

HOTTIP suppresses ferroptosis via mediating DGCR8/miR-214-3p/GPX4 regulatory axis in osteosarcoma

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Abstract. Osteosarcoma (OS) is the most common primary bone malignancy in children and adolescents and the current typical strategy remains unsatisfactory in clinical practice. Ferroptosis has been considered as a novel form of programmed cell death in eukaryotic cells, which is characterized by iron-dependent lipid peroxidation accumulation. The emergence of ferroptosis brings great hope to develop the potential therapeutic targets for OS patients. Long noncoding (lnc)RNA HOXA transcript at the distal tip (HOTTIP) has been identified as an oncogene to facilitate tumorigenesis in OS. Whether ferroptosis participates in this lncRNA mediated OS tumorigenesis is not fully understood. In the present study, HOTTIP was found to be downregulated in the Erastin-treated OS cells. Silence of HOTTIP promoted, while ectopic expression of HOTTIP suppressed, ferroptosis in OS cells *in vitro* and *in vivo*. Mechanically, HOTTIP recruited the RNA binding protein DiGeorge Critical Region 8 (DGCR8) and influenced its protein stability, which disrupted miR-214-3p biogenesis and facilitated the de-repression of glutathione peroxidase 4 transcription, eventually leading to preventing ferroptosis. Taken together, the present study demonstrated that HOTTIP

suppressed ferroptosis in OS cells via DGCR8/micro RNA 214-3p/GPX4 regulatory axis, which might provide insights to develop HOTTIP as a promising therapeutic target for OS patients.

Introduction

As a primary malignant bone tumor, osteosarcoma (OS) often occurs mainly in children and adolescents. Current typically clinical therapy for OS patients is surgery in combination with adjuvant chemotherapeutic agents. However, neither surgical nor non-surgical treatment yields satisfactory effects in OS patients, high mortality and recurrence rates are still obstacles to clinical practice (1). Finding new therapeutic targets is therefore urgently needed to improve the life quality and survival for OS patients. Ferroptosis has emerged as a novel form of programmed cell death in eukaryotic cells and it is morphologically, genetically and biochemically distinct from other types of cell death such as apoptosis, necroptosis and autophagy (2). Ferroptosis is characterized by iron-dependent lipid peroxidation accumulation, which leads to accumulated intracellular production and subsequent cell membrane rupture and death (3,4). Although the phenomenon of ferroptosis has been present in multiple cancers, the detailed mechanism, especially in OS, is largely unknown. Elucidating the regulatory mechanism of ferroptosis is therefore an effective strategy to find the potential targets for OS therapeutics.

Long non-coding RNAs (lncRNAs) have been considered as important regulators in various biological activities and diseases and orchestrate a number of cell biological events ranging from embryogenesis to cell fate determination (5). They have been demonstrated to actively participate in various tumor processes, including carcinogenesis, metastasis, epithelial to mesenchymal transition and drug resistance (6,7). Furthermore, ferroptosis has been reported to be involved in the cancer development, metastasis and drug resistance these lncRNAs mediate. For instance, lncRNA-PMAN or LINC00239 inhibit ferroptosis in gastric and colorectal cancer (8,9). Moreover, lncRNA SNHG14 is markedly upregulated in nutlin3a-resistant OS cells and contributes to drug

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Abbreviations: CHX, cycloheximide; DGCR8, DiGeorge Critical Region 8; Fer-1, ferrostatin-1; GPX4, glutathione peroxidase 4; GSH, glutathione; HOTTIP, HOXA transcript at the distal tip; IKE, Imidazole ketone erastin; MDA, malondialdehyde; miRNA, micro RNA; Necro, necrosulfonamide; OS, osteosarcoma; ROS, reactive oxygen species; xCT, X cystine/glutamate antiporter

Key words: osteosarcoma, HOXA transcript at the distal tip, ferroptosis, DiGeorge Critical Region 8, micro RNA 214-3p

resistance through suppressing ferroptosis (10). HOXA transcript at the distal tip (HOTTIP) is an identified onco-lncRNA and it is upregulated in OS tissues (11). HOTTIP also facilitates cell proliferation, invasion and migration as well as chemoresistance in OS and is associated with poor prognosis in OS patients (12). The present study therefore considered whether ferroptosis is involved in HOTTIP modulated OS progression.

In the present study, a CRISPR/Cas9 system was applied to generate the HOTTIP knockout (KO) mice and the transcriptome analysis from bone marrow revealed that several signaling related ferroptosis were altered, suggesting that HOTTIP may be involved in ferroptosis. It also demonstrated that HOTTIP knockdown promoted, while its overexpression suppressed, ferroptosis in OS cells *in vitro* and *in vivo*. Furthermore, HOTTIP physically interacted with RNA binding protein DiGeorge Critical Region 8 (DGCR8) and influenced its protein stability. As a component of microprocessor complex, DGCR8 interacts with Drosha and facilitates miRNA maturation. The results showed that HOTTIP knockdown potentiated miR-214-3p expression and facilitated the suppression of its target gene glutathione peroxidase 4 (GPX4) transcription, thereby leading to stimulating ferroptosis. Taken together, the results demonstrated that HOTTIP suppressed ferroptosis in OS cells via a novel DGCR8/miR-214-3p/GPX4 regulatory axis, which provided insights to develop HOTTIP as a promising therapeutic target for OS patients.

Materials and methods

Reagents and antibodies. Erastin (cat. no. HY-15763), RSL3 (cat. no. HY-100218A), ferrostatin-1 (cat. no. HY-100579), Z-VAD-FMK (cat. no. HY-16658B) and necrosulfonamide (cat. no. HY-100573) were purchased from MedChemExpress. Imidazole ketone erastin (cat. no. T5523) was purchased from Topscience. Primary antibodies, X cystine/glutamate antiporter (xCT; cat. no. T57046; 1:1,000; Abmart Pharmaceutical Technology Co., Ltd.), GPX4 (cat. no. T56959; 1:1,000; Abmart Pharmaceutical Technology Co., Ltd.), DGCR8 (cat. no. ET1609-32; 1:1,000; HuaBio), Ubiquitin (cat. no. T55965; 1:1,000; Abmart Pharmaceutical Technology Co., Ltd.) and β -actin (cat. no. P30002; 1:1,000; Abmart Pharmaceutical Technology Co., Ltd.). Secondary anti-rabbit antibodies were from Cell Signaling Technology, Inc. (cat. no. 7074S; 1:2,000).

RNA sequencing. RNA sequencing was provided by CloudSeq Biotech Inc. A total of six male HOTTIP KO mice and male WT mice were provided by Cyagen Biosciences. The mice were bred in a specified pathogen-free environment at temperature and humidity controlled (26°C and 50% humidity) environment with 12-h light/dark cycle, and served a standard diet. The mice were 4-6 weeks old and weighed 20-22 g and consisted of three HOTTIP KO mice and three WT mice. They were sacrificed and their bone marrow was isolated, total RNA was extracted using Triquick Reagent (cat. no. R1100; Beijing Solarbio Science & Technology Co., Ltd.). Total RNA was isolated and reverse transcribed into cDNA to generated an indexed Illumina library (Illumina, Inc.), including fragmentation (~300 bp), adapter ligation (unique dual indexes), and PCR amplification (15 cycles). Library quality was validated via Agilent 2100 Bioanalyzer (Agilent

Technologies, Inc.; peak size: 280-320 bp) and quantified by Qubit Flex Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.), followed by sequencing on an Illumina Novaseq platform (Illumina, Inc.).

Cell culture. Human OS cell lines including MG63 and U2OS were provided by Lingnan Medical Research Center, Guangzhou University of Chinese Medicine (Guangzhou, China). Cell lines were authenticated by STR profiling (ATCC) and tested negative for mycoplasma. The two OS cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Shanghai VivaCell Biosciences, Ltd.) with 10% fetal bovine serum (FBS; Shanghai ExCell Biology, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). These cells were incubated and maintained in a humidified atmosphere at 37°C, 5% CO₂.

Cell viability assays. The OS cells were seeded into 96-well plates and cultured for 12 h. Then, cells were starved using 2% FBS for 12 h, the Fer-1 group was preincubated with 2 μ M Fer-1 (MedChemExpress). Subsequently, cells were incubated with 10% FBS and induced by 10 μ M Erastin (MedChemExpress) and 1 μ M RSL3 (MedChemExpress) for 24 h. The cell viability was detected by Cell Counting Kit-8 (Beyotime Institute of Biotechnology) examination and the absorbance was measured at 450 nm using a Hybrid Multi-Mode Microplate Reader (Tecan Group, Ltd.). All the experiments were performed in triplicates.

Colony formation assays. The MG63 and U2OS cells were seeded into 6-well plate and treated with 10 μ M Erastin and 2 μ M Fer-1 at 37°C in a 5% CO₂ incubator for two weeks. At room temperature, the cells were fixed with 4% paraformaldehyde for half an hour and stained with crystal violet staining solution for half an hour. The images were captured using the ImmunoSpot analyzer (Cellular Technology Ltd.) and the colony numbers were counted by ImmunoSpot version 6.0 Academic system (Cellular Technology Ltd.).

Detection of ferroptosis. The OS cells were seeded and ferroptosis was induced by Erastin and RSL3 at 37°C in a 5% CO₂ incubator for 24 h. The samples were collected for the ferroptosis examination. The level of intracellular iron was detected using an Iron Assay kit (Applygen Technologies, Inc.) according to the manufacturer's instructions. For intracellular ROS staining, the lipid ROS level was examined by flow cytometry with a 5 μ M DHE fluorescence probe (Applygen Technologies, Inc.) in a visible spectrum of red range (emission maximum 610 nm). The levels of malondialdehyde and glutathione were evaluated by a lipid peroxidation (MDA) assay kit and a reduced glutathione (GSH) assay kit (Njjcbio).

Reverse transcription-quantitative (RT-q) PCR. Total RNA from 5x10⁵ cells was extracted using Animal Total RNA Isolation Kit (Chengdu Fuji Biotechnology Co., Ltd.) and it was reversely transcribed using PrimeScript RT Reagent Kit (Takara Bio, Inc.) following the manufacturer's instructions. The RT-qPCR examinations were conducted using Power Up TB Green Master Mix (Takara Bio, Inc.) on an ABI-QuantStudio 5 System (Thermo Fisher Scientific, Inc.).

The cycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 25–45 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec (depending on the target gene), and elongation at 72°C for 1 min, with a final extension at 72°C for 5 min. The reactions were stopped during the exponential phase to ensure accurate comparisons. The primer sequences are listed in Table I. GAPDH served as the endogenous control and fold changes were calculated using the relative quantification ($2^{-\Delta\Delta C_q}$) method (13). All the experiments were performed in triplicates.

Western blotting. Total protein was lysed using Radio Immunoprecipitation Assay (RIPA) buffer supplemented with protease and phosphatase inhibitor (Beyotime Institute of Biotechnology). The supernatant fraction was collected by centrifugation and quantified by BCA assay (Thermo Fisher Scientific, Inc.). Subsequently, an equal volume of protein mixture (30 μ g) were separated by 10% SDS-PAGE and transferred to a PVDF membrane (MilliporeSigma). The membranes were blocked with 5% fat-free milk (Bio-Rad Laboratories, Inc.) for 1 h at room temperature and incubated at 4°C overnight with primary antibodies. Next, the membranes were incubated with secondary antibodies in dark at room temperature for 1 h. The expression levels were visualized by chemiluminescence (MilliporeSigma). Densitometric analysis was carried out using ImageJ software (ver. 1.46; National Institutes of Health).

Co-immunoprecipitation (Co-IP). Co-IP was conducted with a magnetic IP kit (cat. no. 88804; Thermo Fisher Scientific, Inc.). Briefly, 1×10^7 cell lysates were gently rotated at 4°C overnight with anti-DGCR8 antibody, or normal rabbit IgG (Wuhan Servicebio Technology Co., Ltd.). Afterwards, pre-washed protein A/G magnetic beads were incubated with rotating at 4°C for 16 h. The beads were collected and washed three times. Western blotting was performed after eluting bound proteins with elution buffer.

RNA immunoprecipitation (RIP). RIP was performed as mentioned before (14) using RNA Binding Protein Immunoprecipitation Kit (cat. no. JKR23003; Wuhan Gene Create Biological Engineering Co., Ltd.). According to the manufacturer's protocol, the A/G protein magnetic beads were pre-incubated with anti-DGCR8 antibody or IgG (Wuhan Servicebio Technology Co., Ltd.) at 4°C overnight. The 1×10^7 cells were washed with cold PBS twice and lysed with 200 μ l lysis buffer. Then, the lysates were incubated with the pre-coated beads at 4°C for 16 h. The total RNA was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and detected by RT-qPCR examination.

Stable cell line generation and miRNA transfection. Stable HOTTIP overexpression or knockdown cell lines were constructed using 2nd lentiviral system as previously reported (15). Briefly, the 4 μ g lentivirus was generated in 293T cells (The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences) by co-transfecting with two packaging vectors (3 μ g psPAX2; 1 μ g pMD2G). Lentiviral particles were harvested 48 h post-transfection, followed by infection of OS cells for 24 h. Cells (30% confluence) were seeded in cell culture

dishes and were infected with lentiviral plasmids at multiplicity of infection (MOI) of 10. After 37°C and screening of the cells for 7 days with medium containing 1 μ g/ml puromycin (Beyotime Institute of Biotechnology), these virus particles infected OS cells and the stable cell lines were developed. The sequence of short hairpin (sh)HOTTIP were Sense: 5'-nnnnnG GCACTTTATATGCTGTAAAnnnnnnnnnTTACAGCATATA AAGTGCCnnnnnn-3'; Anti-sense: 5'-nnnnnnnnnnGGCAC TTTATATGCTGTAAAnnnnnnnnnTTACAGCATATAAAGT GCCn-3'. Has-miR-214-3p mimics were designed and synthesized by Beijing Tsingke Biotech Co., Ltd. These oligoes were transfected in OS cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). The sequence of miR214-3p were Sense: 5'-ACAGCAGGCACAGACAGGCAGU-3'; Anti-sense: 5'-ACUGCCUGUCUGUGCCUGCUGU-3'.

Luciferase activity assays. The GPX4 3'UTR-untranslated region (3'UTR) containing wild-type or mutated miR-214-3p binding sites were respectively synthesized and inserted into pmirGLO luciferase vector (Wuhan Gene Create Biological Engineering Co., Ltd.) and then co-transfected with miR-214-3p mimics into OS cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h. The cell lysates were collected 48 h post transfection and Firefly and Renilla luciferase activities were detected by the Dual-Luciferase Reporter Assay kit (Promega, USA). The luciferase activity was normalized to Renilla luciferase activity.

Immunofluorescence. OS cells were seeded in confocal petri dishes and cultured to 80% confluence. The dishes were then washed twice with phosphate-buffered saline (PBS), fixed with 200 μ l 4% paraformaldehyde at room temperature for 15 min, and permeabilized with 200 μ l 0.1% Triton X for 15 min at room temperature. After washing twice with PBS, the dishes were incubated with the anti-DGCR8 antibody with 1:200 dilution at 4°C overnight. The next day, the dishes were washed twice with PBS, then anti-rabbit IgG-Cy3 Fluor 570 (Beyotime Institute of Biotechnology) was added at 1:1,500 dilution and further incubated at room temperature for 1 h. The nucleus was then counterstained with DAPI (Shanghai Yeasen Biotechnology Co., Ltd.) at room temperature for 5 min and the images were captured using a Zeiss Axiophot 2 microscope (Zeiss AG). The magnification of all images was x40.

Osteosarcoma intra-tibia tumor-bearing model. A total of 20 female Balb/c-nude mice (3–4 weeks old, weight 10–13 g) were purchased from the Laboratory Animal Center, Southern Medical University. The mice were bred in a specified pathogen-free environment at temperature and humidity controlled (26°C; 50% humidity) environment with 12-h light/dark cycle and served a standard diet *ad libitum*. The animals were randomly assigned into four groups (n=5), two groups (Group1 and Group2) were inoculated with $1.5 \times 10^6/100 \mu$ l shHOTTIP infected OS cells through trans-tibia injected into the medullary cavity of the right tibia of mice; the other two groups (Group3 and Group4) were injected with equivalent shNC infected OS cells at the same site. Group1 and Group3 were intraperitoneally (i.p.) injected with 20 mg/kg IKE (Shandong Topscience Biotech Co., Ltd.) and the other two groups were treated with an equal volume of vehicle NaCl.

Table I. The sequences of primer for reverse transcription-quantitative PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
HOTTIP	CCTAAAGCCACGCTTCTTTG	TGCAGGCTGGAGATCCTACT
DGCR8	GCCTCCTCATAGACCCGAAC	CGGTAAAGCTCACGCTAATCTT
GAPDH	GCACCACCAACTGCTTAGCA	TCTTCTGGGTGGCAGTGATG

The IKE or NaCl was added once every other day and tumor formation was monitored. The tumor volume was calculated using the formula $V=0.5 \times L \times W^2$ (L, length and W, width). The largest tumor diameter allowed in this experiment was <20 mm. At the end of the experiment, the mice were anesthetized with a dose of 100 mg/kg pentobarbital (intraperitoneal), followed by cervical dislocation. Mortality was confirmed by the cessation of breathing and heartbeat. And their tumor tissues were isolated. All animal experimental procedures were approved by the Ethics and Animal Research Committee of Southern Medical University (approval no. SMUL2022219; Guangzhou, Guangdong).

Immunohistochemistry examination. Tumor tissues were fixed in 10% neutral formalin fixative solution (BBI Solutions) embedded in paraffin at room temperature overnight, and sectioned at 3- μ m, which were deparaffinized with xylene and then rehydrated with a succession of decreasing alcohol concentrations (100, 95, 85, 70 and 50% ethanol). Tissue sections were then placed in antigen retrieval buffer (pH 9.0; Wuhan Servicebio Technology Co., Ltd.) in a microwave oven on medium power for 10 min until boiling, then cooled for 8 min and switched to medium-low power for 8 min. After natural cooling, the slides were placed in PBS and washed three times on a destaining shaker. Subsequently, 3% hydrogen peroxide solution was added to the sample at room temperature for 25 min to block endogenous peroxidase, followed by blocking with 3% BSA (Sangon Biotech Co., Ltd.) at room temperature for 30 min. The histological sections were then incubated at 4°C for 4 h with Ki67 antibody (cat. no. GB111499; Wuhan Servicebio Technology Co., Ltd.) and GPX4 antibody (cat. no. T56959; Abmart Pharmaceutical Technology Co., Ltd.) with 1:100 dilution, and with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:200; cat. no. SA00001-2; Proteintech Group, Inc.) at 37°C for 30 min. Sections were then counterstained with 0.1% hematoxylin (Boster Biological Technology) at room temperature for 2 min and were stained with the chromogen DAB (Boster Biological Technology). Histological images were captured using a light microscope (cat. no. AE2000; Motic Incorporation, Ltd.), the representative images were taken with at x40 magnification and the positive cells were quantified with ImageJ software (ver. 1.46; National Institutes of Health).

Bioinformatics analyses. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed on differentially expressed genes using the enrich-KEGG function from the clusterProfiler package in R software (ver. 3.20; https://www.genome.jp/kegg/kegg_ja.html). The result of 25 pathways were visualized using the Pathview

package (ver. 3.20, R Core Team, 2024) (16). Gene Ontology (GO) analysis was performed on genes that were increased or decreased by >2-fold in the HOTTIP KO sample compared with the WT sample. The analysis was conducted using the gseGO function in the clusterProfiler package (ver. 4.14.3) in R software (ver. 4.4.1, R Core Team, 2024), based on the Gene Set Enrichment Analysis approach (<https://www.gsea-msigdb.org/gsea/index.jsp>). GO terms which had adjusted P-value <0.01 were considered markedly regulated GO. Up- or down-regulated GO terms were featured by R software. Heatmaps and volcano plots were generated using TBtools (v 1.055; <https://github.com/CJ-Chen/Tbtools/releases>). Venn diagrams showing the intersection between target genes identified in microRNA databases (Targets can. <https://www.targetscan.org/>) and Ferroptosis Marker (FerrDb v2 database. <http://www.zhounan.org/>). The online bioinformatics programs, miRbase (<https://www.mirbase.org/>), Targets can and RNAhybrid (<https://bibiserv.org/>) were applied to predict the binding site of miR-214-3p with GPX4.

Statistical analysis. Data were shown as mean $\pm$ SD from at least three independent experiments. Data were analyzed by two-tailed unpaired Student's t-test between two groups and by one-way ANOVA followed by the Tukey post hoc test for multiple comparisons, Bonferroni correction was applied where appropriate. Statistical analysis was carried out using GraphPad Prism software version 8 (Dotmatics). P<0.05 was considered to indicate a statistically significant difference.

Results

HOTTIP may be a potential regulator of ferroptosis. HOTTIP has been reported to serve as an oncogene in OS and the emergence of ferroptosis provides a novel opportunity to clarify the underlying mechanism. To clarify the relationship between HOTTIP and ferroptosis, the transcriptome alternation between bone marrow derived from KO and wild-type (WT) mice was analyzed (Fig. 1A). The RNA-sequencing data indicated that the differently expressed gene mainly focused on 25 signaling pathways using the KEGG database (Fig. 1B). According to their relative expression level, the changed genes were divided into upregulation and downregulation groups (Fig. 1C). Gene ontology (GO) analysis showed a number of biological processes (BPs) were abnormally influenced such as lipid transport, cellular response to iron ion, cellular response to reactive oxygen species and regulation of lipid metabolic process (Fig. 1D and E). Furthermore, the related ferroptosis genes were predicted from Gene Set Enrichment Analysis (GSEA) website and it was found that Biological Oxidations gene set was clearly upregulated (Fig. 1F). Considering that

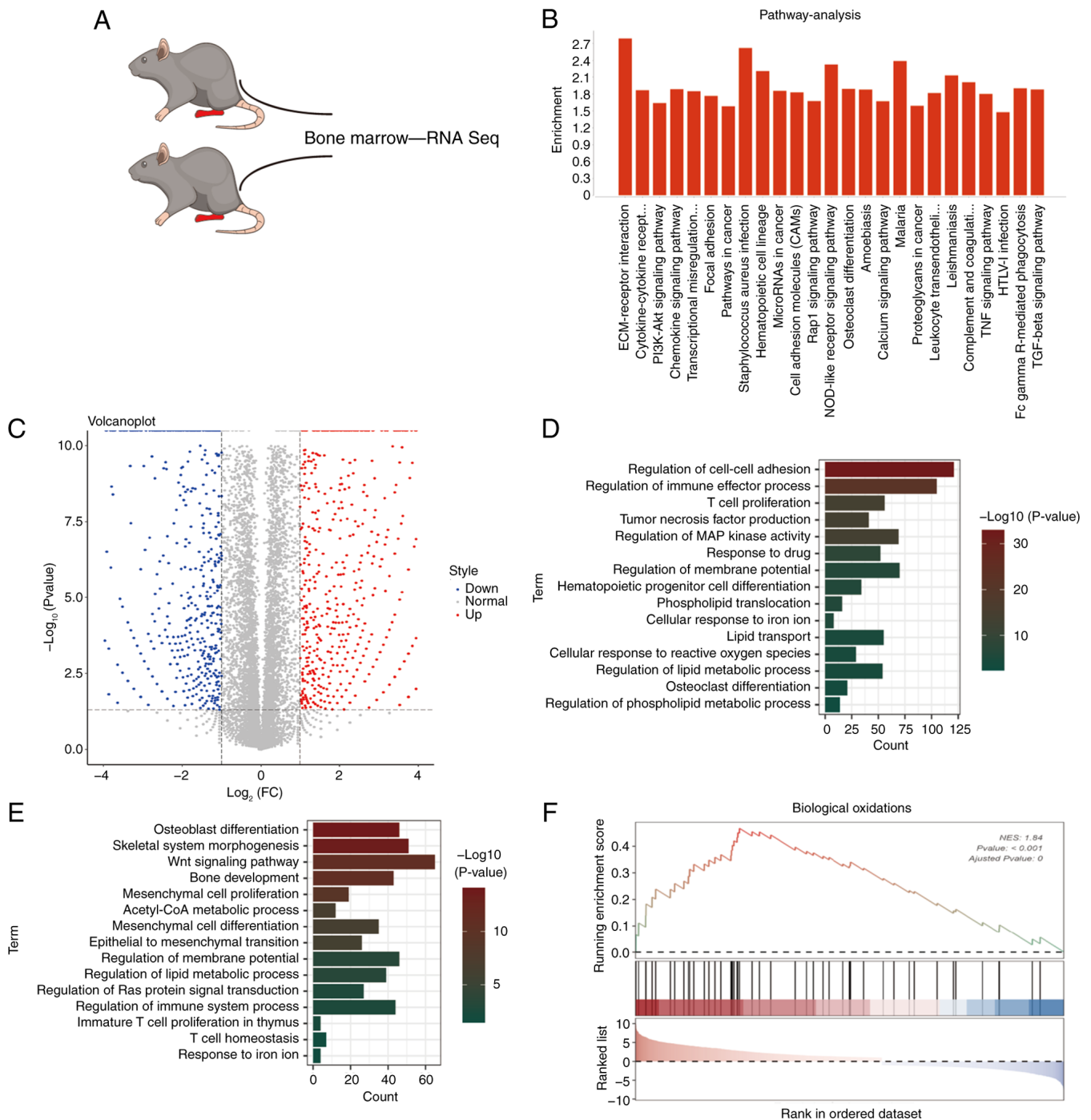


Figure 1. HOTTIP may be associated with ferroptosis. (A) Flow chart of sample preparation. (B) The differently expressed genes mainly enriched in twenty-five signaling pathways. (C) Volcano plot based on the analysis of differentially expressed genes. (D and E) Relevant ferroptotic BPs were markedly influenced. (F) The biological oxidations gene set predicted by GSEA was clearly upregulated. HOTTIP, HOXA transcript at the distal tip; BPs, biological processes; GSEA, Gene Set Enrichment Analysis.

OS is a type of primary malignant bone tumor and most OS come from bone marrow (17), it was therefore hypothesized that HOTTIP may be involved in ferroptosis of OS.

HOTTIP actively participates in the Erastin-induced ferroptosis of OS cells. To determine the involvement of HOTTIP in ferroptosis, the present study successfully established a ferroptotic cell death model with OS cells. As shown in Fig. 2A and B, the cell viability was markedly suppressed after respectively treated with the canonical inducers of ferroptosis Erastin or RSL3. The potent

ferroptosis inhibitor ferrostatin-1 (Fer-1) partially reversed these suppressive effects, whereas the apoptosis inhibitor Z-VAD-FMK and necroptosis inhibitor necrostatin-1 (Necro) appeared to possess no rescue abilities in Erastin or RSL3 treated OS cells (Fig. 2A and B). The colony formation assay also confirmed that Erastin clearly inhibited the numbers and sizes of colonies and Fer-1 markedly attenuated this suppressive effect (Fig. 2C). As a new programmed cell death, reactive oxygen species (ROS) and iron accumulation are two important characters of ferroptosis (18). The level of intracellular iron was determined using

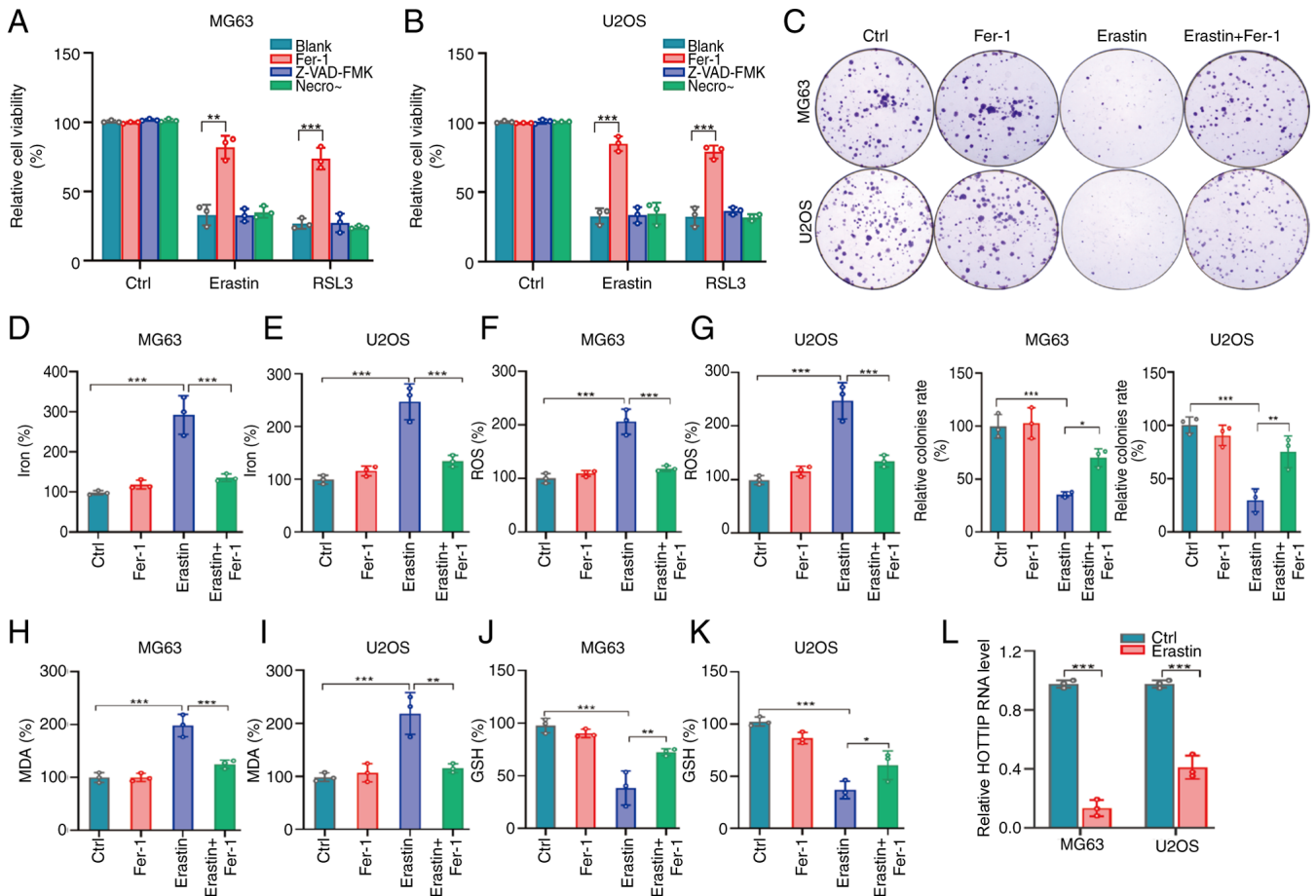


Figure 2. HOTTIP actively participates in the Erastin-induced ferroptosis of OS cells. (A) MG63 and (B) U2OS cells were treated with Erastin (10 μ M) or RSL3 (1 μ M) in the absence or presence of 2 μ M Fer-1, 10 μ M Z-VAD-FMK, or 1 μ M Necro for 24 h, the relative cell viability was measured. (C) MG63 and U2OS cells were treated with 10 μ M Erastin for 14 days and the colony formation was examined. MG63 and U2OS cells were treated with Erastin for 24 h, the levels of (D and E) iron, (F and G) lipid ROS, (H and I) MDA and (J and K) GSH were determined by commercial kits. (L) MG63 and U2OS cells were treated with Erastin for 24 h and HOTTIP expression was monitored by quantitative PCR. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. HOTTIP, HOXA transcript at the distal tip; OS, osteosarcoma; Fer-1, ferrostatin-1; Necro, necrosulfonamide; ROS, reactive oxygen species; MDA, malondialdehyde; GSH, glutathione.

the Iron assay kit and the accumulation of total Fe was promoted by Erastin while Fer-1 markedly rescued this promotion (Fig. 2D and E). The flow cytometry assays were used to detect the intracellular ROS and the results displayed that ROS signal was increased by Erastin and this increase was rescued by Fer-1 in OS cells (Fig. 2F and G). GSH depletion and MDA accumulation are essential for ferroptosis. As expected, the intracellular level of MDA was increased whereas GSH level was suppressed by Erastin in MG63 and U2OS cells (Fig. 2H-K). These results indicated that Erastin successfully triggered the ferroptosis in OS cells, indicating that the ferroptotic cell death model was successfully constructed. Using this cell model, it was found that HOTTIP was markedly suppressed in Erastin-treated OS cells (Fig. 2L), suggesting that HOTTIP may participate in ferroptosis of OS cells.

HOTTIP partly suppresses ferroptosis in OS cells. To identify the real function of HOTTIP in ferroptosis, HOTTIP-overexpressing stable OS cells were established (Fig. S1A). According to cell viability assays, ectopic expression of HOTTIP clearly attenuated the suppressive cell viability triggered by Erastin or RSL3 (Fig. 3A). The accumulation of intracellular iron (Fig. 3B), intracellular ROS signal (Fig. 3C)

and the expression level of MDA (Fig. 3D) were all suppressed while the GSH expression was promoted (Fig. 3E) in this HOTTIP-overexpressing cells. The canonical markers of ferroptosis including xCT and GPX4 were markedly promoted by HOTTIP overexpression in OS cells (Fig. 3F-H). On the other hand, the HOTTIP silencing stable OS cells were developed (Fig. S1B) and it was showed that silencing of HOTTIP markedly enhanced the suppressive effects of the Erastin or RSL3 on cell viability (Fig. 3I). Fer-1 successfully reversed this suppressed cell viability in OS cells. Further investigation showed that the accumulation of intracellular iron, intracellular ROS signal and the expression level of MDA were clearly promoted by HOTTIP knockdown (Fig. 3J-L). Conversely, GSH expression was markedly suppressed in HOTTIP silenced OS cells (Fig. 3M). The expression of xCT and GPX4 was suppressed in HOTTIP silenced OS cells with Erastin or RSL3 treatment (Fig. 3N-P). All these results revealed that HOTTIP inhibited the ferroptosis in OS cells.

Knockdown of HOTTIP clearly promotes ferroptosis in vivo. To further confirm the *in vivo* function of HOTTIP in ferroptosis, an orthotopic intra-tibia tumor-bearing model was established using shHOTTIP infected OS cells. A canonical

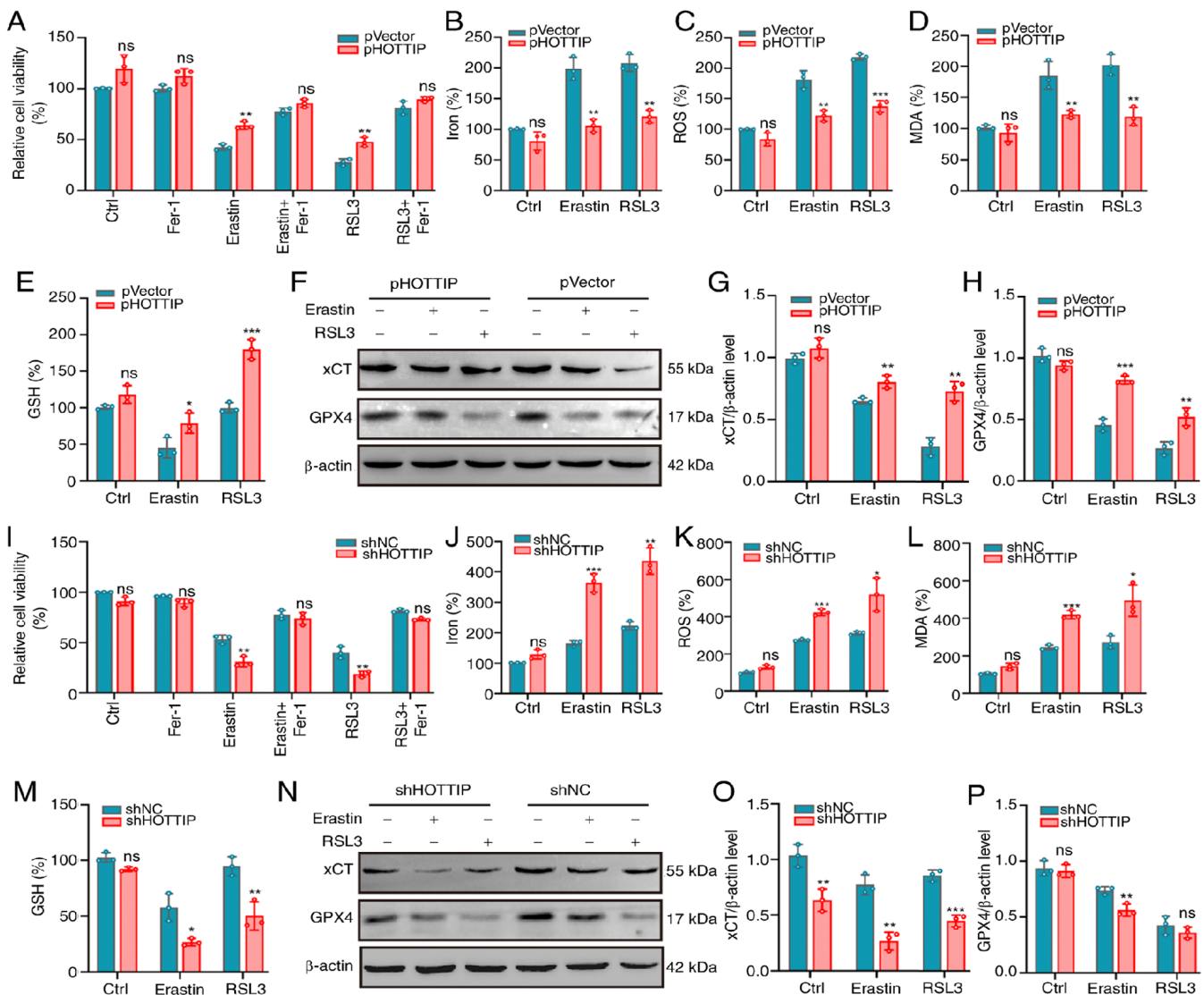


Figure 3. HOTTIP may suppress ferroptosis in OS cells. (A) PHOTTIP or pVector infected MG63 cells were treated with 10 μ M Erastin or 1 μ M RSL3 in the absence or presence of 2 μ M Fer-1 for 24 h and the relative cell viability was measured. PHOTTIP or pVector infected cells were treated with Erastin or RSL3 for 24 h, the levels of (B) iron, (C) lipid ROS, (D) MDA and (E) GSH were determined by commercial kits. (F-H) The ferroptosis relevant proteins expressions of xCT and GPX4 were examined by western blotting. β -actin served as a loading control. (I) The shHOTTIP or shNC infected U2OS cells were treated with 10 μ M Erastin or 1 μ M RSL3 in the absence or presence of 2 μ M Fer-1 for 24 h, the relative cell viability was measured. (J-M) The infected cells were treated with Erastin or RSL3 for 24 h, the levels of Iron (J), lipid ROS (K), MDA (L) and GSH (M) were determined by commercial kits; (N-P) The ferroptosis relevant proteins expressions of xCT and GPX4 were examined by western blotting. β -actin served as a loading control. * P <0.05; ** P <0.01; *** P <0.001; ns: not significant. HOTTIP, HOXA transcript at the distal tip; OS, osteosarcoma; Fer-1, ferrostatin-1; ROS, reactive oxygen species; MDA, malondialdehyde; GSH, glutathione; xCT, X cystine/glutamate antiporter; GPX4, glutathione peroxidase 4; sh, short hairpin; NC, negative control.

in vivo ferroptosis inducer, imidazole ketone erastin (IKE), was given intraperitoneally (i.p.) with 20 mg/kg once every other day. As expected, the IKE groups carried smaller burden when compared with control (NaCl) group and the shHOTTIP group displayed smaller tumor when compared with shNC group (Fig. 4A). Similar results were observed in tumor growth rate (Fig. 4B) and weight (Fig. 4C). The levels of Fe, MDA and GSH were examined in animal tissues and the similar results to the *in vitro* investigation were recorded (Fig. 4D-F). Further immunohistochemical staining indicated that the expressions of Ki-67 and GPX4 were markedly suppressed by IKE treatment and shHOTTIP group exhibited more sensitive to IKE treatment (Fig. 4G and H). These data suggested that HOTTIP knockdown facilitates the ferroptosis-based anti-tumor therapy in OS.

HOTTIP recruits DGCR8 and influences its protein stability. LncRNAs are recognized to exert their function through physically interacting with RNA binding proteins. DGCR8, a known RNA-binding protein, was predicted to directly bind HOTTIP and the RIP assay validated that DGCR8 successfully pulled down HOTTIP RNA transcript (Fig. 5A). Subsequent study demonstrated that overexpression of HOTTIP suppressed while its knockdown promoted DGCR8 expression at protein level (Fig. 5B and C). The immunofluorescence examination also confirmed that the expression of DGCR8 was suppressed by HOTTIP overexpression whereas it was promoted by HOTTIP knockdown (Fig. 5D). However, the mRNA expression level of DGCR8 was inconsistent with HOTTIP overexpression or knockdown (Fig. S2). It was therefore hypothesized that HOTTIP mediated DGCR8 expression via a post-transcriptional

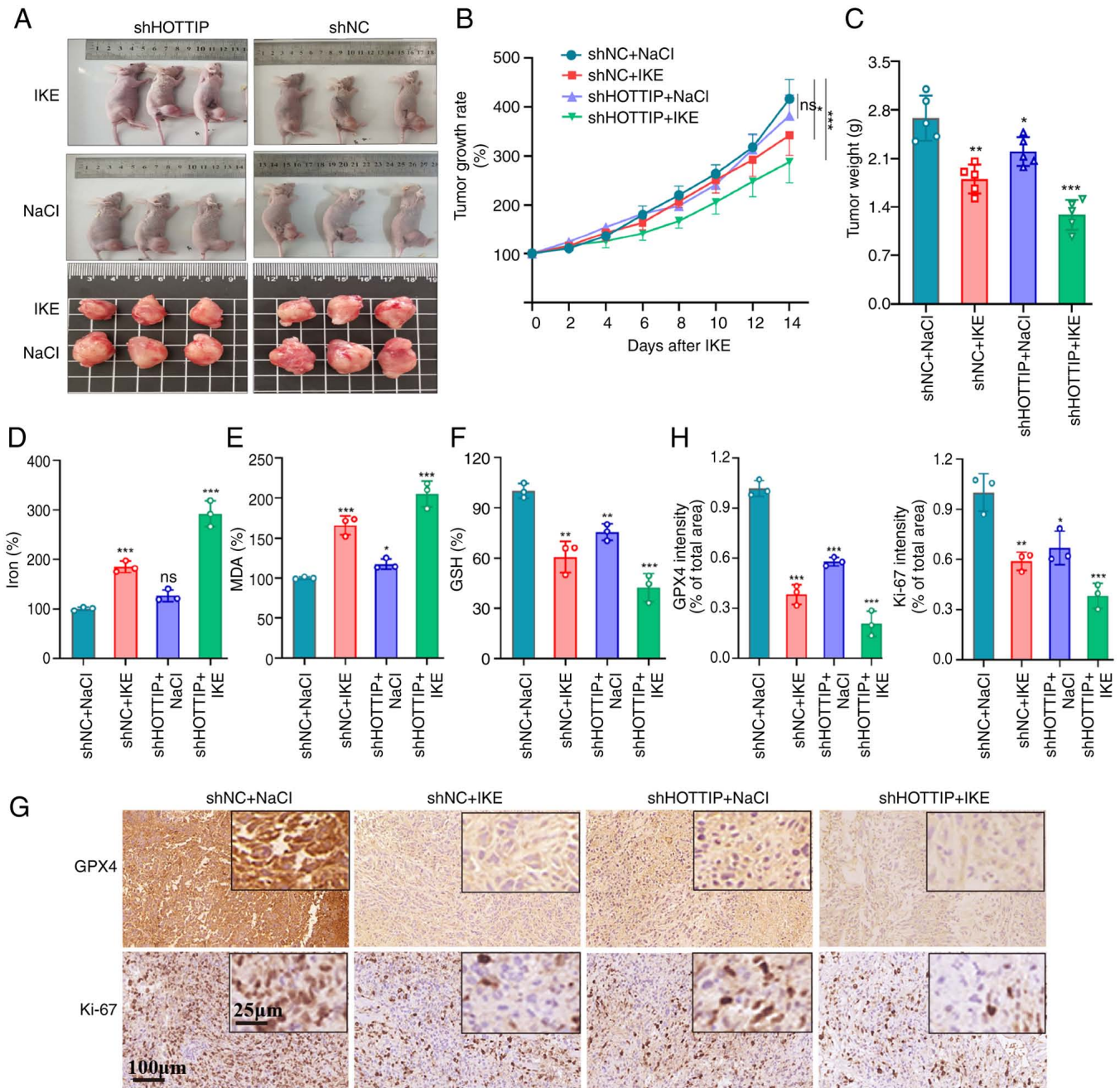


Figure 4. Knockdown of HOTTIP clearly promotes ferroptosis *in vivo*. The shHOTTIP or shNC infected U2OS cells were injected into the medullary cavity of the right tibia of nude mice and 20 mg/kg IKE was administrated with intraperitoneally (i.p.) injection, equal volume of vehicle NaCl as control (n=5). (A) Representative images. The (B) tumor growth rate ($(V_{\text{day}} - V_0) / V_0 \times 100\%$) and (C) tumor weight were calculated. The levels of (D) total iron, (E) MDA and (F) GSH were detected by commercial kits. (G and H) The immunohistochemistry of GPX4 and Ki-67 stained sections followed by counterstaining with hematoxylin. *P<0.05; **P<0.01; ***P<0.001; ns: not significant. HOTTIP, HOXA transcript at the distal tip; sh, short hairpin; NC, negative control; IKE, Imidazole ketone erastin; MDA, malondialdehyde; GSH, glutathione; GPX4, glutathione peroxidase 4.

manner. In order to validate this hypothesis, cycloheximide (CHX) chase assays were conducted to monitor the stability of DGCR8 protein. A shorter half-life was observed in the HOTTIP-overexpressing OS cells (Fig. 5E and F). Given that the ubiquitin-proteasome pathway is the most significant signaling to mediate the protein stability (19), an inhibitor of proteasome MG-132 was added in the HOTTIP-overexpressing OS cells to examine the protein stability of DGCR8. As shown in Fig. 5G and H, MG-132 treatment markedly reversed the suppressive effect of DGCR8 driven by HOTTIP overexpression. Next DGCR8 antibody was utilized to pull down

endogenous DGCR8 proteins and their modification examined by an ubiquitin antibody. Based on the results of Co-IP assays, the decreased DGCR8 ubiquitination was detected in HOTTIP silencing cells (Fig. 5I). This result proposed that destabilization of DGCR8 mediated by HOTTIP may depend on the proteasomal degradation. Collectively, the data demonstrated that HOTTIP physically interacted with DGCR8 and modulated its stability by promoting its ubiquitin-mediated degradation.

HOTTIP influences miR-214-3p biogenesis and suppresses ferroptosis by blocking miR-214-3p expression. Notably,

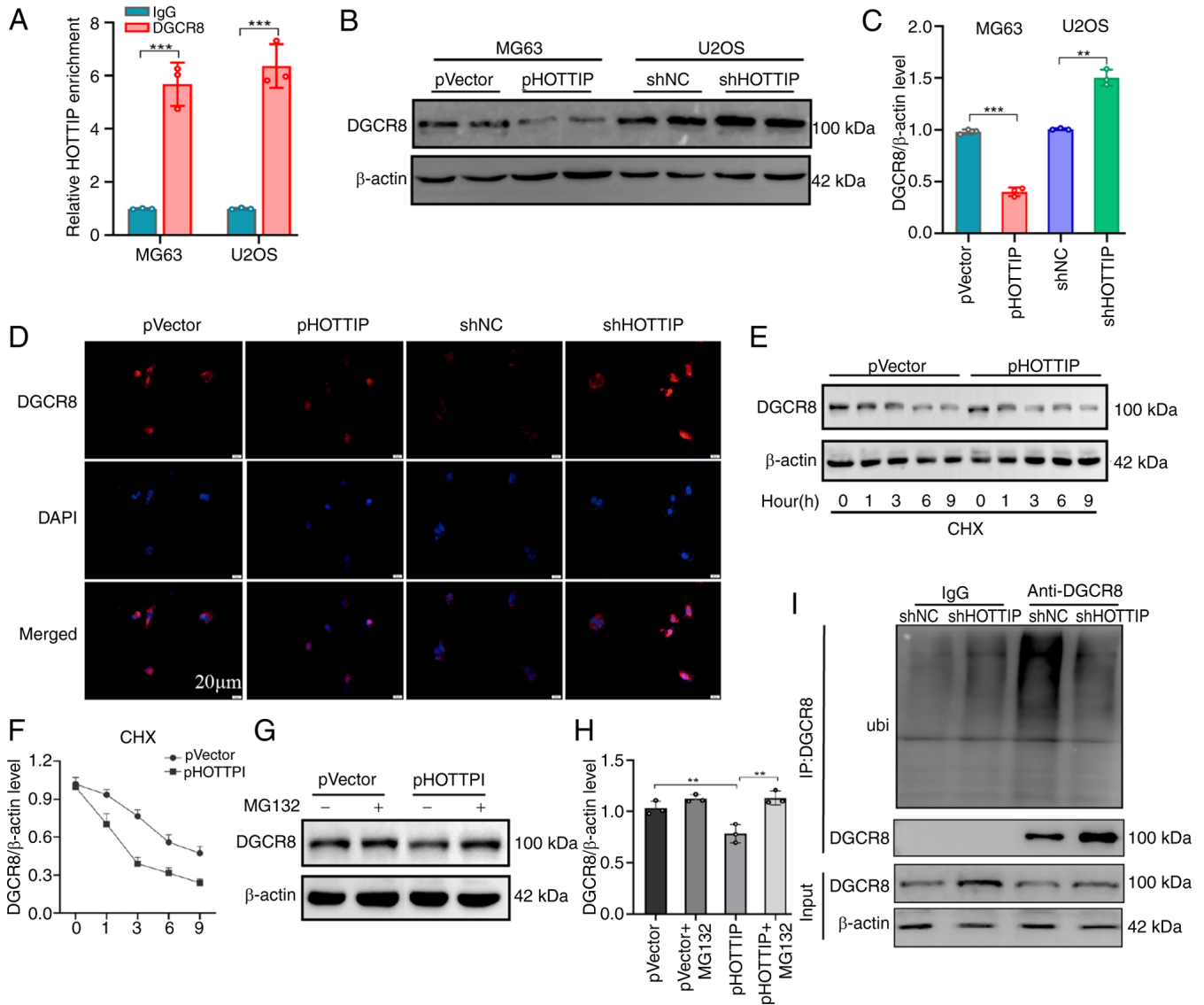


Figure 5. HOTTIP recruits DGCR8 and influences its protein stability. (A) RNA immunoprecipitation assay was conducted to investigate the interaction between HOTTIP and DGCR8. The DGCR8 expression levels were examined in HOTTIP overexpression or knockdown OS cells by (B and C) western blotting and (D) immunofluorescence. The expression levels of DGCR8 were examined by western blotting following (E and F) CHX treatment or (G and H) MG-132 treatment for 3 h in HOTTIP-overexpressing OS cells. (I) HOTTIP knockdown markedly suppressed the ubiquitylation of DGCR8 using Co-IP assay. ** $P < 0.01$; *** $P < 0.001$. HOTTIP, HOXA transcript at the distal tip; DGCR8, DiGeorge Critical Region 8; OS, osteosarcoma; CHX, cycloheximide.

DGCR8 is the RNA-binding partner of the nuclease Drosha. The DGCR8/Drosha complex named by the microprocessor (recognized and cleaved pri-miRNAs) is essential for the processing of primary (pri-)miRNAs in the nucleus (20,21). To identify which miRNA biogenesis was influenced in the present study, miRNA sequencing was conducted using HOTTIP silencing OS cells. As shown in Fig. 6A, among the 10 promising candidates, hsa-miR-214-3p was the most upregulated. Subsequent relevant assays were conducted in the miR-214-3p transfected MG63 cell lines (Fig. S3A), cell viability examination showed that miR-214-3p mimics markedly inhibited cell viability while Fer-1 alleviated this suppressive ability (Fig. 6B). Further investigation of Fe accumulation (Fig. 6C), ROS signal (Fig. 6D) and MDA expression level (Fig. 6E) showed that they were promoted by this miR-214-3p mimics and Fer-1 treatment successfully reversed this promotional effects. In term of GSH expression, the converse results were

observed in miR-214-3p transfected cells and partially rescued by Fer-1 treatment (Fig. 6F).

It is known that miRNAs trigger their function via suppressing their targets expression. To find the targets of miR-214-3p, an online program was searched to predict the potential candidates and GPX4 was identified as the most promising one (Fig. S4). Using the luciferase activity assays, it was found that miR-214-3p mimics markedly suppressed the luciferase activity of the GPX4-3'UTR-WT reporter but the mutagenesis of these binding sites successfully abolished this suppressive effect (Fig. 6G). Further investigation showed that miR-214-3p mimics suppressed GPX4 expression while the suppressive effects were partially reversed by Fer-1 (Fig. 6H and I).

To discover whether miR-214-3p participated in the HOTTIP mediated ferroptosis, ectopic miR-214-3p mimics were applied in the HOTTIP overexpressing cells to examine

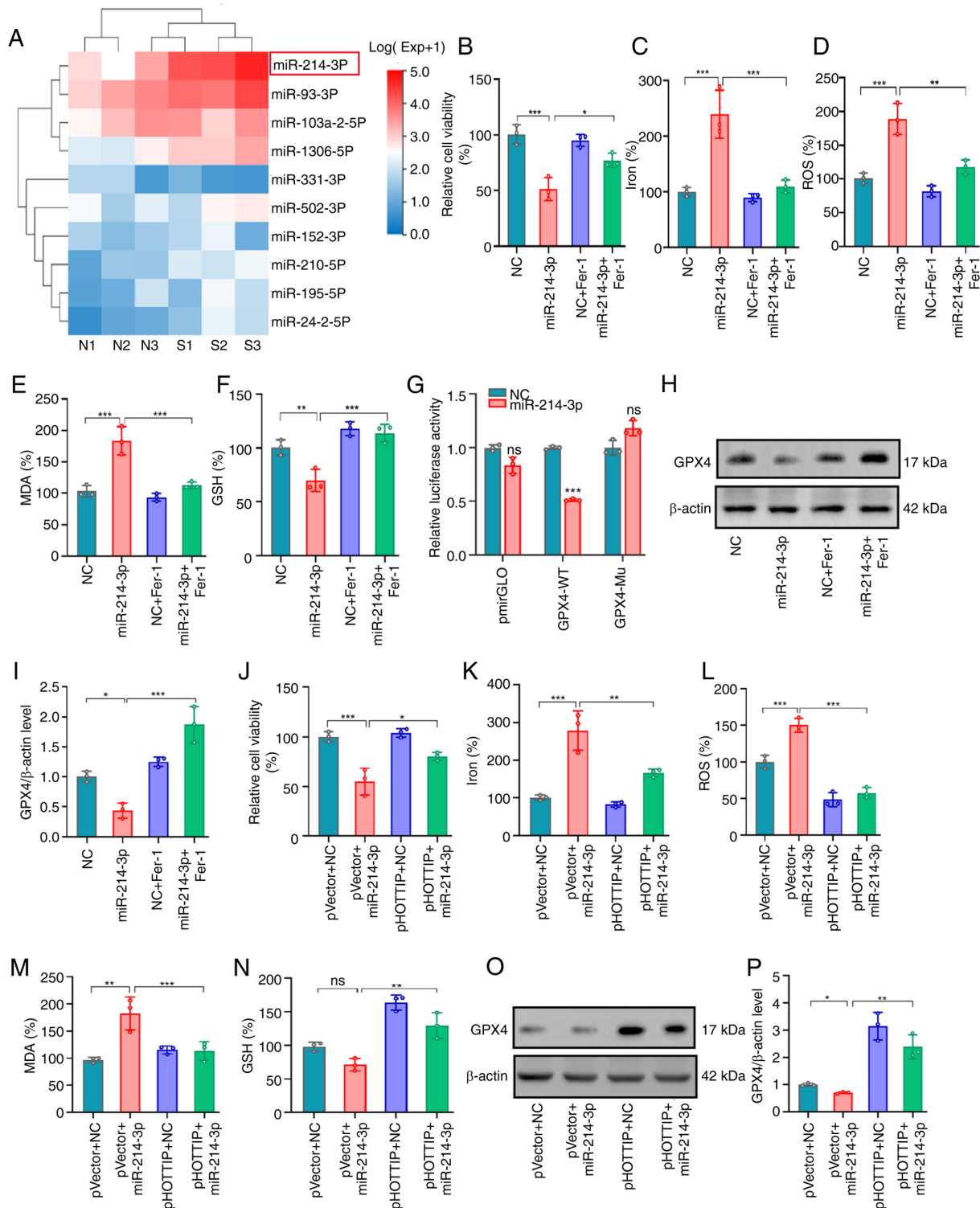


Figure 6. HOTTIP influences miR-214-3p biogenesis to suppress ferroptosis. (A) The miRNA sequencing was conducted using HOTTIP silencing OS cells. (B) Relative cell viability, (C) total iron, (D) lipid ROS, (E) MDA and (F) GSH expression levels were examined in the miR-214-3p transfected OS cells. (G) The interaction between miR-214-3p and GPX4 was determined using luciferase reporter assay. (H and I) GPX4 protein expression was examined in the miR-214-3p transfected OS cells. miR-214-3p mimics were transfected into the HOTTIP overexpressing cells and the (J) relative cell viability, (K) total iron, (L) lipid ROS, (M) MDA and (N) GSH expression levels were measured with or without Fer-1 treatment. (O and P) GPX4 protein expression levels were also examined in the aforementioned described cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns: not significant. HOTTIP, HOXA transcript at the distal tip; miRNA, micro RNA; OS, osteosarcoma; ROS, reactive oxygen species; MDA, malondialdehyde; GSH, glutathione; Fer-1, ferrostatin-1; GPX4, glutathione peroxidase 4.

the rescue effects on ferroptosis (Fig. S3B). The cell viability examination revealed that ectopic expression of miR-214-3p successfully inhibited the viability in OS cells and HOTTIP overexpression reversed this suppressive effect (Fig. 6J). The

similar rescued effects of miR-214-3p mimics on Fe accumulation (Fig. 6K), ROS signal (Fig. 6L), MDA (Fig. 6M) and GSH expression (Fig. 6N) and GPX4 expression (Fig. 6O and P) were also detected in the HOTTIP overexpressing OS cells.

Discussion

OS is a highly malignant tumor due to chemoresistance and the tendency of recurrence following surgery, and the prognosis of patients is not optimistic (22). Therefore, there is an urgent need for novel and effective therapeutics, ferroptosis has also been considered as a promising strategy for OS treatment (23,24). As a new type of iron-dependent programmed cell death, ferroptosis is characterized by iron-dependent lipid peroxidation (25). The results of the transcriptome analysis indicated that the aforementioned biological processes were influenced in bone marrow derived from HOTTIP KO mice, suggesting that HOTTIP may be a potential mediator of ferroptosis. It was therefore hypothesized that HOTTIP might be a potential target for OS treatment possibly via suppressing ferroptosis process.

Increasing evidence has revealed that lncRNAs could serve as an independent regulator for ferroptosis to mediate tumorigenesis in multiple cancers. For example, HOTTIP was identified as an oncogene to facilitate cell proliferation, invasion and migration in osteosarcoma by interaction with PTBP1 to promote KHSRP level (26). It also promoted epithelial-mesenchymal transition via a positive feedback loop with c-Myc and contributed to chemoresistance by activating the Wnt/ β -catenin pathway in OS cells (27,28). However, whether ferroptosis participates in the HOTTIP mediated osteosarcoma remains unknown. The present study showed that HOTTIP was markedly suppressed in OS cells during the Erastin-induced ferroptosis process and HOTTIP overexpression suppressed, while its knockdown promoted, ferroptosis in OS cells. To the best of the authors' knowledge, this is the first report that HOTTIP functions as a negative regulator of ferroptosis in OS.

It has been recognized that lncRNAs usually trigger their functions by physically interacting with RNA binding proteins (29,30). HOTTIP is reported to interact with WDR5 to promote osteogenic differentiation (31). Furthermore, it also directly recruits CTCF/cohesin complex to form R-loops to drive oncogene transcription and leukemia development (32). To find the new direct targets, the present study used bioinformatics analyses to predict the potential binding protein and DGCR8 was the most promising one. The RIP experiment validated the direct interaction between DGCR8 and HOTTIP. Further investigations uncovered that HOTTIP modulated the protein stability of DGCR8 by promoting its ubiquitin-mediated degradation. Although the present study explored the role of HOTTIP in mediating the ubiquitination of DGCR8 in OS cells, the more detailed mechanism needs to be further investigated in the future.

DGCR8 is a member of DGCR8/Drosha dimerization, which recruits and cleaves pri-miRNA to generate pre-miRNA and pre-miRNA is further processed and cut by Dicer to form mature miRNA. DGCR8 has been reported to suppress migration and invasion by facilitating miR-205/200b maturation in HCC cells (33). Similarly, the formation of DDX5/Drosha/DGCR8 complex promotes miRNA-10b processing, which is involved in mammary tumorigenesis and progression (34). Therefore, it was hypothesized that HOTTIP influences pri-microRNA microprocessor by regulating DGCR8 expression in osteosarcoma.

To examine which miRNA biogenesis was influenced, miRNA sequencing was conducted using HOTTIP silencing OS cells. In the present study, miR-214-3p was markedly upregulated by HOTTIP silencing. However, the role of miR-214-3p in tumorigenesis is confusing. miR-214-3p is reported to serve as an oncogene via promoting OS cell proliferation, invasion and migration (35-38). By contrast, it is also demonstrated to suppress cell proliferation and tumor growth in OS by targeting FNDC5 and KCNC4 (39,40). In the terms of ferroptosis, miR-214-3p was found to aggravate ferroptosis by targeting GPX4 in liver cancer, acute kidney injury (41) and cardiovascular diseases (42). The dual function may be related to the diversity of target genes, differences in cellular environment and signaling pathways, the complex relationship between tumor proliferation and inhibition, differences in research background and experimental conditions and potential feedback regulatory mechanisms. For example, in hypoxic core areas of tumor, increased ROS levels may make miR-214-3p more inclined to activate ferroptosis. In the front of oxygen-rich invasion, it may preferentially target pro-transfer genes. The present study showed that miR-214-3p mimics promoted ferroptosis in OS cells by targeting GPX4 while ferroptosis inhibitor Fer-1 reversely attenuated this cell death, suggesting that miR-214-3p may act as a ferroptosis stimulator to suppress OS tumorigenesis. Further rescue experiments validated that the ectopic expression of HOTTIP successfully reversed the ferroptosis induced by miR-214-3p. Based on these results, it was concluded that HOTTIP suppressed ferroptosis in OS, at least partly, through mediating miR-214-3p/GPX4 regulatory axis. Notably, recent clinicopathological studies highlight the prognostic relevance of cancer-testis antigens, including NY-ESO-1 and MAGE-A4, in osteosarcoma (43,44), suggesting that multi-omics approaches integrating lncRNA and CTA networks may uncover novel therapeutic vulnerabilities. Future studies should also evaluate whether HOTTIP silencing potentiates the efficacy of conventional chemotherapeutics, such as cisplatin, by further destabilizing redox homeostasis in osteosarcoma cells.

The present study has some limitations. First, as a critical module in miRNA biogenesis, DGCR8 also probably regulates other miRNAs, such as miR-93-3p and miR-103a-2-5p, which might be involve in HOTTIP mediated ferroptosis suppression and still need further research. Second, there are difficulties in delivering HOTTIP-targeting therapies, including the need for efficient delivery systems and the potential for off-target effects. The importance of further research to develop specific and effective HOTTIP inhibitors should also be acknowledge. Third, only ferroptosis studies were used here, the metastasis mechanism of OS, which involved in local invasion and early metastasis in clinical patients was not investigated.

In conclusion, the present study uncovered a novel function of HOTTIP in inhibiting ferroptosis of OS cells. It discovered that HOTTIP directly recruited DGCR8 and promoted its ubiquitin-mediated degradation to disrupt the miRNA-214-3p/GPX4 regulatory axis. The findings gained from the present study indicated that silencing of HOTTIP sensitized OS cells to ferroptosis-mediated strategy, suggesting that the inhibiting HOTTIP may be developed as an effective intervention for OS patients in clinical practice.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author. The sequence data be found in the NCBI SRA dataset with the accession number is PRJNA1250258 and the URL <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1250258>.

Authors' contributions

JFZ conceived and supervised all the experiments. JFZ and SCD designed the experiments. SCD, CJS and FXP conducted the experiments. RJW, NL, YXM and STZ provided the technical support. SCD and CJS analyzed the data. SCD and FXP confirm the authenticity of all the raw data. JFZ and SCD prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experimental procedures were approved by the Ethics and Animal Research Committee of Southern Medical University (Guangzhou, Guangdong) approval no. SMUL2022219).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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