



Rapamycin-induced small extracellular vesicles under GelMA scaffolds facilitate diabetic wound repair through accelerating angiogenesis and alleviating macrophage-mediated inflammation via PI3K/Akt signaling pathway

Yalu Zhang^{a,1}, Zekun Wang^{c,1} , Liang Wu^{a,1}, Liang Zhu^a, Ming Lu^a, Ziyang Zhang^d, Yuanhao Lv^{b,*} , Xu Zhu^{a,**}, Hanhui Yao^{a,***}

^a Department of General Surgery, The First Affiliated Hospital of USTC, Division of Life Science and Medicine, University of Science and Technology of China, Hefei, 230001, China

^b Department of Orthopaedic Surgery, People's Hospital of Qianxinan Prefecture, Guizhou province, 562400, China

^c Department of Orthopaedic Surgery, Shanghai Putuo District Liqun Hospital, Shanghai, 200060, China

^d School of Nursing, Shenyang Medical College, Shenyang, Liaoning province, 110000, China

ARTICLE INFO

Keywords:

Esenchymal stem cells
Small extracellular vesicles
Rapamycin
Diabetic wound
PI3K/Akt signaling pathway

ABSTRACT

Recently, small extracellular vesicles (sEVs) isolated from mesenchymal stem cells (MSCs) show superior therapeutic potential in diabetic wound repair. Pretreated MSCs with biological or chemical agents could boost the activities of MSC-derived sEVs. This study aims to investigate whether sEVs derived from the human umbilical cord MSCs (hUCMSCs) pretreated with rapamycin (RAPA) exhibit elevated efficacy in improving diabetic wound healing and to elucidate the underlying mechanisms involved. The sEVs extracted from RAPA pretreated hUCMSCs (RAPA-sEVs) were successfully characterized in terms of their morphology, structural features, and concentration. In vitro studies revealed that RAPA-sEVs suppressed the proliferative and migratory capabilities of macrophages and reduced the expression of pro-inflammatory mediators including TNF- α , IL-1 β and iNOS. Meanwhile, they promoted the migration and tube formation of endothelial cells, and increased the level of VEGF. More importantly, full-thickness skin defect models were established in streptozotocin (STZ)-induced diabetic mice. Gelatin methacryloyl (GelMA) carrying sEVs applied to the surface of damaged skin. RAPA-sEVs exhibited exceptional efficacy in accelerating the wound repair via propelling angiogenesis, reducing the percentage of M1-type macrophages, and mitigating excessive inflammatory response under superior biosafety conditions. Mechanistically, the biological activities of RAPA-sEVs were dependent on the PI3K/Akt signaling pathway, and the pro-angiogenic and anti-inflammatory effects of RAPA-sEVs were alleviated after the pathway being inhibited by a PI3K inhibitor PI103. Overall, RAPA-sEVs-based therapy might serve as a promising strategy for diabetic wound healing through fueling angiogenesis and alleviating macrophage-mediated inflammation via activating PI3K/Akt signaling pathway.

1. Introduction

Diabetes is a complex and multifaceted metabolic disorder, and a staggering one-fifth of diabetic patients worldwide have developed diabetic wounds [1]. The persistent challenges of poor wound healing

and pathological healing in diabetic patients after surgery remain pressing issues, as they profoundly impact health-related quality of life and pose substantial risks for long-term complications [2].

Among the multifaceted factors influencing diabetic wound closure, angiogenesis occupies a crucial position, serving as a conduit for the

* Corresponding author.

** Corresponding author.

*** Corresponding author.

E-mail addresses: lyh01090503@163.com (Y. Lv), liu1110150106@163.com (X. Zhu), yhanhui@ustc.edu.cn (H. Yao).

¹ These authors contributed equally to this study.

essential delivery of nutrients and oxygen to the wound site, thereby accelerating collagen synthesis, fibroblast proliferation and re-epithelialization processes [3,4]. Furthermore, inflammatory injury is another important factor affecting wound healing in diabetic patients. Persistent inflammation of wound initiates a sequence of adverse events, including impaired blood perfusion within the wound bed, amplification of inflammatory cytokines, surge in reactive oxygen species production, and a heightened risk for bacterial colonization and infection [5,6]. Macrophage, a vital immunomodulatory cell population, is indispensable in governing the inflammatory dynamics of wound repair. Their phagocytic activities, coupled with the secretion of cytokines and growth factors, macrophage serves as a key orchestrator of inflammation, fibrotic responses, and the intricate wound healing mechanisms [7]. Macrophages are involved in the whole process of wound repair accompanied by transitioning from early pro-inflammatory M1 types to later anti-inflammatory M2 types [8]. Therefore, the novel strategies to stimulate angiogenesis and mitigate the macrophage-mediated inflammation are highly promising for the treatment of diabetic wound.

Mesenchymal stem cells (MSCs), classified as multipotent, non-hematopoietic adult stem cells, have a profound capacity to promote tissue regeneration [9]. *In vitro*, MSCs possess the potential to differentiate into osteoblasts, adipocytes, and chondrocytes [10]. As a predominant stromal cell subset, MSCs are characterized by positive surface markers of CD105, CD166, and CD73, while lacking the expression of hematopoietic and immune-associated markers such as CD45, CD11b, CD19, CD34, CD79a, and HLA-DR [10,11]. Minute, acellular vesicles encapsulated by a lipid bilayer, termed small extracellular vesicles (sEVs), are secreted by cellular entities into the extracellular compartment to mediate cellular communication mechanisms [12]. In the context of angiogenesis during pathological processes, such as tissue repair in wound healing and osseous regeneration in bone repair, MSC-derived sEVs (MSC-sEVs) exhibit a crucial function by enhancing local revascularization via the paracrine release of angiogenic growth factors [13,14]. Previous studies have demonstrated that preconditioning MSCs via the application of chemical, physical, and biological stimuli is a potent strategy to potentiate the biological activities of MSC-sEVs, increasing their therapeutic potential in tissue engineering applications and regenerative medicine therapies [15–17]. For example, CHIR99021, a Wnt signaling pathway agonist, could enhance sEVs production by human umbilical cord MSCs (hUCMSCs). CHIR99021-pretreated hUCMSC-sEVs effectively facilitated cutaneous wound repair in diabetic mice [18]. The hUCMSC-sEVs have desirable biological properties, including abundant sources, low immunogenicity, rapid self-renewal capacity, non-invasive collection method and no tumorigenic outcomes [19]. These characteristics render them promising candidates for applications in tissue regeneration and stem cell therapies. Rapamycin (RAPA), a conventional macrolide antibiotic that also functions as an mTOR inhibitor, exhibits potent antiproliferative and immunosuppressive properties [20]. The dysregulation of mTOR and related proteins within signaling pathway leads to abnormal cellular migration, proliferation, and survival [21]. A study found that RAPA alleviates progression of burn wound through protecting the zone of stasis from further necrosis by propelling autophagy in rats with deep second-degree burns [22]. To date, the influence of RAPA-pretreated hUCMSC-sEVs on diabetic wound healing and the underlying mechanisms involved have yet to be thoroughly investigated.

Consequently, this study aimed to investigate whether GelMA scaffolds equipped with the sEVs originated from hUCMSCs pretreated with RAPA could foster endothelial cell-mediated angiogenesis and restrain macrophage-mediated inflammation during diabetic skin wound repair. Furthermore, we further detected the key signaling pathway PI3K/Akt through sequencing analysis involved in the wound closure-enhancing effects of RAPA-sEVs, and conducted testing and validation *in vivo* and *in vitro*.

2. Materials and methods

2.1. Cell culture and reagents

hUCMSCs (PCS-500-010), human peripheral blood mononuclear cells (PBMC, PCS-800-011) and umbilical vein endothelial cells (HUVEC, PCS-100-010) were obtained from the American Type Culture Collection (ATCC, USA). hUCMSCs were cultured in α -MEM (Gibco, USA) added with 10 % FBS (Gibco, USA) and 1 % penicillin-streptomycin (Gibco, USA). HUVECs were maintained in endothelial cell medium (ECM, ScienCell), supplemented with 5 % FBS and 1 % endothelial cell growth supplement (ECGS, Gibco, USA). PBMCs were incubated in RPMI-1640 (Gibco, USA) with 50 ng/mL GM-CSF (RP00094, Abclonal, China) for 7 days to differentiate into macrophages. All cell types were cultivated at 37 °C with 5 % CO₂.

The multipotency of hUCMSCs was evaluated by inducing their differentiation into chondrogenic, adipogenic, and osteogenic lineages using specialized media (Cyagen, China). Differentiation was confirmed by Oil Red O staining for adipogenesis (Solarbio, China), Alizarin Red staining for osteogenesis (Solarbio, China), and Alcian Blue staining for chondrogenesis (Solarbio, China). Flow cytometry was performed to characterize the hUCMSCs based on their expression profiles, specifically positive for CD105 (323206, BioLegend, USA) and CD166 (343904, BioLegend, USA) and negative for CD11b (340006, BioLegend, USA) and CD45 (304008, BioLegend, USA). Lipopolysaccharide (LPS, S7850, Selleck, USA) was employed as a stimulatory agent to activate primitive macrophages for functional analysis. PI-103 (S1038, Selleck, USA), a PI3K inhibitor, was utilized to investigate the role of the PI3K/Akt signaling pathway in RAPA-sEV-induced anti-inflammatory and pro-angiogenic effects.

2.2. Isolation and identification of RAPA-sEVs

For concentration screening, hUCMSCs were pretreated with complete medium (sEV-free FBS) containing RAPA (S1039, Selleck, USA) at concentrations of 0.5 μ M, 1 μ M and 2 μ M for 72 h and cell counting kit-8 (CCK-8) analysis subsequently identified 1 μ M as suitable for further experiments. The sEVs were isolated from a conditional medium through a series of gradient centrifugation steps. Specifically, the supernatants were centrifuged at 300 $\times$ g for 10 min, 2000 $\times$ g for 10 min, and 10 000 $\times$ g for 30 min at 4 °C to effectively remove cell debris. Subsequently, the conditional medium underwent two additional centrifugations at 100 000 $\times$ g for 70 min each at 4 °C and then the precipitated material was resuspended in PBS to obtain either sEVs or RAPA-sEVs. The morphological characteristics of sEVs were visualized by transmission electron microscopy (TEM, JEM-1400, JEOL, Japan). Nanoparticle tracking analysis (NTA, Malvern, UK) was employed to quantify the particle concentration and assess size distribution. The specific surface markers of sEVs (CD9, CD81, CD63) were detected by Western blotting.

2.3. Synthesis of GelMA scaffolds

GelMA represents a photosensitive biological material. It is capable of undergoing rapid crosslinking and curing upon exposure to ultraviolet light, thereby generating a three-dimensional structure with specific strength. In our study, 10 % gelma liquid was prepared according to reagent instructions, the specific steps are as follows. A photoinitiator stock solution (2.5 mg/ml) was prepared. GelMA was added to a centrifuge tube, followed by the addition of the photoinitiator solution. The mixture was thoroughly vortexed and then incubated in a light-protected water bath at 60 °C for 30 min, with intermittent vortexing every 5 min (a total of three times). The hydrogels were then freeze-dried and cut into pieces in a longitudinal direction. Scanning electron microscopy (SEM) was used to observe the reticular porous structure of the composite hydrogels, and atomic force microscopy (AFM) was used

to detect their morphology and surface roughness.

2.4. Mechanical properties and rheological properties assays

Cylindrical hydrogel specimens (10 mm diameter × 5 mm height) were subjected to uniaxial compression using a Zwick Z050 universal testing machine in press mode. A constant crosshead displacement rate of 1 mm/min was applied until structural failure occurred. Real-time force-displacement data were recorded to calculate the compressive modulus and ultimate strength. The viscoelastic properties of the composite inks were evaluated using a Kinexus ultra and rotational rheometer equipped with a 25 mm parallel plate geometry. Samples were loaded at a fixed gap distance of 1 mm. Steady shear rate sweeps spanning $0.1\text{--}1000\text{ s}^{-1}$ were conducted to determine the shear-thinning behavior and apparent viscosity.

2.5. Cytotoxicity test of GelMA hydrogel and sEVs release analysis

hUCMSCs were seeded in 48-well plates and cultured under standard conditions. For the experimental groups, 200 μl of pre-prepared hydrogel was added to each well and allowed to crosslink. Cells were then introduced onto the hydrogel surface. Cell proliferation viability was assessed using the CCK-8 assay at 1-day and 1-week time points. For the sEVs release test, hydrogel loaded with sEVs (20 $\mu\text{g}/200\text{ }\mu\text{l}/\text{well}$) was placed in 48-well plates, and each well was immersed in 400 μl of PBS. The supernatant was collected at 1, 2, 4, 6, 8, 10, 12, and 14 days. The released sEVs were quantified via BCA protein concentration assay and expressed as a cumulative release percentage relative to the initial sEV loading amount.

2.6. Internalization of sEVs

The sEVs were stained using the PKH-26 staining kit (Sigma, MINI26, Germany) according to the manufacturer's protocol. The 100 μl of sEVs suspension was diluted with Dilution C solution and stained with PKH-26 for 10 min in darkness. The staining reaction was halted by the addition of 1 % bovine serum albumin (BSA). Following this, the mixture was centrifuged at $100\,000\times g$ for 70 min at $4\text{ }^{\circ}\text{C}$. The precipitate was resuspended in cold PBS to obtain the stained sEVs. Macrophages or HUVECs were incubated with the labeled sEVs for 24 h. Then, the cells were fixed with 4 % paraformaldehyde (PFA) and stained with DAPI (Sigma-Aldrich). The stained cells were visualized using fluorescence microscopy.

2.7. Ki67 immunofluorescent staining

Ki67 staining was applied to assess macrophage proliferation. Macrophages underwent fixation with 4 % PFA, permeabilization with 0.1 % Triton X-100, and overnight incubation at $4\text{ }^{\circ}\text{C}$ with a primary Ki67 antibody (1:200, ab16667, Abcam). Then, cells were incubated with Alexa Fluor 594-conjugated secondary antibody (Solarbio, China) for 1 h. Nuclei were stained with DAPI (Sigma-Aldrich), and Ki67-positive cells were counted using Image J software.

2.8. Western blotting

Western blot assays were performed as previously delineated [23, 24]. Briefly, the total cellular lysates were harvested, subjected to electrophoresis, transferred to a membrane, blocked to prevent nonspecific binding, and then incubated with the specified primary antibodies, including β -actin (3700, 1:1000, CST), CD9 (EXOAB-CD9A-1, 1:1000, SBI), CD81 (EXOAB-CD81A-1, 1:1000, SBI), CD63 (EXOAB-CD63A-1, 1:1000, SBI), PI3K (4249, 1:1000, CST), phospho-PI3K (4228, 1:1000, CST), Akt (9272, 1:1000, CST), phospho-Akt (9271, 1:1000, CST). After being washed with TBST, the membranes were incubated in HRP-labeled secondary antibody (Zsbio,

Beijing) and visualized by a Tanon 5200 Multi-imaging system (Tanon, China).

2.9. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured cells or murine skin tissues using Trizol Reagent (9108, Takara). The cDNA was synthesized using PrimeScript RT reagent Kit (RR037A, Takara). Real-time qPCR was administrated by TB Green™ Premix Ex Taq™ Reagent (RR420A, Takara) on the QuantStudio3 (Applied Biosystems). Each experiment was performed in triplicate and fold changes relative to β -actin were calculated using the $2^{-\Delta\Delta\text{CT}}$ method. The primers were shown in Table S1.

2.10. CCK-8, scratch test and transwell assays

The protocol of CCK-8, scratch tests and transwell assays were introduced in detail previously [23,25,26]. CCK-8 assays (Dojindo, Japan) were used to assess the proliferative capability of cells, with OD values determined through absorbance readings at a wavelength of 450 nm. Scratch tests were employed to detect the migrative ability of cells. The relative size of the scratch area was calculated utilizing ImageJ software. In terms of transwell assays, macrophages or HUVECs were seeded in upper chamber at the bottom and different conditional media were added into the lower chamber. After being cultured for 48 h, the migrative cells were stained with 0.1 % crystal violet (Sangon Biotech) and observed under an Olympus IX 70 optical microscope (Japan).

2.11. Tube formation assays

Tube formation assays were utilized to evaluate the angiogenesis *in vitro*. After thawing Matrigel (BD Biosciences) overnight at $4\text{ }^{\circ}\text{C}$, 200 μl /well was added to a pre-cooled 24-well plate and evenly shaken on ice to avoid gelation. The plate was then incubated at $37\text{ }^{\circ}\text{C}$ for 1 h to facilitate Matrigel gelation. HUVECs (1.5×10^5 cells/well) were seeded onto the 24-well plate and incubated with conditional medium. After 8 h, capillary-like structures were observed and counted under an inverted light microscope, including the number of nodes, junctions and branches.

2.12. Wound healing in diabetic murine models

All animal experiments were carried out with the approval of the Animal Ethics Committee of the First Affiliated Hospital of USTC (2024-N(A)-0412). The 8-week-old female C57/BL6 mice were fed in compliance with the Guidelines for the Care and Use of Laboratory Animals of China. The mice received two high-dose injections of STZ solution (80 mg/kg) preoperatively, and mice with blood glucose level above 16.7 mM were selected as diabetic mice. Two wounds with a diameter of 7 mm were created on the back of each mouse with a hole punch under anesthesia. The diabetic model mice were divided into different groups randomly. Three mice in each group. The wounds were treated with PBS (Control), Hydrogel, Hydrogel + sEVs1, Hydrogel + sEVs2, or Hydrogel + sEVs2 + PI103. Finally, 100 μl of hydrogel containing 10 μg sEVs was evenly added to the skin defect, the mice were put back in the cage after solidified. PI103 was administered intraperitoneally daily at a concentration of 10mg/100 μl /kg following the establishment of the model. At day 0, 4, 9, and 14 postoperatively, the diabetic wounds were photographed by a digital camera to record the recovery outcomes. The wound closure rates were calculated by formula: $P(\%) = (A_0 - A_d)/A_0 \times 100$. $P(\%)$ is the percentage of wound healing, A_0 is the initial wound area at day 0 and A_d is the wound area at each time point.

2.13. Histology, H&E and immunofluorescence (IF)

The skin wound tissues were harvested and fixed in 4 % PFA at day

14 postoperatively. The samples were dehydrated, paraffin-embedded, and sectioned into 5- μm slices [23]. Hematoxylin and eosin (H&E) staining was applied for observing wound status, while Masson's trichrome staining (Servicebio, China) was utilized to visualize the collagen synthesis. For tissue immunofluorescence, rehydrated paraffin sections were blocked with 1 % BSA. Primary antibodies CD86 (1:200, 19589, CST) and CD31 (1:200, ab182981, Abcam) were incubated overnight at 4 °C, followed by Alexa Fluor 488 or 594-conjugated secondary antibodies (Solarbio, China) for 1 h. Nuclei were stained with DAPI (Sigma-Aldrich), and images were captured using Zeiss LSM980 confocal microscope.

2.14. Transcriptome profiling

Total RNA was extracted using TRIzol reagent (Takara, Japan). Library construction and sequencing were conducted at BGI Genomics (China) and differential expression analysis was executed using DESeq2 software. The thresholds for statistical significance were set at the corrected p -value ≤ 0.05 and $|\log_2\text{foldchange}| \geq 0.01$.

2.15. Statistical analysis

All data were analyzed and plotted using GraphPad Prism 8 software, and presented as the Mean $\pm$ standard deviation (SD). For comparing two groups, Student's t -test was utilized. For multiple treatment group comparisons, a one-way ANOVA was conducted. Post-hoc analysis was

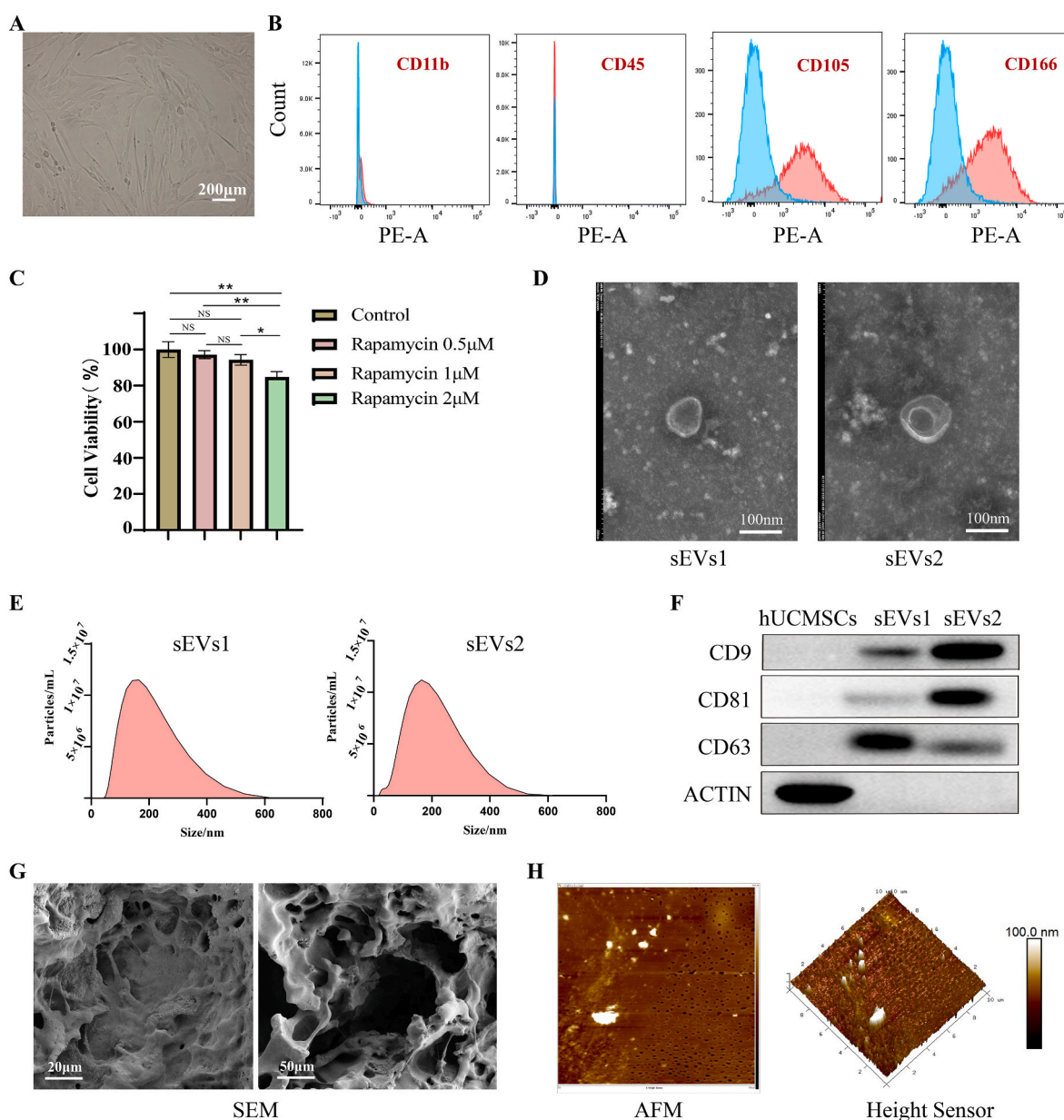


Fig. 1. Identification of hUCMSCs and characterization of RAPA-induced sEVs. (A) hUCMSCs showed spindle-like morphology under light microscope. Scale bar, 200 μm . (B) Assessment of hUCMSC surface markers by flow cytometry, including CD11b, CD45, CD105 and CD166. Blue curve, isotype control; red curve, cell surface marker. (C) CCK-8 assay was used to analyze the effect on the cell viability of hUCMSCs induced by RAPA at different concentration gradients, 0.5 μM , 1 μM , 2 μM . OD values were measured at 450 nm. (D) The morphology of sEVs (sEVs1) and RAPA-sEVs (sEVs2) was observed by TEM. Scale bar, 100 nm. (E) The diameter and particle concentration of sEVs1 and sEVs2 were detected by NTA. (F) Western blot analysis of surface marker (CD9, CD81, CD63). (G-H) The scanning electron microscopy (SEM) and atomic force microscope (AFM) for GelMA. Mean $\pm$ SD. NS, not significant. * $p < 0.05$; ** $p < 0.01$.

performed using Tukey's test. Statistical significance was determined at p -values <0.05 .

3. Results

3.1. Identification of hUCMSCs and characterization of RAPA-induced sEVs

Microscopic photograph showed the typical spindle-like morphology of hUCMSCs (Fig. 1A). hUCMSCs demonstrated multiple differentiation functions including chondrogenic, adipogenic, and osteogenic differentiation potential (Fig. S1A). The flow cytometric data showed that the negative markers on the surface of hUCMSCs included CD11b and CD45, and the positive markers included CD105 and CD166 (Fig. 1B). hUCMSCs was induced using different concentration gradients of RAPA, including 0 μ M, 0.5 μ M, 1 μ M, and 2 μ M, for 72 h. RAPA was found to inhibit the proliferation of hUCMSCs at concentrations up to 2 μ M, while it had little effect on the proliferation ability of hUCMSCs at concentrations of 0.5 μ M and 1 μ M (Fig. 1C). The sEVs1 were isolated from hUCMSC supernatant without RAPA, and sEVs2 were extracted from the

conditioned supernatant of hUCMSCs induced by 1 μ M RAPA (RAPA-sEVs), then characterized by TEM. Like normal sEVs (sEVs1), RAPA-sEVs (sEVs2) displayed typical cup-like shapes with double-layer membrane microstructures (Fig. 1D). NTA indicated that the size distribution of both sEVs1 and sEVs2 showed a single peak at approximately 150–200 nm (Fig. 1E). Western blotting revealed that both sEVs1 and sEVs2 were positive for the surface marker of sEVs, including CD9, CD81, and CD63, compared with the hUCMSCs (Fig. 1F), albeit sEVs2 exhibited down-regulated CD63 level and upregulated CD9 and CD81 expression. Spontaneous osteogenic, chondrogenic, and adipogenic differentiation of hUCMSCs treated with RAPA were reduced (Fig. S1B–C), hinting that RAPA inhibited the differentiative capacity of hUCMSCs. These differences indicated that sEVs1 and sEVs2 likely exhibit distinct biological functions. Collectively, these data confirmed the successful extraction of hUCMSC-derived sEVs from the conditioned medium.

3.2. Characterization of GelMA scaffolds

It has been reported that encapsulating sEVs into GelMA hydrogels can prolong their therapeutic duration and enhance their efficacy in

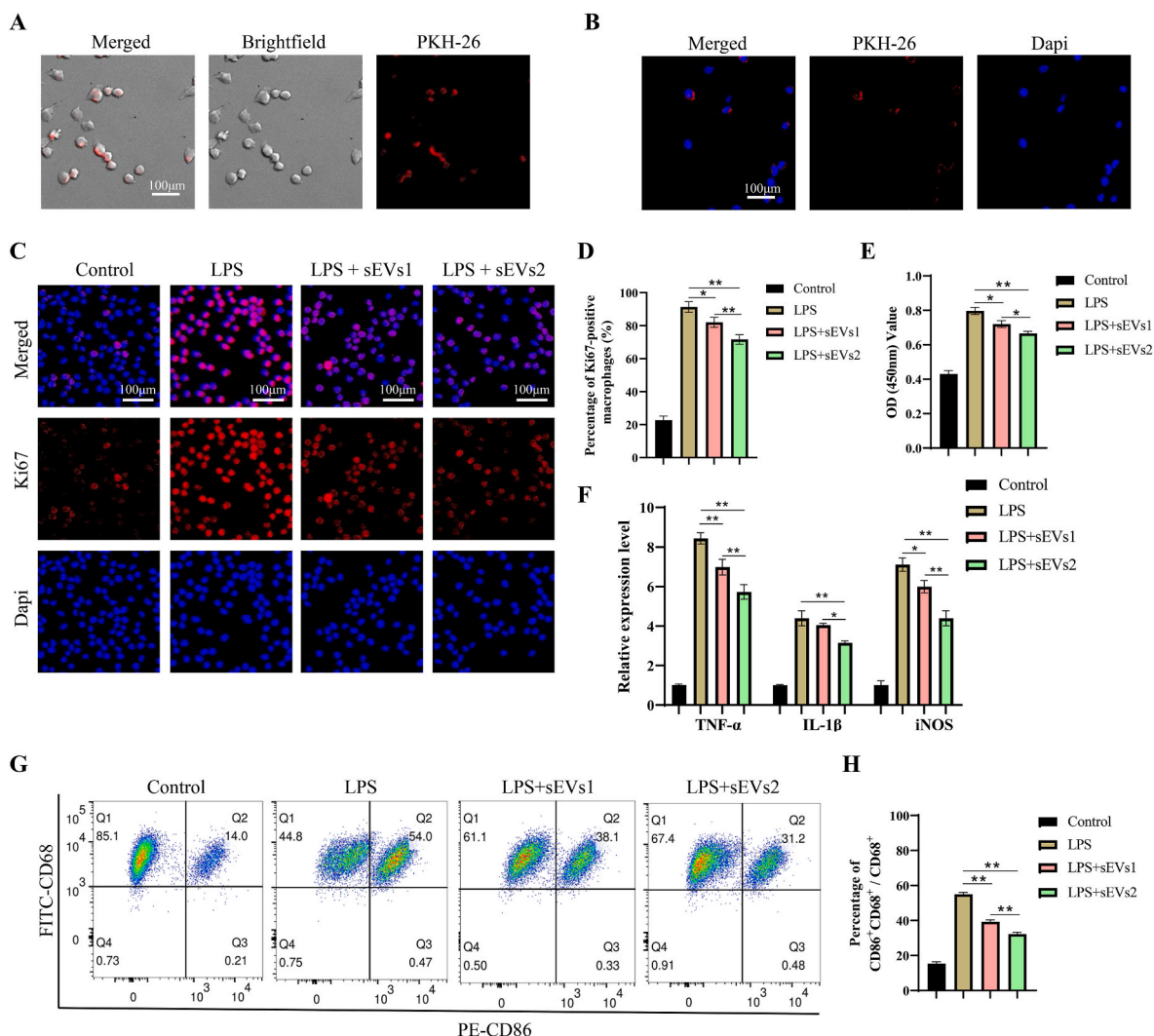


Fig. 2. RAPA-sEVs suppress the proliferation of macrophage and the production of inflammatory cytokines. (A) Macrophages were observed to engulf sEVs under light microscopic examination. Scale bar, 100 μ m. (B) Fluorescent micrograph of sEVs labeled by PKH-26 (red). Scale bar, 100 μ m. (C) Ki67 immunofluorescent staining. Scale bar, 100 μ m. (D) Quantitative analysis of Ki67 positive cells (%). (E) CCK-8 assay was performed to assess the effect of RAPA-sEVs on macrophage proliferation. OD values were measured at 450 nm. (F) The mRNA expression of inflammatory cytokines, including TNF- α , IL-1 β and iNOS. (G–H) Flow cytometry for M1-type macrophages in different groups and corresponding quantitative analysis. CD68, total macrophage marker; CD86, M1-type macrophage marker. Mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.

wound healing [27]. Utilizing SEM and AFM, we observed that the GelMA hydrogel exhibited interconnected porous structures and uneven surface roughness (Fig. 1G and H). Hydrogels with good mechanical strength hold promise for tissue engineering. GelMA hydrogels exhibited favorable mechanical strength (Fig. S2A). Rheological properties assays revealed shear-thinning behavior with increasing shear rate, as GelMA hydrogels are non-Newtonian fluids (Fig. S2B). The cytotoxicity of GelMA hydrogels was evaluated using the CCK-8 assay, and the results showed that no obvious cytotoxicity was observed in the GelMA hydrogels (Fig. S2C). The release behavior of sEVs loaded in hydrogels is shown in Fig. S2D, where both sEVs1 and sEVs2 were released primarily within the first 8 days in a sustained manner when encapsulated into the GelMA hydrogels. These findings demonstrated that GelMA hydrogels serve as an excellent drug carrier for RAPA-sEVs.

3.3. RAPA-sEVs inhibit macrophage cellular function

To clarify whether RAPA-sEVs could be phagocytosed and internalized into macrophages, sEV-labeled with PKH-26 were co-cultured with macrophages. Microscopically, PKH-26-labeled sEVs were endocytosed by macrophages (Fig. 2A). Confocal microscopy further observed that RAPA-sEVs were localized in the cytoplasm of macrophages (Fig. 2B),

substantiating the internalization of sEVs by macrophages. Macrophages being treated with LPS, LPS + sEVs1, and LPS + sEVs2, respectively. IF staining showed that LPS successfully increased the rate of Ki67 positive for macrophages, while both sEVs1 and sEVs2 reduced the proportion of Ki67 positive compared with LPS-treated group (Fig. 2C and D). CCK-8 assays confirmed that both sEVs1 and sEVs2 could suppress the proliferation increased by LPS, while sEVs2 exhibited a stronger anti-proliferative effect than sEVs1 (Fig. 2E). More importantly, RAPA-sEVs dampened the expression of inflammatory factors irritated by LPS, including TNF- α , IL-1 β and iNOS (Fig. 2F). Both sEVs1 and sEVs2 decreased the percentage of pro-inflammatory M1-type macrophages, and RAPA-sEVs showed the more effective reduction than sEVs from hUCMSCs (Fig. 2G and H).

In addition, scratch tests indicated that RAPA-sEVs decreased the migration of macrophages compared with LPS-treated group (Fig. 3A–C). Transwell assays confirmed that RAPA-sEVs curtailed the migration of macrophages provoked by LPS (Fig. 3B–D). These results suggested that the cellular function of macrophages and the inflammatory mediators released by them could be inhibited by RAPA-sEVs.

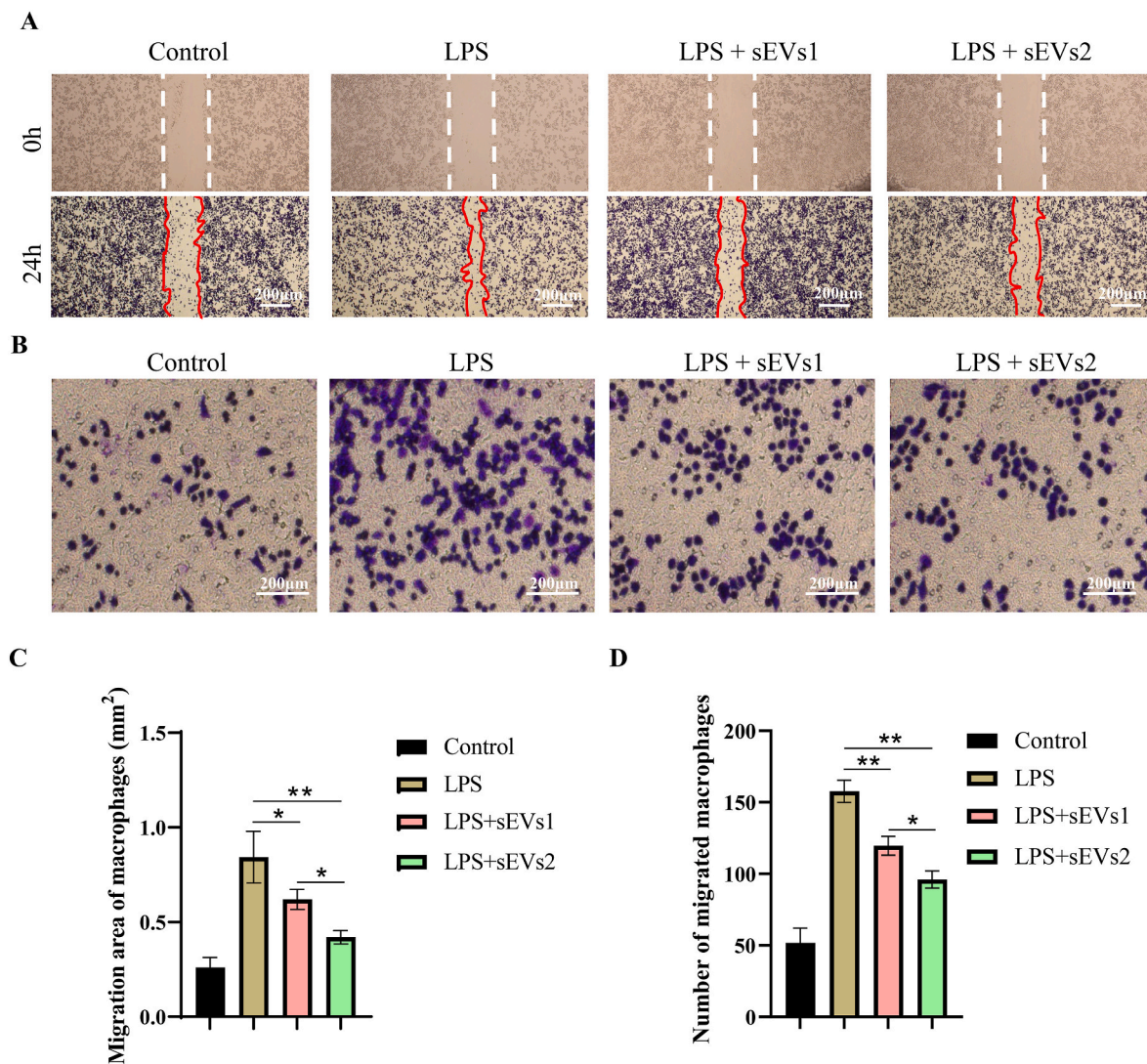


Fig. 3. RAPA-sEVs inhibit the migration of macrophages. (A) Cell migration of macrophages with different treatments using scratch tests. Scale bar, 200 μ m. (B) Transwell assays were employed to evaluate the effect of RAPA-sEVs on the migrative ability of macrophage. Scale bar, 200 μ m. (C–D) Quantitative analysis of migration area and the number of migrative macrophages. Mean $\pm$ SD. * p < 0.05; ** p < 0.01.

3.4. RAPA-sEVs promote the angiogenesis

RAPA-sEVs also could be endocytosed into HUVECs and distributed in the cytoplasm (Fig. 4A). Both sEVs1 and sEVs2 did not affect the proliferation of HUVECs (Fig. 4B), but scratch test and transwell assays showed that they could boost the migration of HUVECs (Fig. 4C–F). Similarly, the sEVs2 exhibited the greater pro-migratory capacity than sEVs1. To investigate the pro-angiogenic ability of RAPA-sEVs, tube

formation assays were performed. More tube structures, including nodes, junctions and branches, in RAPA-sEVs-treated HUVEC were detected than that in the control group, indicating RAPA-sEVs potentiated tube formation capacity of endothelial cells (Fig. 4G–J). Collectively, these finding corroborated the potential of RAPA-sEVs in strengthening the biological functions of HUVECs.

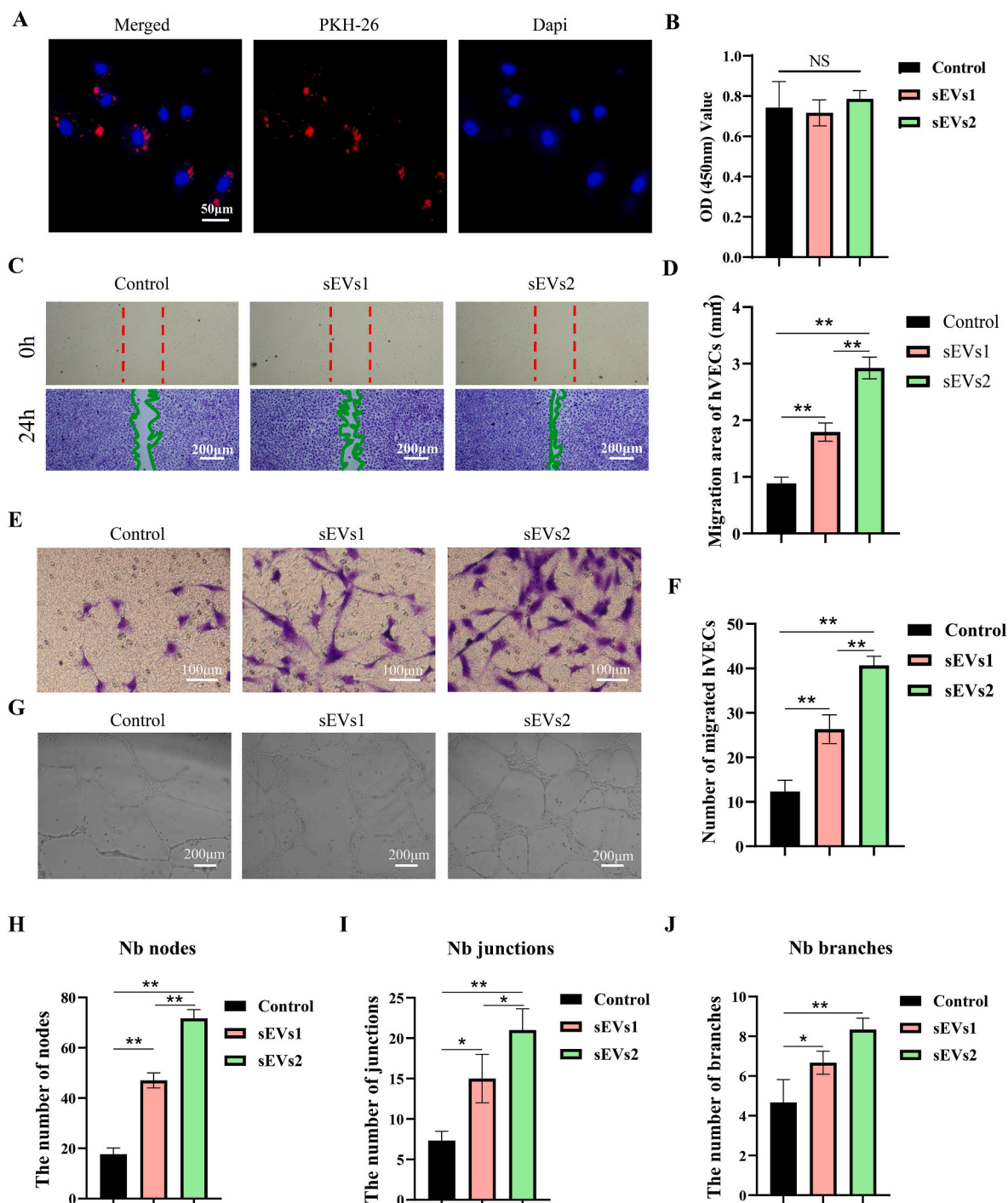


Fig. 4. RAPA-sEVs promote the angiogenesis. (A) HUVECs internalized RAPA-sEVs labeled with PKH-26 (red), which were localized in the perinuclear region as observed via confocal microscopy. Scale bar, 50 μ m. (B) CCK-8 assays were conducted to assess the proliferative capacity of HUVECs. (C) Cell migration of HUVECs with different treatments using scratch tests. Scale bar, 200 μ m. (D) Quantitative analysis of migration area (mm^2). (E) Cell migration of HUVECs after crystal violet staining using transwell assays. Scale bar, 100 μ m. (F) Quantitative analysis of the number of migrated HUVECs. (G) The images of tube formation of HUVECs. Scale bar, 200 μ m. (H–J) Quantitative analysis of tube formation, including nodes, junctions and branches. Mean $\pm$ SD. NS, not significant. * $p < 0.05$; ** $p < 0.01$.

3.5. RAPA-sEVs accelerate diabetic wound closure *in vivo*

Diabetic mice, induced with streptozotocin (STZ), underwent full-thickness skin excision wounds for assessing the therapeutic potential of RAPA-sEVs. From the images of skin defects, no significant difference was observed between control (PBS) and hydrogel group at days 4, 9, and 14 postoperatively, while both sEVs1 and sEVs2 groups promoted diabetic wound healing compared with hydrogel group (Fig. 5A). The sEVs2 exhibited superior wound repair ability than sEVs1. Specific statistical data including wound area and wound closure rates confirmed this finding (Fig. 5B and C). More importantly, throughout the entire experimental period up until euthanasia, the wound area treated with RAPA-sEVs hydrogel remained free of erythema and edema, indicating its superior biosafety. Taken together, these results demonstrated that RAPA pretreatment propelled the hUCMSC-sEVs mediated diabetic wound closure with efficacy and sufficient safety *in vivo*.

3.6. RAPA-sEVs facilitate reepithelization and vascularization, while alleviate macrophage-mediated inflammatory response

Fourteen days later, dorsal skin lesions were harvested for subsequent staining analysis. HE staining showed that the both sEVs1 and sEVs2 groups had better epithelialization and granulation processes in diabetic wounds compared to those treated with hydrogel, including narrower wound widths, thicker epithelial layers, and more abundant epithelial structure (Fig. 6A–C–D). The assessment of wound healing significantly relies on the arrangement and distribution of collagen [28]. Masson staining revealed that sEVs2 possessed thicker collagen fibers accompanied by extensive and neatly arranged collagen deposition (Fig. 6B–E), indicative of potent extracellular matrix (ECM) remodeling potential of RAPA-sEVs.

Besides, IF staining displayed that CD86, a specific marker for M1-type macrophage, was more abundant in both sEVs1 and sEVs2 groups than in control and hydrogel groups (Fig. 7A). Blood vessels are essential for wound repair and CD31 is an important marker for detecting newly formed vessels [29]. The IF analysis targeting CD31 suggested an increased dermal neovascularization at the wound sites following treatment with RAPA-sEVs, as compared to both the control and

hydrogel groups (Fig. 7B). The qPCR analysis of fresh skin lesions confirmed that both sEVs1 and sEVs2 groups expressed less TNF- α , IL-1 β and iNOS, but expressed more PECAM-1 and VEGF compared with control and hydrogel groups (Fig. 7C and D). The sEVs2 exhibited a stronger effect than sEVs1. Consequently, these data underscored that RAPA-sEVs enhanced reepithelization and vascularization processes, while mitigating inflammatory response mediated by macrophages in diabetic skin defects.

3.7. Alterations of transcriptome profile after treatment with RAPA-sEVs

Utilizing high-throughput transcriptome sequencing, alterations in mRNA expression profiles in skin lesions were investigated between the control and sEVs2 groups. As depicted in the volcano plot and heat map, a total of 812 upregulated and 310 downregulated mRNAs were identified in total (Fig. 8A). The relevant heatmap also was performed (Fig. 8B). The outcomes of KEGG pathway analysis indicated that the PI3K/Akt signaling pathway, Rap 1 signaling pathway, and chemokine signaling pathway emerged as the three most pertinent pathways (Fig. 8C). Gene ontology (GO) enrichment analysis hinted that the biological process related to immune regulation, angiogenesis, cell migrate and inflammatory response (Fig. 8D). As the top relevant PI3K/Akt signaling pathway, gene set enrichment analysis (GSEA) was performed (Fig. 8E) and the related networks of protein–protein interaction (PPI) were predicted (Fig. 8F). Further, Western blot verified that the promotion of wound healing by RAPA-sEVs was related to the activation of the PI3K/Akt signaling pathway (Fig. 8G–I).

3.8. Blockade of the PI3K/Akt pathway abrogates RAPA-sEV-mediated inhibition of macrophage activity and promotion of angiogenesis

To further strengthen the above conclusion, we conducted a series of rescue experiments. By administering PI103, a potent inhibitor of the PI3K/Akt pathway, we observed the elimination of the stimulatory effect on macrophage proliferation induced by sEVs2, as evidenced by IF staining for the proliferation marker Ki67 (Fig. 9A and B). As expected, the treatment with PI103 restored the expression of macrophage-associated inflammatory mediators including TNF- α , IL-1 β and iNOS

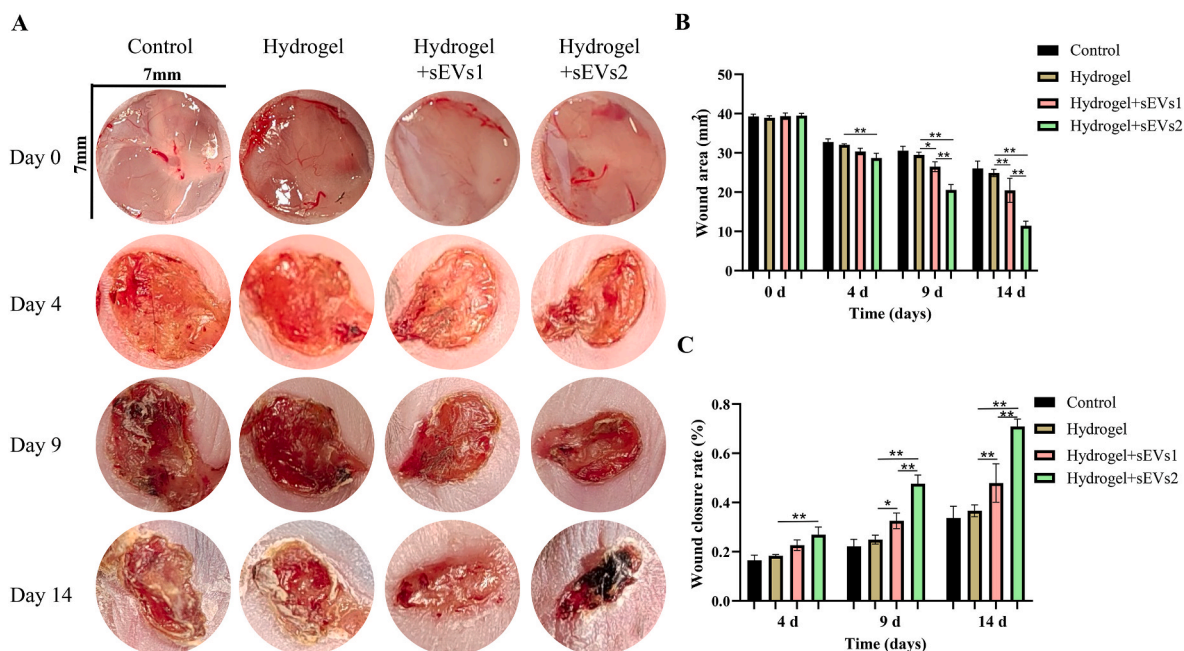


Fig. 5. RAPA-sEVs accelerate diabetic wound closure *in vivo*. (A) Representative images of full-thickness defects (7 mm) of the STZ-induced diabetic mice treated with PBS (control), hydrogel, hydrogel + sEVs1 and hydrogel + sEVs2 at days 0, 4, 9, and 14 postoperatively. (B–C) Quantification of wound area (mm²) and wound closure rate (%). Mean $\pm$ SD. * p < 0.05; ** p < 0.01.

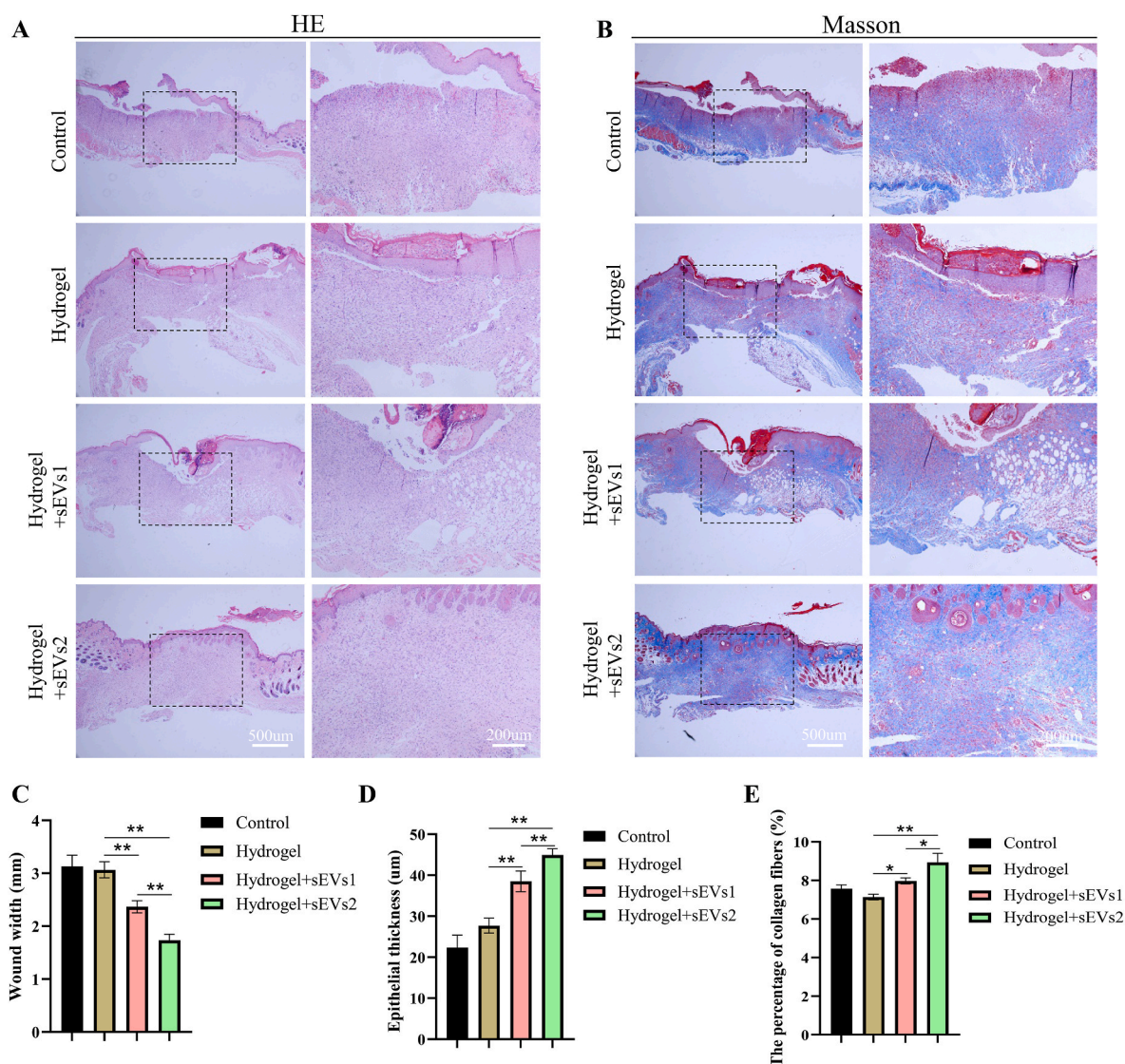


Fig. 6. RAPA-sEVs foster reepithelization and tissue repair. (A–B) H&E and Masson's trichrome staining across various treatment groups at the 14th day post-intervention. Scale bar, 500 μm or 200 μm as indicated in the specific image. (C–E) Quantification of wound width (mm), epithelial thickness (μm) and the percentage of collagen fibers (%). Mean ± SD. * $p < 0.05$; ** $p < 0.01$.

compared with LPS + sEV2 group (Fig. 9C). Similarly, scratch tests and transwell assays revealed that RAPA-sEV-mediated pro-migratory effect on macrophages could be reversed by PI103 (Fig. 10A–D). In addition, PI103 administration effectively abolished the RAPA-sEV-induced enhancement of migration in HUVECs (Fig. 11A–D). The promotional role of RAPA-sEVs on tube formation capacity of endothelial cells could be abated by PI103 (Fig. 11E–H). Altogether, RAPA-sEVs exert biological effect on macrophages and endothelial cells by means of PI3K/Akt signaling pathway.

3.9. PI3K/Akt signaling pathway was responsible for RAPA-sEV-induced diabetic wound repair

To validate the above conclusions *in vivo*, we replicated the establishment of a full-thickness skin wound model in diabetic mice. The results showed that blockade of the PI3K/Akt signaling pathway eliminated the effect of RAPA-sEVs on accelerating skin wound closure at days 4, 9, and 14 (Fig. 12A and B). Additionally, Western blotting validated that the PI3K/Akt signaling pathway was effectively suppressed by PI103 in the skin defect tissues of diabetic mice (Fig. 12C and D). More importantly, *in vivo* experiments confirmed that the expression

of macrophage-related inflammatory factors $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ was increased, while the level of angiogenesis-related markers Pecam-1 and Vegf was decreased following the inhibition of the PI3K/Akt signaling pathway, compared to that of hydrogel + sEVs2 group (Fig. 12E). Overall, these results revealed that the promotion of diabetic wound healing by RAPA-sEVs was dependent on the activation of the PI3K/Akt signaling pathway.

4. Discussion

Delayed diabetic wound repair remains an intractable medical issue with limited therapeutic approaches. The insufficient angiogenesis and exaggerated inflammatory responses are critical causes of delayed and poor wound healing [3,7]. Ordinary extracellular vesicles have been demonstrated to exert a certain positive effect on skin repair. However, their efficacy remains to be further enhanced. We hypothesized that following pre-stimulation of hUCMSCs with the small molecule compound RAPA, the secreted sEVs might possess a more favorable effect on skin repair. Herein, RAPA-sEVs from hUCMSCs were successfully extracted and identified. We investigated the pro-angiogenic and anti-inflammatory properties of hUCMSC-derived sEVs pretreated with

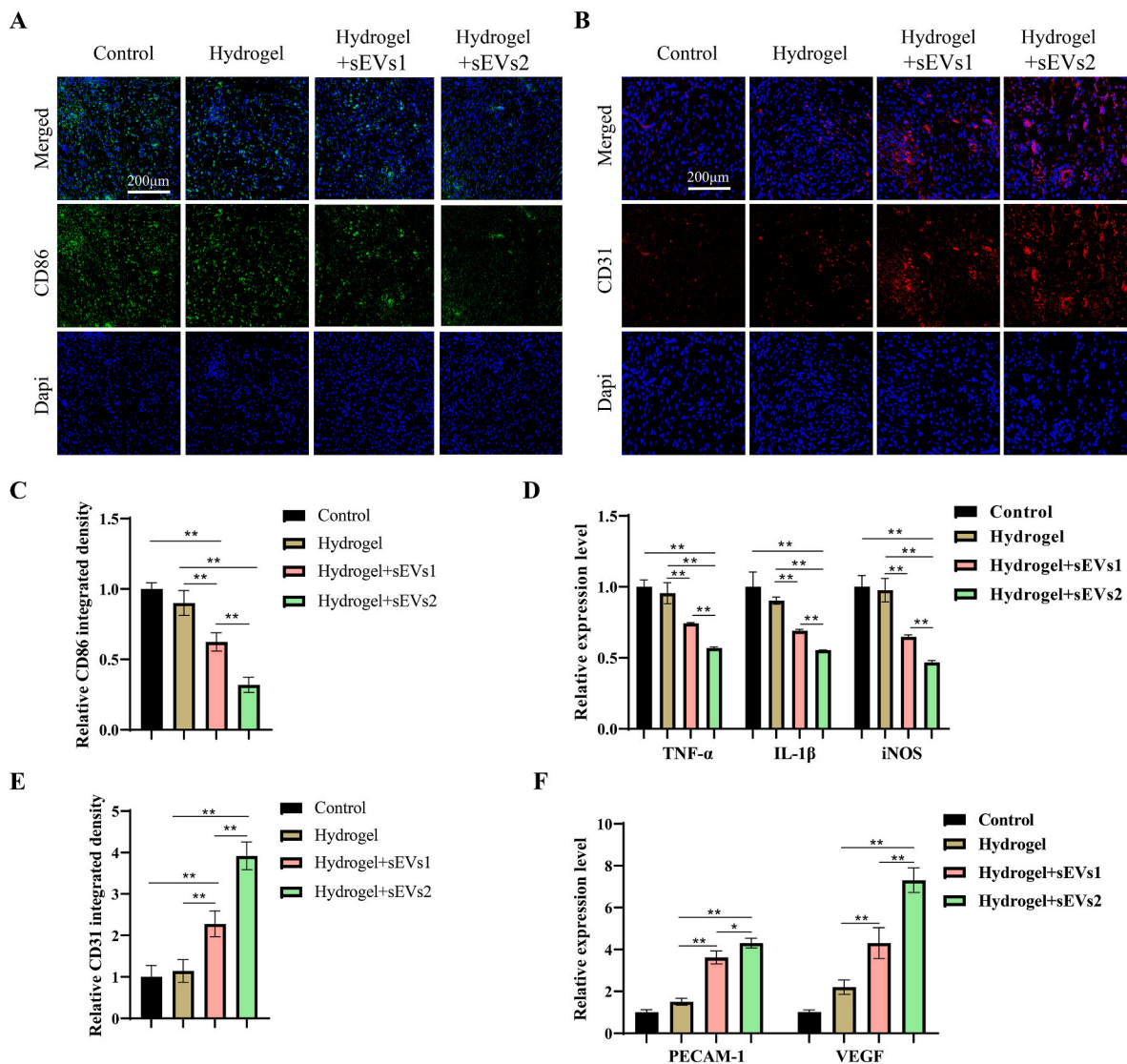


Fig. 7. RAPA-sEVs enhance vascularization while mitigating the macrophage-mediated inflammatory response. (A–B) Tissue immunofluorescence assessment stained by M1-type macrophage marker CD86 and angiogenesis marker CD31. Scale bar, 200 μ m. (C, E) Quantitative analysis of tissue immunofluorescence for CD86 and CD31. (D, F) The mRNA levels of inflammation-related factors (TNF- α , IL-1 β , iNOS) and angiogenesis-related markers (PECAM-1 and VEGF). Mean $\pm$ SD. * p < 0.05; ** p < 0.01.

RAPA *in vitro*. STZ-induced murine models further demonstrated that RAPA-sEVs-loaded GelMA hydrogels exhibit superior wound healing efficacy and excellent biosafety in diabetic wound treatment. Mechanistically, our findings indicated that RAPA-sEVs could promote the angiogenesis of endothelial cells and alleviate the inflammation response of macrophages through activating PI3K/Akt signaling pathway. Specifically, during the wound healing process in diabetic mice, RAPA-sEVs enhanced collagen deposition, vascularization and reepithelization while simultaneously decreasing the local infiltration of macrophages and mitigating the release of inflammatory mediators, such as TNF- α , IL-1 β , and iNOS. These results supported the potential of RAPA preconditioning to improve hUCMSC-sEV-based therapies for diabetic skin lesions.

Angiogenesis plays a pivotal role in driving skin tissue repair and regeneration processes during wound healing, as it ensures the effective transportation of nutrients, oxygen, and immune cells to the wounded area, enhancing cellular proliferation, ECM deposition and infection resistance [30]. Hyperglycemia in diabetes disrupts endothelial cell function, leading to loss of integrity and elevated apoptosis, which subsequently perturbs the delicate process of angiogenesis, manifesting

as damaged microvascular architecture and reduced capillary density [31]. In our study, we observed the RAPA-induced hUCMSC-sEVs promoted the angiogenesis by staining neovascularization marker CD31 in STZ-induced diabetic murine models. In addition, as the crucial factors that promote angiogenesis and maintain vascular stability, VEGF and PECAM-1 were significantly elevated at wound microenvironment, creating favorable conditions for angiogenesis. Recently, investigators have dedicated extensive efforts for developing modified hUCMSC-sEVs to achieve superior wound healing outcomes [32–34]. Zhao et al. [32] uncovered that hUCMSC-sEVs ameliorate HaCaT cell death in skin by hindering the nuclear translocation of apoptosis-inducing factor and promote wound closure. Yang et al. [34] illustrated that sEVs isolated from hUCMSCs stimulated with blue light exhibited enhanced efficacy in promoting wound healing through the upregulation of myocyte enhancer factor 2C (MEF2C). Liu et al. [33] showed that hUCMSC-sEVs facilitate skin wound repair via accelerating angiogenesis via delivering angiopoietin-2 (Ang-2) in rats. Herein, we found that RAPA-induced hUCMSC-sEVs could effectively foster collagen deposition and reepithelization by stimulating angiogenesis, thus accelerating wound closure and skin repair.

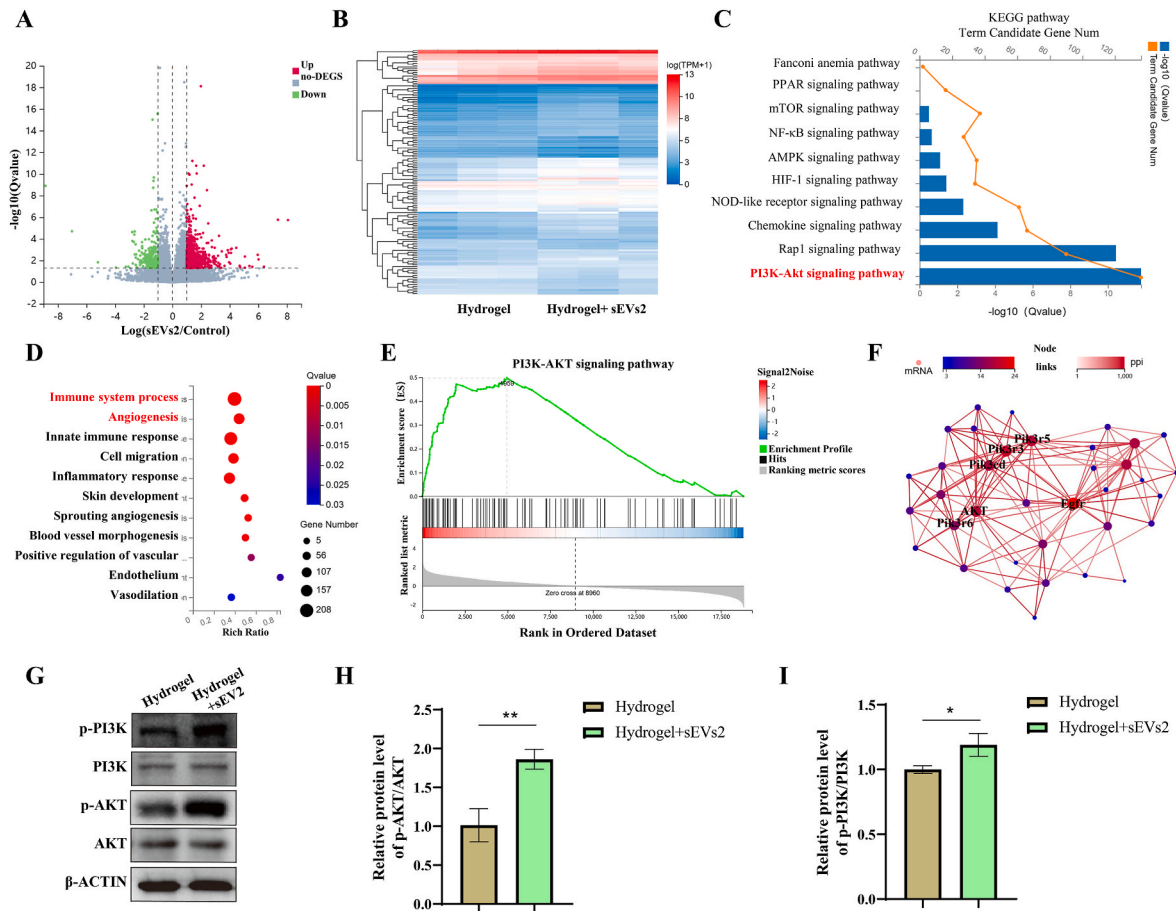


Fig. 8. Alterations of transcriptome profile in diabetic wound tissue after being treated with RAPA-sEVs. (A) Volcano plot exhibited differentially expressed genes (DEGs) between hydrogel and hydrogel + sEVs2 groups. (B) Heatmap of transcriptomics clustering. (C) KEGG pathway analysis. (D) GO enrichment analysis. (E) Gene set enrichment analysis (GSEA) of PI3K/Akt signaling pathway. (F) The networks of protein-protein interaction (PPI). (G-I) The protein levels of p-PI3K, PI3K, p-AKT and AKT and the related quantitative analysis. Mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.

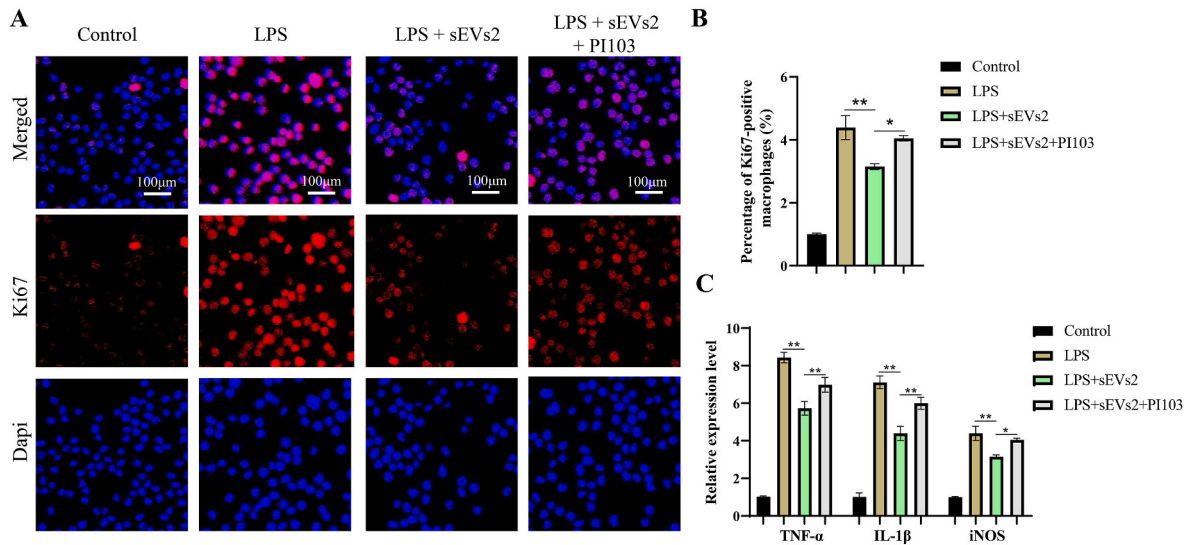


Fig. 9. Blockade of the PI3K/Akt pathway abrogates RAPA-sEV-mediated inhibition of macrophage proliferation and inflammatory factors. (A-B) Ki67 immunofluorescent staining of macrophages and the percentage of Ki67 positive cells (%) after being treated with PI3K inhibitor PI103. Scale bar, 100 μ m. (C) The mRNA expression levels of inflammatory cytokines, including TNF- α , IL-1 β , iNOS, in macrophages subjected to various treatments. Mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.

Hyper-inflammation or sustained inflammation have been shown to delay wound healing, even the failure of wound healing, due to the damage of excessive inflammatory mediators and reactive oxygen

species [35]. Diabetic wounds exhibit a persistent inflammatory state, and alleviating inflammatory response at wound site is important to the management of diabetic wounds. Emerging evidence has indicated that

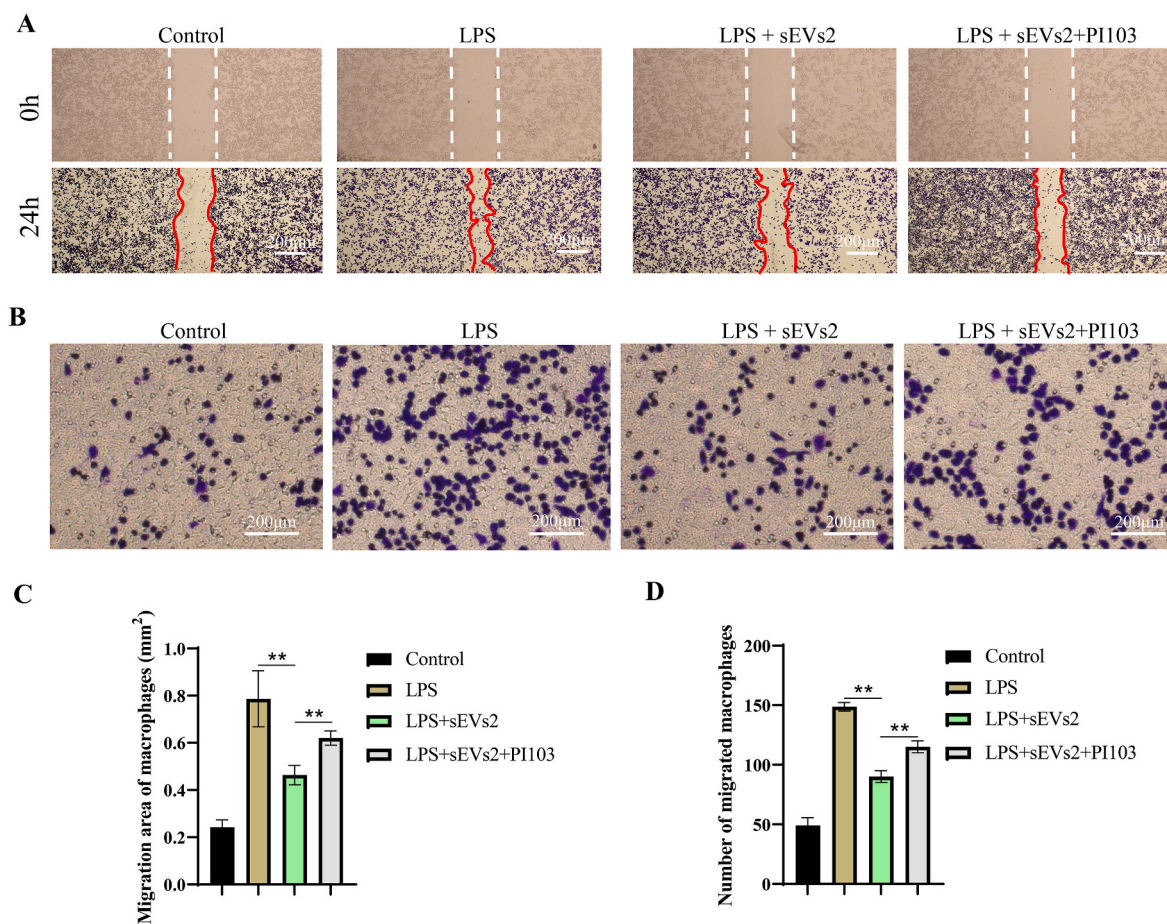


Fig. 10. Blocking the PI3K/Akt pathway attenuates the inhibitory effect of RAPA-sEVs on the migration of macrophages. (A) Scratch tests were utilized to evaluate macrophage migration with different treatments. Scale bar, 200 μ m. (B) Transwell assays were employed to assess the migration ability of macrophage after treatment with PI103. Scale bar, 100 μ m. (C-D) Quantification on the migrative area and number of macrophages with different therapeutic interventions. Mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.

MSC-sEVs exert anti-inflammation and immunoregulation effects to accelerate diabetic wound repair [36]. For example, Long et al. [37] showed that MSC-sEVs inhibited LPS-induced inflammation in macrophages, promoted skin cell proliferation and migration, and accelerated the healing of chronic diabetic wounds. Macrophages are pivotal regulators actively involved in wound healing and tissue repair with a high plasticity and dynamic phenotypic transformations, and their dysfunction results in aggravated inflammation and insufficient healing in diabetic wounds [38]. In the normal progression of wound healing, macrophages undergo a phenotypic shift from a pro-inflammatory state (M1-type) during the initial stages of tissue regeneration to an anti-inflammatory state (M2-type) in the subsequent phases [39]. However, under the diabetic state, this transition is disrupted, with macrophages sustaining the M1-type and failing to polarize into the M2-type, contributing to the persistence of chronic inflammation [40]. Therefore, how to diminish the M1-type of macrophages and reestablish macrophage polarization from M1-type to M2-type becomes the key to the treatment strategy of diabetic wounds. Liu et al. [41] verified that MSC-derived sEVs improve diabetic wound repair by increasing the percentage of M2 to M1 polarization. Similarly, our data showed that RAPA-sEVs could significantly reduce the percentage of pro-inflammatory M1-type macrophages *in vitro*, and STZ-induced diabetic mouse models further verified this result at wound microenvironment. Moreover, proinflammatory cytokines, such as TNF- α , IL-1 β and iNOS, contribute to the persistence of diabetic chronic inflammation, resulting in endothelial cell damage and initiating a cascade of inflammatory factors [42,43]. Here, we discovered that

RAPA-stimulated sEVs from hUCMSCs could effectively reduce the levels of TNF- α , IL-1 β and iNOS at the diabetic wound sites, thereby alleviating excessive local inflammation and favoring wound healing.

Despite MSCs have been shown to exert favorable effects on skin wound repair, there are still some disadvantages impeding their widespread clinical application, especially tumorigenesis and immunogenicity [44]. In contrast to MSCs, MSC-sEVs, as a novel cell-free therapy, exhibit superior stability and augmented biological efficacy. Notably, MSC-sEVs-based treatment can effectively address MSC-related adverse effects, offering robust biosafety, minimal tumorigenesis, and low immunogenicity [45]. Recent studies have revealed that GelMA hydrogels exhibits extensive application in tissue repair owing to its therapeutic optimization capabilities and exceptional biocompatibility [27,46,47]. Our findings demonstrated that GelMA hydrogels exhibited interconnected porous structures, adequate mechanical strength, suitable rheological properties, sustained RAPA-sEVs release capacity, and non-cytotoxicity, suggesting its potential as an ideal delivery vehicle for RAPA-sEVs. Moreover, during the whole animal experiment, we did not observe any detrimental immunoreaction or side-effects in diabetic wounds, including edema and erythema, exhibiting the superior biosafety of GelMA hydrogel formulated with RAPA-sEVs from hUCMSCs.

Accumulated studies have indicated that preconditioning MSCs with different stimuli is an effective means to enhance the therapeutic efficacy of MSC-induced sEVs [15,48,49]. Liang et al. [48] dimethylxaloylglycine-stimulated MSC-sEVs could improve bone regeneration through angiogenesis by activating the AKT/mTOR

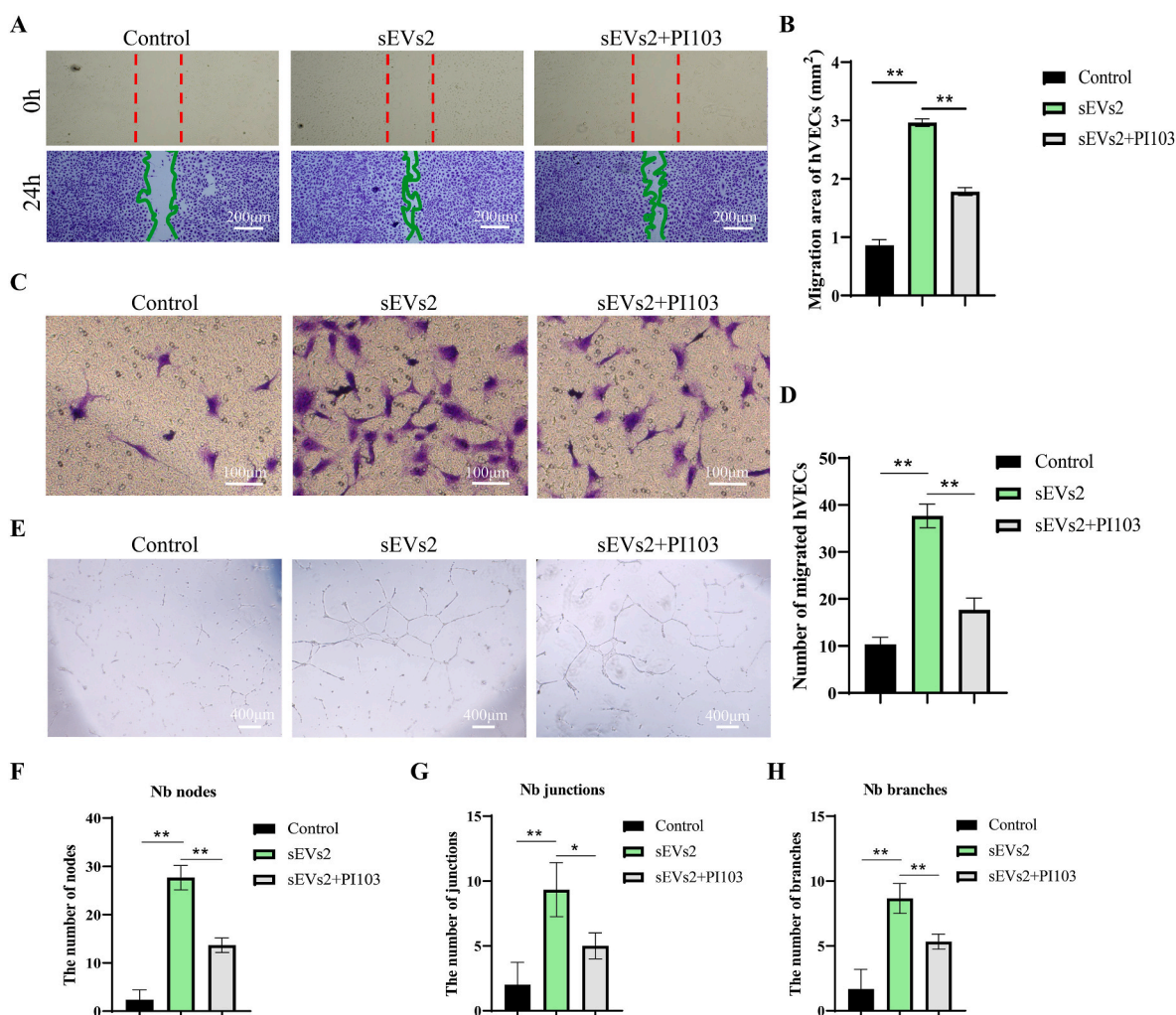


Fig. 11. Inhibition the PI3K/Akt pathway impairs the stimulative effect of RAPA-sEVs on the migration and tube formation abilities of HUVECs. (A) The migration capacity of HUVECs was detected by scratch tests after being treated with PI103. Scale bar, 200 μ m. (B) Quantitative analysis of migrative area (mm²). (C) Transwell assays were utilized to evaluate the migration of HUVECs. Scale bar, 100 μ m. (D) Quantitative analysis of the number of migrated cells. (E) Tube formation assays were applied to assess the angiogenesis with different treatments. Scale bar, 400 μ m. (F–H) Quantification of the number of nodes, junctions and branches. Mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.

pathway. Shi et al. [49] revealed that 3,3'-diindolylmethane stimulates the Wnt11 autocrine signaling of sEVs in hUCMSCs to propel wound repair. However, the biosafety of dimethylolaloylglycine and 3,3'-diindolylmethane remains a matter of concern for clinical application. RAPA, serve as a mTOR inhibitor, has been applied for the treatment of anti-rejection and autoimmune diseases in organ transplantation, and the biosafety has been verified. Furthermore, it has been reported that perioperative RAPA administration in heart transplants increases wound-healing risks [50]. RAPA could impair wound healing by affecting multiple steps of the tissue regeneration, including angiogenesis resistance, decreased infiltration of immune cells, compromised lactate accumulation and ECM remodeling [51,52]. Interestingly, unlike the biological effects of RAPA itself, our findings suggested that sEVs produced by RAPA-stimulated hUCMSCs are able to accelerate diabetic wound healing through proangiogenic and anti-inflammatory effects. Notably, RAPA induction attenuated the differentiation capacity of hUCMSCs and altered the membrane protein expression of secreted RAPA-sEVs, particularly CD63, CD81, and CD9, which play critical roles in cargo sorting, exosome biogenesis, and secretion [53]. We speculate that this may be attributed to alterations in the cargo composition and functional properties of extracellular vesicles. Also, this is very likely an important reason why sEVs2 are more effective than sEVs1 in the wound

healing process. These findings warrant further investigation in the future.

The process of wound healing is inherently dependent on a range of intricate signaling pathways, including VEGF pathway, NF- κ B pathway, PI3K/Akt pathway, MAPK pathway, Wnt/ β -catenin pathway, as well as others [43]. These signaling cascades collectively exert a pivotal influence on the elaborate process of wound repair. Among them, the PI3K/Akt pathway is indispensable for cellular proliferation, viability, metabolism and inflammation during diabetic wound closure, while aberration of this pathway causes induce healing retardation and chronic inflammatory states [43]. Wang et al. [54] warranted that the activation of PI3K/Akt signaling pathway mediated by hypoxia adipose stem cell-derived sEVs promotes the fibroblast proliferation and suppresses inflammation, which increases ECM formation and the secretion of vascular growth factors, thus facilitating diabetic wound healing. Furthermore, insulin-induced PI3K/Akt signaling shifts macrophage from M1 to M2 phenotype, aiding the anti-inflammatory processes and improving chronic healing of diabetic wounds [55]. Analogously, in this study, our findings manifested that RAPA-sEVs induced PI3K/Akt signaling pathway is responsible for the high-quality healing of diabetic wound via promoting angiogenesis and alleviating macrophage-mediated anti-inflammatory effects. These results are

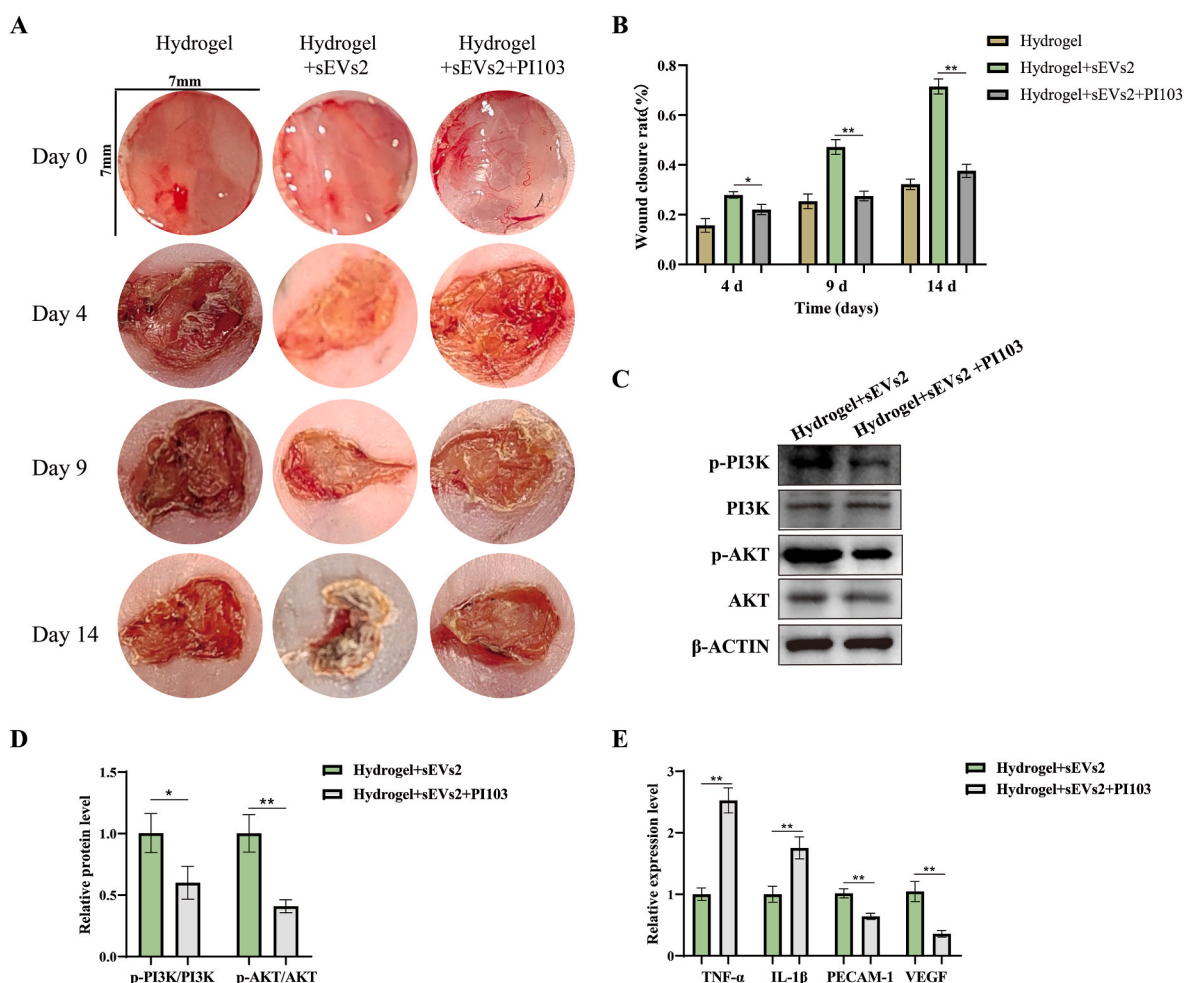


Fig. 12. PI3K/Akt signaling pathway was responsible for RAPA-sEV-induced diabetic wound repair. (A) Diabetic mice with full-thickness defects (7 mm) were treated with hydrogel, hydrogel + sEVs2, and hydrogel + sEVs2 + PI103 at postoperative days 0, 4, 9, and 14. (B) Quantitative analysis of wound closure rate (%). (C) The phosphorylation levels of PI3K and AKT, as well as the relative protein expression levels of wound skin tissues after being treated with PI103 by Western blotting. (D) Quantitative analysis of Western blotting assays. (E) The relative mRNA expressions of inflammation-related factors (TNF- α and IL-1 β) and angiogenesis-related markers (PECAM-1 and VEGF) after treatment with PI103. Mean $\pm$ SD. * p < 0.05; ** p < 0.01.

substantiated through high-throughput sequencing with the help of tissue and a series of reversion experiments. This strategy exhibits encouraging preclinical efficacy, indicating its potential application in the therapeutic interventions of diabetic wound management.

5. Conclusions

Overall, this study firstly reported that RAPA preconditioning heightened the function of sEVs derived from hUCMSCs in facilitating angiogenesis and suppressing macrophage-mediated inflammatory response through activating the PI3K/Akt signaling pathway, resulted in accelerated diabetic wound healing. Our findings provide a novel view for the therapeutic strategy of a diabetic wound.

CRediT authorship contribution statement

Yalu Zhang: Writing – original draft, Visualization, Investigation, Funding acquisition, Data curation, Conceptualization. **Zekun Wang:** Investigation, Formal analysis, Data curation. **Liang Wu:** Validation, Formal analysis, Data curation. **Liang Zhu:** Visualization, Investigation. **Ming Lu:** Investigation, Formal analysis. **Ziyang Zhang:** Visualization, Formal analysis. **Yuanhao Lv:** Writing – review & editing, Supervision. **Xu Zhu:** Writing – review & editing, Supervision, Methodology. **Hanhui Yao:** Writing – review & editing, Supervision, Project administration.

Ethics approval and consent to participate

All animal procedures were approved by the Animal Ethics Committee of the First Affiliated Hospital of USTC (2024-N(A)-0412).

Funding

This study was supported by the Anhui Provincial Natural Science Foundation (2308085QH275), China Postdoctoral Science Foundation (2023M733393) and National Natural Science Foundation of China (82303272).

Declaration of competing interest

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

Acknowledgments

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mtbo.2025.101846>.

Data availability

Data will be made available on request.

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