



Folic acid-conjugated hydroxyapatite nanoparticles induce 4T1 cell apoptosis via multiple pathways to potentiate breast cancer inhibition: a mechanistic study

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Received: 30 September 2025 / Accepted: 17 January 2026 / Published online: 12 September 2026
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Abstract

An increasing number of nanomaterials are being applied in adjuvant tumor therapy, and prior studies have reported that inorganic nanoparticles can exert tumor-suppressive effects. Among these materials, hydroxyapatite (HA) nanoparticles have garnered substantial attention owing to their strong biocompatibility and inhibitory activity against tumor cell proliferation. However, insufficient cancer cell targeting of HA nanoparticles limits their antitumor efficacy. In this study, we developed folic acid (FA)-conjugated HA nanoparticles (HF nanoparticles) that enhance HA-mediated tumor inhibition by binding to overexpressed FA receptors on cancer cell membranes and exploiting the elevated FA demand in cancer metabolism. In *in vivo* experiments, HF nanoparticles significantly inhibited tumor growth compared with HA nanoparticles alone, and the former exhibited a synergistic antitumor effect with doxorubicin. Subsequent *in vitro* mechanistic analyses revealed that tumor-targeted HF nanoparticles induced intracellular calcium overload in 4T1 cells, causing calcium homeostasis imbalance, which disrupted mitochondrial membrane integrity, promoted cytochrome c release, and increased reactive oxygen species accumulation, ultimately activating apoptotic signaling and accelerating apoptosis. Additionally, these exogenous nanoparticles remodeled the immune landscape of the tumor microenvironment: HF nanoparticles promoted M1 macrophage polarization and elevated tumor necrosis factor- α expression, interfering with cancer cell cycle progression and division while activating caspase-related apoptotic genes, further intensifying apoptosis. Overall, this study demonstrated that HF nanoparticles can be engineered as highly targeted antitumor agents and drug delivery carriers, providing nanobiomaterials for more efficient tumor therapy.

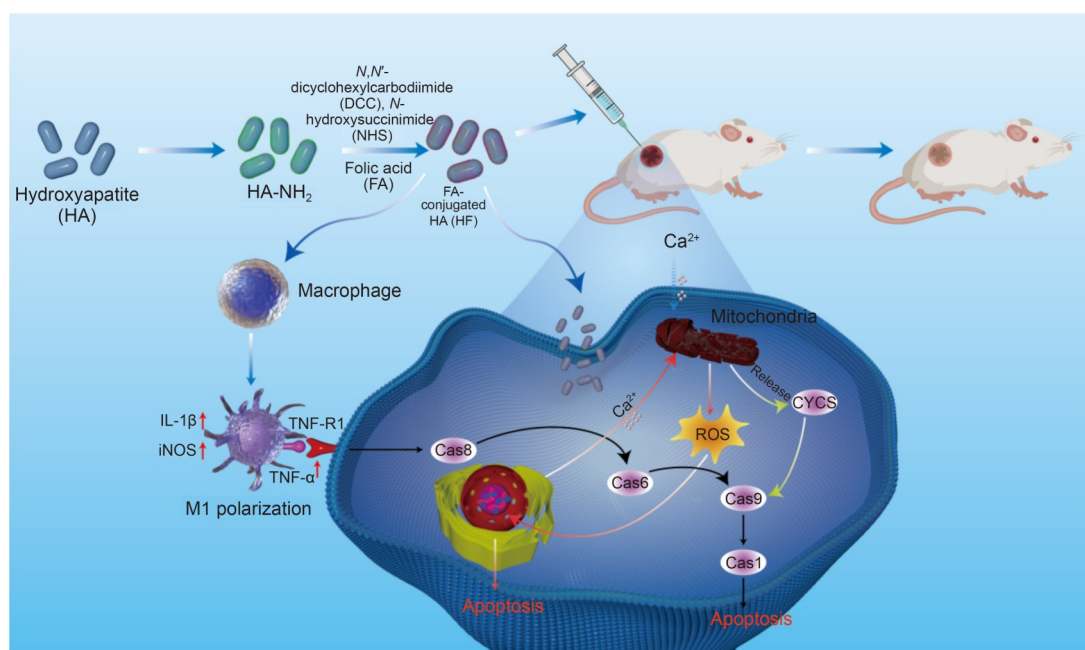
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Graphical abstract



Keywords Hydroxyapatite · Folic acid · Cell apoptosis · Calcium signaling · Inflammatory remodeling

1 Introduction

Breast cancer, one of the most serious threats to human health, poses major challenges due to its complex characteristics, including diverse oncogenic mechanisms, strong metastatic potential, and a high risk of recurrence. The standard treatment strategy for breast cancer primarily involves surgical resection combined with adjuvant radiotherapy or chemotherapy. Although these interventions are effective to some extent, they are associated with numerous severe side effects that substantially reduce patients' quality of life during therapy [1, 2]. In this context, nanomedicine has emerged as a highly promising alternative for cancer treatment. Nanomaterials exhibit dual functionality: they can display intrinsic antitumor activity and serve as efficient carriers for anticancer drugs. Researchers have actively explored the development of nanoscale biomaterials for antitumor applications by exploiting their favorable biocompatibility, which limits damage to healthy organs, and their capacity for drug delivery. In recent years, numerous studies have reported the antitumor activities of diverse nanoparticles, including polymers, metal nanoparticles, carbon-based materials, and biomass derivatives [3–8]. Furthermore, inorganic nanoparticles have attracted increasing attention, particularly in tumor diagnosis and treatment [9, 10]. The antitumor effects of various inorganic nanoparticles have been extensively investigated, with researchers seeking to optimize their

performance as drug delivery carriers while enhancing therapeutic efficacy. However, the nonnegligible side effects associated with these nanomaterials have restricted their clinical translation and widespread application. Consequently, identifying nanomaterials with superior biocompatibility has become a major priority, and systems that combine drug delivery and intrinsic antitumor activity remain a central research focus.

Hydroxyapatite (HA), with the chemical formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, is the principal inorganic component of natural human bone. Owing to its exceptional biocompatibility and tissue inductivity, HA has been widely applied in tissue engineering [9, 11, 12]. In recent years, it has also garnered attention in the antitumor field because of its favorable biocompatibility and distinctive nanoscale effects. Multiple studies have shown that HA nanoparticles can effectively inhibit the growth of various cancer cell types, including breast cancer, malignant melanoma, colon cancer, osteosarcoma, and gastric cancer cells, by inducing apoptosis, suppressing proliferation, and disrupting cellular functions while exhibiting minimal toxicity toward normal cells [12–14]. Several reports have suggested that tumor cell-mediated endocytosis of HA nanoparticles and subsequent oxidative stress induction play key roles in these antitumor effects [15]. Nevertheless, under physiological conditions, the inhibitory activity of HA nanoparticles against solid tumors remains limited, largely because of poor tumor-targeting capability

and interference from the complex immune microenvironment. Therefore, improving the tumor-targeting specificity of HA nanoparticles and clarifying their underlying antitumor mechanisms are urgent research objectives.

Folic acid (FA), an essential vitamin for human metabolism, has attracted considerable interest in cancer research. Overexpressed high-affinity FA receptors on tumor cells allow specific selective FA binding. Additionally, emerging evidence indicates that FA participates in remodeling the immune microenvironment during antitumor responses, such as by regulating macrophage apoptosis and polarization [16]. Based on these characteristics, FA represents an attractive candidate for modifying nanomaterials to improve tumor-targeting efficiency.

In this study, we present a novel strategy for applying HA nanoparticles in antitumor therapy by modifying their surface with FA to generate FA-conjugated HA (HA-FA, hereafter HF) nanoparticles. We successfully synthesized both unmodified HA and HF nanoparticles and systematically assessed their drug-loading and delivery capabilities, as well as their effects on 4T1 breast cancer cells. Our analyses encompassed multiple parameters, including oxidative stress responses, cell proliferation inhibition, and morphological and functional changes, including cell cycle alterations, apoptosis induction, and calcium ion (Ca^{2+}) regulation. We also explored the *in vivo* antitumor efficacy of HF nanoparticles. Moreover, we employed mRNA expression profile microarray analysis to identify mechanisms underlying the enhanced antitumor activity of HF nanoparticles. Gene-level evidence confirmed activation of the caspase-related signaling pathway, thereby clarifying the mechanism of HF nanoparticle-induced apoptosis. We also identified activation of host immune responses closely associated with the improved antitumor performance of HF nanoparticles and demonstrated that these nanoparticles could regulate the immune microenvironment toward a pro-inflammatory state, further strengthening their antitumor efficacy. Collectively, these results highlight the dual-functional advantage of HF nanoparticles: they not only enhance tumor-targeting accuracy but also reshape the immune microenvironment, providing a more effective strategy for eradicating tumor cells.

2 Materials and methods

2.1 Chemicals

Chemicals, including *N*-hydroxysuccinimide (NHS), FA, 3-aminopropyltriethoxysilane, ammonium hydroxide ($\text{NH}_3 \cdot \text{H}_2\text{O}$), calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$), diammonium phosphate ($(\text{NH}_4)_2\text{HPO}_4$), paraformaldehyde (PFA), ethanol, and dimethyl sulfoxide (DMSO), were

purchased from Sinopharm Group Co., Ltd., China. The following reagents and kits were also employed: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT; Beyotime Biotechnology Co., Ltd., China), Dulbecco's modified Eagle medium (DMEM; Gibco, USA), trypsin (Gibco), doxorubicin hydrochloride (DOX·HCl, Sangon Biotechnology, China), fetal bovine serum (FBS; Gibco), phosphate-buffered saline (PBS; Sangon Biotechnology), penicillin–streptomycin (P-SC; Sangon Biotechnology), 4',6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology Co., Ltd.), fluorescein diacetate (FDA; Yuanye Biotechnology, China), crystal violet (Solarbio, China), the cell cycle analysis kit (DAPI-based; Beyotime Biotechnology Co., Ltd.), and the cell apoptosis analysis kit (Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI); Beyotime Biotechnology Co., Ltd.).

2.2 Study design

This study examined the enhancement of HA nanoparticle-based antitumor efficacy through the induction of favorable immune responses in the tumor microenvironment (TME). HF nanoparticles were fabricated to improve tumor cell targeting. *In vivo* experiments were performed using a 4T1 murine breast cancer model ($n=5$ per group), in which HA and HF nanoparticle solutions were intratumorally injected to assess antitumor efficacy. Tumor volume and body weight were recorded every 2 d. After 16 d of treatment, mice were euthanized, and tumor tissues were collected for histological and immunohistochemical analyses.

To elucidate underlying mechanisms, *in vitro* studies were performed at the cellular level, including assessments of nanoparticle effects on 4T1 cell viability, proliferation, morphology, and migration, with drug DOX-loaded HA and HF (HD and HFD, respectively) nanoparticle groups incorporated for comparison. Key endpoints included nanoparticle-induced organelle damage, intracellular calcium homeostasis disruption, reactive oxygen species (ROS) generation, cell cycle arrest, and apoptosis. DOX, a clinically approved anticancer drug, was applied to evaluate nanoparticle drug-loading capacity, release kinetics, and synergistic antitumor effects with HF nanoparticles.

The correlation between nanoparticle-induced immune responses and antitumor efficacy was also explored. Macrophages were cocultured with HA or HF nanoparticles ($n=3$) to simulate the material-modulated immune microenvironment. The effects of immune-polarized macrophages on 4T1 cell viability were systematically evaluated, together with RNA sequencing to identify gene expression changes associated with apoptosis and immune activation. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was performed to validate key genes

and signaling pathways. All experiments were repeated ≥ 3 times to ensure reproducibility.

2.3 HA and HF nanoparticle synthesis

HA nanoparticles were synthesized using a wet chemical approach. Briefly, a 0.2 mol/L $(\text{NH}_4)_2\text{HPO}_4$ solution was added dropwise to a 0.2 mol/L $\text{Ca}(\text{NO}_3)_2$ solution at a Ca/P molar ratio of 1.67 under vigorous stirring. Ammonia solution (25% by volume) was added to maintain the reaction pH at approximately 10. The mixture was stirred for 4 h, kept at room temperature overnight, and subsequently centrifuged (8000 r/min for 10 min) before three washes with deionized water to remove residual ammonia. The precipitate then underwent hydrothermal treatment at 180 °C for 3 h in a Teflon-lined autoclave. After cooling, the product was collected, dried at 60 °C overnight, and milled through a 200-mesh sieve to obtain HA nanoparticles.

For HF nanoparticle synthesis, HA powder was dispersed in a water–ethanol mixture (1:7 by volume) at a solid-to-liquid ratio of 1:40 (g/mL). The suspension was heated to 60 °C and stirred for 8 h to facilitate surface hydration. The precipitated HA was sequentially washed three times with ultrapure water and anhydrous ethanol, followed by drying at 60 °C to yield amino-functionalized HA (HA-NH₂). Separately, *N,N'*-dicyclohexylcarbodiimide (DCC) and NHS were dissolved in DMSO at a molar ratio of 1:1. FA was introduced into the solution and reacted in the dark for 24 h to activate its carboxyl groups. The resulting FA-DCC-NHS conjugate was then mixed with HA-NH₂ under stirring for 12 h at room temperature. The final precipitate was collected via centrifugation (12,000 r/min for 15 min), washed three times with deionized water, and lyophilized to obtain HF nanoparticles.

2.4 Characterization

An X-ray diffractometer (XRD; Philips X'Pert 1, Philips, the Netherlands), a Fourier transform infrared spectroscope (FTIR; Nicolet IS 50/6700, Thermo Scientific, USA), and an X-ray photoelectron spectroscope (XPS; ESCALAB 250Xi, Thermo Scientific) were employed to analyze nanoparticle composition and crystal phase. Size distribution and zeta potential were measured using a laser particle analyzer (90Plus PALS, Brookhaven Instruments Corporation, USA). Morphology was examined using a transmission electron microscope (TEM; Talos F200X, FEI, USA) after dispersing nanoparticles in ethanol.

2.5 Drug loading and release

A 1 mg/mL DOX working solution was prepared, and HA and HF nanoparticles (20 mg of each) were transferred into

1.5-mL centrifuge tubes containing 1 mL of DOX solution. The tubes were incubated overnight at 37 °C with gentle shaking. The following day, samples were centrifuged at 10,000g for 15 min, and the precipitates were collected as drug-loaded materials, denoted HA-DOX and HF-DOX. The absorbance of the remaining supernatants was measured at 480 nm. Residual DOX concentrations were calculated using a pre-established DOX standard curve at 480 nm. Drug-loading efficiency (η_{DL}) was calculated as Eq. (1):

$$\eta_{\text{DL}} = [(m_{\text{total-DOX}} - m_{\text{free-DOX}}) / m_{\text{total-DOX}}] \times 100\%, \quad (1)$$

where $m_{\text{total-DOX}}$ denotes the mass of total DOX and $m_{\text{free-DOX}}$ denotes the mass of free DOX.

For drug release studies, two pH conditions (pH 5.5 and 7.4) were used to simulate the acidic TME and normal physiological environment, respectively. Each drug-loaded sample was placed into a centrifuge tube containing 2 mL of buffer at the designated pH. The tubes were incubated in a thermostatic shaker at 37 °C with agitation at 150 r/min. At predetermined time points, samples were centrifuged, and the entire supernatant was collected for absorbance measurement at 480 nm using an ultraviolet–visible (UV–Vis) spectrophotometer. Released DOX was quantified based on the standard curve. Additionally, 2 mL of fresh buffer was added to the centrifuged pellet, and incubation was continued in the shaker. Cumulative drug release at each time point was calculated accordingly. All experiments were performed with five biological replicates per group.

2.6 Cell lines

Murine breast cancer 4T1 cells (obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were used for in vivo experiments (establishment of a 4T1 xenograft tumor model in mice) and in vitro assays (assessment of nanoparticle effects on tumor cell viability and apoptosis). Cells were cultured in DMEM supplemented with 10% FBS and 1% P-SC, with routine passaging every 72 h. Additionally, mouse macrophages (RAW 264.7, obtained from the same source) were employed to investigate material-induced immune responses and related antitumor mechanisms. These cells were maintained under the same culture conditions (DMEM+10% FBS+1% P-SC). All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

2.7 In vitro evaluation of nanoparticle effects on 4T1 cells

2.7.1 Cytotoxicity evaluation

After seeding 4T1 cells in 96-well microplates at 1×10^4 cells/well, they were exposed to various nanoparticle

concentrations (0, 50, 100, 200, and 400 µg/mL). Following incubation for 1, 3, and 5 d, cell viability and proliferation were evaluated using the MTT assay. Optical density (OD) values at 570 nm were normalized to untreated controls to determine relative cell viability at each time point.

2.7.2 Cell morphology observation

Based on the cytotoxicity results, nanoparticles at 200 µg/mL were selected for subsequent experiments. Experimental groups included HD, HFD, blank HA, and HF nanoparticles, as well as a DOX solution positive control and an untreated control.

For nuclear morphology analysis, 4T1 cells were seeded in 24-well plates containing glass coverslips at 1×10^4 cells/well and treated with experimental formulations for 1, 3, and 5 d. Following treatment, cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and stained with DAPI. Nuclear morphology was visualized using a confocal laser scanning microscope (CLSM; Leica, Germany).

Live cell imaging was conducted using FDA staining. At each time point, cells were incubated with FDA (10 µg/mL) for 15 min at 37 °C, washed with PBS, and immediately imaged using a fluorescence microscope (Observer 3, Zeiss, Germany).

For crystal violet staining, cells cultured on coverslips were fixed with 4% PFA, stained with 0.5% crystal violet solution for 15 min, rinsed thoroughly with water, and imaged using an optical microscope (Eclipse 80i, Nikon, Japan).

Scanning electron microscopy (SEM) was used for high-resolution morphological characterization. Cells were fixed with 4% PFA, dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%; 15 min per step), processed via critical point drying, sputter-coated with gold, and imaged using an SEM (TM4000 PLUS, Hitachi, Japan).

2.7.3 Cell motility

Cell motility was evaluated using a scratch wound healing assay. First, 4T1 cells were seeded into 6-well plates at 5×10^4 cells/well and cultured to approximately 90% confluence. After overnight serum starvation, uniform scratches were created across the monolayer using a sterile 200 µL pipette tip. Detached cells and debris were removed by gently washing with PBS three times. Cells were then treated with complete medium containing nanoparticles (500 µg/mL). Wound closure was imaged at 0-, 12-, and 24-h post-scratching using an inverted phase-contrast microscope. Scratch areas were quantified using ImageJ, and relative migration rates (γ_M) were calculated as Eq. (2):

$$\gamma_M = [(A_0 - A_t) / A_0] \times 100\%, \quad (2)$$

where A_0 and A_t denote the scratch area at 0 h and time t , respectively.

2.8 Effects of nanoparticles on 4T1 cell organelles

2.8.1 Cytoskeleton, mitochondria, and lysosome staining

After 72 h of incubation with nanoparticles, 4T1 cells were fixed with 4% PFA. Cytoskeletons were stained using tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin (Beyotime Biotechnology Co., Ltd.) following the manufacturer's instructions and visualized using a CLSM.

For mitochondrial imaging, cells were treated with nanoparticles for 24 h, rinsed with prewarmed PBS, and incubated with MitoTracker Green FM (Beyotime Biotechnology Co., Ltd.) for 60 min at 37 °C. Nuclei were counterstained with Hoechst 33342 (BBI Life Sciences, China) for 30 min before imaging.

Lysosomal staining was performed by incubating treated cells with LysoTracker Red (Beyotime Biotechnology Co., Ltd.) for 60 min, followed by Hoechst nuclear counterstaining. All samples were imaged using a CLSM system (Leica) under identical acquisition settings.

2.8.2 Intracellular ROS

Intracellular ROS production, including hydroperoxide and superoxide anion, was assessed using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime Biotechnology Co., Ltd.). For CLSM analysis, 4T1 cells were seeded at 2×10^4 cells/well in confocal dishes and treated with nanoparticles for 48 h. Cells were then incubated with 10 µmol/L DCFH-DA in serum-free medium at 37 °C for 30 min, washed three times with PBS to remove excess dye, and immediately imaged using a CLSM (excitation: 488 nm; emission: 525 nm; TCS SP8, Leica).

For flow cytometry (FCM) quantification, cells were seeded at 2×10^5 cells/well in 6-well plates and treated under the same conditions. Following treatment, cells were trypsinized, washed twice with cold PBS, and resuspended in 500 µL of PBS containing 10 µmol/L DCFH-DA. After a 30-min incubation at 37 °C, samples were analyzed using a flow cytometer. At least 10,000 events per sample were acquired, and mean fluorescence intensity was calculated using FlowJo (Celesta, BD, USA). Untreated cells and Rosup-treated cells (positive control) were included in each experiment.

2.8.3 Cellular Ca^{2+} measurement

For intracellular Ca^{2+} detection, 4T1 cells were seeded in 6-well plates at 2×10^5 cells/well and incubated with nanoparticles for 48 h. Cells were then harvested using 0.25% trypsin, washed twice with ice-cold PBS, and resuspended in loading buffer containing 5 µmol/L Fluo-3 AM (Beyotime

Biotechnology Co., Ltd.). Following a 30-min incubation at 37 °C in the dark, cells were washed twice with PBS to remove excess dye, resuspended in PBS, and immediately analyzed via FCM. Fluorescence intensity was recorded at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. A minimum of 10,000 events per sample were acquired, and data were processed using FlowJo. Untreated cells were included as controls in each experiment.

2.8.4 Cell apoptosis

Cell apoptosis was assessed using a double-staining kit (Annexin V-FITC/PI, Beyotime Biotechnology Co., Ltd.) according to the manufacturer's protocol. Briefly, cells were seeded in 6-well culture plates at a density of 2×10^5 cells/well and incubated with nanoparticles for 48 h. Following incubation, cells were harvested, washed twice with PBS, and resuspended in $1 \times$ binding buffer containing 5 μ L of Annexin V-FITC and 5 μ L of PI. The cell suspension was gently vortexed, incubated for 20 min at room temperature in the dark, diluted with $1 \times$ binding buffer to a final volume of 500 μ L, and analyzed via FCM to quantify early and late apoptotic populations. Data were analyzed using FlowJo to determine apoptotic rates.

2.8.5 Cytochrome c (CYCS) detection in 4T1 cells

To clarify the mechanism of nanoparticle-induced apoptosis in 4T1 cells, CYCS expression levels in the cytoplasm and mitochondria were quantified using enzyme-linked immunosorbent assay (ELISA). Briefly, 4T1 cells were seeded into culture plates at 2×10^5 cells/well and treated with HA and HF nanoparticles, whereas control cells were cultured in fresh DMEM. After 24 h of incubation, cells were harvested and washed twice with cold PBS. For subcellular fractionation, cells were resuspended in 500 μ L of pre-cooled cytoplasmic lysis buffer (Mitochondrial Isolation Kit, Beyotime Biotechnology Co., Ltd.), incubated on ice for 10 min to disrupt the plasma membrane, and homogenized 10 times using a micropipette. The lysate was centrifuged at 600g for 10 min at 4 °C, and the supernatant (cytoplasmic fraction) was transferred to a new tube as the cytoplasmic sample. The pellet was resuspended in 500 μ L of mitochondrial isolation reagent, lysed on ice for 15 min to rupture mitochondrial membranes, and centrifuged at 12,000g for 15 min at 4 °C. The resulting supernatant (mitochondrial fraction) was collected as the mitochondrial sample. Total protein concentrations in the cytoplasmic and mitochondrial fractions were measured using a BCA Protein Assay Kit (P0012S; Beyotime Biotechnology Co., Ltd.), and CYCS levels were determined using a Rat/Mouse Cytochrome c ELISA Kit (890020, R&D Systems, Minneapolis, USA). Finally, CYCS expression was normalized to

total protein content and reported as CYCS content per milligram of total protein.

2.9 Impact of nanoparticle-mediated immunity on tumor-killing ability

2.9.1 Coculture of RAW 264.7 macrophages with nanoparticles

RAW 264.7 macrophages were seeded in 24-well culture plates to assess the biocompatibility of various nanoparticles via coculture assays. Cell proliferation and metabolic activity were measured using the MTT assay, whereas morphology and cytoskeletal alterations were examined using CLSM and SEM (as described above). To further assess immunomodulatory effects, RT-qPCR was performed to determine the expression levels of proinflammatory factors, including tumor necrosis factor- α (*TNF- α*) and interleukin-1 beta (*IL-1 β*), as well as inducible nitric oxide synthase (*iNOS*), a phenotypic marker of M1 macrophages. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as the housekeeping gene for normalization. Primer sequences are listed in Table S1 (supplementary information).

2.9.2 Conditioned medium (CM) preparation and inflammatory cytokine evaluation

To evaluate macrophage-mediated immunomodulation of nanoparticle tumor-killing efficacy, CM was prepared from RAW 264.7 macrophages cocultured with various nanoparticles and subsequently applied to 4T1 breast cancer cells. Briefly, RAW 264.7 macrophages were seeded in 6-well plates and incubated for 72 h in DMEM supplemented with 10% FBS and 1% P-SC, either alone (control) or with nanoparticles. After incubation, supernatants were collected, centrifuged at 1000g for 10 min to remove cellular debris, and filtered through a 0.22- μ m membrane. The resulting CMs were defined as follows: control medium without nanoparticles (CM-Con), medium from HA nanoparticle-treated macrophages (CM-HA), and medium from HF nanoparticle-treated macrophages (CM-HF). All CMs were stored at -80 °C until use in subsequent 4T1 cell experiments.

2.9.3 Effects of macrophage-derived CMs on 4T1 cell behavior

The TME comprises complex interactions involving direct cell–cell contact and indirect paracrine signaling (e.g., cytokines, chemokines, or growth factors secreted by macrophages regulating tumor cell proliferation, apoptosis, or invasion). Macrophage-derived CMs were used to specifically evaluate the influence of HF nanoparticles on 4T1 cell behavior mediated through macrophage paracrine pathways.

Initially, 4T1 cells were seeded at appropriate densities, followed by culture with CM-Con, CM-HA, and CM-HF. As a control group, 4T1 cells were maintained in fresh DMEM. Cell proliferation in each group was quantitatively measured using the MTT assay following the manufacturer's protocol.

Live cell visualization 4T1 cells were seeded on glass slides and cultured with various CMs. After the designated incubation period, cells were stained with FDA to label viable cells and imaged via fluorescence microscopy to assess cell viability and morphological characteristics.

Cell morphology To evaluate morphological alterations, 4T1 cells cultured with CMs were prepared for SEM observation following the standard procedure described in Sect. 2.7.2. This enabled detailed analysis of surface structures and overall morphology.

Cytoskeleton organization To visualize the cytoskeleton of 4T1 cells, CM-treated cells were fixed and stained with TRITC-conjugated phalloidin, which selectively binds filamentous actin. Stained cells were then imaged using a CLSM to obtain high-resolution images of cytoskeletal architecture, providing insight into CM-induced structural changes.

2.9.4 Effects of macrophage-derived CMs on 4T1 cell cycle and apoptosis

For apoptosis analysis, cells were stained with Annexin V-FITC and PI to distinguish viable (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+/PI+), and necrotic (Annexin V-/PI+) populations. Cell cycle distribution was determined via PI staining of DNA content, enabling quantification of cells in the G0/G1, S, and G2/M phases. FCM was performed, and data were processed using FlowJo. Experimental procedures followed the methods described in Sect. 2.8.4.

To examine the paracrine effects of RAW 264.7 macrophages on 4T1 cell cycle progression and apoptosis, 4T1 cells were cultured with CM-Con, CM-HA, or CM-HF, as described in Section 2.9.3. Following a 48-h incubation, cells were harvested and analyzed via FCM using the Annexin V-FITC/PI Apoptosis Detection Kit and the Cell Cycle Analysis Kit (Beyotime Biotechnology Co., Ltd.) following the manufacturer's protocols.

2.9.5 Effects of macrophage-derived CMs on 4T1 cell metabolism

The influence of macrophage-derived CMs on intracellular Ca^{2+} levels and ROS production in 4T1 cells was evaluated after 48 h of incubation. Experimental procedures were conducted according to the methods described in Sect. 2.8.2 and Sect. 2.8.3.

The expression levels of apoptosis-related genes in 4T1 cells were quantified using RT-qPCR following a 72-h treatment with CMs. Target genes included caspase family members (e.g., *caspase-1*, -3, -8, and -9). Primer sequences used for RT-qPCR are listed in Table S1 (supplementary information).

2.10 In vivo evaluation of nanoparticle antitumor effects

2.10.1 Experimental animals

All animal experiments were performed in strict accordance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee of Northwestern Polytechnical University, China (ethical approval code: 202301108). Female BALB/c mice (6 weeks old; 20–25 g) were obtained from the Fourth Military Medical University, China. Upon arrival, mice were acclimated to the laboratory environment for one week before any surgical procedures. This acclimation period was intended to reduce stress-related variability and ensure physiological stability before experimental interventions.

2.10.2 Tumor model construction and nanoparticle treatment

All surgical procedures were performed under general anesthesia. To establish the subcutaneous tumor model, mice were placed in the prone position, and the injection site was shaved and sterilized. A 4T1 cell suspension ($5 \times 10^6 \text{ mL}^{-1}$) was prepared in PBS and mixed at a 1:1 ratio (by volume) with type I collagen. In total, 150 μL of the mixture was injected subcutaneously into the dorsal region of each mouse. Following tumor establishment, 30 tumor-bearing BALB/c mice were randomly divided into six treatment groups ($n=5$ per group): control, untreated; HA, intratumoral injection of HA nanoparticles; HF, intratumoral injection of HF nanoparticles; HAD, intratumoral injection of DOX-loaded HA nanoparticles; HFD, intratumoral injection of DOX-loaded HF nanoparticles; and DOX, intratumoral injection of free DOX (positive control).

Nanoparticles were suspended in 35 μL of Matrigel matrix and administered via intratumoral injection every 2 d. Body weight and tumor dimensions were measured every other day. Tumor volume (V) was calculated using Eq. (3):

$$V = \frac{1}{2} \times a \times b^2, \quad (3)$$

where a and b represent the longest and shortest tumor diameters, respectively. After a two-week treatment period, mice were euthanized at Day 16, and tumor tissues were collected for further analyses.

2.10.3 Histological analysis

To assess nanoparticle antitumor efficacy, tumor tissues were collected immediately after euthanasia and fixed in 4% PFA. Samples were then paraffin-embedded, and tissue sections were prepared for hematoxylin and eosin (H&E) staining, immunohistochemical staining, and immunofluorescence staining.

For immunohistochemical staining, tissue sections were first subjected to antigen retrieval through immersion in an ethylenediaminetetraacetic acid solution. Slides were then treated with 3% hydrogen peroxide to quench endogenous peroxidase activity, followed by blocking with 5% bovine serum albumin to reduce nonspecific binding. Sections were incubated overnight at 4 °C with primary antibodies against Ki67. After washing, sections were incubated with corresponding secondary antibodies. Digital images of stained sections were captured using a slide scanner.

For immunofluorescence staining, primary antibodies conjugated with fluorescent dyes against cluster of differentiation 206 (CD206; FITC-conjugated), iNOS (yellow fluorescent protein (YFP)-conjugated), and F4-80 (rhodamine-conjugated) were used to visualize macrophages and their polarization phenotypes in the TME. Cell nuclei were counterstained with DAPI. Fluorescent images were acquired using a fluorescence microscope equipped with appropriate filters and further processed using image analysis software.

2.11 RNA sequence analysis

To further investigate the mechanism underlying the enhanced antitumor efficacy of HF nanoparticles, RNA sequencing was performed to enable comprehensive genomic analysis. Tumor tissues from the HF nanoparticle-treated and control groups were washed with PBS, rapidly frozen in liquid nitrogen, and disrupted using a tissue homogenizer. Total RNA and protein were extracted using TRIzolTM reagent (Sangon Biotechnology) for subsequent sequence analysis. RNA purity and concentration were determined using a spectrophotometer (Nanodrop 2000, Thermo Fisher).

Functional enrichment analyses were performed, including Gene Ontology (GO) analysis to delineate gene regulatory networks and functional categories ($p < 0.05$), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to identify significantly enriched pathways ($p < 0.05$) among differentially expressed genes (DEGs), and protein–protein interaction network analysis to identify functional protein complexes and interaction hubs using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database with visualization via multiple interface string alignment (MISA). The DEGseq algorithm was applied to detect significant transcriptional changes, with

thresholds of $p < 0.05$ and absolute fold-change ≥ 2 . These criteria ensured reliable identification of biologically meaningful expression differences. This integrated strategy enabled systematic characterization of HF nanoparticle-modulated genomic profiles and provided mechanistic insight into its enhanced antitumor activity.

3 Results and discussion

3.1 Physicochemical property characterization

In this study, both HA and HF nanoparticles were used to investigate whether the superior targeting ability of the latter could enhance antitumor efficacy. Particle size distribution analysis (Fig. 1b) showed that most HA nanoparticles were distributed in the 50–70 nm range, whereas HF nanoparticles were mainly distributed around 200 nm. TEM images (Fig. 1c) demonstrated that the average lengths of HA and HF nanoparticles were (62.53 ± 6.21) and (81.49 ± 16.87) nm, respectively, with corresponding widths of (13.3 ± 2.3) and (14.2 ± 1.7) nm, based on quantitative analysis, and both samples exhibited a rod-like morphology.

To determine the crystal phase compositions of HA and HF nanoparticles, XRD analysis was performed. As shown in Fig. 1d, all characteristic diffraction peaks of synthesized HA closely matched the standard HA pattern (JCPDS No. 09-0432) and were indexed to the (001), (100), (110), (112), (211), and (300) crystal planes. Notably, no significant differences were detected between the XRD patterns of HA and HF nanoparticles, indicating that FA incorporation did not alter the crystalline phase of HA. FTIR spectra (Fig. 1e) revealed absorption bands at $551\text{--}603\text{ cm}^{-1}$ attributed to the PO_4^{3-} bending vibrations and at 944 and $1045\text{--}1120\text{ cm}^{-1}$ corresponding to stretching vibrations [17, 18]. Notably, two new peaks appeared at 1609 and 1510 cm^{-1} in the FTIR spectrum of HF nanoparticles.

XPS analysis (Fig. 1f) of HA and HF nanoparticles revealed that both materials were primarily composed of C, O, Ca, and P elements. Notably, the characteristic peak of N 1s was detected in HF nanoparticles (Fig. 1g), indicating the presence of N in HF nanoparticle samples and preliminarily confirming FA grafting onto the HA nanoparticle surface [2]. Fitting analysis revealed that in HA nanoparticles (Fig. 1i), the peak at 284.8 eV corresponded to C–C bonds, which are primarily attributed to adventitious carbon (hydrocarbon contamination) commonly present on sample surfaces during XPS measurements. The characteristic peak at 286.2 eV was assigned to carbon atoms bonded to three oxygen atoms in CO_3^{2-} , forming resonance structures with alternating single and double bonds, whereas the peak position at 288.8 eV was attributed to CO_n species. In HF

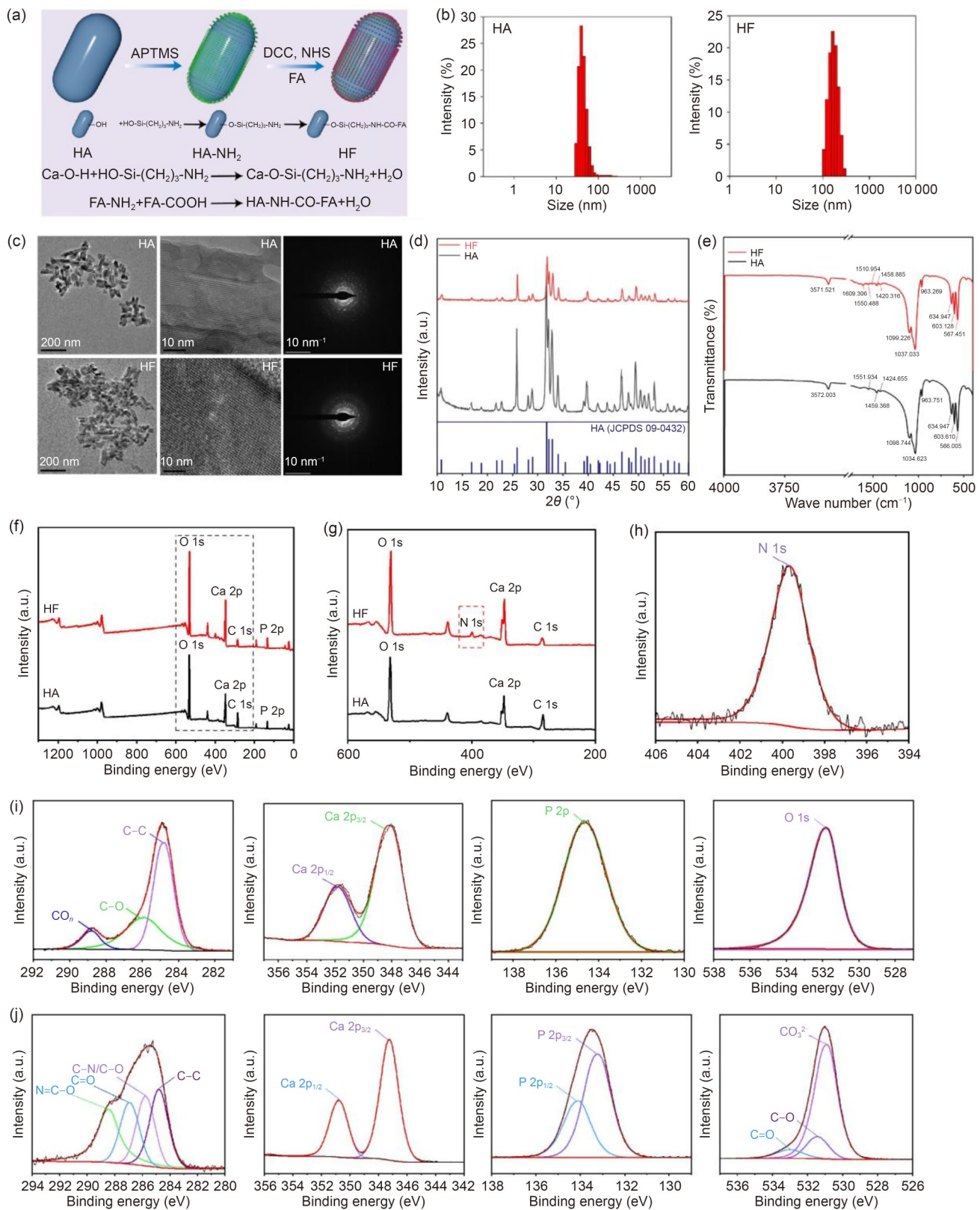


Fig. 1 Preparation and characterization of hydroxyapatite (HA) and folic acid (FA)-conjugated HA (HF) nanoparticles. (a) Schematic illustration of the synthesis process and working principle of HF nanoparticles. (b) Particle size distribution. (c) Transmission electron microscope (TEM) images. (d) X-ray diffractometer (XRD) patterns. (e) Fourier transform infrared spectroscopy (FTIR) spectra. (f) X-ray photoelectron spectroscopy (XPS) survey spectra. (g) High-resolution XPS spectra (200–600 eV). (h) Fitted N 1s spectra of HF nanoparticles. (i, j) Fitted C 1s spectra and fitted Ca, P, and O spectra of HA and HF nanoparticles

nanoparticles (Fig. 1j), the peaks at 284.8, 285.8, 286.9, and 288.4 eV were assigned to N=C–O, C–N/C–O, C=O, and C=O, respectively, indicating the formation of amide bonds [5]. Combined with FTIR results, these findings confirmed successful FA coupling to HA nanoparticles. Owing to spin–orbit coupling, the Ca 2p orbital split into Ca 2p_{1/2} and Ca 2p_{3/2}, corresponding to peaks at 351.8 and 348.2 eV in HA nanoparticles, respectively (Fig. 1i). In HF, Ca 2p_{1/2} and Ca 2p_{3/2} appeared at 350.8 and 347.3 eV, respectively, showing a slight chemical shift (Fig. 1j). The O 1s peak at 531.8 eV in HA nanoparticles corresponded to P–O bonding and matched the P 2p peak of PO₄^{3−} at 134.8 eV [3, 4]. In HF nanoparticles, P 2p orbitals split into P 2p_{1/2} and P 2p_{3/2}, at 134.1 and 133.2 eV, respectively. The peak at 533 eV was attributed to C=O bonding, further supporting amide bond formation. Additionally, a distinct N 1s peak at 399.7 eV was observed in HF nanoparticles, corresponding to amide functional groups.

3.2 Cellular viability and apoptosis induced by nanoparticles

To determine the optimal nanoparticle concentration for in vivo antitumor experiments, tumor (4T1) and normal (L929) cells were coincubated with gradient concentrations of HA and HF nanoparticles. CLSM images and measured OD values are provided in Figs. S1 and S2 (supplementary information).

A significant decrease in 4T1 cell viability was observed when HA and HF nanoparticle concentrations exceeded 200 µg/mL compared with the control group. The inhibition rate (r_i) was used to determine the inhibitory effects of each nanoparticle group on 4T1 cell proliferation:

$$r_i = (1 - \text{OD}_{\text{exp}} / \text{OD}_{\text{control}}) \times 100\%, \quad (4)$$

where OD_{control} represents the control group's OD value and OD_{exp} represents the experimental group's OD value.

The maximum inhibition rates of 4T1 cells reached 27.3% and 40.9% for HA and HF nanoparticles, respectively, after 5 d of coincubation. In contrast, L929 cell viability remained unaffected by coincubation with either nanoparticle type at all tested concentrations.

CLSM imaging of cell nuclei revealed that HA nanoparticle and HF nanoparticle-induced cytotoxicity was concentration-dependent and characterized by nuclear condensation and fragmentation. More severe nuclear damage, including pronounced shrinkage and rupture, was observed at HA and HF nanoparticle concentrations >200 µg/mL. Notably, the HF nanoparticle-treated group exhibited a more substantial reduction in cell density and more severe cellular damage relative to the HA nanoparticle-treated group. Based on these findings, 200 µg/mL was selected as the optimal concentration for subsequent in vivo studies.

3.3 Influence of nanoparticles on tumor cell metastasis and organelle function

3.3.1 Cytoskeletal and nuclear morphological alterations

Treatment of 4T1 cells with HA and HF nanoparticles induced significant changes in cytoskeletal architecture and nuclear morphology. The main features included disorganized cytoskeletal networks and increased nuclear fragmentation (Fig. 2a), with more pronounced changes observed in the HF nanoparticle-treated group. Ultrastructural TEM analysis revealed additional cellular damage in 4T1 cells exposed to HA or HF nanoparticles (Fig. 2b), including chromatin condensation at the nuclear periphery, compromised nuclear membranes, mitochondrial vacuolization, and extensive apoptotic body formation. These findings indicate that both nanoparticle types are internalized by 4T1 cells, accumulate within lysosomes and mitochondria, and effectively trigger apoptotic signaling pathways [19, 20].

3.3.2 Flow cytometry analysis of apoptosis

FCM quantification (Fig. 2d) of apoptosis demonstrated a marked increase in apoptotic 4T1 cells following HF nanoparticle treatment (49.33%) compared with the control (41.17%) and HA nanoparticle-treated cells (36.45%). Specifically, the proportion of late apoptotic cells in the HF nanoparticle group (45.3%) exceeded that in the control (35.9%) and HA nanoparticle (31.0%) groups, whereas the percentage of viable cells in the HF nanoparticle group (48.3%) was significantly lower than that in the control (58.1%) and HA nanoparticle (63.3%) groups. These results indicate that HF nanoparticles induce apoptosis more efficiently and at earlier stages relative to HA nanoparticles. Notably, the apoptosis rate in the HA nanoparticle group was even lower than that in the control group, indicating that HA nanoparticles exhibit intrinsically low cytotoxicity at subthreshold concentrations and cause minimal direct damage to 4T1 cells. Therefore, the enhanced apoptotic effect of HF nanoparticles is not derived from the inherent cytotoxicity of HA but is primarily attributed to the FA-mediated targeting capability.

3.3.3 Intracellular Ca²⁺ homeostasis dysregulation

To elucidate the mechanisms underlying nanoparticle-induced apoptosis, intracellular Ca²⁺ levels were assessed. HA nanoparticle treatment reduced intracellular Ca²⁺ levels compared with controls, whereas HF nanoparticle exposure significantly elevated Ca²⁺ concentrations relative to both the control and HA nanoparticle groups (Fig. 2c). This phenomenon may be explained by several factors. After cellular internalization, HA nanoparticles are likely degraded

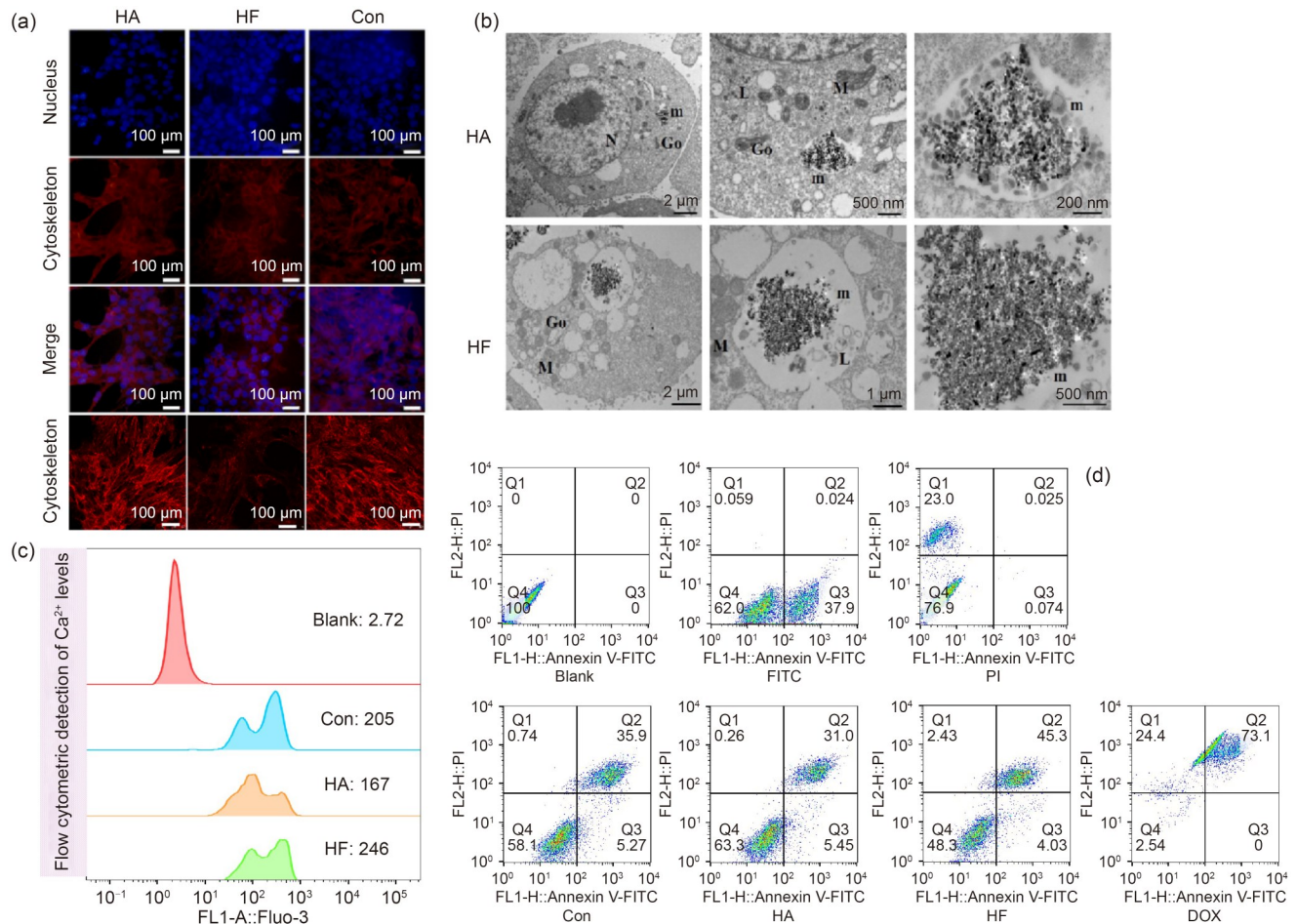


Fig. 2 Effects of hydroxyapatite (HA) and folic acid (FA)-conjugated HA (HF) nanoparticles on the morphology, metabolism, and functions of 4T1 cells. (a) Cytoskeletal organization of 4T1 cells following nanoparticle treatment. (b) Transmission electron microscope (TEM) images showing nanoparticle endocytosis and intracellular distribution. (c) Intracellular calcium homeostasis regulation. (d) Apoptosis analysis of 4T1 cells

within lysosomes and release Ca^{2+} , which is preferentially sequestered by the endoplasmic reticulum (ER) rather than remaining in the cytoplasm, resulting in no significant increase in the detectable cytoplasmic Ca^{2+} levels. In addition, HA nanoparticle stimulation may activate membrane Ca^{2+} efflux channels or promote rapid translocation from the cytoplasm to ER calcium stores, further reducing free cytoplasmic Ca^{2+} concentrations [21–23]. In contrast, the enhanced Ca^{2+} release induced by HF nanoparticles is attributed to FA receptor-mediated endocytosis, which enables more efficient nanoparticle internalization by 4T1 cells. Pure HA nanoparticles are mainly internalized through nonspecific endocytosis, resulting in substantially lower cellular uptake efficiency. Consequently, a larger quantity of HF nanoparticles accumulates intercellularly, providing the basis for extensive Ca^{2+} release [22, 24, 25].

3.3.4 Mitochondrial dysfunction and ROS production

CLSM imaging using Mito-Tracker Green (Fig. 3a) revealed significantly attenuated mitochondrial fluorescence

intensity in HA nanoparticle-treated cells, with even greater reductions in the HF nanoparticle group. As mitochondria play a central role in ROS generation and tumor cell death, reduced mitochondrial abundance and impaired function likely contribute to 4T1 cell apoptosis in this system. Additionally, intracellular Ca^{2+} levels (Fig. 2c) are correlated with mitochondrial dysfunction: Ca^{2+} influx promotes mitochondrial permeability transition pore (mPTP) opening, dissipates membrane potential, and facilitates CYCS release to initiate apoptosis [26, 27].

3.3.5 Lysosomal integrity and apoptotic crosstalk

Lyso-Tracker Red staining (Fig. 3b) was used to assess lysosomal integrity. No significant changes in fluorescence intensity were observed (HA nanoparticle-treated cells vs. controls), whereas a marked reduction was detected in HF nanoparticle-treated cells. This indicates that HF limits lysosomal abundance or activity, which may result from several mechanisms: (1) hydrolase dysfunction—impaired synthesis or transport of acid hydrolases disrupts cellular metabolism

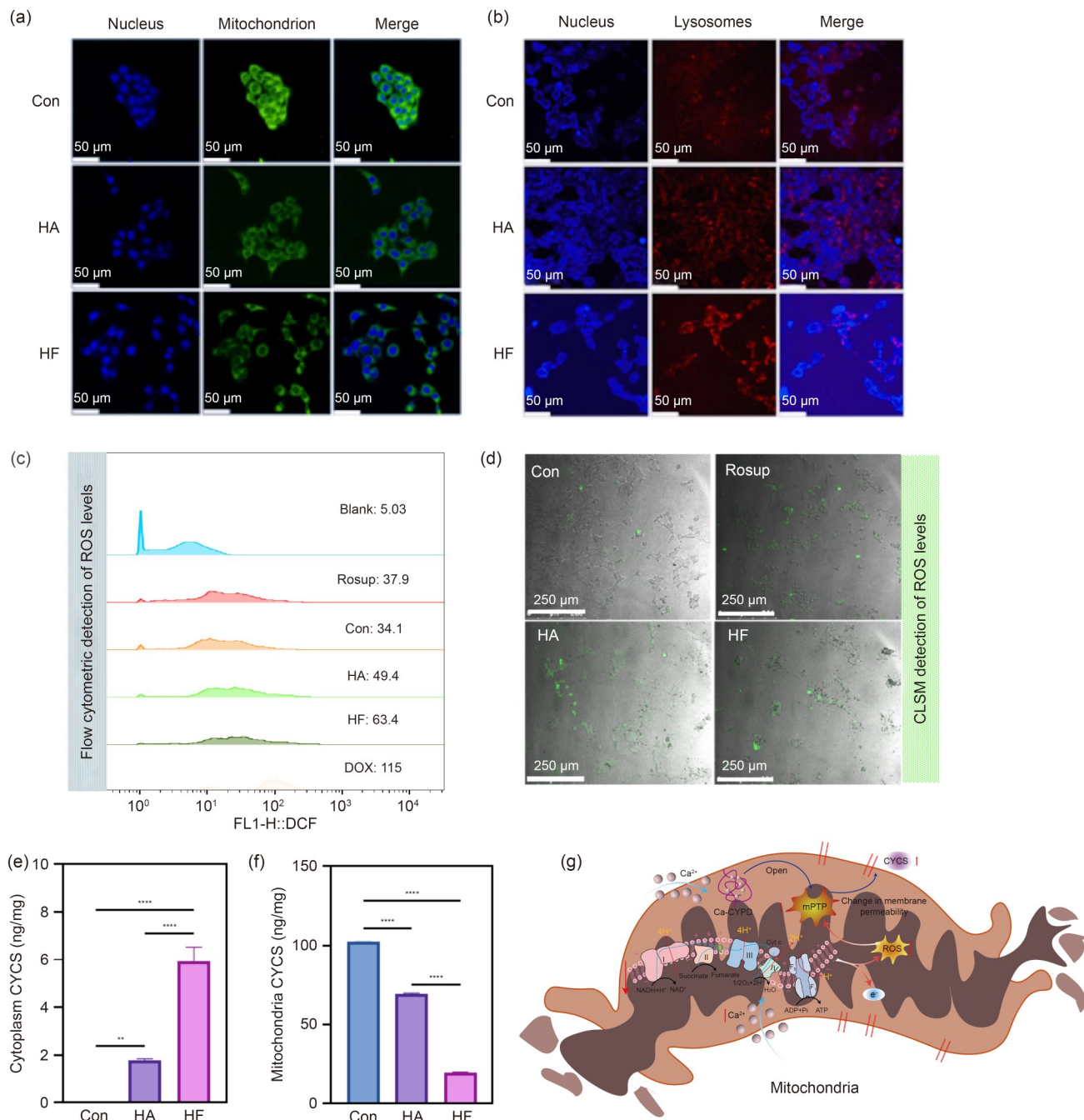


Fig. 3 Effects of hydroxyapatite (HA) and folic acid (FA)-conjugated HA (HF) nanoparticles on organelle function in 4T1 cells. (a) Mitochondrial status of 4T1 cells after nanoparticle treatment. (b) Lysosomal alterations. (c, d) Intracellular reactive oxygen species (ROS) levels measured via flow cytometry (FCM) and fluorescent staining. (e, f) Cytochrome c (CYCS) expression levels in cytoplasm and mitochondria detected via enzyme-linked immunosorbent assay (ELISA). (g) Schematic illustration of the proposed mechanism of calcium homeostasis imbalance-induced mitochondrial permeability transition pore (mPTP). Data are expressed as mean $\pm$ standard deviation ($n=3$). ** $p < 0.01$ and **** $p < 0.0001$

and waste clearance, compromising intracellular homeostasis; (2) membrane permeabilization—lysosomal membrane permeabilization may trigger hydrolase release into the cytoplasm, activating caspase cascades and promoting apoptosis [28]; (3) mitochondrial-lysosomal crosstalk—lysosomal dysfunction may induce mitochondrial ROS overproduction by disrupting energy metabolism and respiratory chain

function, exacerbating macromolecular injury (such as DNA, protein, and lipid damage) and apoptotic signaling [19].

3.3.6 ROS-mediated antitumor mechanism

FCM and CLSM analyses (Figs. 3c and 3d) showed significantly elevated intracellular ROS levels in HA

nanoparticle-treated cells (49.4) vs. controls (34.1), with further enhancement in HF nanoparticle-treated cells (63.4). ROS-mediated oxidative stress suppresses tumor cell proliferation and induces mitochondrial damage, consistent with prior reports [29, 30]. These results identify ROS generation as a key contributor to the superior in vitro antitumor activity of HF nanoparticles.

As shown in Figs. 3e and 3f, CYCS levels in both the cytoplasm and mitochondria of 4T1 cells were quantified following different treatments. CYCS was barely detectable in the cytoplasm of control cells, whereas HA nanoparticle stimulation significantly increased cytoplasmic CYCS content. The increase may result from HA nanoparticle-mediated regulation of the mitochondrial calcium uniporter, which facilitates Ca^{2+} transfer from ER calcium stores into the mitochondrial matrix. Because mitochondrial calcium regulatory mechanisms in 4T1 tumor cells are impaired, excess Ca^{2+} cannot be efficiently removed, leading to mitochondrial calcium overload. This overload disrupts mitochondrial membrane potential ($\Delta\Psi_m$) and causes persistent mPTP opening, leading to the release of CYCS from the intermembrane space into the cytoplasm. Notably, HF nanoparticle stimulation induced a more pronounced increase in cytoplasmic CYCS levels. Correspondingly, mitochondrial CYCS content was decreased after nanoparticle treatment, especially in the HF nanoparticle group, indicating marked CYCS release from mitochondria after HF nanoparticle treatment and indirectly suggesting increased mitochondrial membrane permeability and membrane structure changes [31]. This effect may be attributed to increased endocytosis of HF nanoparticles in 4T1 cells, which elevates intracellular calcium accumulation in the ER and subsequent mitochondrial Ca^{2+} influx. In summary, mitochondrial calcium overload disrupts $\Delta\Psi_m$ and promotes sustained opening of the mPTP. Consequently, CYCS located between the inner and outer mitochondrial membranes is released into the cytoplasm, elevating intracellular CYCS levels and ultimately triggering apoptosis, as illustrated in Fig. 3g.

3.4 Effect of nanoparticles on 4T1 cells via paracrine microenvironment modulation

3.4.1 Influence of nanoparticles on macrophage behavior

To elucidate the mechanism linking nanoparticle-mediated macrophage paracrine signaling to antitumor efficacy, macrophage proliferation, morphology, and cytoskeletal organization were systematically analyzed. SEM images (Fig. 4b) revealed that RAW 264.7 macrophages exhibited increased filopodia formation after nanoparticle exposure compared with controls, indicating altered morphological plasticity. As reported previously, changes in macrophage

morphology often reflect transitions between functional phenotypes [32].

Both MTT assays (Fig. 4a) and FDA staining (Fig. 4c) indicated no significant effects of HA or HF nanoparticles on macrophage proliferation or viability, suggesting minimal direct cytotoxicity at the tested concentrations. Moreover, toxicity assessment requires integration of multiple endpoints, including cell survival, factor secretion, and immune activation. Notably, cytoskeletal staining (Fig. 4d) shows denser actin filament networks in HA nanoparticle- and HF nanoparticle-treated macrophages relative to controls, with occasional cell fusion events observed post-treatment, indicative of heightened cellular activity or intercellular communication.

As shown in Fig. 4e, quantitative analysis of proinflammatory markers revealed significantly upregulated expression of *TNF- α* , *iNOS*, and *IL-1 β* in HA nanoparticle and HF nanoparticle-treated macrophages compared with the control group. This finding aligns with our previous findings that degradation products of biphasic calcium phosphate ceramics promote macrophage polarization toward a proinflammatory (M1) phenotype [33]. Importantly, HF nanoparticles induced a pronounced shift toward the proinflammatory phenotype relative to HA nanoparticles [34, 35].

3.4.2 Impact of macrophage paracrine modulation on 4T1 cell behaviors

Based on these findings, we investigated whether CM derived from macrophages cocultured with HA nanoparticles (i.e., CM-HA) or HF nanoparticles (i.e., CM-HF) affected 4T1 cell apoptosis. Specifically, the cytoskeletal organization, morphology, and proliferation of 4T1 cells cultured in these CMs were assessed, as shown in Fig. 5a. All CM treatments exhibited no obvious changes in 4T1 cytoskeletal structure (Fig. 5b), cell morphology, or spreading area (Fig. 5c). CLSM imaging of FDA-stained cells revealed increased cell count in the CM-Con group, whereas no significant differences were observed among CM-HA, CM-HF, and the control medium (Fig. 5d), consistent with MTT assay results (Fig. 5f).

Apoptosis analysis (Fig. 5e) demonstrated that the proportions of viable cells in the CM-HF (74.9%) and CM-HA (78.9%) groups were significantly lower than those in the control group (81.2%) and the CM-Con group (89.9%). The CM-HF group exhibited the highest total apoptotic cell fraction (approximately 25.1%), with elevated early and late apoptosis rates, indicating that the HF nanoparticle-modulated proinflammatory immune microenvironment strongly promotes tumor cell apoptosis. Previous studies have demonstrated that M1 macrophages exert direct tumoricidal effects and that increased M1 macrophage infiltration promotes tumor cell apoptosis and

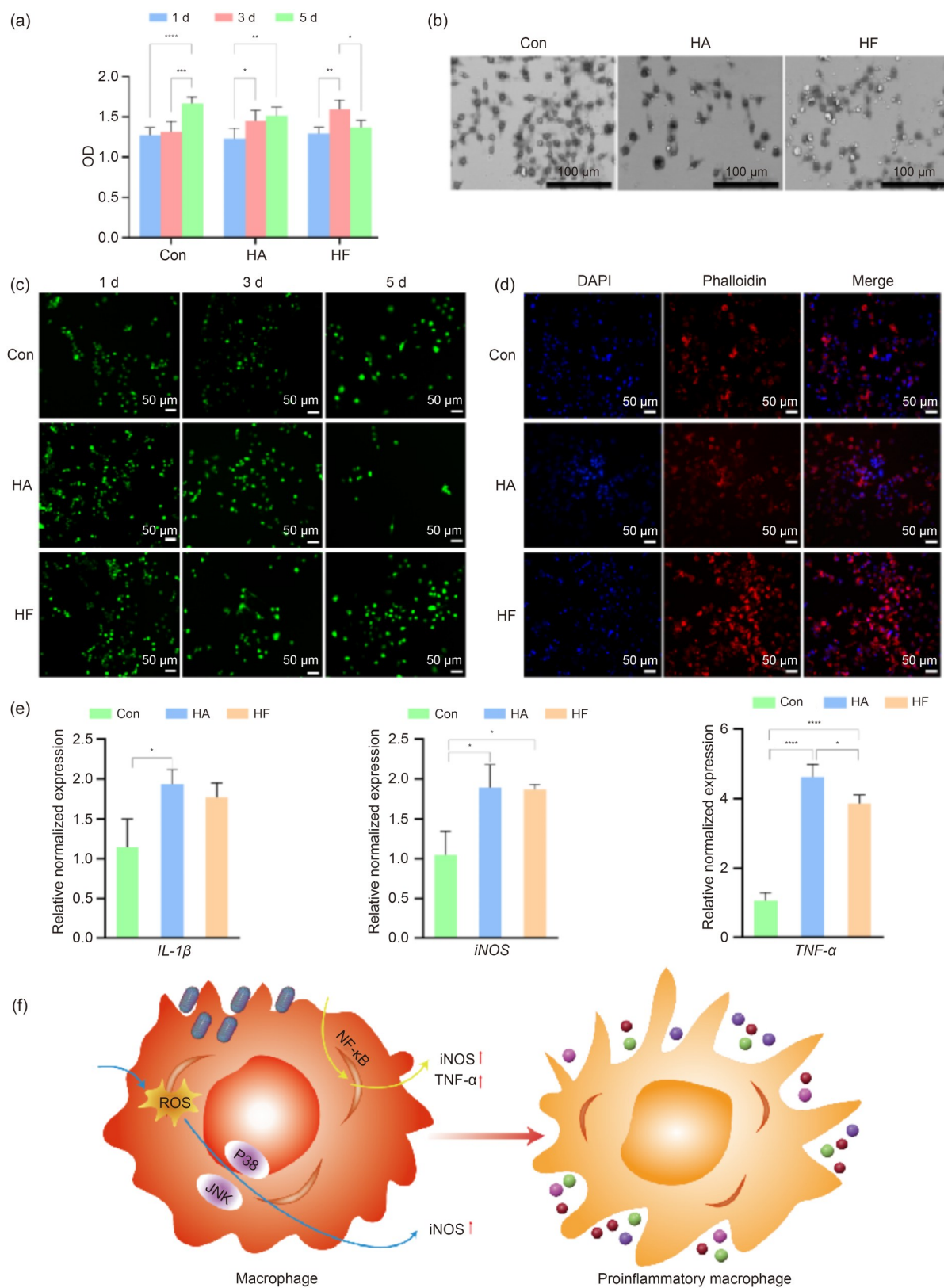


Fig. 4 Effects of hydroxyapatite (HA) and folic acid (FA)-conjugated HA (HF) nanoparticles on macrophage behavior. (a) Macrophage proliferation following nanoparticle stimulation. (b) Scanning electron microscopy (SEM) images showing macrophage morphology. (c, d) Macrophage viability and cytoskeletal organization. (e) Expression levels of proinflammatory genes in macrophages. (f) Schematic illustration of nanoparticle-induced macrophage polarization toward a proinflammatory phenotype. Data are expressed as mean $\pm$ standard deviation ($n=3$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$

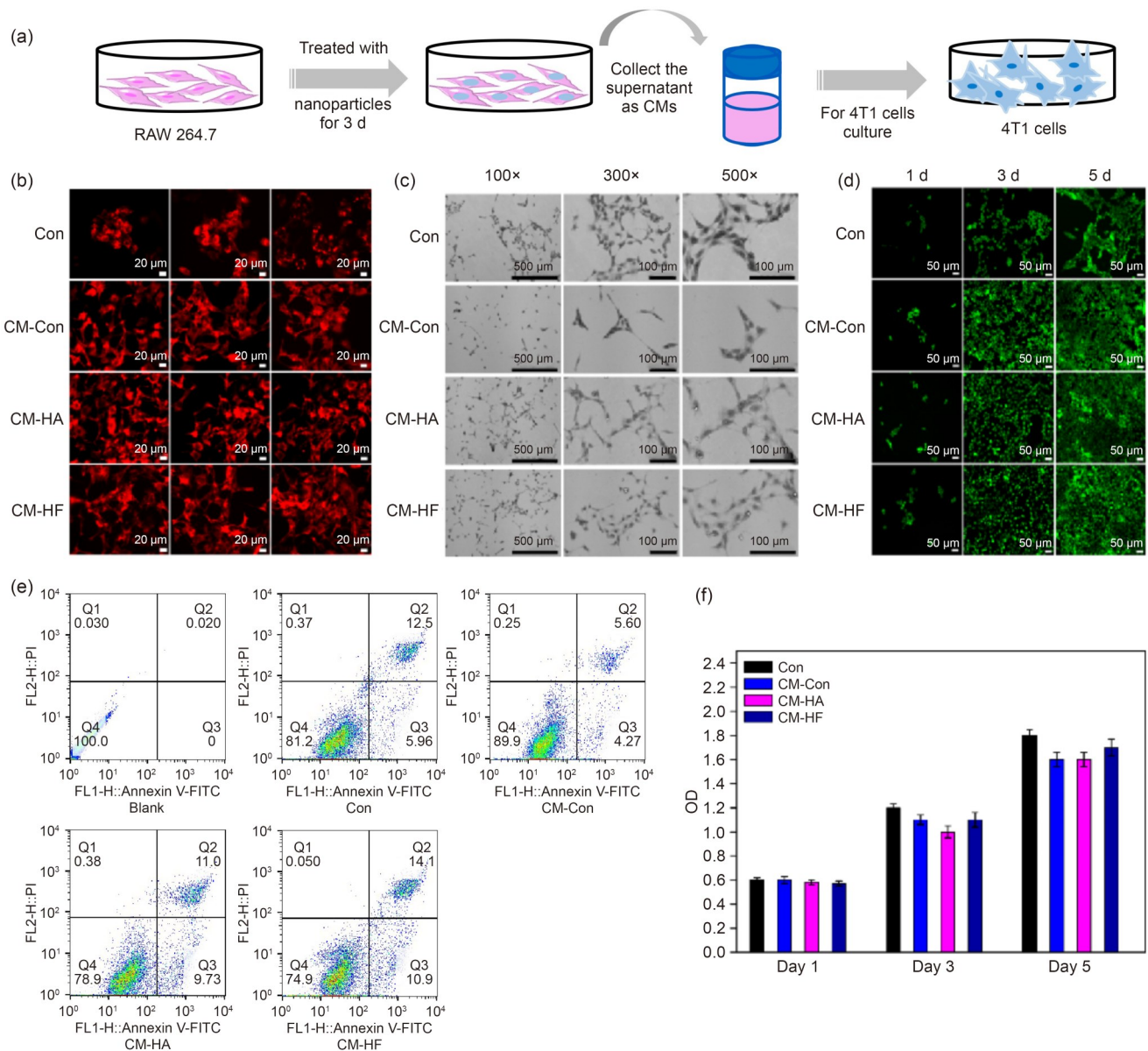


Fig. 5 Effects of hydroxyapatite (HA) nanoparticle and folic acid (FA)-conjugated HA (HF) nanoparticle-modulated immune microenvironment on 4T1 cell behavior. (a) Schematic diagram of the experimental workflow. (b–d) Effects of conditioned media on cytoskeletal organization, morphology, pseudopodia formation, and proliferative activity of 4T1 cells. Apoptosis (e) and proliferation (f) analysis of 4T1 cells treated with conditioned media. Data are expressed as mean ± standard deviation ($n=3$)

suppresses tumor growth [36–38]. Therefore, the observed effects are likely associated with HF nanoparticle-induced macrophage polarization toward a proinflammatory phenotype.

Intracellular Ca^{2+} levels followed the trend $\text{CM-HF} > \text{CM-HA} > \text{CM-Con} > \text{Con}$ (Fig. 6a), indicating that HF nanoparticle-mediated immune modulation significantly increased intracellular Ca^{2+} levels compared with controls. Additionally, intracellular ROS levels in the CM-HA and CM-HF groups were notably higher than those in the Con and CM-Con groups. These findings suggest that HF nanoparticle-induced immune remodeling markedly alters

intracellular Ca^{2+} homeostasis and ROS generation in 4T1 cells. As reported previously, HA-based nanoparticles can be internalized by macrophages, activating relevant signaling pathways and promoting cytokine secretion [33]. Consequently, cytokine concentrations and type changes reshape the immune microenvironment. Signaling molecules (e.g., cytokines and chemokines) in the renewed microenvironment bind to receptors on 4T1 cell surfaces, initiating intracellular cascades that activate Ca^{2+} channels, promote extracellular Ca^{2+} influx, or induce Ca^{2+} release from intracellular stores, thereby elevating cytoplasmic Ca^{2+} levels. Increased Ca^{2+} levels further enhance ROS generation in the

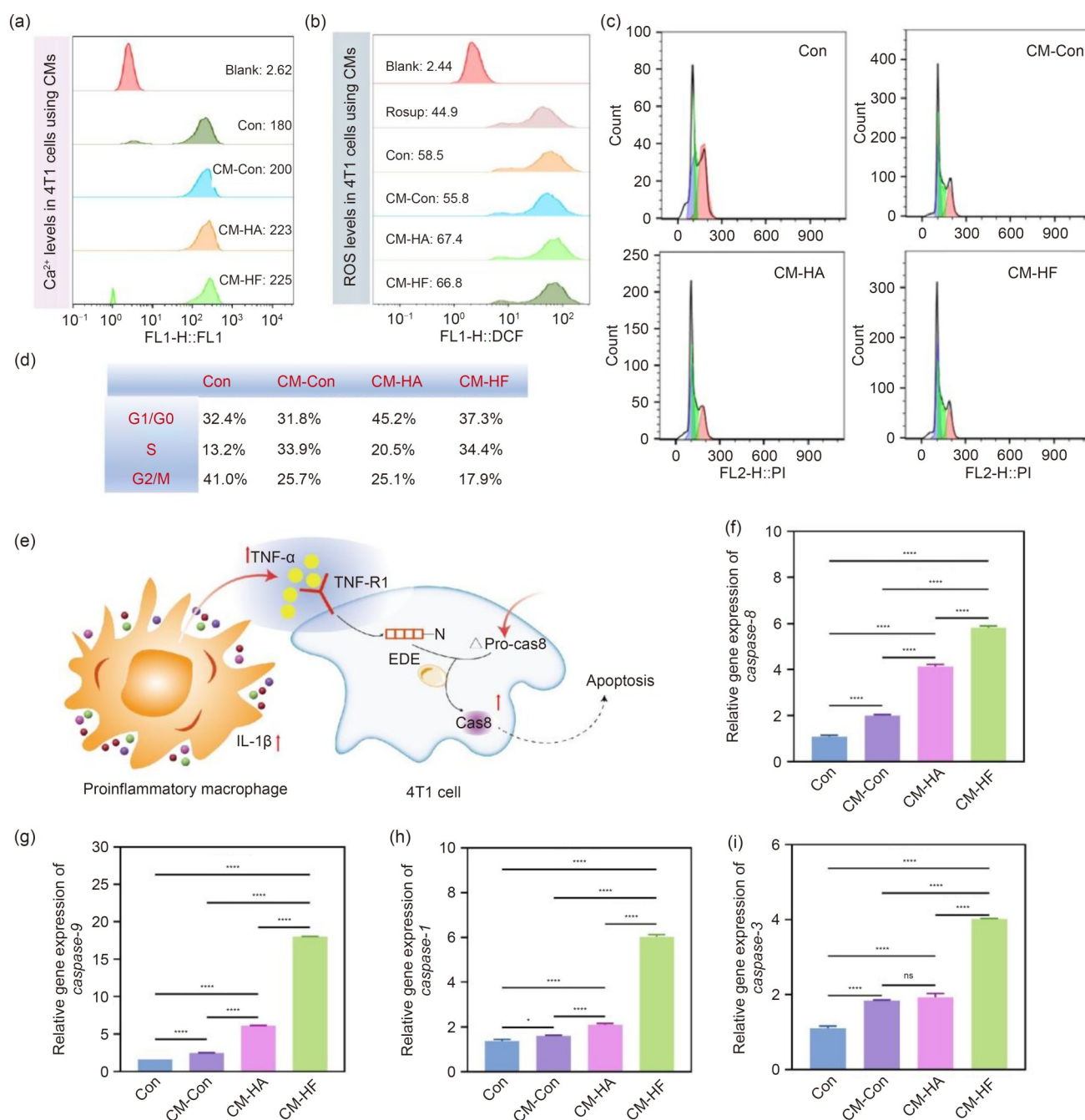


Fig. 6 Effects of hydroxyapatite (HA) nanoparticle and folic acid (FA)-conjugated HA (HF) nanoparticle-modulated immune microenvironment on 4T1 cell metabolism. (a, b) Flow cytometry (FCM) analysis of intracellular Ca²⁺ and reactive oxygen species (ROS) levels. (c, d) Cell cycle distribution and quantitative analysis of 4T1 cells. (e) Schematic illustration of the proposed mechanism by which the nanoparticle-induced proinflammatory microenvironment promotes apoptosis. (f–i) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of caspase family gene expression in 4T1 cells. Data are expressed as mean $\pm$ standard deviation ($n=3$). * $p<0.05$ and **** $p<0.0001$; ns: not significant

mitochondria and ER, ultimately promoting immunogenic cell death [39, 40].

Macrophage-derived paracrine modulation also affected ROS generation in 4T1 cells. As shown in Fig. 6b, both CM-HA and CM-HF treatments increased intracellular ROS levels. Previous studies have indicated that inflammatory mediators activate enzymatic systems (e.g., nicotinamide

adenine dinucleotide phosphate hydrogen (NADPH) oxidase), catalyzing ROS production in 4T1 cells. Upon inflammatory stimulation, NADPH oxidase subunits assemble and generate superoxide anions that are subsequently converted into secondary ROS species (e.g., hydrogen peroxide) [41–43]. In addition, certain cytokines suppress antioxidant enzyme activity, reducing ROS scavenging

capacity and further promoting intracellular ROS accumulation [44, 45]. Excessive ROS accumulation ultimately causes structural damage and functional dysregulation in 4T1 cells, providing a mechanistic basis for apoptosis. Combined with inflammatory gene expression profiles, elevated *TNF- α* expression activates the NF- κ B pathway in cancer cells, inducing mitochondrial dysfunction (e.g., increased electron leakage/membrane potential), thereby accelerating ROS production. Excess ROS accumulation not only damages mitochondria directly but also amplifies apoptotic signaling through activation of the JNK/p38 pathway, further promoting tumor cell apoptosis (Figs. 4f and 6e).

To further evaluate nanoparticle-mediated paracrine inhibition of 4T1 cells, the cell cycle was analyzed (Figs. 6c and 6d). In the control group, cell cycle distributions were 32.4% (G1/G0), 13.2% (S), and 41.0% (G2/M). In macrophage CM without nanoparticles, a higher proportion of cells entered the S phase (33.9%), whereas fewer cells progressed to G2/M (25.7%). Treatment with CM-HA increased the proportion of G1/G0 phase cells (45.2%) and reduced in G2/M phase cells (25.1%) compared with nanoparticle-free groups. Notably, CM-HF treatment significantly increased cell proportions in the G1/G0 (37.3%) and S (34.4%) phases while reducing those in the G2/M phase (17.9%). These results indicate that macrophage-mediated immune regulation strongly influences 4T1 cell survival and cell cycle progression. Both HA and HF nanoparticles suppressed 4T1 cell proliferation by arresting cells at early stages and limiting entry into the G2/M phase, with HF nanoparticles exhibiting the strongest effect.

Gene expression analysis of caspase family members (Figs. 6f–6i) revealed that the nanoparticle-mediated proinflammatory microenvironment significantly upregulated initiator caspase genes (e.g., caspase-8 and caspase-9). Mechanistically, these transcriptional changes may arise from direct activation by proapoptotic transcription factors (e.g., *p53*) or indirect regulation via NF- κ B signaling induced by proinflammatory cytokines (e.g., *TNF- α*) [40, 46]. Concurrently, expression of caspase-3, a central executioner caspase, was markedly increased. This upregulation initiates irreversible apoptotic cascades, driving proteolytic degradation of cytoskeletal proteins, nuclear lamins, and DNA repair enzymes.

Combined with CYCS distribution data (Figs. 3e and 3f), nanoparticle-induced mitochondrial damage resulted in altered membrane permeability, leading to CYCS efflux into the cytoplasm and depletion of intramitochondrial CYCS stores. This process follows the canonical intrinsic apoptotic pathway: released CYCS interacts with apoptotic protease-activating factor 1 to form the apoptosome complex, promoting recruitment and activation of pro-caspase-8. Activated *caspase-8* subsequently triggers downstream effector *caspases*, ultimately leading to coordinated cellular dismantling and programmed cell death (Fig. 6e).

3.5 Synergistic effect of nanoparticles and DOX on antitumor activity

To further explore the potential of the designed nanoparticles as DOX carriers and to determine whether combined administration produces synergistic antitumor effects, the DOX loading efficiencies, release behaviors, and therapeutic performance of HA and HF nanoparticles were assessed. As shown in Fig. S3 (supplementary information), DOX exhibited a distinct adsorption peak at 480 nm. Both HA and HF nanoparticles demonstrated high DOX-loading efficiencies, with HF nanoparticles achieving a significantly higher loading rate (91.07%) vs. HA nanoparticles (88.52%) (Fig. 7a). This high loading capacity is attributed to the nanoscale particle size, which provides a large specific surface area and abundant adsorption sites for DOX.

Figure 7b presents pH-responsive drug release profiles over 28 d. HF nanoparticles exhibited higher cumulative DOX release relative to HA nanoparticles, and acidic conditions significantly accelerated drug release from both carriers. Under physiological conditions (pH 7.4), cumulative release reached only 21.3% and 42.9% for HA and HF nanoparticles, respectively, whereas under tumor-mimicking acidic conditions (pH 5.5), release increased to 28.1% and 47.3%, respectively, over the same period. This behavior is likely driven by H⁺-induced protonation of DOX amino groups at low pH, which enhances DOX's aqueous solubility and facilitates drug desorption [47]. Together, the high loading efficiency and pH-responsive release profile highlight the suitability of HA and HF nanoparticles as smart drug delivery platforms.

Figure 7 further illustrates the nuclear morphology, proliferation, spreading state, and cytoskeleton organization of 4T1 cells treated with nanoparticles and DOX-loaded formulations. In the control group, cell nuclei remained intact at all time points (Fig. 7c). After 3 d of culture, nuclear fragmentation was observed in the HA and HF nanoparticle groups. In contrast, HAD (i.e., HA-DOX) and HFD (i.e., HF-DOX) treatments induced nuclear fragmentation as early as Day 1, with damage intensifying over time. Notably, nuclear disruption in the HAD and HFD groups was comparable to that caused by free DOX. Crystal violet staining of cell morphology (Fig. 7e) corroborated the nuclear findings. Although HA and HF nanoparticles induced moderate cell aggregation and cytoplasmic condensation, HAD and HFD treatments caused extensive apoptosis in 4T1 cells, similar to the free DOX exposure.

Proliferation assays (Fig. 7d) showed continuous growth of control 4T1 cells, whereas HA and HF nanoparticles significantly suppressed proliferation after 3 d. Both HAD and HFD exhibited strong growth inhibition, although slightly weaker than free DOX. SEM imaging of cell morphology (Fig. 7f) further revealed marked cytoplasmic folding,

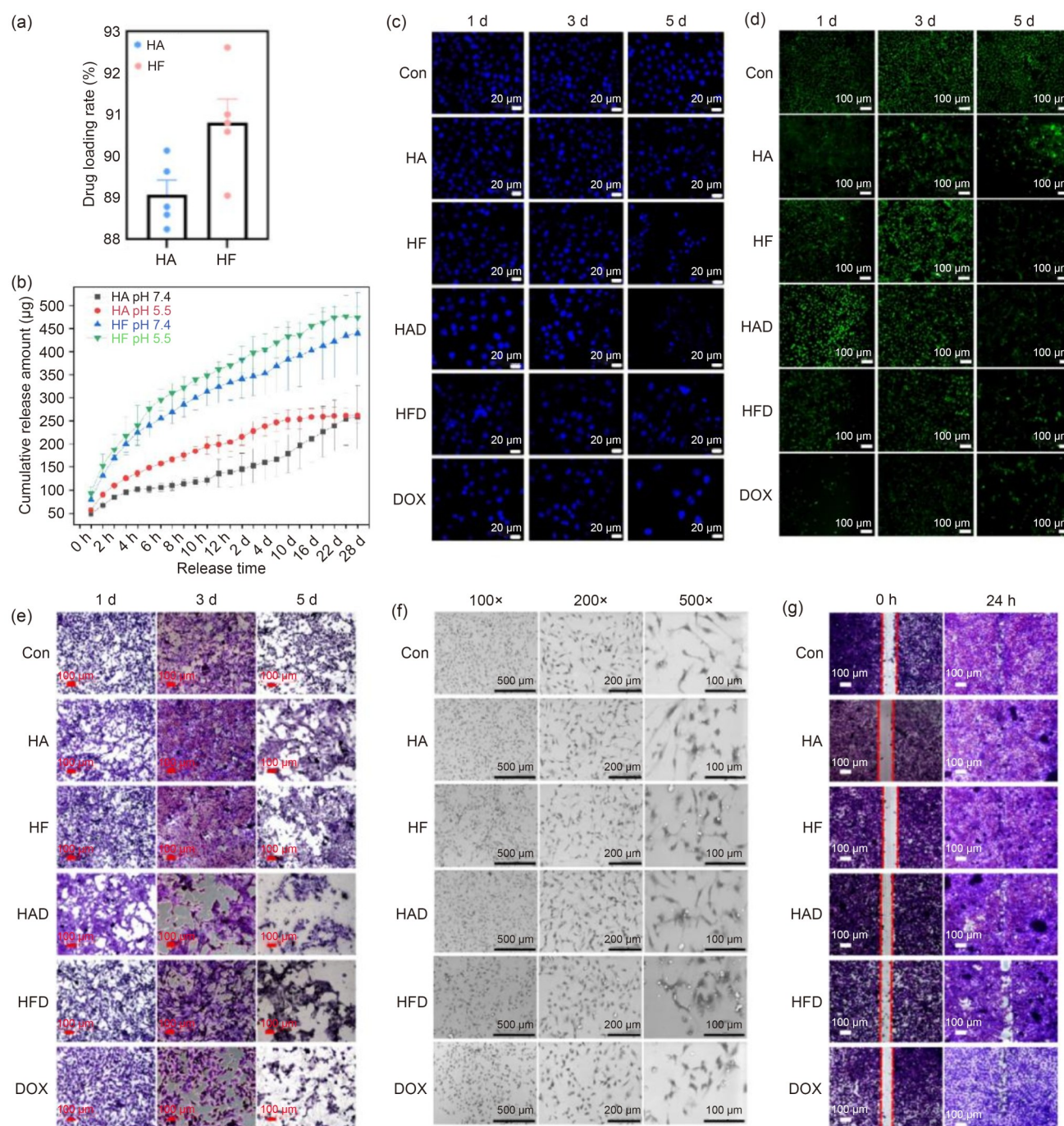


Fig. 7 Evaluation of hydroxyapatite (HA) and folic acid (FA)-conjugated HA (HF) nanoparticles as anticancer drug carriers. (a) Doxorubicin (DOX) loading efficiency of HA and HF nanoparticles. (b) DOX release profiles under various conditions. (c, d) Nuclear morphology, cell viability, and proliferation of 4T1 cells under different treatments. (e) Crystal violet staining of 4T1 cells. (f) Scanning electron microscopy (SEM) images showing cell morphology. (g) Migration assay of 4T1 cells under different treatments. Data in (a, b) are expressed as mean $\pm$ standard deviation ($n=5$)

cellular rounding, and reduced cell protrusion length and count in the nanoparticle- and drug-treated groups.

Cell migration assays (Fig. 7g) showed distinct treatment responses. HA and HF nanoparticles alone produced no significant impact on 4T1 cell migration, whereas HFD suppressed migration, exceeding the inhibitory effect of free DOX.

Collectively, these results demonstrate that HA and HF nanoparticles inhibit 4T1 cell proliferation and induce nuclear damage, and DOX-loading (HAD/HFD) markedly enhances these effects. Notably, HF nanoparticles exhibit strong synergy with DOX, particularly in suppressing tumor cell migration, suggesting that HF-DOX nanocomposites

hold promise as antitumor drug carriers for inhibiting 4T1 cell metastasis by simultaneously targeting proliferation, bioactivity, and motility.

3.6 Effects of nanoparticles on tumor development

Based on the in vitro experimental findings, in vivo experiments were performed to evaluate the antitumor efficacy of HA and HF nanoparticles in 4T1 tumor-bearing mice following the experimental timeline shown in Fig. 8a. Body weight monitoring revealed no significant weight loss among treatment groups, indicating minimal systemic toxicity. Body weight recording results showed that mice in all groups exhibited a trend of slow weight gain (Fig. S4 in the supplementary information). Tumor weight analysis (Fig. 8b) indicated that HA nanoparticles significantly suppressed tumor growth, with HF nanoparticles producing an even stronger inhibitory effect. After drug loading, HAD showed significantly enhanced antitumor efficacy compared with HA nanoparticles alone. Similarly, HFD further amplified HF nanoparticle-mediated antitumor effects, slightly surpassing the inhibitory efficiency of free DOX. These results confirm the synergistic antitumor activity of HF nanoparticles and DOX while highlighting their potential as drug delivery platforms.

H&E staining of tumor tissue sections (Figs. 8c and 8d) revealed reduced blood vessel density (red arrows) and smaller nuclei in nanoparticle-treated tumors compared with controls. Higher-magnification images showed increased infiltration of inflammatory cells (yellow circles), including horseshoe-shaped monocytes (green arrows), segmented neutrophils (black arrows), and eosinophils. This inflammatory infiltration was further amplified in the HAD and HFD groups, suggesting that nanoparticle intervention remodels the tumor immune microenvironment and contributes to tumor suppression.

Immunohistochemical staining for Ki67 is shown in Figs. 8e and 8f. Compared with strong Ki67 positivity in control tumors, HA nanoparticle treatment significantly reduced Ki67 expression, and HF nanoparticles produced a more pronounced suppression. Because Ki67 is expressed during the G1, S, G2, and M cell cycle phases but is absent in quiescent (G0) cells, reduced Ki67 positivity reflects inhibited tumor cell proliferation and growth potential and diminished metastatic potential. Thus, both HA and HF nanoparticles inhibit solid tumor proliferation, with the latter demonstrating a more pronounced effect. In the DOX-loaded nanoparticle groups HAD and HFD, tumor cells exhibited reduced cell volume, approaching that of the DOX-only treatment group, with clear nuclear fragmentation. Abundant, uniformly sized, small black dots, consistent with apoptotic or necrotic debris, were observed in these

sections. These fragments are likely phagocytosed by macrophages and retained during staining, appearing as black dots. Furthermore, Ki67 positivity was significantly lower in drug-loaded nanoparticle groups than in unloaded groups, validating the synergistic antitumor effect of nanoparticles and DOX.

To investigate nanoparticle-induced immune modulation in tumors, immunofluorescence staining of CD206, iNOS, and F4/80 was performed to characterize macrophage phenotypes. As shown in Fig. 8g and quantified in Fig. 8h, total macrophage abundance (F4/80⁺) was comparable among groups. However, the HF nanoparticle group exhibited significantly higher iNOS expression compared with the HA nanoparticle and control groups, indicating increased M1 macrophage polarization in the HF nanoparticle-treated TME. Conversely, M2 macrophage prevalence followed the trend control>HA nanoparticles>HF nanoparticles.

Previous studies have shown that M1 macrophages enhance pathogen clearance, secrete proinflammatory cytokines (e.g., *TNF-α* and *IL-1β*), and exert direct cytotoxic effects on tumor cells, whereas M2 macrophages promote tumor-associated angiogenesis and tissue repair, thereby facilitating tumor progression [48]. In the present study, the enhanced antitumor efficacy of HF is primarily attributed to its ability to induce M1 macrophage polarization, thereby reshaping the tumor immune microenvironment toward a cytotoxic and tumor-suppressive phenotype through coordinated regulation of cytokine profiles and immune cell interactions.

3.7 Additional mechanisms underlying the antitumor effects of HF nanoparticles

To further elucidate the mechanisms underlying HF nanoparticle-mediated antitumor activity, RNA sequencing was performed on HF nanoparticle-treated tumor tissues and control samples. In total, 5299 DEGs were identified, including 1155 upregulated and 4144 downregulated genes, respectively ($p < 0.05$ and fold change ≥ 2 ; Fig. 9a). GO enrichment analysis was conducted to functionally annotate these DEGs (Fig. 9b). Enriched GO terms were primarily clustered into three categories: molecular function (including metal ion binding and cation binding), cellular component (including intracellular organelle, membrane-bound organelle, and organelle subcompartments), and biological process (including organic substance metabolic, cellular metabolic process, primary metabolism, and the regulation of intracellular metabolic pathways).

Further functional classification categorized the DEGs into three major groups (Figs. 9c–9e): molecular function (including genes predominantly linked to molecular transducer activity, signal transduction, transporter activity, enzyme regulation, catalytic activity, molecular function regulation,

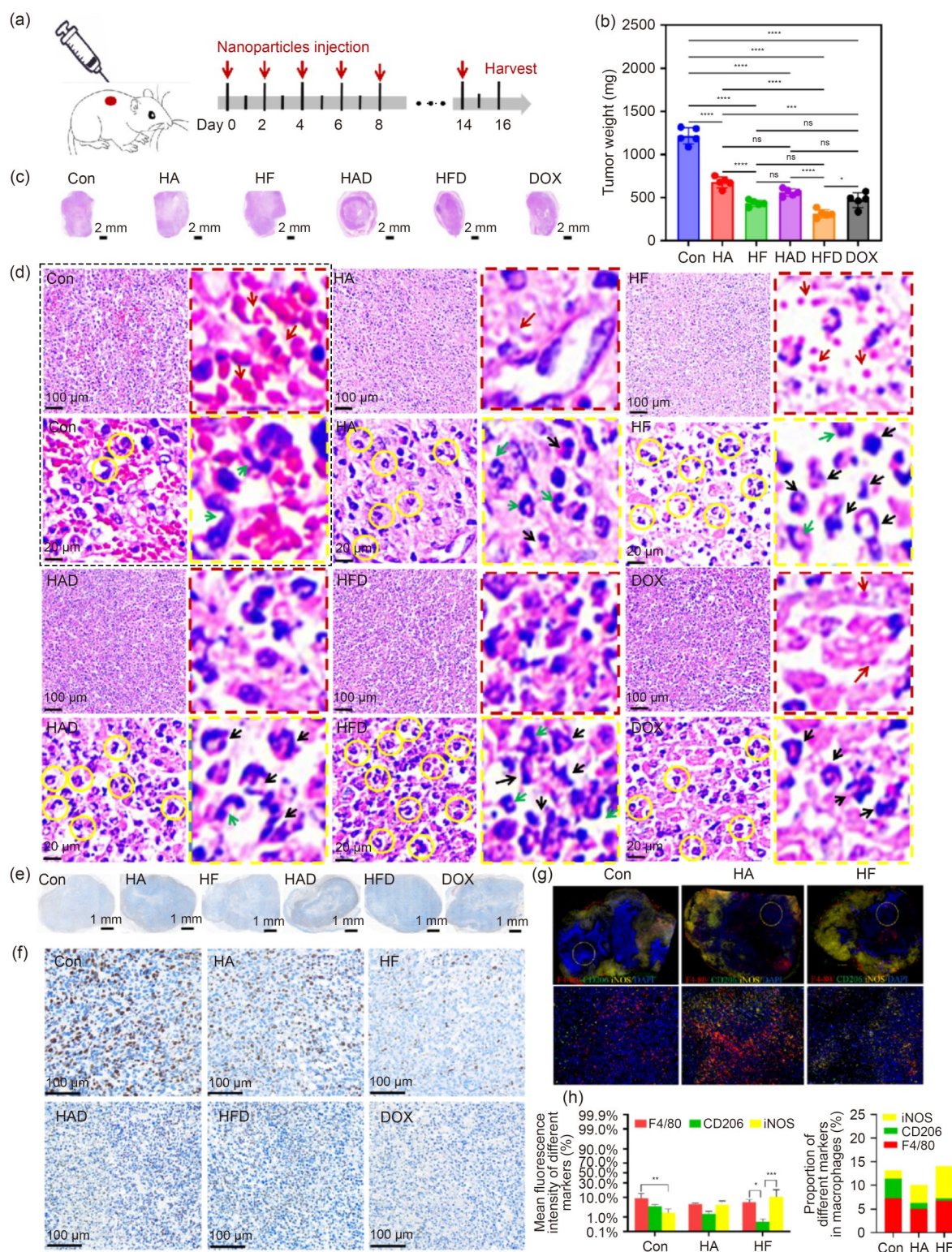


Fig. 8 In vivo antitumor efficacy of hydroxyapatite (HA) and folic acid (FA)-conjugated HA (HF) nanoparticles and evaluation as drug delivery carriers. (a) Experimental timeline and treatment scheme. (b) Tumor weight statistics after treatment. (c, d) Hematoxylin and eosin (H&E) staining of tumor tissues. For each group of four subfigures in (d), upper left shows low magnification, lower left shows inflammatory cell infiltration indicated by yellow circles, lower right shows high-magnification view of inflammatory regions marked by yellow boxes, and upper right shows magnified vascular region outlined by red dashed boxes; red, green, and black arrows indicate blood vessels, monocytes, and neutrophils, respectively. (e, f) Immunohistochemical staining of Ki67 (brown indicates positive expression). Immunofluorescence images (g) and quantitative analysis (h) of macrophage phenotypes in tumor tissues (blue: DAPI-stained nuclei; red: F4/80⁺ macrophages; yellow: iNOS⁺ M1 macrophages; green: CD206⁺ M2 macrophages). Data are expressed as mean ± standard deviation ($n=5$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$; ns: not significant

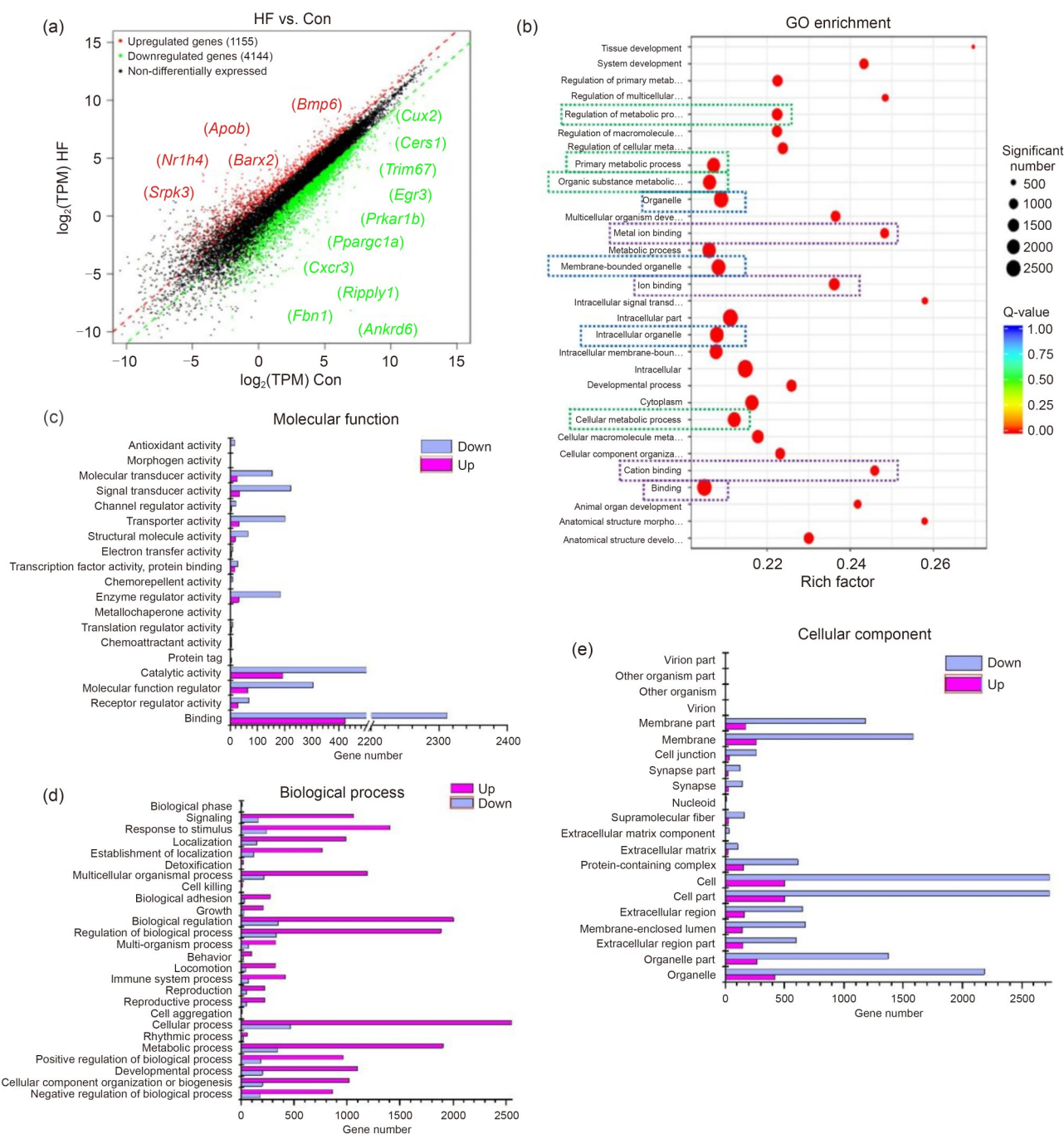


Fig. 9 RNA sequencing analysis of folic acid (FA)-conjugated hydroxyapatite (HF) nanoparticle-treated tumors vs. controls. (a) Volcano plot of the differentially expressed genes (DEGs). (b) Gene Ontology (GO) enrichment analysis of DEGs. (c–e) GO classification of DEGs based on molecular function, biological process, and cellular component categories

and binding), biological process (including enriched pathways primarily involved in signaling transduction, cellular response to stimuli, subcellular localization, multicellular organismal processes, biological regulation, and metabolic pathways), and cellular component (including genes associated with membrane structures, membrane-enclosed lumens, organelles, extracellular regions, and protein-containing complexes).

KEGG pathway enrichment analysis of the DEGs identified 167 upregulated and 245 downregulated pathways, respectively, in HF nanoparticle-treated tumors relative to controls ($p < 0.05$). As shown in Fig. 10a, the most significantly affected pathways included Wnt, TNF, MAPK, Hippo, focal adhesion, calcium signaling, and apoptosis signaling.

Heatmap analyses (Figs. 10b and 10c), visualizing gene expression patterns across the identified pathways, revealed

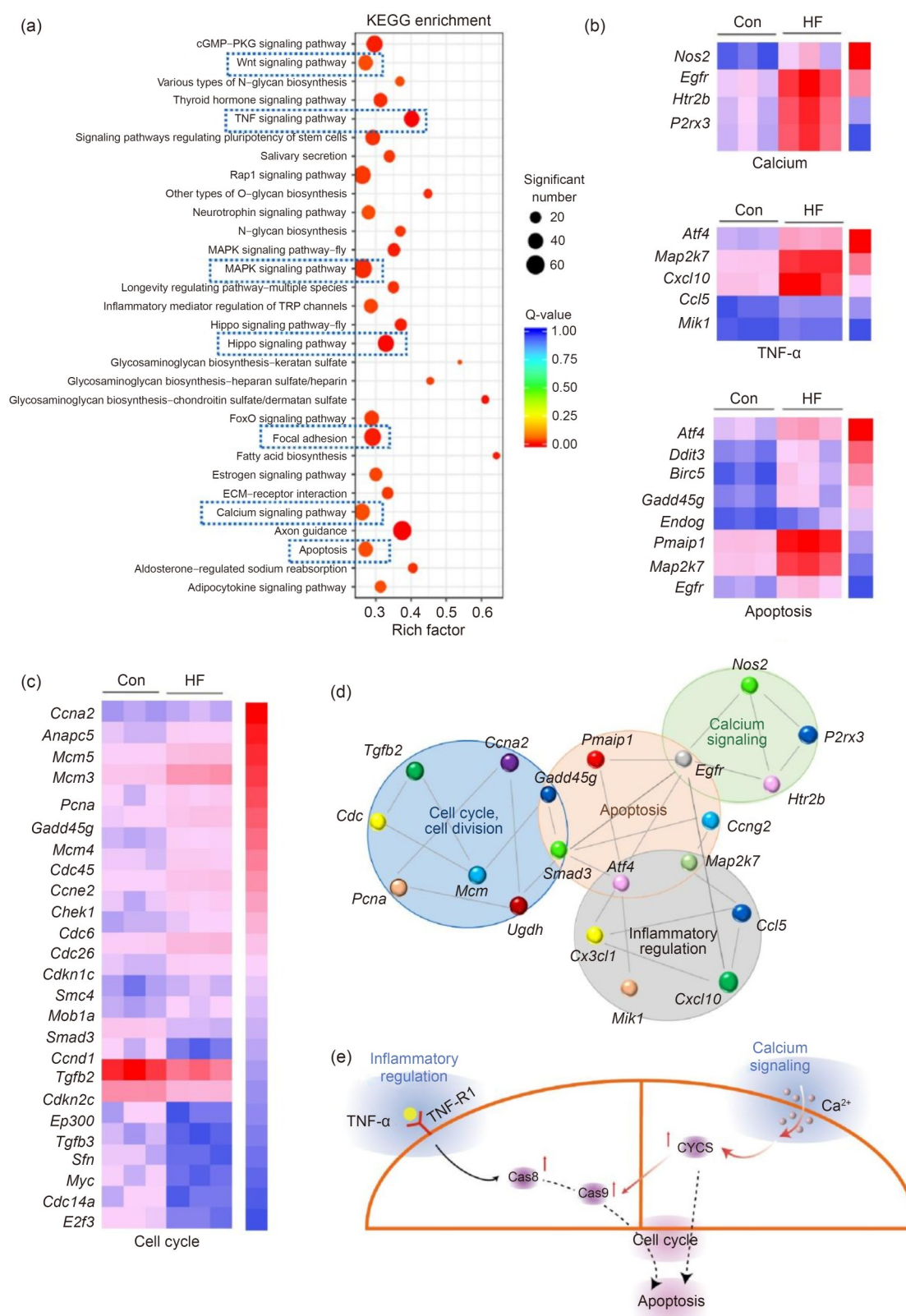


Fig. 10 Transcriptomic analysis revealing the mechanisms underlying folic acid (FA)-conjugated hydroxyapatite (HF) nanoparticle-mediated antitumor effects. (a) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially expressed genes (DEGs) between the HF and hydroxyapatite (HA) nanoparticle groups. (b, c) Heatmaps of DEGs involved in calcium signaling, tumor necrosis factor- α (TNF- α) signaling, apoptosis, and cell cycle regulation. (d) STRING protein-protein interaction network of DEGs. (e) Schematic illustration of the proposed apoptosis-related signaling mechanisms inferred from transcriptomic analysis

pronounced activation of apoptosis-related genes, including *Atf4*, *Ddit3*, *Birc5*, *Gadd45g*, *Endog*, *Pmaip1*, *Map2k7*, and *Egfr*, demonstrating that HF nanoparticles strongly activated apoptotic pathways leading to 4T1 cell death. Genes involved in cellular Ca^{2+} transport (e.g., *Nos2*, *Egfr*, *Htr2b*, and *P2rx3*) showed significant expression changes, indicating that HF nanoparticles disrupt intracellular calcium homeostasis and are detrimental to tumor progression. Additionally, TNF- α signaling-related genes (e.g., *Atf4*, *Map2k7*, *Cxcl10*, *Ccl5*, and *Mkl1*) were differentially regulated, reflecting inflammatory pathway activation. Notably, 25 DEGs associated with the cell cycle pathway were affected, highlighting HF nanoparticle-induced cell cycle arrest.

Interaction network analysis further categorized the DEGs into four major functional modules (Fig. 10d): apoptosis, cell cycle and division, calcium signaling, and inflammatory regulation. Several genes from distinct modules exhibited direct or indirect interactions, with shared nodes identified across categories. For example, *Gadd45* and *Smad3* jointly regulate both apoptosis and cell cycle progression; *Egfr* participates in both apoptosis and calcium signaling pathways; and *Atf4* and *Map2k7* act as crosstalk nodes between apoptosis and inflammatory regulation pathways. These shared genes likely serve as hubs mediating crosstalk among biological processes.

Collectively, these transcriptomic results demonstrate that HF nanoparticles suppress tumor progression by accelerating 4T1 cell apoptosis, inducing cell cycle arrest, disrupting calcium homeostasis, and activating inflammatory signaling, thereby reshaping the TME toward an antitumor state.

4 Conclusions

In this study, HF nanoparticles were engineered as a dual-targeting theranostic platform with enhanced 4T1 tumor cell internalization, achieved via FA receptor-mediated endocytosis. Their administration orchestrated a cascade of cytotoxic events: cell cycle arrest at the G2/M phase, calcium homeostasis dysregulation, mitochondrial membrane depolarization, and sustained ROS accumulation. Mechanistically, HF nanoparticles induced double-strand DNA breaks and mitochondrial outer membrane permeabilization, leading to CYCS release and activation of the caspase-9/-3 apoptotic pathway. In parallel, HF nanoparticles reprogrammed the tumor immune microenvironment by promoting macrophage polarization toward the proinflammatory M1 phenotype, accompanied by increased TNF- α , IL-1 β , and iNOS expression. This immune activation synergized with apoptotic signaling through NF- κ B-mediated caspase-8 activation, further amplifying tumor cell death. In vivo experiments in 4T1 breast cancer mouse models confirmed that HF nanoparticles suppress tumor growth by enhancing

inflammatory cell infiltration, modulating inflammatory cytokine networks, disrupting voltage-gated calcium channels, and inducing oxidative stress-mediated DNA damage. Importantly, DOX-loaded HF nanoparticles (i.e., HFD) exhibited superior antitumor efficacy, achieving a 34.6% greater tumor volume reduction compared with free DOX while minimizing systemic toxicity. These results demonstrate that HF nanoparticles represent a promising precision nanomedicine platform that integrates active tumor targeting with TME modulation to maximize therapeutic outcomes. The proposed design strategy, combining inorganic nanoparticle carriers with multimodal biological functions, provides a robust framework for developing next-generation anticancer drug delivery systems.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1631/bdm.2500501>.

Acknowledgements This work was supported by the National Natural Science Foundation of China (No. 31900948) and the Natural Science Foundation of Shaanxi Province (No. 2024JC-YBMS-168). We would also like to thank the Analytical & Testing Center of Northwestern Polytechnical University.

Author contributions HSL: methodology, data curation, and writing—original draft. WJK: methodology, data curation, and drawing. CHG: data curation. WHL: investigation and methodology. XQH: investigation. JW: resources, supervision, writing—review & editing, project administration, and funding acquisition.

Declarations

Conflict of interest The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval In this study, all methods were performed in accordance with relevant guidelines and regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee of Northwestern Polytechnical University (ethical approval code: 202301108). All animal procedures complied with animal protection agreements and regulations.

Data availability The data used to support the findings of this study are available from the corresponding author upon request.

Use of generative AI tools No generative AI tools were used in the preparation of this manuscript.

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