

RESEARCH PAPER

Ultrasound-targeted cavitation induced tumor vascular normalization and radiotherapy sensitization in breast cancer

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The limited therapeutic efficacy of radiotherapy (RT) in breast cancer is closely linked to hypoxia, which is caused by structurally abnormal vascular networks in the tumor microenvironment (TME). Tumor vascular normalization has emerged as a promising strategy to enhance radiosensitization by alleviating hypoxia. However, current vascular normalization methods face critical limitations, including off-target effects and inadequate spatiotemporal precision. Therefore, developing precise and efficient vascular normalization strategies is crucial for improving RT efficacy. In this study, we developed a tumor vascular normalization strategy based on ultrasound-targeted cavitation for radiosensitization. We synthesized VEGFR2-targeted microbubbles (MB@VEGFR2) to selectively accumulate on tumor vascular endothelial cells. Ultrasound-targeted cavitation enhanced local tumor blood perfusion, increased vascular diameter and maturity, and effectively alleviated hypoxia. By monitoring the time window of vascular normalization and administering RT within this period, tumor growth was significantly suppressed, with a 2.5-fold reduction in tumor volume compared to RT alone. Mechanistically, ultrasound-targeted cavitation promoted ATP release from endothelial cells, activating the PI3K/AKT/eNOS pathway to elevate nitric oxide (NO) production by 4-fold, thereby inhibiting abnormal vascular formation. This study demonstrates that ultrasound-targeted cavitation precisely achieves tumor vascular normalization, effectively improving RT efficacy.

ultrasound-targeted cavitation | vascular normalization | breast cancer | radiotherapy | VEGFR2-targeted microbubble

INTRODUCTION

Radiotherapy (RT) is a mainstay treatment for breast cancer. However, its therapeutic efficacy is constrained by the hypoxic tumor microenvironment (TME) (Liu et al., 2025; Xiong et al., 2025). Aberrant angiogenic signaling in tumor tissues leads to the formation of highly disorganized and abnormally dilated vascular networks, which have been recognized as a critical contributing factor to tumor hypoxia (Viallard and Larrivée, 2017). Vascular normalization improves the hypoxic TME, thereby achieving tumor radiosensitization and serving as a key strategy to enhance the efficacy of RT (Guo et al., 2024a). Combination therapy is gaining increasing attention as a promising approach for cancer treatment (Yan et al., 2024). Low-dose anti-angiogenic drugs can transiently induce tumor

vascular normalization, which is often used in combination with RT to enhance efficacy (Jiang et al., 2023). However, anti-angiogenic drugs are associated with limitations, including poor specificity and systemic adverse effects (Guelfi et al., 2024). Moreover, the brief time window of vascular normalization imposes strict requirements on the drug treatment plan. Therapeutic sensitization can only be achieved when combination therapy is implemented during the critical time window of vascular normalization (Wang et al., 2021). Therefore, there is an urgent need to develop more precise and effective strategies for vascular normalization.

The TME is characterized by remarkable complexity and heterogeneity (Janiszewska, 2020), presenting higher requirements for the precision of novel vascular normalization strategies. Approximately 60% of solid tumors exhibit uneven

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distribution of hypoxic regions, and the lack of localized regulation in these hypoxic regions correlates with reduced overall patient survival (Sun et al., 2012). Furthermore, traditional anti-angiogenic agents, due to their lack of specificity, increase metabolic demands and survival stress on cancer cells when acting on normal vascular regions. This subsequently enhances cellular glycolytic metabolism, consumes more oxygen, exacerbates intratumoral hypoxia, and ultimately diminishes therapeutic efficacy (Yang et al., 2023). Notably, blood vessels in hypoxic tumor regions often remain functionally inactive (Chen et al., 2023). Precisely inducing these dysfunctional vessels not only minimizes off-target adverse effects but, more importantly, enhances vascular normalization efficacy, thereby maximizing therapeutic efficacy.

In recent years, ultrasound cavitation has been extensively explored for inducing vascular function to treat tumors, owing to its unique spatiotemporal specificity and deep tissue penetration capabilities (Guo et al., 2024b; Kooiman et al., 2020). Ultrasound cavitation disrupts the blood-brain tumor barrier, enhancing vascular permeability to augment drug delivery efficiency while avoiding irreversible cerebral tissue damage (Wu et al., 2021). Concurrently, ultrasound cavitation elicits multifaceted inducing effects on vascular endothelial cell functionality, including regulation of oxygenation and nitric oxide biosynthesis, thereby extending its utility to radiosensitization and vascular normalization applications (Karthikesh and Yang, 2021). Targeted microbubbles (MBs) have demonstrated the capacity to specifically and efficiently accumulate at pathological sites, thereby precisely amplifying the therapeutic effects of ultrasound cavitation (Langeveld et al., 2021). Ultrasound-targeted cavitation facilitates the accumulation of

chemotherapeutic drugs in tumor tissue and enhances therapeutic efficacy. In contrast, the application of targeted MBs alone without US exhibited no significant therapeutic effect (Shimamoto et al., 2020). During the early stages of abnormal tumor angiogenesis, vascular endothelial growth factor receptor 2 (VEGFR2) demonstrates characteristically high expression (Shen et al., 2022; Zhu et al., 2017), serving as a therapeutic target for ultrasound-targeted cavitation to precisely induce vascular endothelial cells. Ultrasound-targeted cavitation can overcome intertumoral heterogeneity-induced individual differences, enabling precise regulation of cell composition within complex TME to improve therapeutic effects (Liu et al., 2023a), providing a promising strategy to precisely induce endothelial cell function and address existing challenges in tumor vascular normalization therapies.

In this study, we synthesized MB@VEGFR2 and compared it with conventional MB to verify its excellent vascular endothelial cell targeting ability. By optimizing ultrasound cavitation protocols, we demonstrated effective vascular normalization both *in vivo* and *in vitro* induced by ultrasound-targeted cavitation and monitored the time window of vascular normalization. The spatiotemporal precision of ultrasound irradiation, when strategically synchronized with RT cycles, has demonstrated significant radiosensitization. Furthermore, we explored the mechanisms of ultrasound-targeted cavitation in inducing tumor vascular normalization, including ATP release and the activation of the PI3K/AKT/endothelial nitric oxide synthase (eNOS) pathway, which leads to endogenous NO release. Therefore, we developed a novel ultrasound-induced tumor vascular normalization strategy to enhance the efficacy of RT (Figure 1).

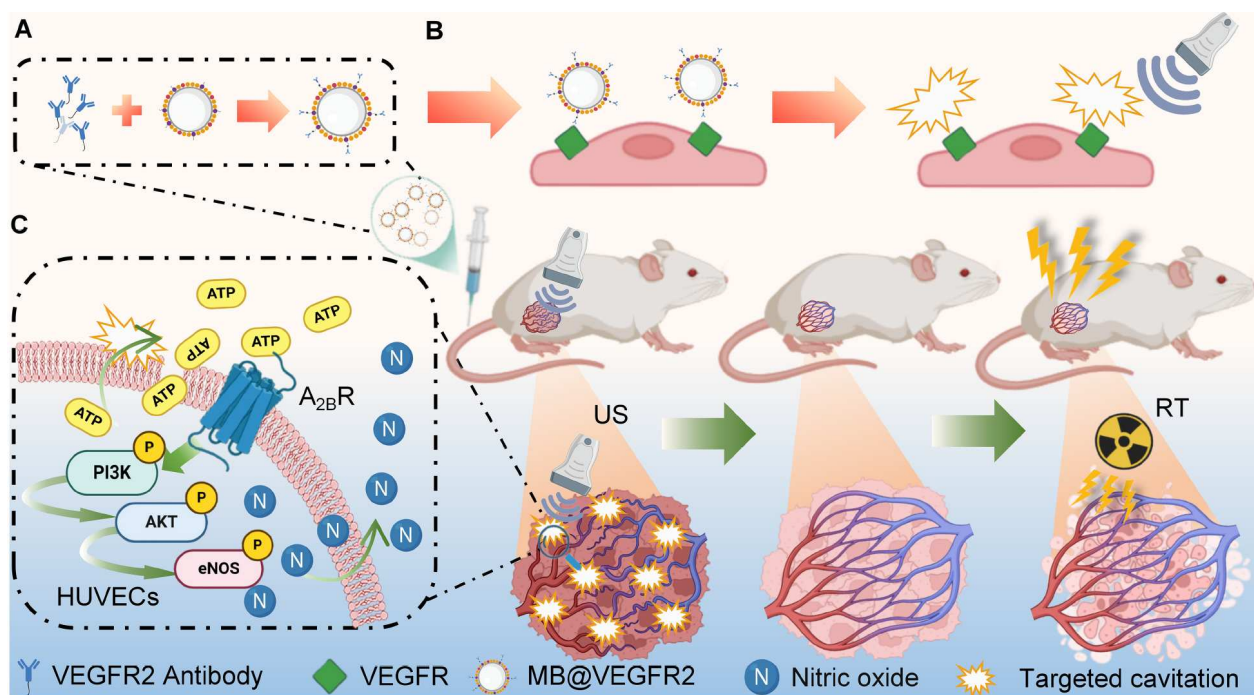


Figure 1 Schematic illustration of ultrasound-targeted cavitation-induced tumor vascular normalization for enhancing the efficacy of RT. A, VEGFR2 antibodies were conjugated to MB via avidin-biotin interactions. B, MB@VEGFR2 selectively accumulated on tumor vascular endothelial cells. Ultrasound-targeted cavitation was applied to induce tumor vascular normalization, improving the hypoxic TME, ultimately enhancing the efficacy of RT in breast cancer. C, Ultrasound-targeted cavitation transiently opens endothelial cell membranes, triggering ATP release into the extracellular space. This activates the A₂B receptors, which drive endogenous NO production through the PI3K/AKT/eNOS signaling pathway, thereby inducing vascular normalization.

RESULTS

Characterization of MB@VEGFR2

The preparation of MB@VEGFR2 was performed as follows: biotinylated lipid MBs were first synthesized using lipid materials and fluorocarbon gas. These MBs were then conjugated with avidin, linked to anti-VEGFR2 antibodies via a biotin-avidin interaction (Figure 2A). The prepared MB@VEGFR2 exhibited an average diameter of $(1,725 \pm 101.4)$ nm and surface zeta potential of (-4.08 ± 0.11) mV (Figure 2B and C). Compared with MB, MB@VEGFR2 had a slightly larger particle size and a lower surface zeta potential. Using light microscopy and SEM, the morphology of MB@VEGFR2 was a regular circular morphology with a translucent middle part (Figure S1). The particle size of MB@VEGFR2 stored at 4°C on the first day vs. the seventh day was detected, and the latter was slightly larger than that on the first day with no significant difference (Figure S2). Ultrasound imaging monitoring showed that MB and MB@VEGFR2 maintained optimal imaging performance during the initial 24 h period at room temperature, followed by rapid signal decay. Both MBs and MB@VEGFR2 demonstrated stability for 24 h under ambient conditions without significant performance discrepancies (Figure S3). Fluorescence microscopy showed that MB@VEGFR2 displayed distinct red fluorescence signals (corresponding to the lipid shell) and green fluorescence signals (corresponding to antibodies), whereas MBs exhibited only red fluorescence, demonstrating successful conjugation of anti-VEGFR2 antibodies to the MB@VEGFR2 (Figure 2D).

The MB@VEGFR2 was successfully synthesized, with surface conjugation of anti-VEGFR2 antibodies confirmed. Furthermore, its targeting capability toward vascular endothelial cells was validated. Cell viability was evaluated using the CCK-8 assay following co-incubation of human umbilical vein endothelial cells (HUVECs) with varying concentrations of MB@VEGFR2. The results demonstrated that cell viability remained consistently above 95% across all tested concentrations, with no statistical differences observed (Figure 2E). HUVECs were stained with DAPI and DiO, while MB@VEGFR2 or MB was labeled with DiI. Following co-incubation and washing, fluorescence microscopy showed minimal red fluorescence signals in the MB group. In contrast, the MB@VEGFR2 group exhibited abundant red fluorescence colocalized with green signals, indicating tight adhesion of MB@VEGFR2 to the cell membrane and demonstrating robust targeting specificity toward HUVECs (Figure 2F). Quantitative analysis of the red fluorescence signal in the MB@VEGFR2 group was approximately 19.04 times compared to the MB group (Figure 2G).

In vivo vascular normalization induced by ultrasound-targeted cavitation

MB@VEGFR2 was irradiated using different ultrasound protocols, and the changes in imaging effect were observed to explore the appropriate ultrasound cavitation parameters. The signal of MB@VEGFR2 gradually decreased with the time of ultrasound irradiation and the increase of ultrasound power (Figure S4). Analysis of the ultrasound images showed that the ultrasound signal of MB@VEGFR2 was significantly reduced after ultrasound irradiation at 0.6 W/cm². Under ultrasonic irradiation at 0.2 and 0.4 W/cm², the imaging signal intensity showed no

significant reduction after 120 s. In contrast, when applying higher-intensity protocols (0.8, 1.0, and 1.2 W/cm²), the signal intensity at 120 s exhibited no statistically significant difference compared to that observed at 0.6 W/cm² (Figure S4). In view of the potential adverse effects of increased ultrasound parameters, 0.6 W/cm², 20% DC, and 120 s were selected as the subsequent ultrasound cavitation protocols. The results confirmed that MB@VEGFR2 co-incubated with cells under ultrasonic irradiation at this protocol did not induce significant cell death (Figure S5).

MB@VEGFR2 was intravenously administered to mice, followed by ultrasound irradiation of the tumor. CEUS was utilized to continuously monitor tumor perfusion signals for 7 consecutive days, aiming to validate the efficacy of vascular normalization and determine the time window of vascular normalization (Figure 3A). Perfusion images of the maximum tumor cross-section demonstrated that, from day 2 post-treatment onward, the MB@VEGFR2+US group exhibited significantly higher perfusion intensity compared to other groups, peaking on day 3. In contrast, the MB+US group showed only mild enhancement, while the Control and US groups exhibited negligible changes in perfusion signals throughout the observation period (Figure 3B). Quantifying the tumor perfusion area and the peak of the ultrasound signal, the results showed that ultrasound combined with MB@VEGFR2 could significantly induce tumor vascular normalization, and the ultrasound signal and the tumor perfusion area reached the peak at day 3, and then gradually declined, and the efficacy was significantly stronger than the other treatments (Figure 3C and D). To confirm the occurrence of vascular normalization, tumor tissues from the peripheral and central regions were subjected to immunofluorescence staining for vascular endothelial cells (CD31) and pericytes (α -SMA) on day 3 post-ultrasound irradiation, aiming to assess vascular morphology and functionality (Figure 3E). In the tumor central region, the MB@VEGFR2+US group exhibited vessel diameters and vascular maturation levels approximately 1.44- and 2.58-fold higher than those in the Control group, respectively, accompanied by a 72.27% reduction in vessel density. Similarly, in the tumor periphery, vessel diameters and maturation levels in the MB@VEGFR2+US group were 1.53- and 3.80-fold greater than those of the Control group (Figure 3F–I). HIF-1 α immunofluorescence staining was performed on tumor tissues to evaluate vascular functional improvement and hypoxia alleviation. Fluorescence analysis showed a significant reduction in HIF-1 α expression in the MB@VEGFR2+US group compared to other treatment groups (Figure 3J). Quantitative analysis demonstrated that the HIF-1 α fluorescence intensity in the MB@VEGFR2+US group decreased by approximately 86.28% vs. the Control group (Figure 3K).

Mechanism of tumor vascular normalization induced by ultrasound-targeted cavitation

We investigated the signaling pathway through which ultrasound-targeted cavitation induces tumor vascular normalization. To assess the potential efficacy of ultrasound-targeted cavitation on angiogenesis, we used 2D Matrigel cultures to simulate vascular formation. Over a 6 h observation period, the Control and US groups displayed similar compact tubular structures, showing no adverse effect of US on tubule formation. In contrast, the MB+US group exhibited a slightly reduced number of tube

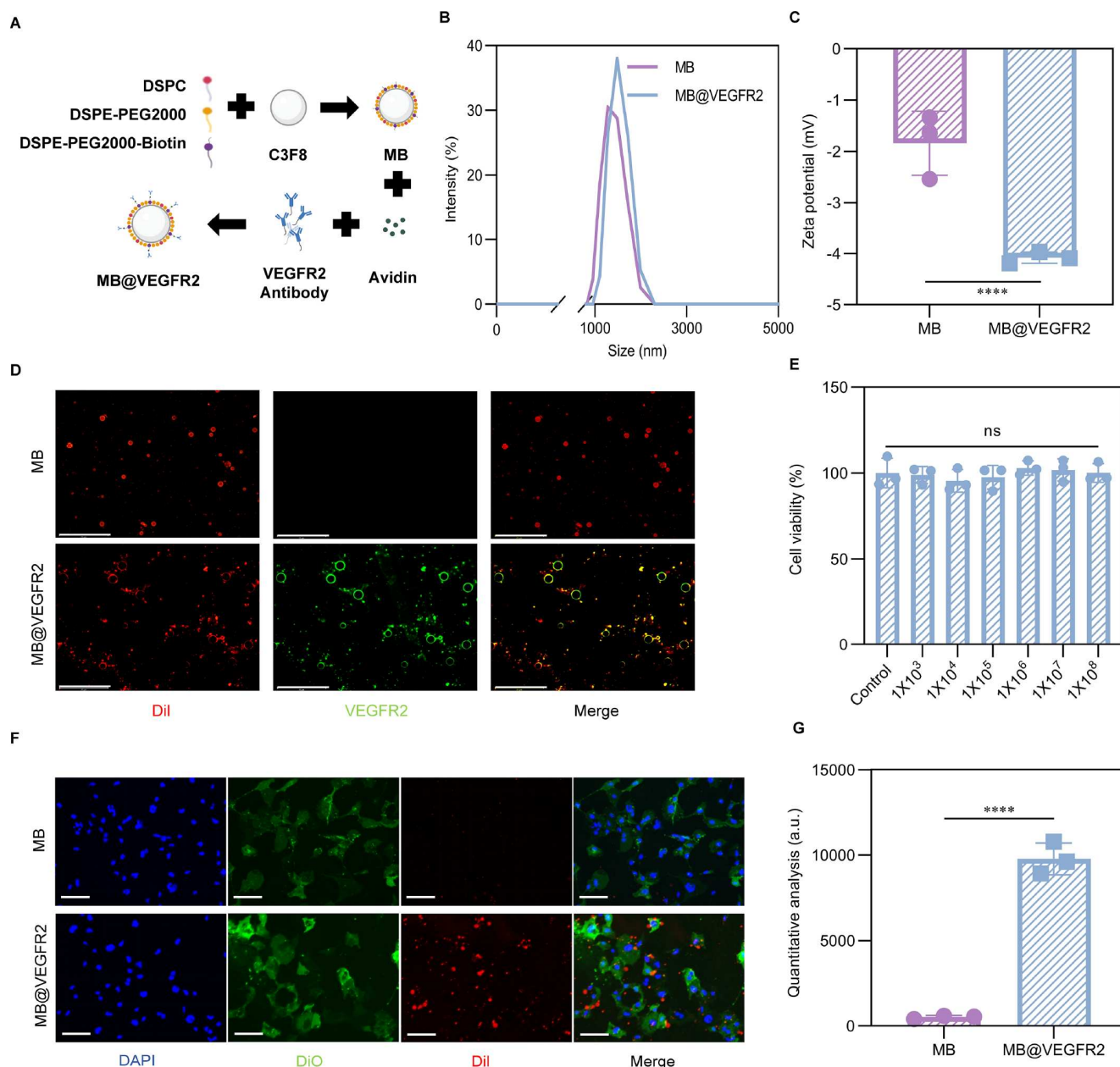


Figure 2 Characteristics of MB@VEGFR2. A, Schematic diagram of MB@VEGFR2 preparation. B, Particle size of MB and MB@VEGFR2 ($n=3$). C, Zeta potential of MB and MB@VEGFR2 ($n=3$). D, Fluorescence image of DiI (red) with FITC-labeled secondary antibody (green) fluorescence images of MB and MB@VEGFR2, scale bar, 200 μ m. E, Cytotoxicity of MB@VEGFR2 ($n=3$). F, Fluorescence images of DAPI (blue), DiO (green) stained HUVECs with DiI (red) stained MB and MB@VEGFR2, scale bar, 100 μ m. G, Quantitative analysis of the red fluorescence signal ($n=3$). Quantitative results were expressed as mean $\pm$ SD and analyzed by one-way analysis of variance (ANOVA) (ns, $P>0.05$; ****, $P<0.0001$).

branches and tube lengths, while the MB@VEGFR2+US group failed to form a typical tube blood vessel network (Figure 4A). Statistical analysis revealed that the number of vascular junctions in the MB@VEGFR2+US group decreased by 92.0% compared to the Control group. This reduction rate was significantly greater than the 55.3% decrease observed in the MB+US group, further confirming the superior anti-angiogenic efficacy of ultrasound-targeted cavitation (Figure 4B). Given the potential role of transient cell membrane perforation caused by ultrasound-targeted cavitation in vascular normalization, we assessed HUVECs' perforation using PI, which is excluded by intact cell membranes. Fluorescence of nuclear DNA indicated

the degree and duration. When PI was added during ultrasound-targeted cavitation, most cells exhibited red fluorescence, whereas adding PI 10 or 20 min after irradiation resulted in red fluorescence in only 5%–10% of cells (Figure 4C and D). This suggests membrane resealing occurred within 10 min post ultrasound-targeted cavitation. Perforation facilitated the release of intracellular substances like ATP. Using commercial kits, we detected ATP levels in cell culture supernatants under different treatments. ATP release in the MB@VEGFR2+US group was 90% and 165% higher than in the MB+US and Control groups (Figure 4E). These findings showed that ultrasound-targeted cavitation-mediated perforation is a prerequisite for ATP release.

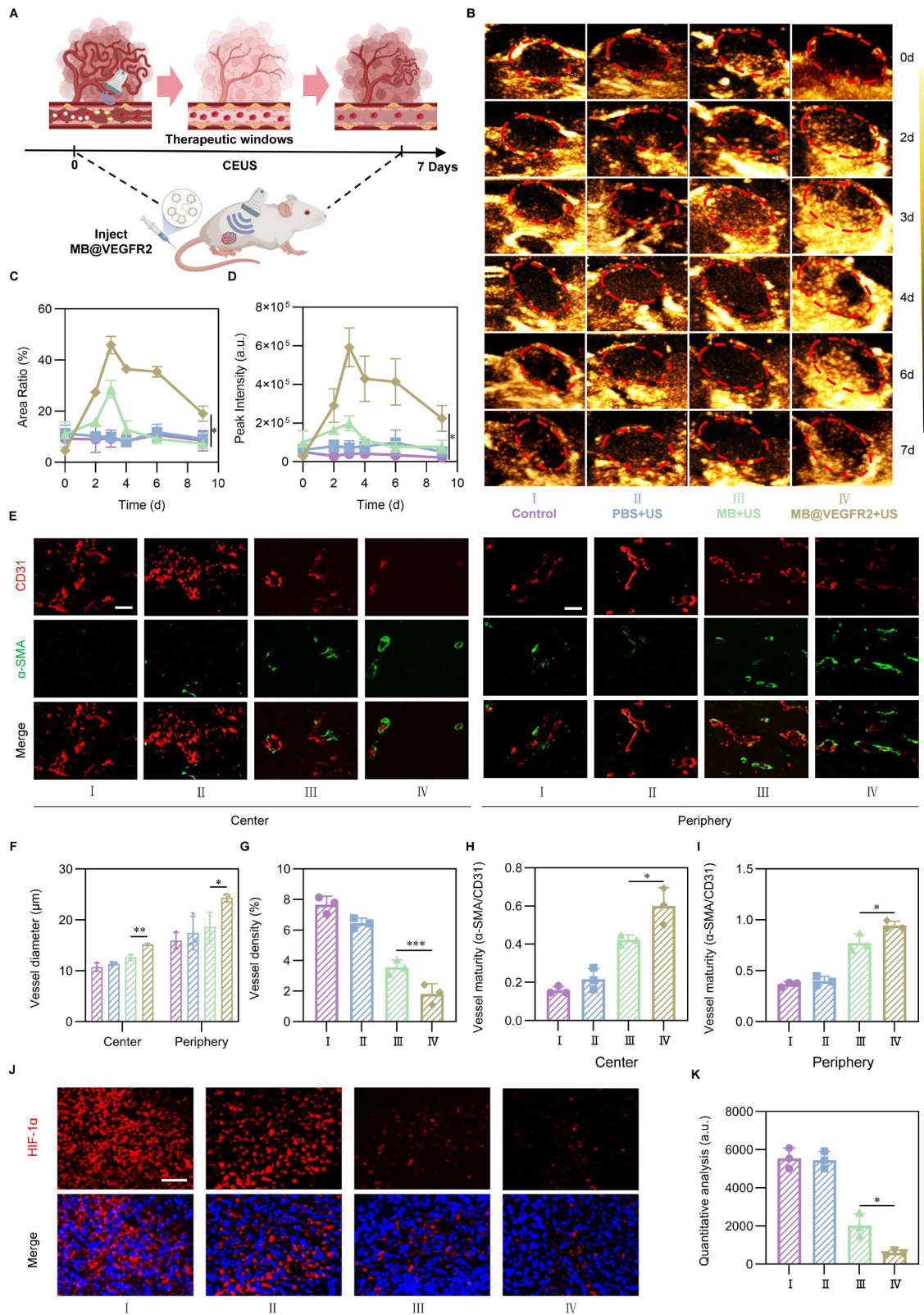


Figure 3 Ultrasound-targeted cavitation induces tumor vascular normalization. A, Schematic representation of CEUS-monitored tumor vascular normalization process. B, Ultrasound perfusion images 0–7 d after ultrasound irradiation in the (I) Control group, (II) US group, (III) MB+US group, and (IV) MB@VEGFR2+US group ($n=3$). C and D, Analysis of (C) signal intensity and (D) signal area of CEUS within 0–7 d ($n=3$). E, CD31 and α -SMA immunofluorescence in the central and peripheral region of the tumors ($n=3$). Scale bar, 100 μ m. F, Vascular vessel diameter in the tumor central and peripheral region ($n=3$). G, Vascular density in the tumor central region of the tumor ($n=3$). H and I, Vascular maturity in the tumor central (H) and peripheral (I) regions ($n=3$). J, HIF-1 α immunofluorescence in the central region of the tumor ($n=3$). K, Quantitative analysis of HIF-1 α fluorescence signal ($n=3$). Scale bar, 100 μ m. Quantitative results were expressed as mean $\pm$ SD and analyzed by one-way ANOVA (*, $P<0.05$; ***, $P<0.001$).

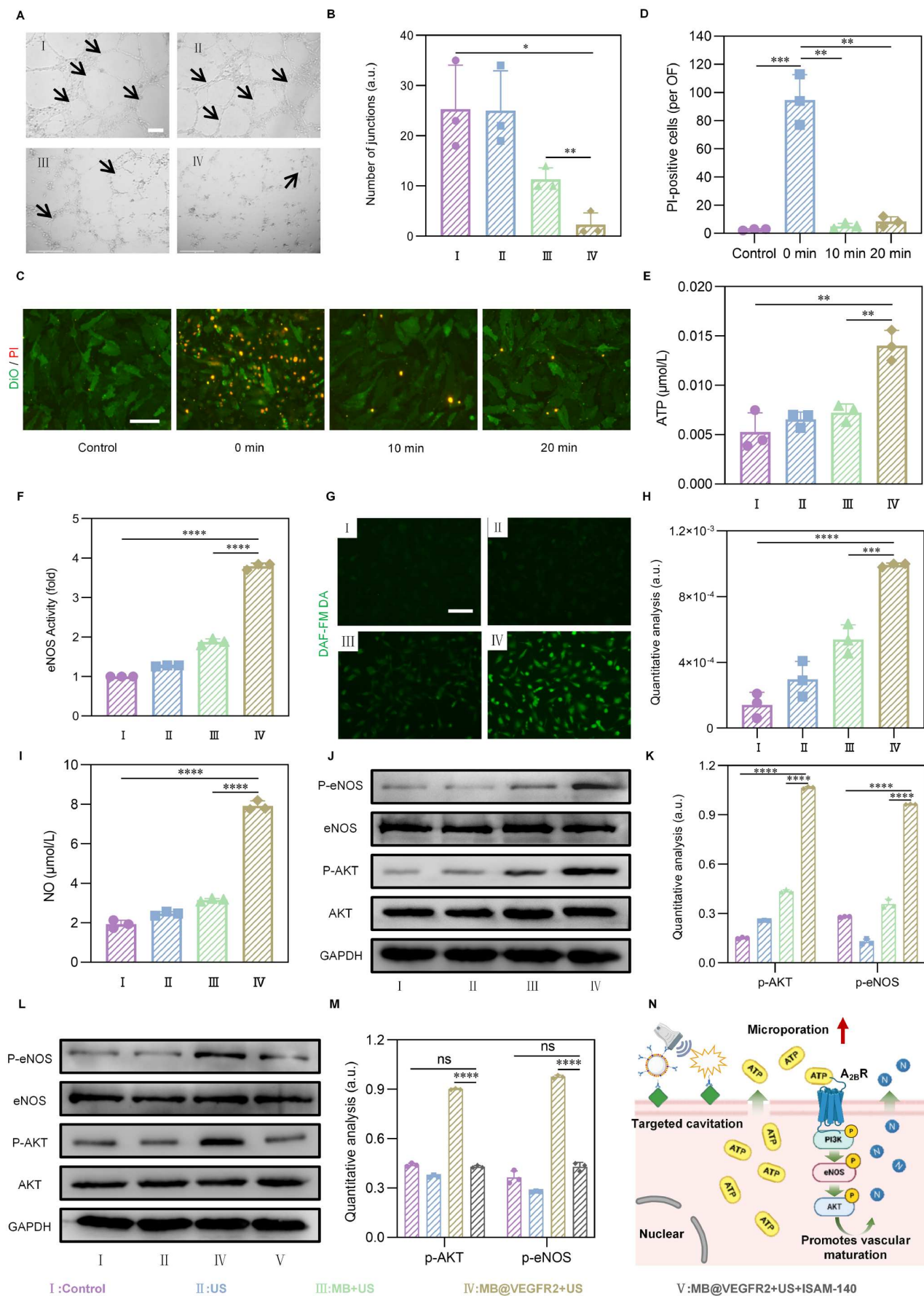


Figure 4 Mechanisms of ultrasound-targeted cavitation in inducing tumor vascular normalization. A and B, Assessment of angiogenesis under different treatment conditions (scale bar, 100 μm), the arrows indicate a representative tubular structure (A) and corresponding quantitative analysis ($n=3$) (B). C, Fluorescence microscopy showing HUVECs with PI-positive nuclei (red fluorescence) and DiO-stained green fluorescence (scale bar, 50 μm). D, The number of cells with PI-positive nuclei per optical field (OF) ($n=3$). E, ATP content in the supernatants of HUVECs under different treatment conditions ($n=3$). F, eNOS activity in HUVECs under different treatment conditions ($n=3$). G and H, Fluorescence images of NO in HUVECs measured with the DAF-FM DA fluorescent probe under different treatment conditions (scale bar, 100 μm) (G) and quantitative analysis of fluorescence intensity ($n=3$) (H). I, NO content in the supernatants of HUVECs under different treatment conditions ($n=3$). J and K, Total and phosphorylated protein levels of AKT and eNOS under different treatment conditions were analyzed by WB analysis (J), and corresponding quantitative analysis ($n=3$) (K). L and M, Total and phosphorylated protein levels of AKT and eNOS after adding the ISAM-140 inhibitor under different treatment conditions, were analyzed by WB analysis (L), and corresponding quantitative analysis ($n=3$) (M). N, The schematic illustrates the pathway of tumor vascular normalization induced by ultrasound-targeted cavitation. Quantitative results were expressed as mean $\pm$ SD and analyzed by one-way ANOVA (ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$).

ATP and its metabolites activate the A_{2B} receptor, mediating intracellular signal transduction, particularly involving critical signaling pathways such as the PI3K/AKT/eNOS pathway. We hypothesized that ultrasound-targeted cavitation could enhance the phosphorylation and activity of these proteins, promoting NO production. Using commercial kits, we found eNOS activity in MB@VEGFR2+US groups-treated cells was twice that of the MB+US group and three times that of the control group (Figure 4F). Increased eNOS activity led to higher intracellular NO production, as detected using the DAF-FM DA probe. Fluorescence analysis showed significantly enhanced green fluorescence on the cells of MB@VEGFR2+US groups (Figure 4G). Quantitative analysis showed fluorescence intensity in the MB@VEGFR2+US group was 1.5 and 5.5 times higher than in the MB+US and Control groups (Figure 4H). NO levels in the cell culture supernatant were measured, showing 7.9 $\mu\text{mol/L}$ in the MB@VEGFR2+US group, compared to 3.1 and 1.9 $\mu\text{mol/L}$ in the MB+US and Control groups (Figure 4I), consistent with intracellular NO diffusion into the extracellular space.

Western blot (WB) analysis further demonstrated activation of the PI3K/AKT/eNOS pathway by ultrasound-targeted cavitation. Phosphorylated AKT and eNOS protein levels increased significantly in the MB@VEGFR2+US group, while total protein levels remained unchanged (Figure 4J). Protein quantification analysis revealed phosphorylated AKT levels were 2.5 and 7 times higher, and phosphorylated eNOS levels were 2.7 and 3.5 times higher, than those in the MB+US and Control groups (Figure 4K). To determine whether ATP and its metabolites are necessary for A_{2B} receptor-mediated activation of the PI3K/AKT/eNOS pathway and NO production, we co-cultured cells with the A_{2B} receptor inhibitor ISAM-140. ISAM-140 is a potent and highly selective A_{2B} receptor antagonist that competitively blocks adenosine binding, thereby inhibiting a series of downstream pathophysiological processes upon receptor activation (El Maaoui et al., 2016). Following ultrasound-targeted cavitation stimulation, phosphorylated AKT and eNOS levels did not significantly increase in the MB@VEGFR2+US+ISAM-140 group (Figure 4L). Protein quantification confirmed these results (Figure 4M), demonstrating that ultrasound-targeted cavitation is essential for A_{2B} receptor-mediated activation of the PI3K/AKT/eNOS pathway (Figure 4N).

Ultrasound-targeted cavitation enhances radiosensitization *in vivo*

To evaluate the *in vivo* radiosensitization of vascular normalization induced by ultrasound-targeted cavitation in breast cancer, the 4T1 murine breast cancer model was subjected to different treatments (Figure 5A). In the MB@VEGFR2+US+RT group, tumor volume was significantly reduced, with an average volume of 212.8 mm^3 on day 12, compared to 384.6 mm^3 in the

MB+US+RT group and 545.5 mm^3 in the RT group. In contrast, tumor volumes in the Control group and the MB@VEGFR2+US group increased to approximately 1,700 mm^3 (Figure 5B–D). The MB@VEGFR2+US+RT group showed the lowest tumor weight (0.2 g), while the Control group exhibited the highest tumor weight (1.14 g), showing a sixfold increase compared to the MB@VEGFR2+US+RT group (Figure 5E). Histopathological examination of tumor necrosis and apoptosis was conducted via H&E staining and TUNEL assay. H&E-stained sections from the MB@VEGFR2+US+RT group exhibited marked nuclear fragmentation and chromatin dissolution, accompanied by widened intercellular gaps (Figure 5F). Consistently, TUNEL fluorescence imaging demonstrated a substantial increase in apoptotic cells in the MB@VEGFR2+US+RT group relative to controls (Figure 5G).

Systemic safety evaluation showed comparable body weight among all groups during the treatment (Figure S6). H&E analysis of major organs from MB@VEGFR2+US+RT-treated animals showed tissue architecture without inflammatory infiltrates or structural anomalies (Figure S7). Quantitative analysis of hepatic (ALT and AST) and renal (BUN and CREA) biomarkers confirmed physiological homeostasis in both treatment and control groups (Figure S8).

DISCUSSION

Tumor vascular normalization, as a critical strategy to improve TME, shows significant clinical value through precise induction to amplify therapeutic sensitization (Zheng et al., 2021). The delicate equilibrium between normalized and abnormal vascular states during therapeutic intervention is termed the “normalization window”. Our findings showed that tumor blood perfusion began to increase on day 2 post ultrasound-targeted cavitation, peaked on day 3, and gradually declined thereafter. Over the seven-day monitoring period, perfusion levels in the MB@VEGFR2+US group remained elevated compared to the MB+US group and other groups, suggesting optimal vascular normalization efficacy on day 3. Studies using low-dose apatinib reported vascular normalization emerging on day 5 and peaking on day 10 in murine models (Jiang et al., 2023). Similarly, although lenvatinib has been demonstrated to induce tumor vessel normalization earlier and more effectively compared to sorafenib, an effect that predominantly manifests on day 4 (Une et al., 2021). This difference in kinetics can be attributed to their distinct modes of action. Anti-angiogenic drugs, such as apatinib, must undergo processes including systemic distribution, cellular uptake, and receptor binding to exert their effects, which inherently delays the peak of therapeutic action (Liu et al., 2023b). In contrast, ultrasound-targeted cavitation acts as a localized physical intervention. Its mechanical energy directly and instantaneously induces endothelial microporation, triggering rapid ATP release and subsequent swift activation of the

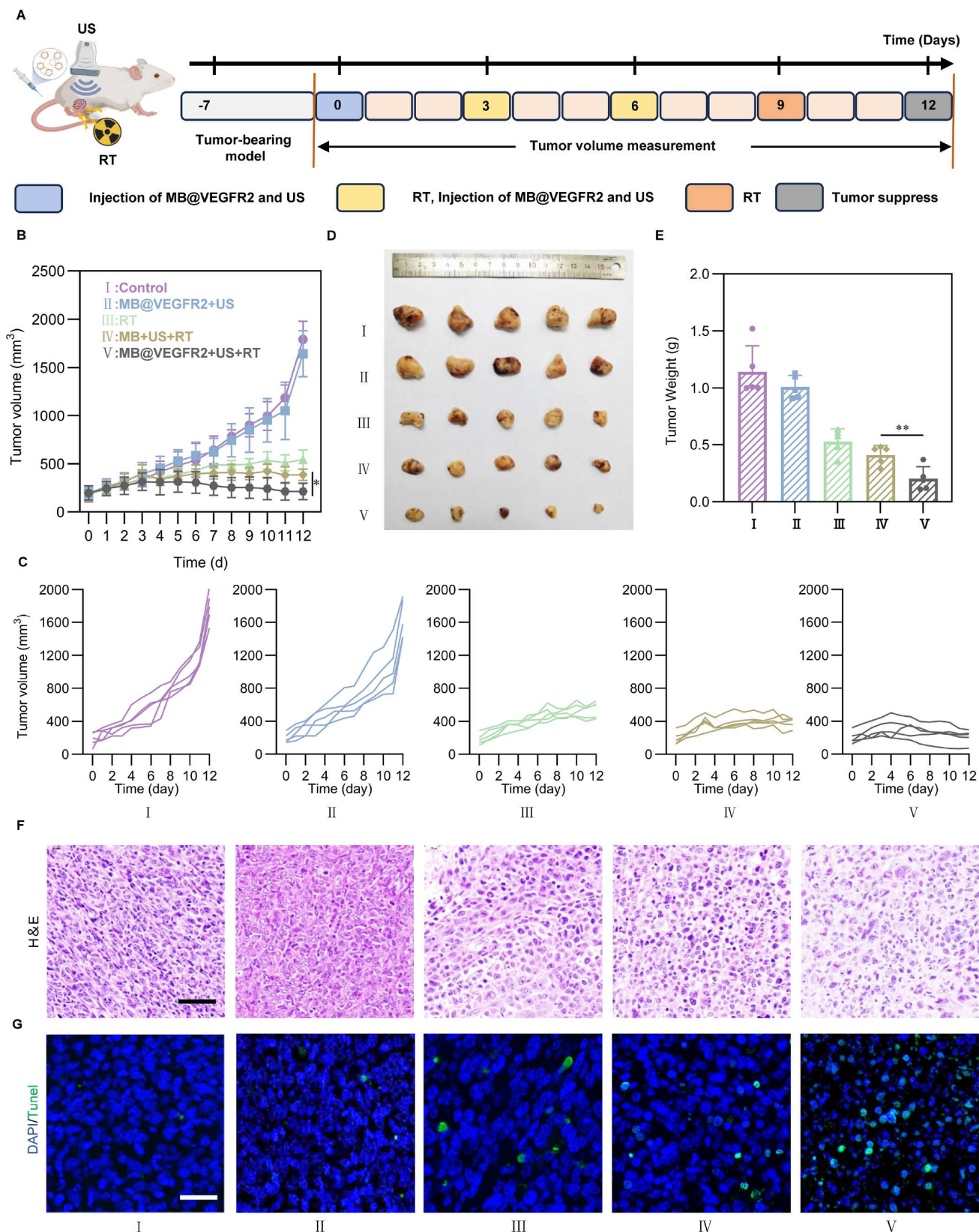


Figure 5 Ultrasound-targeted cavitation induces tumor vascular normalization to enhance radiosensitization *in vivo*. A, Schematic diagram illustrating the therapeutic procedure of ultrasound-targeted cavitation for inducing tumor vascular normalization to enhance radiosensitization. B, Tumor volume curves under different treatment conditions (n=5). C, Individual tumor growth curves under different treatment conditions (n=5). D, Representative tumor images under different treatment conditions (n=5). E, Tumor weights in each treatment group (n=5). F and G, H&E and TUNEL staining of tumors in each treatment group. Scale bar, 50 μ m. Statistical analysis was conducted using one-way ANOVA followed by Tukey's test (**, $P<0.01$).

A₂B/PI3K/AKT/eNOS signaling pathway. This disparity highlights that ultrasound-targeted cavitation enables precise induction of tumor vascular endothelial cells to accelerate the onset of the time window of tumor vascular normalization, thereby conferring clinical advantages for synergistic RT.

However, as a localized physical intervention, the safety and efficacy of ultrasonic cavitation in extending the window of vascular normalization are influenced by the interplay of multiple factors. MBs with a small and uniform size can enhance their ability for direct contact with cells, thereby improving the ultrasonic cavitation effect (Tayler et al., 2019). Simultaneously, the outcomes of stable cavitation exhibit a complex interdependence with MB concentration, ultrasonic peak negative pressure, and pulse intensity (Song et al., 2025). These characteristics complicate the comparison of its tumor vascular normalization effects with drugs administered at varying doses, posing challenges for clinical application. Furthermore, the active targeting and long-term circulation capability of MBs, combined with the deep-tumor penetration efficacy of ultrasound, are closely linked to the permeability and retention of MBs in tumor tissue, as well as the specificity of the treatment (Olsman et al., 2020; Sun et al., 2022). Clinical translation of this strategy necessitates rigorous evaluation of its safety and efficacy in human subjects. The ultrasound parameters employed in our preclinical protocol demonstrated a favorable safety profile in animal models, and the acoustic intensity used was maintained below the safety limit defined by the U.S. Food and Drug Administration for diagnostic ultrasound (Duck, 2007). Furthermore, a comprehensive analysis of molecular subtypes based on biological characteristics and therapeutic responses contributes to the development of more effective therapeutic targets and strategies (Ji et al., 2025). Establishing a comprehensive understanding of the interactive relationships among diverse MB characteristics, tumor types, ultrasonic parameters, and biological effects is a prerequisite for its successful clinical translation.

The mechanisms underlying ultrasound cavitation-induced tumor vascular normalization remain poorly understood, particularly regarding its effects on abnormal vascular endothelial cells. M2 macrophages can express pro-angiogenic factors and contribute to the aberrant angiogenic (Chen and Bonaldo, 2013). Ultrasonic cavitation has been shown to induce macrophages toward the M1 phenotype, thereby achieving tumor vascular normalization (Lin et al., 2023). However, ultrasound cavitation primarily acts on vascular endothelial cells, and macrophage infiltration within tumor tissues remains relatively limited (Kang et al., 2022). Ultrasound cavitation induces vasodilation, enhances intracellular hydrogen peroxide and ATP production, and regulates endothelial metabolic activity (Belcik et al., 2017; Juffermans et al., 2009). Inspired by its applications in stroke and thrombolysis for preventing ischemia-reperfusion injury, we focused on the role of ultrasound-targeted cavitation in facilitating ATP release from vascular endothelial cells during vascular normalization (Belcik et al., 2015; Cui et al., 2017). Our study demonstrates that ultrasound-targeted cavitation induces transient endothelial membrane perforation, triggering ATP release. Subsequent ATP metabolism generates adenosine, which binds to the A₂B receptor, activating the PI3K/AKT/eNOS pathway and enhancing eNOS phosphorylation. As a key enzyme for NO synthesis, activated eNOS promotes endogenous NO production and release. This intervention elevated extracellular NO levels by approximately 4-fold, achieving tumor vascular normalization

and effectively addressing the limitations of existing approaches.

Aberrant tumor vascular network is a critical factor contributing to radioresistance, and achieving tumor vascular normalization is crucial for improving the efficacy of cancer treatment (Guo et al., 2024a). Ultrasound cavitation not only enhances vascular permeability but also suppresses tumor angiogenesis by inducing endothelial cell apoptosis. Studies have shown that RT combined with ultrasound cavitation-induced endothelial apoptosis enhances radiosensitization, achieving a 170% reduction in tumor growth compared to RT alone at 7 d post-treatment (Daecher et al., 2017). However, endothelial apoptosis may paradoxically elevate metastatic risks, revealing clinical implementation challenges (Yang et al., 2019). Additionally, aberrant ATP metabolism in tumor cells may activate the corresponding downstream signaling axis to promote metastasis, and the activation of the PI3K/Akt pathway in tumor cells contributes to tumor metastasis, vascular dysfunction, and therapeutic resistance (Ganesan et al., 2024; Li et al., 2025). Our study demonstrates that precise ultrasound-targeted cavitation can induce transient cell membrane perforation under preserved endothelial viability, triggering ATP release and subsequent endogenous NO production, ultimately achieving tumor vascular normalization. It enhances blood perfusion and oxygen delivery efficiency within tumor tissue, ameliorates the hypoxic micro-environment, downregulates HIF-1 α expression, and consequently increases tumor sensitivity to RT (Zhou et al., 2020). Following three cycles of combination therapy, the MB@VEGFR2+US+RT group showed an average tumor volume of 212.8 mm³, whereas the RT group showed 545.5 mm³.

In summary, we have developed a novel radiosensitization strategy that achieves tumor vascular normalization through ultrasound-targeted cavitation. This approach leverages the spatiotemporal precision of ultrasound-targeted cavitation to selectively enhance endogenous NO production in tumor vascular endothelial cells, thereby effectively realizing tumor vascular normalization in breast cancer, ultimately alleviating hypoxia. This approach demonstrates the mechanisms of ultrasound-targeted cavitation, inducing tumor vascular normalization, offering innovative insights for cancer treatment. Despite these advancements, future studies should prioritize theranostic integration of targeted MBs and investigate synergistic mechanisms involving endothelial function, hemodynamics, and TME. Furthermore, we will further expand the sample size and incorporate more comprehensive experimental groups. Building upon the efficacy assessment, we will monitor long-term survival and recurrence rates to further investigate the stability, safety, and reproducibility of the treatment. This will be integrated with an in-depth exploration of drug delivery strategies and applicability to other tumor types, thereby promoting clinical translation.

METHODS AND MATERIALS

Preparation and characterization of MB and MB@VEGFR2

7.5 mg of 1,2-distearoyl-SN-glycero-3-phosphocholine (DSPC, Aladdin, USA), 1.5 mg of distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG2000, Ruixibio, Xi'an, China), 1.5 mg of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[(polyethylene glycol)-2000]-biotin (DSPE-PEG2000-Biotin, Ruixibio), and 1 mg of distearoylphosphatidylglycerol

(DSPG, AVANTI, USA) were completely dissolved in 1 mL of dichloromethane. The solution was placed in a 60°C water bath to evaporate for 2 h, followed by further evaporation using a rotary evaporator for 4 h to obtain a colorless lipid film. Then, 800 μ L of 5% glycerol solution was added and heated to 65°C with ultrasonic agitation for 15 min. Perfluoropropane gas was introduced to replace the air completely. After 45 s of shaking with a silver-mercury mixer, the MB was obtained.

Next, 20 μ L of 1 mg/mL avidin (Solarbio, Beijing, China) was added, and the mixture was incubated in the dark at 4°C for 30 min. After centrifuging at 400 $\times g$ for 5 min, the lower liquid was removed. The pellet was resuspended in 1 mL of PBS and shaken. This washing step was repeated three times. Then, 20 μ L of Biotin anti-mouse CD309 (VEGFR2) antibody (BioLegend, USA) was added, and the mixture was incubated in the dark at 4°C for 1 h. After centrifugation and resuspension (three times), the MB@VEGFR2 was obtained.

Morphological characterization of MB@VEGFR2 was conducted using optical microscopy and scanning electron microscopy (SEM). Specimens were visualized with an optical microscope (Leica, Germany) and a field-emission SEM (Carl Zeiss, Germany).

For fluorescence microscopic evaluation of VEGFR2-MB conjugation, a fluorescence microscope (Nikon, Japan) was employed. Prior to liposome formation, DiI (Beyotime, Shanghai, China) was incorporated into MBs for fluorescent labeling. Following conjugation with FITC anti-rat IgG2a Antibody (BioLegend), the resultant VEGFR2-FITC-MBs were washed three times with PBS and imaged.

Particle size and zeta potential were assessed using a laser diffraction particle size analyzer (Malvern Panalytical, UK). Each specimen underwent three measurements.

Cell line and tumor model

4T1 cells were cultured in a sterile environment at 37°C with 5% CO₂/95% air, using DMEM (Gibco, USA) containing 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco). HUVECs were cultured in a complete medium specifically for HUVECs (Sunnecell, CN, Wuhan, China) with additional growth factors.

Female BALB/c mice, aged 4 weeks, were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Each mouse received a subcutaneous injection of 1 $\times 10^6$ 4T1 cells into the right hind leg. Tumor growth was monitored, and experiments were conducted when the tumor volume reached approximately 150 mm³.

MB@VEGFR2 targeting specificity toward vascular endothelial cells

DiI was incorporated into MBs to prepare DiI-labeled MB and MB@VEGFR2 complexes. HUVECs in the logarithmic growth phase were seeded into 24-well plates (5 $\times 10^4$ cells/well) and cultured overnight. 1 μ L DiO (Beyotime) cell membrane stain was diluted in 1 mL basal medium to prepare working solution. Each well received 100 μ L of working DiO solution followed by 37°C incubation for 20 min. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Nuclear was performed using 200 μ L DAPI (Beyotime) solution (1 μ g/mL) per well for 5 min. For targeting validation, DiI-labeled MB and MB@VEGFR2 suspensions (1 $\times 10^7$ particles) were added to

designated wells and co-incubated at 37°C for 30 min. Unbound MBs were removed through three gentle PBS washes. Fluorescent imaging was conducted using a fluorescence microscope (Nikon).

Optimization of ultrasound cavitation parameters

A 1% agarose phantom was fabricated to simulate biological tissue. Each well received 1 mL of MB@VEGFR2 suspension (1 $\times 10^8$ particles in PBS). Samples were exposed to varied acoustic intensities (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 W/cm²) using a Sonovibro™ (Shengxiang, Changsha, China) with fixed parameters: 1 MHz fundamental frequency and 20% duty cycle. Real-time monitoring was performed with a handheld ultrasound imager (Suonukang, Guangzhou, China) at 0, 30, 60, 90, and 120 s. Image acquisition parameters remained constant. Quantitative analysis of echogenicity changes was conducted and processed using ImageJ software (1.54g) and GraphPad Prism software version 9.5.0.

Tumor vascular normalization

When the tumor reached 150 mm³, 20 mice with tumors showing no obvious perfusion signals were randomly divided into four groups: (I) Control group, (II) US group, (III) MB+US group, and (IV) MB@VEGFR2+US group. Depending on the treatment group, the animals were injected intravenously via the tail vein with 100 μ L of MB or MB@VEGFR2 (1 $\times 10^8$ bubbles/mL), followed by the corresponding US treatment. Under anesthesia, the tumor was fully exposed to the acoustic field generated by the ultrasound transducer. The transducer was positioned perpendicular to the tumor surface, with its center aligned to the geometric center of the tumor. A slow, continuous scanning motion was applied to cover the entire tumor volume, ensuring comprehensive treatment. An appropriate amount of ultrasound coupling gel was used to minimize air gaps and acoustic attenuation. Contrast-Enhanced Ultrasound (CEUS) was employed to assess tumor blood perfusion over a 7 d period.

On the third day after ultrasound irradiation, CD31 and α -SMA staining were used to evaluate vascular maturity, vascular density, and vessel diameter in the tumor's peripheral and central regions. CD31 was used as an endothelial cell marker, and α -SMA as a pericyte/smooth muscle cell marker. Vascular maturity was assessed by the ratio of pericyte coverage of endothelial cells (α -SMA/CD31). HIF-1 α staining was performed to assess hypoxia in the central region of the tumor. Fluorescence signals were processed using ImageJ software.

Tube formation assay

Matrigel (Corning, USA) was thawed overnight at 4°C, 10 μ L of Matrigel was added to each well of the μ -Slide Angiogenesis (IBIDI, Germany) and incubated at 37°C with 5% CO₂ for 30 min. The HUVEC suspension was prepared and divided into the following groups: (I) Control group, (II) US group, (III) MB+US group, and (IV) MB@VEGFR2+US group. Approximately 2 $\times 10^4$ HUVECs were mixed with MB@VEGFR2 or MB (5 $\times 10^7$ particles), followed by 5 min of co-incubation. The cells were then washed with PBS and subjected to ultrasound irradiation (1 MHz, 0.6 W/cm², 20% duty cycle) for 120 s. The same ultrasound protocols were applied in subsequent experiments.

After the Matrigel gelled, the cell suspension was added, and tube formation was observed by optical microscope (Leica, Germany) after 6 h.

Ultrasound-targeted cavitation effect on cell membrane permeability

HUVECs were seeded onto a 24-well plate and cultured for 24 h. The following groups were set: Control group, 0 min group, 10 min group, and 20 min group. MB@VEGFR2 (5×10^7 particles) was added to each well, followed by ultrasound irradiation (1 MHz, 0.6 W/cm^2 , 20% duty cycle) for 120 s. $0.5 \mu\text{mol/L}$ PI (Applygen, Beijing, China) was added at ultrasound exposure, 10 min post-ultrasound, and 20 min post-ultrasound. After co-incubation for 30 min, the cells were washed and stained with $4 \mu\text{mol/L}$ DiO for 30 min, followed by washing and fluorescence observation.

ATP detection

HUVECs were seeded into a 96-well plate and cultured for 24 h. The cells were divided into four groups: (I) Control group, (II) US group, (III) MB+US group, and (IV) MB@VEGFR2+US group. MB or MB@VEGFR2 (1×10^7 particles) was added to each well and co-incubated with HUVECs. After washing, the cells were subjected to ultrasonic irradiation. Following the irradiation, the cell supernatants were collected. ATP levels in supernatants were quantified using a commercial ATP assay kit (Applygen), according to the manufacturer's instructions.

eNOS activity detection

HUVECs were seeded into a 96-well plate and cultured for 24 h. The cells were divided into four groups: (I) Control group, (II) US group, (III) MB+US group, and (IV) MB@VEGFR2+US group. Each well was added with MB or MB@VEGFR2 (1×10^7 particles) and co-incubated with HUVECs. After washing, the cells were subjected to ultrasonic irradiation. Simultaneously, the eNOS activity in the HUVECs was measured using the eNOS activity assay kit (Beyotime), according to the manufacturer's instructions.

NO generation detection

HUVECs were seeded into a 96-well plate and cultured for 24 h. The cells were divided into four groups: (I) Control group, (II) US group, (III) MB+US group, and (IV) MB@VEGFR2+US group. Each well was added with MB or MB@VEGFR2 (1×10^7 particles) and co-incubated with HUVECs for 10 min. After PBS washing, the cells were subjected to ultrasonic irradiation. Following the irradiation, the cell supernatants were collected. The NO content in the supernatants was measured using a NO detection kit (Applygen), according to the manufacturer's instructions.

WB validation of PI3K/AKT/eNOS Pathway protein expression

HUVECs were divided into five groups: (I) Control group, (II) US group, (III) MB+US group, (IV) MB@VEGFR2+US group, and (V) MB@VEGFR2+US+ISAM-140 group (ISAM-140, MCE, USA). After ultrasound stimulation (1 MHz, 0.6 W/cm^2 , 20% duty

cycle, 2 min), RIPA lysis buffer (Beyotime) was added to the HUVECs to fully lyse the cells, and the total protein was collected for BCA protein quantification. Proteins were denatured with SDS-PAGE loading buffer, and equal amounts of protein were loaded into the wells. Protein separation was performed using 10% SDS-PAGE, followed by transferring to a $0.45 \mu\text{m}$ PVDF membrane. The membrane was blocked with 5% BSA at room temperature for 1 h. The membrane was incubated with primary antibodies against Phospho-eNOS (Thr495), Phospho-AKT1/2/3 (Ser473) (diluted 1:500), eNOS, pan-AKT1/2/3, and GAPDH (diluted 1:1,000) at 4°C for more than 12 h. Afterward, the membrane was incubated with a secondary antibody at room temperature for 1 h. All antibodies were purchased from Affinity (Liyang, China). Chemiluminescence detection was performed by the iBright imaging system (Invitrogen, USA). The results were analyzed using ImageJ software.

In vivo treatment experiment

Tumor-bearing mice were randomly divided into 5 groups ($n=5$): (I) Control group, (II) MB@VEGFR2+US group, (III) RT group, (IV) MB+US+RT group, and (V) MB@VEGFR2+US+RT group. Depending on the assigned treatment group, the animals were intravenously injected with $100 \mu\text{L}$ of MB or MB@VEGFR2 at a concentration of 1×10^8 bubbles/mL via the tail vein. The first ultrasound irradiation was performed on day 0, which was considered the starting point. The body weight and tumor volume of the mice were monitored daily. Three cycles of treatment were performed: ultrasound irradiation on days 0, 3, and 6, followed by RT (6 Gy) using a biological irradiation instrument (CIRP, Taiyuan, China) on days 3, 6, and 9. On day 12, the mice were euthanized, and tumor tissues were collected for imaging and weighing. Tissue samples were analyzed for structural changes using H&E staining and for apoptosis using TUNEL staining. H&E staining was performed on multiple organs, while serum levels of ALT, AST, BUN, and CREA were measured.

Statistical analysis

Experimental data were statistically analyzed using GraphPad Prism software. Paired data were evaluated using Student's *t*-test, and datasets were analyzed using one-way ANOVA. *P*-values were determined using a two-tailed, unpaired Welch's *t*-test. Statistical significance was indicated as ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

Compliance and ethics

The authors declare that they have no conflict of interest. All animal experiments in this study were approved by the Animal Ethics Committee of the University of South China and were in compliance with the ethical standards of the University of South China Animal Welfare and Ethics Committee. The ethics approval number for animal experiments was Ethics Approval No. 2023 (119).

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Supporting information

The supporting information is available online at <https://doi.org/10.1007/s11427-025-3289-5>. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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