Supplementary information

Exosomal *let-7a-5p* derived from human umbilical cord mesenchymal stem cells alleviates coxsackievirus B3-induced cardiomyocyte ferroptosis via the SMAD2/ZFP36 signal axis

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Materials and methods

Flow cytometer

To describe hucMSCs, CD44, CD45, CD90, and CD105 were identified as the previous report (Wu et al., 2021). Cells were collected and fixed with 4% paraformaldehyde. Anti-CD44 (#12-0441-82, eBioscience, USA), anti-CD45 (#12-0459-42, eBioscience, USA), anti-CD90 (#12-0909-42, eBioscience, USA), and anti-CD105 (#12-1057-42, eBioscience, USA) were added and incubated for 30 min. Cells were then analyzed by flow cytometry (Beckman Coulter, USA).

To determine the apoptotic rate, the Annexin V-APC/Propidium Iodide (PI) Apoptosis Detection Kit (#KGA1030-100, KeyGEN BioTECH, China) was performed according to the manufacturer's instructions. Annexin V-FITC and PI working solutions were added and incubated for 10 min. The sample was then analyzed for apoptosis using flow cytometry.

Osteogenic and adipogenic differentiation

As previously described (Wu et al., 2021), hucMSCs were induced for osteogenic and adipogenic differentiation. The Alizarin Red and Oil Red O stainings were performed to evaluate the osteogenic and adipogenic differentiation potential of hucMSCs.

Exosome extraction

The culture supernatants of hucMSCs were collected, and exosomes were isolated by ultracentrifugation as previously described (Mao et al., 2021). The collected supernatants underwent consecutive centrifugations at 3000×*g* and 10 000×*g* for 30 min each. After centrifugation at 64 000×*g*, exosomes were resuspended in sucrose. Finally, the exosomes were

centrifuged at 100 000×*g* for 90 min and resuspended with 100 μL of PBS. The Nanoparticle Tracking Analysis (NTA) was performed to detect the sizes of exosomes, and transmission electron microscopy (TEM) was used to observe exosome morphology as in the previous report (Gu et al., 2020).

TEM scanning

As previously described (Li et al., 2021), the cardiac and cell samples were fixed with glutaraldehyde. The samples were infiltrated with propylene oxide and epoxy resin after treatment with different concentrations of alcohol. Epoxy-embedded samples were then ultrathin sectioned. After staining with uranyl acetate and lead citrate, the TEM was used for observation.

Western blot

Protein levels were detected as previously described (Wu et al., 2021). Tissues, cells, and exosomes were lysed using radioimmunoprecipitation (RIPA) buffer, and protein concentrations were determined using the BCA Protein Assay Kit (#P0012, Beyotime, China). Proteins were separated in 10% polyacrylamide gel containing sodium dodecyl sulphate (SDS-PAGE). The 5% skim milk was used to block the nitrocellulose filter membrane. The membranes were incubated with primary and secondary antibodies for 90 min at room temperature. The protein expression was evaluated with a chemiluminescent imaging system (Chemiscope6100, CLINX, China). The primary antibodies, including anti-CD63 (#25682-1-AP), anti-TSG101 (#28283-1-AP), anti-SMAD2 (#12570-1-AP), anti-ZFP36 (#12737-1-AP), anti-GPX4 (#14432-1-AP), anti-SLC7A11 (#26864-1-AP), anti-p53 (#10442-1-AP), anti-PTGS2 (#12375-1-AP), and anti-GAPDH (#60004-1-Ig), were purchased from Proteintech (USA). The anti-Calnexin (#ab22595) was purchased from Abcam (UK). The anti-COX-2 (#12282), anti-Caspase-3 (#9662), anti-AKT (#9272), anti-p-AKT (#4060), anti-p70S6K (#2708), and anti-p-p70S6K (#9204) were purchased from CST (USA). The secondary antibodies used were HRP goat anti-mouse IgG (#SA00001-1, proteintech, USA) and HRP goat anti-rabbit IgG (#SA00001-2, proteintech, USA). GAPDH was used as a negative control for protein content.

Quantitative real-time PCR (qRT-PCR)

RNA samples were extracted with TRIzol (#15596062, Thermo, MA, USA). RNA was reversely transcribed to cDNA using the HiFiScript cDNA Synthesis Kit (#CW2569, CWBIOTECH, China) and miRNA cDNA Synthesis Kit (#CW2141, CWBIOTECH, China) based on the manufacturers' instructions. The UltraSYBR Mixture (#CW2601, CWBIOTECH, China) was added for PCR amplification. The relative RNA quantities were calculated using the $2^{-\Delta\Delta C_T}$ method after normalization. The primer sequences are shown in Table S1.

Table S1 Primers for qRT-PCR

Gene	Forward	Reverse
M-GAPDH	GCGACTTCAACAGCAACTCCC	CACCCTGTTGCTGTAGCCGTA
R-GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT
R-5S	GCCTACAGCCATACCACCCGGAA	CCTACAGCACCCGGTATCCCA
M-Smad2	AATCATTGCAACAAGAGGCAGT	ATTCCCGTCCCCATCATCCT
R-Smad2	TCCATCTTGCCATTCACTCCG	CAAGCTCATCTAATCGTCCTGT
R-let-7a-5p	TTCACTGTGGGATGAGGTAGT	TGTATAGTTATCTCCCAGTGGTGG
R-ZFP36	CTGACTTCTGCGAACCGACT	TGGGATGGAGTCCGATGAGT
M-ZFP36	GTTTGGGTGGATCCGTTCCT	CCAGGAGACCAGAGTTGCAG
M-GPX4	CATCGACGGGCACATGGTCT	CCACACTCAGCATATCGGGCAT
M-SLC7A11	CATACTCCAGAACACGGGCAG	AACAAAAGCCAGCAAAGGACCA
M-p53	CAGCCCCCTCTCTGAGTAGT	ACCCTATGAGGGCCCAAGAT
M-PTGS2	AATACTGGAAGCCGAGCACCT	ACACCCCTTCACATTATTGCAGA

Determination of the levels of GSH, MDA, and Fe^{2+}

The levels of GSH, MDA, and Fe^{2+} in myocardial tissue and CMCs were detected based on manufacturers' instructions. The kits of GSH (#A006-2-1) and MDA (#A003-1) were purchased from Nanjing Jiancheng Bioengineering Institute (China). The kit of Fe^{2+} (#ab83366) was purchased from abcam (UK).

Immunohistochemistry

As previously described (Dong et al., 2020), myocardial tissue slides were incubated overnight at 4 °C with a primary antibody, including anti-SLC7A11, anti-p53, and anti-PTGS2. The HRP-conjugated secondary antibody at 37 °C was used. Sections were developed with a DAB working solution and counterstained with hematoxylin for microscopy. Image-Pro-Plus was used to analyze images. The ratio of the cumulative optical density of the positive expression site to the sample area under the visual field was used to quantify the levels of SLC7A11, p53, and PTGS2.

Calcein AM/PI staining

To assess CMC cell viability, the Calcein AM (B7755, APExBIO, USA)/PI staining was performed. Cells were washed twice with PBS and then treated with a Calcein AM dye solution at a ratio of 1:1000 medium. After staining for 10 min in the dark, the solution was removed, and cells were washed and photographed using a microscope. PI staining solution was added to the cells, and the cells were stained in the dark for 30 min. Following the removal of the solution, the cells were washed twice with PBS and photographed under a microscope.

ROS detection

The level of ROS was detected using the ROS Assay Kit (#S0033S, Beyotime Biotechnology, China). To perform the assay, cells were first collected with the removal of the growth medium and then immersed in a diluted DCFH-DA solution. Cells were incubated at 37 °C for 20 min and

washed with the serum-free medium three times. Flow cytometry was used for detection after tryptic digestion.

Exosome uptake analysis

Exosomes were labeled with PKH67 and incubated at 37 ℃ for 20 min. The 100 μL labeled exosomes were cocultured with CMCs. After 12 h, cells were fixed with 4% paraformaldehyde and stained with DAPI. A cellular uptake analysis was conducted using an optical microscope.

CCK8 assay

The CCK8 assay was carried out using a CCK8 Kit (#NU679, DOJINDO, Japan). Following the manufacturer's instruction, 30 μL of CCK8 solution was added to each well. Cells were continuously cultured at 37 °C with 5% CO_2 for an additional 4 h. Proliferation activity was evaluated using absorbance at 450 nm.

TUNEL staining

TUNEL staining was adopted to analyze apoptosis of myocardial tissue and CMCs using the TUNEL Apoptosis Detection Kit (#40306ES50, YEASEN, China). After dewaxing, the myocardial tissue sections were treated with xylene and graded alcohol. CMCs cell slides were fixed with 4% paraformaldehyde. Next, 100 μL 1× Proteinase K working buffer and 1× Equilibration buffer were added. The 50 μL TdT incubation buffer was added and incubated at 37 °C for 60 min under dark conditions. The samples were nucleated with the DAPI working solution for 10 min and sealed with glycerin. The results were analyzed under an optical microscope.

Dual-luciferase reporter gene assay

Briefly, the pHG-MirTarget vectors with the wild-type (wt) or mutant-type (mut) sequence of SMAD2 3-untranslated region (UTR), miR-133a mimics and mimics NC were purchased from Honorgene (China). The pHG-MirTarget-SMAD2 wt, pHG-MirTarget-SMAD2 mut, miR-133a mimics, and mimics NC were co-transfected into CMCs via Lipofectamine 2000. The luciferase activity was tested 48 h later.

EdU staining

As previously described (Zhu et al., 2019), EdU staining was operated to analyze cell proliferation. The 50 μmol/L EdU working solution was added to the cultured cells, and 4% paraformaldehyde was used to fix the cells. Fluorescence microscopy was used for observation.

Co-immunoprecipitation (Co-IP) assay

Cells were performed by anti-SMAD2 (#12570-1-AP, Proteintech, USA) and anti-ZFP36 (#12737-1-AP, Proteintech, USA) as previously described (Zhang et al., 2020). Cells were lysed by cell lysis buffer for western and IP (#AWB0144a, Abiowell, China). The supernatant was

mixed with anti-SMAD2 and anti-ZFP36 overnight at 4 ℃ and then co-cultured with beads (Santa Cruz) for 2 h at 4 ℃. The expression of SMAD2 and ZFP36 was detected by western blot.

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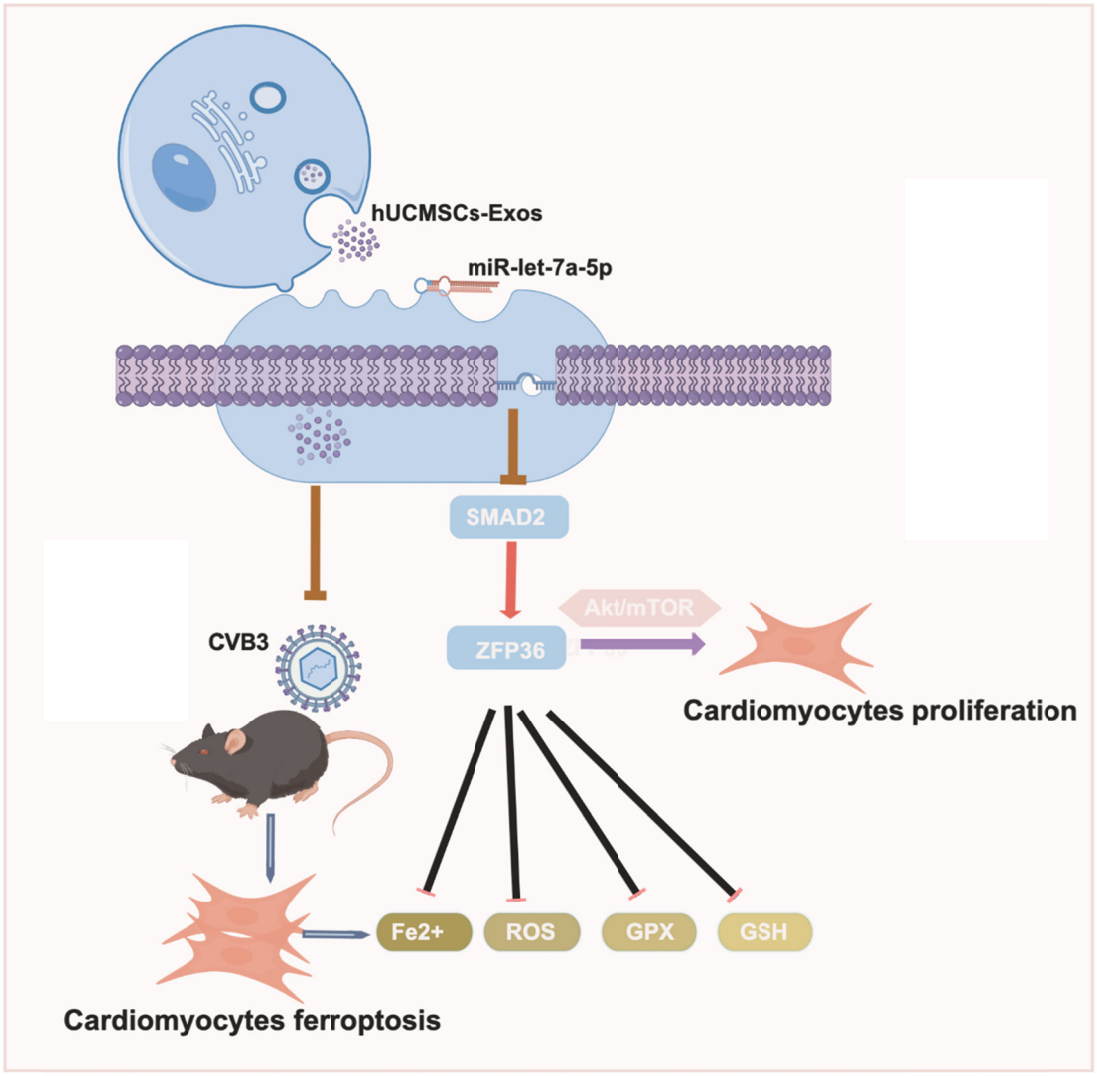

Fig. S1 Potential molecular mechanism of the exo-*let-7a-5p* from hucMSCs on ferroptosis in CVB3-induced VMC.

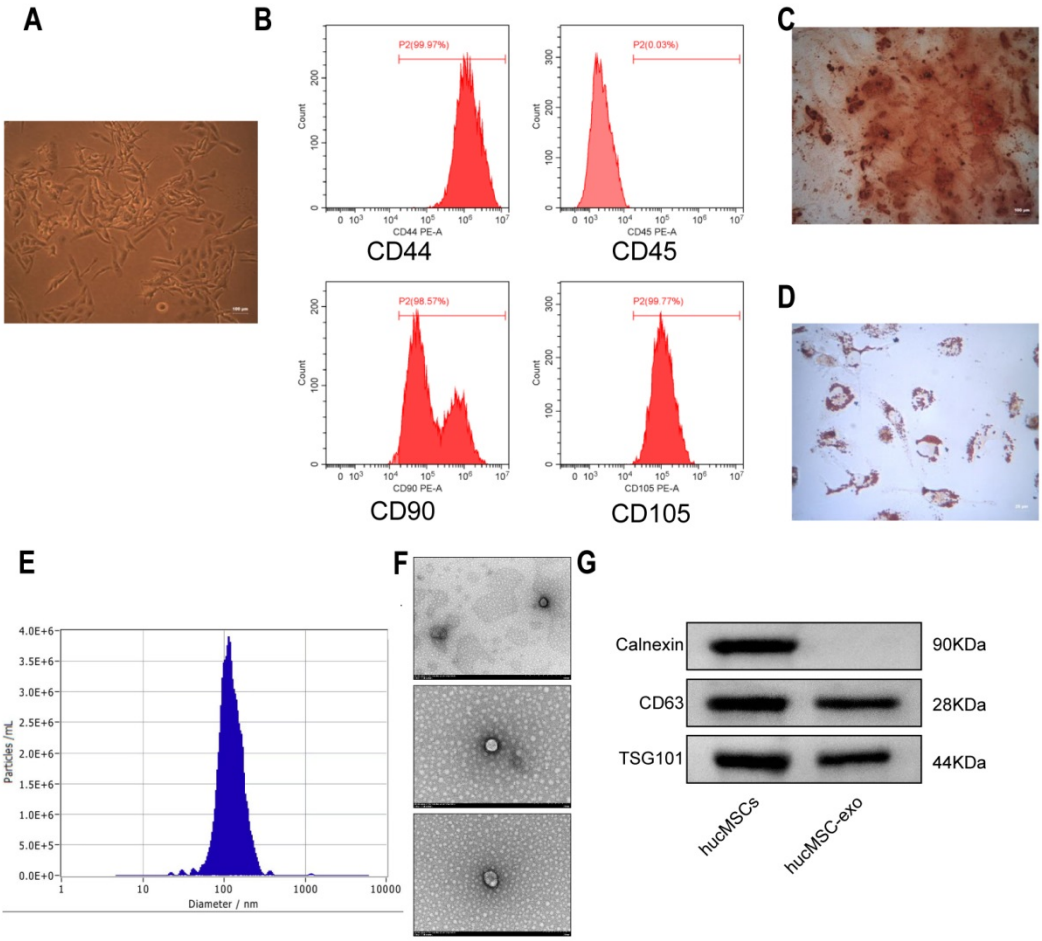

Fig. S2 Identification of hucMSCs and exosomes. (A) The morphology of hucMSCs (scale bar=100 μm). (B) Flow cytometry was used to identify hucMSCs by examining biomarkers (CD44, CD45, CD90, and CD105). The purity of hucMSCs was greater than 98%. (C) Ossification of hucMSCs (scale bar=100 μm). (D) The adipogenic differentiation of hucMSCs (scale bar=25 μm). (E) NTA was performed to detect the sizes and concentration of exosomes. (F) TEM was used to observe exosome morphology (scale bar=500 nm (upper) and 200 nm (middle and lower)). (G) The levels of surface biomarkers of exosomes (Calnexin, CD63, and TSG101) by western blot.

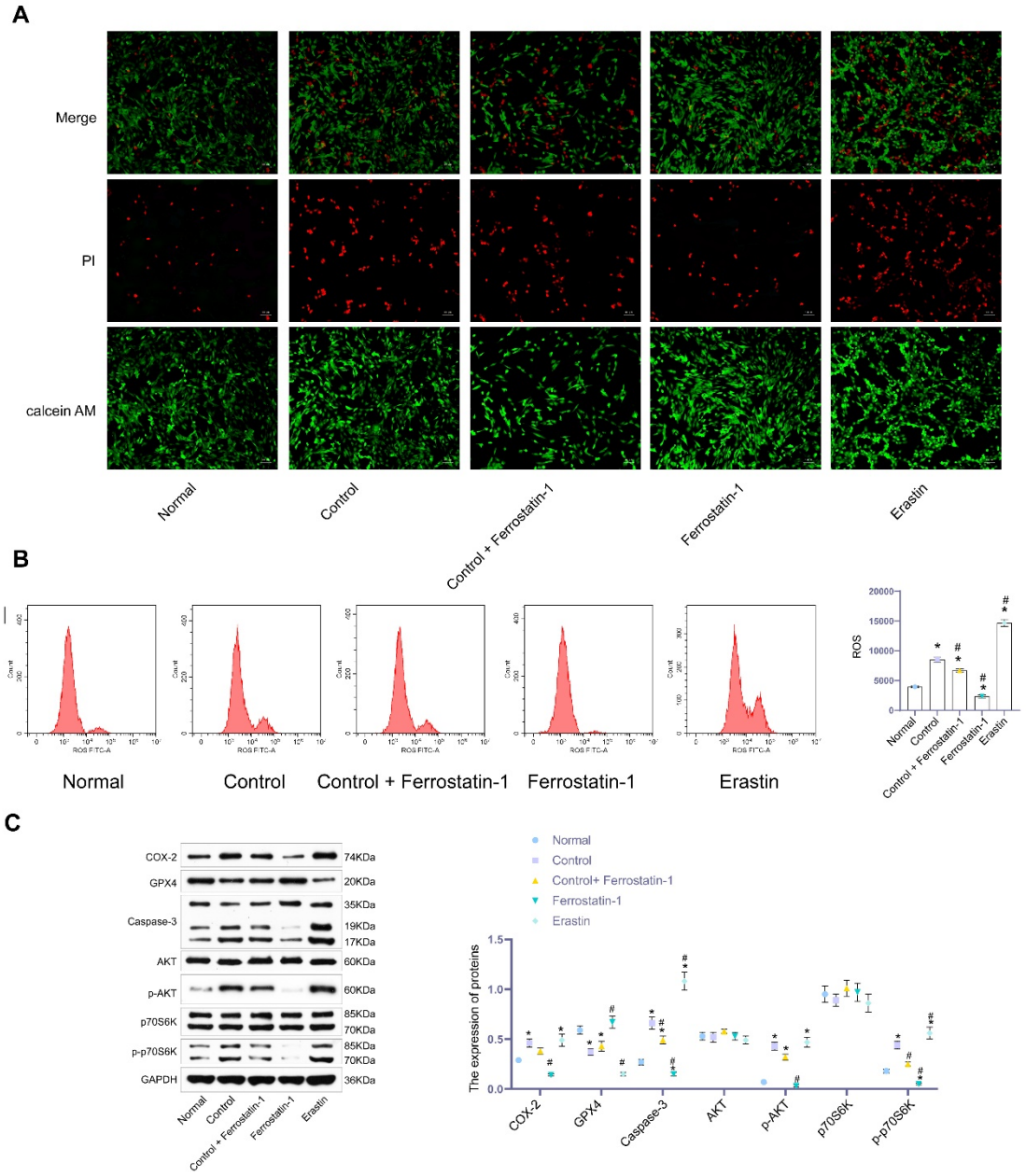

Fig. S3 CVB3 induces ferroptosis in CMCs. (A) Cell viability was assessed by Calcein AM/PI staining (scale bar=100 μm). CMC cell death increased in Control and Erastin groups. Compared with the Control group, the cell death rate in the Control+Ferrostain-1 group decreased. (B) Levels of ROS were higher in the Control and Erastin groups than in the Normal group. The levels of ROS were reduced after ferrostain-1 intervention. $^{*}P$<0.05 vs. Normal and $^{\#}P$<0.05 vs. Control, one-way ANOVA. (C) The protein levels of COX-2, GPX4, Caspase3, AKT, p-AKT, p70S6K, and p-p70S6K were detected. COX-2, Caspase-3, p-AKT, and p-p70S6K increased in the Control and Erastin groups, compared with the Normal group. The ferrostain-1 intervention reduced CVB3-induced increases in protein expression. GPX4 expression was attenuated after CVB3 and erastin treatment. $^{*}P$<0.05 vs. Normal and $^{\#}P$<0.05 vs. Control, two-way ANOVA. Data were presented as mean±standard deviation (SD) of three independent experiments.

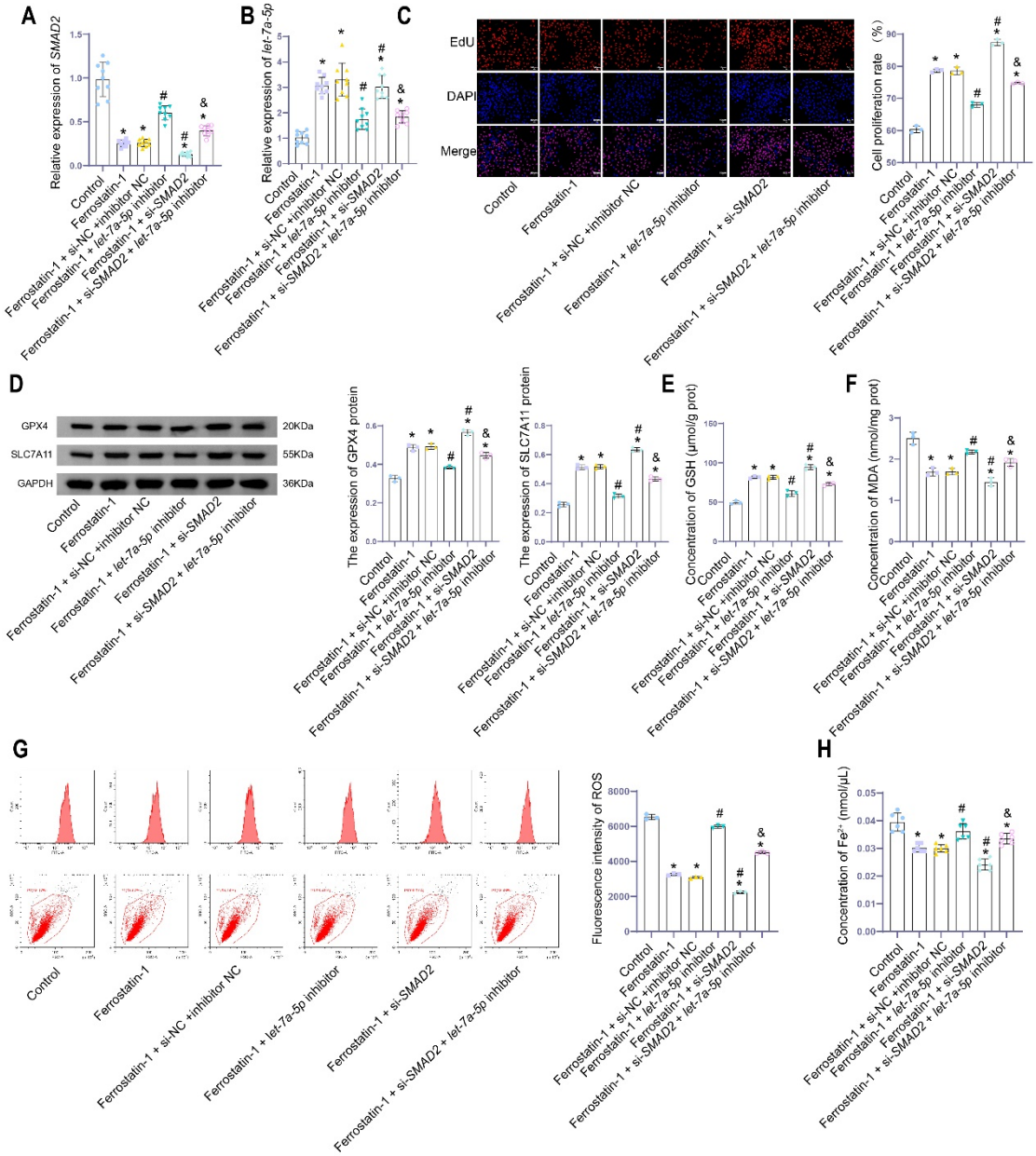

Fig. S4 (A, B) Levels of SMAD2 mRNA and *let-7a-5p*. (C) Proliferation was analyzed by EdU staining (scale bar=50 μm). Compared with Ferrostain-1+si-NC+inhibitor NC group, proliferation ability in the Ferrostain-1+*let-7a-5p* inhibitor group decreased. The proliferation in the Ferrostain-1+si-*SMAD2*+*let-7a-5p* inhibitor group was stronger than that of the Ferrostain-1+*let-7a-5p* inhibitor group. (D–H) Levels of GPX4, SLC7A11 and GSH were higher in the Ferrostain-1 group than in the Control group, while the expression of MDA, ROS, and Fe^{2+} decreased. $^{*}P$<0.05 vs. Control, $^{\#}P$<0.05 vs. Ferrostain-1+si-NC+inhibitor NC, and $^{\&}P$<0.05 vs. Ferrostain-1+si-*SMAD2*, one-way ANOVA. Data were presented as mean±standard deviation (SD) of three to nine independent experiments.