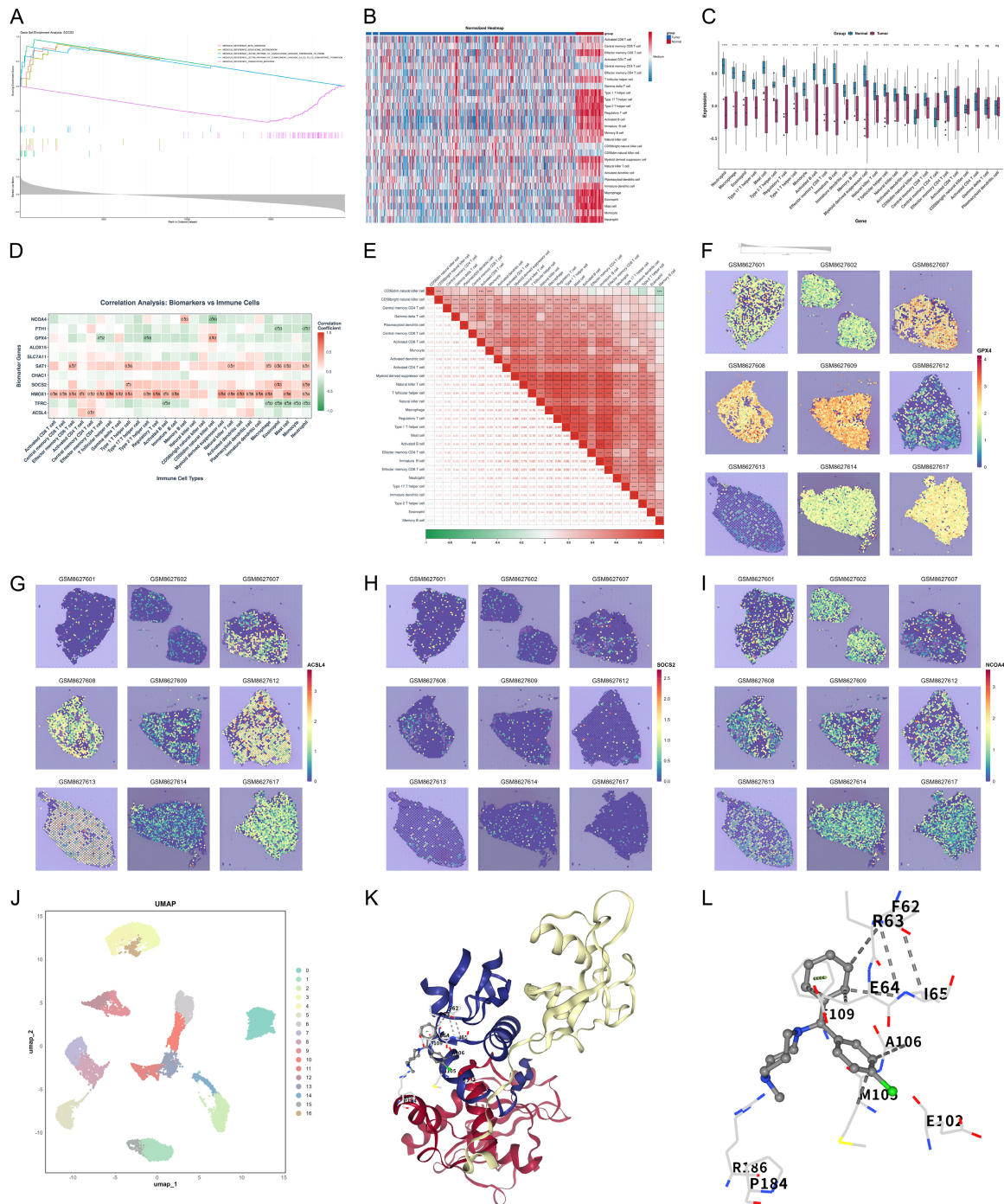


Figure 3. Identification of ferroptosis-related key genes in hepatocellular carcinoma through integrated WGCNA and multi-dimensional analyses, and characterization of their immune and spatial features. (A) WGCNA gene co-expression module dendrogram, with different colors representing distinct co-expression modules. (B) Heatmap of differentially expressed genes between tumor and normal tissues, illustrating global transcriptional differences. (C) Violin plots showing expression levels of differentially expressed genes across samples, demonstrating consistent expression distribution patterns. (D) Correlation heatmap between ferroptosis-related scores and immune cell infiltration levels, indicating associations between ferroptosis activity and the tumor immune microenvironment. (E) Co-expression network analysis of ferroptosis-related genes, where nodes represent genes and edges indicate correlation strength. (F, G) Scatter plots of gene expression versus mean expression and corresponding expression distribution profiles, identifying GPX4, SOCS2, and TP53 as core hub genes. (H, I) Expression patterns of ACSL4 (H) and SOCS2 (I) across individual patient samples, revealing marked inter-patient heterogeneity. (J) UMAP dimensionality reduction analysis showing cellular distribution and subpopulation architecture in HCC single-cell transcriptomic

data. (K) Three-dimensional structural model of GPX4 protein, illustrating its spatial conformational features. (L) Molecular docking analysis between GPX4 and inhibitory compounds, highlighting key binding sites and interaction patterns.

phages, and neutrophils, suggesting a close relationship between ferroptosis and regulation of the tumor immune microenvironment (**Figure 3D**). Co-expression analysis demonstrated significant positive and negative correlations among multiple ferroptosis-related genes, forming an interconnected regulatory network and implying potential synergistic effects in ferroptosis regulation (**Figure 3E**). Single-cell RNA sequencing-based analyses further revealed pronounced expression heterogeneity of key ferroptosis regulators, GPX4 and ACSL4, across different patients and cell subpopulations. Distinct distributions of GPX4-high and ACSL4-enriched cells were observed among samples, suggesting potential inter-patient variability in responses to ferroptosis-inducing therapies (**Figure 3F-I**).

UMAP dimensionality reduction analysis identified multiple transcriptionally distinct cell subpopulations, highlighting the extensive heterogeneity of the HCC tumor microenvironment at single-cell resolution. These findings suggest that different cell subtypes may exhibit differential sensitivity to ferroptosis inducers (**Figure 3J**). At the mechanistic level, three-dimensional structural modeling of GPX4 revealed a canonical glutathione peroxidase fold, including a conserved catalytic active site and substrate-binding region. Molecular docking analysis further elucidated key interactions between GPX4 and its inhibitors, providing structural insights that may facilitate future structure-based drug design targeting GPX4 (**Figure 3K, 3L**).

SOCS2 inhibits proliferation, migration, and invasion of hepatocellular carcinoma cells

To further investigate the role of SOCS2 in hepatocellular carcinoma (HCC), its expression levels were first examined in the normal hepatocyte cell line LO2 and multiple HCC cell lines (SK-Hep-1, Huh-7, and HepG2) using RT-qPCR and Western blot analysis. The results showed that SOCS2 was generally downregulated in HCC cell lines compared with LO2 cells, with the most pronounced decrease observed in SK-Hep-1 cells. Therefore, SK-Hep-1 cells were selected as the primary in vitro model for subsequent functional experiments (**Figure 4A**,

4B). Western blot analysis confirmed the efficiency of SOCS2 overexpression and knockdown. Compared with the control group, SOCS2 protein levels were significantly increased in the overexpression group and markedly reduced in the knockdown group, indicating successful establishment of the corresponding cellular models (**Figure 4C**).

Based on these models, the effects of SOCS2 on HCC cell proliferation, migration, and invasion were systematically evaluated. CCK-8 and colony formation assays demonstrated that SOCS2 overexpression significantly inhibited HCC cell proliferation (**Figure 4D, 4E**). Wound healing assays further revealed that SOCS2 overexpression markedly delayed cell migration, while Transwell assays showed a significant reduction in invasive capacity of SK-Hep-1 cells (**Figure 4F, 4G**). Conversely, SOCS2 knockdown markedly enhanced HCC cell proliferation, colony formation, migration, and invasion. Both CCK-8 and colony formation assays indicated increased proliferative activity, whereas wound healing and Transwell assays demonstrated significantly enhanced migratory and invasive abilities. Overall, these findings indicate that SOCS2 acts as a tumor suppressor in HCC, and its expression level plays a critical regulatory role in controlling the proliferative and invasive behaviors of hepatocellular carcinoma cells.

SOCS2 expression modulates ferroptosis-related phenotypes in HCC cells

To further elucidate the biological function of SOCS2, a stable SOCS2-overexpressing cell line was established, followed by next-generation sequencing (NGS) analysis. The results showed that, compared with the control group, SOCS2 overexpression led to widespread transcriptional alterations. Among the differentially expressed genes, GSDMB, HSPA1A, and HSPA1B were significantly upregulated, whereas CENPB, ODC1, and CEBPA were markedly downregulated (**Figure 5A**). Gene Ontology (GO) enrichment analysis indicated that these differentially expressed genes were predominantly associated with biological processes related to

SOCS2 and ferroptosis-related phenotypes in HCC

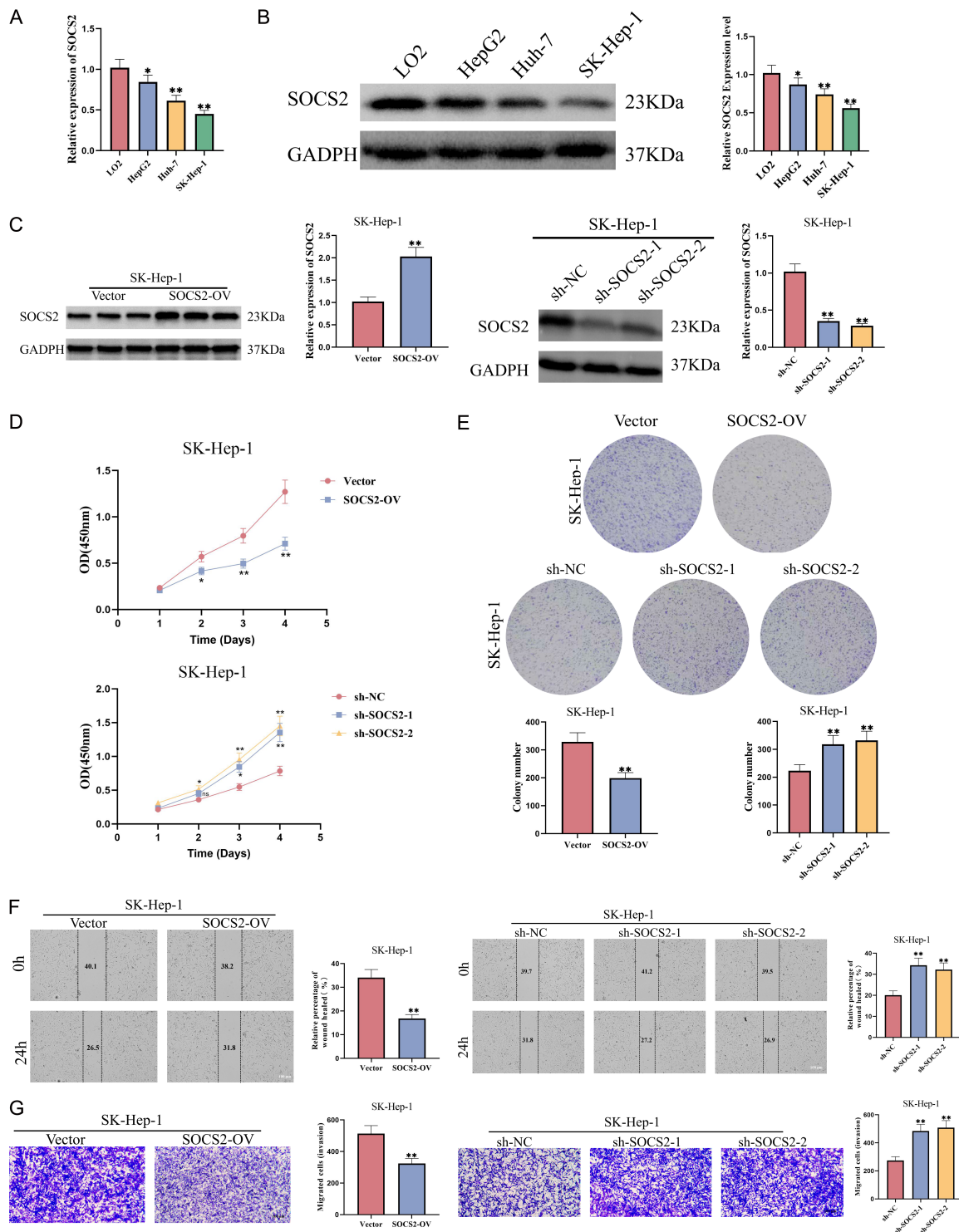


Figure 4. SOCS2 regulates proliferation, migration, and invasion of hepatocellular carcinoma cells. (A) qRT-PCR was performed to determine the relative mRNA expression levels of SOCS2 in the normal hepatocyte cell line (LO2) and hepatocellular carcinoma cell lines (HepG2, Huh-7, and SK-Hep-1). (B) Western blot analysis was conducted to assess SOCS2 protein expression in the above cell lines, followed by densitometric quantification. (C) Western blot was used to validate the efficiency of SOCS2 overexpression (SOCS2-OV) and knockdown (sh-SOCS2-1 and sh-SOCS2-2) in SK-Hep-1 cells, with sh-NC as the control. (D) CCK-8 assays were performed to evaluate the effects of SOCS2 overexpression and knockdown on cell proliferation in SK-Hep-1 cells. (E) Colony formation assays were conducted to assess clonogenic capacity. (F) Wound healing assays were performed to evaluate cell migration, with representative images captured at 0 h and 24 h. Scale bar, 100 μ m. (G) Transwell invasion assays were used to assess invasive

capacity through Matrigel-coated membranes. Scale bar, 100 μ m. Data are presented as mean $\pm$ standard deviation (SD) ($n \geq 3$). Statistical comparisons were performed using Student's t-test for two-group comparisons, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc correction for multiple-group comparisons against the same control, and repeated measures ANOVA for the time-course CCK-8 data in (D). * $P < 0.05$, ** $P < 0.01$.

ferroptosis, reactive oxygen species (ROS) metabolism, and intracellular iron ion homeostasis. They were also enriched in molecular functions such as NADPH oxidase complex activity and heme binding (**Figure 5B**). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis further demonstrated significant enrichment in ferroptosis, glutathione metabolism, and cysteine and methionine metabolism pathways, with the ferroptosis pathway showing the highest enrichment score. Collectively, these findings suggest that SOCS2 may be associated with ferroptosis-related phenotypes by modulating iron metabolism, oxidative stress, and glutathione-related metabolic processes (**Figure 5C**).

Based on the above transcriptomic findings, further phenotypic assays were conducted to validate the regulatory role of SOCS2 in ferroptosis-related processes. The results showed that SOCS2 overexpression significantly reduced intracellular glutathione (GSH) levels, accompanied by an increase in malondialdehyde (MDA) levels, indicating enhanced lipid peroxidation (**Figure 5D, 5E**). Rhodamine 123 staining demonstrated that SOCS2 overexpression led to a marked decrease in mitochondrial membrane potential in HCC cells (**Figure 5F**). Meanwhile, intracellular reactive oxygen species (ROS) levels were significantly elevated, further supporting the association between mitochondrial dysfunction and increased oxidative stress (**Figure 5G**). In contrast, SOCS2 knockdown partially reversed these changes, as evidenced by restored GSH levels, reduced accumulation of MDA and ROS, and partial maintenance of mitochondrial membrane potential stability. These findings suggest that loss of SOCS2 alleviates ferroptosis-related phenotypes in HCC cells. Collectively, these results indicate that alterations in SOCS2 expression are closely associated with mitochondrial function and oxidative stress status in HCC cells, thereby influencing ferroptosis-related phenotypes.

SOCS2 overexpression-induced proliferation inhibition and mitochondrial dysfunction are partially reversed by ferroptosis inhibitors

To further determine whether SOCS2-related cellular phenotypes depend on the ferroptosis pathway, rescue experiments were performed using a ferroptosis inhibitor. Under SOCS2 overexpression conditions, treatment with the ferroptosis inhibitor partially attenuated the inhibitory effect of SOCS2 on HCC cell proliferation. Both colony formation and EdU assays demonstrated a marked recovery of proliferative capacity (**Figure 6A, 6B**). Meanwhile, Rhodamine 123 staining showed that the ferroptosis inhibitor partially restored the decrease in mitochondrial membrane potential induced by SOCS2 overexpression (**Figure 6C**). ROS assays further revealed that intracellular reactive oxygen species accumulation induced by SOCS2 overexpression was significantly reduced upon ferroptosis inhibition (**Figure 6D**). Western blot analysis demonstrated that SOCS2 overexpression increased SOCS2 protein levels, accompanied by decreased GPX4 expression and increased ACSL4 expression (**Figure 6E**). In the SOCS2 overexpression combined with ferrostatin-1 treatment group, the downregulation of GPX4 was partially reversed, while the upregulation of ACSL4 was attenuated, suggesting that ferroptosis inhibition can partially counteract SOCS2-induced changes in ferroptosis-related protein expression at the molecular level. Collectively, both phenotypic and molecular results indicate that ferroptosis inhibition partially rescues SOCS2 overexpression-induced suppression of proliferation, mitochondrial dysfunction, and oxidative stress, suggesting that SOCS2 affects HCC cell behavior at least in part through ferroptosis-related mechanisms.

SOCS2 is associated with ferroptosis-related phenotypes in HCC cells via the Nrf2/GPX4 axis

To identify key candidate genes involved in SOCS2-mediated regulation of ferroptosis, dif-

SOCS2 and ferroptosis-related phenotypes in HCC

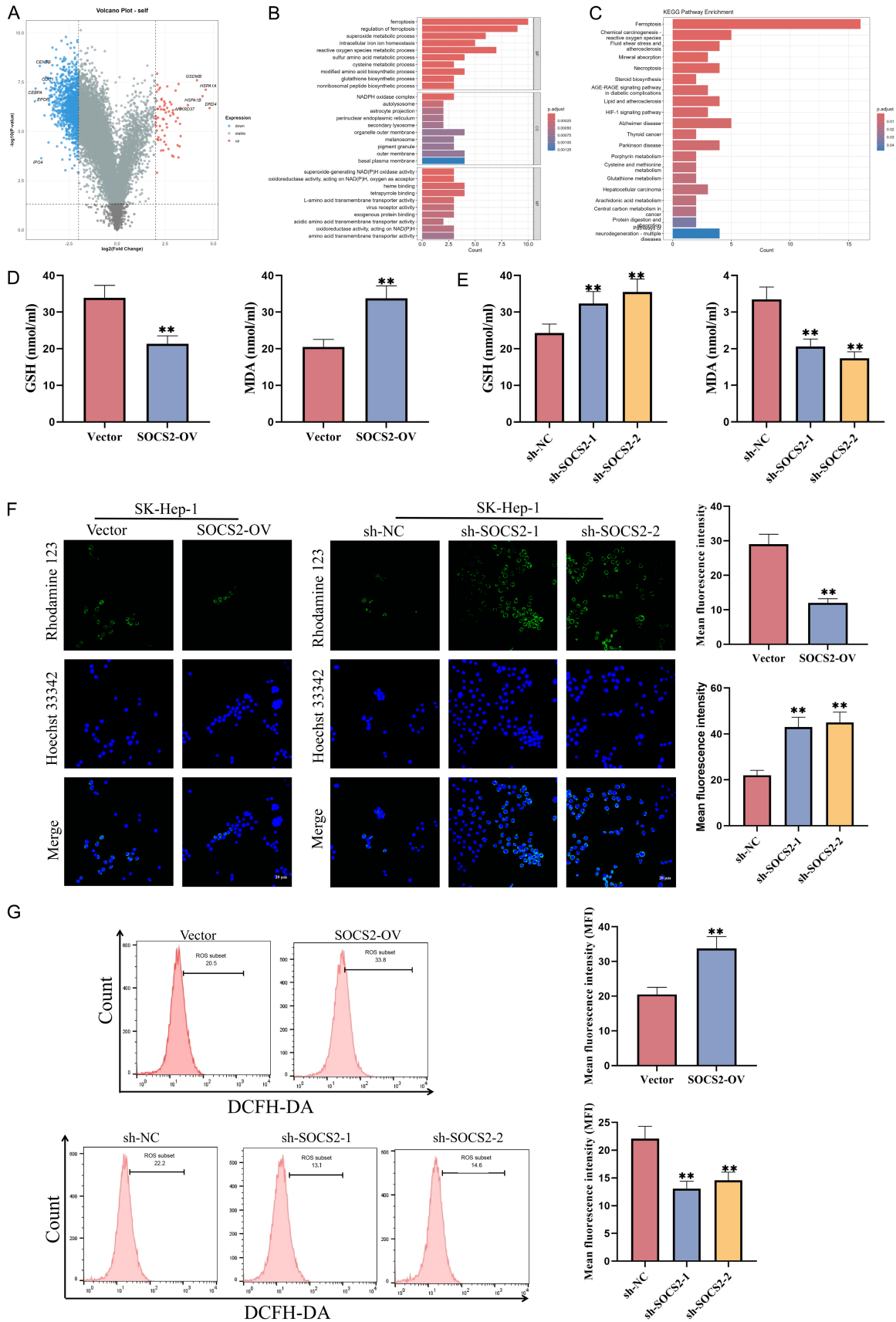


Figure 5. Effects of SOCS2 expression alteration on ferroptosis-related phenotypes in HCC cells. A. Volcano plot showing the distribution of differentially expressed genes (DEGs) in SK-Hep-1 cells following SOCS2 overexpression ($|\log_2FC| > 1$, adjusted $P < 0.05$). B. Gene Ontology (GO) functional enrichment analysis. C. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. D. Measurement of intracellular GSH and MDA levels in SK-Hep-1 cells after SOCS2 overexpression. E. Measurement of intracellular GSH and MDA levels in SK-Hep-1 cells after SOCS2 knockdown. F. Rhodamine 123 fluorescence staining was performed to assess mitochondrial membrane potential following SOCS2 expression alteration, with green fluorescence indicating changes; Hoechst 33342 was used for nuclear staining. Scale bar, 20 μ m. G. Flow cytometry analysis was conducted to detect intracellular reactive oxygen species (ROS) levels using the DCFH-DA probe. Data are presented as mean $\pm$ standard deviation (SD) ($n \geq 3$). Statistical comparisons were performed using Student's t-test for two-group comparisons and one-way analysis of variance (ANOVA) followed by Bonferroni post hoc correction for multiple-group comparisons against the same control. * $P < 0.05$, ** $P < 0.01$.

ferentially expressed genes (DEGs) from SOCS2-overexpressing cells were intersected with a ferroptosis-related gene database. A total of 167 overlapping genes were obtained (Figure 7A). These intersecting genes were further imported into the STRING database to construct a protein-protein interaction (PPI) network. Core genes were identified using Cytoscape based on node degree values. The results showed that NFE2L2 (Nrf2), TP53, NQO1, NFKB1, SLC7A11, SRXN1, PARK7, PRDX2, SESN2, and XBP1 ranked among the top 10 hub genes, exhibiting a highly interconnected network structure. Among the overlapping genes, we identified several hub genes including Nrf2; based on literature evidence, we focused on validating the Nrf2/GPX4 axis as a potential downstream pathway, but the directness of this regulation requires further investigation (Figure 7B). Further validation by RT-qPCR demonstrated that Nrf2 exhibited the most significant expression change among the candidate genes (Figure 7C). Previous studies have shown that Nrf2, a key transcriptional regulator of cellular antioxidant responses, is closely associated with ferroptosis sensitivity [29, 30], indicating that ferroptosis progression may be strongly linked to dysregulation of the Nrf2 signaling pathway. In addition, prior evidence has reported that sodium butyrate (NaB) can regulate ferroptosis in experimental colitis through activation of the Nrf2/GPX4 signaling axis, thereby enhancing intestinal barrier function and exerting a protective effect.

Inspired by this insight, we further investigated whether SOCS2 affects ferroptosis-related phenotypes in HCC cells through the Nrf2/GPX4 signaling axis. The results suggested that SOCS2 may affect tumor cell proliferation and survival via this pathway. Western blot analysis showed that SOCS2 overexpression significant-

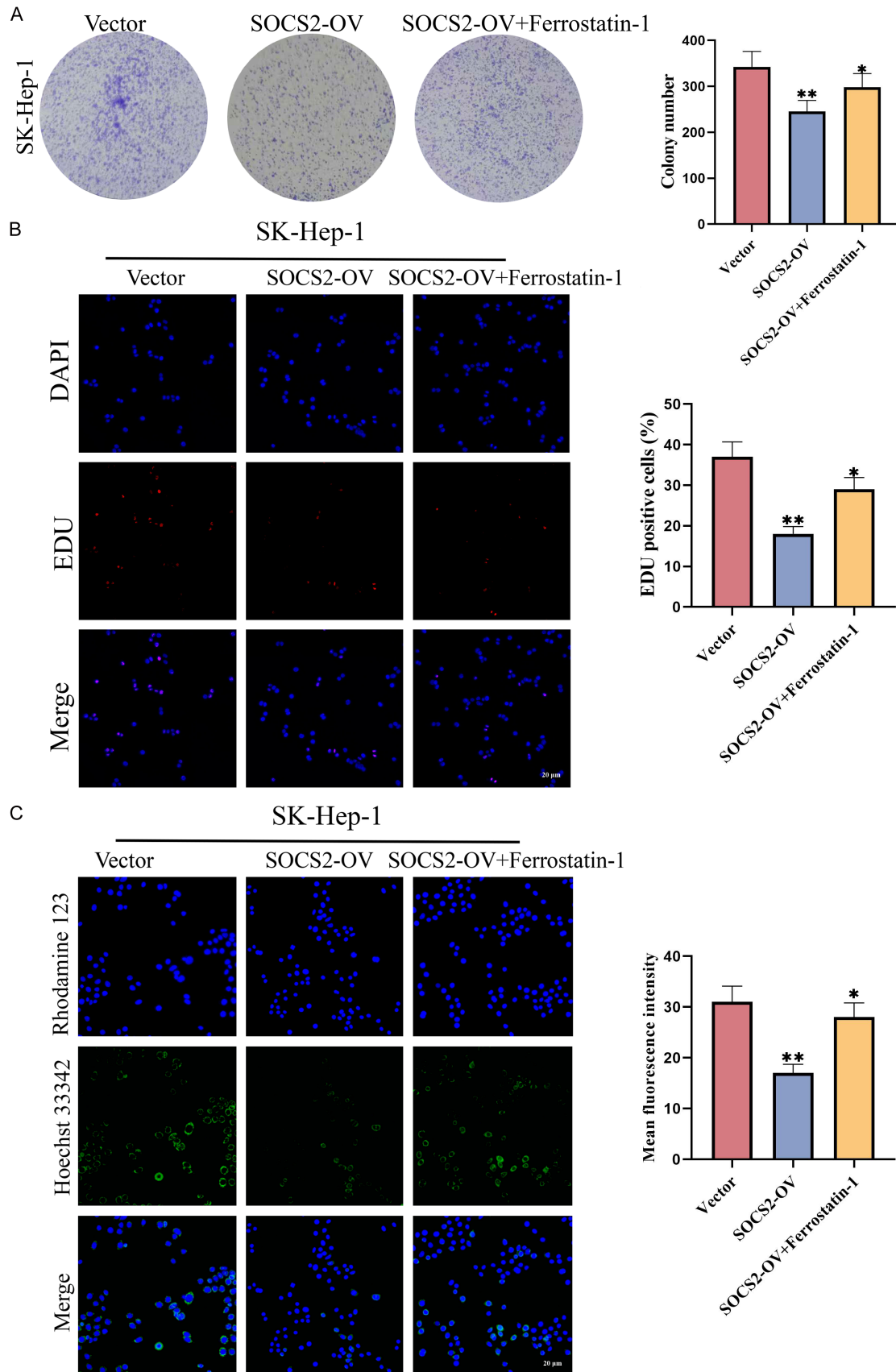
ly upregulated Nrf2 expression while downregulating GPX4 protein levels (Figure 7D). To further evaluate the role of Nrf2 in SOCS2-mediated ferroptosis, experiments were performed in three groups: control, SOCS2 overexpression, and SOCS2 overexpression combined with Nrf2 knockdown (rescue condition) (Figure 7E). Colony formation and EdU assays demonstrated that SOCS2 overexpression markedly inhibited HCC cell proliferation, whereas Nrf2 knockdown partially reversed this inhibitory effect (Figure 7F, 7G). Rhodamine 123 staining revealed that SOCS2 overexpression induced a significant decrease in mitochondrial membrane potential, which was partially restored upon Nrf2 knockdown (Figure 7H). ROS assays showed that SOCS2 overexpression markedly increased intracellular reactive oxygen species levels, while Nrf2 silencing partially reduced ROS accumulation (Figure 7I). Western blot analysis further confirmed that SOCS2 overexpression increased Nrf2 expression and decreased GPX4 levels. In contrast, in the SOCS2 overexpression combined with Nrf2 knockdown group, Nrf2 was effectively suppressed, and GPX4 expression was partially restored (Figure 7J).

Collectively, these findings suggest that SOCS2 is associated with ferroptosis-related phenotypes in HCC cells through the Nrf2/GPX4 axis, and its effects on cell proliferation, mitochondrial function, and oxidative stress are, at least in part, dependent on Nrf2 activity.

SOCS2 inhibits in vivo tumor growth in HCC in association with ferroptosis-related phenotypes and the Nrf2/GPX4 axis

To further verify the role of SOCS2 in regulating tumor growth in association with ferroptosis-related phenotypes, a subcutaneous xenograft mouse model was established, including a con-

SOCS2 and ferroptosis-related phenotypes in HCC



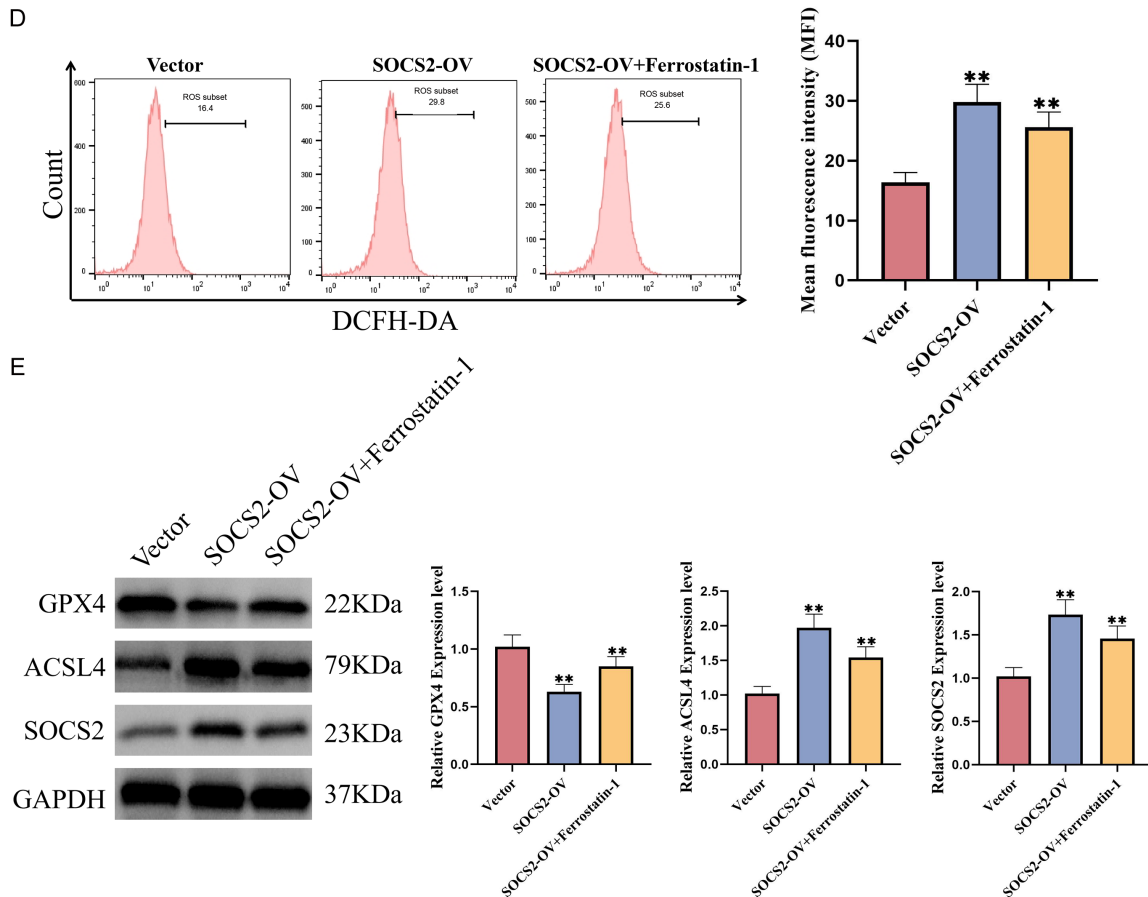


Figure 6. Ferroptosis inhibitor partially reverses SOCS2 overexpression-induced proliferation inhibition and mitochondrial dysfunction. A. Colony formation assays were performed to evaluate the effect of Ferrostatin-1 on SOCS2 overexpression-induced changes in clonogenic capacity. B. EdU incorporation assays were conducted to assess cell proliferation, with representative fluorescence images and corresponding quantitative analysis. Scale bar, 20 μ m. C. Rhodamine 123 staining was used to evaluate mitochondrial membrane potential, followed by quantitative analysis of fluorescence intensity. Scale bar, 20 μ m. D. Intracellular reactive oxygen species (ROS) levels were measured by flow cytometry using the DCFH-DA probe, and mean fluorescence intensity (MFI) was quantified. E. Western blot analysis was performed to detect the expression levels of SOCS2, GPX4, and ACSL4, with densitometric quantification. Data are presented as mean $\pm$ standard deviation (SD) ($n \geq 3$). Comparisons among multiple groups against the same control were performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc correction. * $P < 0.05$, ** $P < 0.01$.

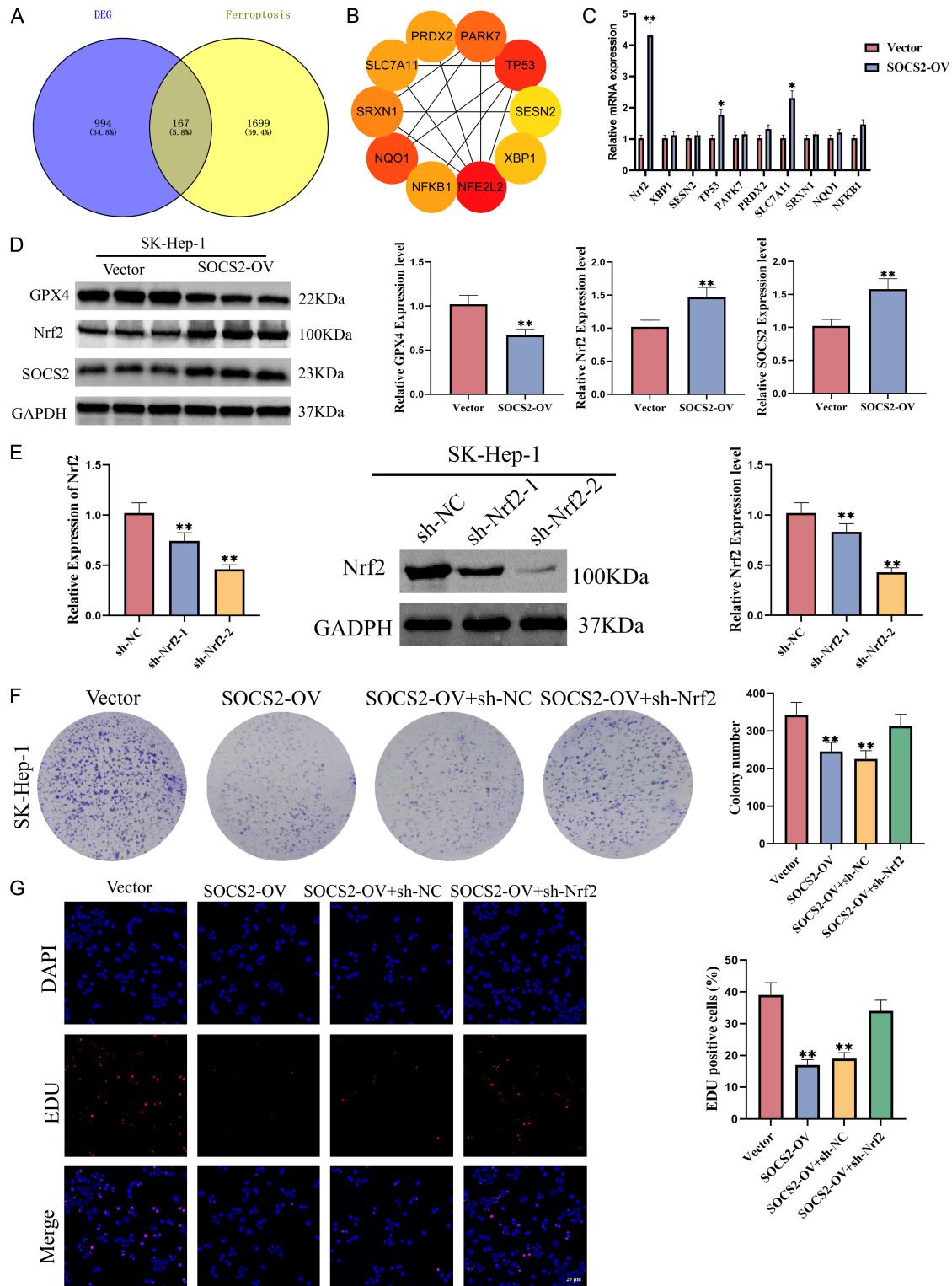
trol group, a SOCS2 overexpression group, a SOCS2 overexpression combined with scramble control (sh-NC) group, and a SOCS2 overexpression combined with Nrf2 knockdown rescue group (**Figure 8A**). Tumor volume and weight measurements showed that SOCS2 overexpression significantly inhibited tumor growth, whereas Nrf2 knockdown partially reversed this suppressive effect (**Figure 8B, 8C**). Western blot analysis revealed that, compared with the control group, SOCS2 overexpression significantly decreased GPX4 protein expression and increased Nrf2 levels. In the SOCS2 overexpression plus Nrf2 knockdown group, Nrf2 expression was effectively suppressed, and GPX4 protein levels were partially

restored relative to the SOCS2 overexpression group, indicating that Nrf2 silencing can, to some extent, reverse SOCS2-mediated regulation of GPX4 expression (**Figure 8D**). Overall, the in vivo findings were consistent with the in vitro results, suggesting that SOCS2 suppresses HCC tumor growth in association with ferroptosis-related phenotypes through the Nrf2/GPX4 axis, with its effects at least partially dependent on Nrf2 signaling.

Discussion

As a member of the suppressor of cytokine signaling (SOCS) family, SOCS2 has been reported to exert tumor-suppressive functions in multi-

SOCS2 and ferroptosis-related phenotypes in HCC



SOCS2 and ferroptosis-related phenotypes in HCC

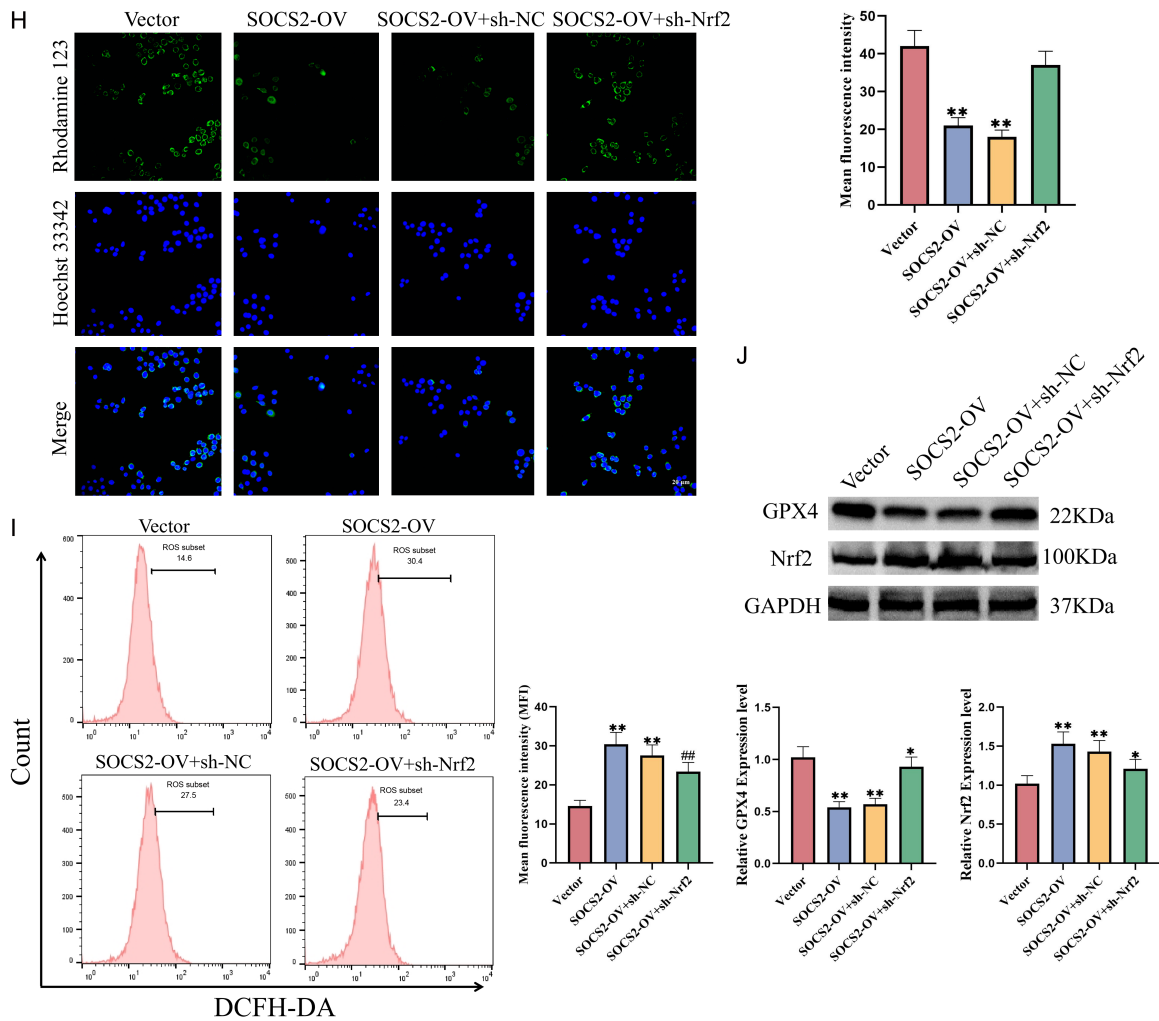


Figure 7. SOCS2 is associated with ferroptosis-related phenotypes in HCC cells via the Nrf2/GPX4 axis. A. Venn diagram analysis showing the intersection between differentially expressed genes (DEGs) in HCC and ferroptosis-related genes. B. Protein-protein interaction (PPI) network constructed using the STRING database, with key hub genes identified by Cytoscape. C. RT-qPCR analysis of candidate gene expression in Vector and SOCS2-overexpressing (SOCS2-OV) groups. D. Western blot analysis of Nrf2, GPX4, and SOCS2 protein expression following SOCS2 overexpression, with densitometric quantification. E. RT-qPCR and Western blot validation of Nrf2 knockdown efficiency and its expression in the SOCS2-OV background. F. Colony formation assays were performed to evaluate the effect of Nrf2 knockdown on SOCS2 overexpression-induced changes in clonogenic capacity. G. EdU incorporation assays were conducted to assess cell proliferation, with quantitative analysis. Scale bar, 20 μ m. H. Rhodamine 123 staining was used to evaluate mitochondrial membrane potential, followed by quantitative analysis of fluorescence intensity. Scale bar, 20 μ m. I. Intracellular reactive oxygen species (ROS) levels were measured by flow cytometry using the DCFH-DA probe, and mean fluorescence intensity (MFI) was quantified. J. Western blot analysis of Nrf2, GPX4, and SOCS2 protein expression in different treatment groups, with densitometric quantification. Data are presented as mean $\pm$ standard deviation (SD) ($n \geq 3$). Statistical comparisons were performed using Student's t-test for two-group comparisons and one-way analysis of variance (ANOVA) followed by Bonferroni post hoc correction for multiple-group comparisons against the same control. * $P < 0.05$, ** $P < 0.01$.

ple malignancies. In breast cancer, reduced SOCS2 expression is associated with aggressive tumor behavior and poor prognosis, whereas restoration of SOCS2 expression suppresses GH/IGF-1-driven proliferative signaling pathways [23, 24]. In prostate cancer, SOCS2 medi-

ates the crosstalk between androgen receptor and growth hormone signaling, and its loss is associated with castration resistance [25]. In colorectal cancer and hematological malignancies, SOCS2 primarily exerts anti-tumor effects by attenuating JAK/STAT-driven oncogenic

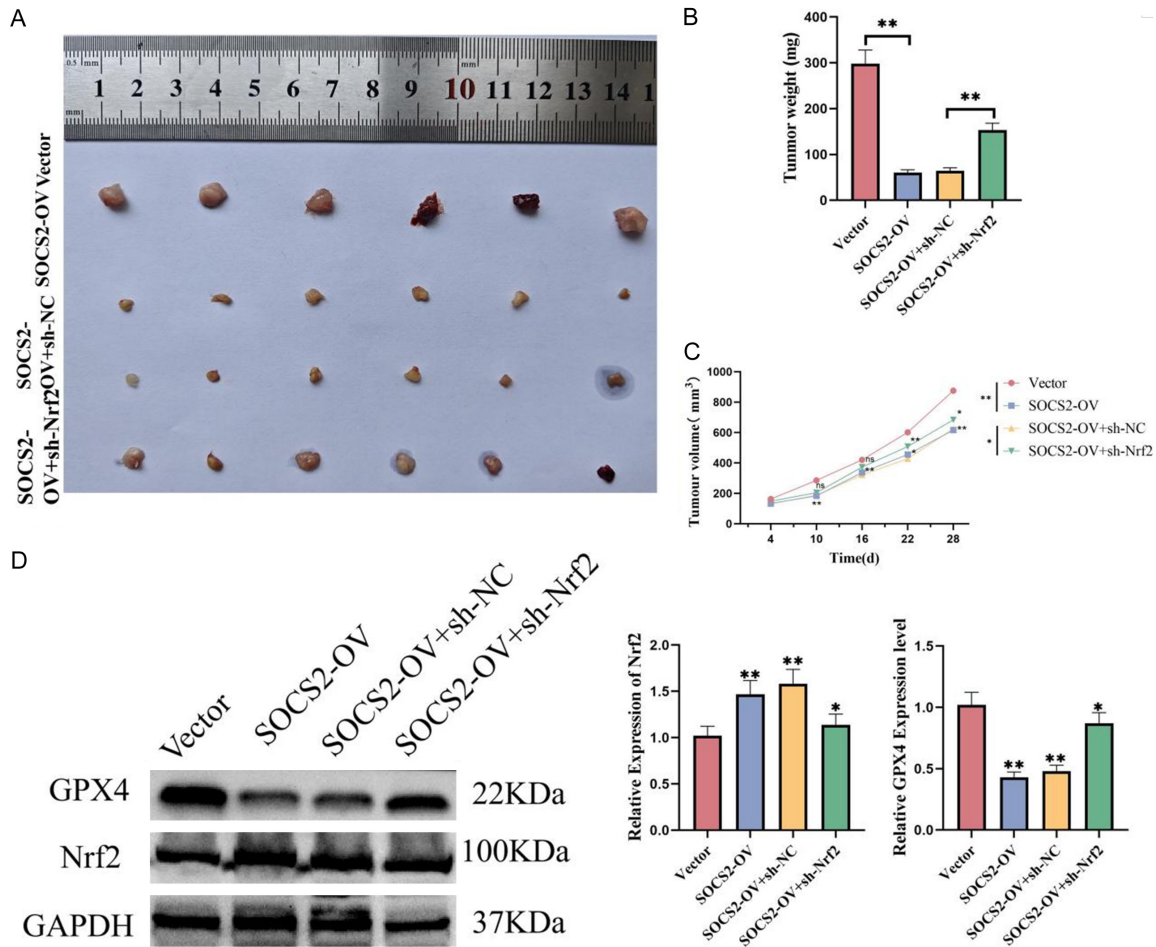


Figure 8. SOCS2 suppresses tumor growth in vivo in association with ferroptosis-related phenotypes and the Nrf2/GPX4 axis. (A) Representative images of subcutaneous xenograft tumors in nude mice. Four-week-old male BALB/c nude mice ($n = 6$ per group) were subcutaneously injected with SK-Hep-1 cells to evaluate tumor growth under different treatment conditions. (B) Quantitative analysis of tumor weight in each group. (C) Tumor growth curves showing changes in tumor volume over time. (D) Western blot analysis of Nrf2 and GPX4 protein expression in tumor tissues, with densitometric quantification; GAPDH was used as a loading control. In vivo data are presented as mean $\pm$ standard error of the mean (SEM) ($n = 6$ per group). Comparisons among multiple groups against the same control were performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc correction, and tumor growth curves in (C) were analyzed using repeated measures ANOVA with time as the within-subject factor, followed by Bonferroni post hoc correction. * $P < 0.05$, ** $P < 0.01$.

transcription [26]. In hepatocellular carcinoma (HCC), Cui et al. demonstrated that SOCS2 suppresses tumor metastasis through inhibition of epithelial-mesenchymal transition (EMT) [28], while Liu et al. reported that decreased SOCS2 expression is associated with shorter patient survival [27]. In the present study, we further demonstrated that SOCS2 overexpression markedly inhibited the proliferation, migration, and invasion of HCC cells, whereas SOCS2 knockdown produced the opposite effects. These findings further support the role of SOCS2 as a broad-spectrum tumor suppressor

and suggest its potential involvement in ferroptosis-related phenotypes.

Chen et al. reported that SOCS2 promotes ferroptosis in hepatocellular carcinoma (HCC) cells by facilitating the ubiquitin-proteasome-mediated degradation of SLC7A11, thereby disrupting the cystine/glutamate antiporter system, suppressing glutathione biosynthesis, and enhancing ferroptotic cell death [31]. This study established the SOCS2-SLC7A11 axis as an important mechanism in ferroptosis regulation and radiosensitization. In contrast, the present

study examined the potential role of the Nrf2/GPX4 axis. Our findings demonstrated that SOCS2 overexpression increased Nrf2 protein levels while reducing GPX4 expression, whereas Nrf2 knockdown partially restored GPX4 expression and attenuated the associated cellular phenotypes. Existing evidence suggests that SOCS2 may affect the GSH pool via SLC7A11, whereas our data indicate that SOCS2 may also influence GPX4 protein levels or transcription; whether these events are linked or independent remains to be determined. Furthermore, while the study by Chen et al. primarily focused on mechanistic validation, the present study integrated multi-omics approaches, including WGCNA, multi-algorithm machine learning, and single-cell transcriptomics, to systematically characterize the role of SOCS2 within the ferroptosis regulatory network of HCC. These findings highlight the need for further investigation into the relationship between SOCS2 and different ferroptosis regulatory pathways.

The core features of ferroptosis include iron-dependent accumulation of lipid peroxides, mitochondrial dysfunction, and disruption of antioxidant defense systems [10]. In this study, SOCS2 overexpression significantly reduced intracellular GSH levels and increased MDA content, accompanied by decreased mitochondrial membrane potential and elevated ROS accumulation, changes that are consistent with ferroptosis-related phenotypes. Moreover, the ferroptosis inhibitor Ferrostatin-1 partially reversed SOCS2 overexpression-induced proliferation inhibition and mitochondrial dysfunction, suggesting that the regulatory effects of SOCS2 on hepatocellular carcinoma (HCC) cells are at least in part associated with ferroptosis-related processes. These findings are in line with recent advances regarding the role of ferroptosis in HCC therapy [32], further supporting ferroptosis as a promising therapeutic target in HCC. However, because the markers examined here (ROS, MDA, GSH, and mitochondrial function) are not specific to ferroptosis and can also be altered in apoptosis and necrosis, and because iron dependence and lipid peroxidation dependence were not directly assessed, these data support the induction of ferroptosis-related phenotypes rather than definitive ferroptosis.

Nrf2 is a key transcriptional regulator of cellular redox homeostasis and plays a central role in determining ferroptosis sensitivity [33]. Under physiological conditions, Nrf2 suppresses lipid peroxidation and protects against ferroptosis by activating the expression of ARE-dependent cytoprotective genes, including SLC7A11, GPX4, and HO-1 [34]. However, in the tumor context, constitutive activation of Nrf2 - commonly driven by KEAP1 or NFE2L2 mutations as well as p62-mediated sequestration of Keap1 - can promote tumor cell survival by enhancing antioxidant capacity and increasing resistance to ferroptosis-inducing stimuli [35]. In the present study, SOCS2 overexpression simultaneously induced Nrf2 upregulation and GPX4 downregulation, suggesting that SOCS2 may disrupt the canonical Nrf2-GPX4 co-regulatory axis. Several potential mechanisms may account for this phenomenon. First, SOCS2 may sequester Nrf2 in the cytoplasm, for example by enhancing its binding to Keap1, thereby reducing its nuclear translocation and transcriptional activity without affecting total Nrf2 protein levels [36]. Second, SOCS2-induced ROS accumulation may trigger compensatory reactive upregulation of Nrf2; however, this response may be insufficient to fully restore GPX4 expression or reverse ferroptosis [37]. In addition, under conditions of severe oxidative stress, certain Nrf2 target genes, such as HMOX1 and NQO1, may paradoxically promote lipid peroxidation and ferroptotic progression [38]. Notably, Nrf2 knockdown only partially restored GPX4 expression and cellular function, indicating that although Nrf2 is an important downstream mediator of SOCS2-induced ferroptosis in HCC cells, it is unlikely to be the sole regulatory factor. However, because only total Nrf2 was measured by Western blot in this study, and nuclear-cytoplasmic fractionation, chromatin immunoprecipitation, and Nrf2 transcriptional activity assays were not performed, the precise molecular mechanism underlying the SOCS2/Nrf2/GPX4 axis remains to be experimentally validated.

This study demonstrated that SOCS2 overexpression significantly reduced GPX4 protein levels, which may be associated with enhanced ubiquitin-mediated GPX4 degradation and Nrf2-dependent feedback regulation. RNA-sequencing analysis further revealed that SOCS2 altered the expression of multiple fer-

roptosis-related genes, including ATF3, CA9, CP, and HMOX1, suggesting that SOCS2 may regulate ferroptosis sensitivity in hepatocellular carcinoma (HCC) cells through a complex transcriptional regulatory network [39]. In addition to the Nrf2/GPX4 axis, SOCS2-associated ferroptosis-related phenotypes may also interact with other key ferroptosis regulatory pathways. Enrichment of the p53 signaling pathway in SOCS2-overexpressing cells suggested a potential association between SOCS2 and the p53-SLC7A11 ferroptosis regulatory axis [40]. Furthermore, the FSP1-CoQ10 pathway, an anti-ferroptotic defense mechanism independent of GPX4 [41], as well as the DHODH-CoQ10 mitochondrial defense pathway that suppresses mitochondrial lipid peroxidation [42], may also contribute to SOCS2-associated ferroptosis-related phenotypes. Given the marked mitochondrial dysfunction induced by SOCS2 overexpression, SOCS2 may amplify ferroptotic signaling by impairing DHODH-CoQ10-mediated mitochondrial antioxidant defense. In vivo experiments further confirmed that SOCS2 is associated with ferroptosis-related phenotypes through the Nrf2/GPX4 axis. SOCS2 overexpression markedly suppressed xenograft tumor growth in nude mice, whereas Nrf2 knockdown partially reversed this inhibitory effect. Western blot analysis demonstrated decreased GPX4 expression and increased Nrf2 expression in SOCS2-overexpressing tumors. In the SOCS2 overexpression combined with Nrf2 knockdown group, GPX4 expression was partially restored, accompanied by attenuation of tumor growth inhibition. Collectively, these findings indicate that SOCS2 is associated with ferroptosis-related phenotypes and suppresses HCC progression through the Nrf2/GPX4 axis, providing experimental evidence supporting SOCS2 as a potential therapeutic target for HCC.

From a clinical translational perspective, the SOCS2-Nrf2-GPX4 regulatory axis identified in this study provides a novel strategy for precision therapy in hepatocellular carcinoma (HCC). Sorafenib, a first-line therapeutic agent for HCC, exerts part of its antitumor activity through the induction of ferroptosis [43], however, the development of therapeutic resistance remains a major clinical challenge. Increasing evidence suggests that activation of the Nrf2/GPX4 axis is a key mechanism underlying sorafenib resistance [44]. In the present

study, SOCS2 was found to downregulate GPX4 expression and be associated with ferroptosis-related phenotypes, suggesting that SOCS2 upregulation or functional mimetics of SOCS2 may represent potential strategies for overcoming sorafenib resistance. Furthermore, combined targeting of Nrf2 may further enhance ferroptosis induction and improve therapeutic sensitivity. Nevertheless, it should be noted that Nrf2 may exert context-dependent dual functions within different tumor microenvironments [45]. Therefore, biomarker-based patient stratification will be essential for the clinical translation of this strategy and for the implementation of personalized therapeutic approaches.

This study has several limitations that should be acknowledged. First, the in vitro functional experiments were conducted exclusively in the SK-Hep-1 cell line, which was selected due to its most pronounced endogenous downregulation of SOCS2. Although suitable for mechanistic validation, whether SOCS2-associated ferroptosis-related phenotypes are broadly applicable across different molecular subtypes of hepatocellular carcinoma (HCC) requires further investigation. Future studies should validate these findings in additional HCC models, including HepG2, Huh7, and patient-derived organoids, to improve the generalizability and clinical relevance of the results [46]. The single-cell RNA sequencing data analyzed in this study were derived from public databases and may therefore be affected by batch effects, differences in cell capture efficiency, and the cross-sectional nature of the datasets. In addition, limited sequencing depth may reduce the sensitivity for detecting low-abundance transcripts, including SOCS2 [47]. Furthermore, the dual role of Nrf2 as both a cytoprotective factor and a potential oncogenic driver adds complexity to the clinical translation of the SOCS2-Nrf2-GPX4 axis. Within the immunosuppressive tumor microenvironment of HCC, Nrf2 activation may enhance ferroptosis sensitivity while simultaneously impairing antitumor immune responses. Therefore, this context-dependent duality should be carefully considered in the design of future therapeutic strategies. The nude mouse xenograft model used in this study lacks a fully competent immune system and thus cannot fully recapitulate the complex immune microenvironment of human HCC. Future studies incor-

porating immunocompetent orthotopic HCC models and patient-derived xenograft (PDX) models are warranted to further evaluate the efficacy and safety of SOCS2-targeted interventions. Despite these limitations, the present study provides preliminary experimental evidence for the association between SOCS2 and ferroptosis-related phenotypes in HCC.

In summary, this study provides preliminary experimental evidence that SOCS2 is associated with ferroptosis-related phenotypes in hepatocellular carcinoma (HCC) through the Nrf2/GPX4 axis. SOCS2 overexpression is associated with increased Nrf2 and decreased GPX4 protein levels, together with enhanced lipid peroxidation, mitochondrial dysfunction, and oxidative stress, and inhibits tumor growth. These findings provide preliminary experimental evidence for the potential role of SOCS2 in ferroptosis-related phenotypes in HCC.

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Disclosure of conflict of interest

None.

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SOCS2 and ferroptosis-related phenotypes in HCC

Supplementary Table 1. RT-qPCR primer sequences

Gene	Sequence (5'-3')
SOCS2	Forward: AGCCACTGCCTGTCCTACTG Reverse: TCTCCACACCACTGCCTTCA
Nrf2	Forward: GAAGCCCTGGATTCACTCA Reverse: CCAGTAGCCGGATTTCCGTA
GPX4	Forward: AACGCCATGAACTACGTCCCG Reverse: GCTGCCACCACGTAGCAGTAG
ACSL4	Forward: CACCACCACTCTGACACCTC Reverse: TGTCCCACTCTGTACACGAG
TP53	Forward: CGCCCCTACTACCTCCATAC Reverse: GAGACGGGCACACTCTGTGTC
SLC7A11	Forward: TCTGCACTGTGCTCACACTCA Reverse: CGAGCACGTCCGACAGATACT
NQO1	Forward: CCTGCCATTCTGAAAGGCTGGT Reverse: GTGGTGATGGAAAGCACTGCCT
NFKB1	Forward: GCAGCACTACTTCTTGACCACC Reverse: TCTGCTCCTGAGCATTGACGTC
PRDX2	Forward: GAGAAGATGGAGCCGTTCAA Reverse: TGTACGCGGTTTGTAATC
PARK7	Forward: CGCAGCTACAGGTGCTGTG Reverse: AGCCACACGTAGCATCTGGT
SESN2	Forward: GGACTCCGATGTATTCCGC Reverse: AGCCGACGAGAAAGAAGTGG
XBP1	Forward: ATGAAACAGAGAAGAGAAGAGAGC Reverse: TGGTCCAACCTGTCCAGAATGC
SRXN1	Forward: GCACTCTCCTGCAGATGGAC Reverse: TTGAAACCCGAATCCCCATC
GAPDH	Forward: GAGTCAACGGATTGGTCGT Reverse: TTGATTTTGAGGGATCTCG