Inhibition of STAT3-ferroptosis negative regulatory axis suppresses tumor growth and alleviates chemoresistance in gastric cancer

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ABSTRACT

Chemotherapy is still one of the principal treatments for gastric cancer, but the clinical application of 5-FU is limited by drug resistance. Here, we demonstrate that ferroptosis triggered by STAT3 inhibition may provide a novel opportunity to explore a new effective therapeutic strategy for gastric cancer and chemotherapy resistance. We find that ferroptosis negative regulation (FNR) signatures are closely correlated with the progression and chemoresistance of gastric cancer. FNR associated genes (GPX4, SLC7A11, and FTH1) and STAT3 are upregulated in 5-FU resistant cells and xenografts. Further evidence demonstrates that STAT3 binds to consensus DNA response elements in the promoters of the FNR associated genes (GPX4, SLC7A11, and FTH1) and regulates their expression, thereby establishing a negative STAT3-ferroptosis regulatory axis in gastric cancer. Genetic inhibition of STAT3 activity triggers ferroptosis through lipid peroxidation and Fe2+ accumulation in gastric cancer cells. We further develop a potent and selective STAT3 inhibitor, W1131, which demonstrates significant anti-tumor effects in gastric cancer cell xenograft model, organoids model, and patient-derived xenografts (PDX) model partly by inducing ferroptosis, thus providing a new candidate compound for advanced gastric cancer. Moreover, targeting the STAT3-ferroptosis circuit promotes ferroptosis and restores sensitivity to chemotherapy. Our finding reveals that STAT3 acts as a key negative regulator of ferroptosis in gastric cancer through a multi-pronged mechanism and provides a new therapeutic strategy for advanced gastric cancer and chemotherapy resistance.

1. Introduction

Gastric cancer is the fifth most diagnosed malignancy worldwide [1]. Gastric cancer patients are usually at advanced stages when diagnosed, thus losing the opportunity for surgery and with the poor prognosis. Currently, chemotherapy is still one of the standard therapies for gastric cancer. 5-fluorouracil (5-FU)-based regimens are first-line treatments for gastric cancer chemotherapy [2]. However, many patients still relapse...
after several courses of 5-FU-based chemotherapy due to the rapid emergence of drug resistance, which has become a major clinical problem. Chemoresistance is a hallmark of malignant tumors and a major hurdle for cancer therapy [3]. During the latest decades, targeted therapies and immunotherapy have provided a new approach for the treatment of gastric cancer [1,4]. However, the therapeutic effect of these treatment strategies is indisposed and many obstacles remain, thus having not been thoroughly applied in the treatment of gastric cancer. Therefore, new and effective therapies are urgent for advanced gastric cancer and chemoresistance.

Ferroptosis is a form of cell death that is caused by the iron-dependent accumulation of lipid peroxide and was termed by Dixon et al. for the first time in 2012 [5,6]. There are three hallmarks of ferroptosis: glutathione (GSH) biosynthesis, lipid peroxidation, and iron metabolism. Iron is an essential reactive element for a variety of biological processes, which exists in two oxidation states (ferrous [Fe2⁺] or ferric [Fe3⁺]), while Fe2⁺ accumulation is an early signal to initiate ferroptosis [7]. Cancer cells resistant to conventional therapy may be susceptible to ferroptosis, in terms of the current research results [6]. Extensive preclinical evidence suggests that the induction of ferroptosis might be an effective therapeutic strategy to alleviate acquired resistance to chemotherapy and targeted therapy [9–11]. Ferroptosis inducers can also work synergistically with traditional drugs (such as cisplatin) to inhibit tumor growth in mouse models of head and neck cancer [12]. More importantly, the susceptibility of different types of cancer cells to ferroptosis was significantly different [13]. Wang et al. revealed that stearyl-CoA desaturase 1 (SCD1) inhibits ferroptosis of cancer cells and promotes tumor growth and migration, potentially by altering cancer stemness and modulating cell cycle-related proteins [14]. A recent study found that cancer-associated fibroblasts (CAFs) secrete exosomal mir-522 to inhibit ferroptosis in cancer cells by targeting arachidonate lipoxigenase 15 (ALOX15) and blocking lipid ROS accumulation in gastric cancer [15]. These studies suggest that gastric cancer may be sensitive to ferroptosis, but the role of ferroptosis in the progression and chemoresistance of gastric cancer has remained largely unexplored.

Signal transducer and activator of Transcription 3 (STAT3) is a key oncogene with dual functions of signal transduction and transcriptional activation [16]. Hyperactivation of STAT3 is a pivotal event in the formation of most human cancers and plays a critical role in cell proliferation, angiogenesis, metastasis, and immunosuppression [17–19]. STAT3 is also aberrantly hyperactivated in gastric cancer and promotes the genesis and development of gastric cancer [20]. Meanwhile, some studies showed that alternatively activated STAT3 plays a prominent role in mediating drug resistance to a broad spectrum of chemotherapies, such as cisplatin [21] and EGFR-TKIs [22,23]. As a transcription factor, STAT3 is also associated with oxidative response [24] and may be a potential regulator of ferroptosis. In a previous study, Gao et al. demonstrated that STAT3 is a positive regulator of ferroptosis in human pancreatic ductal adenocarcinoma (PDAC) cell lines. Furthermore, pharmacological and genetic inhibition of STAT3 blocked erastin-induced ferroptosis in PDAC cells [25]. On the contrary, Liu et al. reported that STAT3 inhibitor BP-1-102 may induce ferroptosis in MGC803/DDP cells after being exposed to cisplatin [26]. Acyl-CoA synthetase long-chain family member 4 (ACSL4), a lipid metabolism enzyme required for ferroptosis, resulting in elevated lipid peroxidation and ferroptosis [27]. Brown et al. found that 46j64-meditated Sre–STAT3 activation repress expression of ACSL4, rendering the cell unable to undergo ferroptosis, while inhibition of STAT3 increases the expression of ACSL4 to trigger ferroptosis in breast cancer cells [28]. According to previous studies, it is controversial whether activation or inhibition of STAT3 induce ferroptosis, and the effect may be different in different cancers. Whether and how STAT3 regulates ferroptosis in gastric cancer has been elusive and runs short of systemic study.

Herein, we report that ferroptosis negative regulation (FNR) signatures are associated with the progression and chemoresistance of gastric cancer. STAT3 mediates ferroptosis through binding to consensus response elements in the SLC7A11, GPX4, and FTH1 gene promoters. Inhibition of STAT3 triggers ferroptosis that is accompanied by lipid ROS increase, Fe2⁺ accumulation, GSH/GSSG depletion, and lipid peroxidation in gastric cancer. Given the importance of STAT3 in ferroptosis, we further develop a potent and selective STAT3 inhibitor W1131, which triggers ferroptosis and possesses potent anti-tumor effects in gastric cancer cell subcutaneous xenograft model, organoids model, and PDX model. Moreover, the combination of W1131 and 5-FU re-sensitizes the chemoresistant cancer cells to 5-FU and demonstrates a significantly synergistic tumor growth regression in the organoids model and resistant gastric cancer cell subcutaneous xenograft model.

2. Materials and methods

2.1. Cell lines and patient specimens

Cells were maintained in 37 °C incubators with 5% CO2. All media used were supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 50 µg/mL penicillin, and 50 µg/mL streptomycin (Gibco, Carlsbad, CA, USA). HEK-293T cells were maintained in a DMEM medium (Gibco, Carlsbad, CA, USA). MGC803, AGS, HGC27, RWPE-1, and A549 cells were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA). 5-fluorouracil-resistant MGC803/5-FU cells were obtained by continuous treatment with a low dosage of 5-fluorouracil for 16 weeks. The gastric tumor tissues and the paraffin-embedded specimens were from Sun Yat-sen Memorial Hospital, Sun Yat-sen University.

2.2. Chemicals

Ferrostatin-1(T6500), Liproxstatin-1(T2376), Deferoxamine(DFO, T1637), Z-VAD-FMK(T6013), Necrosulfonamide(T7129) and 5-FU (T0984) were obtained from TargetMol (Shanghai, China). Bafilomycin A1(S1413) and Erastin(S7242) were obtained from Selleck (Houston, TX, USA). The Synthetic methods and Routes of W1131 are described in detail in Supplemental Information.

2.3. Immunohistochemistry

For immunohistochemistry (IHC) analysis, gastric cancer specimens tissue slides were deparaffinized, rehydrated through an alcohol series followed by antigen retrieval with sodium citrate buffer. Tumor sections were blocked with 5% normal goat serum with 0.1% Triton X-100 and then incubated with appropriate primary antibodies at 4 °C overnight. IHC staining was performed with horseradish peroxidase (HRP) conjugates using DAB detection. Nuclei were counterstained with Hoechst. Images were taken with Nikon microscopy.

2.4. Cell viability, colony formation, EDU proliferation, invasion, and apoptosis assays

The cell viability assay was measured using the Cell Counting Kit-8 (CCK-8, B34304, Bimake, USA), AGS, MGC803, HGC27, RWPE-1, and MGC803/5-FU cells were plated into a 96-well plate and cultured overnight. Then, W1131 at different concentration was added to each well, and incubation was continued for another 72 h. Next, 10 µL of CCK-8 solution was added to each well, subsequently, cells were incubated for around 4 h until the color of cells turned orange. The absorbance (OD) was measured by a microplate reader (FLUOstar Omega-ACU, USA) at test and reference wavelengths of 450 nm. The percentage of growth was calculated as Cell viability (%) = [OD (Compound +)] – [OD (Blank)]/[OD (Compound -) – OD (Blank)] × 100%. Each experiment was repeated in triplicate independently.

The colony formation assay was performed to examine the effect of
Table 1
The sequences of two sense strands of siRNA targeting STAT3.

| Name     | Sequences                                           |
|----------|-----------------------------------------------------|
| siSTAT3#1| Sense: 5'-UCCAGUUUCUAAUUUUUUGGGUC-3'                 |
|          | Antisense: 5'-GACCCGCUAACAAUUAAGGAAGGA-3'            |
| siSTAT3#2| Sense: 5'-AUAGGUCUAAUUCUAIUUGGAUUGCUA-3'             |
|          | Antisense: 5'-UGACAUCCAAUAGGAAAGCGCUAU-3'            |

W1131 on cell colony survival. AGS, MGC803 cells were seeded into 6-well plates and cultured overnight. Different concentrations of W1131 were added to each well. After that, the cell culture medium was changed and maintained with the same dose of compounds or DMSO every 3 days until the colonies were visible. The cells were fixed using 4% paraformaldehyde and stained with crystal violet staining solution in around 10–15 days.

The EDU proliferation assay was performed using the BeyoClick EdU-488 cell proliferation kit (C0075S, Beyotime, Shanghai, China). AGS and MGC803 cells were seeded into a 12-well plate with a corresponding concentration of EDU reagent for 3 h. Cells were washed with PBS for 5 min twice, before incubating with 4% paraformaldehyde for 30 min. Then, samples were permeated with 0.3% TritonX-100 in PBS and dyed with the reaction solution. A fluorescence microscope was used to capture the image.

The cell invasion assay was performed in 24-well Transwell® plates (3422-ND, Costar, USA). AGS and MGC803 cells were seeded in the top chamber of the insert with RPMI-1640 culture medium containing 2% FBS and cultured overnight, while the bottom chamber was filled with 500 μL of the same medium. The cells were treated with W1131 or DMSO for 12 h and the medium of the bottom chamber was changed 2% FBS into 20% FBS. Then, the cells in the top chamber were carefully removed by cotton and then washed with PBS twice, and the invaded cells in the bottom chamber were fixed with 4% paraformaldehyde and stained with crystal violet staining solution. Finally, the invaded cells were photographed and calculated. Three representative fields were captured for each condition. All experiments were set in triplicate.

The cell apoptosis assay was detected using an Annexin V-FITC Apoptosis Detection Kit I (BB-4101, BestBio, Shanghai, China) following the manufacturer’s protocols. Briefly, the cells were washed twice with cold PBS after trypsinization and washed once with the medium. The precipitation was resuspended by 400 μL of 1× binding buffer, and then resuspended cells were transferred into 1.5 mL tubes. Then 3 μL of Annexin V-FITC and 5 μL of propidium iodide were added to the resuspended cells with further incubation at room temperature for 15 min in the dark. The analysis was conducted by Guava easyCyte (USA) and FlowJo 7.6 software.

2.5. Plasmid constructions and cell transfection

For overexpression of STAT3, STAT3 cdNA(NM_139276.3) was cloned into vector pLVX-FLAG-puro. For RNA interference, gastric cancer cells with 80% confluence in 6-well plates were transfected with control siRNA or STAT3 siRNA using Dharmatectm (T-2001-03, Dharmacon, USA) according to the manufacturer’s protocol. A nonspecific oligonucleotide without complementary to any human gene was used as a negative control. The sequences of two sense strands of siRNA targeting STAT3 are listed in Table 1. All above siRNAs were synthesized by Sangon Biotech (Shanghai, China).

2.6. Western blotting and antibodies

Proteins were lysed from cells or tumor tissues using RIPA buffer (Beyotime, Shanghai, China) containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Na-deoxycholate, 1 mM EDTA, 0.1% SDS, and supplemented with protease inhibitors (Beyotime, Shanghai, China) and phosphatase inhibitors (Bimake, USA). Protein concentration was determined using Pierce™ BCA Protein Assay Kit (23225, Thermo, USA) before proteins were equally loaded and separated by polyacrylamide gel. Proteins were then transferred to polyvinylidene fluoride membranes (PVDF, IPVH00010, Millipore, USA) and incubated overnight with primary antibodies against β-Actin(sc-47778, Santa Cruz, USA), STAT3 (10253-2-AP, Proteintech, Wuhan, Hubei, China), Bcl-xl(sc-8392, Santa Cruz, USA), Phospho-STAT3(Tyr705) (#9145, Cell Signaling Technology, USA), c-Myc (#5605, Cell Signaling Technology, USA), Mad-1 (#4572, Cell Signaling Technology, USA), STAT1(10144-2-AP, Proteintech, Wuhan, Hubei, China), Phospho-STAT1(Tyr701)(#9167, Cell Signaling Technology, USA), STAT5(52566, Cell Signaling Technology, USA), Phospho-STAT5 (Y694)(#9351, Cell Signaling Technology, USA), JAK2 (#3320, Cell Signaling Technology, USA), Phospho-JAK2(Tyr1007/1008, USA) (#3771, Cell Signaling Technology, USA), AKT (#4685, Cell Signaling Technology, USA), Phospho-AKT(Ser473)(#4060, Cell Signaling Technology, USA), Flag-tag(66008-3-Ig, Proteintech, Wuhan, Hubei, China), HA-tag (#3742, Cell Signaling Technology, USA), GPX4(ab125066, abcam, UK), SLC7A11(A13685, Abclonal, Wuhan, Hubei, China), TTC11(A1144, Abclonal, Wuhan, Hubei, China), MLKL(ab187091, abcam, UK), SLC7A11(A13685, Abclonal, Wuhan, Hubei, China), 4-HNE(ab48506, abcam, UK), p53(#2524, Cell Signaling Technology, USA), NFR2(66504-1-Ig, Proteintech, Wuhan, Hubei, China), AC804(ab155282, abcam, UK), CathepsinB(12216-1-AP, Proteintech, Wuhan, Hubei, China), MLKL(ab187418, abcam, UK), p358-MLKL(ab187091, abcam, UK), LC-3(14600-1-AP, Proteintech, Wuhan, Hubei, China), PARP-1(#9532, Cell Signaling Technology, USA), Cleaved Caspase-3(#9664, Cell Signaling Technology, USA), HRP-conjugated secondary antibodies were used and the signal was detected on the Bio-rad chemidoc MP system after incubating with ECL solution.

2.7. RT-PCR

The cDNA was prepared using HiFiAir™ II 1st Strand cDNA Synthesis SuperMix Kit (11123ES60, YEASEN, Shangh hai, China) according to the manufacturer’s protocol. PCR was performed by a BIO-RAD CFX96™ (Bio-rad, San Diego, USA) in the presence of SYBR Green (11201ES08*, YEASEN, Shanghai, China). A melting-curve analysis was performed after the fluorescence values were collected. The sequences of primers for the qRT-PCR analysis are listed in Table S1. All the above primers were synthesized by Sangon Biotech (Shanghai, China).

2.8. LDH release assay

The LDH release assay was measured using the LDH Release Assay Kit (C0017, Beyotime, Shanghai, China) according to the manufacturer’s instructions. The samples were prepared and the OD value was measured at 490 nm.

2.9. Transmission electron microscopy

Cells are collected after centrifuge and the precipitation. Then cells were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), followed by the fixation with 1% OsO4. After dehydration, 60–80 nm thin sections were prepared and stained with uranyl acetate and plumbous nitrate before observation under a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan). High-resolution digital images were acquired from randomly selected five different fields at each condition.

2.10. MDA assay

MDA assay was conducted using the Lipid Peroxidation MDA Assay Kit (S0131, Beyotime, Shanghai, China) according to the manufacturer’s instructions. The samples and standards were prepared and the OD value was measured at 532 nm. MDA concentrations (nmol/ml) were expressed as μmol/mg protein.
2.11. GSH/GSSG assay

The levels of GSH (reduced glutathione)/GSSG (oxidized glutathione disulfide) were measured using a GSH/GSSG ratio detection assay kit (ab205811, abcam, UK) according to the manufacturer’s instructions.

2.12. FerroOrange assay

AGS and MGC803 cells were seeded into 15 mm glass-bottom dishes and incubated overnight for detection by confocal microscopy. The cells were plated in a 6-well cell culture plate detection by flow cytometry. Subsequently, cells were pretreated with ferroptosis inhibitors for 1 h and then treated with W1131 or vehicle (DMSO) for 24 h. Plates were washed thrice in HBSS. Next, cells were stained in 1 μM/L Ferroorange (F374, DOJINGO, Japan) in HBSS for 30 min at 37 °C incubator with 5% CO2 and imaged immediately. Treatments were staggered to ensure precise staining duration. Then, images were captured by Laser Scanning Confocal Microscope FV3000 (Olympus, Japan). Five representative fields were captured for each condition under identical exposure times. And Fluorescence intensity was detected by flow cytometry. Data were collected from at least 10,000 cells per sample.

2.13. Liperfluo assay

AGS and MGC803 cells were seeded into 15 mm glass-bottom cell culture dishes and incubated overnight. Then, cells were treated with W1131 or vehicle (DMSO) for 24 h. Cells were stained in 10 μM Liperfluo (L248, DOJINGO, Japan) in RPMI-1640 medium for 30 min at 37 °C incubator with 5% CO2 and imaged immediately. Treatments were staggered to ensure precise staining duration. Images were captured by Laser Scanning Confocal Microscope FV3000 (Olympus, Japan). Five representative fields were captured for each condition under identical exposure times.

2.14. BODIPY C11 assay

Cells were pretreated with ferroptosis inhibitors for 1 h and then treated with W1131 or vehicle (DMSO) for 24 h. Then, cells were incubated with 5 μM C11-BODIPY 581/591 (Invitrogen, Carlsbad, CA, USA) in a serum-free medium at 37 °C for 30 min in the dark. Cells then were washed with HBSS. Lipid ROS levels were analyzed on a flow cytometer using a 617 nm filter for C11-BODIPY 581/591 detection. Data were collected from at least 10,000 cells per sample.

2.15. Molecular docking

Briefly, the crystal structure of STAT3 (PDB code: 6NUQ) was downloaded from Protein Data Bank (http://www.pdb.org). Maestro 11.1 software was employed to dock. Schrödinger’s Protein Preparation Wizard was used to prepare the protein structure and Schrödinger’s LigPrep was used to prepare molecules for docking. Schrödinger’s Receptor Grid Generation was used in the generation of grid files. The grid box was prepared at its SH2 domain, and Schrödinger’s Ligand Docking was used for docking of the protein structure and ligand. Protein was considered rigid and small molecules were flexible during the docking process. The XP extra precision was chosen as the vital docking parameter.

2.16. Cellular thermal shift assay

To determine target engagement of STAT3 by W1131 within cells, AGS cells with 70%-80% confluence in 15 cm culture dish were treated with W1131 or vehicle (DMSO) for 1 h. Cells were harvested and washed once with PBS, then suspended in 1 ml of PBS supplemented with proteinase and phosphatase inhibitors (Beyotime, Shanghai, China) and also maintained with the same dose of W1131 or DMSO as initial treatment. The cell suspension was distributed into seven 0.2 mL PCR tubes at different designated temperature. Samples were heated at different designated temperature for 2 min using a 96-well thermal cycler. Tubes were removed and incubated at room temperature for 3 min immediately after heating. Three freeze and thaw cycles in liquid nitrogen were performed to lyse the cells. The tubes were vortexed briefly after each thawing. The cell lysate was collected and cell debris together with precipitated and aggregated proteins was removed by centrifuging samples at 20,000 × g for 20 min at 4 °C. Cell lysate samples were boiled for 5 min at 95 °C with loading buffer and subjected to western blotting analysis.

2.17. Surface plasmon resonance analysis

The binding affinity between W1131 and STAT3 protein was analyzed by Biacore 8K and Biacore Insight Evaluation software. Purified STAT3 protein (0.17 mg/mL) was dissolved in PBS and immobilized onto the CMS chip (GE Healthcare, USA). Several concentrations of compound dissolved in running buffer (1 × PBS with filtration, 2% DMSO) were flown over the chip to produce response signals. The kinetics and affinities were calculated by the Biacore Insight Evaluation software, and the results were determined as the binding affinity (Kd).

2.18. Immunoprecipitation

For co-immunoprecipitation experiments, 293T cells were co-transfected with plasmids of HA-STAT3 and Flag-STAT3 using Lipofectamine 2000 (1168019, Invitrogen, Carlsbad, CA, USA). Cells were treated with different concentrations of W1131 for 24 h and stimulated with IL-6 (100 ng/mL) for 1 h. Cells were lysed using RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. The cell lysates were incubated with Anti-flag Affinity Gel (B23102, Bimake, USA) overnight at 4 °C. The gel was washed by PBS three times and denatured by heating for 5 min at 95 °C with 1 × loading buffer. Then the proteins were resolved on SDS-PAGE, transferred to PVDF membranes, and analyzed with immunoblotting.

2.19. Nuclear translocation

Stomach cancer cells were seeded into 15 mm glass-bottom dishes and cultured overnight. Cells were treated with compounds or vehicle (DMSO) for 12 h. The cells were added 100 ng/mL IL-6 (PeproTech, USA) for 30 min before fixation. Then, cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Plates were washed three times with PBS. Then cells were permeabilized with 0.3% Triton X-100 for 15 min at room temperature, and plates were washed thrice by PBS. The samples were blocked with goat serum (Boster, Wuhan, Hubei, China) for 1 h at room temperature, and then, incubated with Phospho-STAT3 (Tyr705) (#9145, Cell Signaling Technology, USA, 1:100) primary antibody overnight at 4 °C. Plates were washed thrice in PBS. Next, cells were incubated with Alexa Fluor 488 Conjugate (#4412, Cell signaling Technology, USA, 1:5000) for 1 h in the dark. Plates were washed thrice in PBS. Then, the cells were subsequently stained with DAPI (40728ESS0, Yeasen) for 10 min. Images were captured by Laser Scanning Confocal Microscope FV3000 (Olympus, Japan). Five representative fields were captured for each condition under the same exposure time.

2.20. Transient transfection and luciferase assays

Transient transfections were performed using Lipofectamine 2000 (1168019, Invitrogen, Carlsbad, CA, USA). The STAT3-dependent luciferase reporter, pGL3-STAT3, contained seven copies of the STAT3-specific binding sequence (AATCCCAGAA) in the C-reactive protein gene promoter. For luciferase reporter gene assays, 293T cells were co-transfected with pGL3-STAT3 (50 ng per well) and STAT3C (50 ng per well).
with default parameters. RSeQC (version 2.6.1) was used to analyze the alignment results. Gene expression values of the transcripts were assessed using a NanoPhotometer® manufacturer’s VAHTSTM mRNA-seq V2 Library Prep Kit for Illumina® (IMPLEN, CA, USA). The high-quality RNA samples were subsequently submitted to the Sangon Biotech (Shanghai, China) for library preparation.

Total RNA was extracted by TRizol (ThermoFisher, USA) from AGS cells treated with W1131 or vehicle (DMSO) for 48 h and RNase-free DNase I to remove genomic DNA contamination. RNA integrity was evaluated with a 1.0% agarose gel. Thereafter, the quality and quantity of RNA were assessed using a NanoPhotometer® (IMPLEN, CA, USA). FastQC (version 2.6.1) was used to analyze the quality of sequence data. Clean reads were mapped to the reference genome by HISAT2 (version 2.0) with default parameters. RSeQC (version 2.6.1) was used to analyze the alignment results. Gene expression values of the transcripts were computed by StringTie (version 1.3.3b). Library preparation and high-throughput sequencing were performed by Sangon Biotech (Shanghai, China).

2.2.2. Reporter constructs and reporter-gene assays

GPX4 reporter-gene assays were performed by transfecting 293T cells with pGL3-GPX4, STAT3C, and pCMV-β-gal for normalization. The FTH1 mutant form (FTH1 Mut) contains sequences mutated from CAGCCGAGAAG to CAGCCGAGGGG. Briefly, 293T cells seeded in 96-well plates in phenol red-free DMEM medium were transfected with Lipofectamine 2000 (1168019, Invitrogen, Carlsbad, CA, USA) and the indicated plasmid DNA. 24 h after transfection, transfected cells were treated with W1131 for another 24 h before being harvested for β-galactosidase and luciferase assays. The luciferase and β-galactosidase were then analyzed with a Luciferase Assay Substrate (Promega) and Luminescent β-galactosidase Detection Kit II (Clontech). The primer sequences are listed in Table 2.

Table 2
The sequences of GPX4, SLC7A11, and FTH1 genes in reporter-gene assay.

| Gene           | Primer sequence                                                |
|----------------|----------------------------------------------------------------|
| GPX4 promoter-WT | F: 5'-CTTGGAGGAGGACAAATACACATCCCAAGACGC-3'                      |
| GPX4 promoter-Mut| R: 5'-CAGCCGATTGCTGTTTACCATCGT-3'                               |
| SLC7A11 promoter-WT | F: 5'-GGGTCACCTGACTGTGTTAGCTC-3'                               |
| SLC7A11 promoter-Mut | R: 5'-CCCTCTGCTGAGGATGGTTAGCTC-3'                               |
| FTH1 promoter-WT  | F: 5'-GGGTCACCTGACTGTGTTAGCTC-3'                               |
| FTH1 promoter-Mut | R: 5'-CCCTCTGCTGAGGATGGTTAGCTC-3'                               |

Table 3
The sequences of GPX4, SLC7A11, and FTH1 genes in ChIP-qPCR analysis.

| Gene          | Primer sequence                                                |
|---------------|----------------------------------------------------------------|
| GPX4 promoter | F: 5'-ATTCACAAAACCTCCCTGTGTA-3'                               |
| SLC7A11 promoter | R: 5'-GGGTCACCTGACTGTGTTAGCTC-3'                               |
| FTH1 promoter | F: 5'-GGGTCACCTGACTGTGTTAGCTC-3'                               |

2.23. ChIP-qPCR analysis

AGS cells treated with W1131 or vehicle (DMSO) for 48 h, were then fixed with 1% formaldehyde at room temperature for 10 min and washed with ice-cold PBS. Cells were scraped off in buffer I (0.25% Triton X-100/0.1 mM EDTA/0.5 mM EGTA/10 mM Hepes, pH 6.5). Cell pellets were collected by centrifugation and washed in buffer II (200 mM NaCl/1 mM EDTA/0.5 mM EGTA/10 mM Hepes, pH 6.5). Cell pellets were resuspended in 1 ml of lysis buffer [0.5% SDS/10 mM EDTA/50 mM Tris, pH 8.1/1 × protease inhibitor cocktail (Roche Molecular Biochemicals/1 mg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride) and sonicated four times for a 30-s interval of 0.5-s pulses (Fisher, model 550 Sonic Dismembrator). Cell debris was removed by centrifugation, and the chromatin solutions were diluted 5 × with dilution buffer (1% Triton X-100/2 mM EDTA/150 mM NaCl/20 mM Tris, pH 8.1/1 × protease inhibitor cocktail). Chromatin fragments were immunoprecipitated with specific antibodies overnight at 4 °C. For a 5-ml diluted chromatin solution, the following amounts of antibodies were used: 1 μl of IgG (Millipore, USA) and 2 μg of STAT3 (Proteintech, Wuhan, Hubei, China). Dynabeads TM protein G (1004D, Invitrogen, USA) beads were preincubated with Chromatin solution overnight in dilution buffer and washed three times in dilution buffer before using. Immunocomplexes were recovered and eluted. The DNA fragments were purified with a GencJET Gel Extraction Kit (Thermo, USA) after reverse cross-linking at 65 °C overnight. The immunoprecipitated DNA was analyzed by real-time PCR with SYBR Green on an iCycler instrument. Enrichment of genomic DNA was presented as the percent recovery relative to the input.

The primers are listed in Table 3. All the above primers were synthesized by Sangon Biotech (Shanghai, China).

2.24. Animal experiments

The animal procedures were approved by the Research Ethics Committee of Sun Yat-sen University (SYSU-IACUC-2020-000270; SYSU-IACUC-2021-000139; SYSU-IACUC-2021000149, and SYSU-IACUC-2022-000448) and conducted following the Guide for the Care and Use of Laboratory.

For the MGC803 cell subcutaneous xenograft model, four-week-old BALB/c-nu/nu mice (male, weighing 18–19 g, SPF grade, certification No. SCXK (Nanjing) 2018-0008) were achieved from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Briefly, 2 × 10⁶ MGC803 cells were suspended in a total of 100 μl PBS and matrigel (1:1, v/v) and implanted subcutaneously into the dorsal flank of the mice. When the tumor volume was reached about 50 mm³, the mice were grouped randomly. Then mice were divided into three groups (n = 6) randomly and treated intraperitoneally (i.p.) with 100 μl of either vehicle, W1131 (3 mg/kg), and W1131 (10 mg/kg) for once a day. For gastric cancer PDX tumor model, four-week-old BALB/c-nu/nu mice (male, weighing 16–18 g, SPF grade, certification No. SCXK (Guangzhou) 2016-0029) were achieved from the Experimental Animal Center of Sun Yat-sen University (Guangdong, China). The characteristics of this patient are as follows: male, 42 years, Asian, primary gastric cancer tumor, AJCC IB/grade 3, surgical sample. The PDX tumors were transfecting 293T cells with pGL3-FTH1, STAT3C, and pCMV-β-gal for normalization. The FTH1 mutant form (FTH1 Mut) contains sequences mutated from CAGCCGAGAAG to CAGCCGAGGGG. Briefly, 293T cells seeded in 96-well plates in phenol red-free DMEM medium were transfected with Lipofectamine 2000 (1168019, Invitrogen, Carlsbad, CA, USA) and the indicated plasmid DNA. 24 h after transfection, transfected cells were treated with W1131 for another 24 h before being harvested for β-galactosidase and luciferase assays. The luciferase and β-galactosidase were then analyzed with a Luciferase Assay Substrate (Promega) and Luminescent β-galactosidase Detection Kit II (Clontech). The primer sequences are listed in Table 2.
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propagated in the dorsal flank on both sides of the mice. When the tumor volume was reached about 50 mm³, the mice were grouped randomly. Then mice were divided into four groups (n = 5) randomly and treated intraperitoneally (i.p.) with either vehicle, W1131 alone, ferrostatin-1 alone, or ferrostatin-1 and W1131 together.

For MGC803/5-FU cell subcutaneous xenograft model, four-week-old BALB/c-nu/nu mice (male, weighing 16–18 g, SPF grade, certification No. SCXK (Nanjing) 2018-0008) were achieved from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Briefly, 2 × 10⁶ MGC803/5-FU cells were suspended in a total of 100 µL PBS and matrigel (1:1, v/v), and implanted subcutaneously into the dorsal flank of the mice. When the tumor volume was reached about 50 mm³, the mice were grouped randomly. Then mice were divided into three groups (n = 6) randomly and treated intraperitoneally (i.p.) with 100 µL of either vehicle, 5-FU alone, W1131 alone, erastin alone, or 5-FU in combination with erastin or W1131.

For 5-FU-resistant PDX model, four-week-old NCG mice (male, weighing 16–18 g, SPF grade, certification No. SCXK (Nanjing) 2020-0054) were achieved from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Briefly, the PDX tumors were propagated in the dorsal flank on both sides of the mice. When the tumor volume was reached about 50 mm³, the mice were grouped randomly. Then mice were divided into two groups (n = 5) randomly and treated intraperitoneally (i.p.) with either vehicle or 5-FU.

Tumor volume and body weight were measured two times per week. The volume was calculated with Eq: \( V = \pi r^2 h \), where \( r \) is the radius and \( h \) is the height. The mice were sacrificed at the end of the studies. Tumors were harvested, weighed, and analyzed by immunohistochemistry or immunoblotting assays.

2.25. Organoid culture

Organoids were cultured from PDX xenografts when the tumor size reached ~500 mm³. Briefly, dissected tumors were finely minced and transferred to a 50 mL centrifuge tube, including a digestion mix consisting of Ad-DMEM/F-12 medium ( Gibco, USA) and 1 mg/mL collagenase IV (Sigma, USA), and incubated for 40 min at 37 °C. Isolated organoids were mixed with 5 µL of Matrigel (Costar, USA) and seeded in 96-well plates (Costar, USA). The culture medium contains Ad-DMEM/F-12 with B27 supplement (1 × ), nicotineamide (10 mM), N-acetyl-L-cysteine (1.25 mM), EGF (5 ng/mL), A83-01 (500 nM), SB202190 (10 μM), Y-27632 (10 μM), Noggin (100 ng/mL), R-Spondin 3 (250 ng/mL), FGF2 (5 ng/mL), FGF 10 (10 ng/mL), penicillin/streptomycin (1 × ) and Glutamine (1 × ). Supplementated culture medium (100 µL) was added per well, and organoids were maintained in a 37 °C humidified atmosphere under 5% CO₂.

2.26. Organoid viability

Organoids were seeded into 96-well plates at 300–500 organoids in 5 µL of Matrigel per well in a total volume of 100 µL of the medium. Serially diluted compounds in 100 µL of medium were added to the cells 24 h later. After 4 days of incubation, Cell-Titer Glo reagents (Promega, USA) were added, and luminescence was measured. After 4 days of incubation, the medium was carefully aspirated and 100 µL of live/dead reagents (US EVERBRIGHT) was added followed by 30 min of incubation at room temperature. A fluorescence microscope was used to capture images of calcein AM (494/517 nm) to represent the live cells, of PI (535/617 nm) to identify the dead cells. The above assays were performed in triplicates.

2.27. Bioinformatics analysis

The gene expression profile datasets GSE13911 and GSE27342 were downloaded from Gene Expression Omnibus (GEO) database. The RNA Seq data of gastric cancer tissue in TCGA database and normal gastric tissue or adjacent gastric tissue in GTEx database were downloaded from the UCSC Xena website (http://xena.ucsc.edu/) and subsequently analyzed by R (Version 3.4, http://www.biocoductor.org) with edgeR package using GSEA method. GSEA analysis was performed using the Java desktop software (http://software.broadinstitute.org/gsea/index.jsp). Genes were ranked according to the shrunken limma log2 fold changes, and the GSEA tool was used in ‘pre-ranked’ mode with all default parameters. Bubble chart and volcano plot analysis was performed using the OmicShare tools, an online platform for data analysis (http://www.omicshare.com/tools). A web server for cancer and normal gene expression profiling and interactive analyses, GEPIA (http://gepia.cancer-pku.cn/index.html) and Kaplan-Meier Plotter (https://kmplot.com/analysis/) was recruited to determine the expression of related genes in gastric cancer and the clinical survival of the related genes. The online database of R2: Genomics Analysis and Visualization Platform (https://kmserver1.smc.nl) was applied to determine the correlation between STAT3 and related genes. The open-access database of transcription factor binding profiles JASPAR 2020 (http://jaspar.genereg.net/) was recruited to predict related motifs.

3. Results

3.1. Ferroptosis negative regulation (FNR) signatures are associated with progression and chemoresistance of gastric cancer

We interrogated the TCGA dataset and GTEx dataset (407 gastric cancer tumor tissues and 147 normal gastric tissues or tumor-adjacent tissues) to explore major cancer pathways that are differentially altered in gastric cancer. The analysis showed that the expression of genes in the IL6-JAK-STAT3 signaling pathway and ferroptosis circuit
displayed a significantly different profile in tumor tissues compared with adjacent normal tissues (Fig. 1A). Ferroptosis-related genes are categorized into ferroptosis positive regulation (FPR) signatures that promote ferroptosis, and ferroptosis negative regulation (FNR) signatures that suppress ferroptosis in the FerrDb database. Given the important role of ferroptosis in gastric cancer has remained largely unexplored, we further explored the TCGA and GTEx database and found that the FNR signatures were highly elevated in gastric cancer (Fig. 1B) and significantly associated with low survival rates in gastric cancer patients (Fig. 1C), not FPR or entirety of ferroptosis genes (Figs. S1B and C). Among FNR signatures, GPX4 is a central repressor of ferritin, which prevents Fe from being oxidized by ROS [31]. IHC protein adducts did not increase after long-term treatment of 5-FU with an IC50 of 5.045 μM in MGC803 cells (Fig. S1J). We further detected the level of 4-hydroxynonenal (4-HNE) protein to evaluate the effect of long-term treatment of 5-FU on ferroptosis in vivo. The results demonstrated that 4-HNE protein adducts did not increase after long-term treatment of 5-FU in vivo (Fig. 1F), thus 5-FU may not be a direct ferroptosis inducer even in long-term administration. Together, these results strongly support that the FNR signature is closely related to the development and 5-FU resistance of gastric cancer.

3.2. STAT3 mediates ferroptosis through transcriptional regulation of FNR signatures in gastric cancer

As shown in Fig. 1A, the genes in the IL6-JAK-STAT3 signaling pathway were also highly expressed in tumor tissue of gastric cancer patients. To further explore the potential oncogenic role of STAT3 in gastric cancer, IHC analysis of 30 clinical human gastric tumors and corresponding adjacent normal gastric tissues revealed that pY705-STAT3 is highly expressed in gastric tumor tissue (Fig. 2A and B) and associated with the stages of gastric cancer (Fig. 2C). STAT3 mRNA level is also highly expressed in gastric tumor tissue (Fig. S2A) and associated with the stages of gastric cancer (Fig. S2B) by analysis of the GEO database. Additionally, Kaplan–Meier analysis indicated that gastric cancer patients with higher levels of STAT3 presented worse overall survival (Fig. S2C). Moreover, the expression of STAT3 is positively associated with FNR signatures in gastric cancer tumors (Fig. 2D).

To explore the role of STAT3 in ferroptosis, we knocked down STAT3 and performed transcriptome analysis. Gene-set enrichment analysis (GSEA) showed that FNR signatures were mediated by STAT3 knockdown (Fig. 2E). Furthermore, IHC analysis of gastric cancer tissues also indicated significant positive correlations between pY705-STAT3 and
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Fig. 3. STAT3 inhibition triggers iron-dependent oxidative damage in ferroptosis and suppresses gastric cancer proliferation and survival. A: Gastric cancer cells (AGS and MGC803) were transfected with siRNAs against STAT3 (siSTAT3#1 and siSTAT3#2) or control siRNA (siCont). Viable cells were counted at indicated time points. B: Gastric cancer cells were infected as in (A). Fourteen days later, colonies were counted. C: Gastric cancer cells were infected as in (A). Cell proliferation was determined by Edu cell proliferation assay. Quantification of the signal was shown on the left. Scale bars = 200 μm. D: Electron micrographs of STAT3 knockdown in MGC803 cells for 48 h. Scale bars = 2 μm; Scale bars = 1 μm; from left to right, respectively. E: AGS and MGC803 cells were transfected with siRNAs against STAT3 (siSTAT3#1 and siSTAT3#2) or control siRNA (siCont) for 48 h, and then intracellular MDA were assayed. F: AGS and MGC803 cells were transfected with siRNAs against STAT3 (siSTAT3#1 and siSTAT3#2) or control siRNA (siCont) for 48 h, and then GSH/GSSG ratio were assayed. G: C11-BODIPY 581/591 probe was used to detected lipid peroxidation level in AGS cells transfected with siRNAs against STAT3 (siSTAT3#1 and siSTAT3#2) or control siRNA (siCont) for 48 h by flow cytometry. Quantification of C11-BODIPY 581/591 (FL1) fluorescence was shown at the bottom. H: FerroOrange probe was used to detected intracellular Fe\(^{2+}\) level in AGS cells transfected with siRNAs against STAT3 (siSTAT3#1 and siSTAT3#2) or control siRNA (siCont) for 48 h by flow cytometry. Quantification of FerroOrange (PE) fluorescence was shown at the bottom. I: Liperfluoro and FerroOrange staining for intracellular lipid ROS and Fe\(^{2+}\) in MGC803 cells transfected with siRNAs against STAT3 (siSTAT3#1 and siSTAT3#2) or control siRNA (siCont) for 48 h. Shown is one of five representative fields illustrating fluorescence intensity taken at identical exposures for each condition. Scale bars = 50 μm. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n = 3. Student’s t-test. All data were shown as means ± s.d.

GPX4/FTH1, which are key regulators of FNR signature (Fig. 2G). A similar positive correlation between STAT3 and GPX4/FTH1 was also observed through the analysis of the GEO database (Fig. 2F).

Next, we examined whether STAT3 regulates these FNR signatures genes in gastric cancer cells. We performed RT-PCR analysis and found that GPX4, SLC7A11, and FTH1 were significantly down-regulated by STAT3 knockdown in gastric cancer cells (Fig. 2H). Among them, SLC7A11 is a subunit of the cystine/glutamate transporter, which is called system X\(_c\). X\(_c\)-1 imports cystine into cells and converts it to cysteine, which is used to synthesize GSH [33,34]. Recent studies showed that inhibition of SLC7A11 or GPX4 causes lipid peroxidation and leads to ferroptosis in mammalian cells or tissues [30,35,36]. Moreover, the protein expression of GPX4, SLC7A11, and FTH1 were also inhibited by STAT3 inhibition (Fig. 2I). Therefore, knockdown of STAT3 significantly inhibited GPX4, SLC7A11, and FTH1 at both mRNA and protein levels (Fig. 2H and I). While overexpression of STAT3 promoted the expression of GPX4, SLC7A11, and FTH1 in gastric cancer cells (Fig. 2J). These results imply that the expression of GPX4, SLC7A11, and FTH1 may be regulated by STAT3 in gastric cancer cells.

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The previous reported STAT3 ChIP-seq analysis suggested the potential links between STAT3 and GPX4, SLC7A11, and FTH1 genes [37] (Fig. 2K). To assess the regulation of STAT3 on these FNR genes, we performed Chp-qPCR analysis and found that STAT3 is bound to the promoter of these three genes in gastric cancer cells (Fig. 2L). Then, we cloned the promoter of GPX4, SLC7A11, and FTH1 into a dual-luciferase reporter construct, respectively, and performed luciferase reporter-gene assays (Fig. 2M). We found that these three genes were highly responsive to STAT3-mediated transactivation (Fig. 2N). Finally, we generated the mutant form of GPX4, SLC7A11, and FTH1 promoter by replacing AA with GG in the core motif of human STAT3. Mutations of the putative STAT3 effectively diminished the STAT3-dependent activation (Fig 2N). In addition, it was reported that STAT3 may involve ferroptosis by ACSL4 [28], CathepsinB [25], and STAT3/P53/SLC7A11 pathway [38], we conducted experiments to check these potential mechanisms (Figs. S6A–C). Taken together, these data suggested that STAT3 mediated ferroptosis through transcriptional regulation of FNR signature genes (GPX4, SLC7A11, and FTH1) in gastric cancer.

3.3. STAT3 inhibition triggers iron-dependent oxidative damage in ferroptosis and inhibits gastric cancer cell proliferation and survival

Then, we examined whether STAT3 inhibition contributes to ferroptosis in gastric cancer cells. We knocked down STAT3 in human AGS and MGC803 cells with hyperactivated STAT3 and the knockdown was confirmed by both mRNA and protein levels (Figs. S2D and E). The effects of STAT3 inhibition on cell growth, survival, proliferation, and invasion of gastric cancer cells were also examined (Fig. 3A–C and S2F).

Next, we utilized transmission electron microscopy (TEM) to examine the morphological changes in gastric cancer cells transfected with STAT3 siRNAs. The cells which were knocked down of STAT3 exhibited shrunken mitochondria with increased membrane density, which is a characteristypic morphologic feature of ferroptosis (Fig. 3D). And knockdown of STAT3 upregulated the level of GSH (malondialdehyde), which is the end product of lipid peroxidation (Fig. 3E). Liperfluoro is a lipophilic ROS sensor that provides a rapid, indirect approach to detect lipid ROS [5,6]. The FerroOrange probe is used to detect intracellular Fe\(^{2+}\) levels. The results revealed that STAT3 knockdown resulted in increased lipid ROS and enhanced intracellular Fe\(^{2+}\) levels in gastric cancer cells by measuring the fluorescence in a confocal microscope (Fig. 3F). To further confirm these effects, we also used flow cytometry to determine the signal of lipid ROS and Fe\(^{2+}\). We found that STAT3 knockdown caused an increase of lipid peroxidation level using the C11-BODIPY probe, which is another probe commonly used to measure lipid ROS (Fig. 3G). Similarly, an increase in Fe\(^{2+}\) was also observed in flow cytometry analysis (Fig. 3H). Meanwhile, the knockdown of STAT3 reduced GSH/GSSG ratio in gastric cancer cells, which is considered as a readout for intracellular oxidative damage (Fig. 3F). These results suggested that inhibition of STAT3 could trigger ferroptosis in gastric cancer cells.

3.4. Discovery of a novel and potent STAT3 inhibitor W1131

The above results suggested that STAT3 may serve as a negative regulator of ferroptosis. Therefore, a novel STAT3 inhibitor can act as a ferroptosis-inducing compound for gastric cancer. To this end, we identified a series of compounds based on the privileged structure of 2-phenylimidazo [1,2-a] pyridine for potent and selective STAT3 inhibitors. The detailed structure-activity relationship studies will be reported in a separate publication. W1131 was selected for further study in this article (Fig. 4A and Figs. S3A–B). To determine the potential binding mode of W1131 with STAT3, we performed dock studies using the STAT3 crystal structure (PDB: 6NUQ). The results indicated that the binding mode of W1131 with STAT3 (Fig. 4B and Fig. S3C) is similar to that of compound SI-109 [39] (Fig. S3D). The central moiety of W1131 is located at the ligand-binding pocket. The nitrofuran is bound to the pY705 binding site, in which nitro group forms two hydrogen bonds with the Ser611 and Ser613 residue. In addition, the 1,2,3,6-tetrahydropyridine group targets the Leu706 sub pocket and forms a hydrogen bond with Lys658 residue. We then performed surface plasmon resonance (SPR) assays to determine affinity between W1131 and STAT3 protein, with a K\(_d\) at 7.55 × 10\(^{-6}\) mol/L (Fig. 4C). To further evaluate W1131 target engagement, we performed the cellular thermal shift assay (CETSA). Drug-protein interaction was examined in the native cellular environment basing on ligand-induced changes in protein thermal stability in CETSA assay [40,41]. W1131 can bind and stabilize STAT3 protein in intact AGS cells (Fig. 4D), suggesting the direct interaction between W1131 and STAT3 protein.

We further demonstrated that W1131 inhibited STAT3 Tyr705 phosphorylation in a time- and dose-dependent manner (Fig. 4E and Fig. S4F) without obvious effect on the activation of STAT1 and STAT5 (Fig. 4F), which confirmed the selectivity of W1131. Similarly, W1131 had no obvious effect on phospho-JAK2 or phospho-AKT levels (Fig. 4G and H). W1131 also suppressed the expression of STAT3 target genes.
We next assessed the effects of W1131 on gastric cancer cell growth. W1131 strongly inhibited proliferation of gastric cancer cells with hyperactivated STAT3, whereas it showed weak inhibition on gastric cancer cell HGC27 and human normal cell RWPE-1 without constitutively activated STAT3 (Fig. 5A and Fig. S4B). The basic expression of STAT3 and pY705-STAT3 in these four cells showed in Fig. S4A. Furthermore, W1131 significantly suppressed gastric cancer cell colony formation even at 10 μM (Fig. 5B). The inhibition of cell proliferation by W1131 was demonstrated by the EDU staining assay (Fig. 5C). W1131 manifested stronger and more efficient inhibitory activity of cell viability and survival than a previously reported STAT3 inhibitor SH4-54 [42] (Figs. S4C-D). The effect of W1131 on cancer cell migration and invasion was further investigated. Wound healing assay showed that W1131 significantly reduced cancer cell migration (Figs. 5E and S4E). Transwell assay results demonstrated that W1131 remarkably suppressed the invasion of gastric cancer cells in a dose-dependent manner (Fig. 5D). Next, we performed flow cytometry analysis by Annexin V–FITC staining and found that W1131 indeed induced apoptosis of gastric cancer cells, while the apoptotic percentages were lower than 20% at 3 μM (Fig. S4F), suggesting apoptosis might partly contribute to cancer cell death triggered by W1131. We next carried out LDH release assay with or without inhibitors of apoptosis (Z-VAD-FMK), ferroptosis (ferrostatin-1, liproxstatin-1, and DFO), necrosis (necrosulfonamide), and autophagy (bafilomycin A1) to explore the cell death caused by W1131 treatment. The result demonstrated that the cell death caused by W1131 treatment in gastric cancer cells is varied, and multiple modes of cell death co-exist, which is consistent with the theoretical basis that STAT3 serves as the intersection of multiple signaling pathways and cell functions. Among these cell death caused by W1131, most notably is apoptosis, followed by ferroptosis and necrosis, and autophagy seems to be the least. We further found that W1131-caused cell death could be largely reversed by ferroptosis inhibitor ferrostatin-1, liproxstatin-1, and DFO. Similar results were observed in STAT3 knockdown in gastric cancer cells (Fig. 5G). In addition, we also determined important markers of apoptosis, necrosis, and autophagy by western blotting. The expression of apoptosis-related proteins such as cleaved-PARP-1 and cleaved caspase 7 was increased by W1131 (Fig. S4G). Moreover, W1131 increased LC3-II accumulation and pS358-MLKL expression in gastric cancer cells (Fig. S4H, I), which are markers on autophagy and necrosis, respectively. Therefore, the STAT3 inhibitor W1131 shows strong anti-tumor effects through multiple mechanisms in vitro.

3.5. W1131 strongly inhibits cell survival, migration, and invasion in gastric cancer

To further investigate whether and how W1131 regulate ferroptosis in gastric cancer cells, we performed RNA-seq transcriptome and gene enrichment analysis. We found that the signaling pathways involved cell cycle, DNA damage response, and oxidative phosphorylation, including IL6-JAK-STAT3 pathway and ferroptosis pathway were regulated by W1131 (Fig. 6A). Further GSEA analysis indicated both the IL6-JAK-STAT3 pathway and FNR signature were inhibited by W1131 (Fig. 6B). 1455 differentially expressed genes (DEGs) between control and W1131 treatment were identified in the volcano plot, of which are S85 up-regulated genes and 870 down-regulated genes, including these FNR signature genes GPX4, SLC7A11, and FTH1 (Fig. 6C). We performed RT-PCR analysis and found that GPX4, SLC7A11 and FTH1 were significantly down-regulated by W1131 (Fig. 6D). The protein expression of GPX4, SLC7A11, and FTH1 was further confirmed by western blotting. The expression of apoptosis-related proteins such as cleaved-PARP-1 and cleaved caspase 7 was increased by W1131 (Fig. S4G). Moreover, W1131 increased LC3-II accumulation and pS358-MLKL expression in gastric cancer cells (Fig. S4H, I), which are markers on autophagy and necrosis, respectively. Therefore, the STAT3 inhibitor W1131 shows strong anti-tumor effects through multiple mechanisms in vitro.
3.7. W1131 induces ferroptosis and regresses tumor growth of gastric cancer in vivo

We next evaluated the effects of W1131 on gastric cancer tumor growth. We established the MGC803 subcutaneous xenograft model in BALB/c-nu/nu mice and administrated them with vehicle or W1131 at 3 or 10 mg/kg per day (Fig. 7A). Tumor volume and body weight were monitored every other day. Results showed that W1131 strongly suppressed tumor growth in a dose-dependent manner (Fig. 7B and C), and did not cause significant change of body weight (Fig. 7A) and obvious signs of toxicity, such as loss of appetite, decreased activity, and lethargy during treatment. Histological analysis of the tissues from the lung, heart, liver, kidney, and spleen, further confirmed that there was no obvious toxicity (Figs. 7B–C). Moreover, W1131 significantly decreased STAT3 phosphorylation and its downstream genes including Bcl-xl, Mcl-1, and c-Myc in tumor tissues (Fig. 7F). Immunohistochemical Ki67 staining of the tumor sections suggested that W1131 significantly inhibited cancer cell proliferation (Fig. 7E). W1131 inhibited the expression of GPX4, SLC7A11, and FTH1 in the xenograft tissues, indicating the induction of ferroptosis (Fig. 7E and F). In addition, we performed an iron assay to detect the iron level of tumor tissues and found that W1131 increased iron level in vivo (Fig. 7D). Thus, these results suggested that STAT3 inhibition-triggered ferroptosis played an important role in inhibitory effect of W1131 in gastric cancer.

Next, we treated organoids derived from gastric cancer PDX with W1131 and ferroptosis inhibitor ferrostatin-1. We found that the organoids were sensitive to W1131 treatment, and the ferroptosis inhibitor ferrostatin-1 could mitigate the anti-tumor effect of W1131 (Fig. 7G). Furthermore, we established gastric cancer PDX model in BALB/c-nu/nu mice and administrated it with four regimens (Fig. 7H). Consistent with observations of organoids and in vitro, W1131 efficiently reduced gastric cancer tumor growth, and the inhibitory effect was partly attenuated by ferroptosis inhibitor ferrostatin-1 (Fig. 7I and J). Moreover, W1131 inhibited PDX growth, as indicated by Ki67 (Fig. 7K). To provide further evidence, we carried out MDA assay using tumor tissues and found that W1131 upregulated MDA level and the effect was reversed by ferroptosis inhibitor ferrostatin-1 in vivo (Fig. 7M). These results revealed that W1131, as a potent anti-cancer agent, significantly suppressed tumor growth in gastric cancer xenograft model, organoids, and PDX model through inhibition of STAT3 signaling pathway and induction of ferroptosis.

3.8. W1131 alleviates chemotherapy resistance of gastric cancer in multiple models

Upregulation of GPX4, SLC7A11, FTH1, and pY705-STAT3 in 5-FU resistant cells (Fig. 1H) and 5-FU resistant xenograft models (Figs. 11 and 8A) suggests that ferroptosis regulated by STAT3 might contribute to the chemotherapy resistance of gastric cancer. Moreover, knockdown of endogenous STAT3 significantly inhibited cell growth and colony formation in MGC803/5-FU cells (Fig. 8B and C). W1131 also significantly inhibited cell growth of the 5-FU resistant cells (Fig. 8D). Then, we performed cDNA microarrays analysis to assess the expression of ferroptosis-related genes in MGC803/5-FU cells with or without W1131. We found that GPX4, SLC7A11, and FTH1 were upregulated in 5-FU resistant cells (Fig. 1G), and these three genes were down-regulated after treatment with our STAT3 inhibitor W1131 (Fig. 8E). Next, we performed experiments to determine whether W1131 also enhanced 5-FU sensitivity in 5-FU resistant cell lines. The cell viability assays showed that W1131 significantly enhance the sensitivity of MGC803/5-FU cells to 5-FU (Fig. 8F). Colony formation assay also showed that the combination of 5-FU with W1131 significantly inhibited colony survival of MGC803/5-FU cells compared with single treatment (Fig. 8G). A similar synergistic effect was also observed in the gastric cancer organoids models (Fig. 8H). We further evaluated the therapeutic potential for combining ferroptosis inducer erastin or W1131 with 5-FU in MGC803/5-FU cell-derived xenografts. Remarkably, the combinational treatments led to synergistic tumor growth regression in the 5-FU resistant gastric cancer model (Fig. 8I–K), whereas single treatment of STAT3 inhibitor W1131 or ferroptosis inducer erastin only showed moderate and comparable inhibitory effects on the tumor growth compared to that of 5-FU alone. In addition, IHC analysis of xenograft tumors demonstrated that the combined treatment significantly inhibited tumor growth as indicated by Ki67, suppressed STAT3 signaling as indicated by pY705-STAT3, and triggered ferroptosis as indicated by GPX4 (Fig. 8L). Together, the results from the cell model, organoids model, and animal model suggest that W1131 significantly alleviated chemotherapy resistance in gastric cancer. The combination of W1131 with chemotherapy drugs can be a new strategy for chemotherapy-resistant gastric cancer.

4. Discussion

Gastric cancer is one of the most common and severe cancer worldwide. The primarily curative approach of nonmetastatic gastric
A. Gene enrichment analysis

| Pathway                                      | Gene Number | Rich Factor |
|----------------------------------------------|-------------|-------------|
| p38 signaling pathway                       | 7           | 0.05        |
| DNA damage Response                         | 10          | 0.22        |
| Ferroptosis                                  | 15          | 0.30        |
| Glutathione metabolism                      | 20          | 0.63        |
| Apoptosis                                    | 22          | 0.84        |
| IL6 JAK STAT3 signaling                     | 10          | 0.05        |
| VEGF signaling pathway                      | 5           | 0.13        |
| Cell cycle                                  | 15          | 0.30        |
| T cell receptor signaling pathway           | 20          | 0.63        |
| Ras signaling                               | 25          | 0.84        |
| Oxidative phosphorylation                   | 10          | 0.22        |
| MAPK signaling pathway                      | 15          | 0.30        |

B. HALLMARK_IL6_JAK_STAT3_signalin

- Enrichment Score (ES) for DM5O and W1131
- FNR signature

C. Volcano plot

- log2(Fold Change)
- -log10(p-value)

D. AGS and MGC803

- FGFR1
- FLT1
- FTH1
- SLC7A11
- GPX4
- Beta-Actin

E. W1131 (uM)

F. DM5O and W1131 (1 uM)

G. MDA (μmol/mg protein)

- MGC803
- AGS

H. MDA vs. W1131, Ferrostatin-1, and Ferroxstatin-1

I. C11-BODIPY fluorescence (FL1)

J. FerroOrange fluorescence (FL1)

K. Liperox

- FerroOrange

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cancer is surgical resection, but the 5-year survival rate diminishes rapidly with advancing stage of disease [44]. Moreover, gastric cancer patients are usually at advanced stages when diagnosed, thus losing the opportunity for surgery and with a poor prognosis, and the interventions such as molecular-targeted therapies and immunotherapy show low clinical response. Although significant progress has been made in the field of neoadjuvant chemotherapies, de novo and acquired resistance to these therapies appear inevitable [45]. 5-FU is the first-line drug for the treatment of gastric cancer but 5-FU resistance occurs in clinical practice frequently. 5-FU resistance may develop due to the various mechanisms and pathways, such as the alterations in drug transport, evasion of apoptosis, regulation of autophagy, cancer stem cell involvement, tumor microenvironment interactions, epigenetic alterations, as well as redox imbalances [46]. In recent years, 5-FU has been used along with other modulators for the treatment of many cancers by targeting apoptosis or other cancer signaling pathways. Apoptosis is also one of the major mechanisms of cell death in response to 5-FU [47]. BAX is an indispensable gateway to mitochondrial dysfunction. The down-regulation of BAX plays an important role acquisition of resistance to 5-FU [48]. Wang et al. found that Andrographolide, a natural diterpenoid from Andrographis paniculata, bound to BAX and triggered mitochondria mediated apoptosis, thus reversing 5-FU resistance [49]. Similarly, apigenin[4,5, 7-trihydroxyflavone], a plant flavone, increased the sensitivity of tumors to 5-FU by activating the mitochondria-mediated apoptosis pathway [50]. Toden et al. reported that curcumin and 5-FU combination synergistically induced apoptosis in 5-FU resistant cells by downregulating the HSP-27 and P-gp expression [51].

In recent years, some publications suggested that ferroptosis might be an effective therapeutic strategy to alleviate chemotherapy resistance [9,10]. Cancer cells which are resistant to conventional treatment might be particularly susceptible to ferroptosis, in terms of the current research results [9], thus the development of novel therapeutic strategies based on ferroptosis may alleviate current gastric cancer resistance. There are ever-growing interests to explore the role of ferroptosis in cancer and exploit ferroptosis to improve cancer prevention, diagnostics, treatment, and prognostics [8,10,52]. In this study, we unraveled that the FNR signature is closely related to the development and 5-FU resistance of gastric cancer. We found that the ferroptosis circuit is aberrant expressed in gastric tumors and elevated FNR signature was significantly associated with low survival rates in gastric cancer patients. Furthermore, we found that pY705-STAT3 and GPX4, SLC7A11, and FTH1 were markedly upregulated in 5-FU-resistant gastric cancer cells and xenografts, indicating a potential role of FNR signature in contributing to 5-FU resistance in gastric cancer cells. A combination of 5-FU and ferroptosis inducer erastin demonstrated a significantly synergetic tumor growth regression in the resistant MGC803/5-FU cell and organoids model. It suggests that ferroptosis is closely related to the development and 5-FU resistance of gastric cancer.

We found that there was a positive correlation between STAT3 and FNR signatures in gastric cancer tissue samples. Analysis of the GEO database, TCGA database, and gastric cancer patient tissue arrays suggests that STAT3 and FNR signatures are potential drivers for gastric tumorigenesis. The morphological features of ferroptosis are mainly displayed by decreased or vanished mitochondria cristae, a ruptured outer mitochondrial membrane, and a condensed mitochondrial membrane [13,31]. It is reported that GSH depletion, lipid peroxidation, lipid ROS accumulation, and Fe²⁺ accumulation are critical features in ferroptosis [6]. Mitochondria shrinkage, increased mitochondria membrane density, lipid ROS elevation, GSH depletion, lipid peroxidation, and Fe²⁺ accumulations were observed following STAT3 inhibition. We further found that STAT3 may regulate the gene expression of GPX4, FTH1, and SLC7A11 by binding to the promoters. Our data suggested that a high level of GPX4 in gastric cancer was markedly associated with poor survival. Our study also reveals that down-regulation of SLC7A11 and GPX4 by STAT3 inhibition promotes intracellular lipid ROS and MDA level increment and leads to ferroptosis in gastric cancer. Furthermore, we find that STAT3 transcriptionally regulates iron metabolism-related proteins such as FTH1, and mediates intracellular Fe²⁺ levels to induce ferroptosis.

As an oxidative responsive transcriptional factor, STAT3 was reported to be linked to mediation of stress-related ferroptosis [53]. It was reported that activation of Src and STAT3 suppresses the expression of ASSL4 [28]. Another report showed that genetic blockade of STAT3 limited erasin-induced cathepsin B expression [25]. Our results showed that overexpression of STAT3 inhibited the protein expression of ASSL4 in gastric cancer cells but the expression of cathepsin B had no obvious change, whether STAT3 was overexpressed or knocked down. Luo et al. demonstrated that Bavachin (a bioactive compound extracted from the fruit of Psoralea corylifolia) induced ferroptosis by reducing SLC7A11 and GPX4 expression and promoting ROS and MDA accumulation through down regulation of STAT3 and upregulation of P53. Jiang et al. demonstrated that p53 induced ferroptosis by down regulation of SLC7A11 to inhibit cystine transport [54]. Our results suggested that STAT3 inhibitor W1131 can increase p53 expression and inhibit GPX4 and SLC7A11 expression. Further, our results of chip-qPCR and luciferase reporter gene assay indicated STAT3 directly transcriptional regulates GPX4 and SLC7A11 expression in gastric cancer cells. Therefore, knockdown of STAT3 triggered ferroptosis not only by up-regulating p53 to downregulate GPX4 and SLC7A11 expression but also by directly regulating GPX4 and SLC7A11 expression.

Consistently, we found that STAT3 acts as a key negative regulator of ferroptosis in gastric cancer through a multi-pronged mechanism and...
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inhibition of STAT3 can trigger ferroptosis through a multi-pronged mechanism associated with lipid peroxidation and iron metabolism. To the best of our knowledge, it is the first to illuminate that STAT3 regulates ferroptosis by directly regulating GPX4, SLC7A11 and FTH1 in gastric cancer. Our work may provide a better understanding for mechanisms of regulation of STAT3 on ferroptosis and a new therapeutic strategy for gastric cancer by targeting the STAT3-ferroptosis circuit.

Given its important role in proliferation, survival, and ferroptosis, STAT3 could serve as an attractive drug target in gastric cancer. Meanwhile, ferroptosis-inducing drugs are attracting more attention for cancer treatment. Several non-peptide SH2 domain inhibitors have been identified and shown to inhibit the growth of cancer cells with hyper-activated STAT3, including BP-1-102, STA-21, STATTIC, S3I-201, STX-0119, and WP1066. STAT3 inhibitors have been shown to inhibit the growth of cancer cells with hyper-activated STAT3, including BP-1-102, STA-21, STATTIC, S3I-201, STX-0119, and WP1066. 

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In this study, we systematically demonstrate that the STAT3-ferroptosis circuit plays a critical role in gastric cancer progression and chemoresistance. We discover a novel potent compound W1131, which inhibits STAT3 function, triggers ferroptosis and re-sensitizes the resistant cancer cells to chemotherapy in the organoids model and mouse xenograft model. Our study reveals that STAT3 serves as a key regulator of ferroptosis and chemoresistance. However, the ferroptosis response is regulated by a complex network of epigenetic transcriptional and post-translational mechanisms. Therefore, additional important molecular mechanisms by which STAT3 regulates ferroptosis deserve further exploration. A better understanding of the regulatory mechanisms and signaling pathways of ferroptosis, and the searching for biomarkers to facilitate the detection and tracking of ferroptosis will be an active area in the future. This study reveals that targeting ferroptosis through STAT3 inhibition sheds light on new strategies for gastric cancer therapy and chemoresistance.

Study approval

The animal procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University and followed the Guide for the Care and Use of Laboratory Animals.

Authors’ contributions

Conception, design, and supervising of the study: Xiaolei Zhang, Yuaxiang Wang, and Peiqing Liu. Development of methodology and acquisition of data: Shumin Ouyang, Huaxuan Li, Linlin Lou, Qiuyao Huang, Zhenhua Zhang, Jianshan Mo, Jiaye Lu, and Wen Ding. Analysis and interpretation of data: Shumin Ouyang, Qiuyao Huang, Peibin Yue, James Turkson, Linlin Lou, and Zhenhua Zhang. Technical and material support: Min Li, Kai Zhu, Yunjie Chu, Jianjunian Wang, Peibin Yue, and James Turkson. Study supervision: Xiaolei Zhang, and Yuaxiang Wang.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102317.

Acknowledgments

Fig. 8. W1131 alleviates chemotherapy resistance in multiple models of gastric cancer A: IHC analysis of p70S6-STAT3 expression in MC10803 and MGC803/5-FU xenografts. Scale bars = 100 μm. B: MGC803/5-FU were transfected with siRNAs against STAT3 (siSTAT3#1 and siSTAT3#2) or control siRNA (second). Viable cells were counted at indicated time points. C: MGC803/5-FU cells were infected as in (B), 14 days later, colonies were counted. D: MGC803/5-FU cells were treated with vehicle (DMSO) or the indicated concentrations of W1131 for 72 h. Cells were harvested for determining cell growth by counting viable cells. E: RT-PCR analysis of ferroptosis-related genes with cDNA microarrays in MGC803/5-FU cells treated with vehicle (DMSO) or W1131 (15 μM) for 40 h. Heatmap indicated the mRNA expression level of ferroptosis-related genes (Z-score). F: MGC803/5-FU cells were treated with 5-FU (15 μM) and/or W1131 (1 μM) as indicated. After 72 h, total viable cells were counted with a Coulter cell counter. G: MGC803/5-FU cells were treated with 5-FU (8 μM) and/or W1131 (0.1 μM) as indicated. 14 days later, colonies were counted. H: PDX-derived organoids were treated with DMSO, 5-FU, W1131, erastin, and their combination, as indicated. Four days later, representative images were taken under a fluorescence microscope (top three rows) or standard light microscope (bottom row). Scale bars = 100 μm. Four days later, cell viability in organoids was measured with CellTiter-Glo. I–K: BALB/c-nu/nu mice bearing the MGC803/5-FU xenografts (n = 6 mice per group) received the vehicle, 5-FU (i.p., 20 mg/kg, once every other day), W1131 (i.p., 3 mg/kg, once daily), erastin (i.p., 20 mg/kg, once every other day) and their combination, as indicated. Mean tumor volume ± standard error of the mean (SEM) (J), representative tumor images (I), and mean tumor weight ± SEM (K) are shown. L: H&E and IHC images of the indicated proteins in the randomly selected PDX tumor section. Scale bars = 100 μm. **P < 0.01, ***P < 0.001, ****P < 0.0001. n = 3. Student’s t-test.
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