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MiR-214-3p Confers Ferroptosis Resistance in Gastric Cancer by Targeting the SOCS2/SLC7A11 Axis

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Abstract

Gastric cancer (GC) remains a leading cause of cancer-related death worldwide, with limited therapeutic options for advanced disease. Ferroptosis, an iron-dependent form of regulated cell death, presents a targetable vulnerability. This study investigates how overexpression of the oncogenic miR-214-3p drives ferroptosis resistance in GC. We propose that miR-214-3p directly targets SOCS2, thereby relieving repression of the SLC7A11/glutathione synthetase (GSS) axis, ultimately enhancing glutathione synthesis and antioxidant defense. Analysis of public datasets and our own clinical cohort confirmed miR-214-3p upregulation in GC, which correlated with advanced TNM stage and poor prognosis. Using an integrated discovery strategy—combining Oxford Nanopore Technology long-read sequencing, multi-source bioinformatic prediction, and ferroptosis gene set screening—we identified SOCS2 as a key candidate. Dual-luciferase reporter assays confirmed direct binding of miR-214-3p to the SOCS2 3' UTR. In AGS GC cells, miR-214-3p overexpression suppressed SOCS2 while elevating SLC7A11 and GSS protein levels; conversely, miR-214-3p inhibition reversed these changes. These molecular alterations collectively enhanced cellular antioxidant capacity and conferred resistance to ferroptosis. This work is the first to delineate a complete miR-214-3p/SOCS2/SLC7A11/GSS regulatory axis that suppresses ferroptosis in GC. The study's strengths include systematic elucidation of this novel pathway, an innovative multi-method discovery approach, and a coherent translational link from clinical observation to mechanistic validation. Our findings provide fundamental insights and suggest that

targeting this axis may represent a promising strategy to sensitize GC to ferroptosis-based therapies.

Keywords

Gastric cancer; microRNA-214-3p; SOCS2; SLC7A11; ferroptosis

Highlight

1. First identification of the miR-214-3p/SOCS2/SLC7A11/GSS axis driving ferroptosis resistance in gastric cancer.
2. Integrated long-read sequencing and bioinformatic screening systematically identified SOCS2 as a direct miR-214-3p target.
3. miR-214-3p overexpression correlates with aggressive clinicopathological features and shows diagnostic biomarker potential.
4. Targeting this axis may overcome ferroptosis resistance and enhance the efficacy of ferroptosis-based therapies.

Gastric cancer (GC) remains a significant global health burden, ranking as the fifth most frequently diagnosed malignancy and the fifth leading cause of cancer-related death worldwide **Patel, Sethi, & Park (2026)**. Patients with advanced-stage disease carry a particularly poor prognosis, as conventional modalities such as surgery and chemotherapy offer only modest survival benefits **Van Cutsem, Sagaert, Topal, Haustermans, & Prenen (2016)**. Although the advent of molecular targeted agents, such as anti-HER2 therapies, has improved outcomes for a subset of patients, the inherent heterogeneity and the development of adaptive resistance in GC underscore the urgent need to identify novel therapeutic vulnerabilities and develop innovative treatment strategies **Guan, He, & Xu (2023)**.

In this context, ferroptosis—a regulated form of cell death driven by iron-dependent lipid peroxidation—has garnered significant attention as a promising therapeutic target across various cancer types **Wahida & Conrad (2025)**, including GC **Xu et al. (2025)**. The execution of ferroptosis is intimately linked to the glutathione (GSH)-dependent antioxidant system. The cystine/glutamate antiporter System Xc⁻, whose functional subunit is SLC7A11, is critical for importing cystine for the synthesis of GSH, a key

cofactor for the ferroptosis-inhibiting enzyme GPX4 *Sun, Shen, Jiang, Wang, & Min (2023)*. Therefore, elucidating the upstream regulators of SLC7A11 and GSH biosynthesis is of paramount importance for the development of effective ferroptosis-based therapies.

MicroRNAs (miRNAs) function as pivotal post-transcriptional regulators and are extensively implicated in GC pathogenesis. Among them, miR-214-3p displays a context-dependent duality in its reported functions. It has been shown to promote ferroptosis in certain disease models, such as acute myocardial infarction, by targeting PTEN *Liu et al. (2023)*, and conversely, to inhibit ferroptosis during cerebral ischemia/reperfusion injury *Lu, Xu, & Lu (2020)*. Furthermore, miR-214-3p carried by ADMSC-derived extracellular vesicles was reported to suppress ferroptosis in pulmonary epithelial cells through its target GSTZ1 *Li, Zhong, & Li (2025)*. In GC, our previous work, supported by findings from other groups, has established miR-214-3p as a frequently overexpressed oncomiR that drives GC progression by targeting tumor suppressors, including PTEN *Xin et al. (2016,2025)*. Nevertheless, whether miR-214-3p modulates ferroptosis in GC, and the underlying mechanism, remain completely unexplored.

To address this gap, we adopted a multi-tiered experimental approach. We first generated AGS GC cell lines stably overexpressing or inhibiting miR-214-3p. Oxford Nanopore Technology (ONT) long-read sequencing of these cells provided an unbiased transcriptomic view, enabling the identification of genes responsive to miR-214-3p modulation. By intersecting the resulting differentially expressed genes with computationally predicted miRNA targets and a curated ferroptosis gene set, we pinpointed SOCS2 (suppressor of cytokine signaling 2) as a leading candidate. SOCS2, an E3 ubiquitin ligase adaptor, has been reported to positively regulate ferroptosis in other settings by promoting the degradation of SLC7A11 *King, Norman, & Lichtenstein (1967)*. This finding prompted us to hypothesize the existence of a previously unexplored miR-214-3p/SOCS2 axis that modulates SLC7A11 stability and the ferroptosis defense system in GC. We subsequently validated the direct binding of miR-214-3p to the SOCS2 3' UTR using dual-luciferase reporter assays and assessed the downstream effects of miR-214-3p modulation on the SOCS2/SLC7A11/glutathione synthetase (GSS) pathway.

This work represents the first systematic investigation into the role of miR-214-3p in regulating ferroptosis in GC, specifically through the SOCS2/SLC7A11 axis. By integrating unbiased discovery with targeted validation, we have uncovered a novel regulatory circuit and established a mechanistic foundation for developing ferroptosis-based therapeutic strategies. The identification of miR-214-3p as an upstream modulator of the cystine/GSS defense system opens new avenues for overcoming chemoresistance—a major clinical hurdle in GC treatment—and holds promise for future biomarker development and therapeutic innovation.

Materials and Methods

1.1 Reagents and Instruments

Lentiviral vectors LV-miR-214 inhibitor, LV-miR-214, and LV-NC were purchased from Tsingke Biotechnology (Beijing, China). The human gastric adenocarcinoma cell line AGS was obtained from Procell Life Science & Technology (Wuhan, China). Specific PCR primers for miRNA-214-3p, U6, SOCS2, SLC7A11, GSS, and Beta Actin were synthesized by Tsingke Biotechnology. Key reagents included TRIzol (Invitrogen, USA), total RNA extraction kits, reverse transcription kits, and quantitative PCR kits (TaKaRa, Japan), Dual-Luciferase Reporter Assay kits (Promega, USA), and total protein extraction kits (KeyGEN BioTECH, Jiangsu, China). Primary antibodies included: anti-SOCS2 (Wuhan Cloud-Clone Corp, PAH154Ra01, Rabbit), anti-SLC7A11 (Proteintech, USA, #26864-1-AP, Rabbit), anti-GSS (Proteintech, USA, 15712-1-AP, Rabbit), and anti-Beta Actin (Proteintech, USA, 60008-1-Ig, Mouse). Secondary antibodies included goat anti-mouse IgG (Proteintech, SA00001-1) and goat anti-rabbit IgG (Proteintech, SA00001-2). Major instruments included a quantitative real-time PCR system *Bio – Rad, USA* and a chemiluminescence imaging system (Azure, USA).

1.2 Methods

1.2.1 Cell Culture

AGS cells were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO₂. Cells were passaged every 2 – 3 days, and those in the logarithmic growth phase (passages 2 – 3) were used for subsequent experiments. This study was approved by the Ethics Committee of the

People's Hospital of Ningxia Hui Autonomous Region (Approval No. (2022) -NZR-148).

1.2.2 Establishment of Stable Cell Lines

AGS cells were seeded at a density of 2×10^6 cells per 60 mm dish and allowed to reach 70–80% confluence before lentiviral transduction. The required viral volume was calculated based on the multiplicity of infection (MOI) and viral titer using the formula: Viral Volume = (MOI $\times$ Cell Number) / Viral Titer. For an MOI of 50, a cell number of 2×10^6 , and a viral titer of 1×10^9 TU/mL, 100 μ L of viral suspension was used. At 72 hours post-infection, puromycin was added to the culture medium at a final concentration of 2.5 μ g/mL for selection of positively transduced cells. After 24 hours, the medium was replaced with fresh complete medium overnight. This puromycin selection cycle was repeated three consecutive times. The resulting stable cell populations were expanded for downstream experiments.

1.2.3 Third-Generation Sequencing (Oxford Nanopore Technology)

Long-read sequencing was performed using Oxford Nanopore Technology. Raw reads were filtered by removing those with a minimum average quality score below 7 and a read length below 500 bp; ribosomal RNA reads were also discarded. Full-length non-chimeric (FLNC) transcripts were identified and aligned to the reference genome using minimap2. Consensus isoforms were generated with Pinfish, and redundant transcripts were removed using cDNA Cupcake (min-coverage 85%, min-identity 90%). Fusion transcripts were identified based on multi-locus alignment, coverage, and genomic distance criteria. Transcript structures were characterized using gffcompare for alternative splicing, AStaravista, MISA, TAPIS, and TransDecoder for SSR, APA, and CDS prediction. Transcription factors were predicted using iTAK (for plants) and AnimalTFDB (for animals). Long non-coding RNAs (lncRNAs) were identified using CPC, CNCI, CPAT, and Pfam, retaining transcripts longer than 200 nt with at least two exons. Gene functional annotation was performed against the NR, Pfam, KOG/COG/eggNOG, Swiss-Prot, KEGG, and GO databases. Expression levels were quantified using mapped full-length reads. Differential expression analysis was conducted using DESeq (with replicates) or EBSeq (without replicates), with thresholds of FDR < 0.05 and a fold change ≥ 1.5 . GO and KEGG enrichment analyses were carried out using Goseq and KOBAS, respectively. Protein – protein interaction networks were predicted with STRING and visualized using Cytoscape.

1.2.4 Bioinformatics analysis

To characterize the expression and prognostic significance of miR-214-3p in gastric cancer, a comprehensive bioinformatics analysis was conducted using publicly available data from The Cancer Genome Atlas (TCGA). miRNA-seq expression profiles for the TCGA-STAD (stomach adenocarcinoma) project, processed by the BCGSC pipeline, were retrieved along with corresponding clinical annotations. Raw expression values in reads per million (RPM) were obtained and subsequently transformed using $\log_2(\text{value} + 1)$ normalization. Tumor samples with missing survival or clinicopathological information were excluded, and normal tissue specimens were removed to focus on tumor-specific analyses.

For survival analysis, the R statistical environment (version 4.2.1) was employed. The survival package (version 3.3.1) was used to test the proportional hazards assumption and to fit Cox proportional hazards regression models. Kaplan – Meier survival curves were generated for overall survival (OS) using the survminer (version 0.4.9) and ggplot2 (version 3.4.4) packages. When the optimal cut-off strategy was selected, the surv_cutpoint function from the survminer package was applied to determine the best threshold for dichotomizing miR-214-3p expression into high and low groups. Differences in survival between groups were assessed using the log-rank test. Univariate and multivariate Cox regression analyses were performed to evaluate the independent prognostic value of miR-214-3p.

In addition to survival analysis, differential expression between gastric tumor and adjacent normal tissues was evaluated using the limma package, with a significance threshold of $|\log_2 \text{fold change}| > 1$ and adjusted $P < 0.05$. The associations between miR-214-3p expression and clinicopathological parameters—including T, N, M stage, pathological grade, Helicobacter pylori infection status, and residual tumor classification—were examined using the Wilcoxon rank-sum or Kruskal-Wallis tests. Receiver operating characteristic (ROC) curve analysis was carried out to assess diagnostic performance. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

1.2.5 Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from AGS cells of the empty vector control group and the miR-214 inhibitor group using TRIzol reagent. RNA was reverse-transcribed into cDNA. Quantitative real-time PCR was performed using SYBR Premix Ex Taq II for mRNA detection and the miScript SYBR Green PCR kit for miRNA detection. Beta-actin mRNA and RNU6B were used as internal reference controls. Specific primer sequences were as follows:

miR-214:

Reverse: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTGCC-3';
Forward: 5'-GCGACAGCAGGCACAGACA-3'.

RNU6B:

Reverse: 5'-GTCGTATCGACTGCAGGGTCCGAGGTATTCGCAGTCGATACGACAAAATATG-3';
Forward: 5'-AGCACATATACTAAAATTGGAACGAT-3'.

Beta Actin:

Reverse: 5'-AGGTCTTTGCGGATGTCCACGT-3';
Forward: 5'-CACCATTGGCAATGAGCGGTTC-3'.

SOCS2:

Reverse: 5'-CAGAGATGGTGCTGACGTGT-3';
Forward: 5'-TGCAAGGATAAGCGGACAGG-3'.

SLC7A11:

Reverse: 5'-AGAGGAGTGTGCTTGCGGACAT-3';
Forward: 5'-TCCTGCTTTGGCTCCATGAACG-3'.

GSS:

Reverse: 5'-TGTGACCTCTCCAGCAGTAGAC-3';
Forward: 5'-CCAAGACCGAAGGCTGTTTGTG-3'.

The qRT-PCR cycling protocol was: pre-denaturation at 95 °C for 5 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60°C for 20 s. Each sample was run in triplicate. Data from three independent experiments were analyzed using the $2^{-\Delta\Delta C_t}$ method.

1.2.6 Western Blotting

Total protein was extracted from AGS cells of the empty vector control group, the LV-miR-214-3p group, and the LV-miR-214-3p inhibitor group using a total protein extraction kit. Proteins were separated by SDS-PAGE (gel concentration adjusted according to the target molecular weight) and transferred onto PVDF membranes. Membranes were incubated with primary antibodies overnight at 4 °C, washed three times with Tris-Buffered Saline containing Tween-20 (TBST) for 10 minutes each, and then incubated with horseradish peroxidase-conjugated secondary antibodies for 2 hours at room temperature. After five additional TBST washes (10 minutes each), protein bands were visualized using an ECL substrate and imaged with a chemiluminescence detection system.

1.2.7 Dual-Luciferase Reporter Assay

Wild-type (pmirGLO-SOCS2-WT) and mutant (pmirGLO-SOCS2-MUT) luciferase reporter plasmids containing the SOCS2 3'UTR were constructed. 293T cells were seeded in 6-well plates. When cell density reached 60 – 80%, WT or MUT plasmids were co-transfected with miR-214-3p mimics or negative control plasmids using Lipofectamine 2000. At 48 hours post-transfection, the medium was removed, and cells were washed twice with PBS. Cells were then lysed with 500 µL of Cell Lysis Buffer per well at room temperature for 15 minutes. Lysates were collected into 1.5 mL centrifuge tubes and centrifuged at $12,000 \times g$ for 10 minutes at room temperature. A total of 20 µL of supernatant per well was transferred to a microplate for firefly luciferase activity measurement. Subsequently, 100 µL of Renilla luciferase working solution was added, mixed gently, and Renilla luciferase activity was measured. The ratio of firefly to Renilla luciferase activity was calculated and used as the relative luciferase expression value.

1.3 Statistical Analysis

All data were analyzed using GraphPad Prism 8.0 software. Data were confirmed to follow a normal distribution and are presented as the mean $\pm$ standard deviation (SD). Comparisons between two groups were performed using an unpaired two-tailed Student's t-test. A P-value of less than 0.05 was considered statistically significant.

Result

1. miR-214-3p is upregulated in gastric cancer and associates with aggressive clinicopathological features.

We first profiled miR-214-3p across 33 human malignancies using TCGA pan-cancer data. As shown in Figure 1a, miR-214-3p was dysregulated in a tumor-type – specific manner, and its abundance was significantly higher in gastric adenocarcinoma (STAD) tissues than in their non-malignant counterparts ($P < 0.01$). Within the STAD cohort itself, miR-214-3p expression was markedly elevated in tumor specimens ($n = 439$) compared with adjacent normal mucosa ($n = 44$; $P < 0.001$; Figure 1b).

We next asked whether miR-214-3p levels track with disease progression. Stratification by clinicopathological parameters revealed that tumors at advanced stages (III – IV) expressed higher miR-214-3p than early-stage lesions (I – II; Figure 1c), and expression rose with increasing depth of local invasion (T3/T4 versus T1; Figure 1d). Patients with distant metastasis (M1) also exhibited significantly higher miR-214-3p than those without (M0; $P < 0.05$; Figure 1f), and poorly differentiated (G3) tumors showed elevated expression relative to lower-grade lesions (Figure 1g). Of note, *H. pylori* – positive patients displayed higher miR-214-3p levels than *H. pylori* – negative individuals ($P < 0.05$; Figure 1h). In contrast, no significant difference was observed across lymph node (N) categories (Figure 1e) or among R0/R1/R2 residual tumor groups (Figure 1i).

Taken together, these data identify miR-214-3p as a tumor-enriched miRNA in gastric cancer whose upregulation accompanies advanced stage, deeper invasion, distant metastasis, poorer differentiation, and *H. pylori* infection.

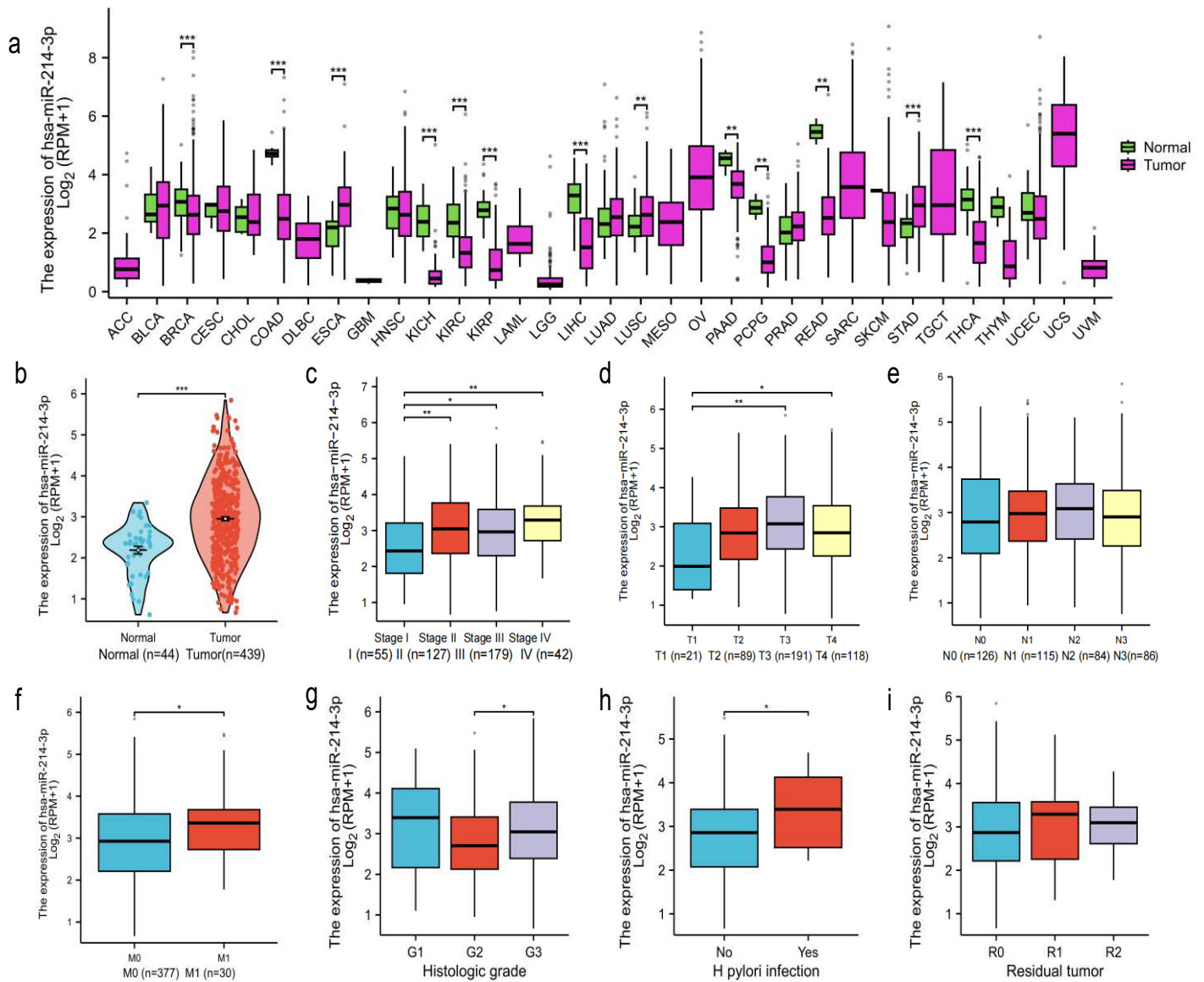


Figure 1 Expression profile and clinical significance of miR-214-3p in gastric cancer. (a) Pan-cancer analysis of miR-214-3p; (b) Expression of miR-214-3p in adjacent normal tissues and gastric cancer tissues; (c) Expression of miR-214-3p at different stages of gastric cancer; (d) Expression of miR-214-3p according to primary tumor (T) classification; (e) Expression of miR-214-3p according to lymph node metastasis (N) classification; (f) Expression of miR-214-3p according to distant metastasis (M) classification; (g) Expression of miR-214-3p in different pathological stages; (h) Expression of miR-214-3p in the presence or absence of Helicobacter pylori infection; (i) Expression of miR-214-3p in residual tumor tissues after gastric cancer surgery. (*P < 0.05; **P < 0.01; ***P < 0.001)

2. miR-214-3p shows moderate diagnostic performance but is not significantly associated with survival outcomes in gastric cancer.

Because miR-214-3p was enriched in gastric tumors, we next examined whether it could discriminate malignant from non-malignant tissue and predict patient outcome. ROC analysis returned an area under the curve (AUC) of 0.736 (95% CI, 0.679 – 0.793; Figure 2e), indicating moderate ability of miR-214-3p to distinguish gastric cancer from adjacent normal mucosa.

In contrast, its prognostic value appeared limited. Patients who had experienced an OS event did not differ from those remaining alive in terms of tumor miR-214-3p levels (Figure 2a), and similar results were obtained when cases were stratified by DSS events (Figure 2b) or PFI events (Figure 2c). Expression was also comparable across primary therapy response categories (PD, SD, PR, and CR; Figure 2d), and Kaplan – Meier survival analysis failed to separate the high- and low-expression groups (HR, 0.93; 95% CI, 0.69 – 1.26; P = 0.653; Figure 2f).

We further asked whether expression differed according to tumor location or histology. miR-214-3p abundance was largely homogeneous across anatomic subsites (antrum/distal, cardia/proximal, fundus/body, and gastroesophageal junction; Figure 2g) and across histological subtypes, including diffuse, mucinous, papillary, signet-ring, and tubular carcinomas (Figure 2h). Taken together, these data support miR-214-3p as a candidate diagnostic marker but not as an independent prognostic indicator of survival in gastric cancer.

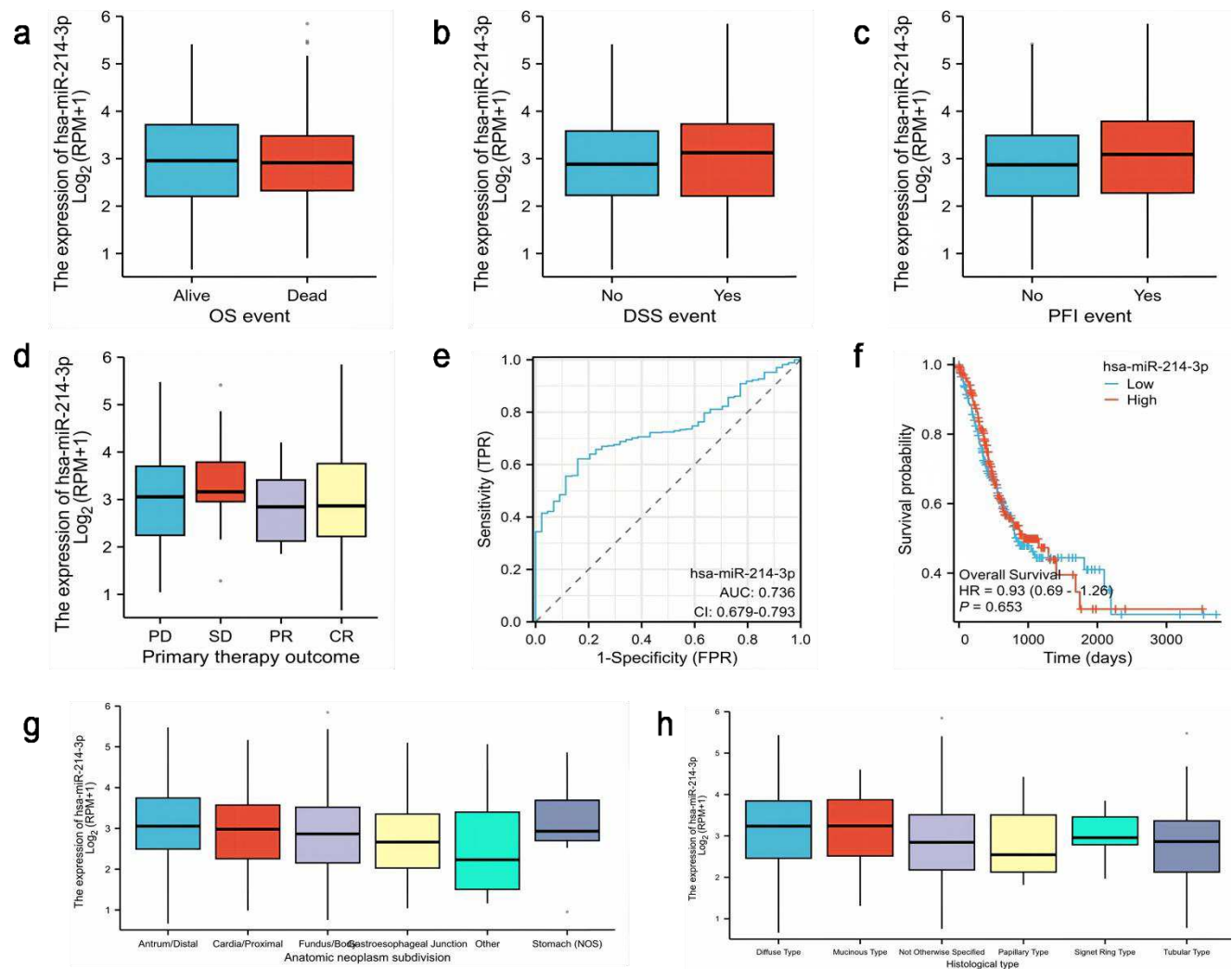


Figure 2 Prognostic value, diagnostic performance, and expression characteristics of miR-214-3p in gastric cancer. (a) Expression of miR-214-3p in relation to overall survival (OS) events in gastric cancer; (b) Expression of miR-214-3p in relation to disease-specific survival (DSS) events; (c) Expression of miR-214-3p in relation to progression-free interval (PFI) events; (d) Expression of miR-214-3p according to initial treatment outcome; (e) ROC curve of miR-214-3p for diagnosing gastric cancer; (f) Correlation between miR-214-3p expression and survival prognosis; (g) Expression of miR-214-3p in different anatomic tumor subtypes; (h) Expression of miR-214-3p in different histological types of gastric cancer

3. Stable manipulation of miR-214-3p in gastric cancer cells reveals its downstream transcriptomic landscape.

To delineate the transcriptional program controlled by miR-214-3p, we first generated stable gastric cancer cell lines in which the miRNA was either overexpressed or knocked down (Figure 3a). Host cells were transfected with the corresponding overexpression or knockdown vectors and subjected to antibiotic selection; surviving colonies were then isolated by single-cell cloning or limiting dilution, expanded under progressively increasing drug concentrations, and validated for stable integration. Following preliminary evaluation, the best-performing clones were further expanded and archived as cell banks, and full-length cDNA libraries were constructed from each line for Oxford Nanopore Technology (ONT) long-read sequencing on the PromethION platform.

During this process, both constructs yielded viable stable clones. However, the overexpression arm consistently produced a more pronounced and reproducible change in miR-214-3p abundance, whereas the knockdown clones, although confirmed to express lower miR-214-3p levels than the controls, exhibited a comparatively modest reduction. Given the stronger perturbation achieved in the overexpression model, we selected this arm for the downstream transcriptomic comparison.

Comparative profiling between miR-214-3p–overexpressing and control cells identified a large cohort of significantly altered transcripts, as illustrated by the volcano and MA plots (Figures 3b, 3c), in which red and green dots denote up- and downregulated genes, respectively. Multi-layer functional annotation was then applied to these differentially expressed genes (DEGs).

Gene Ontology analysis showed that the DEGs were broadly distributed across biological-process categories, with prominent representation in cellular process, metabolic process, biological regulation, and response to stimulus (Figure 3d). At the cellular-component and molecular-function levels, the DEGs were enriched in intracellular and organelle compartments and in binding and catalytic activities (Figure 3d).

KEGG pathway classification further assigned the DEGs to multiple canonical pathways. At the level of organismal systems and human diseases, the most heavily represented sets included Pathways in cancer, MicroRNAs in cancer, and Gastric cancer, together with virus-associated carcinogenesis (Hepatitis B, Hepatitis C, Epstein–Barr virus infection, Viral carcinogenesis) and Proteoglycans in cancer (Figure 4b). Within environmental information processing, the DEGs were mapped to a series of signaling cascades, including MAPK, cAMP, PI3K–Akt, Ras, and Wnt pathways (Figure 4b). The KEGG enrichment scatter plot, ranked by enrichment factor and q-value, additionally highlighted metabolic routes such as cysteine and methionine metabolism, pyruvate metabolism, oxidative phosphorylation, carbon metabolism, biosynthesis of amino acids, glycolysis/gluconeogenesis, and the TCA cycle, together with RNA degradation, the spliceosome, and the proteasome (Figure 4c). A representative pathway diagram is provided in Figure 4a, in which enzymes catalyzing differentially expressed reactions are colored according to whether the corresponding genes were upregulated (red), downregulated (green), or both (blue).

Finally, COG and eggNOG functional clustering allocated the DEGs predominantly to translation, ribosomal structure, and biogenesis; posttranslational modification, protein turnover, and chaperones; signal transduction mechanisms; and general function prediction categories, with a substantial proportion also assigned to function-unknown groups (Figures 4d, 4e).

Together, these findings outline the genome-wide transcriptional consequences of miR-214-3p overexpression in gastric cancer cells and nominate candidate downstream effectors—particularly within metabolic networks and cancer-related signaling pathways—for subsequent mechanistic validation.

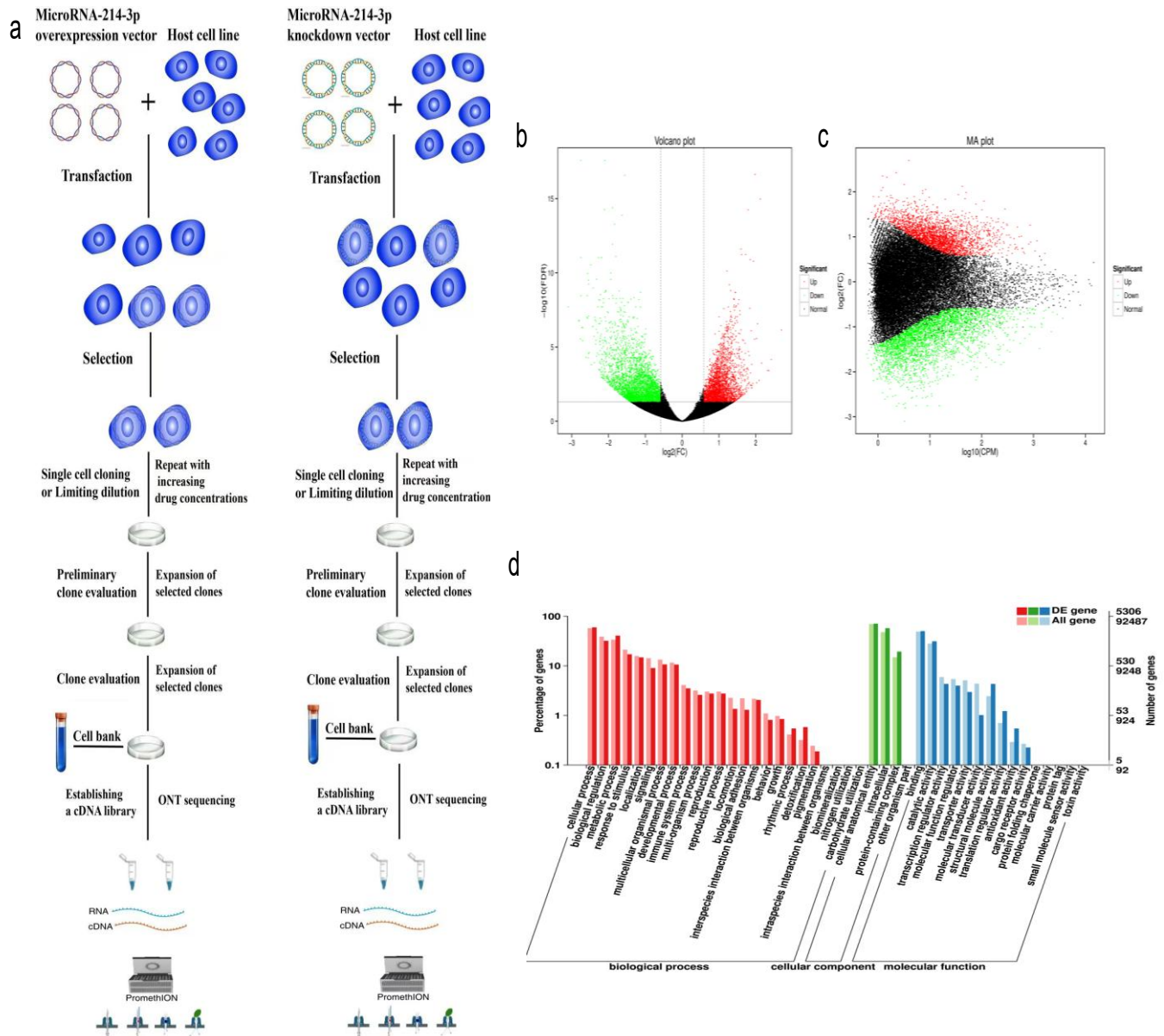


Figure 3 Identification of differentially expressed genes and functional annotation in stable cell lines. (a) Schematic diagram of stable cell line generation and ONT sequencing workflow; (b) Volcano plot of differentially expressed genes. Each dot represents a gene; the x-axis shows $\log_2$ fold change, and the y-axis shows $-\log_{10}(p\text{-value})$. Green, red, and black dots represent downregulated, upregulated, and non-significant genes, respectively; (c) MA plot of differentially expressed genes. Each dot represents a gene; the x-axis shows $\log_2$ (CPM) (average expression level), and the y-axis shows $\log_2(FC)$ (fold change). Color coding is the same as in (b); (d) GO annotation classification of differentially expressed genes

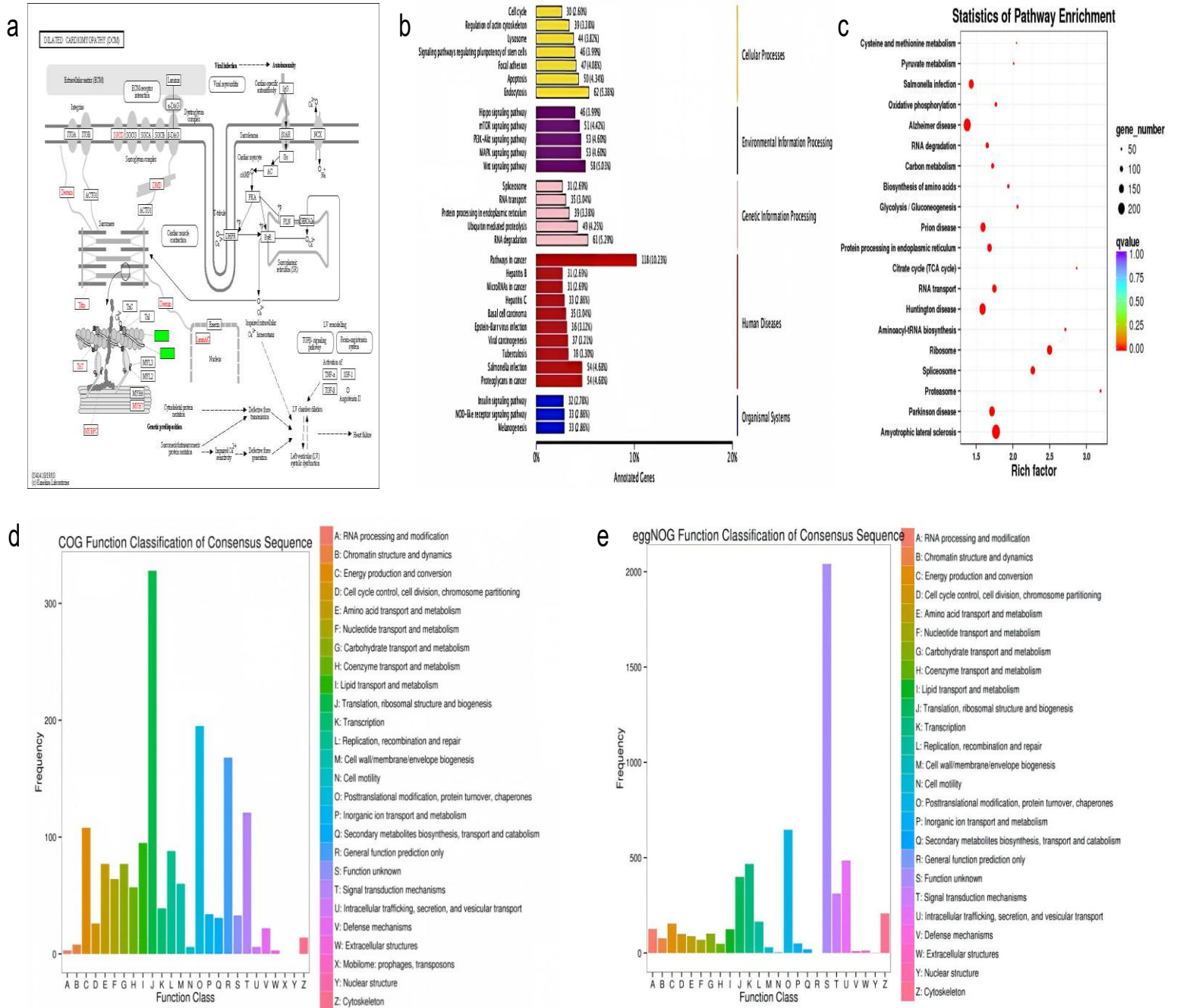


Figure 4 Functional enrichment and annotation classification of differentially expressed genes. (a) Example diagram illustrating KEGG pathway annotations for differential expression (relative to the control group: enzymes marked in red boxes are associated with upregulated genes, those in green boxes with downregulated genes; enzymes in blue boxes are linked to both upregulated and downregulated genes; numbers within boxes

represent enzyme EC numbers). The pathway consists of complex biochemical reactions catalyzed by multiple enzymes. Enzymes related to differentially expressed genes are colored differently across this pathway. Focusing on specific metabolic pathways based on inter-group differences enables more targeted analysis of gene expression variations, thereby elucidating the underlying mechanisms of phenotypic differences through pathway-level interpretation. (b) KEGG pathway classification of differentially expressed genes. The y-axis represents KEGG pathway names, and the x-axis shows the number of genes annotated to each pathway and its proportion relative to the total number of annotated genes. (c) KEGG pathway enrichment scatter plot. Each circle represents a KEGG pathway. The y-axis indicates pathway names, and the x-axis shows the enrichment factor—the ratio of the proportion of differentially expressed genes annotated to a pathway to the proportion of all genes annotated to that pathway. A higher enrichment factor indicates a more significant enrichment level. Circle color represents the q-value (adjusted P-value after multiple hypothesis testing); a smaller q-value indicates more reliable enrichment significance. Circle size reflects the number of enriched genes in the pathway. (d) COG (Clusters of Orthologous Groups) annotation classification of differentially expressed genes. The x-axis represents COG functional categories, and the y-axis shows the number of genes. (e) eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) annotation classification of differentially expressed genes. The x-axis represents eggNOG functional categories, and the y-axis shows the number of genes.

4. Integrated transcriptomic and target prediction identifies SOCS2 as a high-confidence miR-214-3p target linked to ferroptosis.

We first confirmed the robustness of the overexpression transcriptome by unsupervised hierarchical clustering of the differentially expressed genes. As shown in Figure 5a, the six samples separated cleanly into two blocks—the three biological replicates of the control group (Con-1 to Con-3) and the three of the miR-214-3p overexpression group (OE-1 to OE-3)—with replicates within each condition clustering tightly together, indicating that the transcriptomic shift induced by miR-214-3p overexpression was consistent across biological repeats.

To prioritize candidate direct targets among the altered genes, we intersected the DEGs identified by ONT sequencing with predicted miR-214-3p targets from three independent algorithms, TargetScan, miRDB, and miRWalk (Figure 5b). The overlap shared by all

three tools and supported by our RNA-seq data comprised 325 genes, which were retained as a high-confidence candidate target set.

Given the metabolic and redox-related pathways recovered in Figure 4, we next asked whether any of these candidates are known regulators of ferroptosis. Intersecting the 325-gene set with the ferroptosis-related gene collection from the FerrDb database yielded 22 overlapping genes, including SOCS2, RUNX3, MFN2, MTHFR, MAPK8, HSP90AA1, PTEN, GSK3B, PPARG, and CTSB (Figure 5c).

Among these 22 candidates, SOCS2 emerged as the most compelling direct target. TargetScan mapped an 8mer binding site to positions 2400 – 2407 of the SOCS2 3' UTR, with a favorable context++ score of -0.27 (97th percentile) and a predicted relative knockdown of -4.828 (Figure 5d). To place this prediction in structural context, we compared the AlphaFold-modeled three-dimensional structure of SOCS2 (Figure 5e) with the experimentally resolved structure deposited in the Protein Data Bank (Figure 5f); the two models were broadly concordant, supporting the structural feasibility of SOCS2 as a downstream effector amenable to further functional investigation.

Collectively, this multi-layer filtering—differential expression, consensus miRNA target prediction, and ferroptosis-related gene overlap—nominates SOCS2 as a prime downstream mediator through which miR-214-3p may operate in gastric cancer cells.

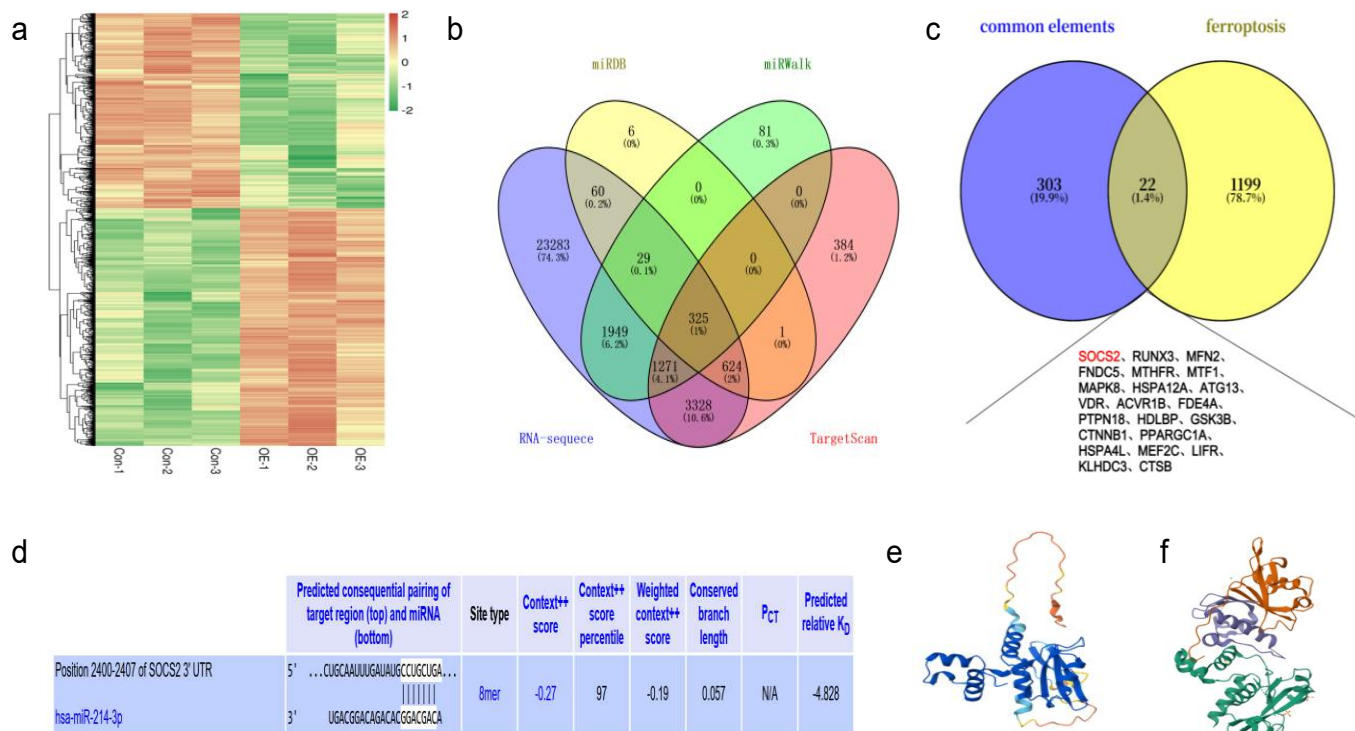


Figure 5 Clustering analysis, target gene prediction, ferroptosis-related gene intersection, and protein structure of SOCS2. (a) Cluster heatmap of differentially expressed genes. The x-axis represents sample names and their clustering results; the y-axis represents differentially expressed genes and their clustering results. Each column corresponds to a sample, and each row corresponds to a gene. Color intensity indicates the expression level ($\log_2[\text{CPM} + 1e^{-6}]$) of each gene across samples. (b) Venn diagram showing the intersection of differentially expressed genes identified by third-generation sequencing with predicted target genes from microRNA target prediction tools (TargetScan, miRDB, and miRWalk). (c) Overlap of the gene set from (b) with ferroptosis-related genes obtained from the FerrDb database (<http://www.zhounan.org/ferrdb/current/>), yielding 22 overlapping genes. (d) Predicted target genes of miR-214-3p by TargetScan, highlighting SOCS2 as a high-confidence target with a high context++ score. (e) Predicted protein structure of SOCS2 by AlphaFold. (f) Predicted protein structure of SOCS2 from the Protein Data Bank (PDB)

4. SOCS2 is downregulated in gastric cancer and correlates with tumor immune cell infiltration.

To validate the transcriptomic prediction that SOCS2 is repressed by miR-214-3p, we examined SOCS2 expression at both the protein and transcript levels. Immunohistochemical staining of tissue microarrays showed that SOCS2 protein was abundant in normal gastric mucosa, with strong cytoplasmic immunoreactivity in

glandular epithelial cells (Figures 6a, 6c), whereas gastric cancer tissues displayed markedly weaker staining (Figures 6b, 6d). Quantitative analysis of an independent transcriptomic dataset confirmed this pattern: SOCS2 expression was significantly lower in tumor specimens (n = 375) than in normal gastric tissues (n = 32; $P < 0.05$; Figure 6e), in keeping with the reciprocal upregulation of miR-214-3p observed in Figure 1.

Given the established links between SOCS family proteins and immune signaling, we further explored the association between SOCS2 expression and tumor immune cell infiltration (Figure 6f). SOCS2 abundance showed positive correlations with a broad panel of infiltrating immune populations, most prominently mast cells ($R = 0.299$, $P < 0.001$), plasmacytoid dendritic cells ($R = 0.282$, $P < 0.001$), effector memory T cells ($R = 0.282$, $P < 0.001$), macrophages ($R = 0.254$, $P < 0.001$), eosinophils ($R = 0.236$, $P < 0.001$), dendritic cells ($R = 0.226$, $P < 0.001$), and NK cells ($R = 0.208$, $P < 0.01$), with additional weaker but significant correlations for CD8⁺ T cells, neutrophils, B cells, and total T cells (all $P < 0.05$). In contrast, Th17 cells showed a modest negative correlation with SOCS2 expression ($R = -0.147$, $P < 0.05$), while $\gamma\delta$ T cells, Tregs, NK CD56^{bright}/dim subsets, and activated dendritic cells did not reach significance.

Together, these data confirm that SOCS2 is downregulated in gastric cancer and indicate that its residual expression is coupled to a broader tumor immune microenvironment, particularly to innate immune cell infiltration.

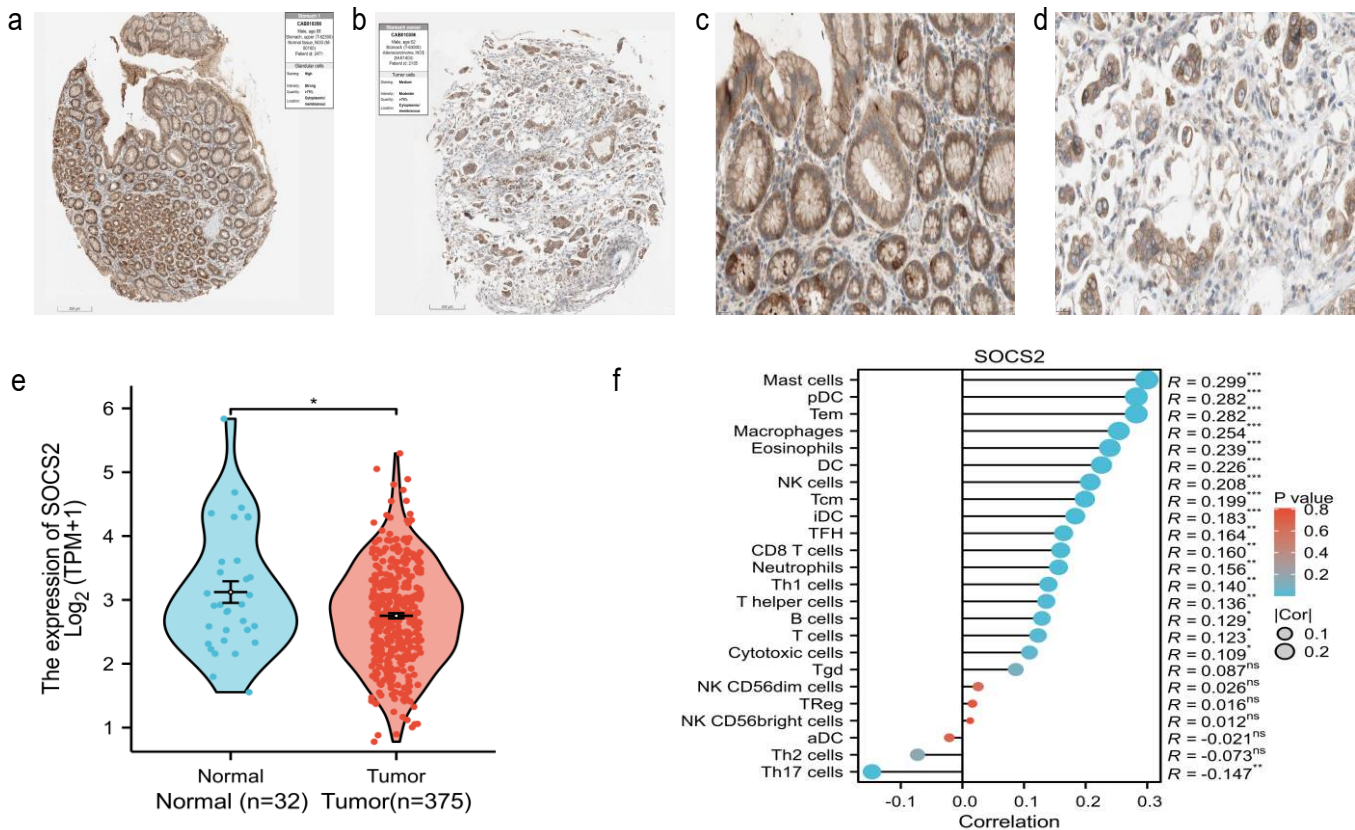


Figure 6 Expression, localization, and immune cell correlation of SOCS2 in gastric cancer. (a) Expression of SOCS2 in normal gastric tissue (scale bar = 200 μm); (b) Expression of SOCS2 in gastric cancer tissue (scale bar = 200 μm); (c) Expression of SOCS2 in normal gastric tissue (scale bar = 25 μm); (d) Expression of SOCS2 in gastric cancer tissue (scale bar = 25 μm); (e) Comparison of SOCS2 expression between adjacent normal tissues and gastric cancer tissues; (f) Lollipop plot showing the correlation between SOCS2 expression and immune cell infiltration.

5. Lentiviral manipulation of miR-214-3p in AGS cells confirms SOCS2 as a direct target and reveals coordinated regulation of ferroptosis-associated genes.

We first validated the AGS gastric cancer cell model used for downstream functional studies. Lentiviral particles carrying either the miR-214-3p inhibitor (LV-miR-214-3p KD) or the miR-214-3p overexpression construct (LV-miR-214-3p OE), together with their corresponding negative control (LV-NC), were transduced into AGS cells. Bright-field and fluorescence imaging confirmed that the majority of cells were successfully infected after selection (Figures 7a, 7c), and qRT-PCR verified that miR-214-3p abundance was markedly reduced upon inhibitor transduction and significantly elevated upon overexpression relative to LV-NC (both $P < 0.001$; Figures 7b, 7d).

We next examined whether SOCS2, the high-confidence candidate target nominated in Figure 5, is actually regulated by miR-214-3p. At the mRNA level, SOCS2 expression was strongly increased after miR-214-3p knockdown and sharply decreased after overexpression (both $P < 0.001$; Figure 8a). Western blot analysis reproduced this direction at the protein level: SOCS2 abundance rose in the KD group and fell in the OE group relative to LV-NC (both $P < 0.01$; Figure 8b). To test whether this regulation is direct, we performed a dual-luciferase reporter assay. Co-transfection of the wild-type SOCS2 3' UTR reporter with miR-214-3p mimics significantly reduced luciferase activity compared with the NC mimics control ($P < 0.01$), whereas mutation of the predicted binding site completely abolished this repression ($P > 0.05$; Figure 8c), confirming that SOCS2 is a direct target of miR-214-3p.

Finally, we asked whether the ferroptosis-related genes identified in Figure 5 are concomitantly altered by miR-214-3p. qRT-PCR showed that both SLC7A11 and GSS mRNA levels were markedly reduced in the miR-214-3p KD group and elevated in the OE group (all $P < 0.001$; Figures 9a, 9b). Western blot validation reproduced this pattern at the protein level: SLC7A11 and GSS were downregulated after miR-214-3p inhibition and upregulated after its overexpression ($P < 0.05$ or $P < 0.01$; Figure 9c), whereas SOCS2 displayed the reciprocal pattern already described.

Together, these gain- and loss-of-function data establish miR-214-3p as a direct negative regulator of SOCS2 in AGS cells and indicate that the miRNA, through this axis, coordinately modulates the ferroptosis-associated genes SLC7A11 and GSS.

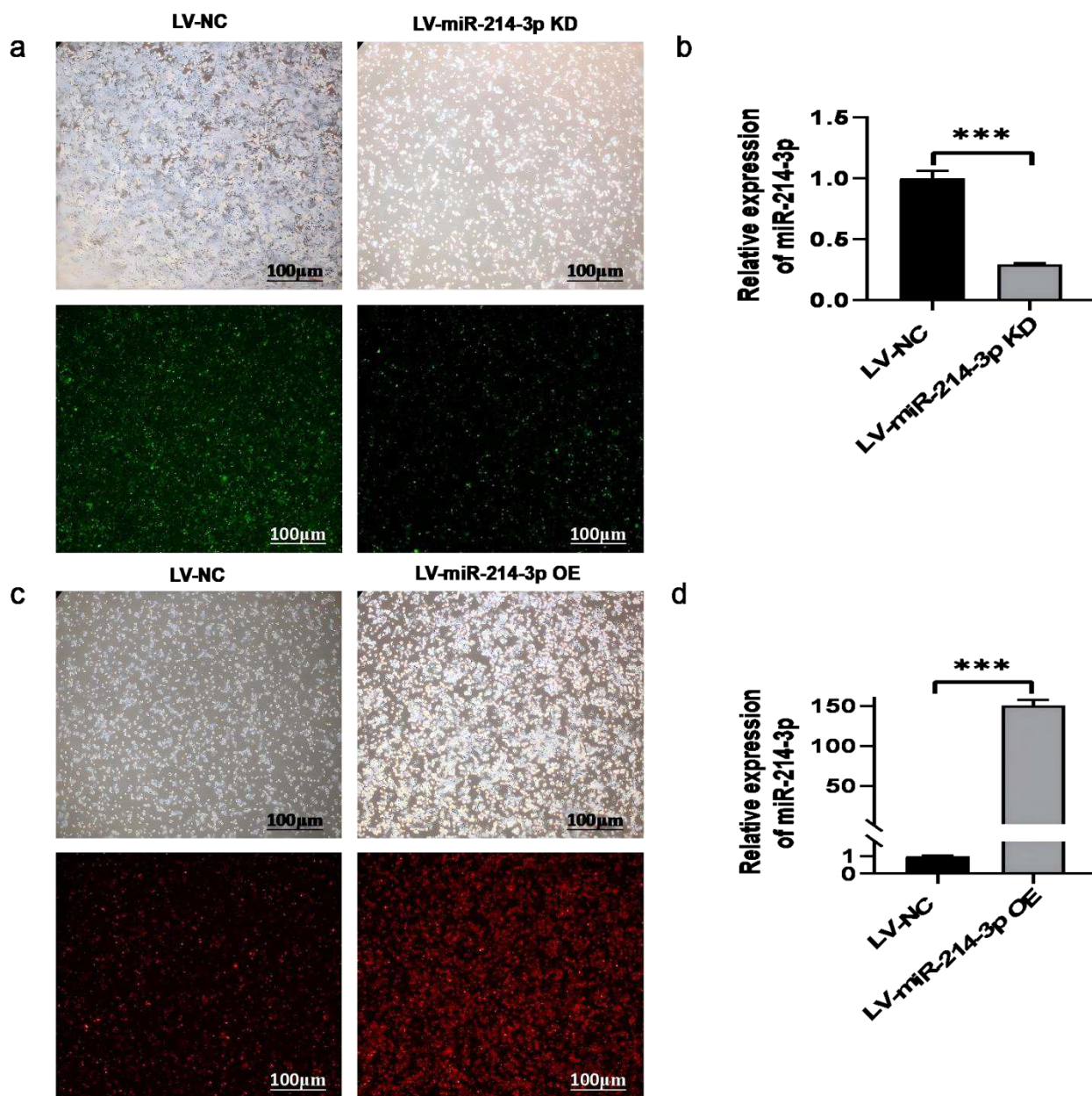


Figure 7 Validation of lentivirus-mediated miR-214 modulation and transfection efficiency in AGS gastric cancer cells. (a) Bright-field and fluorescence images of AGS cells transfected with LV-miR-214 inhibitor; (b) qRT-PCR verification of transfection efficiency after miR-214 inhibition in AGS cells (*** $P < 0.001$); (c) Bright-field and fluorescence images of AGS cells transfected with LV-miR-214; (d) qRT-PCR verification of transfection efficiency after miR-214 overexpression in AGS cells (*** $P < 0.001$)

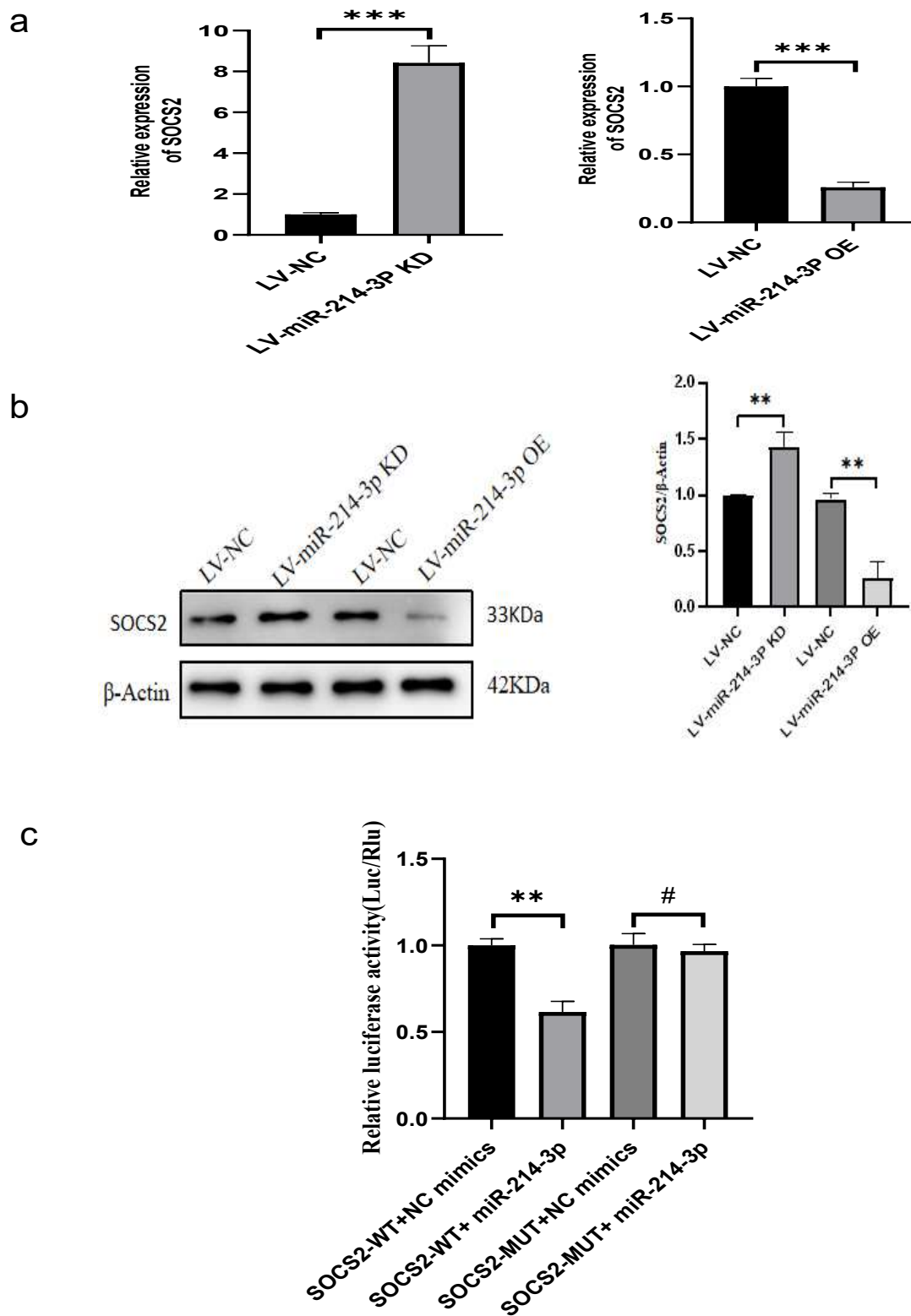


Figure 8 Validation of SOCS2 as a direct target gene of miR-214 in AGS gastric cancer cells. (a) qRT-PCR analysis of SOCS2 mRNA expression in AGS cells after miR-214

downregulation and upregulation ($***P < 0.001$); (b) Western blot analysis of SOCS2 protein expression in AGS cells after miR-214 downregulation and upregulation ($**P < 0.01$); (c) Dual-luciferase reporter assay confirming SOCS2 as a direct target of miR-214 ($**P < 0.01$; # $P > 0.05$, not significant)

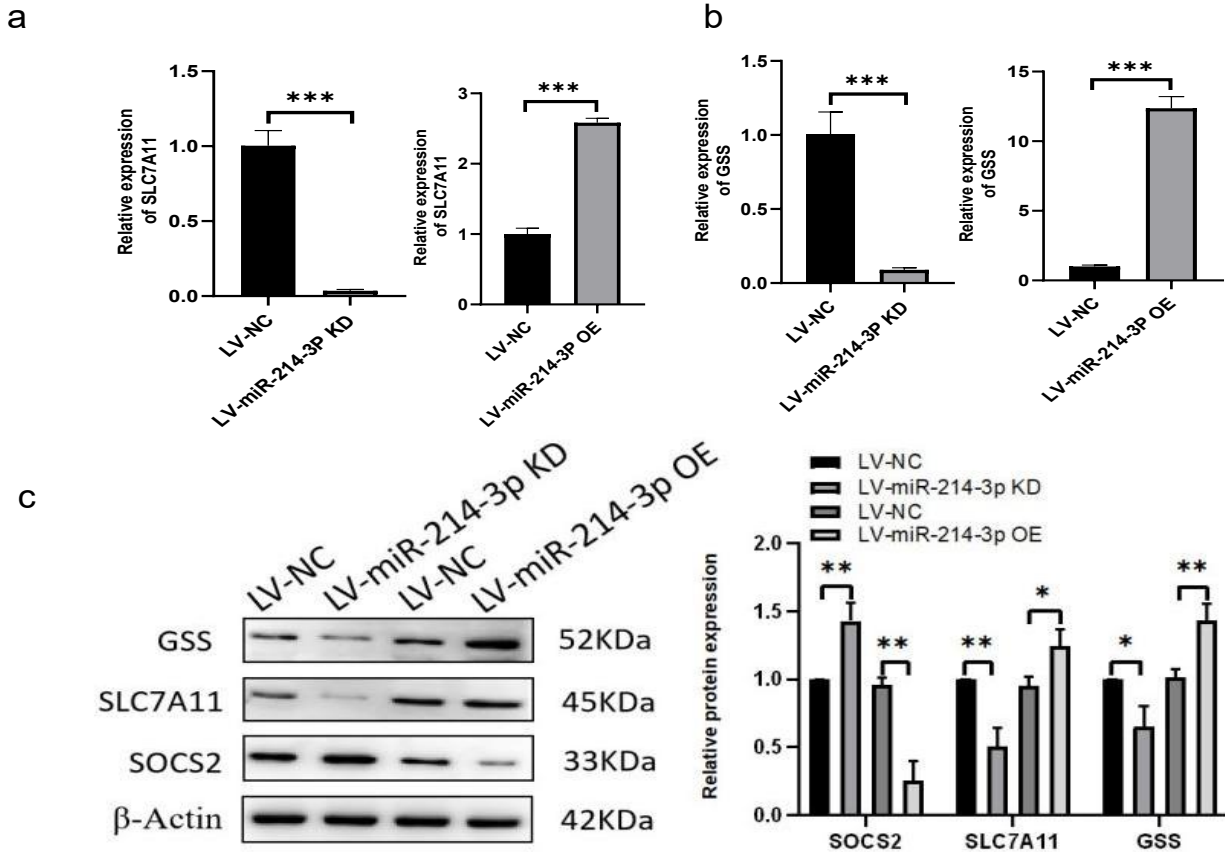


Figure 9 Validation of miR-214 regulation of ferroptosis-related genes SLC7A11, GSS, and SOCS2 in AGS gastric cancer cells. (a) qRT-PCR analysis of SLC7A11 mRNA expression in AGS cells after miR-214 downregulation and upregulation ($***P < 0.001$); (b) qRT-PCR analysis of GSS mRNA expression in AGS cells after miR-214 downregulation and upregulation ($***P < 0.001$); (c) Western blot analysis of SOCS2, SLC7A11, and GSS protein expression in AGS cells after miR-214 downregulation and upregulation ($*P < 0.05$; $**P < 0.01$)

Discussion

Gastric cancer (GC) remains a leading cause of cancer-related mortality worldwide. Patients diagnosed at advanced stages continue to face poor outcomes, largely driven by therapeutic resistance and the scarcity of effective targeted therapies *Patel, Sethi, & Park (2026); Van Cutsem, Sagaert, Topal, Haustermans, & Prenen (2016)* .

The role of microRNA-214-3p (miR-214-3p) in GC has drawn considerable attention; yet, a notable paradox persists in the literature regarding its expression and functional implications. On one hand, substantial evidence supports its upregulation in GC tissues. For instance, a study involving 133 patient-matched tissue pairs reported that high miR-214-3p expression served as an independent prognostic factor for reduced five-year overall survival *Wang, Qiao, Yang, Si, & Xiao (2025)*. On the other hand, an equally compelling body of research points to its downregulation. In a separate cohort of 72 patients, the long non-coding RNA HEIH was found to be significantly upregulated in tumors and promoted cancer cell migration and invasion. Mechanistically, HEIH functioned by directly targeting and suppressing miR-214-3p, which was correspondingly downregulated in the same GC tissues *Jiang et al. (2021)*. This dichotomy—both in expression (“high” vs. “low”) and in inferred function (“oncogenic” vs. “tumor-suppressive”) — highlights the context-dependent complexity of miR-214-3p in gastric carcinogenesis.

To further explore this issue, we analyzed miR-214-3p expression using public databases. Our pan-cancer analysis confirmed its dysregulation across multiple malignancies, with pronounced overexpression specifically in gastric adenocarcinoma. Within GC, elevated miR-214-3p levels were significantly associated with advanced tumor stage, greater depth of invasion, and the presence of distant metastasis, strongly supporting its involvement in driving aggressive disease. Notably, the absence of a significant association with lymph node (N) stage presents an intriguing nuance. This may suggest that miR-214-3p is more closely tied to pathways governing local invasion and hematogenous dissemination rather than lymphatic spread—a distinction that warrants further mechanistic investigation. Collectively, these findings reinforce the view that miR-214-3p is highly expressed in GC and correlates with disease stage, invasive capacity, and metastatic potential.

The observed correlation between miR-214-3p expression and *Helicobacter pylori* (HP) infection status is noteworthy. Given that HP is the primary environmental risk factor for gastric cancer, this association positions miR-214-3p as a potential molecular link between chronic infection and oncogenic progression. It is plausible that the inflammatory microenvironment induced by HP infection drives the dysregulation of specific miRNAs such as miR-214-3p, which in turn modulates downstream targets to promote malignancy.

With regard to prognostic value, our findings present a multifaceted picture. Although miR-214-3p levels correlated with overall survival events, Kaplan – Meier analysis did not reveal a statistically significant difference in survival time based on expression alone. This apparent discrepancy may be explained by viewing miR-214-3p primarily as a marker and mediator of aggressive tumor biology rather than as an independent prognostic factor in multivariate models. Its association with disease stability further suggests a role in maintaining tumor cell viability. The significant differential expression observed in tumors of the gastric fundus and body also underscores the growing importance of anatomical location in GC molecular subtyping, suggesting that the oncogenic function of miR-214-3p may be context-dependent. Importantly, ROC curve analysis indicated that miR-214-3p possesses credible diagnostic discriminatory power. Combined with its stage-related expression, this reinforces its potential utility as a diagnostic and stratification biomarker.

In conclusion, our results establish miR-214-3p as a clinically relevant onco-miRNA in gastric cancer. Its overexpression is linked to key aggressive features and may be modulated by the HP-infected microenvironment. Although it did not serve as an independent prognostic marker in this cohort, its diagnostic potential and association with aggressive biology make it a compelling subject for further investigation. Our subsequent mechanistic studies, which identified its regulation of the SOCS2/SLC7A11-mediated ferroptosis pathway, provide a direct functional link. This explains how miR-214-3p overexpression may confer a survival advantage to GC cells by inhibiting this critical tumor-suppressive form of cell death. Future experiments in our laboratory will continue to explore the relationship between miR-214-3p and HP infection, with the aim of further clarifying the underlying mechanisms.

In recent years, ferroptosis—a form of regulated cell death driven by iron-dependent lipid peroxidation—has been recognized as a therapeutically targetable vulnerability in cancer **Wahida & Conrad (2025)**. In gastric cancer (GC), this process plays a pivotal role in tumor regulation. Combining ferroptosis induction with chemotherapy, targeted therapy, and immunotherapy represents a promising therapeutic strategy. For example, myricetin has been shown to induce ferroptosis and inhibit GC progression by targeting NOX4 **Lu et al. (2024)** , while curcumin suppresses GC growth both in vitro and in vivo through the same mechanism, highlighting its potential as an adjuvant therapeutic agent **Fan, Zhang, Zhao, Chen, & Liang (2024)** .

Conversely, resistance to ferroptosis significantly contributes to GC cell proliferation, metastasis, and chemoresistance, thereby positioning it as a critical therapeutic target. This resistance is largely mediated by aberrant activation of the SLC7A11/GPX4 antioxidant axis **Zhao et al. (2025)**. In support of this, Chen et al **Chen et al. (2024)**. demonstrated that downregulation of the SLC7A11/GPX4 axis induces ferroptosis, markedly inhibiting proliferation, colony formation, and xenograft growth in MKN45 and SGC-7901 cells. Clinical evidence further highlights its relevance: Jiang et al **Jiang, Cui, Cui, & Jing (2023)** found that SLC7A11 overexpression in 126 GC specimens was correlated with advanced T stage, lymph node metastasis, and poor differentiation, and its knockdown suppressed proliferation and enhanced ferroptosis. Similarly, Lin et al **Lin et al. (2025)** reported that elevated GPX4 expression in GC tissues was associated with advanced stage, lymph node metastasis, poor differentiation, and shorter overall survival. Taken together, SLC7A11 and GPX4 serve as key prognostic biomarkers and therapeutic targets linked to ferroptosis resistance in GC.

Given its strong associations with the tumor microenvironment and treatment response, ferroptosis represents a core target for precision therapy in GC **Zhao et al. (2025)**. However, the mechanisms governing ferroptosis resistance, particularly those regulated by non-coding RNAs, remain poorly understood.

The role of miR-214-3p in gastric cancer (GC) exemplifies this complexity, as conflicting reports persist regarding both its expression and function. While the majority of studies associate high miR-214-3p levels with advanced tumor stage, lymph node metastasis, and poor prognosis **Zhao, Fan, Fan, & Gao (2018)**, others have described low expression correlating with tumor size and nodal involvement **Amin, Trevelyan, & Turner (2021)** .

To clarify its role in GC and its potential link to ferroptosis, we designed the present study to systematically investigate its function in regulating this form of cell death. We employed Oxford Nanopore Technology (ONT) long-read sequencing for an unbiased transcriptomic overview and integrated bioinformatic predictions from multiple miRNA target databases (TargetScan, miRDB, miRWalk) with a curated ferroptosis gene set to identify relevant targets. Through this screening strategy, SOCS2 emerged as a key ferroptosis-associated factor suppressed by miR-214-3p. We therefore hypothesized that miR-214-3p promotes ferroptosis resistance in GC by directly repressing SOCS2, thereby protecting cells from lipid peroxidation-induced death—a novel axis connecting an oncogenic miRNA to a metabolic vulnerability.

The core mechanistic finding of this study is the identification of SOCS2 as a direct and functional target of miR-214-3p in GC cells, confirmed by dual-luciferase reporter assays. We demonstrated that miR-214-3p negatively regulates SOCS2 expression. This is significant because SOCS2 acts as a tumor suppressor in the context of ferroptosis; prior research in hepatocellular carcinoma established that SOCS2 functions as an E3 ubiquitin ligase adaptor, promoting the K48-linked polyubiquitination and proteasomal degradation of SLC7A11 *Chen et al. (2023); Yi et al. (2026)*. SLC7A11 is the functional subunit of System Xc⁻, which imports cystine for the synthesis of glutathione (GSH)—the essential cofactor for the ferroptosis inhibitor GPX4 *Liu et al. (2021)*. In line with this established model, our data show that miR-214-3p-mediated suppression of SOCS2 leads to increased protein levels of both SLC7A11 and GSS (glutathione synthetase). This delineates a coherent pathway: miR-214-3p inhibits SOCS2, stabilizes SLC7A11, enhances cystine uptake and GSH synthesis, and ultimately strengthens the cellular antioxidant defense against ferroptosis. This mechanism aligns with studies in other cancers where SOCS2 upregulation promotes ferroptosis *Wang, Jia, Jiang, Lu, & Ning (2024)*, highlighting the context-dependent nature of this regulatory axis.

Limitations and Future Perspectives

This study has several limitations. First, the findings are primarily based on experiments conducted in the AGS cell line. Given the heterogeneity of gastric cancer (GC), the generalizability of these results to other GC subtypes, which may harbor distinct molecular profiles and differential ferroptosis sensitivities, requires validation in a broader panel of cell lines. Second, the work is confined to in vitro models. The absence of in vivo validation using xenograft models and the lack of correlation with clinical

specimens limit the translational relevance of our conclusions. Furthermore, we did not quantify key biochemical hallmarks of ferroptosis, such as intracellular Fe^{2+} and lipid peroxidation (e.g., malondialdehyde, MDA), and glutathione (GSH) levels, which would have provided more direct functional evidence for the induction of ferroptotic cell death.

Despite these limitations, our study offers novel mechanistic insight. We provide the first evidence that miR-214-3p confers ferroptosis resistance in GC by directly targeting SOCS2, thereby upregulating the SLC7A11/GSS axis. This discovery not only identifies SOCS2 as a novel functional target of miR-214-3p but also expands the known regulatory network of non-coding RNAs in ferroptosis. Future investigations should focus on: (1) validating this miR-214-3p/SOCS2/SLC7A11 axis in diverse GC cell lines and in vivo models; (2) assessing the clinical and prognostic significance of this pathway signature in large patient cohorts; (3) directly quantifying ferroptosis markers (Fe^{2+} , MDA, GSH) to solidify the functional link; and (4) exploring the therapeutic potential of co-targeting this axis. Specifically, combining miR-214-3p inhibitors (or SOCS2 mimetics) with established ferroptosis inducers (e.g., Erastin, RSL3) represents a promising strategy for developing novel combination therapies against GC.

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Ethical Approval and Consent to Participate

This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. The retrospective analysis of human tissue microarray specimens was approved by the institutional medical ethics committee, and the requirement for informed consent was waived in view of the retrospective and anonymized nature of the data. The publicly available datasets used in this work—including The Cancer Genome Atlas (TCGA), the FerrDb database, and target predictions generated by TargetScan, miRDB, and miRWalk—were all de-identified and openly accessible, and no additional ethical approval was required for their secondary use. The AGS gastric cancer cell line used in the in vitro experiments was obtained from a standard cell repository and routinely tested for mycoplasma contamination.

Consent for Publication

All figures and data presented in this manuscript are derived from de-identified public databases, anonymized tissue microarray specimens, and in vitro cell-line experiments. No individual patient's personal data, identifiable images, or clinical details are included. Therefore, consent for publication was not required.

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Data availability

All data generated or analyzed during this study have been included in this published article and its supplementary information files. The raw data of RNA-seq are available in the NCBI Gene Expression Omnibus and are accessible through GEO series accession number GSE300926.

Authors' contributions

Fengchen Bi contributed to long-read sequencing data analysis and drafted the initial manuscript. Xue Wang assisted in experiments and participated in bioinformatics analysis. Min Niu engaged in bioinformatics analysis. Qian Hao provided experimental support and validation. Hongliang Li participated in manuscript revision and logical structuring. Ruijuan Xin interpreted the results and approved the final manuscript for submission.