

Calycosin synergistically promotes ferroptosis in cervical cancer cells by inducing epithelial-mesenchymal transition

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Abstract. Cervical cancer treatment faces challenges from chemotherapy resistance. Inducing ferroptosis is a promising strategy. Calycosin (Cal), an active component of *Astragalus membranaceus*, exhibits antitumor activity, but its role in cervical cancer, particularly regarding epithelial-mesenchymal transition (EMT) and ferroptosis, remains unclear. Cell Counting Kit-8, colony formation and Transwell assays assessed Cal's effects on cell viability, migration and invasion. Ferroptosis was evaluated by detecting reactive oxygen species (ROS), malondialdehyde, glutathione and ferrous iron (Fe²⁺) levels, mitochondrial morphology and expression of ferroptosis-related proteins. EMT markers and signaling pathways (TGF- β /Smad and Hippo/YAP) were analyzed using western blot. TGF- β 1 knockdown was used to investigate the functional link between EMT and ferroptosis *in vitro* and *in vivo*. Cal inhibited proliferation and induced ferroptosis in cervical cancer cells, evidenced by mitochondrial shrinkage, lipid peroxidation and altered glutathione peroxidase 4/solute carrier family 7 member 11/acyl-CoA synthetase long-chain family member 4 expression. However, Cal also promoted migration and invasion, upregulated mesenchymal markers, and activated TGF- β /Smad while inhibiting Hippo/YAP signaling. Importantly, TGF- β 1 knockdown suppressed both Cal-induced EMT and ferroptosis, indicating a synergistic relationship. *In vivo* studies confirmed these findings, showing Cal inhibited primary tumor growth but promoted metastasis, effects attenuated by TGF- β 1 knockdown. Cal exerts dual effects in

cervical cancer: Inducing ferroptosis to inhibit growth while promoting EMT-associated metastasis via TGF- β /Smad and Hippo/YAP pathways. Crucially, Cal-induced EMT synergistically enhances ferroptosis sensitivity. These findings highlight the complex pharmacology of Cal and suggest that combining with EMT inhibitors may optimize its therapeutic potential while mitigating pro-metastatic risks.

Introduction

Cervical cancer is the fourth most common cancer in women worldwide and ranks as the leading gynecological malignancy in China (1,2). Although the popularization of human papillomavirus (HPV) vaccines and advances in early screening have markedly reduced its incidence, chemotherapy remains the primary treatment for patients with advanced, recurrent or metastatic cervical cancer (3). However, current platinum-based chemotherapy regimens face two major challenges: Notable adverse drug reactions that severely impact patients' quality of life and the development of drug resistance by tumor cells during treatment, leading to therapeutic failure (4-6). Therefore, identifying novel therapeutic agents with high efficacy, low toxicity and the potential to overcome or reverse resistance is of critical clinical importance for improving the prognosis of patients with cervical cancer.

Ferroptosis, a term first coined by Dixon and Olzmann (7) in 2012, is an iron-dependent, non-apoptotic form of cell death characterized by the accumulation of lipid peroxides. A growing body of research indicates that inducing ferroptosis in tumor cells can effectively inhibit the growth of various cancers (including breast, lung and pancreatic cancers) and enhance their sensitivity to conventional chemotherapy and immunotherapy (8-10). Consequently, discovering agents capable of effectively inducing ferroptosis has become a focal point in antitumor drug development. The susceptibility to ferroptosis is regulated by multiple signaling pathways and cellular states, among which epithelial-mesenchymal transition (EMT) is considered a key modulator (11). EMT is a biological process where epithelial cells lose polarity and cell-cell junctions, acquiring a mesenchymal-like morphology and enhanced migratory capacity, serving a central role in embryonic development, wound healing and tumor metastasis. Recent studies have revealed that cancer cells undergoing

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EMT exhibit distinct metabolic features, such as increased sensitivity to oxidative stress and remodeled lipid metabolism, making them more susceptible to ferroptosis inducers (12-14). In drug-resistant cancer cells, inducing EMT can notably enhance their sensitivity to ferroptosis, suggesting a complex and not yet fully elucidated interplay between EMT and ferroptosis.

Calycosin (Cal), a major isoflavonoid derived from the traditional Chinese herb *Astragalus membranaceus*, has been shown in experimental studies to possess immunomodulatory, anti-inflammatory and antitumor properties, along with a wide availability and low toxicity profile (15,16). In the present study, using human cervical cancer cell lines HeLa and SiHa as models, the effects of Cal on the proliferation, death and migration of cervical cancer cells was first investigated. Subsequently, whether it induces ferroptosis and EMT was explored and the potential signaling pathways involved were elucidated. Finally, by knocking down TGF- β 1 to modulate EMT, the functional link between Cal-induced EMT and ferroptosis was investigated, and the findings were validated *in vivo* using a nude mouse model. The present study aimed to unravel the complex pharmacological effects and mechanisms of Cal in cervical cancer, providing a new theoretical and experimental basis for reassessing its antitumor value and optimizing clinical therapeutic strategies.

Materials and methods

Cell culture. Human cervical cancer HeLa, SiHa and normal human cervical epithelial ECT1/E6E7 cells were purchased from Wuhan Pricella Biotechnology Co., Ltd. HeLa and SiHa cells were cultured in DMEM (cat. no. 11965092; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.) under the conditions of 5% CO₂ at 37°C. ECT1/E6E7 cells were cultured in keratinocyte serum-free medium (cat. no. 17005042; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 44.1 mg/l calcium chloride (final concentration: 0.4 mM; cat. no. C1016; Sigma-Aldrich; Merck KGaA), 0.1 ng/ml human recombinant EGF (cat. no. PHG0311; Gibco; Thermo Fisher Scientific, Inc.), 0.05 mg/ml bovine pituitary extract (cat. no. 13028014; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (cat. no. 15140122; Gibco; Thermo Fisher Scientific, Inc.) under the same culture conditions (37°C, 5% CO₂). Cal (purity, >98%; cat. no. A0514; Chengdu Must Bio-Technology Co., Ltd.) was dissolved in DMSO for storage.

Transfection and knockdown. For transient knockdown of TGF- β 1 in HeLa cells, small interfering RNA (siRNA) duplexes targeting TGF- β 1 (sense: 5'-GCCUGGACACCAACUAUUG-3', antisense: 5'-CAAUAGUUGGUGUCCAGG C-3') and a non-targeting scrambled siRNA (sense: 5'-UUCUCCGAACGUGUCACGU-3', antisense: 5'-ACGUGACACGUUCGGAGAA-3') were used. Cells were seeded in 6-well plates and transfected at 70-80% confluence using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, for each well, 50 pmol siRNA was diluted in 125 μ l Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) and mixed with 5 μ l Lipofectamine® 3000 and 2.5 μ l P3000 reagent in 125 μ l

Opti-MEM. The transfection complexes were incubated at room temperature for 15 min and then added to the cells in complete medium. Cells were incubated at 37°C in a 5% CO₂ incubator for 6-8 h, after which the transfection mixture was replaced with fresh complete medium. Transfection efficiency was determined by western blotting at 48 h post-transfection and subsequent functional assays were performed at 48-72 h post-transfection.

Cell viability detection. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay. HeLa cells were seeded in 96-well plates and treated with Cal at concentrations ranging from 0-105 mg/l (0, 15, 30, 45, 60, 75, 90 and 105 mg/l) for 24 h at 37°C. Cells treated with DMSO (the solvent for Cal) served as the solvent control and untreated cells served as the negative control; wells containing medium alone were used as blank controls for background subtraction. Following the 24-h treatment, the medium was replaced with fresh medium containing 10% CCK-8 reagent and the plates were incubated for a further 2 h at 37°C. The absorbance at 450 nm was detected using a Multiskan FC Microplate Reader (Thermo Fisher Scientific, Inc.).

Colony formation assay. HeLa and SiHa cells in the logarithmic growth phase were digested and seeded into 6-well plates at 500 cells per well. After overnight culture, cells were treated with medium containing different concentrations of Cal (0 and 90 mg/l) for 24 h at 37°C in a 5% CO₂ incubator, then replaced with fresh complete medium and cultured for 10-14 days until visible colonies appeared. The medium was discarded, cells were washed with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min and stained with 0.1% crystal violet at room temperature for 20 min. The staining solution was gently washed away with running water, the plates were air-dried and images were captured using an inverted light microscope (Olympus Corporation). Colonies (defined as clusters containing $\geq$ 50 cells) were counted in five randomly selected fields per well.

Migration, invasion and wound healing assays. For wound healing assays, HeLa and SiHa cells were cultured in 6-well plates to ~90% confluence and a sterile 200- μ l pipette tip was used to create a linear scratch across the monolayer. Cells were washed twice with PBS to remove detached cells and then cultured in serum-free medium with or without Cal (90 mg/l) for 24 h at 37°C in a 5% CO₂ incubator. Wound closure was photographed at 0 and 24 h using an inverted microscope (Olympus Corporation) and the migration rate was calculated as: [(wound width at 0 h - wound width at 24 h)/wound width at 0 h] x 100% using ImageJ software (version 1.54p; National Institutes of Health).

For invasion assays, Transwell chambers (8- μ m pore size; Corning, Inc.) were pre-coated with Matrigel (diluted 1:8 in serum-free medium; cat. no. 356234; Corning, Inc.) at 37°C for 1 h to solidify. HeLa and SiHa cells (5x10⁴ per well) suspended in serum-free medium were seeded into the upper chambers, while the lower chambers contained complete medium with 10% FBS as a chemoattractant. After incubation for 24 h at 37°C in a 5% CO₂ incubator, cells on the upper surface of the membrane were carefully removed with a cotton swab.

Cells that had invaded through the Matrigel to the lower surface were fixed with 4% paraformaldehyde for 15 min at room temperature, stained with 0.1% crystal violet for 20 min at room temperature, washed with PBS, air-dried and imaged under a light microscope. Invaded cells were counted in five randomly selected fields per well. Each experiment was performed in triplicate.

Transmission electron microscopy. After drug treatment, HeLa cells were collected and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 2 h. The cells were then post-fixed with 1% osmium tetroxide at 4°C for 1 h, dehydrated through a graded ethanol series (30, 50, 70, 90 and 100%), and embedded in Epon 812 resin at 60°C for 48 h. Ultrathin sections (70 nm thick) were cut using an ultramicrotome (Leica EM UC7; Leica Microsystems), mounted on copper grids and stained with 2% uranyl acetate at room temperature for 15 min, followed by lead citrate at room temperature for 8 min. The ultrastructure of the cells was observed by transmission electron microscope (JEM1400; JEOL, Ltd.).

Intracellular reactive oxygen species (ROS) detection. ROS levels were detected using a DCFH-DA probe (cat. no. S0033S; Beyotime Biotechnology). After Cal treatment, the culture medium was discarded, and cells were incubated with serum-free medium containing 10 μ M DCFH-DA at 37°C for 20 min in the dark. After washing three times with serum-free medium to remove the probe that had not entered the cells, cells were trypsinized, resuspended in PBS and observed under a fluorescence microscope.

Biochemical index detection. Contents of malondialdehyde (MDA) and glutathione (GSH) in cells were detected using the corresponding kits (cat. nos. S0131S and S0053; Beyotime Biotechnology) and the experiments were carried out strictly in accordance with the manufacturer's instructions.

Western blotting. HeLa and SiHa cells were seeded in 6-well plates and treated with 90 mg/l Cal or vehicle control for 24 h at 37°C in a 5% CO₂ incubator. Cells were then harvested and lysed on ice for 30 min using RIPA lysis buffer (strong; cat. no. P0013B; Beyotime Biotechnology) supplemented with 1 mM PMSF and a protease inhibitor cocktail. Lysates were centrifuged at 12,000 x g for 15 min at 4°C and the supernatants were collected. Protein concentrations were determined using a BCA protein assay kit (cat. no. P0012S; Beyotime Biotechnology). Equal amounts of protein (20 μ g per well) were separated by 10% SDS-PAGE using an SDS-PAGE gel preparation kit (cat. no. P0012A; Beyotime Biotechnology) and transferred onto PVDF membranes (cat. no. IPVH00010; Immobilon-P; 0.45 μ m; Merck KGaA).

Membranes were blocked with 5% (w/v) non-fat milk (cat. no. P0216-300g; Beyotime Biotechnology) in TBST (0.1% Tween-20) for 1 h at room temperature, followed by incubation with primary antibodies overnight at 4°C. The primary antibodies used were as follows: Anti-SLC7A11 (cat. no. ab175186; 1:1,000; Abcam), anti-glutathione peroxidase 4 (GPX4; cat. no. ab125066; 1:1,000; Abcam), anti-acyl-CoA synthetase long-chain family member 4

(ACSL4; cat. no. ab155282; 1:1,000; Abcam), anti-ferritin heavy chain 1 (FTH1; cat. no. ab75973; 1:1,000; Abcam), anti-transferrin receptor 1 (TFR1; cat. no. ab84036; 1:1,000; Abcam), anti-NADPH oxidase 1 (NOX1; cat. no. ab131088; 1:1,000; Abcam), anti-E-cadherin (cat. no. ab40772; 1:5,000; Abcam), anti-N-cadherin (cat. no. ab18203; 1:1,000; Abcam), anti-vimentin (cat. no. ab8069; 1:1,000; Abcam), anti-TGF- β 1 (cat. no. ab179695; 1:1,000; Abcam), anti-Snail (cat. no. ab216347; 1:1,000; Abcam), anti-twist family BHLH transcription factor 1 (cat. no. ab50887; 1:500; Abcam), anti-zinc finger E-box binding homeobox 1 (ZEB1; cat. no. ab203829; 1:1,000; Abcam), anti-phosphorylated (p)-Smad2 (cat. no. ab280888; 1:1,000; Abcam), anti-p-Smad3 (cat. no. ab52903; 1:1,000; Abcam), anti-p-MST1/2 (cat. no. ab76323; 1:2,000; Abcam), anti-p-large tumor suppressor kinase 1 (LATS1; cat. no. ab305030; 1:1,000; Abcam), anti-p-YAP (cat. no. ab76252; 1:1,000; Abcam) and anti- β -actin (cat. no. ab8226; 1:5,000; Abcam). After washing with TBST, membranes were incubated with an HRP-conjugated secondary Goat Anti-Rabbit IgG H&L antibody (cat. no. ab6721; 1:5,000; Abcam) for 1 h at room temperature. Protein bands were visualized using ECL substrate and imaged using a chemiluminescence imaging system. Band intensities were quantified using ImageJ software (version 1.54p; National Institutes of Health).

Animal experiments. A total of 16 female BALB/c nude mice (4-6 weeks old; 17-20 g) were purchased from Hangzhou Ziyuan Laboratory Animal Technology Co., Ltd. The mice were housed under specific pathogen-free conditions in individually ventilated cages with sterile bedding, at a controlled temperature of 22-26°C, relative humidity of 40-70% and a 12-h light/12-h dark cycle (lights on at 07:00). Autoclaved commercial rodent chow and filtered tap water were provided *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee of Jiangsu Health Vocational College (Nanjing, China; approval no. JHVC-IACUC-2025-B0192) and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (17).

HeLa-luc cells (fluorescently labeled human cervical cancer cells stably expressing firefly luciferase) were obtained from Wuhan Pricella Biotechnology Co., Ltd. (cat. no. CL-0277) and cultured in DMEM (cat. no. 11965092; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (cat. no. 15140122 Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO₂ incubator. HeLa-luc cells stably expressing short hairpin RNA (shRNA) targeting TGF- β 1 [target sequence: 5'-ATGCTGTGTGTACTCTGCTTG-3' (sh-TGF- β 1)] or a scrambled negative control [target sequence: 5'-CCTAAGGTTAAGTCGCCCTCG-3' (sh-NC)] were generated by lentiviral transduction using the pLKO.1 vector (plasmid no. 136035; Addgene, Inc.).

Lentiviral particles were produced in 293T cells by co-transfection of the pLKO.1 construct with packaging plasmids psPAX2 and pMD2.G using Lipofectamine® 3000 (cat. no. L3000015; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. HeLa-luc cells were transduced with the lentiviral supernatant in the presence of

8 $\mu\text{g/ml}$ polybrene (cat. no. TR-1003; MilliporeSigma) for 24 h at 37°C. Stably transduced cells were selected with 2 $\mu\text{g/ml}$ puromycin (cat. no. P8833; MilliporeSigma) for 7 days, and knockdown efficiency was determined by western blotting prior to *in vivo* experiments (Fig. S1). The cell suspension concentration was adjusted to 5×10^7 cells/ml and 0.1 ml per mouse was inoculated subcutaneously into the left forelimb armpit of BALB/c nude mice. When the tumor volume reached $\sim 100 \text{ mm}^3$, mice were randomly divided into four groups ($n=4$ per group): sh-NC group, sh-NC + Cal group, sh-TGF- $\beta 1$ group and sh-TGF- $\beta 1$ + Cal group. The drug treatment groups received daily intraperitoneal injections of Cal (40 mg/kg), while the non-treatment groups received an equal volume of solvent. Body weight and tumor volume were measured every 3 days. *In vivo* imaging was performed at weeks 2, 4 and 6. After 6 weeks, all mice were humanely euthanized by cervical dislocation under isoflurane anesthesia and tumor tissues were harvested for subsequent analysis. Anesthesia was induced with 4% isoflurane in oxygen delivered through a precision vaporizer in an induction chamber. Once deep anesthesia was determined by the absence of a pedal withdrawal reflex, mice were removed from the chamber and anesthesia was maintained with 1.5-2.0% isoflurane delivered through a nose cone. Cervical dislocation was performed swiftly while the mouse remained in a surgical plane of anesthesia. Mortality was determined by the absence of heartbeat and respiration.

Immunohistochemistry and H&E staining. For immunohistochemistry, tumor tissues were fixed in 4% (w/v) paraformaldehyde in PBS (pH 7.4) at 4°C for 24 h, dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin wax. Serial sections were cut at a thickness of 4 μm using a microtome (Leica RM2235; Leica Microsystems) and mounted onto poly-L-lysine-coated glass slides. Sections were dewaxed in xylene and rehydrated through a graded ethanol series, followed by antigen retrieval in citrate buffer (10 mM, pH 6.0) at 95°C for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol at room temperature for 10 min. Non-specific binding was blocked by incubation with 5% (v/v) normal goat serum (cat. no. C0265; Beyotime Biotechnology) in PBS containing 0.3% (v/v) Triton X-100 (cat. no. ST795; Beyotime Biotechnology) for 1 h at room temperature. The sections were then incubated with primary antibodies overnight at 4°C: Anti-GPX4 (cat. no. ab125066; 1:200; Abcam) and anti-ACSL4 (cat. no. ab155282; 1:200; Abcam). After washing with PBS, the sections were incubated with an HRP-conjugated goat anti-rabbit secondary antibody (cat. no. ab6721; 1:1,000; Abcam) for 1 h at room temperature. Immunoreactivity was visualized using DAB substrate (cat. no. P0203; Beyotime Biotechnology) according to the manufacturer's instructions and sections were counterstained with hematoxylin. Slides were dehydrated, cleared, coverslipped and examined under a light microscope (Olympus BX53; Olympus Corporation). For H&E staining, sections were rehydrated, stained with hematoxylin at room temperature for 3 min, washed in running tap water for 5 min, differentiated in 1% acid alcohol (1% hydrochloric acid in 70% ethanol) for 5 sec, blued in Scott's tap water substitute for 2 min and counterstained with eosin at room temperature for 2 min. After dehydration and clearing,

sections were mounted and observed under the same light microscope (Olympus BX53; Olympus Corporation).

Statistical analysis. Data were processed using GraphPad Prism 8.0 software (Dotmatics). The data are expressed as mean $\pm$ standard deviation. Unpaired Student's t-tests were used for comparisons between two independent groups and one-way ANOVA followed by Tukey's post hoc test was used for multiple comparisons among three or more groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cal inhibits proliferation and induces ferroptosis in cervical cancer cells. To evaluate the effect of Cal on cervical cancer cells, its cytotoxicity on HeLa, SiHa, and normal cervical epithelial cells was first assessed using a CCK-8 assay. The results showed that Cal inhibited the viability of HeLa and SiHa cells in a concentration- and time-dependent manner. At a concentration of 90 mg/l, the inhibition rate exceeded 50% in both cancer cell lines (HeLa, 60.38%; SiHa, 50.26%), whereas the inhibition rate in normal cervical epithelial cells was $< 50\%$ (46.16%) (Fig. 1A). Based on these concentration-dependent effects, 90 mg/l was selected for subsequent *in vitro* experiments. The colony formation assay further confirmed that treatment with 90 mg/l Cal significantly reduced the number of colonies formed by HeLa and SiHa cells (Fig. 1B), indicating an inhibitory effect on the long-term proliferation of cervical cancer cells. To explore the mode of cell death, the ultrastructure of HeLa cells was observed using transmission electron microscopy. Compared with the control group, cells in the Cal-treated group exhibited typical morphological features of ferroptosis: Shrunken mitochondria with increased membrane density, reduced or disappeared cristae, while the nuclear morphology remained relatively normal, with no obvious chromatin condensation or apoptotic bodies (Fig. 1C). This suggested that Cal might induce ferroptosis.

Subsequently, biochemical indicators related to ferroptosis were measured. ROS staining revealed markedly increased green fluorescence intensity in HeLa and SiHa cells after Cal treatment (Fig. 1D), indicating elevated ROS levels. Concurrently, the content of the lipid peroxidation product MDA increased significantly, while the level of the antioxidant GSH decreased and intracellular Fe^{2+} content also markedly increased (Fig. 1E-G). Western blot results showed that Cal treatment downregulated the expression of key negative regulators of ferroptosis, SLC7A11, GPX4 and FTH1, while upregulating the expression of positive regulators ACSL4, TFR1 and NOX1 (Fig. 1H). These results collectively demonstrate that Cal effectively induces ferroptosis in cervical cancer cells.

Cal promotes cell migration and induces EMT in cervical cancer cells. Given that Cal's effect on cell motility while inhibiting proliferation was unclear, Transwell and wound healing assays were performed. Notably, after treating HeLa and SiHa cells with Cal, the number of migrating and invading cells was markedly higher than in the control groups (Fig. 2A and B). This indicates a potential risk of Cal promoting metastasis in cervical cancer. Enhanced migratory

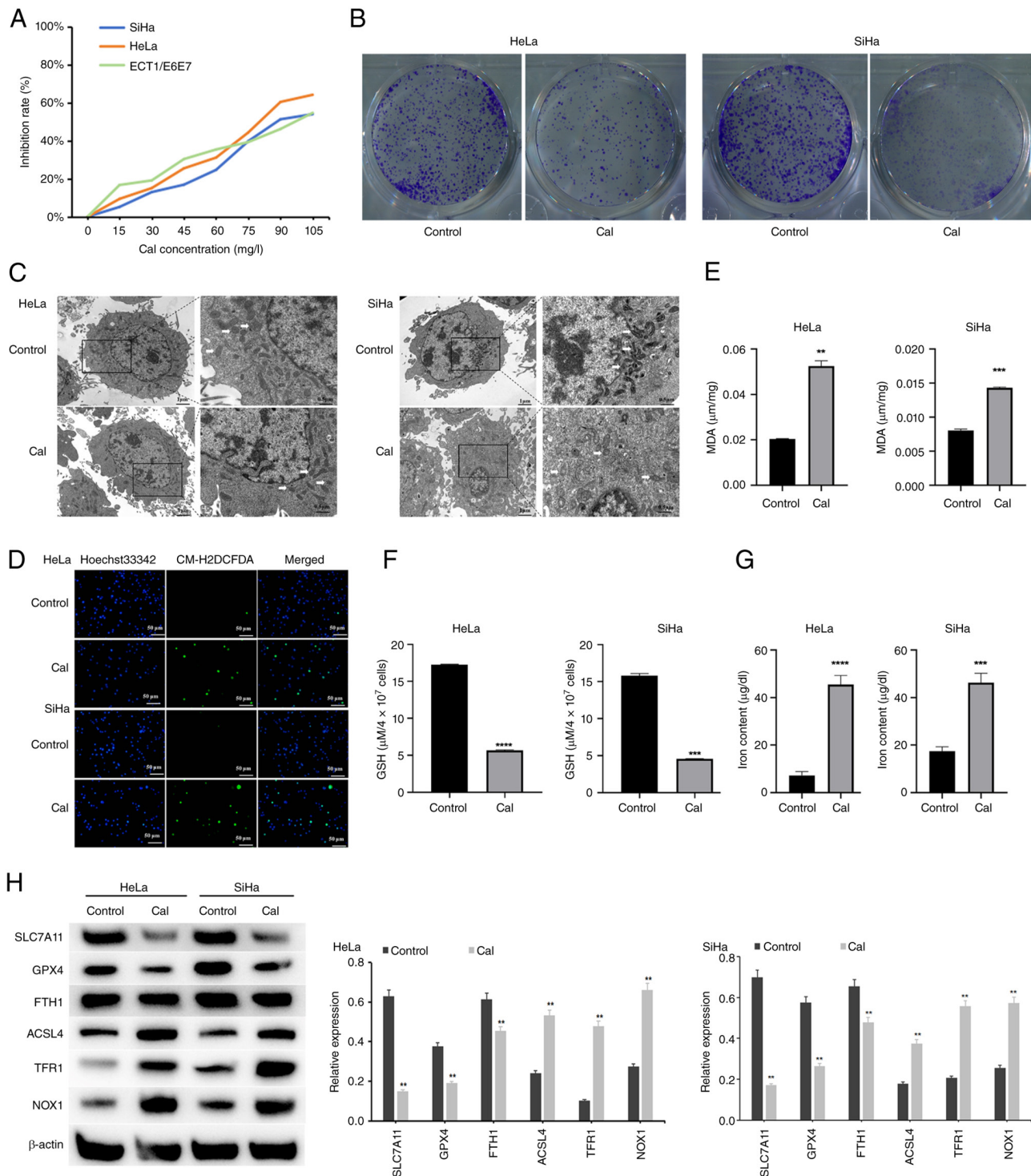


Figure 1. Cal inhibits proliferation and induces ferroptosis in cervical cancer cells. (A) Cell Counting Kit-8 assay showed Cal suppressed HeLa and SiHa cell viability in a concentration-dependent manner over the range of 0-105 mg/l, with the inhibition rate exceeding 50% in both cancer cell lines at 90 mg/l (HeLa: 60.38%; SiHa: 50.26%), while the inhibition rate in normal cervical epithelial cells remained below 50% (46.16%). (B) Colony formation assay with 90 mg/l Cal treatment reduced clonogenicity of HeLa/SiHa cells. (C) Transmission electron microscopy showed Cal induced ferroptotic mitochondrial changes (shrinkage, dense membrane and lost cristae) in HeLa and SiHa cells, with normal nuclei. (D) DCFH-DA staining showed Cal elevated intracellular reactive oxygen species levels in HeLa/SiHa cells. (E) Biochemical assay for MDA: Cal significantly increased the MDA content in HeLa and SiHa cells, indicating elevated lipid peroxidation. (F) Biochemical assay for GSH: Cal significantly decreased the GSH level in HeLa and SiHa cells, indicating reduced antioxidant capacity. (G) Biochemical assay for ferrous iron (Fe^{2+}): Cal significantly elevated intracellular Fe^{2+} levels in HeLa and SiHa cells. (H) Western blots showed Cal downregulated SLC7A11/GPX4/FTH1 and upregulated ACSL4/TFR1/NOX1 in HeLa/SiHa cells. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. control. Cal, calyculin; MDA, malondialdehyde; GSH, glutathione; SLC7A11, solute carrier family 7 member 11; ACSL4, acyl-CoA synthetase long-chain family member 4; GPX4, glutathione peroxidase; FTH1, ferritin heavy chain 1; TFR1, transferrin receptor 1; NOX1, NADPH oxidase 1.

capacity is often associated with the occurrence of EMT. The expression of EMT-related markers was examined. Western

blot results showed that Cal treatment significantly promoted the expression of upstream EMT regulators TGF- β 1, Snail,

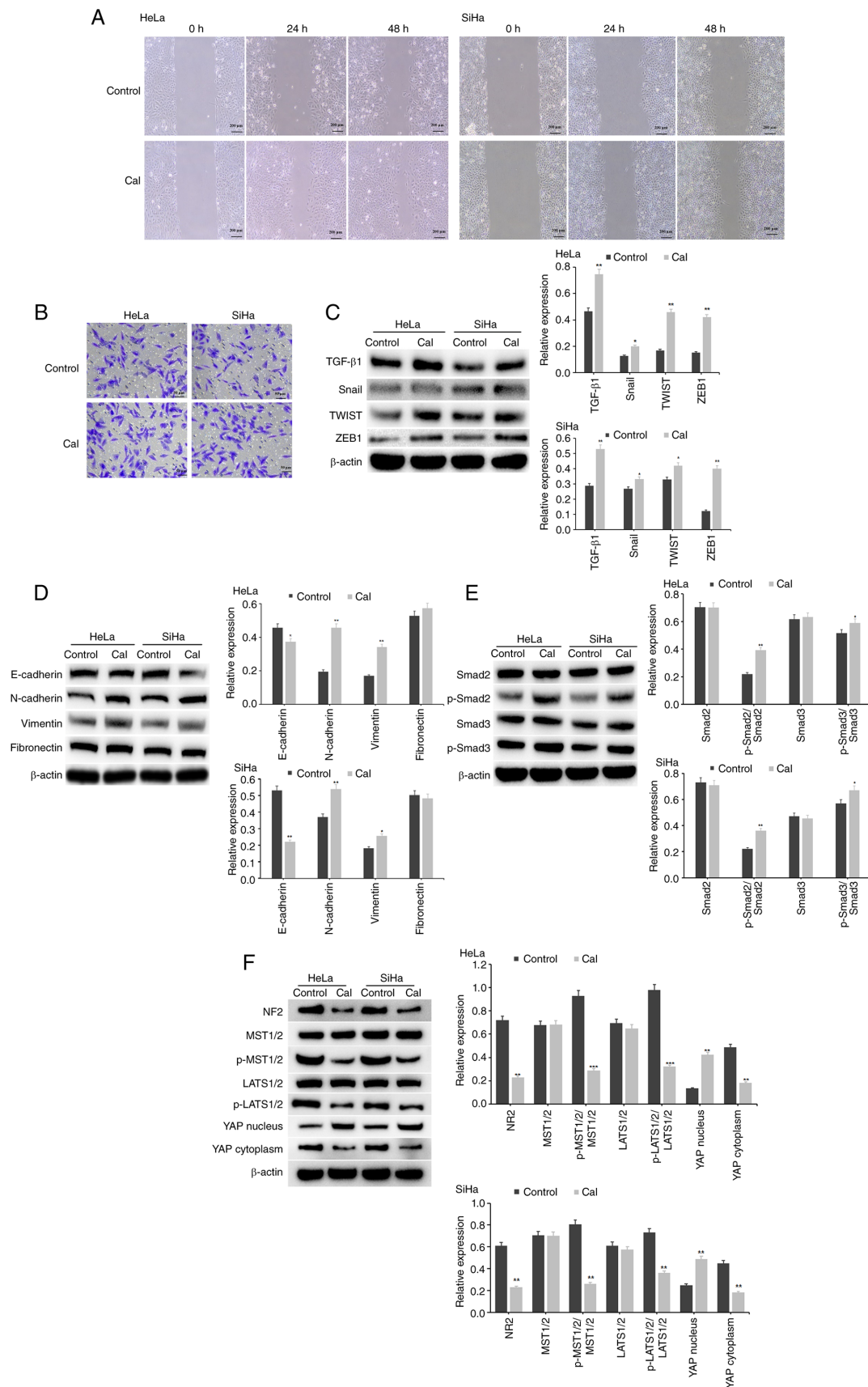


Figure 2. Cal promotes migration and induces epithelial-mesenchymal transition via the TGF- β /Smad and Hippo/YAP pathways. (A) Wound healing assay showing Cal enhanced migration of HeLa/SiHa cells. (B) Matrigel-coated Transwell invasion assay showing Cal enhanced migration of HeLa/SiHa cells. (C) Western blot showing Cal upregulated TGF- β 1/Snail/TWIST/ZEB1 in cervical cancer cells. (D) Cal increased N-cadherin/Vimentin and decreased E-cadherin in HeLa/SiHa cells. (E) Cal elevated p-Smad2/3, activating TGF- β /Smad pathway. (F) Western blot showing Cal reduced p-MST1/2/p-LATS1/2/p-YAP and promoted YAP nuclear translocation, inhibiting Hippo/YAP pathway. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control. Cal, calycosin; ZEB1, zinc finger E-box binding homeobox 1; TWIST, Twist-related protein 1; p-, phosphorylated; MST1/2, mammalian sterile 20-like kinase 1 and 2; LATS1/2, large tumor suppressor kinases 1 and 2.

TWIST and ZEB1 (Fig. 2C), while also increasing the protein levels of mesenchymal markers N-cadherin and vimentin, and decreasing the expression of the epithelial marker E-cadherin (Fig. 2D). To explore the upstream mechanisms, the classical EMT-inducing TGF- β /Smad pathway was examined. The results showed that Cal increased the phosphorylation levels of Smad2/3 (Fig. 2E). Additionally, the Hippo signaling pathway, crucial for regulating organ size and cell fate, is also involved in EMT regulation. After Cal treatment, the levels of the core Hippo pathway kinases p-MST1/2 and p-LATS1/2 were found to be decreased, and the phosphorylation level of its downstream effector YAP was reduced, leading to increased translocation of YAP from the cytoplasm to the nucleus (Fig. 2F), indicating inhibition of the Hippo pathway. In summary, Cal can induce EMT in cervical cancer cells by activating the TGF- β /Smad pathway and inhibiting the Hippo/YAP pathway, thereby enhancing their migratory capabilities.

Cal-induced EMT promotes ferroptosis. To investigate whether there is a functional link between the seemingly contradictory phenomena of Cal-induced EMT and ferroptosis, siRNA was used to knock down TGF- β 1 expression in HeLa cells to inhibit EMT. Western blotting confirmed the knockdown efficiency of TGF- β 1 (Fig. 3A). Functional assays demonstrated that TGF- β 1 knockdown markedly attenuated the Cal-promoted cell migratory abilities (Fig. 3B and C), confirming TGF- β 1 as a key mediator of Cal-induced EMT. Subsequently, the effect of inhibiting EMT on ferroptosis was examined. Notably, in TGF- β 1 knockdown cells, the Cal-induced decrease in cell viability was partially reversed (Fig. 3D). More importantly, compared with the Cal-only treatment group, the increases in ROS levels and MDA content, as well as the decrease in GSH level induced by Cal, were significantly attenuated upon TGF- β 1 knockdown (Fig. 3E-G). Western blot results also demonstrated that in TGF- β 1 knockdown cells, Cal's downregulation of GPX4 and upregulation of ACSL4 were weakened (Fig. 3H). These results reveal that Cal-induced EMT contributes to the induction of ferroptosis in cervical cancer cells, and inhibition of EMT attenuates its effect of promoting ferroptosis.

In vivo validation that Cal enhances ferroptosis by inducing EMT. To validate the aforementioned findings *in vivo*, HeLa cell lines with stable TGF- β 1 knockdown and corresponding control cell lines were constructed (Fig. S1) and subcutaneously inoculated into nude mice to establish a xenograft tumor model. There was no significant change in body weight among the mice in each group during treatment, indicating that Cal at this dose had no obvious systemic toxicity (Fig. 4A). Consistent with the *in vitro* results, Cal treatment significantly inhibited tumor growth in the sh-NC group (Fig. 4B). However, the tumor inhibitory effect of Cal was markedly diminished in the sh-TGF- β 1 group. H&E staining showed that tumor tissue in the Cal-treated sh-NC group exhibited extensive necrosis, structural disorganization and vacuolization, while the degree of tissue necrosis in the sh-TGF- β 1 group was milder (Fig. 4C). Immunohistochemical staining results demonstrated that in the sh-NC group, Cal treatment significantly reduced the expression of the key ferroptosis protein GPX4 and increased ACSL4 expression. However, in the sh-TGF- β 1 group, Cal's

downregulation of GPX4 and upregulation of ACSL4 were both significantly weakened (Fig. 4D). Furthermore, distant tumor metastasis was monitored using an *in vivo* imaging system. The results showed that in the Cal-treated sh-NC group, the fluorescence signal at the orthotopic inoculation site was suppressed, but the fluorescence signal from distant metastases was notably enhanced, demonstrating its side effect of promoting metastasis. However, in the sh-TGF- β 1 group, Cal's ability to inhibit orthotopic tumor growth and promote distant metastasis was reduced (Fig. 4E). These *in vivo* results are highly consistent with the *in vitro* findings, collectively demonstrating that Cal can synergistically enhance its ferroptosis-inducing effect on cervical cancer cells via TGF- β 1-mediated EMT both *in vitro* and *in vivo*, while its pro-metastatic risk cannot be ignored.

Discussion

The present study systematically investigated the complex biological effects and underlying mechanisms of Cal, an active component of the traditional Chinese herb *Astragalus membranaceus*, in cervical cancer. Cal was shown to serve a 'double-edged sword' role: On the one hand, it effectively induces ferroptosis in cervical cancer cells, thereby inhibiting tumor growth; on the other hand, it promotes EMT in tumor cells by activating the TGF- β /Smad pathway and inhibiting the Hippo/YAP pathway, enhancing their migratory capability, which clinically implies a potential pro-metastatic risk. More importantly, through *in vitro* functional rescue experiments and *in vivo* validation, a functional synergy was uncovered between Cal-induced EMT and its effect of promoting ferroptosis. This finding provides a new perspective for understanding the complex actions of drugs and the interplay between tumor cell plasticity and modes of cell death.

The antitumor effects of Cal have been demonstrated in various cancer models, with mechanisms involving the induction of apoptosis and cell cycle arrest (18,19). The present study extends this understanding to the novel cell death modality of ferroptosis. Following Cal treatment, cervical cancer cells exhibited typical morphological and biochemical features of ferroptosis, including mitochondrial shrinkage, accumulation of ROS and MDA, GSH depletion, and downregulation of GPX4/SLC7A11 expression. This aligns with the recent trend of numerous natural compounds exerting anticancer effects via ferroptosis induction (20,21). This suggests that inducing ferroptosis may be a core mechanism by which Cal inhibits cervical cancer growth. How it specifically regulates the expression of SLC7A11 and GPX4 warrants further investigation, whether by directly acting on promoter regions or by affecting upstream transcription factors (such as nuclear factor erythroid 2-related factor 2).

Concurrently, the finding that Cal promotes EMT and migratory ability in cervical cancer cells is strikingly similar to the report by Zhang *et al* (22) in pancreatic cancer, suggesting this might be a common 'off-target' effect or an inherent property of the drug itself across different tumor types. That study attributed Cal's pro-metastatic effect to the activation of TGF- β and its downstream Raf/MEK/ERK signaling pathway. The present results also confirm that activation of TGF- β 1 and its downstream Smad pathway is key to Cal-induced EMT.

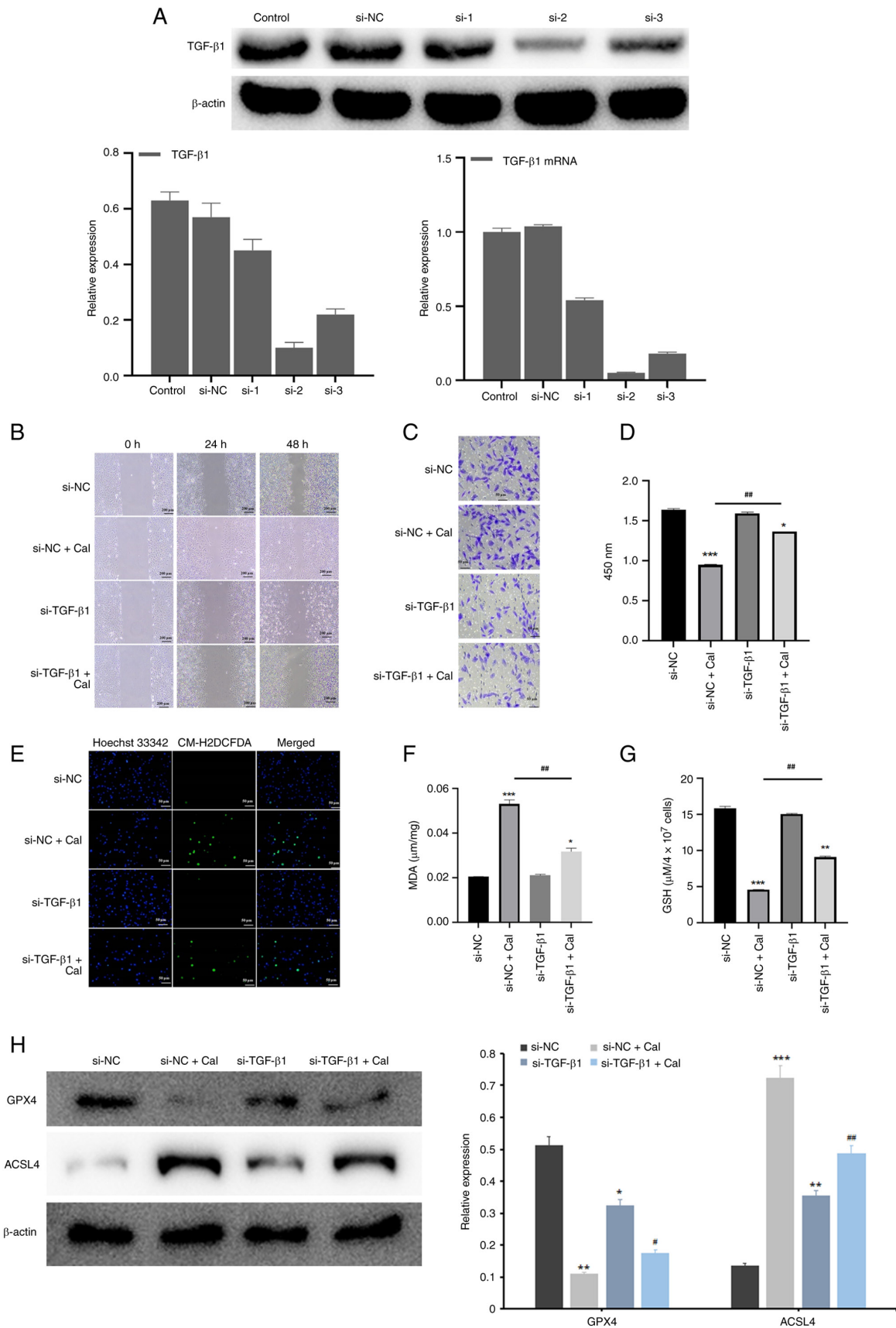


Figure 3. TGF-β1 knockdown attenuates Cal-induced epithelial-mesenchymal transition and ferroptosis. (A) Western blot showing efficient TGF-β1 knockdown in HeLa cells by siRNA. (B) Wound healing and (C) Transwell assays showing TGF-β1 knockdown reversed Cal-enhanced HeLa migration. (D) Cell Counting Kit-8 assay showing TGF-β1 knockdown partially restored Cal-reduced HeLa viability. (E) TGF-β1 knockdown attenuated Cal-induced reactive oxygen species elevation in HeLa cells. (F) TGF-β1 knockdown attenuated Cal-induced MDA elevation in HeLa cells. (G) TGF-β1 knockdown attenuated Cal-induced GSH reduction in HeLa cells. (H) Western blot showing TGF-β1 knockdown weakened Cal's regulation of GPX4/ACSL4. *P<0.05, **P<0.01, ***P<0.001 vs. si-NC; #P<0.05, ##P<0.01 vs. si-NC + Cal. Cal, calycosin; MDA, malondialdehyde; GSH, glutathione; ACSL4, acyl-CoA synthetase long-chain family member 4; GPX4, glutathione peroxidase; si/siRNA, small interfering RNA; NC, negative control.

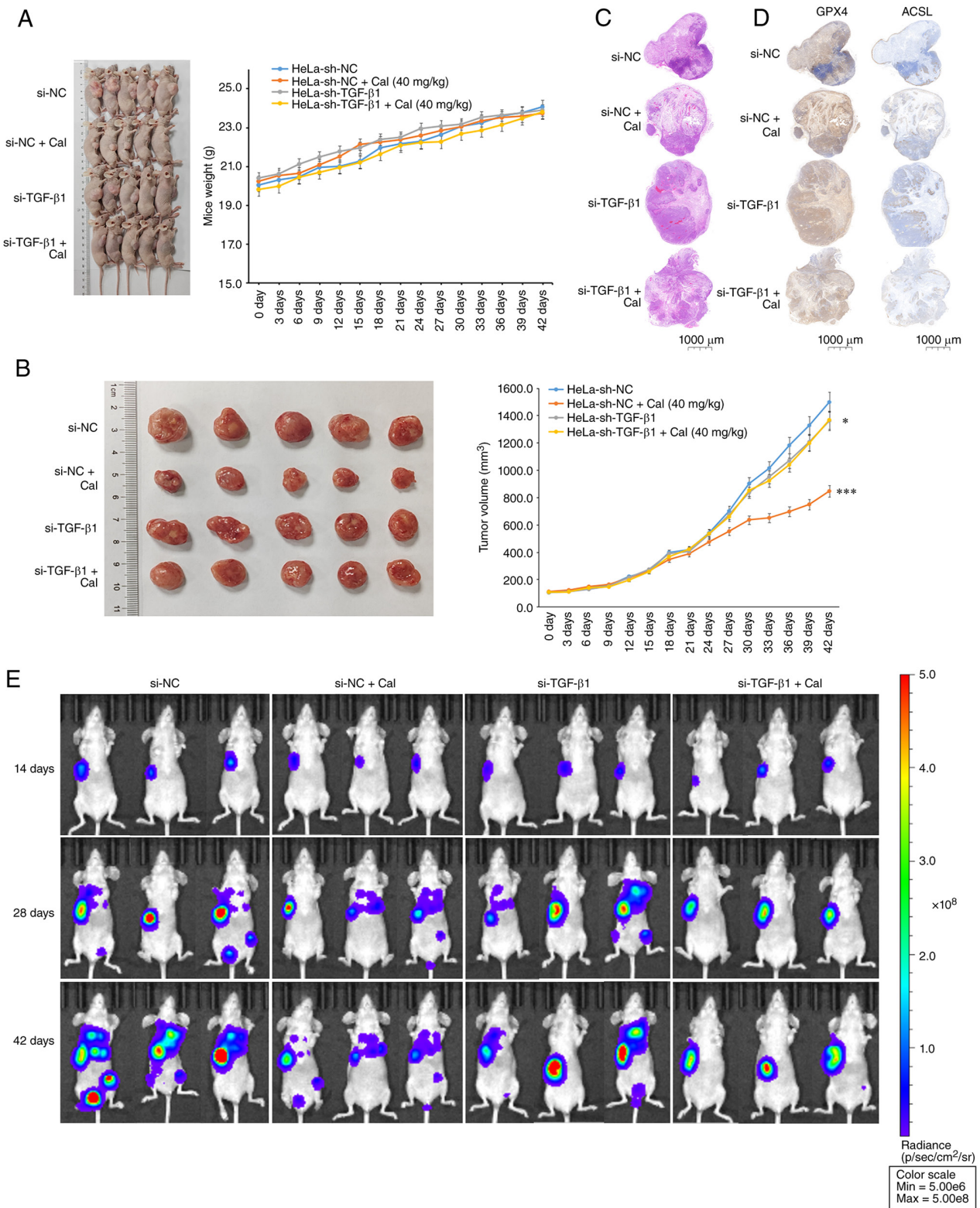


Figure 4. Cal enhances ferroptosis and promotes metastasis via TGF-β1-mediated epithelial-mesenchymal transition *in vivo*. (A) Body weight of nude mice: 40 mg/kg Cal showed no systemic toxicity. (B) Tumor volume measurements showing Cal inhibited sh-NC tumor growth, with diminished effect in sh-TGF-β1 tumors. (C) H&E staining showing severe necrosis in sh-NC + Cal tumors, and milder necrosis in sh-TGF-β1 + Cal group. (D) Immunohistochemistry staining showing Cal reduced GPX4 and increased ACSL4 in sh-NC tumors, with the effects weakened in sh-TGF-β1 tumors. (E) *In vivo* imaging showing Cal suppressed orthotopic fluorescence but enhanced distant metastatic signals in the sh-NC group; TGF-β1 knockdown abrogated these effects. n=4 per group. *P<0.05, ***P<0.001 vs. sh-NC. Cal, calycosin; ACSL4, acyl-CoA synthetase long-chain family member 4; GPX4, glutathione peroxidase; sh/shRNA, short hairpin RNA; NC, negative control; d, day.

Furthermore, Cal was found to inhibit the Hippo pathway, leading to increased nuclear translocation of YAP. YAP, as a

transcriptional co-activator, can bind to transcription factors such as transcriptional enhancer factor domain upon nuclear

entry, promoting the expression of mesenchymal phenotype-related genes (such as vimentin and connective tissue growth factor), thereby driving EMT (23). Extensive cross-talk exists between the Hippo and TGF- β /Smad pathways; for example, YAP can interact with Smad2/3 in the nucleus to synergistically promote the transcription of EMT-related genes (24,25). Therefore, Cal may synergistically amplify its pro-EMT effect by concurrently intervening in these two pathways.

The core innovative aspect of the present study lies in revealing the synergistic relationship between Cal-triggered EMT and ferroptosis. Recent research has presented conflicting views on the connection between EMT and ferroptosis susceptibility. Some studies indicate that cancer cells in a mesenchymal state (such as drug-resistant cells) are highly dependent on GPX4 to counteract oxidative stress and ferroptosis, thus being particularly sensitive to GPX4 inhibitors (26,27). This implies that inducing or being in an EMT state renders cancer cells less capable of resisting ferroptosis, making them easier to kill. The present experimental results support this notion: Cal itself acts both as an inducer of EMT and an inducer of ferroptosis. When the EMT program was turned off by knocking down TGF- β 1, the cells' sensitivity to Cal's pro-ferroptotic effect decreased. This suggests that in cervical cancer cells, Cal-induced EMT might remodel cellular metabolism and redox status, for instance, by increasing the synthesis of polyunsaturated fatty acids (consistent with the upregulation of ACSL4) or downregulating parts of the antioxidant system, pushing them into a state more prone to ferroptosis. At this point, Cal, by simultaneously triggering ferroptosis through mechanisms such as direct GPX4 inhibition, achieves a 'multiplied' effect. This model of 'first inducing a plastic state, then triggering a specific death' offers a new strategy for antitumor drug design. However, how EMT specifically regulates ferroptosis sensitivity at the molecular level, whether by altering membrane lipid composition, iron metabolism or antioxidant capacity, is a direction for future in-depth exploration.

The *in vivo* results further reinforce the clinical relevance of this discovery. Cal indeed inhibits orthotopic tumor growth, but its side effect of promoting metastasis is also notable. Knocking down TGF- β 1, while partially diminishing the antitumor effect, simultaneously reduced its pro-metastatic capacity, providing insights for optimizing potential therapeutic strategies. In the future, if Cal is considered for clinical application, it might be necessary to combine it with EMT inhibitors (such as TGF- β receptor kinase inhibitors) or utilize specific drug delivery systems for targeted delivery, aiming to avoid its pro-metastatic risk without affecting or even enhancing its antitumor efficacy.

The present study has some limitations. First, although the necessity of EMT for ferroptosis was demonstrated through TGF- β 1 knockdown experiments, the deeper molecular mechanisms underlying their interaction were not fully elucidated, such as whether a key 'node' molecule simultaneously regulates both EMT and ferroptosis. Second, the specific role of the Hippo/YAP pathway and its interactive mechanisms with the TGF- β pathway require further investigation, for example, by using YAP-specific inhibitors (such as verteporfin) for reverse validation. Finally, the present study was primarily based on

cell line-derived xenograft models; future validation in more clinically relevant patient-derived xenograft models or spontaneous tumor models, along with assessment of combination therapy effects, is needed.

In summary, the present study systematically elucidated the dual role of Cal in cervical cancer: It induces ferroptosis to suppress tumor growth while concurrently promoting EMT and metastasis through the TGF- β /Smad and Hippo/YAP pathways. Notably, Cal-induced EMT serves a synergistic role in sensitizing cancer cells to ferroptosis, as TGF- β 1 knockdown attenuates both EMT and ferroptotic responses. The present findings reveal the complex interplay between tumor cell plasticity and ferroptosis sensitivity and suggest that combining Cal with EMT inhibitors may represent a promising strategy to harness its antitumor potential while mitigating its pro-metastatic risks, though careful evaluation of its translational applicability is warranted.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LZ and WHW conceived and designed the present study. QS performed the cell culture, transfection, western blotting and biochemical assays and was a major contributor in acquiring and interpreting the experimental data. JYZ performed the software-based data analysis and statistical evaluations. PW validated the experimental results and performed the immunohistochemical and H&E staining analyses. JZ contributed to the formal analysis and interpretation of the data and provided the necessary resources for the study. JYZ and QS carried out the investigation, including the cell viability, colony formation, migration, invasion and animal experiments. PW curated the data. LZ prepared the original draft of the manuscript. WHW reviewed and edited the manuscript. WHW and JZ supervised the study. WHW administered the project. WHW acquired the funding. LZ and WHW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by Jiangsu Health Vocational College (approval no. JHVC-IACUC-2025-B0192) and was strictly compliant with all relevant national ethical regulations. Involved animal experiments were performed based on the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Khojaste E and Ahmadizadeh C: Catechin metabolites along with curcumin inhibit proliferation and induce apoptosis in cervical cancer cells by regulating VEGF expression in-vitro. *Nutr Cancer* 74: 1048-1057, 2022.
- Zhang J, Chen XW, Shu LS and Liu CD: The correlation and role analysis of SLC30A1 and SLC30A10 in cervical carcinoma. *J Cancer* 13: 1031-1047, 2022.
- Li X, Chen B, Huang A, Ren C, Wang L, Zhu T, Xiong J, Ding W and Wang H: LncRNA HCP5 enhances the proliferation and migration of cervical cancer via miR-216a-5p/CDC42 axis. *J Cancer* 13: 1882-1894, 2022.
- Surendiran A, Balamurugan N, Gunaseelan K, Akhtar S, Reddy KS and Adithan C: Adverse drug reaction profile of cisplatin-based chemotherapy regimen in a tertiary care hospital in India: An evaluative study. *Indian J Pharmacol* 42: 40-43, 2010.
- Sabt A, Kitsos S, Ebaid MS, Furlan V, Pantiora PD, Tsolka M, Elkaeed EB, Hamissa MF, Angelis N, Tsitsilonis OE, *et al*: Novel coumarin-6-sulfonamide-chalcone hybrids as glutathione transferase P1-1 inhibitors. *PLoS One* 19: e0306124, 2024.
- Zhang H and Zheng Y: LPCAT1 is transcriptionally regulated by FOXA1 to promote breast cancer progression and paclitaxel resistance. *Oncol Lett* 25: 134, 2023.
- Dixon SJ and Olzmann JA: The cell biology of ferroptosis. *Nat Rev Mol Cell Biol* 25: 424-442, 2024.
- Li Y, Zhang X, Wang Z, Li B and Zhu H: Modulation of redox homeostasis: A strategy to overcome cancer drug resistance. *Front Pharmacol* 14: 1156538, 2023.
- Chen T, Ding L, Zhao M, Song S, Hou J, Li X, Li M, Yin K, Li X and Wang Z: Recent advances in the potential effects of natural products from traditional Chinese medicine against respiratory diseases targeting ferroptosis. *Chin Med* 19: 49, 2024.
- Xu J, Zheng B, Wang W and Zhou S: Ferroptosis: A novel strategy to overcome chemoresistance in gynecological malignancies. *Front Cell Dev Biol* 12: 1417750, 2024.
- Yin S, Li Z and Ou WB: Ferroptosis: Mechanisms, comparison with cuproptosis and emerging horizons in therapeutics. *Oncol Res* 34: 8, 2025.
- Dalseno D, Gajic N, Flanagan L and Tait SWG: Cell death and cancer: Metabolic interconnections. *Cell Rep* 44: 115804, 2025.
- Allela OQB, Shareef A, Jyothi SR, Nayak PP, Janney JB, Singh G, Chauhan AS, Oripov F, Sameer HN, Yaseen A, *et al*: Engineering CAR T NK and NKT cell therapies to target cancer stem cells and overcome stem like resistance. *Discov Oncol* 17: 113, 2025.
- Gong D, Chen M, Wang Y, Shi J and Hou Y: Role of ferroptosis on tumor progression and immunotherapy. *Cell Death Discov* 8: 427, 2022.
- Naselli F, Bellavia D, Costa V, De Luca A, Raimondi L, Giavaresi G and Caradonna F: Osteoarthritis in the elderly population: Preclinical evidence of nutrigenomic activities of flavonoids. *Nutrients* 16: 112, 2023.
- Afroze N, Sundaram MK, Haque S and Hussain A: Long non-coding RNA involved in the carcinogenesis of human female cancer - a comprehensive review. *Discov Oncol* 16: 122, 2025.
- National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals: Guide for the Care and Use of Laboratory Animals. 8th edition. National Academies Press, Washington, DC, 2011.
- Wu Q, Bai Z, Xin M, Wang W, Chen Y, Zhai X, Chen J, Zhang C and Wang Y: Calycosin alleviates blood-brain barrier disruption after cerebral ischemia-reperfusion injury by inhibiting NLRP3-mediated pyroptosis. *Acta Biochim Biophys Sin (Shanghai)*: doi:10.3724/abbs.2025225.
- Liu Y, Piao XJ, Xu WT, Zhang Y, Zhang T, Xue H, Li YN, Zuo WB, Sun G, Fu ZR, *et al*: Calycosin induces mitochondrial-dependent apoptosis and cell cycle arrest, and inhibits cell migration through a ROS-mediated signaling pathway in HepG2 hepatocellular carcinoma cells. *Toxicol In Vitro* 70: 105052, 2021.
- Chen GQ, Benthani FA, Wu J, Liang D, Bian ZX and Jiang X: Artemisinin compounds sensitize cancer cells to ferroptosis by regulating iron homeostasis. *Cell Death Differ* 27: 242-254, 2020.
- Wang F, Liu Y, Wang L and Bai Y: Ginkgetin reverses cisplatin resistance in cervical cancer by regulating the Nrf2/HO-1 signaling pathway to induce ferroptosis. *Sci Rep* 16: 1542, 2025.
- Zhang Z, Auyeung KK, Sze SC, Zhang S, Yung KK and Ko JK: The dual roles of calycosin in growth inhibition and metastatic progression during pancreatic cancer development: A 'TGF- β paradox'. *Phytomedicine* 68: 153177, 2020.
- Zhou T, Li X, Liu J and Hao J: The Hippo/YAP signaling pathway: the driver of cancer metastasis. *Cancer Biol Med* 20: 483-489, 2023.
- Taiyab A, Belahlou Y, Wong V, Pandi S, Shekhar M, Chidambaranathan GP and West-Mays J: Understanding the role of yes-associated protein (YAP) signaling in the transformation of lens epithelial cells (EMT) and fibrosis. *Biomolecules* 13: 1767, 2023.
- Wang N, Wang Y, Zhang L, Yang W and Fu S: Molecular mechanisms of epithelial-mesenchymal transition in retinal pigment epithelial cells: Implications for age-related macular degeneration (AMD) progression. *Biomolecules* 15: 771, 2025.
- Kram H, Prokop G, Haller B, Gempt J, Wu Y, Schmidt-Graf F, Schlegel J, Conrad M and Liesche-Starnecker F: Glioblastoma relapses show increased markers of vulnerability to ferroptosis. *Front Oncol* 12: 841418, 2022.
- Sun Y, Xue Z, Huang T, Che X and Wu G: Lipid metabolism in ferroptosis and ferroptosis-based cancer therapy. *Front Oncol* 12: 941618, 2022.



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