**Engineered exosomes for the targeted delivery of a novel therapeutic cargo to enhance sorafenib-mediated ferroptosis in hepatocellular carcinoma.**

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**Supplementary Figure**

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**Figure S1 Suppressed ferroptotic activity during sorafenib treatment is associated with compromised therapeutic efficiency. Related to figure 1.**

(**A**) Colony formation ability of HepG-2 cells from the control, sorafenib, sorafenib plus vehicle and sorafenib plus ferrostatin-1 groups. (**B**) GSVA was conducted to calculate the score for enrichment of ferroptosis suppressor genes. Hepatocellular carcinoma cells with sorafenib sensitivity or sorafenib resistance were obtained from the Gene Expression Omnibus (GSE176151). (**C**) Kaplan–Meier progression-free survival analysis of the ferroptosis suppressor gene signature in HCC patients who received sorafenib treatment. (**A, B**) The data are shown as the means ± S.E.M. (**A**) ANOVA with Dunnett’s *t*-test. (**B**) Unpaired *t*-test. (**C**) Log-rank test. \**p* <0.05, \*\**p* <0.01.



**Figure S2 Gene-silencing activities of multi-siRNA against GPX4 and DHODH genes. Related to figure 2.**

(**A**) Real-time PCR was conducted to detect GPX4 mRNA levels in HepG-2 cells transfected with scramble or GPX4 siRNAs. (**B**) Real-time PCR was conducted to detect the DHODH mRNA level in HepG-2 cells transfected with scramble or DHODH siRNAs. (**A, B**) The data are shown as the means ± S.E.M. ANOVA with Dunnett’s *t*-test. \*\*\**p* <0.001.



**Figure S3 SP94-Lamp2b-RRM-functionalized exosomes could efficiently target hepatocellular carcinoma *in vivo.* Related to figure 6.**

(**A**) Relative fluorescence intensity of DiR-labeled exosomes in different organs and tumors in Figure 6D.

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**Figure S4 Systemic toxicity evaluation. Related to figure 8. (A)** The body weight of each mouse was recorded every seven days. (**A**) The data are shown as the means ± S.E.M. ANOVA with Dunnett’s *t*-test. ns *p* >0.05.

**Supplementary Methods**

**Cell proliferation assay.**

The viability of cells was measured by Cell Counting Kit-8 (CCK-8) solution (7sea biotech, China). Briefly, cancer cells were seeded on 96-well plates (Corning, USA) and incubated at 37 °C overnight. The CCK-8 reagents were then added to a subset of wells. After the cells were incubated for 2 h at 37 °C, we quantified the absorbance at 450 nm using a microplate reader (Bio–Rad, USA). Each group was made in quintuplicate.

**Western blot analysis**

The cells were lysed on ice, according to the instructions. Protease inhibitor cocktail (MedChem Express, USA) was used in cell lysates to increase protein stability. After SDS–PAGE, the proteins were transferred to PVDF membranes (0.22 µm, Invitrogen) using a Bio–Rad Semi-Dry Electrophoretic Transfer Cell. Western blot analyses were performed using corresponding specific antibodies, followed by HRP-conjugated IgG antibody. Enhanced chemiluminescence against HRP was used for the visualization of immunoreactive proteins.

**qRT–PCR**

Total RNA was isolated from cultured cells with RNAiso Plus (Takara, Dalian, China), and cDNA was synthesized with the PrimeScript RT Reagent Kit (Vazyme, Nanjing, China). Then, cDNA and SYBR Green Ex Taq (Vazyme, Nanjing, China) were used for real-time PCR in a Prism 7500 real-time thermocycler (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The results were analyzed by the relative quantitation 2-ΔΔCT, and GAPDH was used as an internal control. The primers (Table S1) for GPX4 and DHODH were designed by Tsingke (Beijing, China). Each group was analyzed in triplicate.

**Cell transfection**

siRNA molecules (Gene Pharma, China) were used to knockdown GPX4 or DHODH expression (Table S2). siRNAs were transfected into cells using Lipofectamine 3000 (Invitrogen, USA) following the manufacturer’s instructions. The sequence of multi-siRNAs was listed in Table S3.

**Immunofluorescence staining**

Cells for immunofluorescence were seeded on glass plates at 37 °C overnight. The cells were then washed twice with cold PBS, fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature, and blocked with goat serum at room temperature for 20 min. After blocking, cell samples were incubated with primary antibody overnight at 4 °C. Then, the nuclei were counterstained with 4’-6-diamidino-2-phenylindole (DAPI, Invitrogen, USA). Images were captured using a confocal microscope (Olympus, Japan).

**Kaplan–Meier plotter analysis**Survival analysis in HCC patients who received sorafenib treatment was performed on the Kaplan–Meier plotter website (www.kmplot.com), an online database that can assess the effect of 54,675 genes on the prognosis of breast cancer, liver cancer, lung cancer and gastric cancer patients.

**GEO Datasets**

GSE109211 is published array data of human hepatocellular carcinoma. Tumor tissues from 64 patients (sorafenib responder, n=21; sorafenib nonresponder, n=43) who received sorafenib treatment were collected. Ferroptosis suppressor genes (GPX4, DHODH, FTH, FTL, NFS1, NQO1) were used in the GSVA analysis.

**Exosome purification**

For exosome isolation from HEK-293T cells, cells were cultured in DMEM with 10% exosome-depleted FBS (ultracentrifugation at 120,000 ×g for 16 h) for 24–36 h. Then, the culture medium was precleared by centrifugation at 500×g for 15 min and then at 10000×g for 20 min. Exosomes were isolated by ultracentrifugation at 110000×g for 70 min at 4 °C and washed in PBS using the same centrifugation conditions. The concentration of exosomes was determined by measuring total exosomal protein using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufacturer’s instructions. NanoSight and transmission electron microscopy were used to determine the size distribution, concentration and morphology of the exosomes. For siRNA loading experiments, 100 nM corresponding siRNAs were transfected with Lipofectamine 3000. Twenty-four hours later, the cells were further cultured in DMEM with 10% exosome-depleted FBS for another 24 h, followed by exosome isolation.

**Plasmid Construction**

Briefly, the sequences for SP94 peptide (SFSIIHTPILPL), the CDS sequences of Lamp2b and the CDS sequences of the N-terminal RNA recognition motif (RRM) of U1-A were orderly arranged from N-terminal to C-terminal. Then the sequence encoding the above fusion protein was digested with BamHI-XhoI and cloned into pcDNA3.1(+) vector. The clones were confirmed by DNA sequencing and stored for the following experiments.

**Animal study**

Six-week-old male nude mice were used. All animal experiments were carried out under protocols approved by the Animal Care and Use Committee of Fourth Military Medical University.

For *in vivo* tracking of exosomes, purified exosomes with the indicated modifications were labeled with the fluorescent dye DiR at a final concentration of 8 μM (Invitrogen). Labeled exosomes were collected by ultracentrifugation after washing with saline and stored in saline before use. Mice were injected with labeled exosomes (100 μg at the protein level in 100 μL) via tail vein injection. Mice were subjected to fluorescent living imaging 6 h after injection with an *in vivo* imaging system (IVIS lumina II).

For orthotopic implantation, six-week-old male nude mice were anesthetized with 3% (w/v) pentobarbital sodium by intraperitoneal injection. Then, 2×106 sorafenib-resistant HepG-2 cells stably expressing luciferase were surgically implanted into the left liver lobes of mice. Tumor growth was monitored by bioluminescence with an *in vivo* imaging system (IVIS lumina II). Two weeks after inoculation, mice were randomized to each group and began to receive different treatments. In the sorafenib treatment group, sorafenib (30 mg/kg) was given every 3 days. In the sorafenib and exosome combination treatment group, sorafenib was administered at the same dose, and 100 μg of the indicated exosomes (at the protein level) was injected via the tail vein 24 h after every sorafenib administration.

**Table S1: The sequence of primer sets for qPCR**

|  |  |  |
| --- | --- | --- |
| ID | Forward | Reverse |
| GPX4 | GAGGCAAGACCGAAGTAAACTAC | CCGAACTGGTTACACGGGAA |
| DHODH | GTTCTGGGCCATAAATTCCGA | TCTGGGTCTAGGGTTTCCTTC |

**Table S2: The sequence of siRNAs**

|  |  |  |
| --- | --- | --- |
| **ID** | **sense（5'-3'）** | **antisense（5'-3'）** |
| GPX4#1 | GGAGUAACGAAGAGAUCAA | UUGAUCUCUUCGUUACUCC |
| GPX4#2 | GCCAUCAAGUGGAACUUCA | UGAAGUUCCACUUGAUGGC |
| GPX4#3 | GACCGAAGUAAACUACACU | AGUGUAGUUUACUUCGGUC |
| DHODH#1 | GGGCCAUAAAUUCCGAAAU | AUUUCGGAAUUUAUGGCCC |
| DHODH#2 | CGGGAUUUAUCAACUCAAA | UUUGAGUUGAUAAAUCCCG |
| DHODH#3 | CAGGUAUGGAUUUAACAGU | ACUGUUAAAUCCAUACCUG |
| Scramble | UUCUCCGAACGUGUCACGU | ACGUGACACGUUCGGAGAA |

**Table S3: The sequence of multi-siRNAs**

|  |  |  |
| --- | --- | --- |
| **ID** | **sense（5'-3'）** | **antisense（5'-3'）** |
| multi-siRNA#1 | GGAGUAACGAAGAGAUCAACAAUGGGCCAUAAAUUCCGAAAU | AUUUCGGAAUUUAUGGCCCAUUGCACUUGAUCUCUUCGUUACUCC |
| multi-siRNA#2 | GGAGUAACGAAGAGAUCAACAAGGGCCAUAAAUUCCGAAAU | AUUUCGGAAUUUAUGGCCCUUGAUCUCUUCGUUACUCC |