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Targeted delivery of engineered extracellular vesicles to simultaneously promote vascularization and muscle regeneration in ischemic limbs

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Abstract

Critical limb ischemia (CLI) leads to a high rate of limb amputation. Regenerating vasculature and skeletal muscles can save the affected limbs. Therapy using stem cell-derived extracellular vesicles (EVs) has emerged as a promising approach. However, the therapeutic efficacy is limited because EVs were not engineered to simultaneously possess the optimal composition of proangiogenic and promyogenic factors necessary to effectively support the survival, migration, and morphogenesis of endothelial and skeletal muscle cells under ischemic conditions. We discovered that the proangiogenic and promyogenic factors, including miR-126, miR-21, miR-296, miR-182, PDGF-BB, VEGF, bFGF, and HGF, can be concurrently upregulated in EVs derived from human iPSC-derived mesenchymal stem cells (iMSCs) by enhancing either N-cadherin-mediated or RGD-mediated interactions between the cells and matrix. Notably, enhancing N-cadherin interaction was more effective in upregulating these factors. The EVs from enhanced N-cadherin interaction markedly improved survival, migration, and morphogenesis of endothelial cells and myoblasts under the CLI-like conditions. To ensure targeted delivery to ischemic limbs, these EVs were cloaked with platelet membranes modified with an ischemia-homing peptide. Following intravenous delivery in a murine model of ischemic hindlimb, the EVs fully restored blood perfusion within 28 days, and significantly promoted skeletal muscle regeneration. These results

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underscore the potential of EVs with simultaneously upregulated proangiogenic and promyogenic factors in effectively treating CLI.

Keywords

Critical limb ischemia; Extracellular vesicles; N-cadherin interaction; Angiogenesis; Muscle regeneration

1. Introduction

Peripheral arterial disease (PAD) constitutes a significant global health burden, affecting over 200 million individuals. It is a leading cause of amputation (3 %–4 %) and is associated with a considerably elevated mortality risk, as evidenced by an adjusted crude 5-year death rate of 82.4 per 1000 patient-years [1]. The most severe form of PAD is critical limb ischemia (CLI), for which revascularization procedures like bypass grafting and angioplasty are primary treatments. However, less than 45 % of patients are eligible for these procedures due to comorbidities, frequently resulting in short-term amputations [2]. This underscores a critical need for alternative treatments for these “no-option” patients to re-establish vascularization and regenerate skeletal muscles.

Stem cell therapy is a promising approach for treating CLI [3]. Preclinical and clinical studies have shown that stem cells can stimulate angiogenesis to increase blood perfusion in the ischemic limbs, mainly through paracrine effects [4]. Despite its potential to promote revascularization, current stem cell therapy shows limited efficacy due to poor cell survival and engraftment in ischemic limbs. Additionally, concerns about the safety and scalability of stem cell therapy limit its widespread clinical adoption [5]. An emerging alternative is the use of stem cell-derived EVs, which offer similar therapeutic benefits in a cell-free format [6]. EVs deliver bioactive molecules such as microRNAs (miRNAs) to endothelial and skeletal muscle cells in ischemic limbs, regulating their functions and thus promoting vascularization and muscle regeneration [7]. Using EVs for CLI treatment offers advantages over therapies based solely on growth factors and miRNAs, as EVs can simultaneously deliver multiple essential factors required for these processes [8–12].

Despite the promising therapeutic effects of stem cell-derived EVs for CLI treatment [13–15], their current efficacy remains suboptimal [8]. This is partly because these EVs are not tailored with an optimal composition of proangiogenic and promyogenic factors necessary for rapid vascularization and myogenesis. Current methods to modify EV composition include genetic modification of parent cells or the EVs themselves, manipulation of cell culture conditions, and exposure to biochemical or growth factors. While gene transfection into parent cells [16–18] or EVs [19,20] can enhance the therapeutic potential of EVs by producing those with highly expressed specific miRNAs, it also carries risks of off-target effects. Adjusting cell culture conditions like media composition, temperature, and oxygen levels can modulate the biomolecular profile of EVs [21–23]. Hypoxia preconditioning, for instance, has been shown to increase the expression of proangiogenic factors in EVs from mesenchymal stem cells (MSCs) [24–27]. However, our previous studies suggest that hypoxia-induced EVs alone were not sufficiently effective for revascularization in a

diabetic CLI model [27]. Exposing parent cells to specific biochemicals or growth factors can also alter cargo content [28–30]. However, this approach faces challenges in specifically upregulating proangiogenic and promyogenic factors necessary for effective vascularization and muscle regeneration. Moreover, using soluble biochemicals and growth factors can complicate both the composition of EVs and their therapeutic effects [31–33]. Therefore, there is a critical need for new strategies that can specifically and simultaneously enhance the expression of these crucial factors in stem cell-derived EVs.

To address these challenges, we developed a strategy to specifically and simultaneously upregulate proangiogenic and promyogenic factors in stem cell-derived EVs. This strategy is based on enhancing N-cadherin-mediated cell-cell interactions and RGD-mediated integrin-matrix interactions, both of which have been shown to modulate the cellular secretome [28,34–36]. N-cadherin, a key adhesion molecule, plays a critical role in cell-cell communication and the regulation of cellular secretory profiles [28,34,37,38], while integrins, which mediate cell-matrix interactions, have also been implicated in modulating the composition of the cellular secretome [35,36]. While previous studies have focused on the growth factor composition in conditioned media, little is known about the specific microRNA composition inside EVs secreted under these conditions. To bridge this gap, we systematically analyzed EV composition in the secretome of iMSCs, modulated by HAVDI-mediated cell-cell interactions (N-cadherin) and RGD-mediated cell-matrix interactions (integrins). To achieve this, we immobilized two functional peptides on a matrix: Ac-HAVDIGGGK (HAVDI), which mimics the extracellular domain of N-cadherin to enhance cell-cell interactions, and GRGDS, which recognizes integrin receptors to strengthen cell-matrix adhesion. We then investigated how these interactions influenced EV secretion and enriched EV cargo with proangiogenic and promyogenic factors in human-induced pluripotent stem cell-derived mesenchymal stem cells (iMSCs). The rationale of using iMSCs is that they offer greater proliferation potential than adult tissue-derived MSCs, making them suitable for large-scale production of EVs. Additionally, iMSCs can be derived from the induced pluripotent stem cells of a specific patient, allowing to produce personalized EVs. These EVs can then be applied therapeutically to the same patient, ensuring compatibility and potentially reducing the risk of immune rejection. The EVs with upregulated proangiogenic and promyogenic factors were modified for targeted delivery to ischemic limbs after intravenous administration. We evaluated their efficacy in promoting survival, migration, and morphogenesis of endothelial cells and myoblasts in vitro under CLI-mimicking conditions and investigated their role in regenerating vasculature and skeletal muscles in murine ischemic hindlimbs.

2. Materials and methods

All peptides, including Ac-HAVDIGGGK (HAVDI), GRGDS (RGD), tetramethylrhodamine (TMR) functionalized-HAVDI (Ac-HAVDIGGGK-K-TMR, TMR-HAVDI), TMR functionalized-RGD (GRGDS-K-TMR, TMR-RGD), CSTSMLKAC (CST), and TMR functionalized-CSTSMLKAC (TMR-CST), were purchased from Genemed Synthesis Inc. All other chemicals and bioreagents were supplied by Sigma-Aldrich unless otherwise noted.

2.1. Cell lines and culture

Induced pluripotent stem cell-derived mesenchymal stem cells (iMSCs, MSC-301–010-001-PT) obtained from FUJIFILM Cellular Dynamics were maintained in the serum-free medium recommended by the manufacturer, as detailed in Table S1. According to the supplier, these cells exhibit high CD105 expression (>98 %) and low CD31 expression (<2 %) based on flow cytometry analysis, confirming their MSC identity and minimal endothelial cell contamination. Additionally, they can be induced to differentiate into adipocytes, chondrocytes, and osteocytes using the specified differentiation media.

The C2C12 myoblast cell line (CRL-1772, ATCC) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10 % fetal bovine serum (FBS, Atlanta Biologicals) and 1 % penicillin/streptomycin (Gibco™). Human umbilical vein endothelial cells (HUVECs, PCS-100–013, ATCC) were grown in vascular cell basal medium (ATCC) supplemented with the endothelial cell growth kit-BBE (ATCC) and 1 % penicillin/streptomycin. The THP-1 cell line (TIB-202, ATCC) was cultured in RPMI 1640 medium (ATCC) supplemented with 10 % FBS and 0.5 mM 2-mercaptoethanol (Gibco™). All cells were maintained in a humidified environment at 37 °C with 5 % CO₂, unless otherwise specified.

2.2. Functionalization of glass dish with glycine, RGD peptide, or HAVDI peptide

To functionalize glass dishes with glycine, RGDS, or HAVDI, the process outlined in Fig. 1A was followed [39,40]. Initially, the glass dishes were treated with a freshly prepared Piranha solution (H₂SO₄/H₂O₂ (30 %) = 3:1) at 95 °C for 2 h to introduce hydroxyl groups on the surface. After thorough rinsing with DI water, the dishes were incubated with a 10 % solution of (3-Aminopropyl)triethoxysilane (APTES) in methanol at 60 °C for 2 h. The silanized dishes were then rinsed with 70 % ethanol and exposed to UV light for 1 h. Subsequently, a 2.5 % glutaraldehyde (GA) solution was applied at 37 °C for 1 h to introduce aldehyde groups on the surface. Solutions containing 30 mg/mL of glycine, 8.5 mg/mL of RGD, or 10 mg/mL of HAVDI were added to the dishes and left to react overnight at 37 °C. After the peptide binding, the dishes were further treated with a 30 mg/mL glycine solution at 37 °C for 2 h to block any remaining reactive aldehyde groups. Finally, the functionalized dishes were rinsed with DPBS before use.

To assess the conjugation ratio of HAVDI, silanized glass dishes were incubated with TMR-RGD (10 mg/mL) and TMR-HAVDI solution (13 mg/mL in 1× DPBS) at 37 °C overnight. Subsequently, the residual amount of TMR-RGD and TMR-HAVDI in the solution was measured using a SpectraMax iD3 plate reader (Molecular Devices) with excitation and emission wavelengths of 550 and 580 nm, respectively, and calculated using a standard curve.

2.3. Isolation of extracellular vesicles (EVs) derived from iMSCs under various induction

iMSCs were plated onto glass dishes functionalized with glycine, RGD, or HAVDI at a density of 1.2×10^6 cells per dish (Fig. 1A). After 24 h of incubation, the maintenance medium (composition listed in Table S1) was removed, and the cells were washed three times with DPBS. Each dish was then supplemented with 10 mL of serum- and growth

factor-free medium (composition listed in Table S2) that had been pre-conditioned under 1 % O₂. The dishes were subsequently incubated at 37 °C in a humidified environment with 1 % O₂ and 5 % CO₂ for 3 days. Following this period, the culture medium was collected and centrifuged at 2000 ×g for 30 min to eliminate cell debris.

For the EV isolation, the supernatant was treated with Total Exosome Isolation Reagent (Invitrogen™) as per the manufacturer's guidelines. The reagent was mixed with the supernatant in a 1:2 ratio and incubated at 4 °C overnight. EVs were then concentrated by centrifugation at 10,000 ×g for 1 h at 4 °C. The resulting pellet of EVs was resuspended in DPBS and passed through a 0.22-µm sterile filter. The concentration of EVs was determined using a protein assay dye reagent concentrate (Bio-Rad).

2.4. EV biomarker profiling

The analysis of biomarkers on EVs was performed using the Exo-Check™ Exosome Antibody Array (System Biosciences) following the manufacturer's guidelines. Initially, 50 µg of EVs (G-EVs, R-EVs, or H-EVs) were lysed using 10× lysis buffer. The lysate was then labeled with 1 µL of labeling reagent at room temperature for 30 min. After labeling, the lysate was purified using the provided column and then incubated with the supplied membrane at 4 °C overnight. After incubation, the membrane was washed with wash buffer and then exposed to detection reagent at room temperature for 30 min. Detection was conducted using WesternBright™ ECL HRP substrate (Advansta), and signals were captured with a ChemiDoc™ XRS+ Gel Imaging System (Bio-Rad) equipped with Image Lab™ software.

2.5. Analysis of EV composition

MicroRNAs (miRNAs) within EVs were isolated using the Total Exosome RNA and Protein Isolation Kit (Invitrogen™) following the manufacturer's instructions. Initially, the EV pellets were resuspended in ice-cold resuspension buffer and lysed using an equal volume of 2× denaturing solution on ice for 5 min. miRNAs were then extracted using acid-phenol/chloroform and collected via filter cartridges. Subsequently, the enriched miRNAs were polyadenylated and converted into cDNA using the MystiCq® microRNA cDNA Synthesis Mix.

For quantitative PCR (qPCR), the MystiCq® microRNA qPCR Universal PCR Primer and specific assay primers for miR-21-5p, miR-126-3p, miR-296-3p, and miR-182-5p were used at a concentration of 300 nM. RNU6-1 served as the normalization control. The miRNA expression levels were quantified using the MystiCq® microRNA® SYBR® Green qPCR ReadyMix® on a QuantStudio™ 5 Real-Time PCR Detection System (Applied Biosystems™). Relative expression levels were calculated using the standard $\Delta\Delta C_t$ method.

The growth factors and cytokines present in EVs, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF-BB), hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF), were assessed using corresponding ELISA kits. First, the pelleted EVs were lysed with RIPA buffer and quantified with a protein assay dye reagent concentrate. Subsequently, segments of the lysed EVs underwent analysis through

ELISA assays employing human VEGF165 (PeproTech), PDGF-BB (PeproTech), HGF (R&D Systems), and bFGF (PeproTech), respectively.

2.6. Western blotting analysis

Cells were lysed using RIPA buffer supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). The protein concentrations of the lysates were determined using a protein assay dye reagent concentrate (Bio-Rad). Proteins from the lysates were then separated on 10 % Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad) and transferred to polyvinylidene-fluoride (PVDF) membranes (Bio-Rad). The membranes were blocked with 5 % milk (Bio-Rad) before incubation with primary antibodies as listed in Table S3. This was followed by incubation with Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, HRP (G-21234, Thermo Fisher Scientific). Immunoblots were visualized using WesternBright™ ECL HRP substrate (Advanta) and images were captured with a ChemiDoc™ XRS+ Gel Imaging System (Bio-Rad).

2.7. Preparation of ischemia-targeting EVs

The ischemia-targeted G-EVs and H-EVs were engineered by coating them with a platelet membrane modified with the ischemia-targeting peptide CST (Fig. 2A). The CST-modified platelet membrane was prepared using established methods [27]. Bovine blood platelets were isolated via centrifugation at $250 \times g$ for 25 min at room temperature and then washed with DPBS containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 2 μ M prostaglandin E1. The washed platelets were resuspended in DPBS with 1 mM EDTA and Pierce™ Protease Inhibitor Tablets (Thermo Scientific). The platelet membrane was then harvested after 4 freeze-thaw cycles, followed by centrifugation and sonication. For CST conjugation, 2.5 mL of platelet membrane (PM) suspension (0.5 mg protein/mL in $1 \times$ DPBS) was mixed with 0.5 mL of CST peptide (5 mg/mL in $1 \times$ DPBS) and stirred at room temperature for 30 min. Then, 0.3 mL of suberic acid bis (N-hydroxysuccinimide ester) (DSS, 25 mM in dimethyl sulfoxide) was added dropwise, and the mixture was stirred at room temperature for 2 h. To quench unreacted N-hydroxysuccinimide groups, 0.2 mL of 1 M glycine solution was added, followed by an additional 30-min incubation at room temperature. The resulting CST-conjugated platelet membranes (CST-PM) were dialyzed against $1 \times$ DPBS using a 10 kDa molecular weight cut-off (MWCO) dialysis membrane for 24 h, with buffer changes every 6 h. Finally, the CST-PM suspension was sonicated until clear and stored at 4 °C before use.

To coat the CST-modified platelet membrane (CST-PM) onto EVs, they were mixed at equal protein concentrations in the presence of 5 % poly(ethylene glycol) (PEG, molecular weight 1000), following previously established protocols [41,42]. Specifically, 2 mL of EVs (0.5 mg/mL in $1 \times$ DPBS, based on protein concentration) were combined with 2 mL of CST-PM (0.5 mg/mL in $1 \times$ DPBS, based on protein concentration), along with 0.2 mL of PEG 1000. The mixture was then extruded 20 times through a 400 nm membrane using a mini-extruder (Avanti® Polar Lipids) to ensure thorough integration of the platelet membranes onto the EVs.

2.8. Characterization of EVs

The size distribution of the EVs was analyzed using nanoparticle tracking analysis (NTA) performed on a NanoSight NS300 system (Malvern Panalytical). The particle concentration was 4.53×10^9 particles/mL, and the protein-to-particle ratio was 6.62 μg per 10^9 particles. The morphology of the EVs was examined using a JEOL JEM-1400Plus transmission electron microscope (TEM). Additionally, the presence of CST (TMR labeled) on the EVs was confirmed by imaging the samples with a Zeiss LSM 880 II Airyscan FAST confocal microscope.

2.9. Cellular uptake of EVs

To evaluate cellular uptake of EVs, C2C12 and HUVECs were used. Both cell types were seeded onto collagen-coated coverslips in 24-well plates, with densities of 3×10^4 cells/well for C2C12 cells and 4×10^4 cells/well for HUVECs. After 24 h of incubation, the maintenance medium was removed and replaced with serum-free medium containing 100 $\mu\text{g/mL}$ of iH-EVs (based on EV protein concentration), which had been pre-conditioned under 1 % O_2 and 5 % CO_2 at 37 °C. For C2C12 cells, the medium used was DMEM supplemented with 1 % penicillin/streptomycin, while for HUVECs, the medium was vascular cell basal medium (ATCC) supplemented with 1 % penicillin/streptomycin. The cells were then incubated in a humidified atmosphere with 1 % O_2 and 5 % CO_2 at 37 °C for 2 h. Following incubation, the cells were stained with Phalloidin-iFluor™ 488 conjugate (ab176753, Abcam) and DAPI for visualization under an Olympus FV1200 scanning confocal microscope. The uptake of EVs by the cells was quantified by calculating the percentage of cells that internalized the EVs.

To assess EV uptake by THP-1-derived macrophages, THP-1 cells were seeded onto collagen-coated coverslips in 24-well plates at a density of 2×10^5 cells/well and differentiated by treatment with 100 ng/mL phorbol 12-myristate 13-acetate (PMA). After 3 days, the maintenance medium was replaced with serum-free RPMI 1640 medium containing 100 $\mu\text{g/mL}$ of iH-EVs (based on EV protein concentration), which had been pre-conditioned under 1 % O_2 and 5 % CO_2 at 37 °C. The cells were then incubated in a hypoxic environment (1 % O_2 and 5 % CO_2 at 37 °C) for 2 h. Following incubation, the cells underwent the same staining and imaging process as described for the C2C12 cells and HUVECs.

2.10. Hemocompatibility of EVs

The hemocompatibility of EVs coated with CST-modified platelet membranes was assessed by determining their hemolysis rate [43]. Bovine red blood cells (RBCs) were first isolated from blood by centrifuging at $500 \times g$ for 10 min. After 3 saline washes, the RBCs were diluted in saline at a 1:9 ratio. Next, 0.3 mL of this RBC suspension was mixed with 1.2 mL of either deionized water or EV suspension (100 $\mu\text{g/mL}$, based on EV protein concentration), followed by incubation at 37 °C for 1 h. The mixtures were then centrifuged at $500 \times g$ for 10 min, and the hemoglobin absorbance of the supernatant was measured using a SpectraMax iD3 plate reader (Molecular Devices). The hemolysis rate was calculated as: hemolysis rate (%) = $(A_{\text{sample}}/A_{\text{DI water}}) \times 100$.

To evaluate the thromboresistance of platelet membrane-coated EVs, fresh bovine blood was diluted with saline in a 1:9 ratio. For each test, 200 μ L of the diluted blood was mixed with 125 μ L of either saline or an EV suspension (100 μ g/mL, based on EV protein concentration). Then, 30 μ L of a 0.2 M CaCl_2 solution was added to initiate clotting. The mixtures were incubated at 37 $^{\circ}\text{C}$ for 15 min, 1 h, or 3 h, followed by centrifugation at 400 $\times g$ for 3 min. The supernatants were then transferred into 1 mL of DI water to liberate hemoglobin from the un-clotted red blood cells. Hemoglobin absorbance was subsequently measured using a SpectraMax iD3 plate reader (Molecular Devices).

2.11. Cell migration under hypoxia

HUVECs and C2C12 cells were seeded in 6-well plates at a density of 3×10^4 cells/well, respectively. Once confluency was achieved, scratches were made on the cell monolayers using a 200- μ L pipet tip. After scratching, each well was supplemented with 2 mL of basal medium containing either DPBS, iG-EVs (100 μ g/mL, based on EV protein concentration), or iH-EVs (100 μ g/mL, based on EV protein concentration). The cells were cultured in a hypoxic environment (1 % O_2). The gaps were imaged at 12-h intervals using a bright-field microscope (Olympus IX70). The wound areas were quantified using ImageJ software, and the relative wound areas were calculated with the following formula: Relative wound area (%) = [(Remaining scratch areas at each time point) / (Scratch area at 0 h)] $\times$ 100.

2.12. Cell survival under hypoxia

HUVECs and C2C12 cells were cultured in 96-well plates at densities of 8×10^3 cells/well, and 3.5×10^4 cells/well, respectively. After a 24-h period to allow for cell attachment, the original medium was replaced with basal medium specific to each cell type (vascular cell basal medium for HUVECs and DMEM for C2C12), supplemented with 1 % penicillin/streptomycin. The basal medium was pre-conditioned under hypoxic conditions (1 % O_2 and 5 % CO_2) and contained either DPBS, iG-EVs (100 μ g/mL, based on EV protein concentration), or iH-EVs (100 μ g/mL, based on EV protein concentration). After 3 days after incubation under 1 % O_2 condition, the cells were lysed using a papain solution. The dsDNA content in the lysates was then quantified using a Quant-iTTM PicoGreen dsDNA Assay Kit (Invitrogen) according to the manufacturer's instructions.

2.13. Endothelial tube formation under hypoxia

To assess the impact of iH-EVs on endothelial tube formation under hypoxic conditions, a serum-free collagen gel model was used. Initially, rat tail Type I soluble collagen solution (Corning) was prepared on ice by diluting it with 5 $\times$ DMEM and 1 $\times$ DMEM to achieve a final concentration of 2 mg/mL collagen and 1 $\times$ DMEM. The pH was adjusted to 7.4 using 1 M sodium hydroxide (NaOH). Next, 500 μ L of the collagen mixture was dispensed into each well of a 24-well plate pre-equipped with glass coverslips and allowed to gel at 37 $^{\circ}\text{C}$ under hypoxic condition (1 % O_2 and 5 % CO_2) for 1 h. HUVECs were suspended in vascular cell basal medium, which was supplemented with 1 % penicillin/streptomycin and pre-conditioned under 1 % O_2 and 5 % CO_2 . For the iG-EVs and iH-EVs groups, iG-EVs or iH-EVs were added to the cell suspension to achieve a final concentration of 100 μ g/mL (based on EV protein concentration). For the PBS control group, an equivalent volume of PBS was added to the cell suspension. The resulting cell suspension were seeded onto the

gels at a density of 5×10^4 cells/well. Following a 20-h incubation under hypoxic conditions (1 % O₂ and 5 % CO₂ at 37 °C), the cells were stained with 2.5 μM CellTracker™ Green CMFDA Dye (Invitrogen™) and visualized using an Olympus FV1200 scanning confocal microscope. Tube density was quantified after imaging.

2.14. Myoblasts myotube formation under hypoxia

To evaluate the impact of iH-EVs on myoblast myotube formation under hypoxic conditions, the C2C12 cells were plated on collagen-coated glass coverslips within a 24-well plate at a density of 5×10^4 cells/well. When they reached about 80 % confluence, the medium was replaced with DMEM supplemented with 1 % penicillin/streptomycin, which had been pre-conditioned under hypoxic conditions (1 % O₂ and 5 % CO₂). The medium was further supplemented with either DPBS, iG-EVs (100 μg/mL, based on EV protein concentration), or iH-EVs (100 μg/mL, based on EV protein concentration). After incubating for 5 days under 1 % O₂, the cells were fixed with 4 % paraformaldehyde (PFA, Thermo Scientific™) and stained with myosin heavy chain antibody (MHC, MAB4470, R&D Systems). The stained cells were then imaged using an Olympus FV1200 scanning confocal microscope. Quantification was specifically focused on myotubes containing five or more nuclei.

2.15. Murine critical limb ischemia model

Female C57BL/6 mice (Charles River Laboratories) aged 20 weeks were used for the study. After anesthetizing with 2 % isoflurane, ligation was performed on the proximal and distal sections of the left femoral artery and vein of each mouse using 6.0 silk thread, and then the vessels were completely severed between the two sutures. The contralateral hindlimb served as a control. To evaluate therapeutic efficacy, four hours post-surgery, the mice were divided into three groups ($n = 6$ per group), receiving an intravenous injection of 200 μL of either DPBS (PBS group), 0.3 mg/mL of iG-EVs in PBS (based on EV protein concentration iG-EVs group), or 0.3 mg/mL of iH-EVs in PBS (based on EV protein concentration, iH-EVs group).

For the biodistribution analysis of iH-EVs, mice received an intravenous injection of 200 μL of PBS or 0.3 mg/mL of iH-EVs in PBS (based on EV protein concentration) four hours after surgery. Two days later, the mice were euthanized, and both hindlimbs, along with major organs (heart, lung, liver, spleen, and kidney), were collected for ex vivo imaging. This imaging was conducted using an IVIS® 50 In Vivo Bioluminescence Imaging System (PerkinElmer) equipped with a dsRed emission filter. The captured images were analyzed using Living Image® 4.4 software.

2.16. Blood perfusion measurement

Blood flow in both the surgical and contralateral hindlimbs was assessed using a laser Doppler imager (Perimed) one day post-surgery, with follow-up measurements conducted every seven days. The images captured during these sessions were analyzed to quantify blood perfusion. Subsequently, the perfusion ratio between the surgical hindlimb and the contralateral hindlimb was calculated.

2.17. Histology and immunohistochemistry analysis

Four weeks post-surgery, mice were euthanized and the surgical hindlimbs were harvested. The limbs were fixed in 4 % paraformaldehyde and then embedded in paraffin. Sections of 5- μ m thickness were prepared for histological and immunohistochemical analysis. To evaluate the microanatomy of skeletal muscles, sections were stained with hematoxylin and eosin (H&E) and examined under a Zeiss LSM 880 Confocal Microscope using bright-field illumination. For assessing collagen distribution, tissue sections were stained with Picrosirius red and analyzed under polarized light microscopy (Zeiss Axio Imager 2). Cell apoptosis in ischemic regions was detected using the Click-iT™ Plus TUNEL Assay for In Situ Apoptosis Detection Kit (Invitrogen™) and visualized with the same Zeiss microscope.

For immunohistochemical analysis, the paraffin sections were deparaffinized and subjected to heat-induced epitope retrieval in Tris-EDTA buffer (pH = 8). Sections were then blocked with 10 % goat serum and 0.3 % Triton X-100 in DPBS, followed by overnight incubation at 4 °C with primary antibodies (Table S4). Subsequently, sections were co-stained with Alexa Fluor™ 488- or 647-conjugated secondary antibodies (Invitrogen™) and Hoechst 33342 for nuclear staining. The stained sections were imaged using a Zeiss LSM 880 confocal microscope.

2.18. mRNA expression analysis

Four weeks post-treatment, total RNA was extracted from ischemic muscle tissues using Trizol (Invitrogen) and subsequently converted into cDNA utilizing the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™). The expression levels of the genes Actb, Vegfa, Pdgfb, Fgf2, Hgf, and Igf1 were quantified using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific™) on a QuantStudio™ 5 Real-Time PCR Detection System (Applied Biosystems™). Primer sequences are detailed in Table S5. The qPCR protocol included an initial denaturation at 95 °C for 10 min, followed by 40 amplification cycles of 15 s at 95 °C and 1 min at 60 °C. Relative mRNA expression levels were calculated using the standard $\Delta\Delta C_t$ method, with β -actin serving as the reference gene.

2.19. Statistical analysis

Data are presented as mean $\pm$ standard deviation. Statistical significance was determined using one-way ANOVA. Significance was defined as $p < 0.05$.

3. Results

3.1. Enhancing N-cadherin interaction of cell-matrix more significantly enriched angiogenic and myogenic factors in stem cell-derived EVs than enhancing integrin-matrix interaction

We investigated whether enhancing cell-matrix interactions can augment the angiogenic and myogenic factors in EVs derived from iMSCs, potentially improving vascularization and myogenesis in ischemic limbs. Two strategies were employed to boost these interactions. First, N-cadherin interaction was enhanced by immobilizing an N-cadherin mimicking peptide (Ac-HAVDIGGGK, HAVDI) onto a glass culture dish at an average density of $(6.1 \pm 0.4) \times 10^{-8}$ mol/cm² (Fig. 1A). Second, cell integrin-matrix interaction was improved by

conjugating a GRGDS peptide onto the dish at an average density of $(8.7 \pm 2.2) \times 10^{-8}$ mol/cm². The control involved a glass dish attached with glycine. Cells were cultured under 1 % O₂ conditions, which we previously demonstrated can increase EV secretion [27]. To verify that the isolated EVs exhibit the characteristic features of typical extracellular vesicles, we performed the Exo-Check™ Exosome Antibody Array. Representative blots are shown in Fig. 1B. The G-EVs, R-EVs, and H-EVs displayed established EV markers, including ALIX, TSG101, ICAM-1, and CD63, but did not express the cell membrane marker GM130. The EVs from iMSCs cultured on HAVDI-functionalized dishes (H-EVs) showed significantly elevated levels of proangiogenic miR-126 [44–46], miR-21 [47,48], and miR-296 [49] (Fig. 1C-E), as well as the promyogenic miR-182 [50] (Fig. 1F), compared to those from cells on glycine-immobilized dishes (G-EVs) ($p < 0.001$). Conversely, EVs from iMSCs on RGDS-conjugated dishes (R-EVs) displayed a significant increase only in miR-296 expression compared to G-EVs ($p < 0.001$). Notably, H-EVs presented significantly higher levels of miR-126, miR-21, and miR-182 compared to R-EVs ($p < 0.0001$). In addition to microRNAs, H-EVs also demonstrated significantly higher amounts of the proangiogenic factors PDGF-BB [51], VEGF [52], and bFGF [53], the promyogenic factor HGF [54,55], compared to both R-EVs and G-EVs (Fig. 1G-J). When comparing R-EVs to G-EVs, R-EVs exhibited significantly higher levels of VEGF, PDGF, and HGF.

The above findings suggest that enhancing N-cadherin-mediated cell-matrix interactions in iMSCs is more effective in upregulating proangiogenic and promyogenic microRNAs and growth factors than enhancing integrin-mediated interactions. To investigate the underlying mechanisms, Western blot analysis was performed on iMSCs cultured on both HAVDI- and glycine-conjugated dishes (Fig. 1K & L). The results demonstrated that N-cadherin expression was significantly upregulated on HAVDI-conjugated dishes, consistent with the observation that increased N-cadherin expression correlates with enhanced cadherin-based cell-cell interactions in the presence of HAVDI [56,57].

3.2. Fabrication and characterizations of ischemia-targeting HAVDI-induced EVs

The H-EVs, which contained higher levels of proangiogenic and promyogenic factors compared to R-EVs, was used to evaluate their effectiveness in promoting angiogenesis and myogenesis in ischemic limbs after intravenous (IV) injection. To enhance their circulation time and ensure targeted delivery to ischemic limbs, we cloaked the H-EVs in non-thrombotic platelet membrane modified with an ischemia-targeting peptide, CSTSMLKAC (CST). While the exact mechanism remains unclear, CST has been demonstrated to effectively target ischemic regions [27,58–60]. These modified EVs, referred to as iH-EVs (Fig. 2A), displayed a unilamellar membrane structure (Fig. 2C), in contrast to the cup-shaped morphology of the unmodified H-EVs (Fig. 2B). To verify CST conjugation, TMR-labeled CST was utilized, and confocal imaging confirmed its presence on iH-EVs (Fig. 2D). The cloaking process increased the diameter of EVs from 108.7 ± 1.8 nm for H-EVs (Fig. 2E) to 136.9 ± 10.4 nm for iH-EVs (Fig. 2F).

To assess the hemocompatibility of iH-EVs intended for IV use, we performed hemolysis and thromboresistance tests. In the hemolysis test, red blood cell rupture was minimal (<10 %) after a one-hour exposure to iH-EVs, with the degree of hemolysis comparable

to that observed in saline-exposed samples (Fig. 2G & H). In the thromboresistance test, blood samples exposed to iH-EVs for durations of 0.25, 1, and 3 h exhibited hemoglobin absorbance levels similar to those of samples treated with saline (Fig. 2I & J). These findings collectively confirm the excellent hemocompatibility of iH-EVs.

The targeting capability of iH-EVs was evaluated by administering them via IV injection 4 h post-critical ischemic limb surgery. Two days later, IVIS imaging of EV biodistribution showed a 3.4-fold increase in fluorescent signals in the ischemic limbs compared to negligible signals in the contralateral non-operated limbs (Fig. 2K & L), indicating a preferential accumulation in the ischemic regions. It is possible that the iH-EVs accumulated in the ischemic limbs via the vasculature bordering the ischemic area and leaky vasculature in the ischemic area. The iH-EVs showed minimal distribution to major organs like the liver, spleen, kidneys, heart, and lungs. In addition, comparable fluorescence was observed in organs of mice injected solely with PBS and those receiving iH-EVs injections.

To evaluate the contributions of platelet membrane and CST to the targeting capability of iH-EVs, we included unmodified H-EVs and platelet membrane-coated H-EVs without CST (PM-H-EVs) as control groups. Both H-EVs and PM-H-EVs exhibited comparable fluorescence signals in the ischemic and contralateral limbs (Fig. S2), similar to the PBS group. These findings indicate that neither H-EVs nor platelet membrane alone confer ischemia-targeting ability to iH-EVs, and confirm that the CST peptide is primarily responsible for the targeting function.

Cellular uptake of iH-EVs was investigated in major cell types present in ischemic limbs: endothelial cells, skeletal muscle cells, and macrophages. Human umbilical vein endothelial cells (HUVECs), mouse myoblast cells (C2C12), and human THP-1-derived macrophages (THP-1 MØs) were cultured on collagen-coated plates. The rationale of using collagen coating was to mimic the ischemic limb environment where exposed collagen binds platelet membranes [41]. After a 2-h incubation in hypoxia (1 % O₂), 25.8 % of HUVECs, 32.1 % of C2C12 cells, and 36.1 % of THP-1 MØs had internalized iH-EVs (Fig. 2M & N).

3.3. iH-EVs promoted the survival, migration and morphogenesis of endothelial cells and myoblasts under ischemic conditions

Under CLI conditions, the severely reduced blood flow creates an ischemic environment that leads to widespread cell death. Enhancing the survival of endothelial and skeletal muscle cells under such conditions is crucial for promoting vascularization and muscle regeneration in ischemic limbs. We investigated the cytoprotective effects of iH-EVs on HUVECs and C2C12 myoblasts under ischemic conditions via a double-stranded DNA (dsDNA) assay to quantify the number of viable cells remaining after treatments [61]. The control was iG-EVs, prepared by cloaking G-EVs with CST-conjugated platelet membrane using the same approaches as for iH-EVs. Following a 3-day incubation in serum-free medium at 1 % O₂, HUVECs in the control PBS group underwent extensive cell death, with dsDNA content decreasing to 25 % of day 0 level (Fig. 3A). Treatment with iH-EVs significantly preserved HUVEC viability, maintaining dsDNA content at 54 % of day 0 level ($p < 0.001$). Although control EVs (iG-EVs) also improved cell survival, iH-EVs enhanced survival more significantly than iG-EVs ($p < 0.0001$ iG-EVs and iH-EVs vs. PBS; $p < 0.001$ iH-EVs vs.

iG-EVs). Similarly, iH-EVs treatment notably increased the survival and proliferation of C2C12 cells under the same conditions. After 3 days, the dsDNA content in the iH-EVs group was 208 % of the day 0 level (Fig. 3B), significantly higher than both the iG-EVs group (183 %) and the PBS group (148 %) ($p < 0.0001$ iG-EVs and iH-EVs vs. PBS; $p < 0.0001$ iH-EVs vs. iG-EVs). These findings suggest that iH-EVs more effectively enhanced the survival of endothelial cells and myoblasts under ischemic conditions compared to control treatments.

In ischemic limbs, the migration and morphogenesis of surviving endothelial and skeletal muscle cells are essential for promoting vascularization and muscle regeneration. We evaluated the effects of iH-EVs on the migration of HUVECs and C2C12 cells using a 2D scratch assay. Over 12, 24, and 48 h of incubation, HUVECs treated with iH-EVs demonstrated significantly faster wound closure compared to those treated with iG-EVs and the PBS control groups (Figs. 3C & D). Although iG-EVs treatment also enhanced HUVEC migration, iH-EVs was more effective. Among C2C12 cells, those treated with iH-EVs exhibited the quickest migration, achieving complete closure of the scratch within 24 h (Figs. 3E & F). These results confirm that iH-EVs have the capability of effectively enhancing the migration of both endothelial cells and skeletal muscle cells, supporting their role in facilitating recovery processes in ischemic limbs.

The ability of iH-EVs to stimulate tube formation of HUVECs was assessed on a 2D collagen gel (Figs. 3G & H). After a 20-h incubation in serum-free media under hypoxic conditions (1 % O₂), HUVECs in the control group (PBS) displayed limited tubular structure formation. In contrast, treatment with either iH-EVs or iG-EVs led to a significant increase in tube density ($p < 0.0001$ for both iG-EVs and iH-EVs vs. PBS; $p < 0.0001$ for iH-EVs vs. iG-EVs). Notably, tube density in the iH-EVs group was 1.5 times higher than in the iG-EVs group ($p < 0.0001$). Morphogenesis of C2C12 cells was evaluated by assessing myotube formation after a 5-day incubation in serum-free media under hypoxic conditions (1 % O₂) (Figs. 3I & J). In the PBS-treated group, C2C12 cells rarely formed myotubes. Both iG-EVs and iH-EVs treatments significantly induced myotube formation. Remarkably, the density of myotubes containing more than 5 nuclei in the iH-EVs group was 5.9 times higher than in the PBS group and 1.7 times higher than in the iG-EVs group ($p < 0.0001$ for both iG-EVs and iH-EVs vs. PBS; $p < 0.0001$ for iH-EVs vs. iG-EVs). These results highlight that iH-EVs more effectively facilitated both endothelial and myoblast morphogenesis in ischemic environments.

3.4. iH-EVs promoted cell survival, proliferation, and mitochondrial metabolism in a murine CLI model

To evaluate the therapeutic efficacy of iH-EVs in ischemic limbs, we utilized a murine model of CLI where ischemia was induced by unilateral femoral artery ligation in the hindlimbs of C57BL/6 mice (Fig. 4A). Six mice per group were administered either iH-EVs, iG-EVs, or PBS via tail vein injection. Cell survival in the ischemic limbs was assessed using TUNEL analysis four weeks post-exosome administration (Fig. 4B & C). Significant cell apoptosis was noted in the PBS-treated group. In contrast, apoptosis was significantly reduced in both the iH-EVs and iG-EVs treated groups ($p < 0.0001$), indicating that both

types of EVs promoted cell survival in ischemic limbs. Furthermore, cell proliferation was enhanced in the ischemic limbs, with higher densities of Ki67⁺ proliferating cells observed in the iH-EVs and iG-EVs groups compared to the PBS group (Fig. 4D & E). Notably, the iH-EVs group exhibited the highest density of Ki67⁺ cells, highlighting its superior efficacy in promoting cell proliferation compared to iG-EVs. To investigate whether the improved cell survival and proliferation were linked to enhanced mitochondrial biogenesis, we measured the expression of the transcription coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) in the ischemic limbs. The iH-EVs group showed significantly elevated PGC-1 α expression compared to both the iG-EVs and PBS groups ($p < 0.0001$, Fig. 4F & G), suggesting that iH-EVs more effectively boosted mitochondrial metabolism.

3.5. iH-EVs promoted angiogenesis in ischemic limbs

To assess the efficacy of iH-EVs in promoting angiogenesis, we monitored blood perfusion, measured vessel density, and analyzed mRNA levels of angiogenic growth factors in the ischemic limbs. In the PBS-treated group, angiogenesis was sluggish, with blood perfusion reaching only 48 % after 28 days (Fig. 5A & B). Treatment with iH-EVs and iG-EVs significantly accelerated angiogenesis. Both treatments achieved significantly higher blood perfusion compared to the PBS group ($p < 0.0001$ for both iG-EVs and iH-EVs vs. PBS; $p < 0.0001$ for iH-EVs vs. iG-EVs, Figs. 5A & B). Specifically, blood perfusion in the iG-EVs group reached 72 % at day 28, whereas the iH-EVs group achieved complete blood perfusion restoration ($p < 0.0001$, iH-EVs vs. iG-EVs). Correspondingly, blood vessel density results mirrored the blood perfusion findings. Sparse isolectin⁺ blood vessels were observed in the PBS-treated limbs, while limbs treated with iH-EVs or iG-EVs exhibited abundant isolectin⁺ blood vessels (Fig. 5C & D). The highest vessel density was in the iH-EVs group, with total vessel density being 1.9-fold and 1.2-fold higher than in the PBS and iG-EVs groups, respectively ($p < 0.0001$ for iG-EVs and iH-EVs vs. PBS; $p < 0.01$ for iH-EVs vs. iG-EVs, Fig. 5C & D). Moreover, both iH-EVs and iG-EVs significantly promoted vessel maturation compared to the PBS group. The density of mature vessels (isolectin⁺ and α -SMA⁺) was 4.7-fold higher in the iH-EVs group and 3.3-fold higher in the iG-EVs group relative to the PBS group ($p < 0.01$ for iG-EVs vs. PBS; $p < 0.0001$ for iH-EVs vs. PBS, Fig. 5C & E). Additionally, iH-EVs treatment significantly upregulated the mRNA expression of key proangiogenic growth factors such as *Vegfa*, *Pdgfb*, *FGF2*, *Hgf*, and *Igf1*, compared to the iG-EVs and PBS groups (Figs. 5F-J). These comprehensive results collectively demonstrate that iH-EVs effectively expedite angiogenesis in ischemic limbs.

3.6. iH-EVs enhanced skeletal muscle regeneration in ischemic limbs

To evaluate skeletal muscle regeneration following treatments with iH-EVs, we assessed central nucleus density and muscle fiber diameter in skeletal muscles of ischemic limbs harvested on day 28. H&E staining indicated significant muscle degeneration in the PBS group, characterized by highly fragmented and isolated muscle fibers. Treatments with iH-EVs and iG-EVs notably mitigated muscle fiber separation and facilitated muscle regeneration (Figs. 6A & E). Both iH-EVs and iG-EVs treatments resulted in significantly higher central nuclei densities compared to the PBS group ($p < 0.0001$ for iH-EVs vs. PBS;

$p < 0.001$ for iG-EVs vs PBS, Fig. 6B & F). Additionally, the iH-EVs group exhibited significantly larger muscle fiber diameter than both the PBS and iG-EVs groups ($p < 0.0001$ for iH-EVs vs PBS; $p < 0.001$ for iH-EVs vs iG-EVs, Fig. 5E). These findings suggest that iH-EVs treatment effectively enhanced muscle regeneration in ischemic limbs.

Resident satellite cells are crucial for skeletal muscle regeneration, as they can activate and differentiate into myofibers [62]. To investigate the impact of iH-EVs treatment on satellite cell activation, immunostaining for MyoD and Pax7 was performed. Cells positive for both markers (MyoD⁺/Pax7⁺) were identified as activated satellite cells (Fig. 6C & G). The iH-EVs group displayed a significantly higher density of MyoD⁺/Pax7⁺ cells, showing a 2.5-fold increase over the PBS group and a 6.6-fold increase over the iG-EVs group ($p < 0.0001$ for iH-EVs vs PBS; $p < 0.001$ for iH-EVs vs iG-EVs, Fig. 6C & G). This demonstrates that iH-EVs can effectively activate resident satellite cells for myogenesis in ischemic limbs.

Myogenin plays an essential role in the later stages of myogenesis, particularly in regulating myoblast fusion [63]. Immunohistochemical staining for myogenin was conducted, and cells positive for myogenin (myogenin⁺) were identified as differentiated myoblasts (Fig. 6D & H). Treatment with iH-EVs resulted in a 1.3-fold increase in myogenin⁺ density compared to iG-EVs and a 1.9-fold increase compared to PBS ($p < 0.0001$ for iH-EVs vs PBS; $p < 0.001$ for iH-EVs vs iG-EVs). These results indicated that iH-EVs promoted myoblast fusion in ischemic muscles, further enhancing muscle regeneration.

3.7. iH-EVs reduced inflammation and fibrosis in a murine CLI model

To assess the effect of iH-EVs treatment on inflammation in ischemic limbs, we evaluated the density of pro-inflammatory M1 macrophages (CD68⁺) and reparative M2 macrophages (CD206⁺) following treatment. Both iH-EVs and iG-EVs treatments significantly reduced the presence of M1 macrophages compared to the PBS control group ($p < 0.0001$ for both iH-EVs and iG-EVs vs PBS, Figs. 7A & B). Notably, iH-EVs treatment led to a substantial decrease in M1 macrophage density. Furthermore, iH-EVs treatment significantly increased the density of reparative CD206⁺ M2 macrophages compared to both the PBS and iG-EVs groups ($p < 0.0001$ iH-EVs vs PBS; $p < 0.01$ iH-EVs vs iG-EVs, Figs. 7C & D). These findings demonstrated that iH-EVs mitigated inflammation in ischemic limbs by reducing pro-inflammatory M1 macrophages while simultaneously enhancing reparative M2 macrophages.

To determine the impact of iH-EVs on tissue remodeling in ischemic limbs, PicroSirius red staining was performed 4 weeks post-treatment to measure total collagen content. The collagen content in both the iH-EVs and iG-EVs groups was approximately 7.0 %, which was significantly lower than that in the PBS group ($p < 0.0001$ for both iH-EVs and iG-EVs vs PBS, Figs. 7E & F). This suggests that iH-EVs treatment effectively reduced collagen deposition in the ischemic areas.

4. Discussion

The speed of vascularization and muscle repair under CLI conditions largely depends on the availability and morphogenesis of endothelial cells and skeletal muscle cells. Enhancing the recruitment of these cells and promoting their morphogenesis are crucial for accelerating vascularization and muscle repair. However, these processes are often impaired by the ischemic conditions prevalent in ischemic limbs [64–67]. In this work, we demonstrated that EVs from iMSCs, engineered to specifically and simultaneously upregulate proangiogenic and promyogenic growth factors and microRNAs, can stimulate the survival and migration of endothelial cells and myoblasts, and the formation of endothelial tubes and myotubes, thus expediting vascularization and muscle repair.

While EVs have been explored as a treatment for limb ischemia, their therapeutic efficacy has often been limited. [68] A major challenge is that current EVs formulations are not optimally tailored with a suitable composition of proangiogenic and promyogenic factors needed for accelerated vascularization and myogenesis. Although EVs can be engineered to carry specific cargos, current methods predominantly utilize soluble biochemicals and growth factors, complicating the composition and therapeutic effects of the EVs [31–33]. Approaches that can upregulate the expression of proangiogenic and promyogenic factors in EVs using insoluble factors are attractive, but still in their infancy. In this work, we enhanced cell-matrix interactions in iMSCs to augment the levels of proangiogenic and myogenic factors in the EVs. We employed two strategies: first, by immobilizing an N-cadherin mimicking peptide (HAVDI) onto a glass culture dish, and second, by conjugating a GRGDS peptide to enhance integrin-matrix interaction. Previous research indicates that these enhancements can amplify the paracrine effects of MSCs [28,34,69,70]. Yet, no previous study has investigated how these two interactions modulate EV composition, especially in terms of proangiogenic and promyogenic factors for regeneration of vascularization and skeletal muscles under CLI conditions.

We demonstrated that enhancing these two interactions significantly upregulated multiple proangiogenic and promyogenic miRNAs and growth factors in the iMSC-derived EVs (Fig. 1). EVs from iMSCs cultured on HAVDI-functionalized dishes (H-EVs) exhibited significantly elevated levels of proangiogenic miR-126, miR-21, miR-296, PDGF-BB, VEGF, and bFGF, alongside promyogenic miR-182 and HGF (Fig. 1C-J). Meanwhile, EVs from iMSCs on RGDS-conjugated dishes (R-EVs) showed enhanced levels of miR-296, PDGF-BB, VEGF, and HGF (Fig. 1E, G, H, & J). Notably, H-EVs possessed significantly elevated levels of both proangiogenic (miR-126, miR-21, PDGF-BB, VEGF, and bFGF) and promyogenic (miR-182 and HGF) factors compared to R-EVs. These findings suggest that N-cadherin-mediated cell-matrix interactions are more effective at enhancing the expressions of key proangiogenic and promyogenic factors in iMSC-derived EVs than RGD-mediated integrin interactions. This underscores the potential of targeting cell-matrix interactions to enhance the therapeutic properties of stem cell-derived EVs for applications in regenerative medicine. Our strategy of immobilizing peptides on substrates to modulate EV composition offers several advantages, including enhanced specificity, controllability, stability under cell culture conditions, and simplified operations for large-scale applications.

To ensure that the selected H-EVs delivered by IV injection have prolonged circulation duration and specifically target ischemic limbs, they were cloaked with a CST-functionalized platelet membrane. Previous studies have shown that platelet membranes can effectively “disguise” cloaked nanoparticles in the circulation [60,71], and CST is a peptide that specifically targets ischemic areas [60]. In our study, comparative biodistribution analysis showed that unmodified H-EVs and platelet membrane-coated H-EVs (PM-H-EVs) exhibited similar distributions between ischemic and contralateral limbs, indicating a lack of intrinsic targeting ability. In contrast, the ischemic limb-targeting EVs (iH-EVs) demonstrated significantly enhanced accumulation in the ischemic limbs (Fig. 2K and Fig. S2), underscoring the critical role of CST modification in mediating ischemia-specific targeting. These findings support the notion that while platelet membrane cloaking primarily optimizes systemic biodistribution by reducing nonspecific organ uptake, targeted delivery to ischemic regions is primarily mediated by the CST peptide. The preferential accumulation of iH-EVs in ischemic limbs likely occurs through enhanced interaction with vasculature bordering the ischemic tissue and via leaky blood vessels within the ischemic region. Our results further demonstrated that iH-EVs significantly improved endothelial cell and myoblast survival, migration, and morphogenesis under CLI-like conditions (Fig. 3A-J). After being delivered by IV injection, the iH-EVs was found to completely restore blood perfusion 28 days after surgery (Fig. 5A & B), primarily through reduced cell apoptosis, enhanced cell survival and mitochondrial metabolism, and increased expression of angiogenic growth factors in the ischemic limbs (Fig. 4B-G). In addition to promoting angiogenesis, iH-EVs also facilitated skeletal muscle regeneration, as indicated by larger myofiber diameters and higher central nucleus densities (Fig. 6A, B, E & F). It is likely that iH-EVs activated satellite cells and differentiated myoblasts (Fig. 6C-G). Importantly, the delivered EVs did not induce fibrosis or inflammation (Fig. 7A-F).

This study presents a strategy to specifically and simultaneously upregulate proangiogenic and promyogenic factors in stem cell-derived EVs for promoting vascularization and muscle regeneration under CLI conditions (Fig. 8). However, we acknowledge several limitations in our research. First, while we have demonstrated that EVs with upregulated miR-126-3p, miR-296-3p, miR-21-5p, PDGF-BB, VEGF, and bFGF exhibited angiogenic effects both in vitro and in vivo, the precise role of miR-126-3p remains undefined, as its gene targets were not identified in this study. miR-126-3p is primarily recognized as a pro-angiogenic microRNA. However, under certain conditions, it may exert anti-angiogenic effects [72]. Future studies will explore the specific role of miR-126-3p in the EVs for the angiogenesis of ischemic limbs. Second, the dose-response effect of EVs was not explored in this study, which leaves open the possibility that higher dosages could yield greater therapeutic effects. This aspect will be investigated in our future studies. Third, while we did not directly evaluate CST-conjugated EVs without membrane cloaking in this study, future work will explore this approach as a streamlined alternative for enhancing ischemic tissue targeting while maintaining delivery efficiency. Moreover, while we used a well-established murine model of CLI, this may not fully replicate the conditions of chronic limb ischemia observed in patients. To address this, future studies will incorporate large animal models, such as swine, whose vascular pathophysiology closely resembles that of humans. The apolipoprotein E (ApoE) knockout mouse model, which is among the most clinically

relevant models mimicking patients with CLI, [73–75] may also be utilized in our future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Data availability

Data will be made available on request.

Appendix A.: Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2025.113938>.

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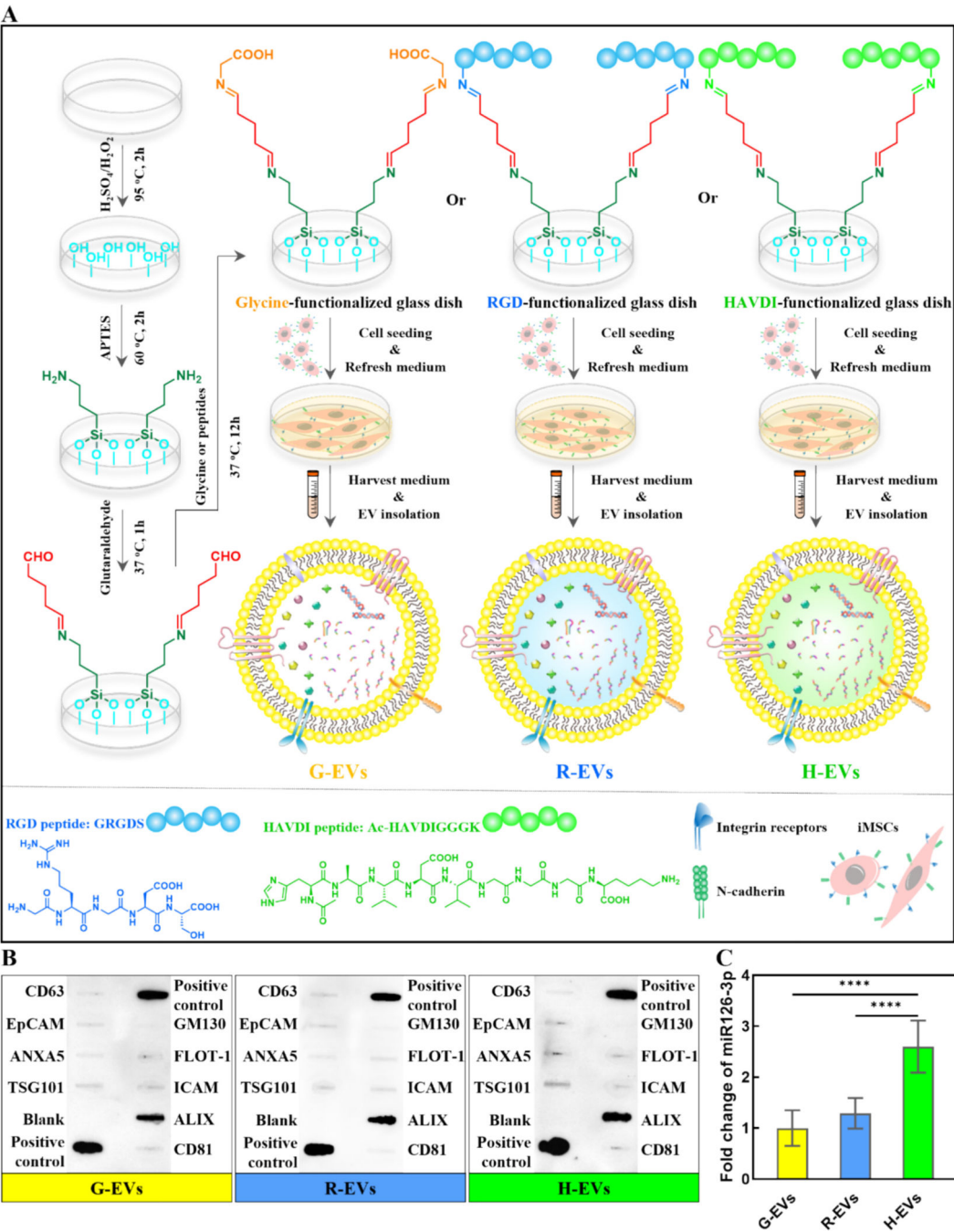
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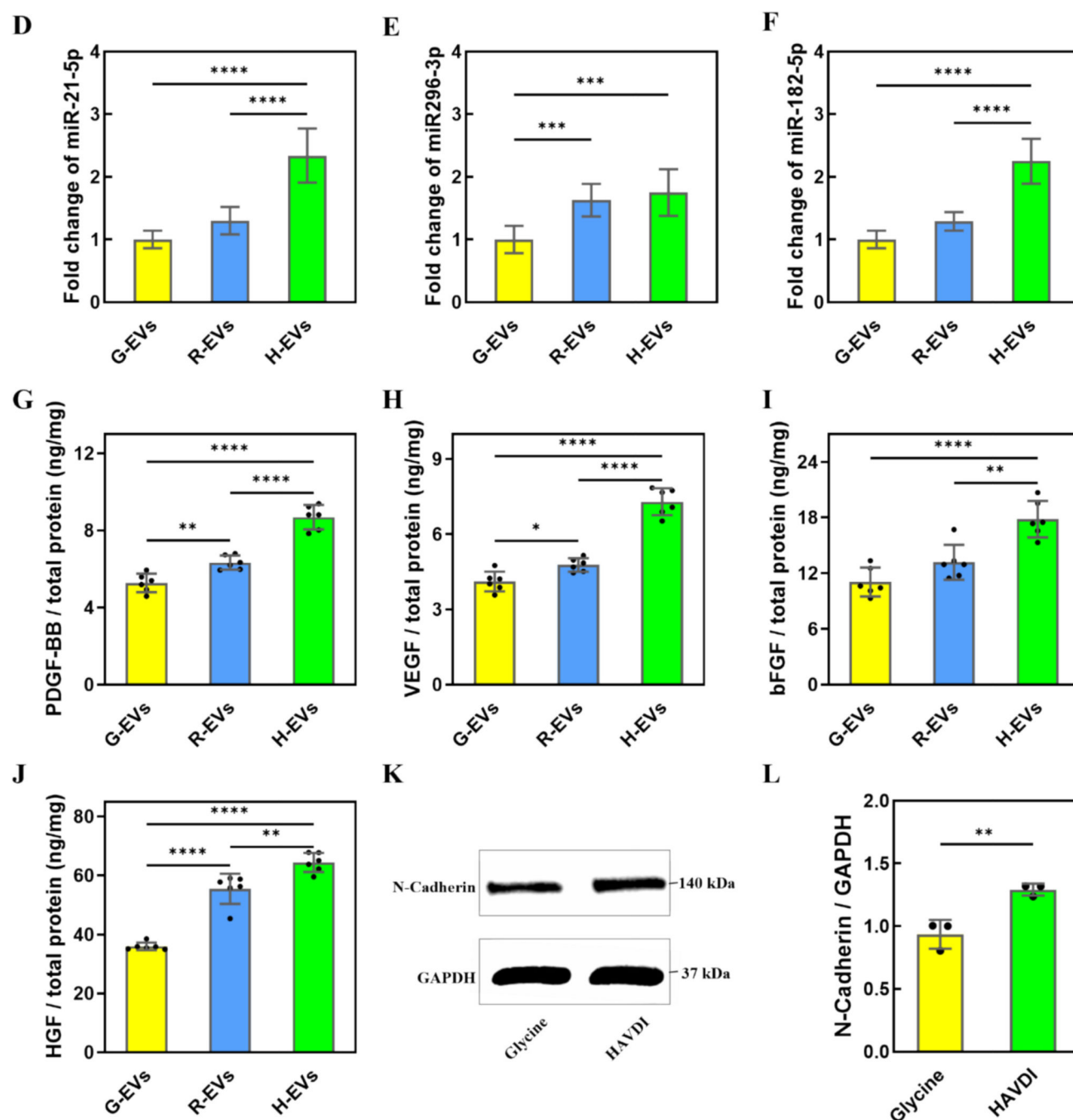
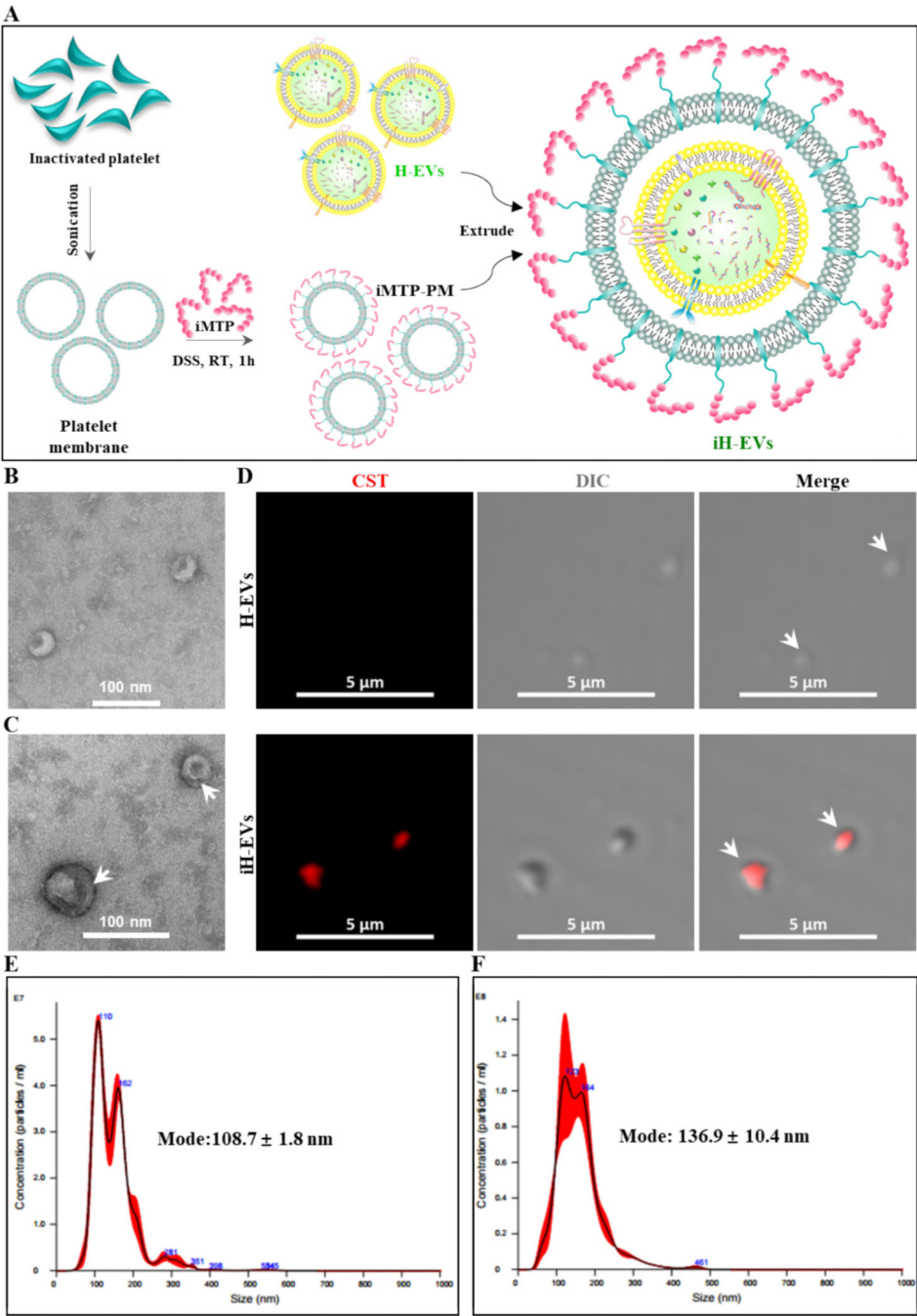


Fig. 1. Peptide-functionalization of glass dishes altered iMSCs-derived EV cargos under hypoxia. (A) Schematic illustration depicting the functionalization of glass dish surfaces with glycine, RGD peptide, and HAVDI peptide, designed for the culture of iMSCs and subsequent EV isolation under various induction conditions. (B) Biomarkers on glycine-induced EVs (G-EVs), RGD-induced EVs (R-EVs), and HAVDI-induced EVs (H-EVs) via an Exo-Check™ Exosome Antibody Array. (C–F) Comparative analysis of miRNA profiles showing the contents of miR126–3p (C), miR21–5p (D), miR296–3p (E), and miR182–5p (F) in EVs

secreted by iMSCs following induction with glycine, RGDpeptide, or HAVDI peptide under hypoxia (1 % O₂), $n = 6$. (G-J) Quantitative analysis of growth factors and cytokines, including VEGF (G), PDGF-BB (H), HGF (I), and bFGF (J) in EVs isolated from iMSCs cultured in differently functionalized glass dishes under hypoxic condition (1 % O₂), $n = 6$. (K) Western blotting of N-cadherin in iMSCs induced with glycine or HAVDI peptide under hypoxia for 3 days. (L) Quantification of N-cadherin normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



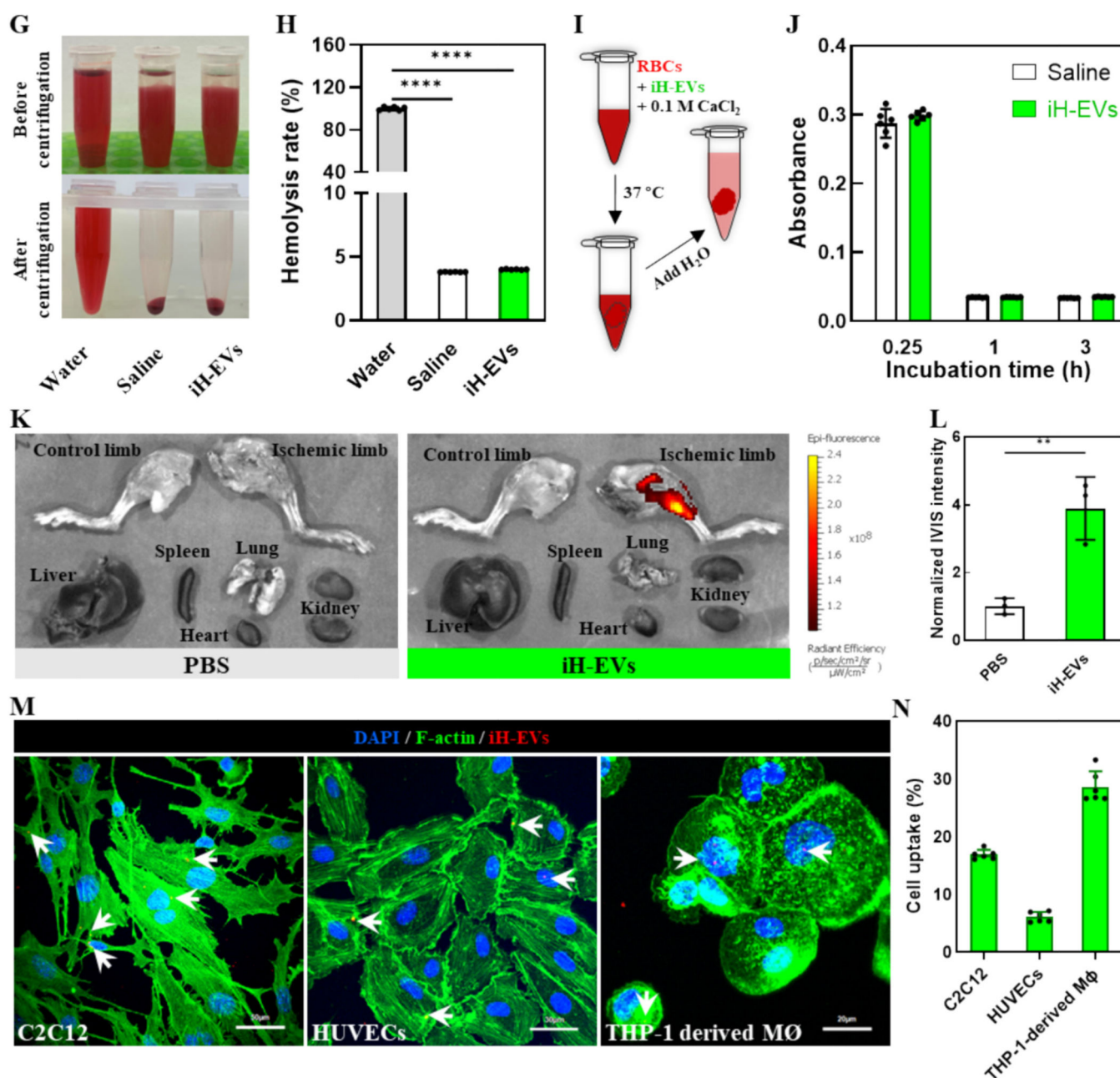


Fig. 2. Fabrication and characterizations of ischemia-targeting HAVDI-induced EVs (iH-EVs). (A) Schematic representation of iH-EVs fabrication. (B–C) TEM images of HAVDI-induced EVs (H-EVs, B) and iH-EVs (C). The arrow indicates the platelet membrane. Scale bar = 100 nm. (D) Fluorescent image (middle) corresponding to differential interference contrast (DIC) images (left) and merged images (right) for both H-EVs (top) and iH-EVs (bottom) with a 5 μ m scale bar. The images were acquired to showcase aggregated EVs, given the limited resolution of confocal microscopes in clearly imaging individual EVs. (E–F) Size distribution of H-EVs (E) and iH-EVs (F) using nanoparticle tracking analysis (NTA). (G) Appearance of bovine blood after incubation with iH-EVs for 2 h at 37 °C and after

centrifugation of the mixture at 500 *g* for 10 min. (H) The absorbance of hemoglobin (540 nm) released from broken red blood cells after 2 h incubation and centrifugation (*n* = 6). (I) Illustration of thromboresistance test for iH-EVs. (J) Hemoglobin absorbance (540 nm) from unclotted red blood cells after incubation with iH-EVs or saline for 0.25, 1, and 3 h, *n* = 6. (K) IVIS images of hindlimbs and main organs harvested 2 days after CLI surgery and IV injection of iH-EVs. (L) Quantification of IVIS intensity in ischemic limbs normalized to control limbs. (M) Cell internalization of iH-EVs by C2C12 cells, HUVECs, and THP-1 derived macrophages following 2 h of hypoxic incubation (1 % O₂). Scale bars: 50 μm for C2C12 cells, 50 μm for HUVECs, 30 μm for THP-1 derived macrophages. (N) Quantification of cell uptake efficiency of iH-EVs by three types of cells under hypoxia (*n* = 6). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

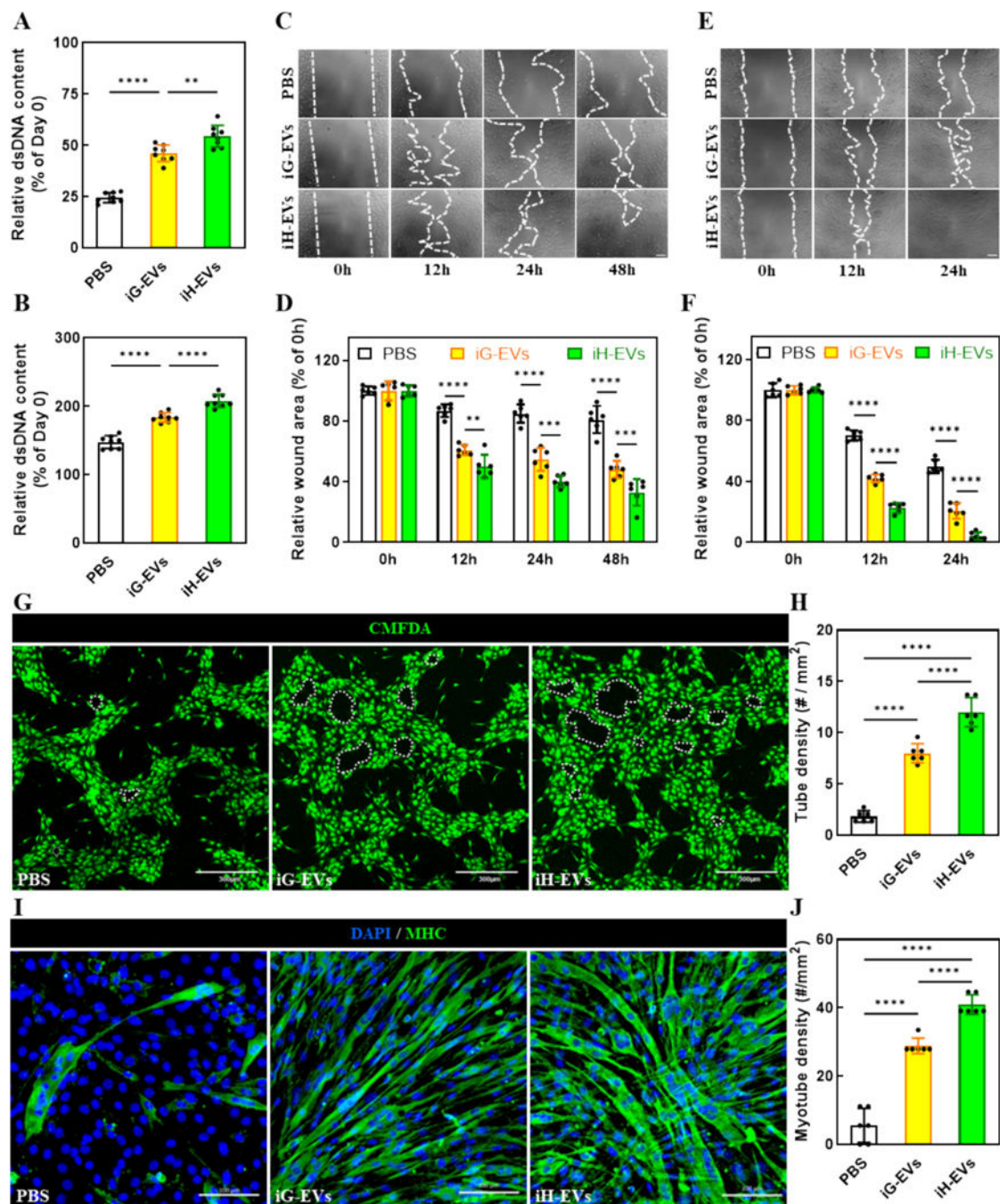


Fig. 3. iH-EVs promoted HUVECs and C2C12 cell migration, survival, and morphogenesis in hypoxic conditions. (A-B) Double-stranded DNA (dsDNA) content of HUVECs (A) and C2C12 (B) remained post 3-day hypoxic incubation with or without iH-EVs, $n = 8$. (C) HUVECs migration with or without iH-EVs treatment under hypoxia, scale bar = 200 μ m. (D) Scratch area quantification of HUVECs under hypoxic incubation, $n = 6$. (E-F) Migration of C2C12 cells (E, scale bar = 200 μ m) and quantified relative scratch areas (F, $n = 6$) after incubation with or without iH-EVs under hypoxia. (G) Tube formation by

HUVECs after 20 h incubation under hypoxia. Scale bar = 300 μm . (H) Tube density formed by HUVECs following 20 h hypoxic incubation, $n = 6$. (I) C2C12 myotube formation post-5-day of iH-EVs treatment under hypoxia. Scale bar = 100 μm . (J) Myotubes density, defined as cells containing five or more nuclei, $n = 6$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

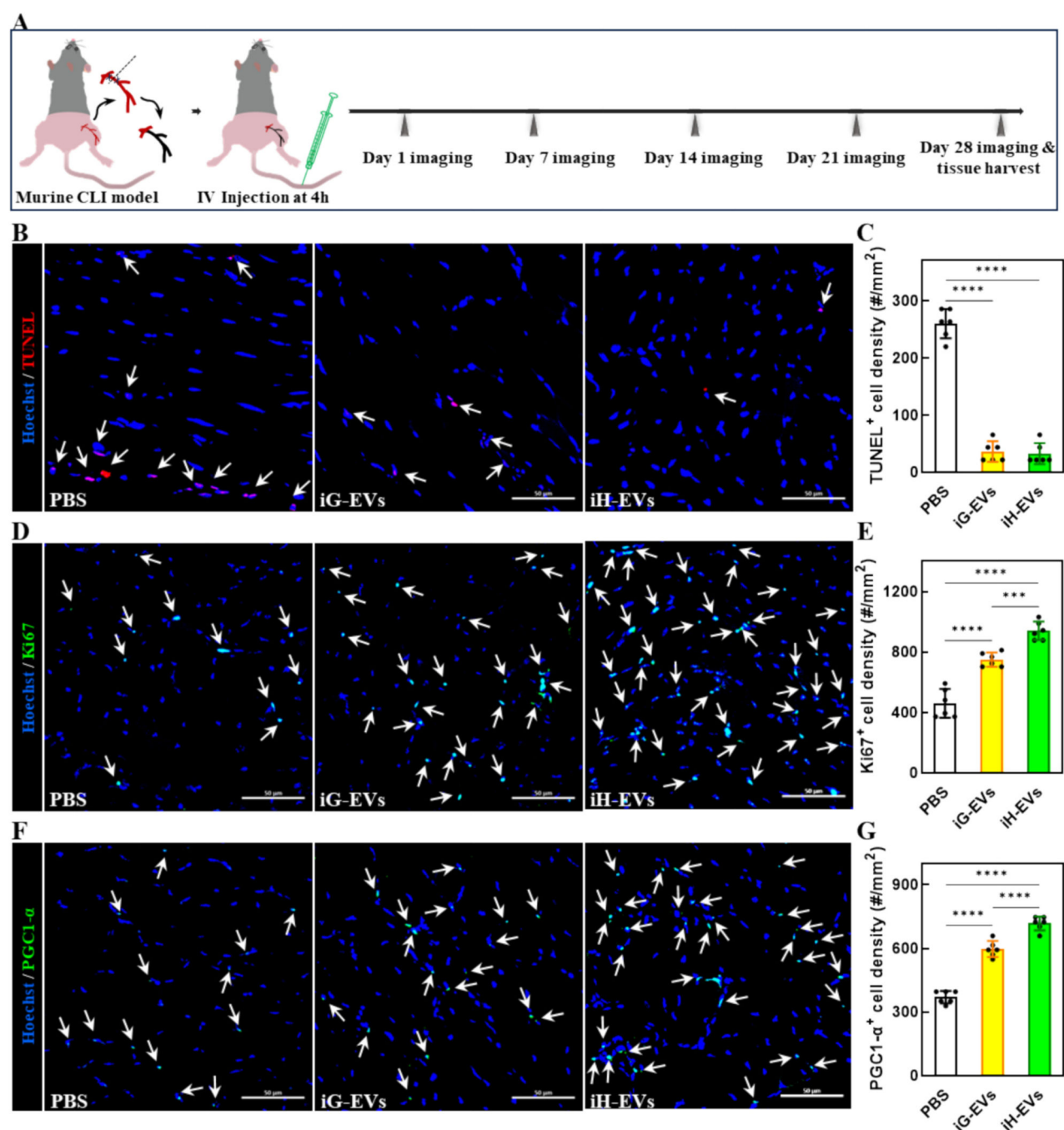


Fig. 4. Intravenous (IV) injection of iH-EVs promoted cell survival, proliferation, and mitochondrial metabolism in a murine CLI model. (A) The diagram depicts CLI surgery and the timeline for in vivo assessment following iH-EVs IV injection. (B–C) TUNEL staining reveals cell apoptosis (red, B), with quantification of TUNEL⁺ apoptotic cells presented in (C), in ischemic muscle sections 28 days post-CLI surgery. (D–E) Immunohistochemical staining of proliferation-associated protein Ki67 (green, D) and quantification of Ki67⁺ proliferating cells (E) in ischemic muscle sections 4 weeks after IV injection. (F–G) PGC1-α

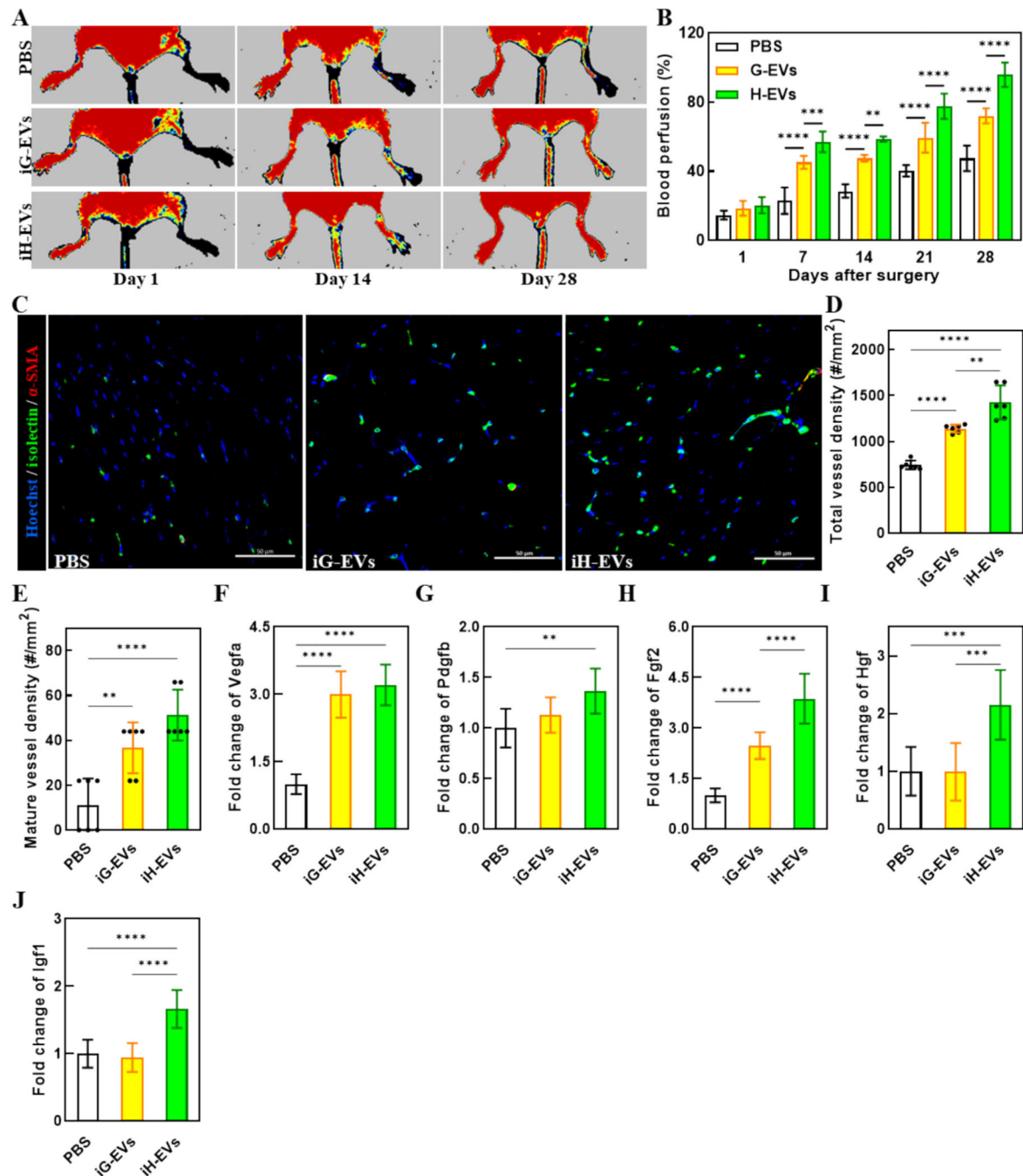
staining (green, F) and quantification of PGC1- α^+ cells (G) in ischemic limbs 4 weeks after treatment. All nuclei were stained with Hoechst 33342 (blue) and positive signals were indicated by arrows. Scale bar = 50 μm . $n = 6$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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**Fig. 5.**

IV injection of iH-EVs promoted angiogenesis in a murine CLI model. (A) Post-surgery Laser Doppler perfusion images in mice receiving various treatments. (B) Normalized blood perfusion in ischemic limbs compared to contralateral limbs after treatments, $n = 6$. (C) Isolectin (green) and α -SMA (red) staining in ischemic muscles 4 weeks post-injection. Nuclei were stained with Hoechst 33342 (blue). Scale bar = 50 μ m. (D-E) Quantification of total vessel density (D) and mature vessel density (E) in the ischemic limbs 28 days after CLI surgery, $n = 6$. (F-J) Gene expression of *Vegfa* (F), *Pdgfb* (G), *Fgf2* (H), *Hgf* (I), and

Igf1 (J) in ischemic muscles, $n = 6$. *Actb* was used as a housekeeping gene. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

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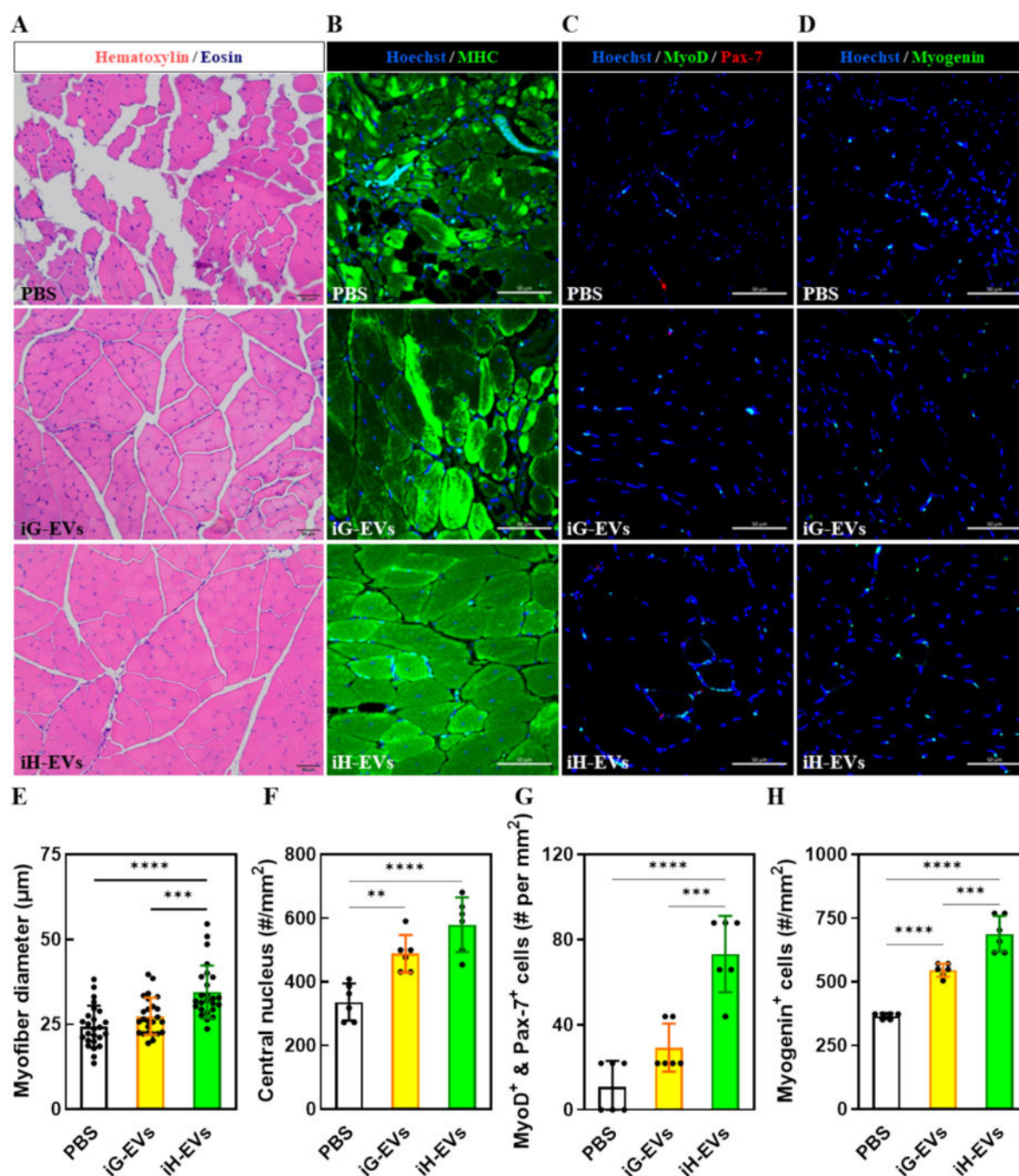


Fig. 6. IV injection of iH-EVs enhanced skeletal muscle regeneration in a murine CLI model. (A) H&E staining for ischemic limbs 4 weeks post-CLI surgery, scale bar = 50 μ m. (B–D) Immunohistochemical staining of MHC (green, B), MyoD/Pax-7 (green/red, C), and myogenin (green, D) in the ischemic sections 4 weeks post-surgery. All Nuclei were stained with Hoechst 33342 (blue). Scale bar = 50 μ m. (E–H) Quantification of the myofiber diameter (E), central nucleus (F), dual staining of MyoD⁺ and Pax-7⁺ cells (G), and

myogenin⁺ cells (H) in ischemic muscle regions 28 days after CLI surgery, $n = 6$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

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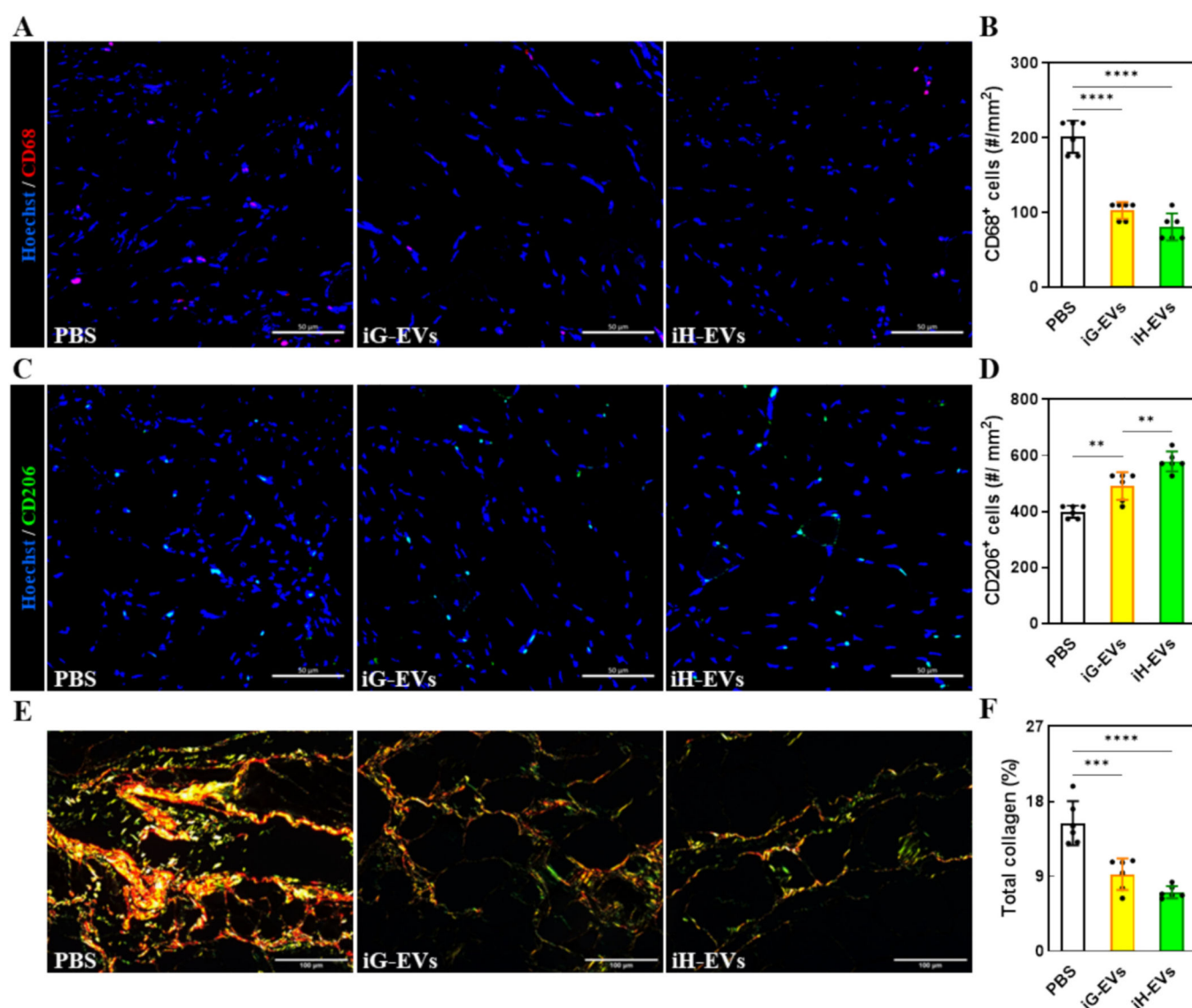


Fig. 7.

IV injection of iH-EVs reduced fibrosis and inflammation in a murine CLI model. (A-B) Immunohistochemical staining of CD68 (A, green) and quantification of CD68⁺ cells (B) in the ischemic muscle sections 28 days after various treatments. Nuclei were stained with Hoechst 33342 (blue). Scale bar = 50 μ m. $n = 6$. (C-D) Immunohistochemical staining of C206 (C, green) and quantification of CD206⁺ cells (D) in the ischemic muscle sections 28 days post-treatment. Nuclei were stained with Hoechst 33342 (blue). Scale bar = 50 μ m. $n = 6$. (E-F) PicroSirius red staining of collagen (E) and quantification of total collagen content (F) in the ischemic muscles 4 weeks post CLI surgery and receiving various administration. Scale bar = 100 μ m. $n = 6$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

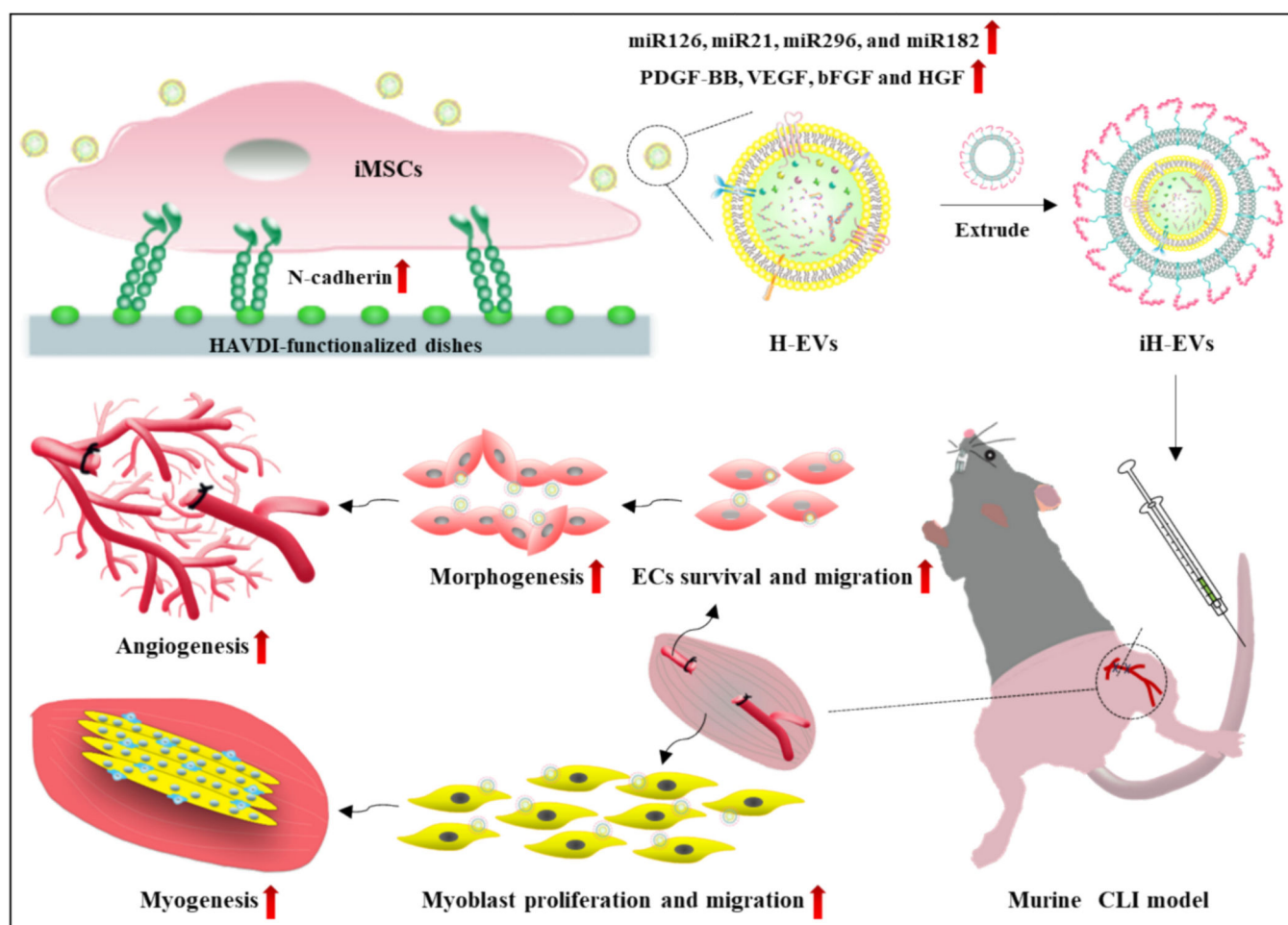


Fig. 8. Mechanisms elucidate the simultaneous enhancement of proangiogenic and promyogenic factors in stem cell-derived EVs through augmented N-cadherin interaction, fostering vascularization and muscle regeneration in ischemic hindlimbs. N-cadherin-mediated cell-matrix interactions are effective at enhancing the expression of key proangiogenic and promyogenic factors in iMSC-derived EVs. The iH-EVs plays a pivotal role in recruiting endothelial as well as skeletal muscle cells and stimulating the morphogenesis of these cells, thereby expediting vascularization and muscle regeneration and in ischemic limbs.