



Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

Iron and siRNA co-encapsulated ferritin nanocages induce ferroptosis synergistically for cancer therapy



Danni Liu^{a,b,c}, Yaoqi Wang^{a,b,c}, Qi Sun^{a,b,c}, Dong Mei^{c,d},
Xiaoling Wang^{c,d}, Yan Su^{c,e}, Jie Zhang^{a,b,c}, Ran Huo^{a,b,c},
Yang Tian^{a,b,c}, Siyu Liu^{a,b,c}, Shuang Zhang^{a,b,c,*}, Chunying Cui^{a,b,c,*}

^aSchool of Pharmaceutical Sciences, Capital Medical University, Beijing 100069, China

^bBeijing Area Major Laboratory of Peptide and Small Molecular Drugs, Engineering Research Center of Endogenous Prophylactic of Ministry of Education of China, Beijing Laboratory of Biomedical Materials, Beijing 100069, China

^cLaboratory for Clinical Medicine, Capital Medical University, Beijing 100069, China

^dDepartment of Pharmacy, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing 100045, China

^eMedical Oncology Department, Pediatric Oncology Center, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing 100045, China

Received 9 August 2024; received in revised form 28 August 2024; accepted 15 September 2024

KEY WORDS

Ferroptosis;
Ferritin;
Iron homeostasis;
siRNA;
Gene silencing;
Glutathione peroxidase 4;
Antitumor;
Lipid peroxidation

Abstract Ferroptosis has received great attention as an iron-dependent programmed cell death for efficient cancer therapy. However, with the accumulation of iron in tumor cells, the antioxidant system is activated by reducing glutathione (GSH) with glutathione peroxidase 4 (GPX4), which critically limits the ferroptosis therapeutic effect. Herein, an iron and GPX4 silencing siRNA (siGPX4) co-encapsulated ferritin nanocage (HFn@Fe/siGPX4) was developed to enhance ferroptosis by disruption of redox homeostasis and inhibition of antioxidant enzyme synergistically. The siGPX4 were loaded into the nanocages by pre-incubated with iron, which could significantly improve the loading efficiency of the gene drugs when compared with the reported gene drug loading strategy by ferritin nanocages. And more iron was overloaded into the ferritin through the diffusion method. When HFn@Fe/siGPX4 was taken up by human breast cancer cell MCF-7 in a TfR1-mediated pathway, the excess iron ions in the drug delivery system could for one thing induce ferroptosis by the production of reactive oxygen species (ROS), for another promote siGPX4 escaping from the lysosome to exert gene silencing effect more effectively. Both

*Corresponding authors.

E-mail addresses: zshuang@ccmu.edu.cn (Shuang Zhang), ccy@ccmu.edu.cn (Chunying Cui).

Peer review under the responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

<https://doi.org/10.1016/j.apsb.2024.10.006>

2211-3835 © 2025 The Authors. Published by Elsevier B.V. on behalf of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

the *in vitro* and *in vivo* results demonstrated that HFn@Fe/siGPX4 could significantly inhibit tumor growth by synergistical ferroptosis. Thus, the developed HFn@Fe/siGPX4 afforded a combined ferroptosis strategy for ferroptosis-based antitumor as well as a novel and efficient gene drug delivery system.

© 2025 The Authors. Published by Elsevier B.V. on behalf of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Ferroptosis is an iron-dependent cell death caused by unrestricted lipid peroxidation and plasma membrane rupture¹⁻⁴. Ferroptosis has been associated with a wide range of biological processes such as inflammation, aging, and cancer⁵⁻⁷. As ferroptosis plays an important role in tumorigenesis and tumor progression, it has been implicated in the development and therapeutic responses of various types of tumors, which provides a new direction for efficient cancer therapy⁸⁻¹¹. Currently, several FDA-approved agents have shown ferroptosis-promoting activity, such as sorafenib and cisplatin^{12,13}. However, these molecular agents have also shown significant limitations in preclinical analyses, such as *in vivo* instability and lack of tumor selectivity, hence, there is a need to develop effective ferroptosis-based tumor treatment strategies¹⁴⁻¹⁶.

Tumor cells have been proven to have a higher iron requirement than normal cells, often referred to as "iron addiction"^{17,18}. The excessive iron load may lead to ferroptosis in cancer cells^{14,19,20}. Researches has shown that iron accumulation in lysosomes can lead to the production of reactive oxygen species (ROS) over time through Fenton chemistry, resulting in lipid membrane peroxidation and lysosome membrane permeabilization (LMP)^{17,19}. Ferroptosis is mediated by multiple signaling pathways, but a single stimulus is usually ineffective²¹. When there is an imbalance in the oxidation/reduction system within the tumor cell, the antioxidant program spontaneously initiates to reduce the oxidative damage triggered by ferroptosis, and this defense mechanism weakens the anti-tumor effect of ferroptosis^{22,23}.

As an innate protective mechanism, tumor cells overexpress an antioxidant system with reduced glutathione (GSH) as the core to eliminate ROS, while glutathione peroxidase 4 (GPX4) is the key enzyme that catalyzes the antioxidant reaction²⁴⁻²⁶. Therefore, GPX4 is considered a promising prognostic marker and therapeutic target for a variety of cancers^{15,27,28}. Adjusting the expression of GPX4 can work synergistically with iron enrichment to improve the therapeutic efficiency and precise controllability of ferroptosis-mediated cancer treatment. Inhibition of GPX4 has been demonstrated to be effective in cancer therapy, and desirable approaches include GPX4 inhibitors, gene or protein expression, and others^{26,29,30}. RNA gene therapy is an important pathway for protein expression regulation and has a great prospect in the treatment of diseases such as tumor, cardiovascular disease and viral infection³¹⁻³⁵. It was found that siRNA had a better GPX4 gene silencing effect compared with shRNA³⁰. However, the characteristics of siRNA such as large molecular weight, negative electronegativity, and susceptibility to degradation by RNA enzymes limit the application of siRNA³⁶⁻³⁸. So, finding an ideal drug carrier for *in vivo* delivery of siRNA is an urgent problem.

Ferritin nanocages are currently a hotspot in the study of drug delivery systems³⁹⁻⁴¹, which are stable, highly water-soluble,

biocompatible, and can be disassembled/assembled under certain conditions, and their appropriate nano-size facilitates enhanced permeability and retention effect (EPR)⁴¹⁻⁴³. Ferritin heavy chains (HFn) can specifically bind to transferrin receptor 1 (TfR1), which is overexpressed on the surface of many tumor cells^{44,45}, and have been reported to be widely used for delivering anticancer drugs and diagnostic reagents⁴⁶⁻⁴⁸. Ferritin nanocages have a natural affinity for metal ions and one ferritin can be loaded with nearly 4000 iron atoms^{49,50}. In addition, the pH method has been successfully employed to load siRNA into HFn, it is worth noting that the direct loading of the surface negatively charged HFn with equally negatively charged siRNA did not show satisfactory results⁵¹. However, the assistance of metal ions such as Ca²⁺ can promote the loading of siRNA⁵². It has also been shown that metal ions compete with other drugs to enter into HFn at the same time⁵³, so an improved preparation method is needed to ensure maximum siRNA encapsulation when assisted with metal ions. Also, lysosomal escape of siRNA is an issue that limits its application⁵⁴⁻⁵⁶. Previous studies have shown that except for inducing ferroptosis, the iron ions could promote the lysosomal escape of the gene drugs into the cytoplasm to exert the gene-silencing effect more efficiently⁵⁷. Combining administration with siGPX4 and iron ions is a promising strategy to enhance the effects of both drugs to play a tumor-inhibiting effect.

In this study, a ferritin nanocage-based co-encapsulated drug delivery system for iron ions and antioxidant-regulated genes (HFn@Fe/siGPX4) was prepared to solve the challenge that single iron enrichment cannot effectively induce ferroptosis. The loading capacity of the gene drug was greatly increased by pre-incubation of the gene drugs with iron ions, and the overloading of iron ions was achieved by diffusion. When this nano-delivery system was administrated *in vivo*, owing to the tumor-targeting and drug-protecting effects of ferritin nanocages, iron ions and gene drugs could be effectively delivered to tumor tissues and cells. Not only overloaded iron ions played a role in inducing ferroptosis, but they also promoted the escape of gene drugs from lysosomes to modulate antioxidant systems, enhance cellular sensitivity to iron ions, and synergistically promote ferroptosis. Through these works, the drug-loading capacity of gene drugs could be improved and the application of HFn-loaded gene drugs could be expanded, and at the same time, it also provided new insights for inducing ferroptosis synergistically.

2. Materials and methods

2.1. Materials

Ferritin heavy chain (HFn) was purchased from MCE (HY-P70246, Monmouth Junction, NJ, USA). siGPX4/Cy5-siGPX4 (sense strand: 5'-GGAUGAAGAUGCAACCAATT-3'; anti-sense strand: 5'-UUGGGUUGGAUCUUCAUCCAC-3') and GPX4

primer were purchased from GenePharma (Shanghai, China). Ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) was purchased from Acme (Shanghai, China). Ferrous sulfate (FeSO_4) was purchased from YAANDA Biology (Beijing, China). DEPC water, 4% paraformaldehyde, agarose, methylene blue (MB) solution and RIPA were purchased from Solarbio (Beijing, China). RNase A and Protease K were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3,3',5,5'-Tetramethylbenzidine (TMB) solution and LysoTracker Green were purchased from Beyotime (Shanghai, China). Hoechst 33342, LipoTM2000 and Trizol were purchased from Life Technologies (Gaithersburg, MD, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Aladdin (Shanghai, China). H_2DCFDA was purchased from MCE (Monmouth Junction, NJ, USA).

2.2. Preparation and characterization of HFn@Fe/siGPX4

siGPX4 was internalized into HFn following the below steps. Briefly, siGPX4 was incubated with 20 mmol/L FeCl_3 for 20 min at room temperature (RT) and then ultrafiltered using an Amicon filter (Merck Millipore, MWCO = 10 kDa, Billerica, MA, USA) to remove excess Fe^{3+} . HFn was dissolved in buffer (20 mmol/L Tris, 0.15 mol/L NaCl, pH 8.0) and the Fe-siGPX4 complex was added, then was achieved by lowering the pH to 2.0 by adding 0.1 mol/L HCl following shaking at $0.1 \times g$ on a shaking incubator (IKA, KS 260 basic, Staufen, Germany) for 20 min at RT. After that, the pH of the mixture was adjusted to 8.0 with 0.1 mol/L NaOH which was added dropwise slowly. The mixture was shaken at $0.1 \times g$ on a shaking incubator (IKA) for 2 h continuously at RT. Then, 20 mmol/L FeSO_4 was added and was shaken for another 2 h at RT. Finally, the unencapsulated siGPX4 and Fe^{3+} were removed by ultrafiltration using an Amicon filter (Merck Millipore, MWCO = 50 kDa, Billerica, MA, USA). The particle size and zeta potential of HFn@Fe/siGPX4 were measured using a Zetasizer (Malvern Panalytical, Nano ZS 90, Malvern, UK). The morphology of HFn was determined by transmission electron microscopy (JEOL, JEM-2100, Tokyo, Japan). The morphology and energy dispersive spectroscopy mapping of HFn@Fe/siGPX4 were determined by a high-resolution transmission electron microscopy (HRTEM, FEI, Tecnai G2 F30, Hillsboro, OR, USA). 2% Agarose gel electrophoresis (AGE) was used to demonstrate the successful loading of siGPX4 and was imaged with ChemiDocTM (Bio-Rad, Hercules, CA, USA).

2.3. Internalization efficiency

Particularly, to quantitatively evaluate the internalization efficiency of siGPX4 in HFn, Cy5-siRNA was used as an indicator. For the analysis, HFn@Fe/siGPX4 were disassembled by adjusting the pH of the solution to 2.0 with 0.1 mol/L HCl, and the fluorescence intensity of Cy5 was determined using a fluorescence spectrophotometer (SHIMADZU, RF-6000, Kyoto, Japan). And the loading efficiency of siGPX4 was also examined by AGE. Additionally, the total iron content of NPs was determined by an Iron Assay Kit (LEAGENE, TC1015, Beijing, China). The internalization efficiency of siRNA was calculated using Eq. (1):

$$\text{Internalization efficiency (\%)} = \frac{\text{Sample content}}{\text{Total content}} \times 100 \quad (1)$$

2.4. RNase stability, serum stability and storage stability

Protecting siRNA from RNAase degradation is essential for siRNA delivery *in vivo*⁵⁸. To investigate the siRNA protection of HFn@Fe/siGPX4, naked siGPX4 and HFn@Fe/siGPX4 were incubated with RNase A for different times. To check the stability of HFn@Fe/siGPX4 in the serum, HFn@Fe/siGPX4 was incubated with serum-containing PBS solution (50% FBS, pH 7.4). To check the stability of HFn@Fe/siGPX4, HFn@Fe/siGPX4 was stored in PBS at 4 °C for 14 days. 2% AGE was used to monitor the siGPX4 of HFn@Fe/siGPX4. The gel was run at 100 V for 30 min and subsequently imaged using ChemiDocTM (Bio-Rad). Meanwhile, the particle size and zeta potential of HFn@Fe/siGPX4 were determined for 14 days. The amount of Fe^{2+} and Fe^{3+} in HFn after storing in PBS at 4 °C for 14 days was detected by X-ray photoelectron spectrometry (Thermo Fisher Scientific, K-Alpha, Waltham, MA, USA).

2.5. Evaluation of the catalytic activity at solution level

Briefly, HFn@Fe/siGPX4 (the concentration of Fe was 1.25 $\mu\text{g/mL}$) was added to 200 μL of TMB solution or 50 μL of MB solution, and the pH of the solution was adjusted to 2.0 by adding 0.1 mol/L HCl, the concentration of H_2O_2 was 50 nmol/L, followed by reacting at 37 °C for 5 min, and then spectroscopic detection was carried out by a UV spectrophotometer (SHIMADZU, UV-2600, Kyoto, Japan), and the detection wavelength was from 400 to 800 nm. The effects of different concentrations of HFn@Fe/siGPX4 and reaction times on the absorbance of TMB at 652 nm and the effects of different reaction times on the UV absorption of MB were also examined. In addition, the ability of the nanoparticles (the concentration of Fe was 1.25 $\mu\text{g/mL}$) to generate $\cdot\text{OH}$ was examined using electron paramagnetic resonance (JEOL, FA-300, Tokyo, Japan).

2.6. Cell culture

MCF-7 cells, RAW264.7 cells and THP-1 cells were obtained from the Chinese Academy of Sciences Shanghai Cell Bank. Dulbecco's modified Eagle's medium (DMEM) medium and 1640 medium were purchased from Servicebio (Wuhan, China). Fetal bovine serum (FBS) was purchased from PAN-Seratech (Aidenbach, Germany). Trypsin, Penicillin and streptomycin were purchased from HyClone (South Logan, UT, USA). MCF-7 cells and RAW264.7 cells were cultured in DMEM containing 10% (v/v) FBS and 1% antibiotics (penicillin, 100 U/mL; streptomycin, 100 $\mu\text{g/mL}$). THP-1 cells were cultured in 1640 containing 10% (v/v) FBS and 1% antibiotics (penicillin, 100 U/mL; streptomycin, 100 $\mu\text{g/mL}$). The culture condition was at 37 °C and under 5% CO_2 atmosphere.

2.7. Cellular uptake

The cellular uptake of siGPX4 and HFn@Fe/siGPX4 was qualitatively evaluated by confocal laser scanning microscopy (CLSM, Leica, TCS SP5, Wetzlar, Germany). Different types of cells were seeded into Petri dishes (Biosharp, BS-20-GJM, Hefei, China) at a density of 1.5×10^5 cells per dish. After incubation for 12 h, Cy5-siGPX4 and HFn@Fe/Cy5-siGPX4 (Cy5-siRNA, 20 nmol/L) were added into dishes for 4 h. Then the cells were treated for 10 min with Hoechst 33342 (10 $\mu\text{g/mL}$) at RT to stain the cell

nuclei. Finally, the cells were washed with PBS and observed using a CLSM (Leica). In addition, the cellular uptake efficiency of Cy5-siGPX4 and HFn@Fe/Cy5-siGPX4 were examined using flow cytometry (FCM, BD, LSRFortessa SORP, San Jose, CA, USA). Different cells were seeded in 24-well plates at 1×10^5 cells per well. After incubation for 12 h, the cells were treated with Cy5-siGPX4 and HFn@Fe/Cy5-siGPX4 (Cy5-siRNA, 20 nmol/L) for 4 h and then collected for FCM. The data were analyzed using the Flowjo software (TreeStar, v10.8.1, Ashland, OR, USA).

An antibody-blocking assay was performed to investigate the uptake behavior of HFn@Fe/Cy5-siGPX4 *via* TfR1 in MCF-7 cells following a general protocol. Briefly, cells were incubated for 4 h with 20 nmol/L of HFn@Fe/Cy5-siGPX4 in the presence or absence of 1 μ L antiTfR1 mAbs (Beyotime, Beijing, China). After that, the cells were washed three times with PBS, and the fluorescence signal was measured by CLSM and FCM similarly as done in the cellular uptake study.

2.8. Lysosomal escape

To verify that HFn@Fe/Cy5-siGPX4 could escape from lysosomes, HFn@Cy5-siGPX4 and HFn@Fe/Cy5-siGPX4 (Cy5-siRNA, 20 nmol/L) were incubated with MCF-7 cells for 1 and 4 h. After incubation for different times, the cells were washed with PBS three times and stained with LysoTracker Green as the indicator of lysosomes. And the cells were treated for 10 min with Hoechst 33342 (10 μ g/mL) to stain the nuclei. Finally, the cells were washed with PBS and the lysosomal escape behavior was determined and analyzed using STimulated Emission Depletion (STED, Leica, TCS SP8, Wetzlar, Germany).

2.9. Cytotoxicity evaluation

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was applied to evaluate the cytotoxicity and biocompatibility of HFn, Fe, siGPX4, HFn@Fe, HFn@siGPX4 and HFn@Fe/siGPX4. MCF-7 cells were seeded into a 96-well plate at a density of 5×10^3 cells per well and incubated for 24 h. Then the cells were incubated with different concentrations of samples for 48 h. After incubation, 25 μ L MTT solution (5 mg/mL in sterile PBS) was added to each well and incubated for another 4 h at 37 °C. The medium was removed carefully and replaced by 150 μ L DMSO in each well. The plate was shaken for 5 min and the absorbance was measured using a microplate reader (Thermo Fisher Scientific, Multiskan Spectrum 1500, Waltham, MA, USA) to calculate the cell viability of each group. The untreated cells were taken as a negative control. Cell viability was calculated using Eq. (2):

$$\text{Cell viability (\%)} = \frac{(\text{OD value of the sample} - \text{OD value of the blank})}{(\text{OD value of the control} - \text{OD value of the blank})} \times 100 \quad (2)$$

2.10. Cell apoptosis analysis

The Annexin V-FITC/PI apoptosis detection kit (Beyotime, Beijing, China) was used to detect the apoptosis of MCF-7 cells. MCF-7 cells were placed into 24-well plates at a density of 1×10^5 cells per well for 12 h. Then, the cells were treated with

the medium containing different nanoparticles at a siGPX4 concentration of 80 nmol/L and co-incubated for 48 h. The cells were washed with cold PBS three times, and the following processing and staining of the cells were performed in accordance with the manufacturer's protocol. Finally, the apoptosis of the cells was detected by flow cytometry (BD LSRFortessa). The data were analyzed using the Flowjo software (TreeStar).

The HFn@Fe/siGPX4-induced cancer cell death was observed directly by Calcein-AM/PI staining. MCF-7 cells were seeded on 24-well plates at a density of 1×10^5 cells per well and cultured for 24 h. Subsequently, the cells were treated with different formulations (siGPX4, HFn@siGPX4 and HFn@Fe/siGPX4) at a concentration of 80 nmol/L siGPX4 for 24 h. After being stained with Calcein-AM (Beyotime, Beijing, China) and PI (Life Technologies, Gaithersburg, MD, USA) for 30 min, the cells were imaged by an inverted fluorescence microscope (Nikon, Ts2RFL, Tokyo, Japan).

2.11. ATP level assessment

The pretreatment of MCF-7 cells was basically the same as the cell apoptosis analysis experiment. ATP levels were measured according to the manufacturer's protocol of the ATP Assay Kit (Beyotime, Beijing, China). A multifunctional enzyme labeling instrument (Molecular Devices, SpectraMax Mini, Sunnyvale, CA, USA) was used for the detection of luminescence (RLU).

2.12. RT-qPCR

MCF-7 cells were placed into a 24-well plate at a density of 1×10^5 cells per well and cultured for another 12 h. Then, the cells were treated with different formulations (siGPX4, HFn@siGPX4, HFn@Fe/siGPX4 and lipo2000@siGPX4) at a concentration of 80 nmol/L siGPX4 for 48 h. For gene expression analysis, total RNA was isolated using Trizol and reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Gaithersburg, MD, USA) for RT-qPCR (Applied Biosystems, Real-Time PCR System 7500, Carlsbad, CA, USA). Quantitative real-time PCR was performed using the TaqMan® Gene Expression Master Mix (Life Technologies, Gaithersburg, MD, USA), and gene expression was normalized to *GAPDH*.

2.13. ELISA assay for GPX4

To detect intracellular GPX4, MCF-7 cells were incubated with treatments as the upper RT-qPCR experiment. For protein expression analysis, total proteins were extracted with RIPA and GPX4 protein assay was performed with a Human GPX4 ELISA Kit (FANKEW, Shanghai, China). The BCA kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify protein concentration.

2.14. Western blot analysis for GPX4

MCF-7 cells were processed and total proteins were extracted as per ELISA assay. Western blot (WB) was used to detect the relative expression of GPX4. Electrophoresis was carried on with 12% gradient Tris-glycine polyacrylamide gels (Beyotime, Beijing, China) for SDS-PAGE and electroblotted onto

polyvinylidene fluoride (PVDF, Beyotime, 0.22 μm , Beijing, China). PVDF membranes were blocked for 30 min at RT in QuickBlock™ blocking buffer (Beyotime, Beijing, China). Then, the membranes were incubated overnight at 4 °C with GPX4 rabbit polyclonal antibody (1:1000, Beyotime, Beijing, China). On the next day, the membranes were washed three times with TBST and incubated at RT for 2 h with HRP-labeled goat anti-rabbit IgG(H + L) (1:1000, Beyotime, Beijing, China) as the secondary antibody. The HRP-conjugated recombinant anti-beta actin antibody (1:1000, Servicebio, Wuhan, China) was used for the β -actin detection. The ECL signal was recorded with the BeyoECL Star (Beyotime, Beijing, China). The quantification of the signal intensity was carried out with the ChemiDoc™ (Bio-Rad) and the grayscale analysis was carried out with Image J software (National Institutes of Health, v1.8.0, Bethesda, MD, USA).

2.15. ROS evaluation

ROS probe H_2DCFDA was utilized to evaluate intracellular ROS levels. MCF-7 cells were processed as per ELISA assay. Next, drugs were abandoned and cells were washed by PBS. After that, H_2DCFDA (1×10^{-5} mol/L) was added and incubated for 30 min. The free fluorescence probe was removed by triple washing with PBS. Fluorescence images were obtained from an inverted fluorescence microscope (Nikon, Ts2RFL, Tokyo, Japan) and quantified using ImageJ software (National Institutes of Health).

2.16. Evaluation of intracellular iron ion

To measure the intracellular content of Fe in different treatments, MCF-7 cells were seeded in 24-well plates with a density of 1×10^5 cells per well. After attachment, cells were incubated with different formulations Fe, HFn@Fe and HFn@Fe/siGPX4 at a concentration of 0.5 $\mu\text{g/mL}$ Fe for 48 h. After that, cells were harvested to measure the iron ion content with the Iron Assay Kit (LEAGENE, Beijing, China). The BCA kit was used to quantify protein concentration.

2.17. Evaluation of intracellular GSH

To assess the intracellular concentration of GSH in various groups, MCF-7 cells were processed as per ELISA assay. Next, cells were harvested to detect the concentration of GSH using the GSH Content Assay Kit (LEAGENE, Beijing, China). The BCA kit was used to quantify protein concentration.

2.18. Lipid oxidation assay

For analyzing the intracellular concentrations of malondialdehyde (MDA) in various groups, MCF-7 cells were incubated with treatments as the upper GSH evaluation experiment. Next, cells were harvested and the MDA concentration was measured with a micro-MDA assay kit (Beyotime, Beijing, China). The BCA kit was utilized to measure protein concentration.

2.19. Ethics approval statement

All animal care and experiments were conducted in line with the Guide for the Care and Use of Laboratory Animals proposed by the National Institutes of Health (AEEI-2019-132). All experimental procedures were executed according to the protocols

approved by the Institutional Animal Ethics Committee of Capital Medical University.

2.20. Animals

BALB/c nude mice (female, about 4–6 weeks old) were used for animal studies. The animal research center was raised under a standard condition at 25 ± 2 °C and $60 \pm 10\%$ humidity environment with a 12 h light/dark cycle. For all the animal studies, mice were randomly allocated to each group. The investigator was aware of the group allocation during the animal studies as demanded by the experimental designs. For the establishment of MCF-7 tumor-bearing mice models, Female BALB/c nude mice were injected intraperitoneally with estradiol benzoate (10 mg/kg, dissolved in DMSO:corn oil = 1:9) every three days for two weeks. And then were subcutaneously injected with MCF-7 cells (5×10^6 /mice) at the right forelimb. The mice were also injected intraperitoneally with estradiol benzoate (10 mg/kg) every three days starting on the alternate day⁵⁹.

2.21. In vivo tumor targeting assay

For the analysis of the *in vivo* tumor targeting efficiency of HFn@Fe/siGPX4, MCF-7 tumor-bearing mice were randomly divided into different groups and injected intravenously with free Cy5-siGPX4 or HFn@Fe/Cy5-siGPX4, (Cy5-siGPX4 = 2 mg/kg). Then, an *in vivo* fluorescence imaging system (PerkinElmer, IVIS Spectrum, Waltham, MA, USA) was used to detect the Cy5 fluorescent signal at 1.5, 3, 6 and 7.5 h post-injection. In addition, the Cy5 fluorescent signals in major tissues of MCF-7 tumor-bearing mice were detected at 24 h post-injection.

2.22. In vivo antitumor efficacy

The MCF-7 tumor-bearing mice models were established. When the tumor volume reached 200 mm^3 , mice were randomly divided into four groups ($n = 6$). The mice were intravenously injected with 200 μL of different formulations (siGPX4, HFn@siGPX4 and HFn@Fe/siGPX4, siGPX4 = 1 mg/kg) or saline every three days for seven times. The tumor growth and body weight were monitored every two days, and the tumor volume was calculated as Eq. (3):

$$\text{Tumor volume (mm}^3\text{)} = (\text{Tumor length}) \times (\text{Tumor width})^2/2 \quad (3)$$

For tumor survival, the mice were monitored daily and considered dead when the tumor volume reached 1000 mm^3 for 50 days after the first treatment. At the end of observation, all of the tumors were collected, imaged and preserved for subsequent research. Tumor tissues were excised to perform H&E and TUNEL stain.

2.23. In vivo ferroptosis induction

Tumor tissues were used for GPX4 immunofluorescence analysis and ROS staining and quantified using ImageJ software (National Institutes of Health). Tumor tissues were additionally milled and assayed for GPX4 levels, iron content, MDA levels and GSH levels using the previously mentioned methods.

2.24. *In vivo* biosafety evaluation

The body weight of mice of each group was measured every other day to evaluate the general toxicity after the treatment. After 14 days treatment, mice were sacrificed. Their blood samples and major organs (heart, liver, spleen, lung and kidney) were collected. Part of their organs was sectioned into slices and stained with H&E staining. Pathology was examined by using a fluorescence microscope (Nikon, Eclipse Ti-SR, Tokyo, Japan). Five important hepatic indicators (*i.e.*, ALT, AST, ALP, ALB and TP), and three renal indicators (*i.e.*, UREA, UA and CREA) were measured by using a blood biochemical auto analyzer (Mindray, BS-430, Shenzhen, China).

2.25. Statistical analysis

All the results in this study are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were performed with GraphPad Prism (GraphPad Inc., v9.5, La Jolla, CA, USA) using one-way ANOVA. *P* values < 0.05 were considered to indicate significance.

3. Results and discussion

3.1. Characterization of HFn@Fe/siGPX4

HFn@Fe/siGPX4 were constructed using pre-complexation of siGPX4 with Fe^{3+} to neutralize the negative charge of siGPX4, and after removal of uncompensated free Fe^{3+} , Fe-siGPX4 loading was carried out using the pH method, followed by diffusion method for overloading Fe^{2+} . HFn@Fe/siGPX4 were dispersed in Tris-NaCl buffer (pH 7.4) with a clear Tyndall effect (Fig. 1A). Dynamic light scattering (DLS) indicated that the diameter of HFn@Fe/siGPX4 was 107.6 ± 7.2 nm (Fig. 1A), and the zeta potential was -11.85 ± 0.49 mV (Fig. 1B). As shown in Fig. 1B, obvious charge inversion could be observed when HFn@Fe/siGPX4 were dispersed in different pH (the pH values changed from 7.4 to 2.0). When the pH values were set at 7.4, the zeta potentials were negative but changed to positive at 5.0 and 2.0. The results showed that HFn@Fe/siGPX4 could dissolve into individual subunits in acidic environments⁶⁰, which may exhibit a positive charge due to the absorption of iron ions. Moreover, the dissolution of HFn@Fe/siGPX4 and charge inversion in low pH will favor the release and lysosomal escape of siGPX4, enhancing the silencing effect of gene drugs. The TEM image showed that HFn had a hollow cage structure with a particle size of 10–20 nm (Fig. 1C), while HFn@Fe/siGPX4 encapsulating iron ions had a rounded nanoparticle morphology with a particle size of 100–200 nm and were homogeneously distributed in size (Fig. 1D). The results of EDS mapping (Fig. 1E) showed that HFn@Fe/siGPX4 contained iron and the elements were uniformly distributed, indicating the successful preparation of HFn@Fe/siGPX4.

To investigate the gene encapsulating ability of HFn with or without the assistance of Fe^{3+} , Cy5 labeled siGPX4 was applied to load into HFn nanocages. The addition of Fe^{3+} could promote the loading of siGPX4 into HFn (Fig. 1F), and the encapsulation rate of siGPX4 of HFn-Fe@Cy5-siGPX4 ($74.43 \pm 0.38\%$) was 1.34-fold higher than that of HFn@Cy5-siGPX4 without Fe^{3+} ($55.41 \pm 2.97\%$). It suggested that pre-incubating siGPX4 with iron ions could promote the encapsulation of gene drugs by HFn nanocages. Meanwhile, the results (Fig. 1G and H) showed that

the maximum encapsulation was achieved when the molar ratio of Cy5-siGPX4 to HFn was 1:6 with Fe^{3+} assistance. It was noteworthy that by continuing to increase the amount of HFn, the encapsulation efficiency of siGPX4 did not increase, was even decreased. The possible reason for this was the increased chance of interaction with HFn subunits at high concentrations and the decreased interaction with Fe-siGPX4. The results were in keeping with the findings of Huang et al.⁵², who used calcium ions to promote the loading of siRNA to HFn, and Pang et al.⁶¹, who used iron ions to promote the loading of ATP. In addition, the iron loading capacity of HFn@Fe/siGPX4 was tested by a total iron assay kit, and the iron content was calculated to be 0.824 ± 0.003 $\mu\text{g/mL}$, indicating the successful load of iron ions in nanocages.

3.2. Stability of HFn@Fe/siGPX4 *in vitro*

The stability of HFn@Fe/siGPX4 against RNase A was tested by AGE. The results (Fig. 1I) showed that the band of siGPX4 remained bright for HFn@Fe/siGPX4 after 8 h of co-incubation with RNase A, whereas the band for naked siGPX4 almost disappeared. It suggested that HFn could protect siGPX4 from degradation by RNase A. Besides, the stability of HFn@Fe/siGPX4 against serum was also evaluated. As seen in Supporting Information Fig. S1, when incubated with 50% serum for 2 h, the band of naked siGPX4 was significantly weakened. While for HFn@Fe/siGPX4 the band remained clear even when incubated with 50% serum for 24 h. All these results ensured that the HFn@Fe/siGPX4 could protect the gene drugs from degradation by RNase A or serum when administrated *in vivo*, making it a promising system for gene drug delivery.

And the long-term stability of HFn@Fe/siGPX4 was also evaluated. The bright bands of siGPX4 were basically unchanged when HFn@Fe/siGPX4 were stored in PBS (pH 7.4) at 4 °C for 14 days as tested by AGE (Supporting Information Fig. S2), which indicated that the HFn@Fe/siGPX4 had good stability under this storage condition, and no obvious drug leakage was found. The particle size and zeta potential of HFn@Fe/siGPX4 were measured continuously, and the results (Supporting Information Fig. S3) showed that the particle size and zeta potential of HFn@Fe/siGPX4 remained unchanged under long-term storage conditions. The long-term stability experiments suggested that HFn@Fe/siGPX4 had good storage stability, which was of great significance to its application in the clinic.

It is well known that Fe^{2+} is easily oxidized. HFn@Fe/siGPX4 was stored in PBS (pH 7.4) at 4 °C for 14 days and the Fe^{2+} content was detected using XPS. As shown in Fig. 1J, Fe^{2+} content was 19.50% of total Fe, indicating that Fe^{2+} in HFn@Fe/siGPX4 was not fully oxidized, which was conducive to inducing ferroptosis *in vitro* and *in vivo*.

3.3. *In vitro* catalytic activities

High levels of H_2O_2 are one of the most prominent symbols and the major etiological factors of tumors⁶², meanwhile, most anti-cancer drugs exert the anti-tumor effect by increasing intracellular ROS⁶³. Fe^{2+} has the ability to catalyze H_2O_2 to produce ROS by Fenton reaction⁶⁴. The Fe ions encapsulated in HFn@Fe/siGPX4 could serve as Fenton catalysts to transform H_2O_2 into $\cdot\text{OH}$. The results of EPR (Fig. 1K) demonstrated that Fe^{2+} contained in HFn@Fe/siGPX4 could react with H_2O_2 to produce $\cdot\text{OH}$. And the colorimetric method based on H_2O_2 oxidation of TMB was used

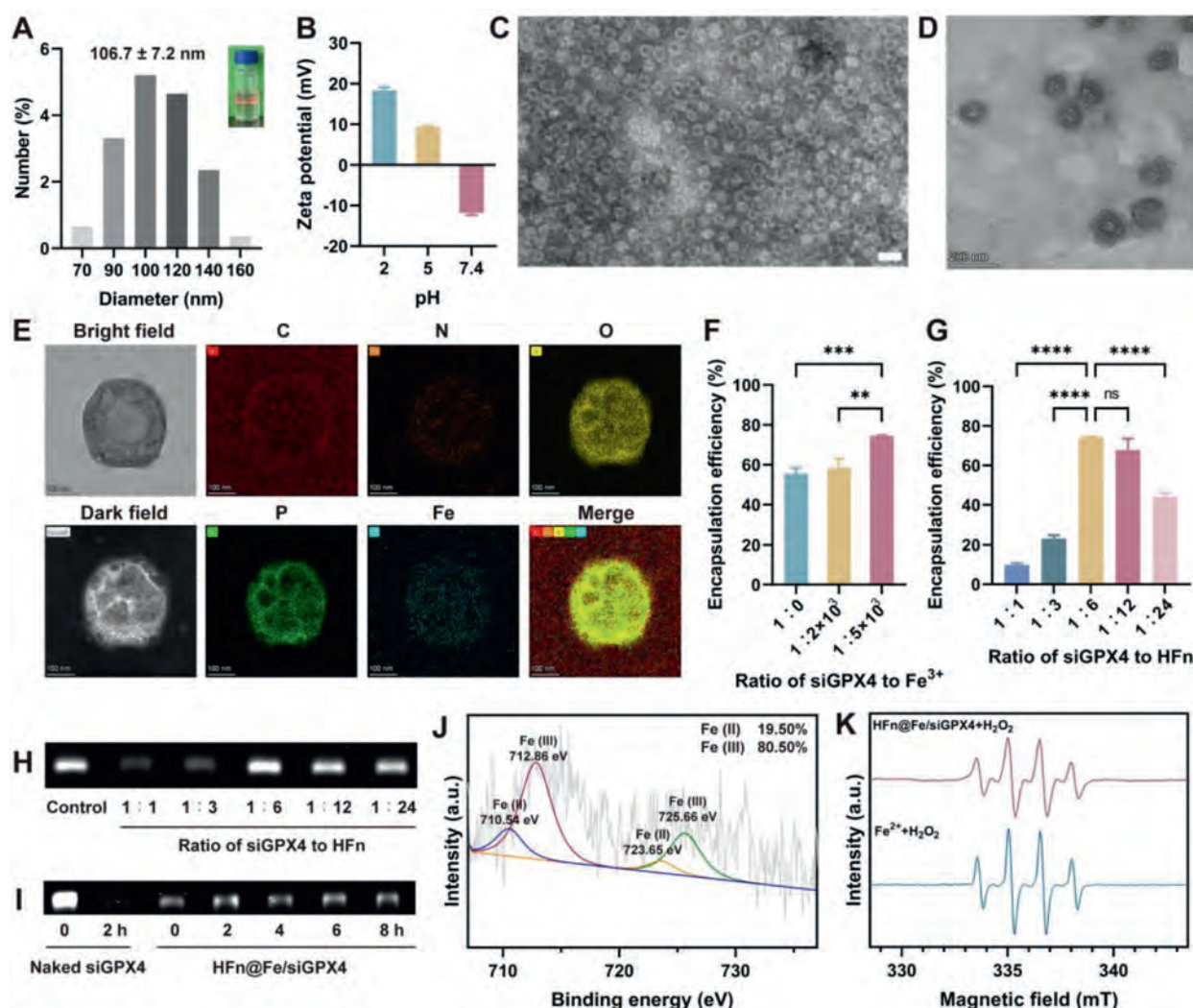


Figure 1 Characterization of HFn@Fe/siGPX4. (A) Particle diameter distribution of HFn@Fe/siGPX4. (B) Zeta potential of HFn@Fe/siGPX4 at different pH. Data are presented as mean $\pm$ SD ($n = 3$). (C) TEM image of HFn in pH 7.4 buffer with negative staining using 2 % phosphotungstic acid. Scale bar = 20 nm. (D) HRTEM image of HFn@Fe/siGPX4 without negative staining. Scale bar = 200 nm. (E) EDS mapping of HFn@Fe/siGPX4. Scale bar = 100 nm. (F) Internalization efficiency of HFn@Fe/siGPX4 with different Fe^{3+} to siGPX4 ratios. Data are presented as mean $\pm$ SD ($n = 3$). (G) Internalization efficiency of HFn@Fe/siGPX4 with different siGPX4 to HFn ratios. Data are presented as mean $\pm$ SD ($n = 3$). (H) Gene encapsulation efficiency of different siGPX4 to HFn ratios detected by AGE. (I) RNase stability of naked siGPX4 and HFn@Fe/siGPX4, co-incubated with RNase A at 37 $^{\circ}\text{C}$ for 0, 2, 4, 6 and 8 h, detected by AGE. (J) Fe 2p XPS spectrum of HFn@Fe/siGPX4 after storing in PBS at 4 $^{\circ}\text{C}$ for 14 days. (K) EPR spectra showing the production of $\cdot\text{OH}$ by $\text{Fe}^{2+} + \text{H}_2\text{O}_2$ and HFn@Fe/siGPX4 + H_2O_2 . ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant.

to evaluate the catalytic activity of HFn@Fe/siGPX4⁶⁵. The result was shown in Supporting Information Fig. S4A. The addition of H_2O_2 or HFn@Fe/siGPX4 alone failed to oxidize TMB into oxTMB (blue color). And when both H_2O_2 and HFn@Fe/siGPX4 were added, a clear blue color was observed, which indicated that HFn@Fe/siGPX4 had the activity to catalyze the reaction of H_2O_2 to generate ROS. Notably, the catalytic efficiency of this HFn@Fe/siGPX4 was concentration- and time-dependent, and more H_2O_2 was catalyzed into ROS as the concentration of HFn@Fe/siGPX4 or the incubating time increased (Fig. S4B). Besides, the catalytic activity of HFn@Fe/siGPX4 was also evaluated by the MB degradation test⁶⁶. The results (Fig. S4C) showed that the characteristic absorption peak of MB was significantly decreased when incubated with HFn@Fe/siGPX4

and H_2O_2 simultaneously. And the oxidation of MB was attributed to ROS produced by HFn@Fe/siGPX4 and H_2O_2 incubation. Moreover, the MB degradation test also confirmed that the catalytic effect of HFn@Fe/siGPX4 was time-dependent (Fig. S4D). All the above results demonstrated that HFn@Fe/siGPX4 had an excellent ability to catalyze the Fenton reaction, which provided a strong support for the ferroptosis effect of HFn@Fe/siGPX4 both *in vitro* and *in vivo*.

3.4. Cellular uptake

Cellular uptake was performed using CLSM and FCM. HFn had been shown to be taken up into cells *via* TfR1 on the surface of MCF-7 cells⁴⁵. In order to investigate the uptake mechanism of

HFn@Fe/siGPX4, the TfR1 receptors on MCF-7 cells were blocked by a TfR1 antibody. As seen in Fig. 2A, a small amount of the free siGPX4 was taken up by MCF-7 cells, and the efficiency of HFn@Fe/siGPX4 uptake by MCF-7 was high when TfR1 was not blocked, and decreased when TfR1 was blocked by the antibody of TfR1, suggesting that the uptake of HFn@Fe/siGPX4 in MCF-7 cells was TfR1 mediated. To further prove the active targeting of HFn@Fe/siGPX4, cellular uptake was performed using three types of cells (RAW264.7, THP-1, and MCF-7) with different levels of TfR1 expression. MCF-7 expressed TfR1 at a high level on its surface and was significantly different from the other two types of cells (Supporting Information Fig. S5). As shown in Fig. 2B, HFn@Fe/siGPX4 had the highest uptake efficiency in MCF-7 cells, again demonstrating that cellular uptake of HFn@Fe/siGPX4 was TfR1 mediated. The results of FCM (Fig. 2C and D) similarly demonstrated that HFn@Fe/siGPX4 enters the cell *via* TfR1.

3.5. Lysosomal escape

The lysosomal escape behavior of HFn@Fe/siGPX4 was observed by STED. As presented in the results (Fig. 2E), HFn@siGPX4 co-localized with the lysosome at both 1 and 4 h, indicating failure to escape. In contrast, HFn@Fe/siGPX4 partially co-localized with the lysosome at 1 h and hardly co-localized with the lysosome at

4 h, indicating that HFn@Fe/siGPX4 successfully escaped from the lysosome. These results demonstrated that HFn@Fe/Cy5-siGPX4 were transported into lysosome when incubated with cells, but could be effectively transported to the cytoplasm by lysosomal escape. It has been reported that increasing intracellular iron leads to endo-lysosomal defects, which may benefit the escape of contents from lysosomes⁵⁷. We speculated that the iron ions in HFn@Fe/siGPX4 were attributed to the lysosomal escape behavior of the nanoparticles, which was essential for siRNA to exert an RNA interference effect.

3.6. *In vitro* cellular ferroptosis mechanism of HFn@Fe/siGPX4

Ferroptosis is an iron-dependent, non-apoptotic form of programmed cell death characterized by abnormally elevated iron-mediated oxidative stress and GSH depletion. GPX4 is a key enzyme in the regulation of ferroptosis, and increased concentrations of iron ions promote oxidative stress, which in turn triggers ferroptosis⁶⁷. In this work, the ferroptosis mechanism of HFn@Fe/siGPX4 was studied systematically. Firstly, the gene silencing effect of HFn@Fe/siGPX4 was evaluated. As shown by the results of RT-qPCR (Fig. 3A), free siGPX4 could not effectively exert RNAi effect on *GPX4* expression in MCF-7 cells. In contrast, HFn@siGPX4 treated cells had a significant gene silencing effect by down-regulating the mRNA of *GPX4* (the gene

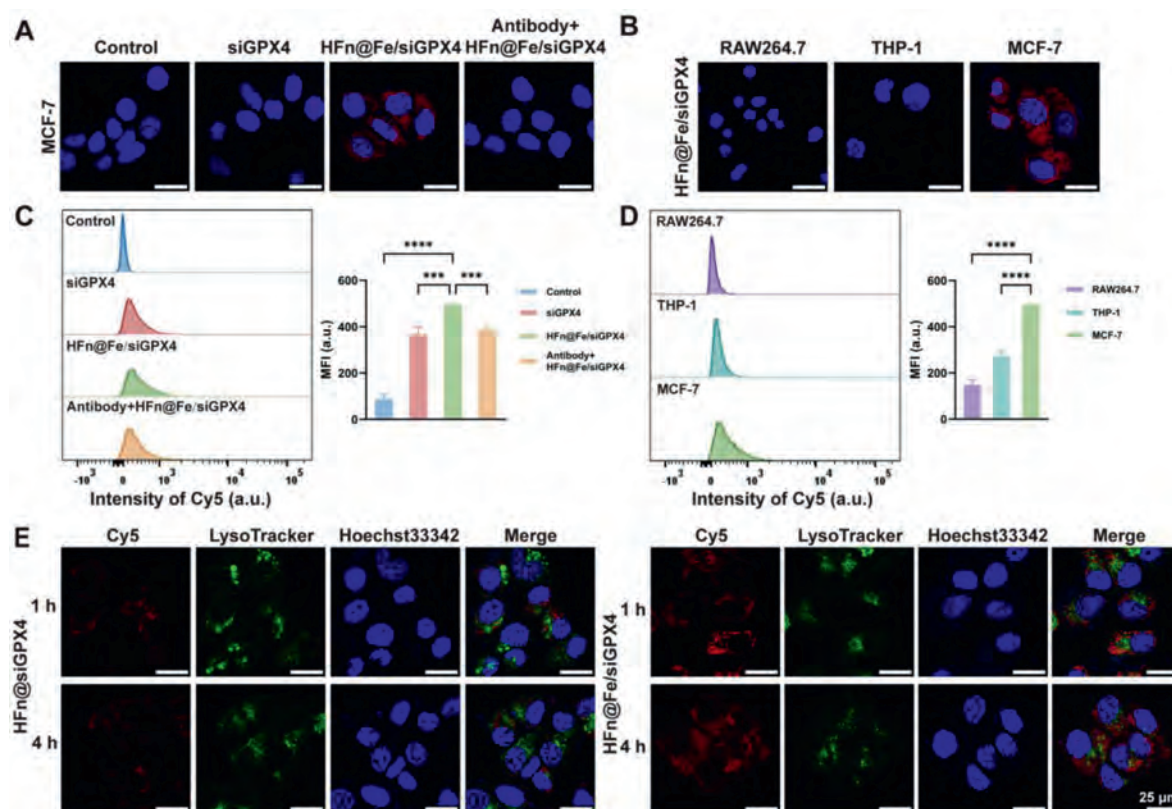


Figure 2 *In vitro* cellular uptake and lysosomal escape. (A) Cell uptake of siGPX4, HFn@Fe/siGPX4 and Antibody + HFn@Fe/siGPX4 in MCF-7 cells for 4 h by CLSM. (B) Uptake of HFn@Fe/siGPX4 in RAW264.7 cells, THP-1 cells and MCF-7 cells for 4 h by CLSM. (C) Cell uptake of siGPX4, HFn@Fe/siGPX4 and Antibody + HFn@Fe/siGPX4 in MCF-7 cells for 4 h by FCM and MFI of Cy5. (D) Uptake of HFn@Fe/siGPX4 in RAW264.7 cells, THP-1 cells and MCF-7 cells for 4 h by FCM and MFI of Cy5. (E) Lysosomal escape of HFn@siGPX4 and HFn@Fe/siGPX4 after incubation with MCF-7 cells for 1 and 4 h by STED. Scale bar = 25 μm. Data are presented as mean ± SD ($n = 3$). *** $P < 0.001$, **** $P < 0.0001$.

silencing efficiency of siGPX4 was $58.0 \pm 2.5\%$ for HFn@siGPX4), which may be due to the targeting effect of HFn towards MCF-7 cells. More impressively, the mRNA level of *GPX4* was further down-regulated when treated with HFn@Fe/siGPX4 (the gene silencing efficiency was $78.3 \pm 1.5\%$ for HFn@Fe/siGPX4). It was speculated that the improved gene silencing effect was attributed to the lysosomal escape behavior of HFn@Fe/siGPX4 with the assistance of iron ions. And the gene silencing effect of HFn@Fe/siGPX4 was even stronger than that of lipo2000@siGPX4 (the gene silencing efficiency was $73.2 \pm 2.0\%$ for lipo2000@siGPX4), suggesting that the iron ions coated HFn nanoparticles were an efficient gene drug delivery system.

And the gene silencing effect of HFn@Fe/siGPX4 was further confirmed at the protein level by ELISA and Western blot (WB). As seen in Fig. 3B, the expression of GPX4 protein was down-

regulated to $27.85 \pm 2.74\%$ and $30.88 \pm 12.85\%$ for HFn@Fe/siGPX4 and lipo2000@siGPX4 treated groups, which was significantly lower than the siGPX4 or HFn@siGPX4 treated ones. As expected, the results of WB (Fig. 3C) were consistent with those of ELISA. The relative expression of GPX4 in the HFn@Fe/siGPX4 group was substantially reduced compared to the control group and comparable to the lipo2000@siGPX4 group, indicating that HFn@Fe/siGPX4 significantly reduced the GPX4 protein level in MCF-7 cells.

Since GPX4 is a crucial protein for regulating cell ferroptosis, and the silencing of *GPX4* expression is expected to induce ferroptosis to play an anti-tumor effect²⁶. Moreover, ferroptosis is regulated by a variety of molecules. Expect elevating levels of lipid oxidation by silencing *GPX4*, the increase of concentration of iron ions in cells is another important way to induce ferroptosis. Therefore, it was expected that HFn@Fe/siGPX4 would be a

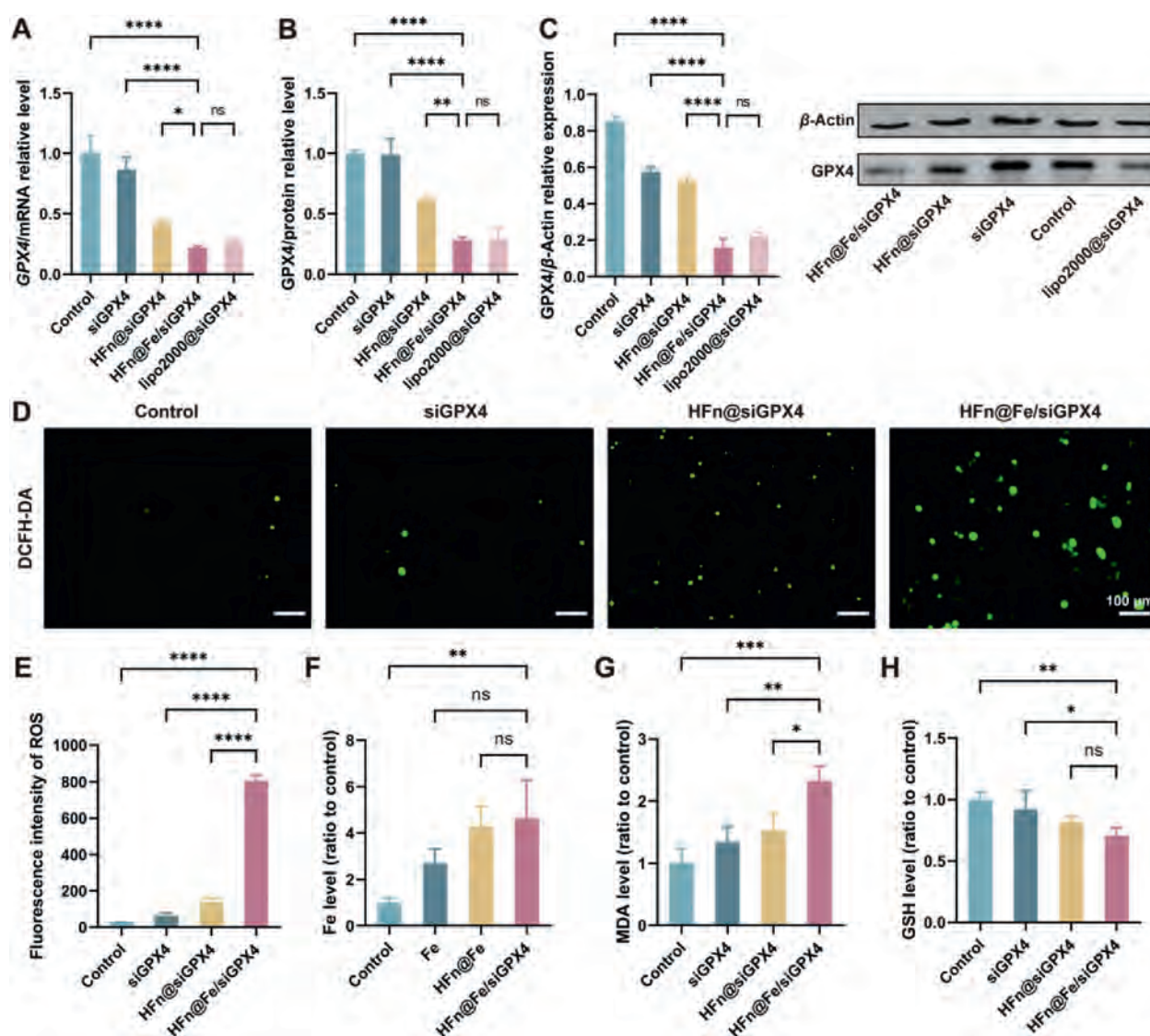


Figure 3 *In vitro* gene silencing effects and cellular ferroptosis induction. (A) The expression level of *GPX4* mRNA in MCF-7 cells by RT-qPCR. (B) The expression level of the GPX4 protein in MCF-7 cells by ELISA. (C) The expression level of the GPX4 protein in MCF-7 cells by WB. (D) Intracellular ROS concentration of MCF-7 cells detected by ROS probe DCFH-DA. Scale bar = 100 μ m. (E) Quantification of fluorescence intensity based on (D) by ImageJ. (F) Intracellular Fe level of MCF-7 cells. (G) MDA level of MCF-7 cells. (H) Intracellular GSH level of MCF-7 cells. Data are presented as mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant.

promising system for tumor treatment by cellular ferroptosis mechanism.

Increased cellular ROS is a prominent marker of ferroptosis and a prerequisite for its induction⁶⁸, so the intracellular levels of ROS were detected by DCFH-DA probe. The results (Fig. 3D) showed that the fluorescence intensity of the negative control group and the free siGPX4 group were extremely weak indicating that the intracellular ROS level was low, while the HFn@siGPX4 treated group exhibited a relatively strong fluorescence intensity suggesting that encapsulated into HFn was helpful for siGPX4 to exert treatment effect. Moreover, the fluorescence intensity of HFn@Fe/siGPX4 treated group was remarkably increased when compared with HFn@siGPX4. The quantification of DCFH-DA fluorescence suggested that the fluorescence intensity of DCFH-DA for HFn@siGPX4 treated group was 5-fold higher than for control group, but for HFn@Fe/siGPX4 treated one was 27-fold higher (Fig. 3E). All these results suggested that HFn@Fe/siGPX4 could increase the intracellular ROS level to induce ferroptosis, which for one thing was due to the enhanced gene silencing effect of siGPX4 when loaded into HFn@Fe/siGPX4, for another was because of the synergistic effect of iron ions for ferroptosis induction.

And the increase of intracellular iron ion levels when treated with HFn@Fe/siGPX4 was also confirmed. HFn@Fe/siGPX4 drastically increased the intracellular Fe ion level after entering the cells (Fig. 3F), which was 4-fold compared with the blank control group and 1.5-fold compared with the free iron ion treated group, proving that HFn@Fe/siGPX4 had a high iron ion loading and delivery efficiency to induce ferroptosis. MDA is also an important test indicator for ferroptosis⁶⁹, and was tested to further verify the ferroptosis inducing effect of HFn@Fe/siGPX4. The results of the MDA content assay (Fig. 3G) showed that the lipid oxidation level of HFn@Fe/siGPX4 treated group was 2.5-fold compared with that of the blank control group, which was also significantly higher than that of the HFn@siGPX4 treated group, suggesting that the HFn@Fe/siGPX4 could successfully induce lipid oxidation so as to induce ferroptosis. In addition, by the gene silencing effect of siGPX4, HFn@Fe/siGPX4 could inhibit the activity of GPX4, which was correlated with the content of GSH⁷⁰. And the results of the intracellular GSH content assay showed that the level of GSH for the HFn@Fe/siGPX4 treated group was significantly lower than for the blank control group (Fig. 3H), indicating that lower intracellular antioxidant level and reduced resistance of cells to ferroptosis.

3.7. *In vitro* inhibition effect of HFn@Fe/siGPX4 on MCF-7 cells

The production of high levels of membrane lipid peroxides is a defining feature of ferroptosis associated with other forms of cell death. When these membrane lipid peroxides accumulate at high levels, they cause the plasma membrane to rupture, which is lethal to the cell²³. These facts highlight the potential value of ferroptosis in tumor therapy². Considering the impressive ferroptosis inducing effect of HFn@Fe/siGPX4, they were hypothesized with an outstanding anti-tumor effect. The inhibition effect of HFn@Fe/siGPX4 on MCF-7 cells was evaluated. Firstly, the toxicity of the vector was evaluated by MTT assay, and the survival rate of MCF-7 cells was above 80% after treatment with different concentrations of HFn, indicating that HFn had no killing effect on tumor cells (Supporting Information Fig. S6). However,

HFn@Fe/siGPX4 exhibited anti-proliferation effect for MCF-7 cells *in vitro* in a dose-dependent manner, with significantly stronger compared with siGPX4 or HFn@siGPX4 (Fig. 4A). The IC₅₀ value of HFn@Fe/siGPX4 was 88.06 nmol/L (Supporting Information Fig. S7), indicating that tumor cells could be effectively killed at low concentrations of HFn@Fe/siGPX4. It was noteworthy that iron ion alone and HFn@Fe did not produce a killing effect on MCF-7 cells, which reflected the importance of the drug vectors and the synergistic effect of the gene drugs (Fig. 4B).

Mitochondria, as the powerhouse of cells, play an important role in generating energy in the form of adenosine triphosphate (ATP), maintaining metabolic homeostasis and regulating cell death⁷¹. High levels of ATP in the tumor cells satisfy the energy requirements for tumor cell growth. A decrease in ATP level can lead to mitochondrial dysfunction, thus promoting cellular death⁷². Therefore, the ATP level is an important indicator of cell viability and was tested in this work. The results of the ATP assay (Fig. 4C) showed that the intracellular ATP level was reduced to $52.24 \pm 2.32\%$ after HFn@Fe/siGPX4 treatment, which was distinctly lower than that treated with siGPX4 ($98.66 \pm 0.01\%$) or HFn@siGPX4 ($82.03 \pm 1.48\%$). These results implied that HFn@Fe/siGPX4 could effectively reduce ATP in tumor cells and thus promote cellular death. Furthermore, the results of apoptosis analysis investigated by FCM (Fig. 4D) showed that the apoptosis rate in the HFn@Fe/siGPX4 group could reach $39.13 \pm 4.57\%$, which was obviously higher than that in the control ($8.00 \pm 2.85\%$), free siGPX4 ($10.68 \pm 1.21\%$) and HFn@siGPX4 ($15.28 \pm 3.43\%$) groups, and proved the effective cell-apoptosis promoting effect of HFn@Fe/siGPX4.

Besides, the tumor cell killing effect of HFn@Fe/siGPX4 was further studied by Calcein-AM/propidium iodide (PI) live/dead staining with Calcein-AM staining the live cells in green and PI staining the dead cells in red. When treated with HFn@Fe/siGPX4, the red fluorescence indicating the dead cells was significantly increased, demonstrating that HFn@Fe/siGPX4 had a strong cell-killing ability (Fig. 4E). All the above results suggested that HFn@Fe/siGPX4 had an excellent inhibiting ability for tumor cells, which established the foundation for their anti-tumor effect *in vivo*.

3.8. *In vivo* tumor targeting effect of HFn@Fe/siGPX4

The mice were injected with free Cy5-siGPX4 and HFn@Fe/Cy5-siGPX4 by *i.v.*, and NIR fluorescence imaging was recorded at different time points. As shown in Fig. 5A and B, at 1.5 h, HFn@Fe/Cy5-siGPX4 was predominantly distributed in the tumor site and was significantly different from free Cy5-siGPX4, suggesting that HFn@Fe/Cy5-siGPX4 had targeting properties, and with the change of time, free Cy5-siGPX4 was partially distributed in the tumor site at 3 h, but free Cy5-siGPX4 was not distributed in the tumor site at 6 and 7.5 h, suggesting that Cy5-siGPX4 was cleared from tumor tissues. HFn@Fe/Cy5-siGPX4 consistently showed higher tumor accumulation. These results suggested that HFn@Fe/Cy5-siGPX4 had desirable tumor-targeting properties and tumor accumulation ability. Moreover, the tumor-bearing mice were intravenously injected with free Cy5-siGPX4 and HFn@Fe/Cy5-siGPX4, and after 24 h of administration, the fluorescent distribution of Cy5-siGPX4 in different organs was observed. As seen in Fig. 5C, compared with free Cy5-siGPX4, the fluorescence intensity of Cy5-siGPX4 in tumor tissues of HFn@Fe/Cy5-siGPX4 treated mice was

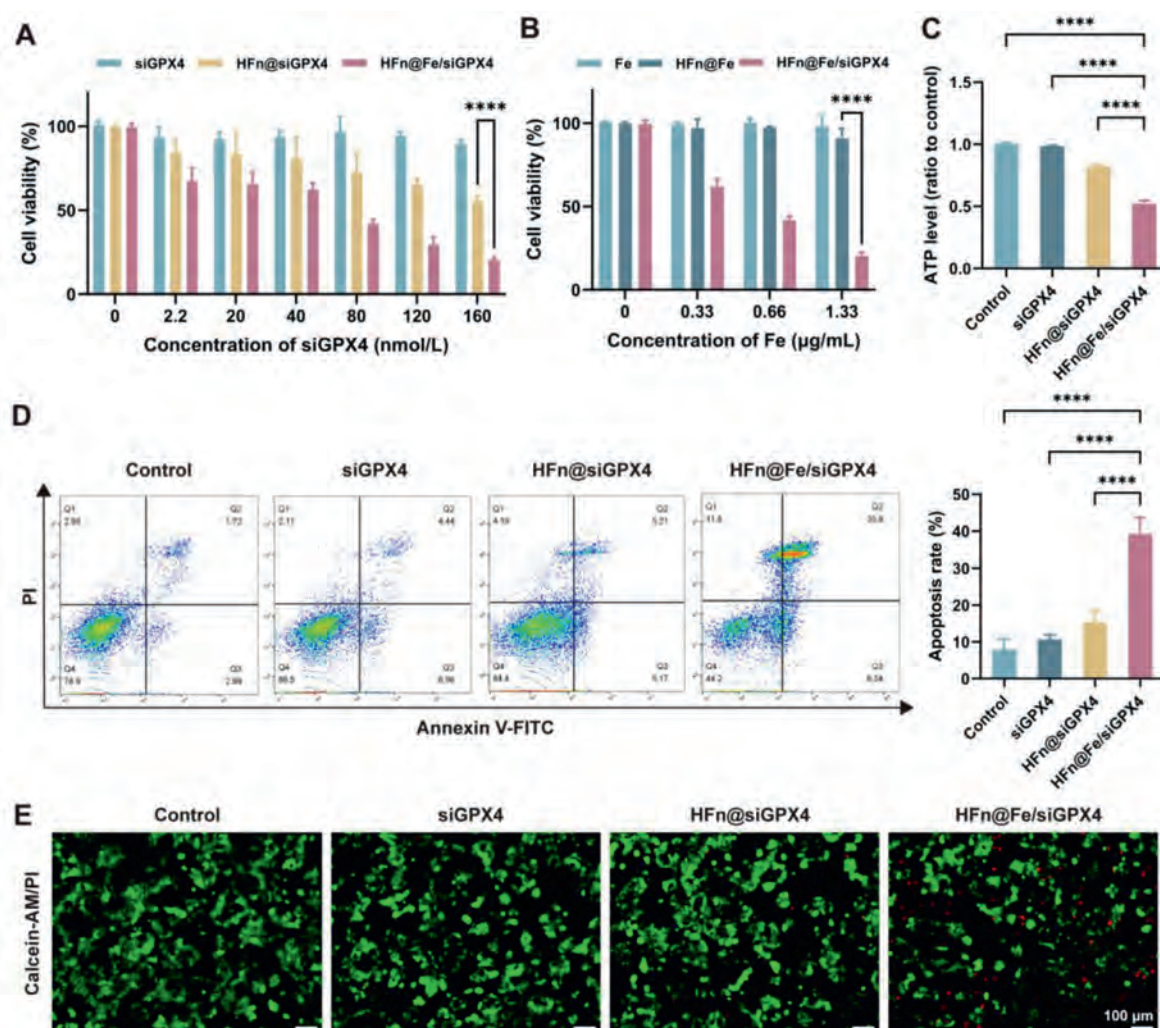


Figure 4 *In vitro* anti-tumor activity of HFn@Fe/siGPX4. (A) *In vitro* cell viability of MCF-7 cells after 48 h of exposure to siGPX4, HFn@siGPX4 and HFn@Fe/siGPX4 at different concentrations of siGPX4. (B) *In vitro* cell viability of MCF-7 cells after 48 h of exposure to Fe, HFn@Fe and HFn@Fe/siGPX4 at different concentrations of Fe. (C) The ATP level in MCF-7 cells of different groups. (D) Flow cytometry analysis of MCF-7 cell apoptosis induced by siGPX4, HFn@siGPX4, and HFn@Fe/siGPX4 NPs for 48 h using Annexin V-FITC/PI staining. (E) Fluorescence images of Calcein-AM/PI. Scale bar = 100 μ m. Data are presented as mean $\pm$ SD ($n = 3$). **** $P < 0.0001$.

impressively enhanced, and was increased by 11-fold as calculated by the quantitative result (Fig. 5D). These results confirmed that HFn@Fe/siGPX4 could be targeted to the tumor site so as to exert anti-tumor effect more effectively.

3.9. *In vivo* anti-tumor effect of HFn@Fe/siGPX4

The anti-tumor efficacy of HFn@Fe/siGPX4 was also assessed with an MCF-7 xenograft model. The model mice were randomly divided into 4 groups and respectively injected with saline, free siGPX4, HFn@siGPX4 or HFn@Fe/siGPX4 by *i.v.* every three days for seven doses. The tumor volume was monitored for two weeks after the first treatment. As depicted in Fig. 5E and F, following the initiation of treatment, the tumor volumes in mice administered with saline and free siGPX4 continued to enlarge progressively. After treatment, the tumor volumes in these groups had escalated to a range of 500–600 mm^3 . In the group treated with HFn@siGPX4, there was a modest deceleration in the rate of tumor volume increase, yet the volume continued to

expand, reaching a size of $402.2 \pm 31.4 \text{ mm}^3$. In contrast, mice treated with HFn@Fe/siGPX4 exhibited a significant reduction in tumor volume. By the end of the two-week period, the tumor volume had dramatically decreased to $99.4 \pm 19.0 \text{ mm}^3$. This reduction suggested that HFn@Fe/siGPX4 not only retarded tumor growth but even eliminated already existing tumors, highlighting the promising *in vivo* antitumor effect of HFn@Fe/siGPX4.

The survival outcomes post-treatment were meticulously monitored and documented in our study. The Kaplan-Meier survival curves (Fig. 5G) graphically represent the survival rates of the experimental groups over the study period. Notably, there was a significant disparity in the survival profiles between the HFn@Fe/siGPX4 treatment group and other groups. Mice in the saline group, which received no intervention, exhibited a median survival time of approximately 27 days. And the medium survival time in the siGPX4 group was 30 days. The HFn@siGPX4 treatment could prolong the median survival time of tumor-bearing mice to 36 days. In stark contrast, the median survival

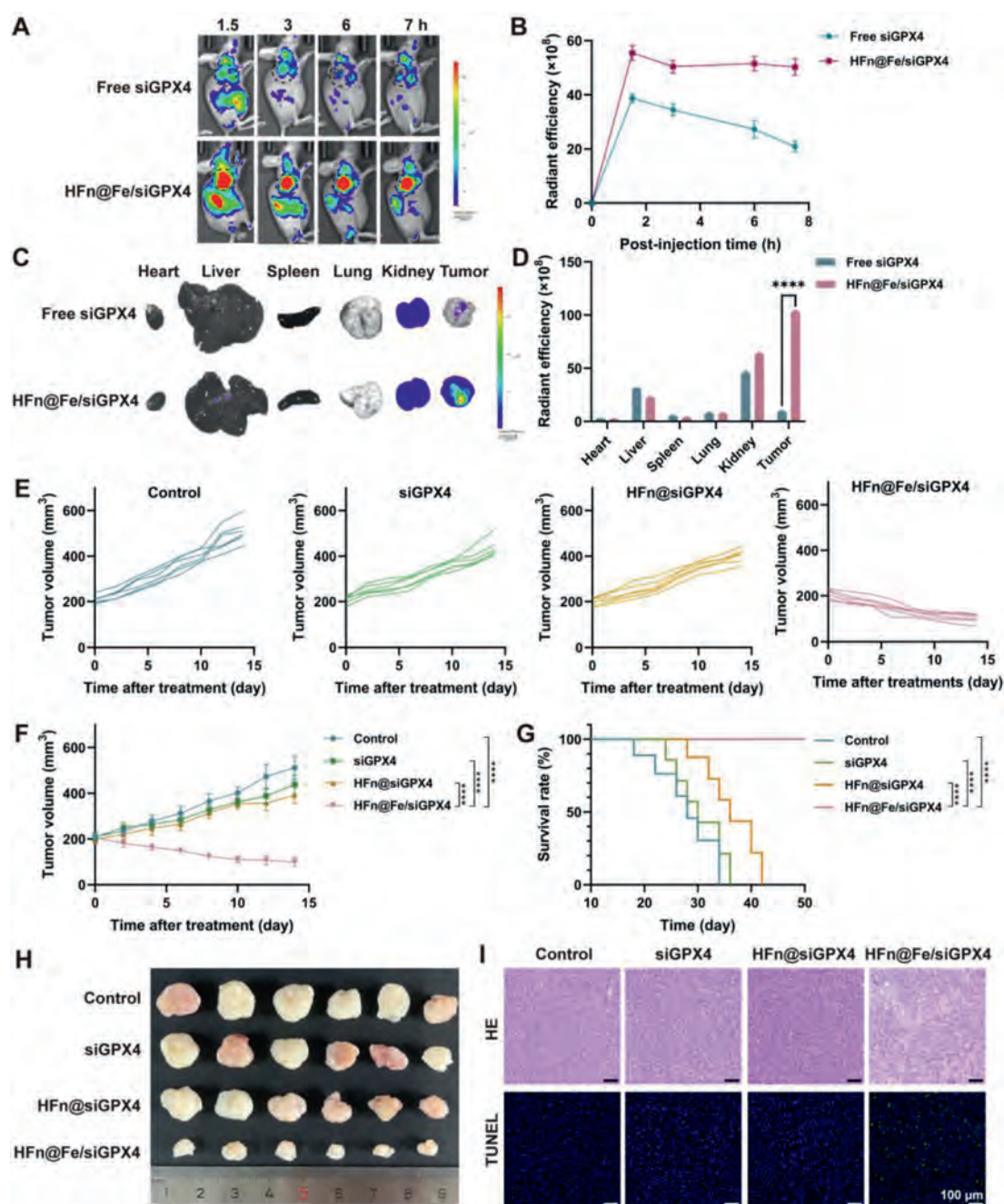


Figure 5 *In vivo* targeting and anti-tumor ability of HFn@Fe/siGPX4. (A) Near-infrared fluorescence *in vivo* imaging images at different time points post-injection. (B) Radiation efficiency of tumor sites at different time points post-injection. Data are presented as mean $\pm$ SD ($n = 3$). (C) Near-infrared fluorescence images of major organs and tumors at 24 h post-injection. (D) Radiation efficiency of different tissues at 24 h post-injection. Data are presented as mean $\pm$ SD ($n = 3$). (E) Tumor growth curves of each MCF-7 tumor over time following different treatments. (F) Tumor growth curves of different groups of MCF-7 tumor-bearing mice after various treatments indicated. Data are presented as mean $\pm$ SD ($n = 6$). (G) Kaplan–Meier survival curve of MCF-7-bearing mice following different treatments. ($n = 6$). (H) The isolated MCF-7 tumors from the sacrificed mice following treatments. (I) Representative H&E and TUNEL stained tumor slice images after the treatment. Scale bar = 100 μ m. **** $P < 0.0001$.

time was markedly extended in the HFn@Fe/siGPX4 treatment group, surpassing 50 days, indicative of a pronounced therapeutic effect. Moreover, the tumor tissues were removed at the end of the observation, which showed that the tumor volume of the HFn@Fe/siGPX4 group was significantly reduced when compared with other groups (Fig. 5H).

In addition, the tumor-inhibiting effect was further confirmed by histopathological analysis using H&E staining as well as immunofluorescence analysis using TUNEL staining (Fig. 5I). H&E staining results revealed that the tumor tissues in the HFn@Fe/siGPX4 group were loose in structure and the cells were not tightly arranged, and TUNEL staining results showed that

there were a large number of dead cells in the tumor tissues in the HFn@Fe/siGPX4 group, which indicated that HFn@Fe/siGPX4 achieved a potent tumor-inhibiting effect *in vivo*.

3.10. *In vivo* tumor ferroptosis-inducing ability of HFn@Fe/siGPX4

In order to investigate the mechanism of the tumor-inhibiting effect of HFn@Fe/siGPX4, we examined its ferroptosis-inducing ability *in vivo*. Firstly, ROS level in tumor tissues of different drug administration groups was evaluated by immunofluorescent staining (Fig. 6A and B). Compared with the control group, both the groups treated with HFn@siGPX4 and HFn@Fe/siGPX4 had an obvious improvement in ROS level. More impressively, the ROS level of HFn@Fe/siGPX4 was significantly higher than that of HFn@siGPX4, which proved the synergistic effect of iron ion and siGPX4 for catalyzing the Fenton reaction.

The expression of GPX4 proteins in tumor tissues was also evaluated by immunofluorescent staining (Fig. 6A and C) and ELISA (Fig. 6D), which indicated that the GPX4 expression level for HFn@Fe/siGPX4 treated group was obviously decreased when compared with the control group. What's more, the *in vivo* gene silencing effect of HFn@Fe/siGPX4 was remarkably stronger than that of HFn@siGPX4, which was due to the lysosomal escape promotion of iron ions so as to improve the gene silencing

efficiency *in vivo*. GPX4 inactivation leads to uncontrolled lipid peroxidation, causing damage to cell membranes, which in turn triggers ferroptosis²⁶. Studies have shown that excessive iron ions in cells was another leading cause of ferroptosis, so the iron ion content in tumor tissues was analyzed. As expected, for HFn@Fe/siGPX4 treated group, there was an extreme accumulation of iron ions when compared with other groups, which may also contribute to the excellent ferroptosis-inducing ability of HFn@Fe/siGPX4 *in vivo* (Fig. 6E).

Moreover, the ferroptosis-inducing effect of HFn@Fe/siGPX4 was further proved by MDA and GSH assay. It is reported that MDA is an end product of lipid oxidation that accumulates to promote ferroptosis and GSH can inhibit ferroptosis by decreasing the production of lipid peroxides⁷³. So, these two molecules are considered as two important indicators of ferroptosis. As expected, HFn@Fe/siGPX4 had the strongest moderating effect on the expression of MDA and GSH levels for tumor tissues (Fig. 6F and G), which was also consistent with the results of MDA and GSH tests *in vitro*. To sum up, all these results suggested that HFn@Fe/siGPX4 was a promising nano-drug delivery system for tumor treatment by inducing ferroptosis efficiently. The impressive effect of HFn@Fe/siGPX4 was for one thing because of the synergistically ferroptosis inducing effect of iron ions and siGPX4, and for another contributed to the enhanced gene silencing effect of siGPX4 which may be due to the targeting effect of ferritin

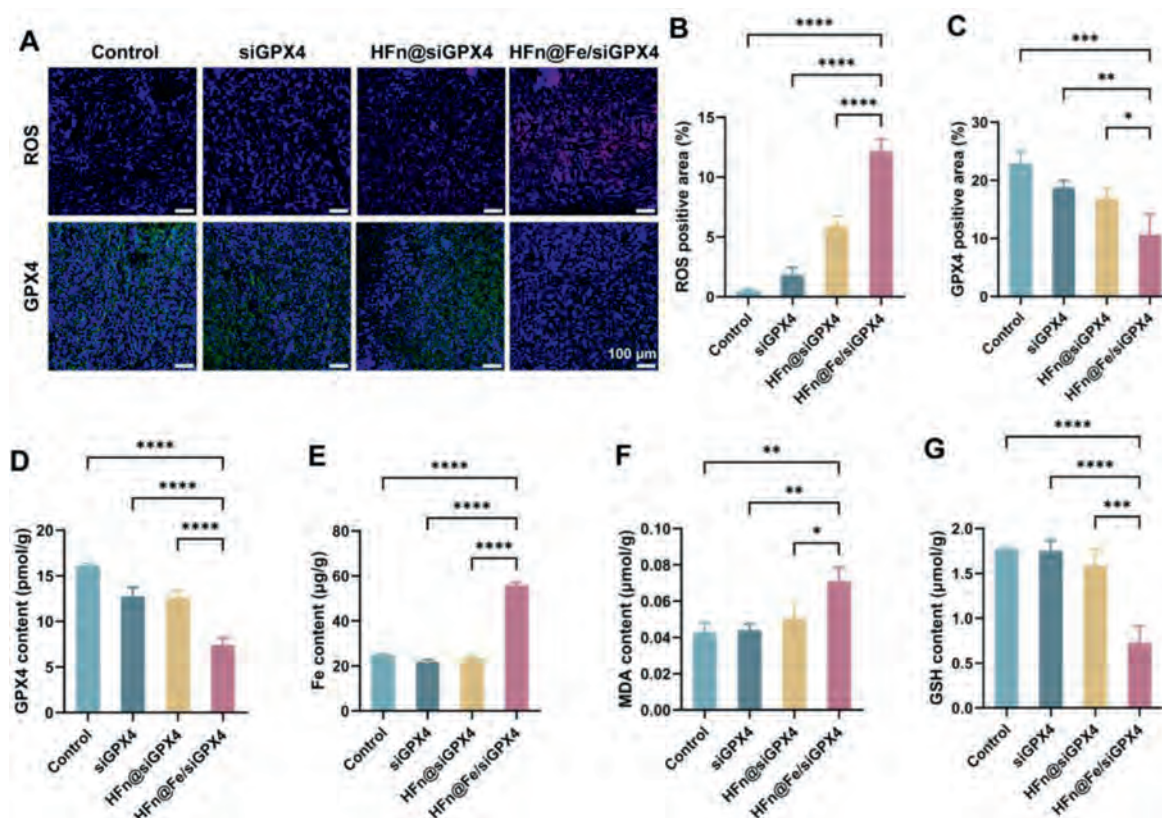


Figure 6 *In vivo* ferroptosis induction. (A) ROS staining and immunofluorescence staining of GPX4 in tumor sections. Scale bar = 100 μm. (B) Quantification of positive area based on ROS staining by ImageJ. (C) Quantification of positive area based on GPX4 staining by ImageJ. (D) Tumor tissue GPX4 content measured by ELISA. (E) Tumor tissue Fe content. (F) Tumor tissue MDA content. (G) Tumor tissue GSH content. Data are presented as mean ± SD (*n* = 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

nanocages for tumor tissues and the lysosomal escape behavior with the exist of iron ions in the nanosystems.

3.11. *In vivo* safety evaluation

Finally, the *in vivo* safety of HFn@Fe/siGPX4 was evaluated. During the treatment process, there was no significant change in the body weight of the mice in all the treated groups, which verified the biosafety of the nano-drug vector primarily (Fig. S8). And in order to further investigate the biocompatibility and systemic response of the different formulations, histopathology and hematologic tests were carried out. As shown in Supporting Information Figs. S9 and S10, there were no differences between the saline-treated group and the HFn@Fe/siGPX4-treated group either in the H&E staining of the major organs or the blood biochemical data. Thus, these results confirmed that it was safe to apply HFn@Fe/siGPX4 *in vivo*.

4. Conclusions

In summary, we successfully prepared a ferritin-based nano vehicle HFn@Fe/siGPX4 encapsulated with iron ions and siGPX4 for inducing ferroptosis synergistically. In order to encapsulate more siGPX4 into ferritin nanocages, the gene drugs were pre-incubated with iron ions to form Fe-siGPX4 complexes which could be loaded into the nanocages more easily. Then excess iron ions were overloaded by the diffusion method to prepare siGPX4 and iron ions co-loaded ferritin nanocages. The overloaded iron ions in the nanocages could not only induce ferroptosis together with siGPX4 for synergistic tumor treatment but also assist with the lysosomal escape of siGPX4 so as to enhance the gene silencing effect. With the tumor-targeting effect of ferritin, HFn@Fe/siGPX4 could be effectively delivered into tumor cells with a TfR1-mediated pathway to inhibit tumor growth by promoting the ferroptosis of tumor cells as proved both *in vitro* and *in vivo*. Altogether, this study not only offered a proof-of-concept for facilitating the loading of siRNA into ferritin nanocages through adding iron ions but also provided a new strategy of synergistic ferroptosis induction for cancer treatment as well as a new insight for the development of anti-tumor nanomedicines based on the ferroptosis mechanism.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81502688), Cooperation Research Funding of Capital Medical University (2020KJ000514, China), Cooperation Research Funding of Capital Medical University (2023KJ000814, China), and R&D Program of Beijing Municipal Education Commission (KM202210025024, China).

Author contributions

Danni Liu and Shuang Zhang designed the research. Danni Liu carried out the experiments and performed data analysis. Jie Zhang, Ran Huo, Yang Tian and Siyu Liu participated part of the experiments. Yaoqi Wang, Qi Sun, Dong Mei and Xiaoling Wang provided experimental drugs and quality control. Danni Liu wrote the manuscript. Shuang Zhang and Chunying Cui revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2024.10.006>.

References

- Jiang XJ, Stockwell BR, Conrad M. Ferroptosis: mechanisms, biology, and role in disease. *Nat Rev Mol Cell Biol* 2021;**22**:266–82.
- Yan HF, Zou T, Tuo QZ, Xu S, Li H, Belaidi AA, et al. Ferroptosis: mechanisms and links with diseases. *Signal Transduct Target Ther* 2021;**6**:49.
- Fearnhead HO, Vandenabeele P, Vanden Berghe T. How do we fit ferroptosis in the family of regulated cell death? *Cell Death Differ* 2017;**24**:1991–8.
- Wang X, Hua P, He CW, Chen MW. Non-apoptotic cell death-based cancer therapy: molecular mechanism, pharmacological modulators, and nanomedicine. *Acta Pharm Sin B* 2022;**12**:3567–93.
- Toyokuni S, Yanatori I, Kong YY, Zheng H, Motooka Y, Jiang L. Ferroptosis at the crossroads of infection, aging and cancer. *Cancer Sci* 2020;**111**:2665–71.
- Chen Y, Fang ZM, Yi X, Wei X, Jiang DS. The interaction between ferroptosis and inflammatory signaling pathways. *Cell Death Dis* 2023;**14**:205.
- Stockwell BR. Ferroptosis turns 10: emerging mechanisms, physiological functions, and therapeutic applications. *Cell* 2022;**185**:2401–21.
- Chen X, Kang R, Kroemer G, Tang DL. Broadening horizons: the role of ferroptosis in cancer. *Nat Rev Clin Oncol* 2021;**18**:280–96.
- Lei G, Zhuang L, Gan BY. Targeting ferroptosis as a vulnerability in cancer. *Nat Rev Cancer* 2022;**22**:381–96.
- Zhao L, Zhou XX, Xie F, Zhang L, Yan HY, Huang J, et al. Ferroptosis in cancer and cancer immunotherapy. *Cancer Commun (Lond)* 2022;**42**:88–116.
- Song G, Li MH, Fan SM, Qin MM, Shao B, Dai WB, et al. Boosting synergism of chemo- and immuno-therapies via switching paclitaxel-induced apoptosis to mevalonate metabolism-triggered ferroptosis by bisphosphonate coordination lipid nanogranules. *Acta Pharm Sin B* 2024;**14**:836–53.
- Wang K, Zhang ZY, Tsai HI, Liu YF, Gao J, Wang M, et al. Branched-chain amino acid aminotransferase 2 regulates ferroptotic cell death in cancer cells. *Cell Death Differ* 2021;**28**:1222–36.
- Kim DH, Choi HI, Park JS, Kim CS, Bae EH, Ma SK, et al. Farnesoid X receptor protects against cisplatin-induced acute kidney injury by regulating the transcription of ferroptosis-related genes. *Redox Biol* 2022;**54**:102382.
- Yang HC, Yao XM, Liu YQ, Shen XK, Li MH, Luo Z. Ferroptosis nanomedicine: clinical challenges and opportunities for modulating tumor metabolic and immunological landscape. *ACS Nano* 2023;**17**:15328–53.
- Liu KX, Huang L, Qi SY, Liu SC, Xie WN, Du LY, et al. Ferroptosis: the entanglement between traditional drugs and nanodrugs in tumor therapy. *Adv Healthc Mater* 2023;**12**:e2203085.
- Gu YL, Li YZ, Wang JX, Zhang LL, Zhang JF, Wang YX. Targeting ferroptosis: paving new roads for drug design and discovery. *Eur J Med Chem* 2023;**247**:115015.
- Rodriguez R, Schreiber SL, Conrad M. Persister cancer cells: iron addiction and vulnerability to ferroptosis. *Mol Cell* 2022;**82**:728–40.
- Basuli D, Tesfay L, Deng Z, Paul B, Yamamoto Y, Ning G, et al. Iron addiction: a novel therapeutic target in ovarian cancer. *Oncogene* 2017;**36**:4089–99.

19. Zhao N, Huang YJ, Wang YH, Muir RK, Chen YC, Wei JN, et al. Ferromagnetic: measuring tumoral ferrous iron with PET to predict sensitivity to iron-targeted cancer therapies. *J Nucl Med* 2021;**62**:949–55.
20. Tang BT, Zhu JY, Li J, Fan K, Gao Y, Cheng SM, et al. The ferroptosis and iron-metabolism signature robustly predicts clinical diagnosis, prognosis and immune microenvironment for hepatocellular carcinoma. *Cell Commun Signal* 2020;**18**:174.
21. Hassannia B, Vandenabeele P, Vanden Berghe T. Targeting ferroptosis to iron out cancer. *Cancer Cell* 2019;**35**:830–49.
22. Chen X, Li JB, Kang R, Klionsky DJ, Tang DL. Ferroptosis: machinery and regulation. *Autophagy* 2021;**17**:2054–81.
23. Dixon SJ, Olzmann JA. The cell biology of ferroptosis. *Nat Rev Mol Cell Biol* 2024;**25**:424–42.
24. Zheng XG, Jin XD, Ye F, Liu XX, Yu BY, Li Z, et al. Ferroptosis: a novel regulated cell death participating in cellular stress response, radiotherapy, and immunotherapy. *Exp Hematol Oncol* 2023;**12**:65.
25. Chen X, Yu CH, Kang R, Kroemer G, Tang DL. Cellular degradation systems in ferroptosis. *Cell Death Differ* 2021;**28**:1135–48.
26. Liu Y, Wan YC, Jiang Y, Zhang L, Cheng WJ. GPX4: the hub of lipid oxidation, ferroptosis, disease and treatment. *Biochim Biophys Acta Rev Cancer* 2023;**1878**:188890.
27. Du YX, Guo Z. Recent progress in ferroptosis: inducers and inhibitors. *Cell Death Discov* 2022;**8**:501.
28. Wang Y, Sun T, Jiang C. Nanodrug delivery systems for ferroptosis-based cancer therapy. *J Control Release* 2022;**344**:289–301.
29. Hangauer MJ, Viswanathan VS, Ryan MJ, Bole D, Eaton JK, Matov A, et al. Drug-tolerant persister cancer cells are vulnerable to GPX4 inhibition. *Nature* 2017;**551**:247–50.
30. Yang WS, SriRamaratnam R, Welsch ME, Shimada K, Skouta R, Viswanathan VS, et al. Regulation of ferroptotic cancer cell death by GPX4. *Cell* 2014;**156**:317–31.
31. Revia RA, Stephen ZR, Zhang MQ. Theranostic nanoparticles for RNA-based cancer treatment. *Acc Chem Res* 2019;**52**:1496–506.
32. Paunovska K, Loughrey D, Dahlman JE. Drug delivery systems for RNA therapeutics. *Nat Rev Genet* 2022;**23**:265–80.
33. Lucas T, Bonauer A, Dimmeler S. RNA therapeutics in cardiovascular disease. *Circ Res* 2018;**123**:205–20.
34. Kang H, Ga YJ, Kim SH, Cho YH, Kim JW, Kim C, et al. Small interfering RNA (siRNA)-based therapeutic applications against viruses: principles, potential, and challenges. *J Biomed Sci* 2023;**30**:88.
35. Zhang XC, Hai L, Gao YB, Yu GC, Sun YL. Lipid nanomaterials-based RNA therapy and cancer treatment. *Acta Pharm Sin B* 2023;**13**:903–15.
36. Lee SJ, Kim MJ, Kwon IC, Roberts TM. Delivery strategies and potential targets for siRNA in major cancer types. *Adv Drug Deliv Rev* 2016;**104**:2–15.
37. Zhou Z, Liu X, Zhu D, Wang Y, Zhang Z, Zhou X, et al. Nonviral cancer gene therapy: delivery cascade and vector nanoproperty integration. *Adv Drug Deliv Rev* 2017;**115**:115–54.
38. Yu AM, Choi YH, Tu MJ. RNA drugs and RNA targets for small molecules: principles, progress, and challenges. *Pharmacol Rev* 2020;**72**:862–98.
39. He JY, Fan KL, Yan XY. Ferritin drug carrier (FDC) for tumor targeting therapy. *J Control Release* 2019;**311–312**:288–300.
40. Zhang BL, Tang GH, He JY, Yan XY, Fan KL. Ferritin nanocage: a promising and designable multi-module platform for constructing dynamic nanoassembly-based drug nanocarrier. *Adv Drug Deliv Rev* 2021;**176**:113892.
41. Tesarova B, Musilek K, Rex S, Heger Z. Taking advantage of cellular uptake of ferritin nanocages for targeted drug delivery. *J Control Release* 2020;**325**:176–90.
42. Song NN, Zhang JL, Zhai J, Hong JJ, Yuan C, Liang MM. Ferritin: a multifunctional nanopatform for biological detection, imaging diagnosis, and drug delivery. *Acc Chem Res* 2021;**54**:3313–25.
43. Lee NK, Cho S, Kim IS. Ferritin-a multifaceted protein scaffold for biotherapeutics. *Exp Mol Med* 2022;**54**:1652–7.
44. Li L, Fang CJ, Ryan JC, Niemi EC, Lebrón JA, Björkman PJ, et al. Binding and uptake of H-ferritin are mediated by human transferrin receptor-1. *Proc Natl Acad Sci U S A* 2010;**107**:3505–10.
45. Budiarta M, Roy S, Katenkamp T, Feliu N, Beck T. Overcoming non-specific interactions for efficient encapsulation of doxorubicin in ferritin nanocages for targeted drug delivery. *Small* 2023;**19**:e2205606.
46. Ren E, Chen HM, Qin ZN, Guan SW, Jiang L, Pang X, et al. Harnessing bifunctional ferritin with kartogenin loading for mesenchymal stem cell capture and enhancing chondrogenesis in cartilage regeneration. *Adv Healthc Mater* 2022;**11**:e2101715.
47. Zhang JL, Cheng DF, He JY, Hong JJ, Yuan C, Liang MM. Cargo loading within ferritin nanocages in preparation for tumor-targeted delivery. *Nat Protoc* 2021;**16**:4878–96.
48. Monti DM, Ferraro G, Merlino A. Ferritin-based anticancer metal-lodrug delivery: crystallographic, analytical and cytotoxicity studies. *Nanomedicine* 2019;**20**:101997.
49. Palombarini F, Masciarelli S, Incocciati A, Liccardo F, Di Fabio E, Iazzetti A, et al. Self-assembling ferritin-dendrimer nanoparticles for targeted delivery of nucleic acids to myeloid leukemia cells. *J Nanobiotechnology* 2021;**19**:172.
50. Seo J, Do Yoo J, Kim M, Shim G, Oh YK, Park RW, et al. Fibrinolytic nanocages dissolve clots in the tumor microenvironment, improving the distribution and therapeutic efficacy of anticancer drugs. *Exp Mol Med* 2021;**53**:1592–601.
51. Li L, Muñoz-Culla M, Carmona U, Lopez MP, Yang F, Trigueros C, et al. Ferritin-mediated siRNA delivery and gene silencing in human tumor and primary cells. *Biomaterials* 2016;**98**:143–51.
52. Huang HQ, Sha K, Veroniaina H, Wu ZH, Wu ZH, Qi XL. Ca²⁺ participating self-assembly of an apoferritin nanostructure for nucleic acid drug delivery. *Nanoscale* 2020;**12**:7347–57.
53. Zhen ZP, Tang W, Chen HM, Lin X, Todd T, Wang G, et al. RGD-modified apoferritin nanoparticles for efficient drug delivery to tumors. *ACS Nano* 2013;**7**:4830–7.
54. Van de Vyver T, Bogaert B, De Backer L, Joris F, Guagliardo R, Van Hoeck J, et al. Cationic amphiphilic drugs boost the lysosomal escape of small nucleic acid therapeutics in a nanocarrier-dependent manner. *ACS Nano* 2020;**14**:4774–91.
55. Kim HJ, Kim A, Miyata K, Kataoka K. Recent progress in development of siRNA delivery vehicles for cancer therapy. *Adv Drug Deliv Rev* 2016;**104**:61–77.
56. Kim B, Sun S, Varner JA, Howell SB, Ruoslahti E, Sailor MJ. Securing the payload, finding the cell, and avoiding the endosome: peptide-targeted, fusogenic porous silicon nanoparticles for delivery of siRNA. *Adv Mater* 2019;**31**:e1902952.
57. Rizzollo F, More S, Vangheluwe P, Agostinis P. The lysosome as a master regulator of iron metabolism. *Trends Biochem Sci* 2021;**46**:960–75.
58. Kim B, Park JH, Sailor MJ. Rekindling RNAi therapy: materials design requirements for *in vivo* siRNA delivery. *Adv Mater* 2019;**31**:e1903637.
59. Kyprianou N, English HF, Davidson NE, Isaacs JT. Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. *Cancer Res* 1991;**51**:162–6.
60. Wang J, Ding HY, Zhu Y, Liu YN, Yu ML, Cai HL, et al. Iron-siRNA nanohybrids for enhanced chemodynamic therapy via ferritin heavy chain downregulation. *Angew Chem Int Ed Engl* 2023;**62**:e202302255.
61. Pang J, Feng X, Liang Q, Zheng XY, Duan YM, Zhang X, et al. Ferritin-nanocaged ATP traverses the blood–testis barrier and enhances sperm motility in an asthenozoospermia model. *ACS Nano* 2022;**16**:4175–85.
62. Li CT, Wang JX, Lu XM, Ge H, Jin X, Guan QH, et al. Hydrogen peroxide-response nanoprobe for CD44-targeted circulating tumor cell detection and H₂O₂ analysis. *Biomaterials* 2020;**255**:120071.
63. Laurent A, Nicco C, Chéreau C, Goulvestre C, Alexandre J, Alves A, et al. Controlling tumor growth by modulating endogenous production of reactive oxygen species. *Cancer Res* 2005;**65**:948–56.
64. Wang YQ, Chen J, Lu JX, Xi JQ, Xu ZL, Fan L, et al. Metal ion-s/nucleotide coordinated nanoparticles comprehensively suppress

- tumor by synergizing ferroptosis with energy metabolism interference. *J Nanobiotechnology* 2022;**20**:199.
65. Zhang X, Yang Q, Lang YH, Jiang X, Wu P. Rationale of 3,3',5,5'-tetramethylbenzidine as the chromogenic substrate in colorimetric analysis. *Anal Chem* 2020;**92**:12400–6.
66. Satoh AY, Trosko JE, Masten SJ. Methylene blue dye test for rapid qualitative detection of hydroxyl radicals formed in a Fenton's reaction aqueous solution. *Environ Sci Technol* 2007;**41**:2881–7.
67. Sepand MR, Bigdelou B, Maghsoudi AS, Sanadgol N, Jim QH, Chauhan P, et al. Ferroptosis: environmental causes, biological redox signaling responses, cancer and other health consequences. *Coordin Chem Rev* 2023;**480**:215024.
68. Xu M, Zha HD, Han R, Cheng YX, Chen JM, Yue LD, et al. Cyclodextrin-derived ROS-generating nanomedicine with pH-modulated degradability to enhance tumor ferroptosis therapy and chemotherapy. *Small* 2022;**18**:e2200330.
69. Zhang X, Hou LC, Guo Z, Wang GC, Xu JT, Zheng ZH, et al. Lipid peroxidation in osteoarthritis: focusing on 4-hydroxynonenal, malondialdehyde, and ferroptosis. *Cell Death Discov* 2023;**9**:320.
70. Yang SLX, Wong KH, Hua P, He CW, Yu H, Shao D, et al. ROS-responsive fluorinated polyethyleneimine vector to co-deliver shMTHFD2 and shGPX4 plasmids induces ferroptosis and apoptosis for cancer therapy. *Acta Biomater* 2022;**140**:492–505.
71. Leist M, Single B, Castoldi AF, Kühnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* 1997;**185**:1481–6.
72. Yu M, Zeng WW, Ouyang YQ, Liang S, Yi YF, Hao HS, et al. ATP-exhausted nanocomplexes for intratumoral metabolic intervention and photoimmunotherapy. *Biomaterials* 2022;**284**:121503.
73. Zeng FT, Nijati S, Tang LG, Ye JM, Zhou ZJ, Chen XY. Ferroptosis detection: from approaches to applications. *Angew Chem Int Ed Engl* 2023;**62**:e202300379.