

Mechanism and regulatory strategy study on promoting vascularized bone regeneration via intracellular zinc ion transport

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ABSTRACT

Bone regeneration is a major clinical challenge. The main obstacles to bone injury repair are local blood flow disorders and hypoxic microenvironments. Mesenchymal stem cell (MSC) therapy has notable advantages in promoting bone-tissue regeneration. In this study, we established a mouse model of skull bone injury treated with bone marrow mesenchymal stem cells (BMSCs). We found that local BMSC transplantation stimulated vascularized bone regeneration and matrix metalloproteinase (MMP)10 was the major regulatory protein. Local hypoxic microenvironment-induced mitochondrial permeability increased, resulting in cytoplasmic Zn^{2+} accumulation, which is a key factor in activating the JAK1/STAT1/MMP-10 pathway. The cytoplasmic Zn^{2+} enrichment caused ZRT/IRT-like protein 6 (ZIP6) inhibition was the key initiating factor in this process. Based on these findings, we designed and engineered CD90@ZIF-8-ICG, with an outer membrane chimeric CD90 antibody containing indocyanine green (ICG), to achieve increased intracellular zinc ion content by targeted delivery of the particles into local MSCs, so that local MMP-10 production and angiogenesis are regulated at the early stage of healing. ICG provided BMSCs with continuous photothermal stimulation in response to the laser intervention, which successfully achieved stable improvement of bone-defect regeneration. This study innovatively describes the regulatory importance of intracellular zinc ion homeostasis and ZIP proteins in the function of transplanted MSCs, as well as the related efficiency strategy development, which elucidates MSC therapy treatment mechanisms and provides strategies for the design and development of stem-cell-based biomaterials.

1. Introduction

Compared with soft tissue healing and repair, bone regeneration is a major challenge in clinical therapies, especially for large-bone-defect healing, which cannot be repaired spontaneously by host healing processes [1]. Tissue engineering technology, which has been widely used in numerous disease therapies, provides a good strategy for bone-defect repair by replanting cells, scaffold materials, and growth factors [2–4]. Local blood flow disorders and hypoxic microenvironments are major

obstacles to bone injury recovery because they are not conducive to host cell homing and differentiation [5,6] and cause oxidative stress in local cells [7]. Therefore, early repair of local blood supply in tissue-engineered bone regeneration is critical because it provides sufficient oxygen and nutrients to local cells [8]. Moreover, gradual neovascularization and infiltration of blood vessels mark the initiation of osteogenesis [9]. During this process, host stem cells actively and densely surround the neovascularization and colonize, regulate, and promote each other through endothelial cells during this process [10].

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Such complex spatiotemporal regulatory networks between osteoblasts, osteoclasts, and vascular endothelial cells are known as angiogenic-osteogenic coupling [8].

Recently, mesenchymal stem cell (MSC) therapy has shown notable benefits in promoting bone-tissue regeneration and been linked to significant therapeutic effects in multiple bone-related diseases, including bone fracture [11], bone-defect repair [12], osteoarthritis [13], and osteoporosis [14]. However, local hypoxic microenvironments are believed to inhibit the proliferation and survival of MSCs, thereby lowering their differentiation ability [15,16], including osteogenic differentiation functions [17]. Therefore, the regulatory mechanism of MSC therapy in promoting bone-tissue repair is unclear, and an investigation into whether transplanted MSCs directly participate in tissue regeneration as seed cells is warranted. The oxygen partial pressure in the surrounding environment substantially changes after MSCs are transplanted into a bone defect, changing from a 21 % oxygen-rich culture environment to an extremely hypoxic environment [6,18,19]. Moreover, our previous study indicated that the local *Hif1a* expression level in the bone-defect tissues was significantly higher than that in healthy tissues, confirming that the bone-defect tissue microenvironment was hypoxic *in vivo* [19]. Such a microenvironment may alter the functions of the transplanted MSCs, resulting in their involvement in the active regulation of host cells and vascular reconstruction to quickly reverse hypoxia. Therefore, some studies have hypothesized that transplanted MSCs mobilize host cell homing and other functions through paracrine signaling, indirectly promoting tissue reconstruction and regeneration, rather than directly [20]. However, further research is required to elucidate the molecular mechanisms and key regulatory factors involved in this process.

The endoplasmic reticulum (ER) and mitochondria are closely connected and interact in a hypoxic microenvironment; the completion of this process depends on mitochondrial-ER coupling [21]. The mitochondria-associated ER membrane (MAM) [22] at the junction activates and triggers the opening of the mitochondrial membrane permeability transition pore (mPTP), ultimately leading to mitochondrial dysfunction and cell death [21,23]. Information exchange between mitochondrial and cytoplasmic pools is crucial for intracellular homeostasis, and mitochondria-associated metal ions play an important role. Mitochondria are one of the cellular “zinc banks” [24,25]. Zinc ion (Zn^{2+}), an essential trace element and protease catalyst, is involved in the regulating cellular protein synthesis and plays a critical role in cell development and metabolism [26–30]. Typically, several large “zinc banks” in the cell (including mitochondria, ER, and metallothionein) maintain perfect zinc ion pool homeostasis, which is mediated by zinc-binding proteins and zinc transporters, providing a stable free zinc concentration essential for normal cellular function [31]. However, in response to certain physiological or pathological stimuli, sudden changes in cytoplasmic Zn^{2+} concentrations can trigger signal transduction, thus regulating cellular functions [32]. The opening of the mPTP in a hypoxic microenvironment may result in the passive transfer of endogenous zinc ion pools without the use of zinc transporters. Although many studies have focused on zinc ion supplementation to promote bone-defect repair [33–35], the regulatory effect of outgoing mitochondrial Zn^{2+} on transplanted MSCs remains unknown, leaving a gap in MSC therapy studies.

Zinc transporters within cells are closely related to many cellular functions. Typically, zinc is taken up as Zn^{2+} , which is hydrophilic and cannot penetrate cell membranes via non-facilitated-passive diffusion [36]. Therefore, specialized transporters are required to transport Zn^{2+} into the cytoplasm, and the Zrt/Irt-like protein (ZIP) family proteins are responsible for this process [26]. The ZIP family consists of 14 protein subtypes (ZIP1–ZIP14) that are distributed in different locations, including plasma and organelle membranes [37]. Although the traditional view holds that their main role is to transfer Zn^{2+} , in recent years, multiple evidence has shown that ZIPs also play a key role in the functional regulation of eukaryotic cells. Certain intracellular zinc storage

organelles, including mitochondria, are responsible for releasing Zn^{2+} into the cytoplasm when needed, thus regulating cell metabolism and signal transduction [38]. In lipopolysaccharide-stimulated macrophages, the expression of ZIP8 is notably upregulated to enhance the inflammatory response by mediating an increase in cytoplasmic zinc levels [39]. Conversely, ZIP9 facilitates the polarization of M2 macrophages and suppresses inflammatory progression by enhancing STAT6 phosphorylation [40]. During bone remodeling, the expression of ZIP1 is markedly upregulated in human MSCs and osteoblasts, playing a pivotal role in promoting local cell osteogenic differentiation [41]. For osteoclasts, studies have shown that both ZIP1 and ZIP14 are detectable, and exert inhibitory effects on osteoclast function [42,43]. However, it remains unclear whether ZIP plays a regulatory role in the process by which transplanted MSCs promote bone-defect repair.

This study found that the local transplantation of bone marrow mesenchymal stem cells (BMSCs) promoted angiogenesis in the early stages of bone-defect repair in mouse skull bone-defect models. Further proteomic chip analysis revealed that the transplanted MSCs promoted endothelial cell chemotactic and angiogenic differentiation in a local hypoxic microenvironment by upregulating MMP-10 expression. In addition, using multiple technologies, including transmission electron microscopy (TEM), structural illumination microscopy (SIM), metal ion probe labeling, and flow cytometry, we found that the mitochondrial membrane permeability of BMSCs increased in a hypoxic microenvironment, resulting in mitochondrial zinc ion (mito- Zn^{2+}) transport to the cytoplasm and changes in zinc ion transporter protein expression, thus affecting the downstream JAK1/STAT1/MMP-10 pathway. Notably, further exploration indicated that cytoplasmic Zn^{2+} enrichment causing ZIP6 inhibition is the key initiating factor in this process.

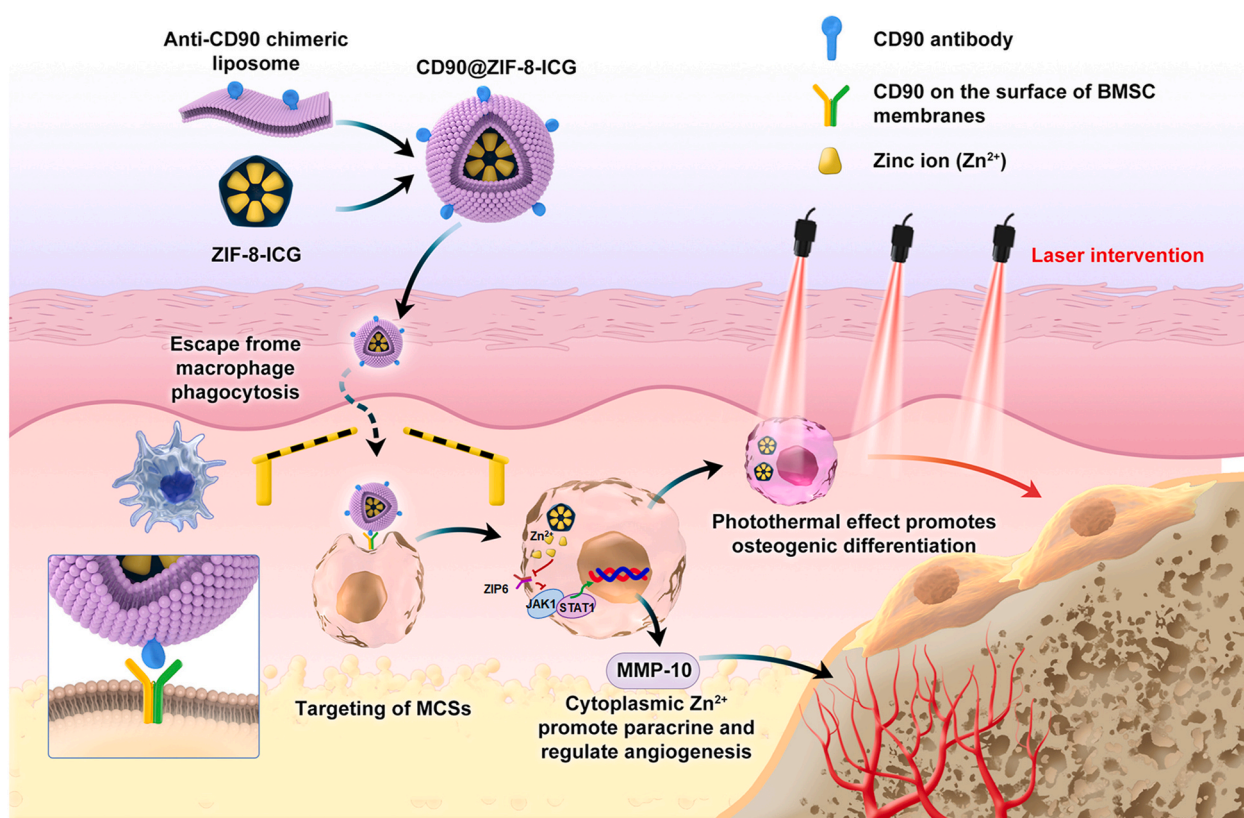
Based on the above findings, we designed and engineered CD90@ZIF-8-ICG, to achieve multilevel regulation during bone-defect repair. As a representative metal-organic framework (MOF), zeolitic imidazolate frameworks (ZIFs) have emerged as a prominent research focus owing to their unique topological structures and exceptional stabilities [44]. ZIF-8 has garnered extensive attention since its discovery. Its structural core mimics the classic sodalite (SOD) topology of zeolites, forming a highly ordered three-dimensional microporous network through the self-assembly of divalent Zn^{2+} as metal nodes and 2-methylimidazole anions (2-MIM[−]) as organic linkers [45]. Zn^{2+} is the main metallic elements released during the degradation process of ZIF-8 [46] and is also one of the essential elements for the human body [26]. This is one reason the *in vivo* metabolism of ZIF-8 is considered safe. In this study, by preparing ZIF-8 particles coated with CD90 antibody-chimeric liposomes, we achieved targeted delivery of particles to local MSCs, increasing their intracellular zinc ion concentration, and achieved primary regulation of angiogenesis by promoting MMP-10 expression in the early stage of bone-defect repair. In addition, by loading indocyanine green (ICG) into ZIF-8 and continuously applying low-energy laser intervention within one week after delivery, we improved the osteogenic differentiation ability of local stem cells in the repair process through secondary photothermal effect regulation and gained a stable bone regeneration promotion effect (Scheme 1). Our study elucidates the mechanism of MSC therapy-based tissue engineering and provides a potential strategy for the further development of clinical techniques for the treatment of bone-defect regeneration.

2. Materials and methods

2.1. Isolation and culture of BMSCs

Male C57BL/6 mice aged 6–8 weeks were obtained from Vital River Laboratory Animal Technology Co. (Beijing, China). All animal experiments were approved by the Animal Ethics Committee of Beijing Stomatological Hospital (KQYY-202008-005; Beijing, China).

The C57BL/6 mice were sacrificed and placed in 75 % alcohol for 1 min. Clean femur surfaces were collected using sterile instruments and



Scheme 1. Schematic illustration of regulation mechanism of CD90@ZIF-8-ICG during bone defect healing process.

rinsed several times with phosphate-buffered saline (PBS). After both ends of the femurs were cut off with sterile scissors, BMSCs were collected by washing out the internal bone marrow tissue and passing the fluid through a 70- μ m cell strainer. The cells were then centrifuged at 1100 rpm for 6 min. The single-cell suspension was re-suspended in α -MEM medium (Invitrogen, Carlsbad, CA, USA) containing 20 % fetal bovine serum (Equitech-Bio, Kerrville, TX, USA), 5 % penicillin/streptomycin (Invitrogen), and 5 % glutamine (Invitrogen). The cells were cultured at 37 °C under a humidified atmosphere with 5 % CO₂ and the medium was replaced every three days. The cells were passaged when they reached 70–80 % confluence. All the cells used in this study were obtained from passage 2 (P2).

2.2. Establishment of mouse skull bone-defect models

After routine disinfection and anesthesia, a flap of skin on the head was opened and the skull was exposed. A 4-mm diameter cut was made using a bone trephine (Nobel Biocare AB, Karlskoga, Sweden) to create a skull defect. Following suturing, 1×10^6 BMSCs/0.2 mL of saline and 0.2 mL of saline alone were injected into the defects in the BMSCs and Blank group, respectively. The animals in both groups were sacrificed one month after the operation. After injecting the mice with tetramethylrhodamine isothiocyanate-dextran, local vascular remodeling was observed using a two-photon fluorescence microscope (Leica TCS SP8; Leica Microsystems, Wetzlar, Germany). A micro-CT scan of 14.93- μ m thickness was used to assess the extent of healing of the skull bone defect. The degree of new bone volume and angiogenesis was assessed using H&E and CD31 immunohistochemical staining. The principle for selecting the positions of the slices was based on the middle of the defect (midline).

2.3. In vitro hypoxic induction

After the BMSCs had passed through P2, they were seeded in a medium dish at a density of 2×10^6 cells/well. After 24 h, the cells were transferred to serum-free culture medium and cultured for another 6 h in either a normoxic (control group) or hypoxic environment (hypoxia group) using Aneropack C-1 (deoxidizer) and a 2.5-L sealed culture tank, C-31 (culture tank) (Mitsubishi Gas Chemical Company; Niigata, Japan). This system was composed of a sealed culture tank with a deoxidizer and an indicator. Therefore, when cells were cultured in the box, the surrounding environment was completely oxygen-deficient (0 % oxygen, confirmed by the indicator inside). The conditioned media and cell samples from both groups were collected for further analysis.

2.4. Cell scratch migration assays

Human vascular endothelial cells (HUVECs) were obtained from Cyagen Biosciences Inc. (Jiangsu, China). HUVECs were seeded at a density of 2×10^5 cells/well in six-well plates. Following a 24-h period, the medium was discarded, and scratches were made using a 200- μ L sterile gun tip. The scratched cells were washed with PBS and photographed under a microscope (0 h). Conditioned medium (CM) was added to the control and hypoxic CM groups and the cells were subsequently cultured at 37 °C under 5 % CO₂ in a humidified atmosphere for 48 h before observation.

2.5. Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) after induction. cDNA was synthesized according to the kit instructions, and a real-time PCR was performed using *Ang1*, *Vegf*, *Zip1-14*, and *Gapdh* primers (Table S1) in each group with a Power SYBR Green PCR Master Mix kit (Life Technologies, Carlsbad, CA, USA).

2.6. Western blotting

After induction, the cells were washed twice with pre-cooled PBS and proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA). Mitochondrial proteins were extracted using a mitochondrial protein extraction kit (Solarbio, Beijing, China), according to the manufacturer's instructions. Electrophoresis was performed using 25- μ g protein samples at a constant voltage of 80 V. The transmembrane reaction was performed at 250 mA for 1.5 h. The membranes were then incubated for 1 h at room temperature with 5 % skim milk. Using the primary antibodies, MMP-10 (bs-23106R; Bioss, Beijing, China), P-JAK1 (66245S; Cell Signaling Technology [CST], Danvers, MA, USA), JAK1 (3332; CST), P-STAT1 (9167S; CST), STAT1 (14994S; CST), β -actin (3700; CST), MFN1 (bsm-60386M; Bioss), COX IV (4844; CST), Cytochrome *c* (Cyt-C, 4280; CST), ZIP6 (14236-1-AP; Proteintech, Beijing, China), H90 (#4877; CST), and Tubulin (11224-1-AP; Proteintech), the membranes were incubated overnight at 4 °C. The following day, a diluted secondary antibody (Pierce, Malibu, CA, USA) was added and incubated at room temperature for 1 h before adding ECL (New Cell & Molecular Biotech Co. Ltd., Suzhou, Jiangsu, China) for chemiluminescence analysis.

2.7. Protein array

Normoxic and hypoxic BMSC-CM were collected and delivered to RayBiotech Inc. (Guangzhou, China) for protein array analysis. Data analyses included original data normalization, differential protein screening, differential protein clustering, and GO/KEGG pathway enrichment analysis.

2.8. Visualization of organelles and zinc ions

A mitochondrial staining kit (AAT Bioquest, Sunnyvale, CA, USA) was used to label mitochondria. A live cell ER staining kit (AAT Bioquest) was used to label the endoplasmic reticula of the cells. Zinc ions were labeled with cell-permeable (MK Bio, Shanghai, China) or cell-impermeable zinc ion probes (AAT Bioquest) and the nuclei were labeled with Hoechst (Beyotime, Beijing, China). Images were captured using a structural illumination microscope (Nikon, Tokyo, Japan).

2.9. Tube formation assay

Each well of a 96-well plate received 50 μ L of Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). The plates were then incubated for 30 min at 37 °C. Each well was seeded with HUVECs stained with PKH26 (Solarbio). After 6 h, the cells were examined under a microscope (OLYMPUS, Tokyo, Japan).

2.10. mPTP detection

The opening of the mPTP was observed using an mPTP assay kit (Beyotime), after which the cells were treated with hypoxia medium, an mPTP activator (Ionomycin from the mPTP assay kit) or inhibitor (ER-000444793; MCE, Shanghai, China), according to the manufacturer's instructions.

2.11. ELISA analysis

The concentration of MMP-10 in the CM was determined using an MMP-10 ELISA kit (Mlbio, Shanghai, China), according to the manufacturer's instructions.

2.12. Preparation of CD90@ZIF-8-ICG

The 2-methylimidazole (Adamas, Shanghai, China) and zinc nitrate hexahydrate (Adamas) were separately dissolved in methanol. The zinc

nitrate hexahydrate solution was slowly added to the 2-methylimidazole solution, followed by the addition of ICG (Aladdin, Shanghai, China). The mixture was stirred at room temperature for 1 h. Subsequently, the products were centrifuged at 12,000 rpm for 10 min and washed thrice with methanol. Finally, the precipitate was re-dispersed in ultrapure water to obtain ZIF-8-ICG. CD90 (Proteintech) and DSPE-PEG-NHS (Xi'an Ruixi Biological Technology, Xi'an, China) were reacted at a pH of 8.0, followed by dialysis to obtain the DSPE-PEG-CD90 solution. Subsequently, SPC (Xi'an Ruixi Biological Technology) and cholesterol (Xi'an Ruixi Biological Technology) were dissolved in 3 mL of chloroform and evaporated under reduced pressure to form a thin film within a sample bottle. Next, 2 mL of the DSPE-PEG-CD90 solution was added to the film to prepare the liposome solution. The ZIF-8-ICG was incorporated into the liposome solution and subjected to ultrasonication and extrusion through a polycarbonate membrane (Whatman, Cardiff, UK) with a pore size of 100 nm. Finally, deionized water was added to adjust the volume to 3 mL, after which a cryoprotectant was added before freeze-drying.

2.13. Characterization of CD90@ZIF-8-ICG

The morphologies of ZIF-8-ICG and CD90@ZIF-8-ICG were characterized using TEM (JEM-2100Plus, JEOL Ltd., Akashima, Japan). The particle size and zeta potential were determined using dynamic light scattering and a Zetasizer Nanoseries instrument (Zetasizer Nano ZS90, Malvern, UK). Ultraviolet (UV)–vis–near-infrared absorption spectra were recorded using a UV–vis–NIR spectrometer (UV-3600, SHIMADZU, Japan). The photothermal effect of the samples was evaluated using an 808 nm NIR laser (LASERWAVE, China) at 1.0 W/cm² for 3 min, and the temperature was recorded using an infrared thermal imager (InfiRay, China). Anti-CD90 antibody (BioLegend, San Diego, USA) was incubated with the samples at room temperature for 30 min and centrifuged at 12000 rpm for 10 min. The positivity rate of the anti-CD90 antibody was detected using flow cytometry.

2.14. Cytotoxicity evaluation of CD90@ZIF-8-ICG

The *in vitro* cytotoxicity of BMSCs was investigated using a Cell Counting Kit-8 (CCK-8) viability assay. Cells were seeded in the 96-well plate and incubated in 5 % CO₂ at 37 °C for 24 h. The medium was replaced by 100 μ L of α -MEM medium (blank control) and fresh medium containing different concentrations of CD90@ZIF-8-ICG (10, 30, 50, 70, and 90 μ g/mL). After further incubation with the samples for 24 h, 10 μ L of CCK-8 solution was added into the wells. The absorbance was measured at 450 nm using a microplate reader. Apoptosis was assessed using flow cytometry. BMSCs were seeded in 12-well plates and cultured for 24 h. Subsequently, the cells were treated with different concentrations of CD90@ZIF-8-ICG (0, 10, 30, and 50 μ g/mL) for 24 h. Each group of cells were collected, and apoptosis was detected using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, New Jersey, USA) and analyzed using flow cytometry within 1 h.

2.15. Targeting effect evaluation

PKH 67 labeled-BMSCs and RAW264.7 cells were seeded on 12-well plates incubated and cultured for 24 h. Following the incubation, the cells were treated with Cy5-se labeled-CD90@ZIF-8-ICG (30 μ g/mL) for 24 h. After the incubation period, the cells were washed three times with PBS and fixed with 4 % paraformaldehyde for 30 min. Subsequently, the images of the cells were captured using a microscope (OLYMPUS) to visualize and analyze the uptake of CD90@ZIF-8-ICG.

2.16. Alkaline phosphatase (ALP) staining

The BMSCs were cultured in 12-well plates and exposed to 30 μ g/mL of CD90@ZIF-8-ICG + NIR intervention. After 7 days of osteogenic

induction, the samples were fixed in 4 % paraformaldehyde for 30 min and washed three times with PBS. A BCIP/NBT ALP staining kit (Beyotime) was used for staining.

2.17. Alizarin red staining

BMSCs were cultured according to the conditions described for ALP staining, after 21 days of osteogenic induction, the cells were fixed in 4 % paraformaldehyde for 30 min and washed with distilled water. Subsequently, the samples were incubated in the dark with 0.2 % alizarin red S solution (Solarbio) for 30 min.

2.18. In vivo therapeutic effects

In addition to establishing models of skull bone defects as previously mentioned, the hair above the bone defect was depilated to reduce interference from the NIR laser.

The mice were randomly assigned to five groups ($n = 6$): Blank, BMSC therapy, CD90@ZIF-8-ICG, NIR, and CD90@ZIF-8-ICG + NIR. Five days after bone-defect modeling, PBS was injected into the bone defects of the Blank and NIR groups. BMSCs (1×10^6 cells) were injected in the BMSC therapy group, and 100 μ g of protein CD90@ZIF-8-ICG was injected in the CD90@ZIF-8-ICG and CD90@ZIF-8-ICG + NIR groups. For the following 7 days, the NIR and CD90@ZIF-8-ICG + NIR groups were exposed to an 808-nm NIR laser (1.5 W/cm^2) intervention for 3 min each day, and an infrared thermal imager was used to determine the local temperature. All animals were sacrificed one month after the intervention. Routine and biochemical blood tests were performed in the laboratory of the Beijing Stomatological Hospital to evaluate safety *in vivo*. A micro-CT scan of 14.93- μ m thickness was used to assess the extent of healing of the skull bone defect. The degree of new bone volume and angiogenesis was assessed using H&E and CD31 immunohistochemical staining.

2.19. Statistical analysis

All experiments were independently repeated in triplicate, and the data were presented as means $\pm$ standard deviations. GraphPad Prism 9 software was used for statistical analysis. Comparisons between groups were evaluated by Student t-test or one-way analysis of variance, and the least significant difference (LSD) post-test was performed to confirm the normality and homogeneity of variance of the data. Data that did not show a normal distribution were compared using the Kruskal-Wallis test followed by the Bonferroni's test for multiple comparisons or the Mann-Whitney *U* test for two group comparisons. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. The paracrine activity of transplanted MSCs in a hypoxic microenvironment promotes vascularized bone regeneration

Bone defect healing has always been a clinical challenge owing to various problems, including lack of blood supply and local cell dysfunction. Recently, many related strategies have been proposed. For instance, some studies used biomaterials equipped with relevant active factors to promote bone-tissue repair [47–49]. With the rapid development of tissue engineering, MSC therapy has provided an innovative approach for bone-defect repair and regeneration. Multiple studies have confirmed the therapeutic effects of MSC transplantation in bone-defect therapies [50–52]. In this study, mouse skull bone-defect models were used to investigate the effects of MSC therapy on bone-defect repair. The micro-CT showed that BMSC injection significantly increased the rate and area of bone-defect repair after one month, compared with those in the Blank group (Fig. 1a). Neovascularization at the edge of the bone defect was observed using two-photon laser confocal microscopy. BMSC

therapy improved vascular reconstruction during the early stages of bone-defect repair (Fig. 1b). Histological examination and CD31 immunohistochemical staining corroborated these findings (Fig. S2).

The local hypoxic microenvironment in bone defects is a key factor in regulating transplanted MSCs [5,53,54]; however, its regulatory relevance has not been fully clarified. Hypoxia is essential for MSC paracrine functions [55,56] and hypoxic pre-treated MSCs have a greater effect than that of untreated cells [57,58]. In our previous studies [19,59], we found that most transplanted stem cells underwent apoptosis within 24 h *in vivo* owing to local hypoxic stimulation, indicating that the most critical period for transplanted stem cell paracrine regulation is the first 24 h after transplantation. Therefore, the therapeutic mechanisms of transplanted MSCs during the early stages after transplantation need to be investigated before MSC therapy or stem cell-based agents can be applied in clinical treatments [20]. Given the extremely hypoxic local microenvironment of bone defects, we induced BMSCs *in vitro* under hypoxic conditions for 24 h and extracted the CM, which was then co-cultured with HUVECs to investigate the presence of key regulatory proteins that promote HUVEC function (Fig. 1c). Cell migration (Fig. 1d), chemotaxis (Fig. 1e), and angiogenesis induction (Fig. 1f–h) assays showed that hypoxic CM increased HUVEC homing and differentiation. Real-time PCR and ELISA results showed that hypoxic CM upregulated the expression of vasculogenic genes, such as *Ang1* and *Vegf* (Fig. 1f), and the critical factor, VCAM-1 (Fig. 1g), during the induction of angiogenesis in HUVECs. These results suggested that transplanted BMSCs produce important regulatory factors that increase the vasogenic function of HUVECs in a hypoxic microenvironment, facilitating vascular remodeling and bone-defect repair.

3.2. MMP-10 is a crucial regulatory protein that promotes vascular regeneration in transplanted BMSCs

The quantitative results of the bicinchoninic acid (BCA) assay supported our hypothesis that BMSCs cultured in a hypoxic environment express more proteins than those cultured in a normoxic environment (Fig. 2a). Protein arrays were used to identify various proteins in the two CM samples. Under hypoxic culture conditions, the expression levels of multiple proteins in BMSCs increased. The activities of the differentially expressed proteins were primarily focused on growth- and chemotactic-related functions (Fig. 2b), as revealed by the molecular functional enrichment analysis. Among these proteins, MMP-10 expression was the most significantly altered (Fig. 2c). ELISA was performed to demonstrate that hypoxia increased MMP-10 expression in BMSCs (Fig. 2d). The JAK/STAT signaling pathway was most likely an associated pathway (Fig. S3a). Western blotting verified that following hypoxia induction in BMSCs, the JAK1/STAT1 pathway was activated and MMP-10 expression increased (Fig. S3b–c).

We used MMP-10 siRNA transfection to inhibit MMP-10 expression in hypoxia-treated BMSCs (Fig. 2e) to investigate whether MMP-10 is the key regulatory protein in transplanted BMSCs promoting HUVEC angiogenesis. The upregulatory effect of hypoxic CM on HUVEC migration (Fig. 2f) and angiogenesis-related genes (Fig. 2g) ceased. Moreover, local injection of MMP-10 siRNA-transfected BMSCs had less of an impact on the promotion of vascular regeneration and bone repair in mouse skull defect models, as confirmed using micro-CT (Fig. 2h), histological staining (Fig. 2i), and CD31 immunohistochemical staining (Fig. 2j). These findings indicate that MMP-10 is a crucial regulatory protein in transplanted BMSCs during the promotion of vascular regeneration.

3.3. BMSC mitochondrial permeability increased in a hypoxic microenvironment, leading to the cytoplasmic enrichment of mito- Zn^{2+} , which is a key factor affecting the MMP-10 production

The mitochondria and ER are key organelles that maintain cell homeostasis and are sensitive to variations in oxygen content; they are

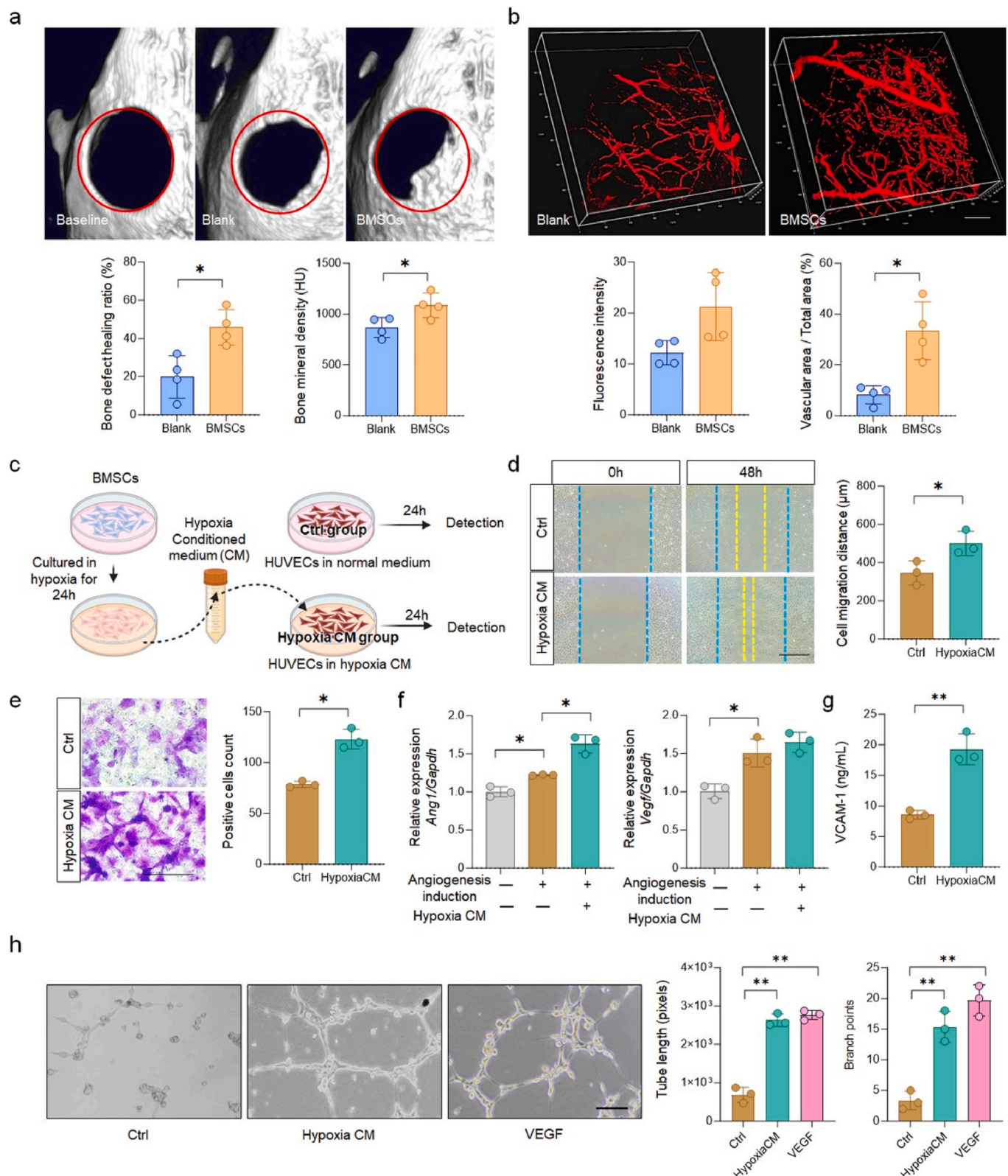


Fig. 1. Transplanted BMSCs promoted HUVEC angiogenesis in the hypoxic microenvironment (a) The results of micro-CT showed that BMSC injection significantly increased the rate and area of bone defect repair ($n = 4$). (b) BMSC therapy improved vascular reconstruction during the early stages of bone defect repair ($n = 4$). (c) Schematic illustration of conditioned medium preparation and *in vitro* intervention process. (d) The results of cell migration in the control and hypoxia CM groups. (e) The results of the transwell assay in the control and hypoxia CM groups. (f) Real-time PCR results showed that hypoxic CM upregulated the gene expression of *Ang1* and *Vegf* in HUVECs. (g) ELISA results showed that hypoxic CM upregulated the expression level of VCAM-1 in HUVECs. (h) The results of the angiogenesis induction assay. All *in vitro* results are representative of at least three independent experiments ($n = 3$ for each experiment). Scale bar in (b) = 100 μm . Scale bar in (d) = 200 μm . Scale bar in (e) = 20 μm . Scale bar in (h) = 100 μm . Data are presented as mean $\pm$ SD. * $p < 0.05$. ** $p < 0.01$. Ctrl = Control. CM = conditioned medium. BMSC = bone marrow mesenchymal stem cell.

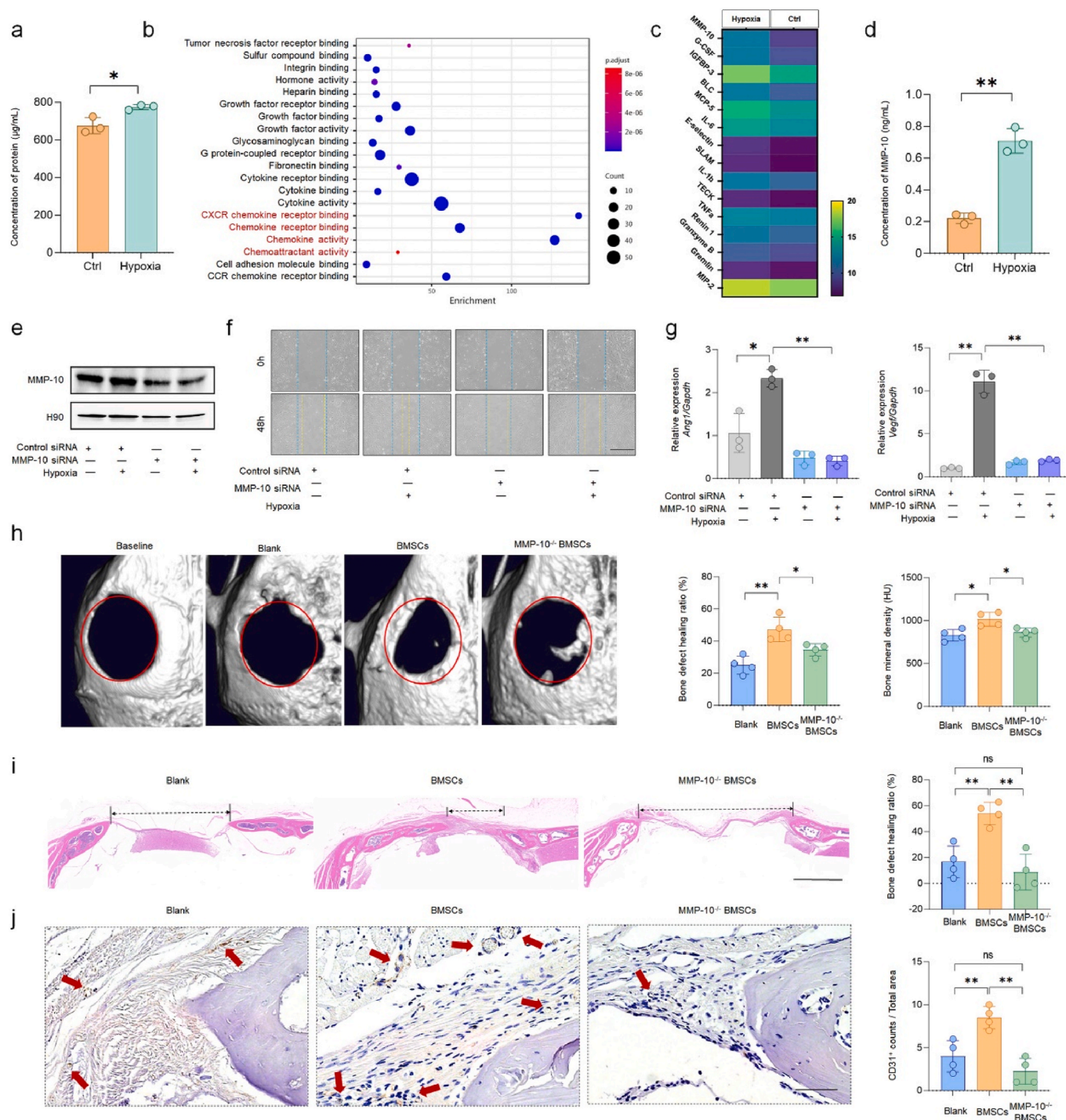


Fig. 2. MMP-10 is a crucial regulatory protein that promotes vascular regeneration (a) The BCA quantitative results of the secreted protein in control CM and hypoxia CM. (b) GO enrichment results of the different proteins between control CM and hypoxia CM. (c) Protein array results showed that the expression levels of multiple proteins in BMSCs increased under hypoxic culture conditions, in which MMP-10 was the most significantly altered protein. (d) ELISA results demonstrated that hypoxia increased MMP-10 expression in BMSCs. (e) MMP-10 siRNA transfection inhibited MMP-10 expression in hypoxia-treated BMSCs. (f) The absence of MMP-10 led to a weakening of the promoting effect of hypoxia CM on HUVEC migration. (g) The results of real-time PCR confirmed that MMP-10 was the key factor in hypoxia CM-induced upregulation of *Ang1* and *Vegf*. (h) The results of micro-CT showed that local injection of MMP-10 siRNA-transfected BMSCs had less therapeutic effects in mouse skull defect models ($n = 4$). (i,j) Histologic staining and CD31 immunohistochemical staining confirmed that MMP-10 knockout BMSCs had less of an impact on the promotion of bone repair and vascular regeneration ($n = 4$). All *in vitro* results are representative of at least three independent experiments ($n = 3$ for each experiment). Scale bar in (f) = 200 μm. Scale bar in (i) = 1000 μm. Scale bar in (j) = 50 μm. Data are presented as mean $\pm$ SD. ns $p > 0.05$. * $p < 0.05$. ** $p < 0.01$.

closely related structurally and functionally [60]. The physical functional coupling of the ER and mitochondria allows the direct transfer of Ca^{2+} between these organelles, which is important for rapidly increasing the mitochondrial metabolic activity [61]. In response to environmental stress, cells increase mitochondrial ER coupling to promote ATP synthesis and maintain functional homeostasis [61]. The linked membrane components between the ER and mitochondria are MAMs, which mediate Ca^{2+} transfer from the ER to the mitochondria, resulting in Ca^{2+} overload in the mitochondrial matrix and subsequent opening of a cell damage-associated channel, mPTP [23]. To examine the major organelles or cell components that changed throughout this process, TEM was used to continuously observe BMSCs in a hypoxic environment. The results indicated that ER-mitochondria coupling occurred quickly after hypoxia induction, followed by mitochondrial swelling, structural destruction, and cell apoptosis (Fig. 3a). Flow cytometry (Fig. S4a) also showed that the mitochondrial membrane potential of BMSCs gradually decreased as the hypoxia-induced time increased, indicating mitochondrial dysfunction and increased cell apoptosis. Western blotting was used to detect the expression of MFN1, an ER-mitochondrial coupling-associated protein, in the mitochondria of hypoxia-treated BMSCs (Fig. 3b, top) and the expression of Cyt-C, a mitochondrial intermembrane protein, in the cytoplasm of hypoxia-treated BMSCs (Fig. 3b, bottom). Based on these results, we concluded that ER-mitochondrial coupling peaks 6 h after hypoxia induction, resulting in increased mitochondrial permeability [21–23], leading to mitochondrial intermembrane protein transportation into the cytoplasm. The mPTP detection results also confirmed that the mitochondrial permeability of BMSCs increased after 6 h of hypoxic induction (Fig. 3c). Opening of the mPTP increases the permeability of the mitochondrial membrane and is highly correlated with autophagy and other cellular functions [62].

Mitochondria are reservoirs of various metal ions, including Mn, Fe, Ca, and Zn. Mitochondria participate in intracellular ion homeostasis, which is critical for several cellular functions. Under pathological conditions, the overload or depletion of mitochondrial metal ions interferes with cells and leads to cell death [63]. Continuous opening of the mPTP and excessive stromal swelling associated with other mPTP-independent processes can impair mitochondrial function and integrity, resulting in an imbalance in intracellular ion homeostasis [64]. To explore the effect of increased mitochondrial permeability on the composition of metal ions in the cytoplasm, we used differential centrifugation to extract the cytoplasmic components of BMSCs and detected a variety of metal ions. The cytoplasmic zinc ions significantly and stably increased during hypoxia (Fig. 3d). To confirm the results, we used a cell-permeable zinc ion probe to detect cytoplasmic Zn^{2+} . This probe could only visualize the concentration of Zn^{2+} after being hydrolyzed to ZnAF-2F by esterase in the cytoplasm. The results showed that the concentration of cytoplasmic Zn^{2+} in BMSCs significantly increased after hypoxia induction (Fig. 3e and Fig. S4b–c). By labeling the mitochondria, ER, and cytoplasmic zinc ions with the corresponding fluorescent probes, we visually demonstrated the enrichment of cytoplasmic Zn^{2+} in hypoxia-induced BMSCs (Fig. 3f). When we artificially increased mitochondrial permeability using the mPTP activator, lonomycin, under normal oxygen culture conditions, we observed a transient increase in cytoplasmic Zn^{2+} (Fig. 3g), demonstrating that zinc ions are stored in the mitochondria and can flow into the cytoplasm when the mPTP is open.

To investigate whether cytoplasmic Zn^{2+} enrichment was the key mechanism influencing the expression of regulatory proteins in hypoxia-treated BMSCs, an mPTP inhibitor was used to block intracellular zinc ion transport during hypoxic induction (Fig. 3h and i). The lack of zinc ions inhibited the hypoxia-induced upregulation of secreted proteins (Fig. 3j). More importantly, the levels of the key regulatory protein, MMP-10, were significantly downregulated (Fig. 3k). We also detected JAK1/STAT1 activation using western blotting and demonstrated that this pathway was suppressed, accompanied by blockade of cytoplasmic Zn^{2+} enrichment (Fig. S5). These findings suggest that cytoplasmic Zn^{2+} enrichment is essential for JAK1/STAT1/MMP-10 activation in BMSCs

under hypoxic conditions.

3.4. Cytoplasmic zinc ions regulate MMP-10 expression by inhibiting the ZIP6-related pathway

The ability of Zn^{2+} transporters to enable intercellular Zn^{2+} pool redistribution is closely associated with several cellular functions in eukaryotic cells [36]. To explore the mechanism by which Zn^{2+} affects protein kinase activity, we used real-time PCR to assess the gene expression levels of the zinc transporter ZIP family [26]. The highly expressed ZIP genes in BMSCs were *Zip 1*, *Zip 6*, *Zip 7*, *Zip 9*, *Zip 10*, *Zip 13*, and *Zip 14*, among which *Zip 6*, *Zip 7*, *Zip 9*, and *Zip 13* showed significant changes during hypoxia induction (Fig. S6a). The fold change of *Zip 6* was the most significant (fold change 2.29 for *Zip 6*, 1.51 for *Zip 7*, 1.62 for *Zip 9*, 1.48 for *Zip 13*). In addition, this inhibition was rescued by treatment with an mPTP inhibitor (Fig. S6b), suggesting that ZIP6 may play a key role in hypoxia-induced functional changes in BMSCs. ZIP6 is a zinc ion transport channel located in the plasma membrane of cells and abundantly expressed on the surface of activated T cells, mediating zinc inflow into the cytoplasm, thereby activating signals essential for the regulation of T cell responses, including T cell receptor stimulation [65]. However, no studies have been conducted on the regulatory functions of ZIP6 in stem cells. To demonstrate this mechanism, lentiviral transfection was used to knock out ZIP6 expression in BMSCs cultured under normal oxygen conditions (Fig. S6c). The elimination of ZIP6 caused significant activation of JAK1/STAT1/MMP-10 in BMSCs, even under normal oxygen conditions (Fig. S6d) and their CM promoted HUVEC angiogenesis (Fig. S6e). In contrast, lentiviral overexpression of ZIP6 reduced the activation of the JAK1/STAT1/MMP-10 pathway (Fig. S6f). It suppressed the hypoxic CM enhancement of HUVEC angiogenesis (Fig. S6g). These results demonstrate the negative regulatory effect of ZIP6 on hypoxia-induced MMP-10 expression in BMSCs.

ZIP6 gain- and loss-of-function were investigated *in vivo* using a mouse skull defect model. Untreated BMSCs (BMSCs group), lentiviral vectors transfected for ZIP6-knockout ($\text{ZIP6}^{-/-}$ BMSCs group), or ZIP6-overexpressing BMSCs (ZIP6^{+} BMSCs group) were injected into the bone defects, whereas the Blank group received saline. Micro-CT one month after establishment showed that $\text{ZIP6}^{-/-}$ BMSCs had more noticeable therapeutic effects, whereas ZIP6^{+} BMSCs had essentially no therapeutic effect, which was consistent with the *in vitro* results (Fig. S7a). Furthermore, the results of histological (Fig. S7b) and CD31 immunohistochemical staining (Fig. S7c) were similar, indicating that the stimulatory effect of ZIP6^{+} BMSCs on local vascularized bone regeneration was significantly weakened. We also noticed that the promoting effect of $\text{ZIP6}^{-/-}$ BMSCs *in vivo* was not very stable, which could be attributed to ZIP6 dysfunction caused by the local hypoxic microenvironment in untreated BMSCs, which therefore showed no significant differences from ZIP6-knockout BMSCs. Therefore, we conclude that the enhancement strategy of this process should be based on the direct regulation of local cell function, and modification of transplanted stem cells may not be the best choice.

3.5. Fabrication and characterization of CD90@ZIF-8-ICG

As a well-known member of the MOF family, ZIF-8 is composed of 2-methylimidazole and zinc ions [66–68], therefore, it was selected as a biomaterial component to supplement intracellular zinc ions. It has potential for use in biology because of its degradability and substantial drug-carrying capability [69]. Based on the previous findings, engineered CD90@ZIF-8-ICG particles were designed to enhance angiogenesis and bone repair *in vivo*. Using the natural process of bone-defect healing, we targeted the internalization of CD90@ZIF-8 into local BMSCs to immediately activate the cytoplasmic Zn^{2+} /JAK1/STAT1/MMP-10 pathway to enhance blood vessel formation in the early stages of healing. The low-energy, laser-induced ICG photothermal

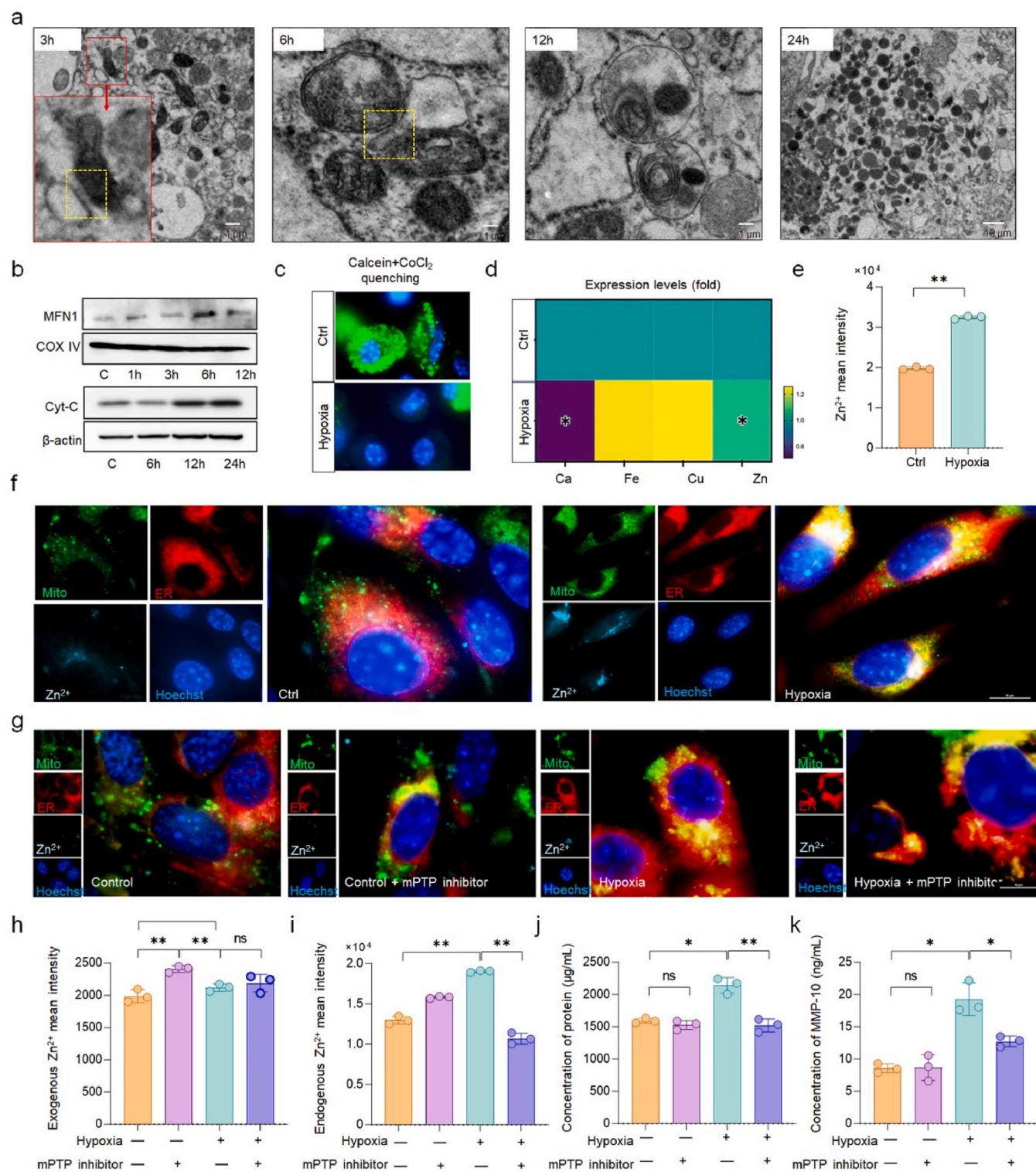


Fig. 3. BMSC mitochondrial permeability increased and cytoplasmic Zn²⁺ accumulated in the hypoxic microenvironment (a) TEM results of BMSCs cultured in a hypoxic milieu for different time periods. (b) Western blotting detecting the expression of MFN1 in the mitochondrial protein (up) and the expression of Cyt-C in the cytoplasmic protein (bottom). (c) mPTP detection results. (d) Multiple metal ion detection in the cytoplasmic components of BMSCs during the process of hypoxia induction. (e) Flow cytometry detection of cytoplasmic Zn²⁺ in BMSCs under hypoxia induction. (f,g) Fluorescence colocalization of cellular mitochondria, endoplasmic reticulum, and cytoplasmic Zn²⁺. (h,i) Flow cytometry detection of exogenous or endogenous Zn²⁺ in BMSCs. (j) The BCA quantitative results of the secreted protein in CM. (k) ELISA results of MMP-10 expression in BMSC CM. All *in vitro* results are representative of at least three independent experiments (n = 3 for each experiment). The yellow rectangle in (a) indicates the site of endoplasmic reticulum-mitochondria coupling. Scale bar in (f) & (g) = 10 μm. Data are presented as mean ± SD. *p < 0.05. **p < 0.01. Ctrl = Control. CM = conditioned medium. BMSC = bone marrow mesenchymal stem cell.

effect continued to promote temperature-dependent osteogenic differentiation of BMSCs in the subsequent process, thus promoting bone-tissue regeneration at the later stage of healing. ICG-loaded ZIF-8 (ZIF-8-ICG) was prepared using a simple one-step method according to the literature [70]. The morphology of ZIF-8-ICG was characterized using TEM, and the elemental mapping analysis results showed that ZIF-8-ICG was mainly composed of zinc, nitrogen, carbon, and oxygen (Fig. 4a). Additionally, the hydrodynamic size of ZIF-8-ICG determined by the dynamic light scattering (DLS) was 123.29 ± 6.10 nm (Fig. 4b).

To confirm the successful preparation of ZIF-8-ICG, the UV–vis–NIR absorption spectra were recorded to determine the effect of ICG modification on ZIF-8. The ICG modification did not have a negative effect on the optical properties of ZIF-8, as evidenced by the absorption peak near 800 nm of ZIF-8-ICG, whereas no absorption was observed in the pure ZIF-8 nanoparticles (Fig. 4c). X-ray photoelectron spectroscopy (XPS) spectrum analysis (Fig. 4d) showed that the surface of ZIF-8-ICG mainly contained signals from carbon (C), nitrogen (N), and zinc (Zn) originating from ZIF-8. Sodium (Na) and sulfur (S) elements were also detected, indicating the successful introduction of ICG. Oxygen (O) originates from ICG as well as pollution during synthesis. High-resolution spectra confirmed $\text{Zn}2\text{p}3/2$ at a binding energy (BE) of 1022.17 eV and $\text{Zn}2\text{p}1/2$ at BE = 1045.21 eV (Fig. S8a), confirming the tetrahedral coordination of Zn^{2+} . The presence of C–N (399.68 eV) and $=\text{N}-(+)$ (401.50 eV) was also confirmed (Fig. S8b). The ZIF-8-ICG surface had a high C–N proportion of 79.62 %. This high ratio is the key to promoting cell adhesion because the primary amine group provides binding sites. The high-resolution spectra also confirmed the presence of BE 284.80 eV (C–C/C=C), 286.42 eV (C–O/C–N), 287.50 eV (C=O), 532.82 eV (C–O/S–O), and 531.85 eV (C=O) (Fig. S8c and 8d). Considering the chemical formula composition of ICG ($\text{C}_{43}\text{H}_{47}\text{N}_2\text{NaO}_6\text{S}_2$) and ZIF-8 ($\text{Zn}(\text{C}_4\text{H}_5\text{N}_2)_2$), the N content in ICG was 3.62 %, whereas in ZIF-8, it was 24.62 %. High-resolution N1s spectra of ZIF-8, ICG, and ZIF-8-ICG (Fig. 4e, Fig. S8b) show that only C–N bonds exist in ZIF-8. In ICG, the bonds present are C–N (71.4 %) and $=\text{N}-(+)$ (28.6 %). For ZIF-8-ICG, the percentages are 79.62 % for C–N and 20.38 % for $=\text{N}-(+)$. Calculating the nitrogen source in ZIF-8-ICG revealed that the drug loading of ICG in ZIF-8-ICG was 94.41 % (Fig. 4f).

The *in vitro* photothermal properties of ZIF-8-ICG were evaluated using an NIR laser (808 nm, $1.0 \text{ W}/\text{cm}^2$) for 3 min. The temperature changes were monitored using an Infrared Thermal Imager (Fig. 4g). ZIF8-ICG underwent a rapid increase in temperature after exposure to NIR irradiation, from 36.0 to 42.6 °C. To evaluate the photothermal stability of ZIF8-ICG, we observed the photothermal effect of the nanoparticles in α -MEM medium, 5 % CO_2 , 37 °C for 9 days. After 3 min of NIR irradiation on day 9, ZIF8-ICG could still reach a temperature of approximately 40 °C (Fig. 4g). The temperature did not substantially decrease as the culture time increased, suggesting that ZIF8-ICG had good photothermal stability and could be used repeatedly and successfully in the photothermal treatment process.

To improve the biocompatibility and targeting of ZIF-8-ICG, CD90-liposome was modified on the ZIF-8-ICG surface. TEM and DLS revealed that CD90@ZIF-8-ICG grew in size (139.98 ± 8.17 nm) and had a core-shell structure (Fig. 4h and i). The polydispersity index (PDI) of CD90@ZIF-8-ICG was 0.18 (data not shown). The zeta potential of CD90@ZIF-8-ICG was similar to that of CD90-liposome, indicating the successful preparation of CD90@ZIF-8-ICG (Fig. 4j). The encapsulation efficiency was 66.00 % (data not shown). The flow cytometry results demonstrated that CD90 was highly expressed on the surface of CD90@ZIF-8-ICG (Fig. 4k). These results showed that we successfully constructed CD90@ZIF-8-ICG with a stable photothermal effect. According to the results of CCK8 (Fig. 4l) and cell apoptosis detection (Fig. 4m), CD90@ZIF-8-ICG based on an effective ZIF-8 concentration of $30 \mu\text{g}/\text{mL}$ was used for follow-up *in vitro* experiments (equivalent to $8.62 \mu\text{g}/\text{mL}$ of zinc ion concentration). As expected, we demonstrated that CD90@ZIF-8-ICG targeted BMSCs by constructing an *in vitro* co-culture system of BMSCs and RAW264.7 (Fig. 4n). In addition, flow

cytometry was performed to quantify the co-localization efficiency of CD90@ZIF-8-ICG and BMSCs. The results showed that 82.26 % of the CD90@ZIF-8-ICG particles were phagocytosed by BMSCs 24 h after intervention in such *in vitro* co-culture system (Fig. 4o).

Despite multiple reports proving that heat can promote the osteogenic differentiation of BMSCs [71], the duration of NIR intervention in the CD90@ZIF-8-ICG system needs to be discussed. Therefore, we used different durations of NIR stimulation while conducting osteogenic induction of BMSCs *in vitro* and detected the expression of various osteogenic differentiation genes using real-time PCR. The NIR intervention for seven consecutive days steadily promoted the upregulation of osteogenic genes in BMSCs, including *Alp*, *Runx2*, *Osx*, and *Opn* (Fig. 5a–d). ALP and alizarin red staining further demonstrated that CD90@ZIF-8-ICG + 7-day NIR stimulation significantly improved the osteogenic differentiation of BMSCs (Fig. 5e). Moreover, consecutive days of photothermal stimulation did not cause any changes in cell proliferation (Fig. 5f) or apoptosis (Fig. 5g). To quantify the regulatory effect of the photothermal treatment on BMSCs, *hsp70* was detected, which has been proven to be an important factor regulating stem cell osteogenic differentiation in response to temperature [72]. The results indicated that photothermal intervention for over five consecutive days could stably promote the expression levels of *hsp70* in BMSCs, which also indirectly proved the promotion of the photothermal effect on related functions in BMSCs (Fig. S9). Furthermore, the zinc ions released by the internalized CD90@ZIF-8-ICG vesicles were enriched in the cytoplasm of the BMSCs (Fig. 5h), increasing their MMP-10 production (Fig. 5i and j), which ultimately promoted the angiogenic differentiation of endothelial cells (Fig. 5k). This effect was similar to that of the previous *in vitro* hypoxia stimulation and comparable to those of the positive control groups with additional 20 ng/mL VEGF [73] or 100 ng/mL rhMMP-10 [74] added to the BMSC CM (Fig. 5k).

Our results on zinc ion-promoting stem cell osteogenesis are consistent with previous studies [75,76]. Both *in vitro* [77–82] and *in vivo* [83] studies have shown that zinc promotes osteoblast differentiation and proliferation and upregulates ALP activity. Zinc induction *in vitro* [84–88] showed the effect of promoting the expression of runt-related transcription factor 2 (RUNX2) in MSCs. Considering that zinc ions show a regulatory effect on the osteogenesis of stem cells, we compared the osteogenic differentiation of BMSCs treated with different concentrations of CD90@ZIF-8-ICG, with or without NIR intervention. We found that within the concentration range that does not affect cell viability ($<30 \mu\text{g}/\text{mL}$), CD90@ZIF-8-ICG promotes the osteogenic ability of BMSCs in a concentration-dependent manner. As expected, additional NIR irradiation promoted these effects (Fig. S10). Previous studies have demonstrated that ZIF-8 can stimulate angiogenesis and bone repair *in vitro* and *in vivo* [89–91], and our study provides a possible mechanistic explanation.

3.6. *In vivo* safety and efficacy evaluation of CD90@ZIF-8-ICG

CD90@ZIF-8-ICG particles were injected *in vivo* to treat skull bone defects in mice, and their safety and efficacy were evaluated (Fig. 6a). Routine and biochemical blood test results 24 h after CD90@ZIF-8-ICG injection showed it was safe and did not cause significant changes in the blood parameters (Fig. 6b, Fig. S11). The immunohistochemical staining results of the local tissue of bone defect indicated that neither CD90@ZIF-8-ICG nor CD90@ZIF-8-ICG + NIR intervention caused an increase in local inflammatory factors, including $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ (Fig. 6c–d). In addition, the targeting of CD90@ZIF-8-ICG was verified *in vivo*, and most of the particles were internalized by $\text{CD}105^+$ BMSCs 24 h after injection (Fig. 6e). Flow cytometry was performed to assess the distribution of CD90@ZIF-8-ICG in the local MSCs ($\text{CD}105^+\text{CD}146^+$) and immune cells ($\text{CD}45^+$). The results showed that 57.64 % of the CD90@ZIF-8-ICG was located in MSCs and 31.71 % of the CD90@ZIF-8-ICG was located in immune cells 24 h after injection, indicating the MSC-targeting property of the CD90@ZIF-8-ICG (Fig. 6f). As a result of

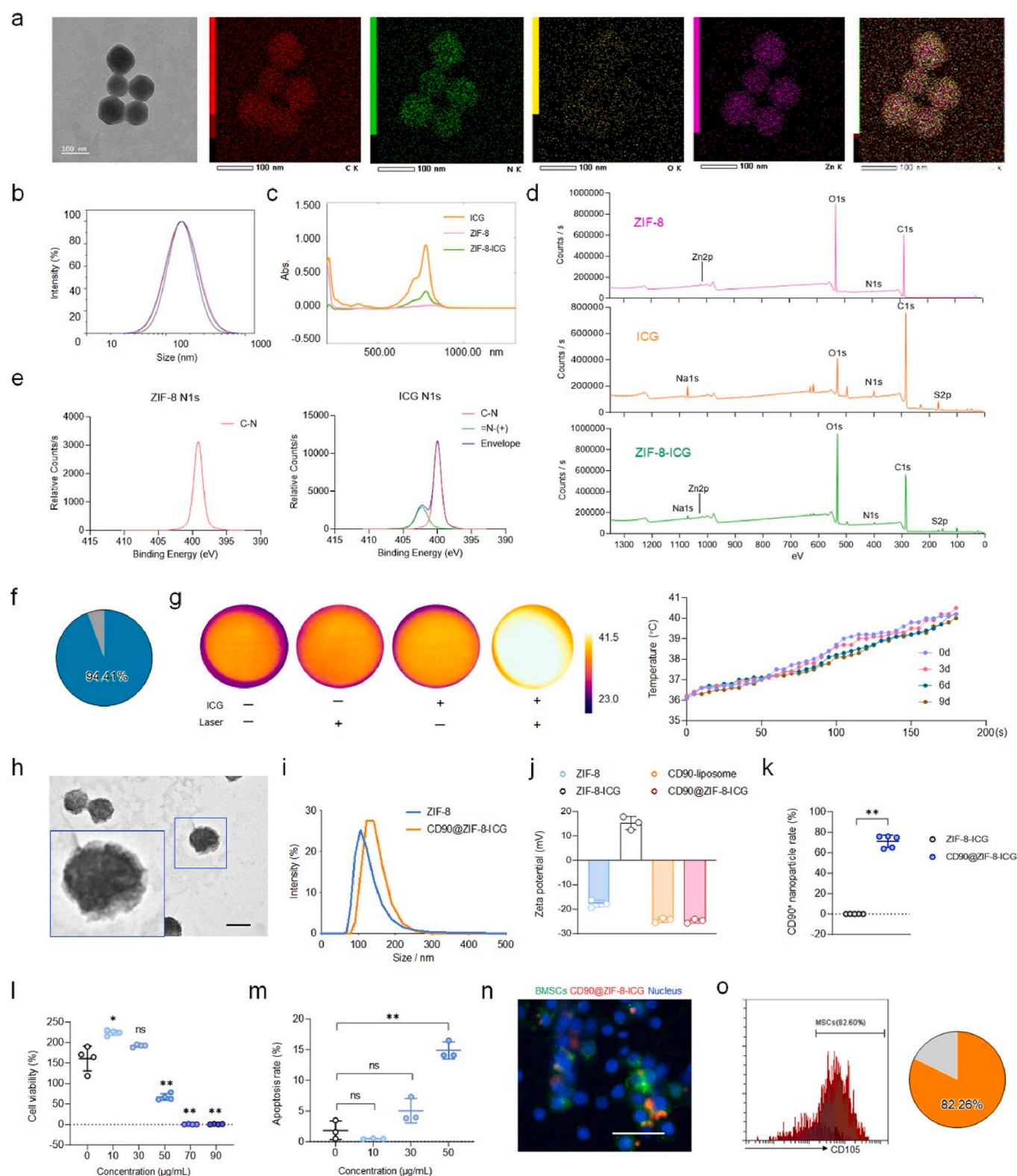


Fig. 4. Preparation and characterization of CD90@ZIF-8-ICG (a) TEM and the elemental mapping analysis results showed that ZIF-8-ICG was mainly composed of zinc, nitrogen, carbon and oxygen. (b) Dynamic light scattering detection of ZIF-8-ICG. The two DLS datasets were derived from the results of different batches of samples. (c) UV-vis-NIR absorption spectra detection of ZIF-8-ICG. (d) XPS survey spectrum of ZIF-8, ICG and ZIF-8-ICG. (e) High-resolution N1s spectrum of ZIF-8 and ICG. (f) The drug loading of ICG in ZIF-8-ICG was 94.41 %. (g) The photothermal effect evaluation of the ZIF-8-ICG under 808 nm NIR laser. (h) TEM results of CD90@ZIF-8-ICG. (i) Dynamic light scattering detection of ZIF-8 and CD90@ZIF-8-ICG. (j) Zeta potential of ZIF-8, ZIF-8-ICG, CD90-liposome and CD90@ZIF-8-ICG. (k) Flow cytometry detection of CD90 expression. (l) Cell Counting Kit-8 results. (m) Cell apoptosis detection. (n) Fluorescence colocalization of BMSCs (green), CD90@ZIF-8-ICG (red), and nucleus of BMSCs and RAW264.7 (blue). (o) The co-localization efficiency of CD90@ZIF-8-ICG in BMSCs *in vitro*. All *in vitro* results are representative of at least three independent experiments ($n = 3$ for each experiment). Data are presented as mean $\pm$ SD. Scale bar in (a) = 100 nm. Scale bar in (h) = 100 nm. Scale bar in (n) = 50 μ m ns $p > 0.05$. * $p < 0.05$. ** $p < 0.01$. CM = conditioned medium. BMSC = bone marrow mesenchymal stem cell.

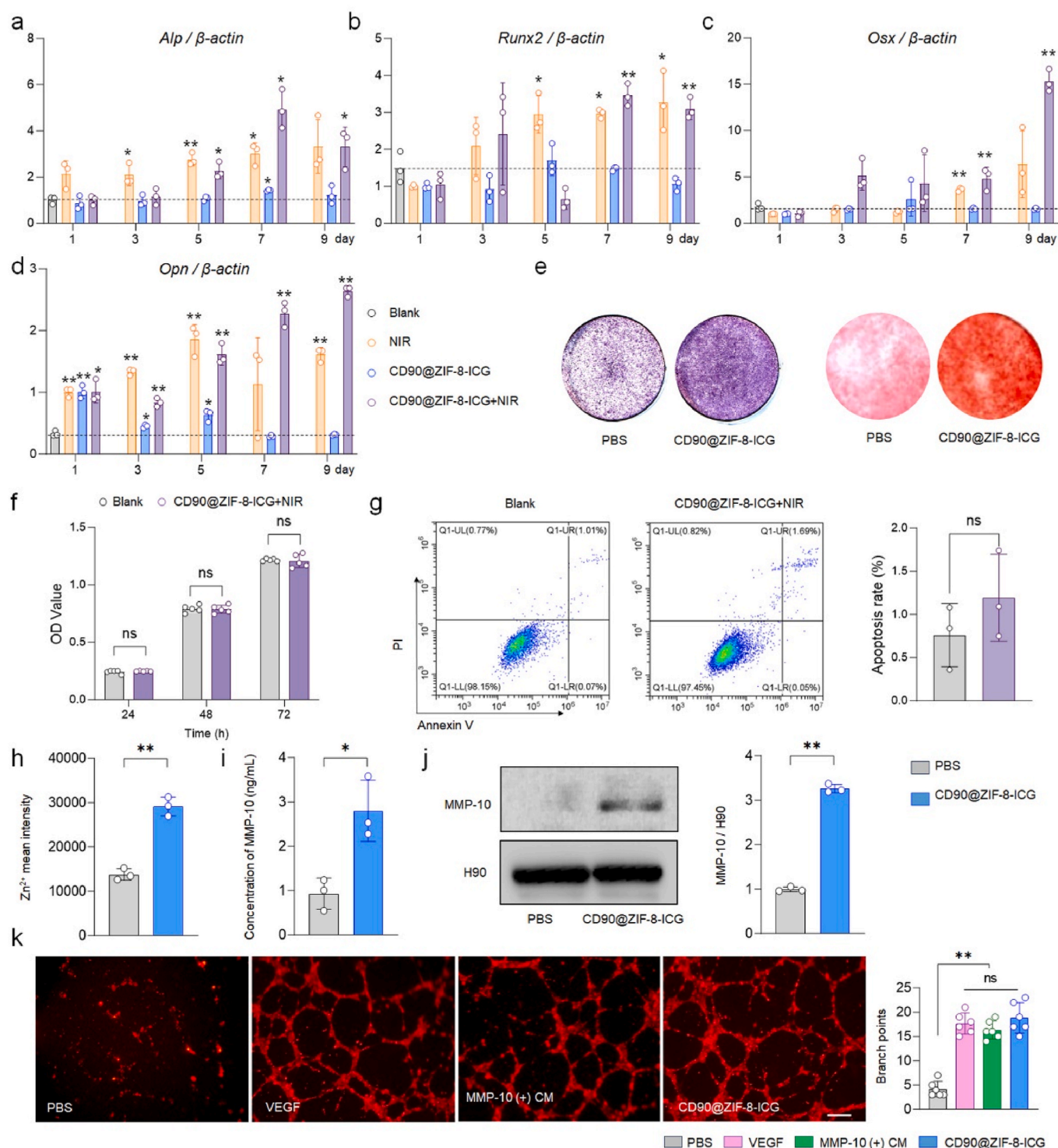


Fig. 5. Regulatory effect evaluations of CD90@ZIF-8-ICG (a–d) Real-time PCR detection of osteogenic genes of BMSCs, including *Alp*, *Runx2*, *Osx* and *Opn*. (e) Alkaline phosphatase staining (left) and alizarin red staining (right). (f) Cell Counting Kit-8 results. (g) Cell apoptosis detection. (h) Flow cytometry detection of cytoplasmic Zn^{2+} in BMSCs. (i) ELISA and (j) western blotting results of MMP-10 expression. (k) The results of the angiogenesis induction assay. All *in vitro* results are representative of at least three independent experiments ($n = 3$ for each experiment). Data are presented as mean $\pm$ SD. Scale bar in (k) = 100 μ m ns $p > 0.05$. * $p < 0.05$. ** $p < 0.01$. CM = conditioned medium. BMSC = bone marrow mesenchymal stem cell.

the CD90@ZIF-8-ICG intervention, the gene expression of the key regulatory factor in local tissues, *Mmp10*, was significantly increased at 3 days after injection (Fig. 6g). The expression levels of genes related to local angiogenesis was also increased 7 days after injection, including *Vegf* and *Ang1* (Fig. 6h). The residual CD90@ZIF-8-ICG remained in the tissue for at least one week, which was confirmed by *in vivo* fluorescence

imaging of the visualized Cy7se-labeled CD90@ZIF-8-ICG (Fig. 6i). Similarly, the photothermal effect of CD90@ZIF-8-ICG under NIR irradiation could also be maintained for 7 days (Fig. 6j), which met the needs of *in vivo* osteogenesis promotion. These results indicate that the CD90@ZIF-8-ICG particles were effectively delivered to the local stem cells in the bone-defect tissues and remained at the defect site for the

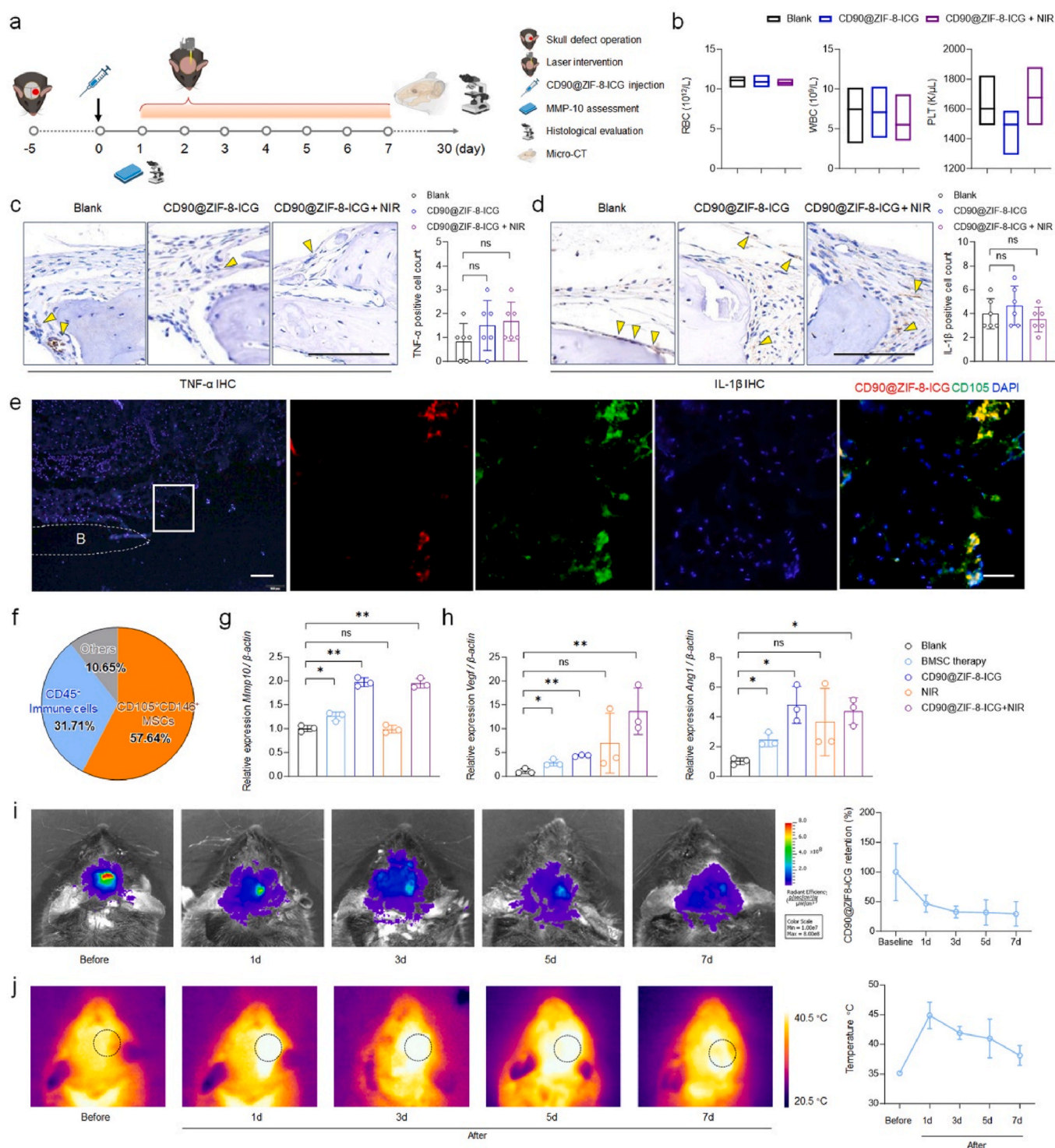


Fig. 6. *In vivo* safety and regulatory effect evaluation of CD90@ZIF-8-ICG (a) Schematic illustration of the establishment and sampling process of *in vivo* study. (b) Blood routine and blood biochemical test results 24 h-post injection (n = 6). (c) TNF- α detections using immunohistochemical staining results. (d) IL-1 β detections using immunohistochemical staining results. (e) Fluorescence colocalization of CD105⁺ BMSCs (green), CD90@ZIF-8-ICG (red), and DAPI (blue). B = bone. (f) The co-localization efficiency of CD90@ZIF-8-ICG in CD105⁺CD146⁺ BMSCs or CD45⁺ immune cells *in vivo* (n = 6). (g) Real-time PCR detection of local *Mmp10* gene expression 3 days after injection (n = 3). (h) Real-time PCR detection of local *Vegf* (left) and *Ang1* (right) gene expression 7 days after injection (n = 3). (i) The *in vivo* fluorescence imaging results of the visualized Cy7se-labeled CD90@ZIF-8-ICG (n = 4). (j) *In vivo* photothermal effect of CD90@ZIF-8-ICG under the NIR intervention (n = 4). Scale bar in (c) and (d) = 100 μ m. Scale bar in (e) = 100 μ m (left) and 50 μ m (right). Data are presented as mean $\pm$ SD. ns p > 0.05. * p < 0.05. ** p < 0.01.

following time to exert photothermal regulatory effects.

As an FDA-approved NIR fluorescent dye, ICG absorbs NIR light and transforms the light energy into thermal effects [92]. Therefore, the CD90@ZIF-8-ICG achieved continuous NIR-dependent heating of the

bone defect during the later stage of healing, promoting bone regeneration since low localized heat (40–43 $^{\circ}$ C) can promote BMSC proliferation, ALP activity, and mineralization [93–95]. Localized heat stimulation may raise safety concerns, while our *in vivo* and *in vitro*

results proved the safety of the CD90@ZIF-8-ICG + NIR intervention. Other studies have demonstrated the effectiveness and safety of regulating bone repair at photothermal temperatures. Liao et al. [96] presented a treatment modality that combined a mild photothermal therapy

hydrogel with a pulsed drug delivery mechanism. They active mild photothermal nanoparticles in the temperature range of 40–43 °C (for 3 min), which enhance the precision and efficacy of drug delivery. Consequently, these systems addressed infection control and promoted

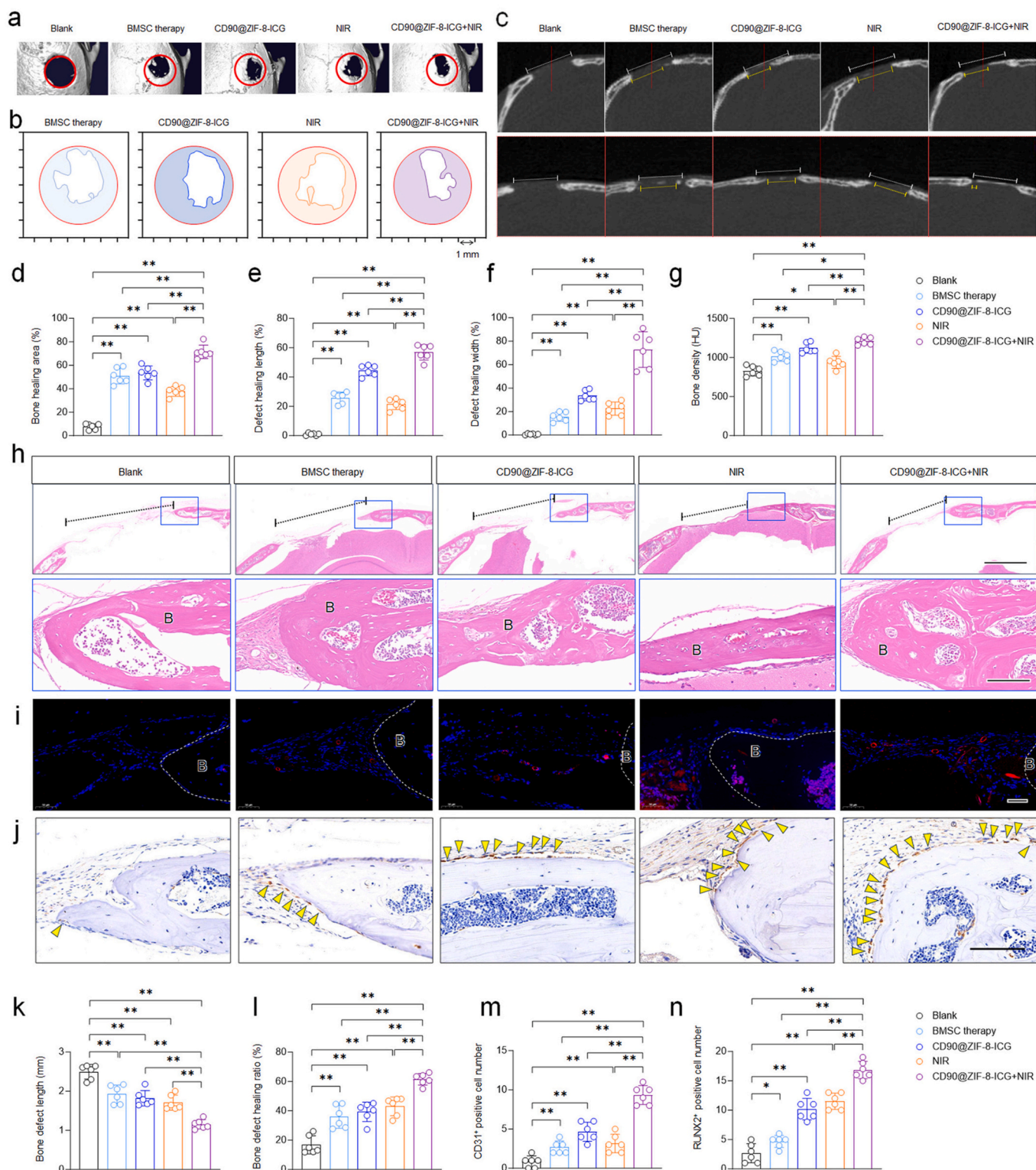


Fig. 7. *In vivo* efficacy evaluation of CD90@ZIF-8-ICG (a) 3D reconstruction images of micro-CT (up) and bone defect area ratio analysis (bottom) one month-post injection. (b) Bone defect length measurements. (d–g) Detection results of bone mineral density, bone healing area, defect healing length (%) and defect healing width (%) (n = 6). (h) Histological staining results. (i) CD31 immunofluorescence results. (j) RUNX2 detection results using immunohistochemical staining. (k,l) Data analysis for (h) (n = 6). (m) Data analysis for (i) (n = 6). (n) Data analysis for (j) (n = 6). Scale bar in (h) = 1000 μ m (up) and 100 μ m (bottom). Scale bar in (i) = 50 μ m. Scale bar in (j) = 100 μ m. Data are presented as mean $\pm$ SD. ns $p > 0.05$. * $p < 0.05$. ** $p < 0.01$. B = bone.

bone regeneration in infectious cranial defect models.

The bone defect healing effects in each group were evaluated 1 month after injection using micro-CT. The 3D reconstruction images directly show the promoting effect of the CD90@ZIF-8-ICG + NIR intervention on local bone regeneration (Fig. 7a). Bone defect length and mineral density measurements were performed, and the results confirmed that the CD90@ZIF-8-ICG + NIR intervention significantly and stably promoted the healing of bone defects, and the therapeutic effect was superior to that of the BMSC therapy group (Fig. 7b–g). Histological staining results showed that the healing effect of bone defects was the best in the CD90@ZIF-8-ICG + NIR group, and new bone and neovascularization structures were observed at the defect margins (Fig. 7h–k,l). To more accurately determine the formation of local blood vessels, CD31 immunofluorescence staining was performed. As expected, the results showed that CD90@ZIF-8-ICG + NIR increased vascularized bone regeneration (Fig. 7i–m). The RUNX2 immunohistochemical staining results showed that the CD90@ZIF-8-ICG + NIR intervention promoted local osteoblast differentiation *in vivo* (Fig. 7j–n). These results indicate that CD90@ZIF-8-ICG is a potential biomaterial candidate for promoting vascularized bone regeneration based on the natural mechanism regulated by transplanted stem cells.

It is particularly noted that many studies have shown that local application of metal ions can enhance the efficiency of bone regeneration [97]. The application of a zinc-combined scaffold *in vivo* significantly promotes the homing of BMSCs and endothelial cells to the bone-defect area, thus promoting bone regeneration by improving BMSC migration and vessel formation [98]. However, the regulatory roles and mechanisms of metal ions in bone-tissue repair require more in-depth exploration. It is necessary to clarify the regulatory mechanisms of metal ions on host cells as well as develop corresponding precise delivery and intervention strategies before actual clinical application [99]. This study innovatively describes the regulatory importance of intracellular zinc ion homeostasis and ZIP proteins in the function of transplanted MSCs, as well as the related efficiency strategy development, which elucidates MSC therapy treatment mechanisms and provides strategies for the design and development of stem cell-based biomaterials.

This study had some limitations. The mechanism by which ZIP6 regulates the activation of molecular pathways remains unknown. Metal ion transporters cause changes in protein conformation when sensing variations in ion concentration, which is a prerequisite for their ability to transport ions unidirectionally [100]. Whether changes in protein conformation can alter the manner in which they bind to downstream kinases requires in-depth exploration. Based on these findings, innovative agonists or drugs also need to be developed in the future. In addition, the lack of large animal experiments and limited *in vivo* observation time for bone repair make the clinical translatability of CD90@ZIF-8-ICG questionable. Further large-animal model validation and more observation time points need to be performed to evaluate the scalability. For *in vitro* studies, owing to the excessive differences within the groups, the results for Fe²⁺ and Cu²⁺ were not statistically significant. However, we cannot exclude the possibility that hypoxia may have short- or long-term effects on these ions in the mitochondria or cytoplasm as well as on calcium ions [101,102]. In addition, MMP-10 was screened due to its most significant fold change, resulting in protein array data, which did not mean that it was the only regulatory factor. Therefore, the functions of other secretory proteins need to be further explored. In the future, larger sample sizes, more diverse animal models, and more comprehensive explorations are required.

4. Conclusion

In conclusion, our findings show that transplanted BMSCs in bone defects have increased mitochondrial permeability in response to local hypoxia, resulting in passive transport and cytoplasmic enrichment of mitochondrial zinc ions, which eventually inhibit ZIP6 protein and

promote activation of the JAK1/STAT1/MMP-10 pathway. MMP-10 secretion enhances the angiogenic function of the host endothelial cells, thereby promoting vascularized bone regeneration. Based on this process, an intelligent biomimetic material with simultaneous two-stage regulatory effects was designed and demonstrated to promote vascularized bone regeneration *in vivo*. This study elucidates the treatment mechanisms of transplanted MSCs and offers strategies for designing and developing stem cell-based biomaterials.

CRediT authorship contribution statement

Yitong Liu: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition. **Siyan Liu:** Writing – review & editing, Visualization. **Juan Du:** Project administration. **Junji Xu:** Writing – review & editing. **Jing Li:** Writing – review & editing, Writing – original draft, Supervision. **Lijia Guo:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition. **Yi Liu:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Ethics Committee of the Beijing Stomatological Hospital (KQYY-202008-005; Beijing, China).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioactmat.2025.07.020>.

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