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Natural compound So-2 suppresses triple-negative breast cancer through inducing ferroptosis via downregulating transcription factor E2F7.

Na Liu
Jinan University
Jing Zhang
Jinan University
Wenqi Duan
Jinan University
Tingting Luo
Jinan University
Cong Wu
Jinan University
Lina Han
Jinan University
Fengying Yang
Jinan University
Hongwei Yue (talatala@126.com)
Qilu Hospital of Shandong University https://orcid.org/0000-0002-1839-4699
Di Ge
Jinan University

Research Article

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Abstract

Background

Siegesbeckia orientalis L. have been used as a traditional Chinese medicine “Xi-Xian-Cao” for centuries with multiple medicinal benefits including cancerous treatment. Breast cancer is one of the leading causes of death in women worldwide. And the Triple-negative breast cancer (TNBC), accounting for about 15 ~ 18% of all breast cancers, is notorious for its poor prognosis, high rate of relapse and short overall survival. Because of lacking effective therapeutic targets or drugs, treatment of TNBC in clinical encounters great obstacle. Recently, we have reported the isolation of twenty-seven germacranolides including So-2 from the aerial parts of *S. orientalis* with potent cytotoxicity against breast cancer cells. However, the specific mechanism needs to be elucidated. The aim of this study is to verified the anti-tumor function of the natural compound So-2 and uncover the underlying mechanism.

Methods

We investigated the anti-tumor role of So-2 both in vitro and in vivo. An orthotopic transplantation tumor model was established to assess the in vivo antitumor effects of So-2. Two TNBC cell lines MDA-MB-231 and BT-549 cells were employed to study the cytotoxicity effect and specific mechanism of So-2 in vitro. We studied the influence of So-2 on TNBC cell proliferation, migration and ferroptosis in detail.

Results

So-2 was shown to cause cell cycle arrest and suppress TNBC cell proliferation and migration. Also, So-2 was identified to be a bona fide ferroptosis inducer in TNBC cells. We also characterized the oncogenic role of the transcription factor E2F7 in TNBC. And E2F7 was downregulated by So-2 while inducing ferroptosis. E2F7 was demonstrated to be involved in the ferroptosis-inducing and tumor suppression effect of So-2.

Conclusion

So-2 exhibits inhibitory effect on TNBC growth and migration both in vitro and vivo by inducing TNBC ferroptosis via downregulating the expression of E2F7. These findings provide valuable insight into the pathogenesis of TNBC. The natural compound So-2, isolated from Chinese traditional medicine, might be a prospective drug candidate in TNBC therapy.

1. Background

Breast cancer ranks as the fifth leading cause of cancer mortality worldwide. In 2020, the incidence of female breast cancer has surpassed lung cancer to become the world's largest cancer, with about

2.3 million new cases, accounting for 11.7% of all cancer cases[1]. Triple-negative breast cancer (TNBC), characterized by uniformly negative expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2), is the most malignant clinical subtype of all breast cancers with poor prognosis, accounting for about 15% of all breast cancer cases[2, 3]. Currently, chemotherapy is the key approach used for TNBC therapy [4]. However, high rate of reoccurrence and short overall survival make it still a great challenge to cure TNBC in clinical[5]. Therefore, it is of great clinical significance to explore new potential targets or identify novel compounds to treat TNBC.

(1 (10) E, 4Z, 6α, 8β, 9α)-9-ethoxy-6, 15-dihydroxy-8-(2-methylacryloxy)-14- oxogermacra-1 (10), 4, 11 (13)-trieno-12, 6-lactone (So-2, Figure S1) is a natural germacrane-type sesquiterpenoid separated from *Sigesbeckia orientalis*[6]. This sesquiterpenoid contains a decane cyclopean pentacolide structure and exhibits anti-inflammatory, antitumor, and anti-angiogenesis activities[7, 8]. In our previous study, So-2 was demonstrated to be cytotoxic to MDA-MB-231 cells[9], while the specific anti-TNBC mechanism of So-2 is poorly understood. Here in the current investigation, we explored the effect and mechanism of So-2 on TNBC cells in vitro, and further verified the role of So-2 on TNBC in vivo by establishing an orthotopic tumor-bearing mouse model.

Ferroptosis is a new type of programmed cell death characterized in recent years which is closely related to diverse diseases [10, 11]. This form of cell death is driven by intracellular iron accumulation and overwhelming lipid peroxidation[12]. Once the antioxidant activity of glutathione-dependent peroxidase (GPX4) in cells is lower than the level of lipid reactive oxygen species, it will cause a breakdown of cellular redox homeostasis, thereby leading to cell death[13, 14].TNBC cells were reported to be highly sensitive to oxidative stress and ferroptosis, thus making ferroptosis as one of the most promising intervention target for TNBC treatment[15]. Herein, we reported a natural compound So-2 that could promote ferroptosis in TNBC cells both in vitro and vivo.

Mammalian E2F transcription factors family (E2F1-E2F8) is a class of important transcription factors. Through binding to hundreds of target genes, they are associated with a lot of cellular functions, such as cell proliferation, apoptosis, and differentiation[16–18]. E2F transcription factor 7 (E2F7) is a major transcriptional repressor in the E2F family[19] and has been found to be involved in various malignant tumors. For example, E2F7 is highly expressed in acute myeloid leukemia, gallbladder cancer, endometrial cancer, and esophageal cancer, promoting cell proliferation and metastasis[20–23]. With regard to breast cancer, limited findings have suggested that high expression of E2F7 correlates with the degree of malignancy of breast cancer. Patients with higher Scarff Bloom & Richardson (SBR) grades have higher E2F7 mRNA levels, and the transcript level of E2F7 is positively correlated with mortality in TNBC patients[24]. Furthermore, studies have shown that E2F7 was overexpressed in tamoxifen-resistant MCF-7 cells, and knockdown of E2F7 resensitized the resistant MCF-7 cells to tamoxifen. These results all indicated that E2F7 may play a role of proto-oncogene in breast cancer [25, 26].

In this study, we investigated the effect of So-2 on TNBC cell growth and found that So-2 promoted G2/M phase arrest of TNBC cell cycle and inhibited cell proliferation and migration. By establishing an

orthotopic tumor-bearing mouse model of TNBC, So-2 was found to suppress the growth of TNBC in vivo without obvious side effects. We further found that So-2 induced TNBC ferroptosis both in vitro and in vivo, featured by ROS and lipid peroxide accumulation, GSH depletion. Mechanistically, E2F7 was shown to be an oncogene in TNBC cells, while acting as a negative regulator in So-2-induced ferroptosis. Collectively, our findings suggested that the natural product So-2 promotes ferroptosis and suppress metastasis of TNBC cells by downregulating E2F7.

2. Materials And Methods

2.1. Chemicals

The structure of (1 (10) E, 4Z, 6α, 8β, 9α)-9-ethoxy-6, 15-dihydroxy-8- (2-methylacryloxy)-14-oxogermacra-1 (10), 4, 11 (13)-trieno-12, 6-lactone (So-2), isolated from *S. orientalis* was appraised by interpretation of spectral data (MS, 1H NMR, 13C NMR). The compound was dissolved in dimethyl sulfoxide (DMSO), prepared as a 20 mM stock solution, and diluted according to experimental needs when used.

2.2. Reagents and antibodies

Deferoxamine (S5743) and ferrostatin-1 (S7243) were purchased from Selleck. Necrostatin-1 (HY-15760), 3-Methyladenine (HY-19312), N-Acetyl-L-cysteine (HY-B0215), Z-VAD-FMK (HY-16658B), and glutathione (GSH, HY-D0187) were bought from MedChemExpress. Antibodies against GPX4 (A1933), HO-1 (A1346), SLC40A1 (A14884), transferrin (A19130), SLC7A11 (A13685), Glutaminase (A3885), N-Cadherin (A19083), E-cadherin (A11492), Snail (A5243), Vimentin (A19607), Ki-67 (A17341), E2F7 (A15211), and β-Actin (AC026) were purchased from ABclonal.

2.3. Cell lines and cell culture

Cell lines MDA-MB-231, BT-549, MCF-7, MCF-10A, MCF-10F and 4T1-Luc were purchased from the American Type Culture Collection. The human breast cancer cell lines MCF-7 and MDA-MB-231 were cultured in DMEM medium supplement with 10% fetal bovine serum at 37°C in a humidified environment with 5% CO_2. The cell lines BT-549 and 4T1-Luc were cultured in 1640 medium supplement with 10% fetal bovine serum at 37°C in a humidified environment with 5% CO_2. The MCF-10A and MCF-10F cell line was cultured in DMEM/F12 (1:1) supplemented with 5% FBS, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin and 0.5 mg/ml hydrocortisone under a 5% CO_2 humidified atmosphere at 37°C.

2.4. Cell viability assay

A density of 6×10^3 cells were added to 96-well plates and cultured for 24 h. Different concentrations of So-2 or cytostatics were added for 48 h. The medium was discarded, 100 μl of 10% TCA was added to each well and fixed at 4°C for 1 h. Gently wash 5 times with ultrapure water. After natural drying, 100 μl of 0.4% SRB (L812440, MACKLIN) was added and shaken for 5 min. Then, the plate was gently washed 5 times with 1% acetic acid. After natural drying, 150 μl of Tris buffer was added to dissolve and shaken for

5 min. Absorbance was performed at 540 nm by using a microplate reader (TECAN, Switzerland). Cytotoxicity (%) =(Control-Experimental)/Control×100%. The results were shown as cell viability percentages.

2.5. Cell colony formation assay

The cells were planted in 6-well plates at a density of 800 cells/ml/well. After 8–12 h, different concentrations of So-2 were added and cultured in the incubator for 2–3 weeks. the cells were immobilized with methanol at 37°C for 15 min. After washing with PBS, the cells were stained with crystal violet solution (C0121, Beyotime) at room temperature for 30 min. The excess crystal violet was sucked out and rinsed with water for 3 times. The number of colonies was counted.

2.6. Cell Cycle Analysis

A density of 5×10^5/ml cells were seeded into 6 cm dishes. cultured for 24 h, and treated with different concentrations of So-2 for 24 h. Cells were collected and rinsed twice with pre-cooled PBS. Pre-chilled 75% ethanol was added and fixed overnight at 4ºC. Stained with cell cycle staining kit (KGA512) purchased from KeyGEN BioTECH Company for 30 min at room temperature in a dark room. Analysis of cell cycle distribution by flow cytometry (ACEA, CA, USA).

2.7. Transwell assays

The cells with a density of 1×10^5/ml were resuspended in 200 µl medium containing So-2 with different concentrations, added to the transwell chamber (Corning, USA), and 500 µl medium containing 10% FBS was added to the lower chamber. After incubation for 24 h, the chamber was removed and fixed with methanol for 30 min. Washed with PBS and stained with 1% crystal violet for 20 min. Five wild cells were randomly observed and photographed by the microscope (Nikon Corporation, Japan).

2.8. Wound healing assay

Logarithmic growth cells were seeded into 6-well plates with 4×10^5 cells/ml. After the cells have covered the bottom of the dish, scratch the cells with a 200 µl pipette tip. The medium containing So-2 was replaced, and the scratch healing at 0 h and 24 h was recorded with the digital microscope software ZEN (Carl Zeiss IMT, Oberkochen, Germany), respectively. Images were processed with Image J (NIH, MD, USA) and the migrated area of cells was calculated. The experiment was repeated 3 times.

2.9. Western blot assay

The cells were collected and lysed with RIPA buffer (P0013B, Beyotime) containing 1 mM of PMSF (ST506, Beyotime) for 30 min on ice. The protein content was measured with the BCA protein concentration assay kit (PC0020, Solarbio). Proteins (20 µg) in the samples were isolated by 10% polyacrylamide gel electrophoresis and transferred to PVDF membranes (IPVH07850, Millipore). The PVDF membrane was sealed in 5% nonfat milk powder blocking solution at room temperature for 1 h. The primary antibody was diluted with 3% BSA solution, and the PVDF membrane was incubated at 4ºC overnight. After washing 5 times with TBST, the PVDF membrane was placed in horseradish peroxidase-

conjugated IgG secondary antibody diluted in 3% nonfat milk, and incubated at room temperature for 1 h. After washing the membrane 3 times with TBST, ECL reagent (WBKLS0100, Millipore) was added and developed under the chemiluminescence system (Bio-Rad, Hercules, CA, USA).

2.10. Measurement of ROS

The cells in logarithmic phase were pretreated with various concentrations of So-2 for 24 h and then incubated with 10 μM C11 BODIPY 581/591 (D3861, Thermo Fisher) for 1 h. After washing twice with PBS, trypsin was added, and the supernatant was removed after centrifugation. The cells were resuspended in PBS and detected by flow cytometry (Agilent, CA, USA).

2.11. MDA assay

The cells were treated with various concentrations of So-2 for 24 h, digested with trypsin, and the supernatant was discarded by centrifugation. According to the instructions, 1 ml of extraction solution should be added for every 5 million cells, and the cells were disrupted by ultrasonic waves. The supernatant was discarded by centrifugation and placed on ice until assayed. Add the corresponding reagents in accordance with the instructions of the MDA assay kit (BC0025, Solarbio) and mix well, incubate in a 100 ºC water bath for 1 h, after cooling, centrifuge to take the supernatant, and measure the absorbance of each sample at 532 nm and 600 nm with a microplate reader, respectively. The MDA content was performed according to the stated formula.

2.12. GSH assay

After treating cells with different concentrations of So-2 for 24 h, they were trypsinized and harvested. According to the instructions of the glutathione assay kit (A00621, Nanjing Jiancheng), add the reagents in turn, mix well, let stand for 5 min, and use a microplate reader to detect the absorbance at 420 nm. Calculate the GSH content according to the instructions.

2.13. Cell transfection

E2F7 siRNA and negative control were both produced from Shanghai GenePharma Co. E2F7 overexpression and the empty control vectors were established by Shanghai GenePharma Co. The cells in logarithmic growth phase were inoculated to 6 cm dishes at 1×10^5 cells/ml, and the degree of cell fusion reached about 50%. The transfection complex was prepared in keeping with the instructions of Lipofectamine 2000 (2030910, Thermo Fisher), and each transfection solution was added to the petri dish and mixed, incubated for 6 h, then replaced with the medium containing So-2 and incubated for 24 h, and the next step was carried out according to the experimental requirements.

2.14. qPCR analysis

After cells were treated, total RNA was obtained by using TRIzol reagent (10296010, Thermo Fisher). In accordance with the specific operation method, cDNA was produced by ABscript ll cDNA First Strand

Synthesis Kit (RK20400, Abclonal). The CFX96 Real-Time system (Bio-Rad, Hercules, CA, USA) was used to amplify the PCR products and examine the expression of the target gene. The primer sequences were as follows: E2F7, forward 5-CTCCTGTGCCAGAAGTTTC-3 and reverse 5-CATAGATGCGTCTCCTTTCC-3; GAPDH, forward 5-GCCTCAAGATCA TCAGCCAATGCCT-3 and reverse 5-TGTGGTCATGAGTCCTTCCACGAT-3; E-cadherin, forward 5-CGACCCAACCCAAGAATCTA-3 and reverse 5-CTCCA AGAATCCCCAGAATG-3; Snail, forward 5-AGCTCTCTGAGGCCAAGGATCT-3 and reverse 5-TGTGGCTTCGGATGTGCAT-3; N-Cadherin, forward 5-AACGCC AGGCCAAACAAC-3 and reverse 5-TCGTCGGATTCCCACAGG-3. These primers were purchased from Shanghai Gene Pharmaceutical Co., Ltd. The relative expression level of the target gene was calculated by $2^{-\Delta\Delta Ct}$ method.

2.15. In vivo anti-tumor activity assay

Female BALB/c mice aged 6 weeks were fed under standard conditions according to the approved regimen. Following acclimation, 4T1-Luc cells (1×10^6/0.1 ml PBS) were inoculated into the fourth pair of mammary fat pads of mice. When tumors reached 50–100 mm^3, mice were randomly divided into control group and So-2 treatment group, with 6 mice in each group. In the treatment group, mice were intraperitoneally injected with So-2 (10 mg/kg/d) at a dose of 10 ml/kg/d, once a day. The control group was injected with PBS containing the same amount of DMSO. The tumor diameter was measured with calipers every 3 days, and the tumor volume was calculated according to the formula ($length\times width^2\times0.5$). Tumor growth in mice was detected using the PerkinElmer IVIS Lumina XRMS Series III (MA, USA) in vivo imaging system. The mice were sacrificed 21 days after administration, and the tumors were resected for histological analysis. Female BALB/c mice were bought from the Experimental Animal Center of Shandong University. All the animal experiments used in this study comply with the Institutional Animal Care and Use Committee of Shandong University (Shandong, China).

2.16. Statistical analysis

Data analysis and graphing were performed using GraphPad Prism 8. 2. 1 (GraphPad Software, USA). The results are expressed in the form of mean ± SD. The results of at least three independent experiments were taken and t-tests were performed. Differences were considered statistically significant at $P < 0.05$ for all analyses.

3. Results

3.1. So-2 arrested cell cycle at G2/M phase and inhibited the proliferation in TNBC cells

We first isolated and the natural compound So-2 from the aerial parts of S. orientalis and analyzed its chemical structure and properties (Fig. S1-3.). To determine the effect of So-2 on cell viability, we treated MDA-MB-231 and BT-549 cells with various concentrations of So-2 for 48 h and assessed cell viability

using SRB assay. The results demonstrated that So-2 markedly inhibited the viability of two types of TNBC cells in a manner of dose-dependent compared to the untreated cells (Fig. 1A). Meanwhile, we observed obvious inhibitory effect of So-2 on MDA-MB-231 and BT-549 cell growth (Fig. 1B). And then, we tested the effect of So-2 on the proliferation of TNBC cells by cell clone formation assay, the results demonstrated that So-2 dramatically suppressed the clone formation ability of TNBC cells (Fig. 1C), indicating that So-2 inhibited the proliferation of TNBC cells. And flow cytometry was used to detect the role of So-2 on TNBC cell cycle. The results showed that So-2 caused a dramatic increase in number of cells arrested in G2/M phase and reduced cells in G0/G1 and S phase (Fig. 1D). These results demonstrated that So-2 significantly inhibited TNBC cell proliferation and promoted cell cycle arrest in G2/M phase.

3.2. So-2 suppressed migration in TNBC cells

To explore the effect of So-2 on the migration of TNBC cells, we examined the migratory capacity of MDA-MB-231 and BT-549 cells after So-2 treatment for 24 h by wound healing and transwell migration assay. The wound-healing assay illustrated that So-2 significantly inhibited TNBC cell migration compared with that in control (Fig. 2A and C). The transwell migration assay results further confirmed that So-2 inhibited the migration ability of TNBC cells (Fig. 2B and D). To further explore the potential mechanism of So-2 on TNBC cell migration, we measured several of the key proteins involved in cell migration signal pathway using western blotting. The results indicated that So-2 suppressed the migration of TNBC cells by decreasing the expression of mesenchymal markers Vimentin, N-cadherin, Snail while increasing the expression of epithelial marker E-cadherin (Fig. 2E). Thus, all these data concluded that So-2 suppressed TNBC cell migration by inhibiting epithelial mesenchymal transition (EMT).

3.3. So-2 inhibits tumor growth in vivo

In order to evaluate the in vivo antitumor effects of So-2, an orthotopic transplantation tumor model of BALB/c mice carrying 4T1-Luc cells was established. Tumors in the breast and other organs of mice were detected by IVIS Lumina XRMS III in vivo imaging system. When the tumors grew to 50–100 mm^3, the mice were randomly divided into two groups (6 mice per group) and intraperitoneally injected with PBS (DMSO) and So-2 (10 mg/kg/d), respectively. After 21 days of administration, the fluorescence signal in the So-2 treatment group weakened compared with the control group (Fig. 3A and C). In addition, the tumor volume of the mice in the So-2 treatment group was significantly smaller (Fig. 3B and E), indicating that So-2 inhibited the growth of TNBC in vivo. We observed no significant change in the body weight of the mice in So-2 treated group(Fig. 3D). To further evaluate the toxicity of So-2, two groups of major organs including heart, liver, spleen, lung, and kidney were subjected to pathomorphological analysis by H&E staining. No significant histological differences were found in the So-2 treatment group (Fig. 3F), indicating no apparent toxicity. These results demonstrated that So-2 effectively suppressed tumor growth of TNBC in vivo.

3.4. So-2 is a bona fide ferroptosis inducer in TNBC cells

Since dramatic downregulation of So-2 on TNBC viability was observed, we further investigated the role of So-2 on TNBC cell death usinginhibitors of diverse types of programmed cell death. According to our result, treatment with Z-VAD-FMK, 3-Methyladenine, or Necrostin-1 did not abrogate So-2 induced TNBC cell death (Fig. S4), suggesting that So-2 might not induce apoptosis or necroptosis in TNBC cells.

Ferroptosis is a novel form of cell death with potential for cancer therapy. Therefore, we investigated whether So-2 promotes TNBC cell death by inducing ferroptosis. We detected several key ferroptotic events in TNBC cells, including intracellular ROS accumulation, GSH depletion and MDA accumulation. As was shown by the results, So-2 caused a significant accumulation of ROS and MDA in MDA-MB-231 and BT-549 cells (Fig. 4A 4C), while GSH level were decreased (Fig. 4B). Next, the effect of So-2 on the expression of ferroptosis-related proteins was detected using western blotting. And the data suggested that HO-1 and transferrin were up-regulated in MDA-MB-231 and BT-549 cells after So-2 treatment. On the contrary, the levels of negative regulators of ferroptosis (GPX4, SLC7A11, glutaminase, and SLC40A1) were down-regulated (Fig. 4D). These results indicated that So-2 could activate the ferroptosis signal in TNBC cells. Furthermore, ferrostatin-1 (Fer-1) and deferoxamine (DFO) blocked part of So-2-induced TNBC cell death (Fig. 5A and B) and migration (Fig. S5). Subsequently, when the ROS inhibitor NAC was added to reduce intracellular ROS levels (Fig. 5C), and GSH was additionally added to enhance intracellular reduction process, part of So-2-induced cell death was rescued (Fig. 5D and E). All these results confirmed that So-2 is a bona fide ferroptosis inducer in TNBC cells.

3.5. E2F7 acts as an oncogene in TNBC cells

In previous studies, GEPIA online analysis of the TCGA database indicated that E2F7 was significantly up-regulated in BRCA tissue (n = 1085) compared to normal breast tissue (n = 291) (Fig. 6A). To specific the role of E2F7 in breast cancer and the anti-tumor effect of So-2 in TNBC, we first examined the expression of E2F7 using qPCR and western blotting in two breast cancer cell lines (MDA-MB-231 and MCF-7), and two normal breast epithelial cell lines (MCF-10A and MCF-10F). The results showed that the expression of E2F7 in MDA-MB-231 cells was significantly higher than that in MCF-7, MCF-10A and MCF-10F cells (Fig. 6B and C).

To further determine the role of E2F7 in the malignancy of breast cancer cells, we detected the effect of E2F7 knock down and overexpression on migration of MDA-MB-231 cells and MCF-7 cells by use of wound healing assay. As shown in Fig. 6D, E2F7 knockdown suppressed the migration capacity of MDA-MB-231 cells, while E2F7 overexpression enhanced cell migration in MCF-7 cells. We then explored the regulatory effect of E2F7 on EMT-related genes in MDA-MB-231 cells (Fig. 6E) and the results demonstrated that the expression of mesenchymal markers Snail and N-cadheri decreased after E2F7 knockdown, while the expression of epithelial marker E-cadherin increased, suggesting that E2F7 knockdown inhibited cell migration. On the contrary, the expression of Snail and N-cadherin increased after E2F7 overexpression in MCF-7 cells, while the epithelial marker E-cadherin decreased, indicating that E2F7 overexpression promotes cell migration. All these results concluded that E2F7 might be a pro-oncogene in TNBC cells.

3.6. So-2 induced ferroptosis in TNBC cells by down-regulating the expression of E2F7

We further investigated whether E2F7 is involved in So-2-induced ferroptosis in TNBC cells. We first examined the influence of So-2 on E2F7 mRNA and protein level in TNBC cells. qPCR results demonstrated that So-2 reduced the mRNA level of E2F7 in MDA-MB-231 and BT-549 cells (Fig. 7A). Similarly, at the protein level, So-2 also reduced E2F7 expression (Fig. 7B). We then overexpressed E2F7 in TNBC cells and found that overexpression of E2F7 antagonized the inhibitory effect of So-2 on TNBC cell viability (Fig. 7C). Additionally, overexpression of E2F7 reduced the promoting effect of So-2 on HO-1 expression and its inhibitory effect on GPX4 and SLC7A11 expression (Fig. 7D, E and F), suggesting that E2F7 suppressed So-2-induced ferroptosis in TNBC cells.

We then verified the expressions of Ki-67, GPX4, transferrin and E2F7 in orthotopic transplantation tumor by immunohistochemical staining. As shown in Fig. 7G, Ki-67 signal was downregulated by So-2 in tumors, confirming that So-2 suppressed tumor growth in vivo. Meanwhile, So-2 downregulated the negative regulator of ferroptosis GPX4, but increased the expression of transferrin, indicating that So-2 induced ferroptosis in TNBC. Likewise, So-2 suppressed the expression level of E2F7 in TNBC tissues. Therefore, ourresults demonstrated that So-2 might suppress triple-negative breast cancer through inducing ferroptosis via downregulating transcription factor E2F7.

4. Discussion

Siegesbeckia orientalis L. have been used as a traditional Chinese medicine “Xi-Xian-Cao” for centuries with several medicinal benefits, including cancerous treatment. Recently, we have reported the isolation of twenty-seven germacranolides including So-2 from the aerial parts of S. orientalis with potent cytotoxicity against breast cancer cells[9]. However, the specific role and mechanism of So-2 in TNBC tumors remains obscure. In this research, we detected the in vitro and in vivo effects of So-2 on TNBC cells. And the results showed that So-2 suppressed cell proliferation, promoted G2/M phase arrest, blocked cell migration and induced TNBC cell ferroptosis.

Ferroptosis is a new way of regulated cell death, implicated in metabolism, redox biology and disease states[27]. In cancer therapy, a subset of so-called "persistent cells" that survive treatment with several chemotherapeutic drugs often undergo metabolic reprogramming to acquire ferroptosis sensitivity [28, 29]. Emerging evidence shows that targeting ferroptosis is appearing as a new potential strategy for cancer treatment[30]. In this study, we found that several death inhibitors Z-VAD-FMK, 3-MA, or Necrostin-1 did not prevent So-2-induced TNBC cell death, while both Fer-1, a lipid peroxidation inhibitor, and DFO, an iron chelating agent, could suppress So-2-induced cell death, suggesting that ferroptosis might be the major decisive factor of So-2-induced cell death. We further verified the presence of ferroptosis in TNBC cells during So-2 treatment. According to our results, So-2 triggered several key ferroptosis biochemical events in TNBC cells, including ROS accumulation, GSH consumption and lipid peroxide accumulation. At the same time, So-2 down-regulated the expression of SLC7A11, GPX4, SLC40A11 and Glutaminase, and

up-regulated transferrin and HO-1, key proteins that are involved in ferroptosis pathway. Furthermore, So-2 downregulated the expression of GPX4 and upregulated transferrin in TNBC tissues in vivo. Therefore, we concluded that So-2 induced TNBC ferroptosis both in vitro and in vivo.

E2F7, as a transcription factor, plays a prominent regulatory part in various malignant tumors and acts as a promising therapeutic target for cancer therapy[19]. In breast cancer research, the results of Kaplan-Meier plotter database analysis showed that E2F7 and E2F8 were overexpressed in breast cancer patients. Meanwhile, The expression levels of E2F7 and E2F8 were inversely related to the overall survival, recurrence-free survival and distant metastasis-free survival in breast cancer patients[31]. In our study, TCGA database analysis showed that E2F7 expression was higher in breast cancer tissues in compare with normal breast tissues. We also detected the transcript and protein levels of E2F7 in normal and malignant breast cancer cell lines including MCF-10A, MCF-10F, MCF-7 and MDA-MB-231 cells. two breast cancer cell lines (MDA-MB-231 and MCF-7), and two normal breast epithelial cell lines (MCF-10A and MCF-10F). The results confirmed that E2F7 expressed in a higher level in malignant cells including MDA-MB-231 and MCF-7 than that in normal breast epithelial cell lines like MCF-10A and MCF-10F. Thus, the expression of E2F7 is proportional to the degree of tumor deterioration progression in TNBC. Overexpressing E2F7 promotes MCF-7 migration, while interference with E2F7 inhibited the migration of TNBC cells. Therefore, our research provide evidence for the oncogenic role of E2F7 in TNBC, and targeting E2F7 maybe a potential treatment for TNBC in the future.

Importantly, we here proved that the natural compound So-2, isolated from Chinese traditional medicine, could down-regulate the expression of E2F7 in TNBC cells both in vitro and vivo, and overexpression of E2F7 weakened So-2-induced ferroptosis of TNBC. Thus, the ferroptosis-inducing and antitumor effect of So-2 might dependent on regulating the transcription factor E2F7, which is beneficial for us to further understand the role and mechanism of So-2 in TNBC, and help to evaluate its potential to be a drug candidate.

As was exhibited by our data, overexpressing E2F7 antagonized the role of So-2 in up-regulating HO-1 or down-regulating GPX4 and SLC7A11 expression in TNBC cells. Studies have shown that transcription factors E2F7/8 antagonize the activity of E2Fs by recruiting chromatin modification repressor complexes, thereby inhibiting gene expression[32]. Thus, there is a possibility that E2F7 may modulate the transcription of genes involved in ferroptosis pathway. However, the specific regulatory role of transcription factor E2F7 on ferroptosis genes (HO-1, GPX4, SLC7A11) needs to be further studied.

5. Conclusion

In summary, we uncovered the anti-tumor role and the underlying mechanism of So-2 in TNBC cell. Our results demonstrated that So-2 caused the cell cycle arrest of TNBC cell, and could inhibit TNBC cell proliferation and migration. We then clarified the role of So-2 in inducing ferroptosis in TNBC cell, potentially through down-regulating E2F7 expression (Fig. 8). Therefore, So-2 might be a novel ferroptosis

inducer. This study has important implications for the development of novel anti-TNBC drugs based on ferroptosis induction.

Declarations

Author contributions

Na Liu and Jing Zhang performed the experiments, analyzed the data, and wrote the first draft of the manuscript. Wenqi Duan, Tingting Luo, Lina Han and Cong Wu participated in the performance of experiments. Fengying Yang revised the manuscript. Di Ge and Hongwei Yue designed the research, revised, and approved the manuscript.

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Ethics approval and consent to participate

No applicable

Consent for publication

No applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interest

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

Acknowledgments

No applicable

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Figures

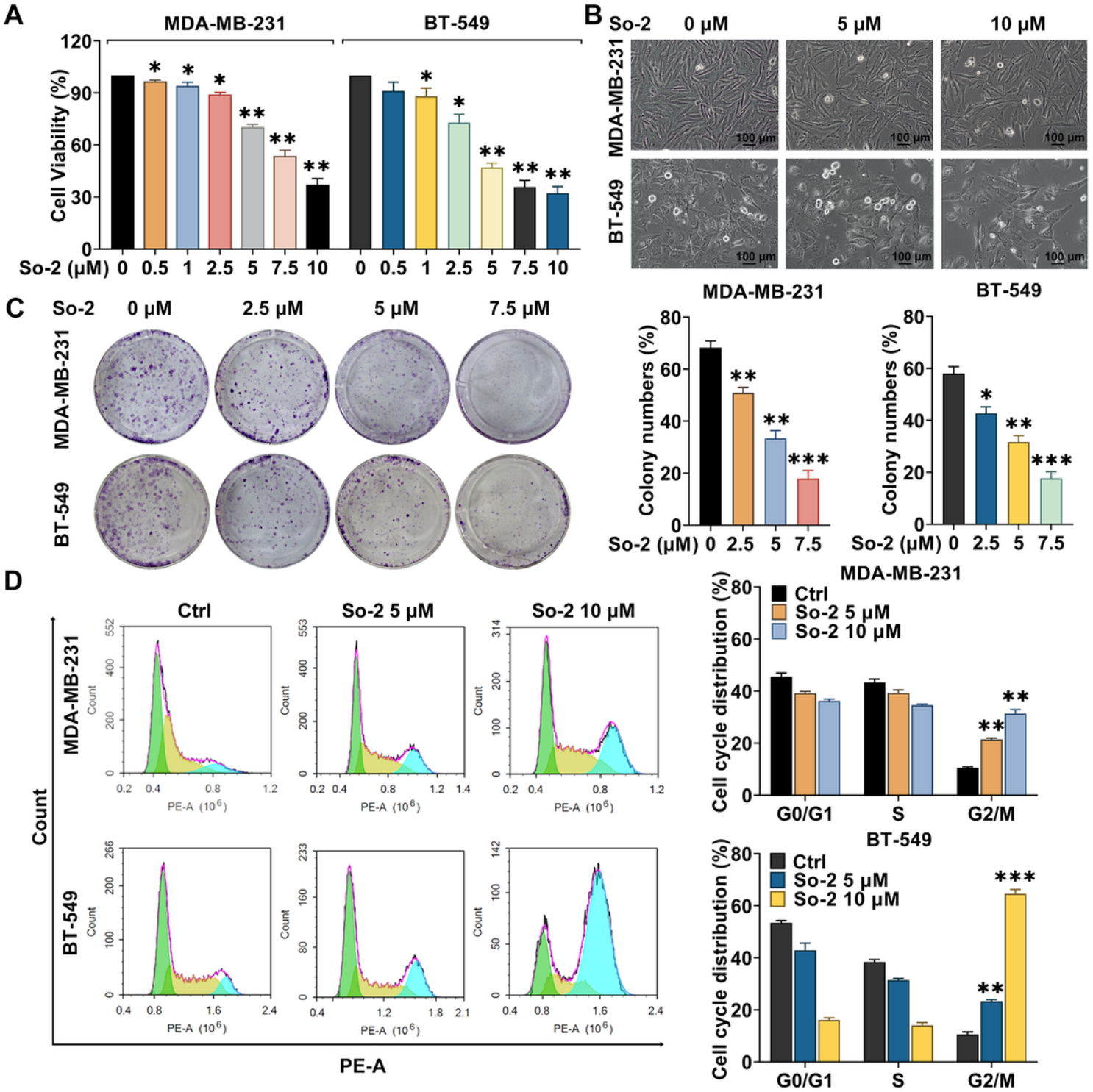

Figure 1

So-2 inhibited proliferation and arrested cell cycle at G2/M phase in TNBC cells.

(A) Cell viability was detected by SRB assay. (B) The typical morphological changes were observed under light microscope. Scale bars: 100 μm. (C) The colony formation was detected by crystal violet staining, and quantitative analysis. (D) The cell cycle distribution was analyzed by flow cytometry, and quantitative analysis. Values represent the mean±SD (n=3). *p<0.05, **p<0.01, ***p<0.001.

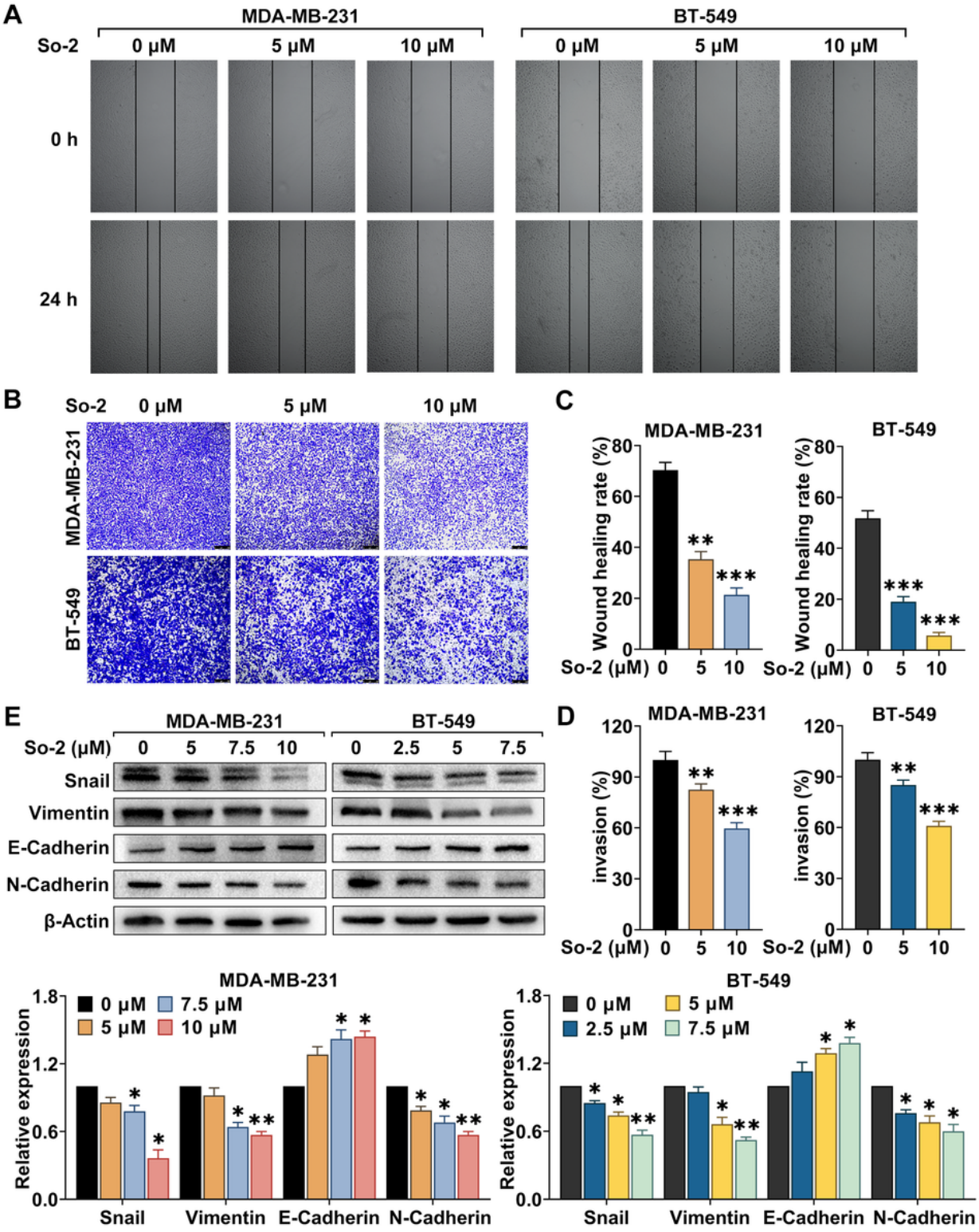

Figure 2

So-2 suppressed migration in TNBC cells.

(A) and (C) The cell migration was detected by wound healing assay, quantitative analysis was performed by Image J. (B) and (D) The cell migration was detected by transwell assay, quantitative analysis was performed by Image J. Scale bars: 250 μm. (E) The expression of several key cell metastatic

signal regulators, Vimentin, E-cadherin, N-cadherin, and Snail, were examined by western blotting, and quantitative analysis was performed by Image J. Values represent the mean±SD (n=3). *p<0.05, **p<0.01, ***p<0.001.

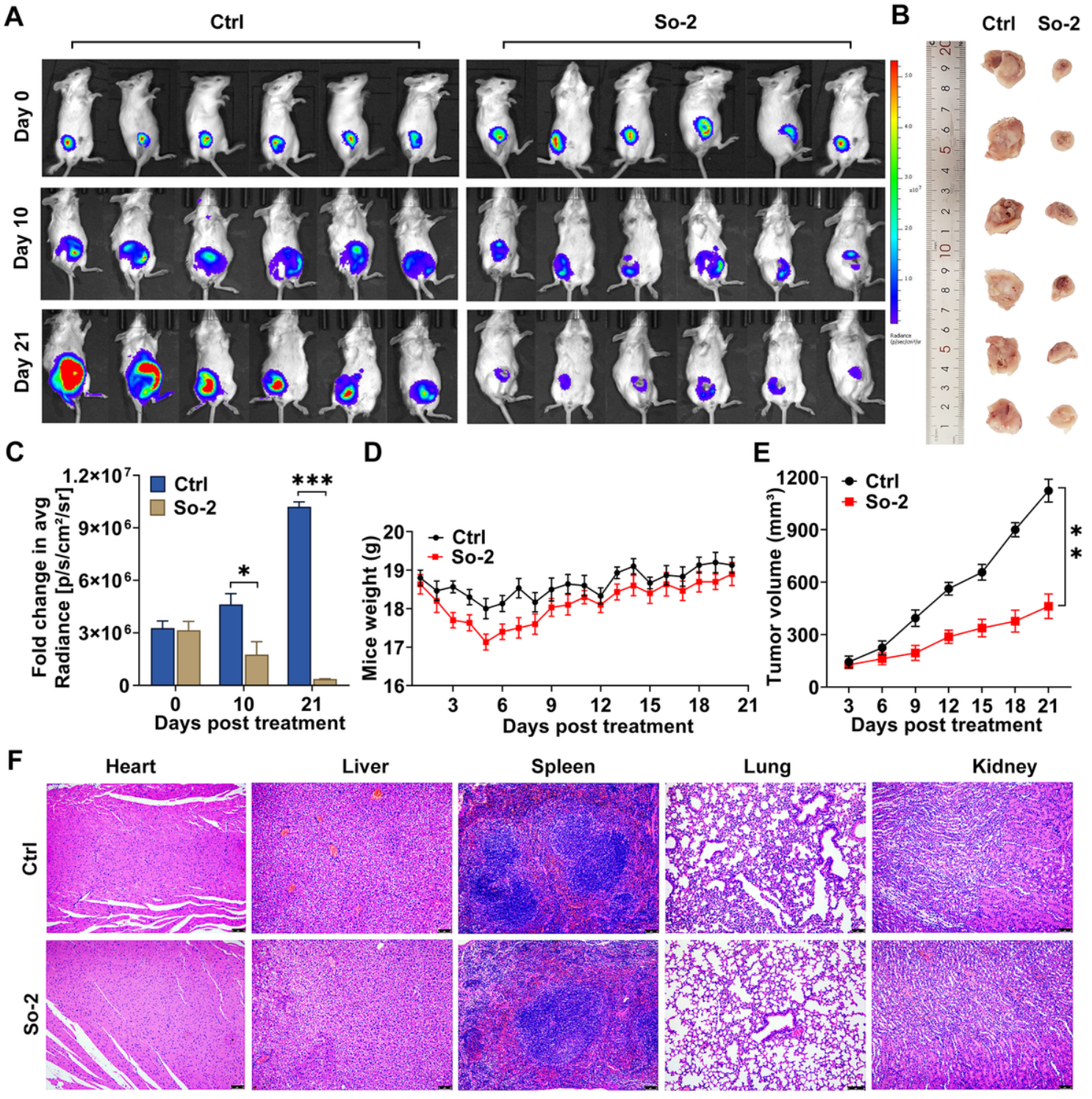

Figure 3

So-2 inhibits tumor growth in vivo.

(A) Representative bioluminescence images. (B) Images showed the tumors removed in each group (n=6). (C) Bioluminescence values plotted as a function of time in days to assess tumor growth (days 0, 10 and 21). (D) Body weight of mice from each group during the whole observation period. (E) Tumor volume of mice was measured every three days. (F) Representative images of H&E staining of heart, liver, spleen, lung, and kidney from mice in each group at the end of the observation period. Scale bars: 75μm. Values represent the mean±SD (n=3). *p<0.05, **p<0.01.

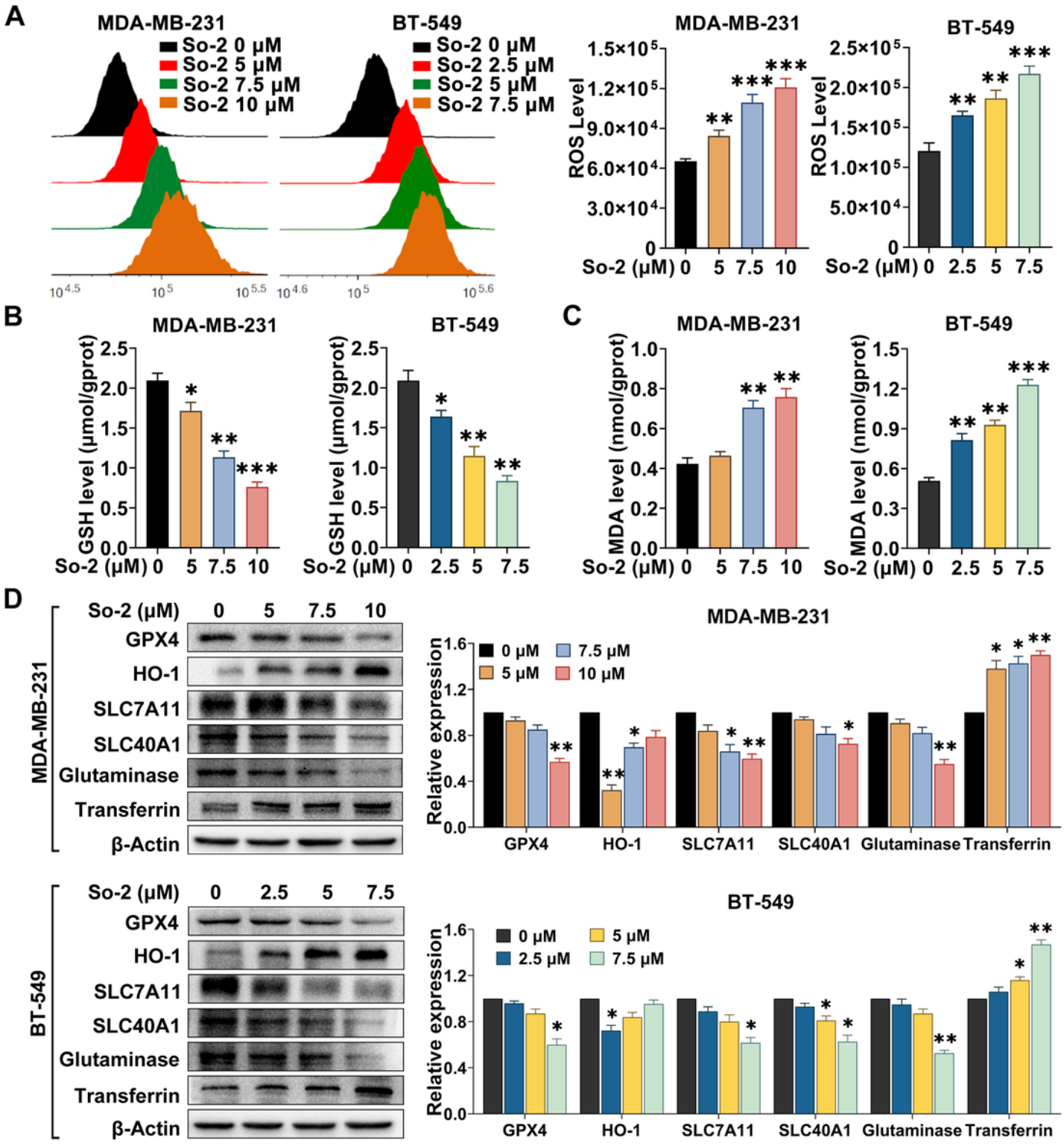

Figure 4

So-2 induced ferroptosis in TNBC cells.

(A) The intracellular ROS levels were analyzed by flow cytometry, and quantitative analysis. (B) Intracellular GSH levels. (C) Intracellular MDA levels. (D) The expression of several key ferroptosis regulators was examined by western blotting, and quantitative analysis was performed by Image J. Values represent the mean±SD (n=3). *p <0.05, *p<0.01, ***P < 0.001.

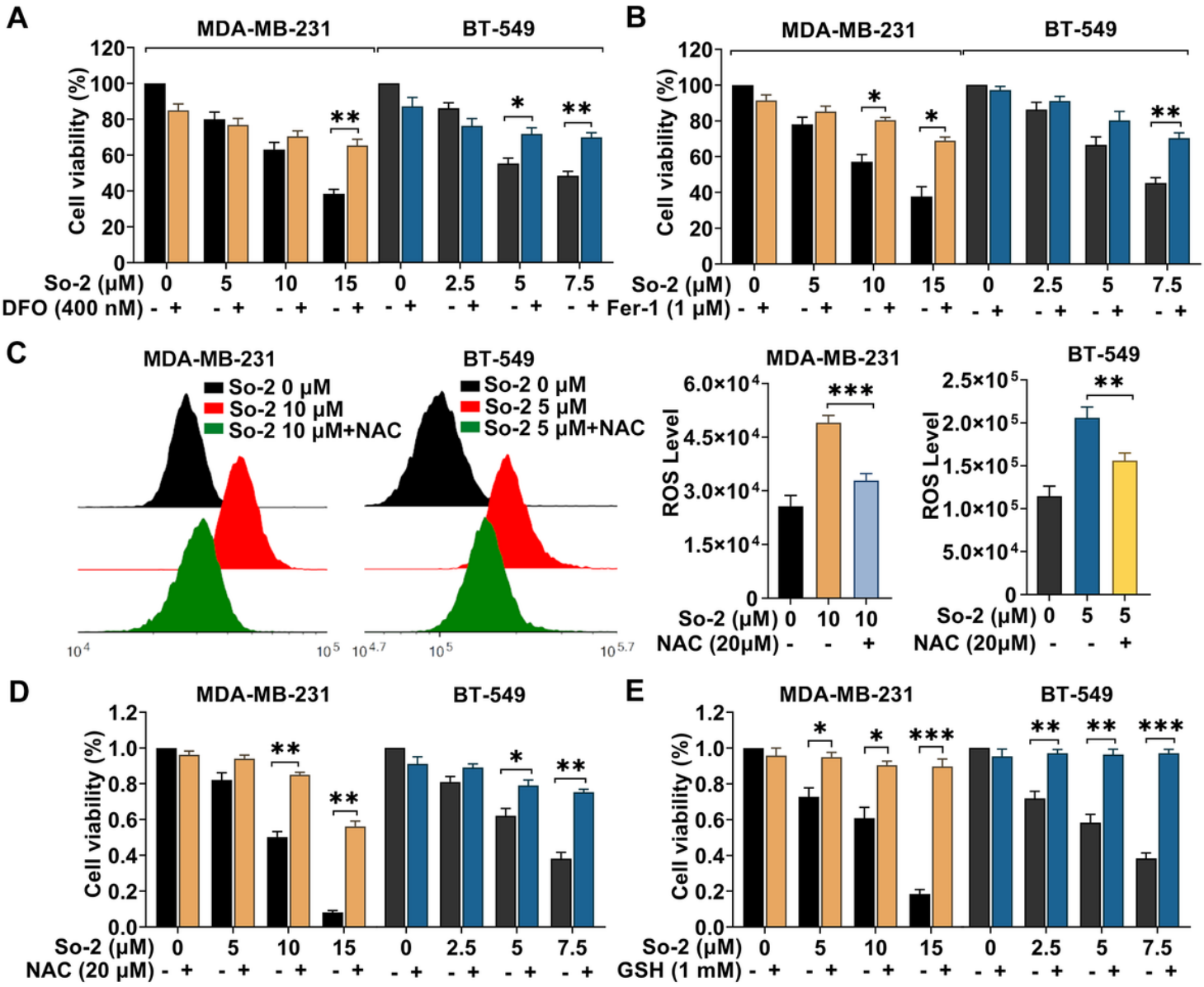

Figure 5

So-2 induced TNBC cell death could be reversed by ferroptosis inhibitors.

(A), (B), (D) and (E)The cell viability was detected by SRB assay. (C) The intracellular ROS levels were analyzed by flow cytometry. Values represent the mean±SD (n=3). *p <0.05, **p< 0.01, ***P < 0.001.

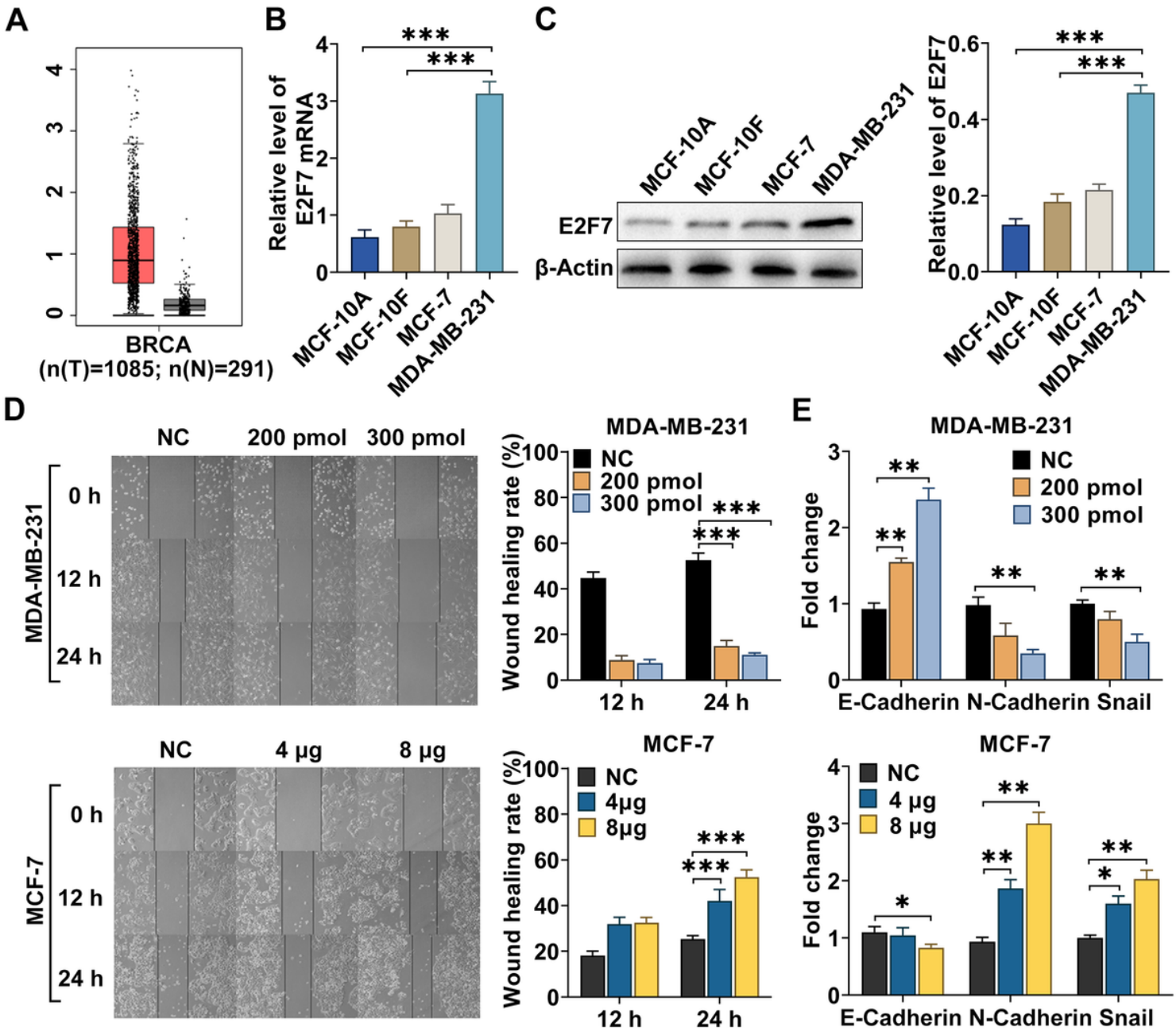

Figure 6

E2F7 acted as an oncogene in TNBC cells.

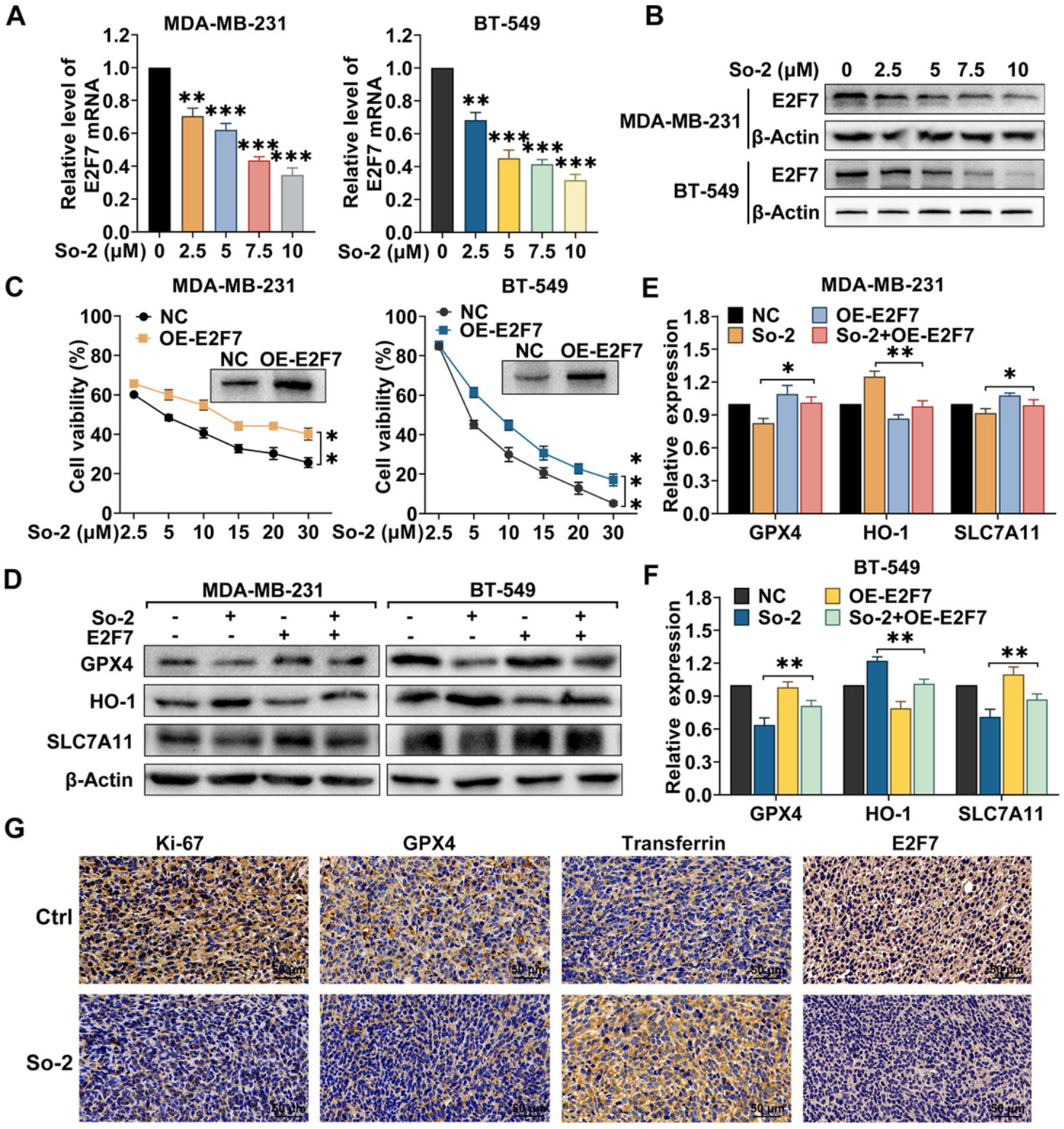

Figure 7

So-2 induced ferroptosis in TNBC cells by down-regulating the expression of E2F7.

(A) The expression level of E2F7 was examined by qPCR, and quantitative analysis. (B) The expression level of E2F7 was examined by western blotting. (C) The cell viability were detected by SRB assay. (D) The expression of ferroptosis related proteins was detected by western blotting. (E) and (F) Quantitative

analysis of ferroptosis related protein expression. (G) Images of immunohistochemical staining for Ki-67, GPX4, transferrin and E2F7 in tumors from each group as indicated, scale bars: 50 μm. Values represent the mean±SD (n=3). *p <0.05, **p< 0.01, ***P < 0.001.

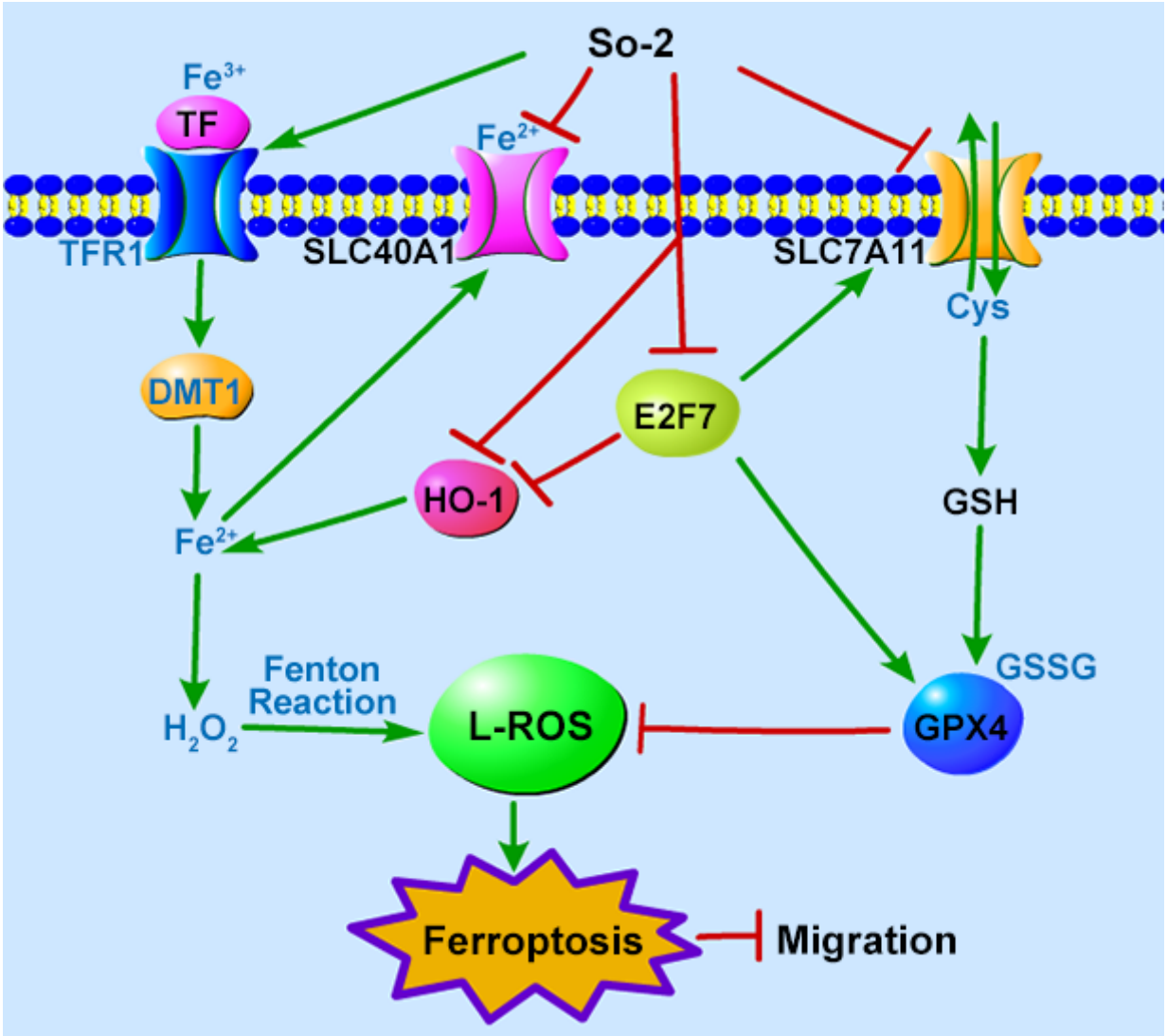

Figure 8

Scheme shown the essential role of So-2 in ferroptosis induction and cell migration inhibition.

Several signaling pathway proteins of ferroptosis are marked with blue and black fonts, and the proteins marked with black are involved in the experiments of our research.

Supplementary Files

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- SupplementaryMaterials.docx