

Engineered sEVs encapsulated in GelMA facilitated diabetic wound healing by promoting angiogenesis via targeting thrombospondin-1

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Abstract

Background: Chronic nonhealing wounds are major complications in diabetic patients, with impaired angiogenesis playing a critical role in the delayed healing process. Current treatments for diabetic wounds are inadequate. The dysregulation of endothelial cell genes, particularly thrombospondin-1 (TSP-1), impairs neovascularization and delays wound repair. In recent years, hydrogel-based wound dressings have gained widespread application in biomedicine. The study introduced a new therapeutic approach, embedding miR-221-3p-loaded small extracellular vesicles (miR-221^{OE}-sEVs) within gelatin methacryloyl (GelMA) hydrogels to reduce TSP-1 levels and improve healing in diabetic wounds.

Methods: First, we observed upregulated TSP-1 expression in human umbilical vein endothelial cells (HUVECs) when cultured in a high-glucose (HG) environment. We employed small interfering RNA (siRNA) and miR-221-3p to suppress TSP-1 expression and then evaluate the functional effects on HUVECs. Subsequently, miR-221-3p was encapsulated in sEVs via lentiviral transfection. The effects of miR-221^{OE}-sEVs on HUVECs under HG conditions were evaluated. Finally, miR-221^{OE}-sEVs were incorporated into a GelMA hydrogel (G-miR-221^{OE}-sEVs) and applied to a diabetic murine wound model to evaluate their effects on wound closure and angiogenesis.

Results: Under HG conditions, the use of siTSP-1 to silence TSP-1 enhanced the proliferation, migration, and tube formation capabilities of HUVECs. Similarly, miR-221-3p treatment exerted proregenerative effects via the targeting of TSP-1. We successfully generated miR-221^{OE}-sEVs that exhibited a 28-fold increase in miR-221-3p expression, which significantly enhanced HUVEC functionality under HG conditions. Encapsulation within the GelMA hydrogel enabled G-miR-221^{OE}-sEVs to significantly accelerate diabetic wound healing via increased angiogenesis.

Conclusions: This study demonstrated the successful fabrication of a novel bioactive wound dressing (G-miR-221^{OE}-sEVs), which promotes diabetic wound healing by promoting angiogenesis through the regulation of TSP-1. This approach offers a potential therapeutic option for enhancing the management of diabetic wounds.

Keywords: Thrombospondin-1; Angiogenesis; miR-221-3p; Diabetic wounds; Small extracellular vesicles

Highlights

- A novel bioactive dressing (G-miR-221^{OE}-sEVs) to enhance angiogenesis by targeting thrombospondin-1 (TSP-1) for diabetic wound therapy is developed.
- Engineered miR-221^{OE}-sEVs ameliorate the regenerative abilities of HUVECs under HG conditions by targeting TSP-1.
- GelMA hydrogel is utilized to construct a sustained release system of sEVs and barrier similar to extracellular matrix to maximize the therapeutic efficacy of sEVs.

Background

Chronic refractory wounds represent a major clinical challenge due to their complex pathophysiology. With the increasing number of diabetic patients, the occurrence rate of chronic refractory wounds caused by diabetes has correspondingly increased on an annual basis [1]. A report from the International Diabetes Federation showed that ~500 million

individuals worldwide were diagnosed with diabetes in 2021, among whom ~19%–34% will experience a lifelong risk of enduring chronic refractory wounds exemplified by diabetic foot ulcers, thus causing considerable suffering to patients [2]. A sufficient blood supply is essential for tissue repair. However, in the local hyperglycemic environment of diabetic wounds, the endothelial cells' ability to proliferate and migrate is limited. This disruption subsequently hampers

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the process of wound angiogenesis [3, 4]. Therefore, the restoration of impaired endothelial function and an increase in the blood supply are indispensable for promoting wound healing in diabetic patients [5, 6].

Thrombospondin-1 (TSP-1), which is a matricellular protein, acts as a strong antiangiogenic agent by suppressing endothelial cell migration and proliferation, along with inducing endothelial apoptosis [7]. The interaction of TSP-1 with its receptor (CD47) further inhibits angiogenesis by antagonizing nitric oxide signaling in endothelial and vascular smooth muscle cells [8, 9]. A high-glucose (HG) environment has been reported to induce a relatively high level of TSP-1 expression, leading to delayed healing of diabetic wounds [10]. Several previous studies have concentrated on the targeting of TSP-1 as an effective approach for managing diabetic wounds. For example, treatment with metformin markedly attenuates TSP-1 in endothelial precursor cells, thus improving angiogenesis and promoting diabetic wound healing [11]. Zhu *et al.* reported that alizarin protects against vascular endothelial dysfunction in type 2 diabetes through suppression of TSP-1 generation and activation of AMPK-mediated signaling networks [12]. Therefore, therapies that target the inhibition of TSP-1 can be used as an effective means of treating diabetic wounds.

MicroRNAs (miRNAs) exist naturally in the human body and can bind to the messenger RNAs (mRNAs) of multiple target genes, thereby exerting regulatory effects and effectively reversing the pathophysiological states of cells [13, 14]. The miRNA-based approach offers high efficiency and cost-effectiveness, thus making it a highly favorable method for experimental research and clinical treatment [15–17]. Recent investigations highlight miRNAs' angiogenic modulation through endothelial cell functional restoration, particularly in vascular pathophysiology [18]. Experimental evidence shows that extracellular vesicle-encapsulated miR-17-5p improves cellular performance in hyperglycemic environments, enhancing human umbilical vein endothelial cell (HUVEC) and human dermal fibroblast viability [19]. Additionally, miR-221-3p is widely recognized as a functional molecule that exerts regulatory control over a diverse range of biological processes [20], such as cardiac fibrosis [21], osteogenesis [22], and papillary thyroid cancer [23]. Previous studies have demonstrated that in an HG environment, miR-221-3p can accelerate diabetic wound healing through targeted inhibition of TSP-1 expression; however, the downstream pathway involved in this effect has not been elucidated [24]. These findings provide a novel strategy for the management of diabetic wounds. Therefore, miR-221-3p can be used as an effective functional molecule to promote angiogenesis.

Due to their exceptional biocompatibility, minimal immunogenicity, efficient delivery capabilities, capacity to traverse various physiological barriers, and targeting precision, small extracellular vesicles (sEVs) can serve as crucial carriers of bioactive molecules, including nucleic acids and proteins [25, 26]. These characteristics enable sEVs to protect miRNAs from degradation in enzyme-rich environments, thereby ensuring their stability and effective delivery to target cells [27, 28]. Thus, they possess immense potential as tools for repair and delivery purposes [29]. Mesenchymal stem cell (MSC)-originated extracellular vesicles (EVs) have resulted in significant advancements in the field of tissue repair [30]. However, natural EVs exhibit certain

challenges, including complex compositions, low contents of effective components, and limited clinical translational efficiency. Therefore, researchers have engineered EVs by incorporating specific proteins or nucleic acid molecules to further enhance their biological functions, thereby increasing their therapeutic efficacy for targeted diseases. For example, Huang *et al.* administered engineered EVs loaded with cir-CDK13 to diabetic wounds in preclinical models and observed accelerated wound healing [31]. These findings indicate that engineered EVs encapsulating sufficient quantities of miR-221-3p may be promising therapies for promoting diabetic wound healing.

The GelMA hydrogel serves as a fundamental component in developing and studying multifunctional dressings for tissue regeneration. This material is an optical crosslinking hydrogel that exhibits excellent biocompatibility, cell adhesion, and remarkable molding capabilities [32–34]. Due to its adjustable physical and chemical properties, as well as its biodegradability, GelMA demonstrates extensive applications in tissue regeneration, drug delivery, and other related fields [35–38]. Yoon *et al.* incorporated chemokines into a hydrogel, which demonstrated immunomodulatory and prohealing properties by recruiting MSCs to severe cutaneous injuries in diabetic mice [39]. The safe and effective sustained release system offers a potential solution to the issue of rapid drug clearance in the body following the conventional administration of free EVs or local drug injections, thereby enabling sustained release throughout all stages of wound healing.

In this study, miR-221^{OE}-sEVs were prepared and incorporated into GelMA hydrogels to create an innovative therapeutic dressing for diabetic wound management. Initial experiments demonstrated elevated TSP-1 expression under hyperglycemic conditions, while suppressing TSP-1 expression enhanced HUVEC proliferation and migratory capacity. We further verified that miRNA-221-3p could target TSP-1 and act on the downstream AKT/eNOS pathway, thus ameliorating the impaired functions of HUVECs in an HG environment. Finally, we generated engineered EVs encapsulating miR-221-3p and loaded miR-221^{OE}-sEVs in GelMA hydrogels to treat the diabetic wounds in Db/Db mice. We subsequently reported that the G-miR-221^{OE}-sEVs markedly improved the healing of diabetic wounds through enhancing neovascularization. These results may propose a promising combination therapy leveraging engineered EVs and hydrogel delivery systems for diabetic wound management.

Methods

Cell culture

HUVECs and human umbilical cord mesenchymal stem cells (hucMSCs) were sourced from the Cell Bank of the Chinese Academy of Sciences (China), while human microvascular endothelial cells (HMECs) were acquired from Shanghai Fuheng Biotechnology Co., Ltd (China). These cells underwent expansion, characterization, and classification as the primary (P1) culture. HUVECs were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 5 mM glucose (normal glucose, NG), followed by incubation at 37°C with 5% CO₂. HMECs were maintained in Modified Chee's Dulbecco's Medium (MCDB) 131 enriched with 10% FBS, 10 ng/ml epidermal growth factor, 1 pg/ml hydrocortisone,

2 mM L-glutamine solution, and 1% P/S. Under HG conditions, the glucose concentrations were 25 mM for the HMECs [40] and 35 mM for HUVECs [19, 31]. HucMSCs were specifically cultured using serum-free mesenchymal stem cell medium (Yocon, China) under standard culture parameters (5% CO₂ at 37°C).

siRNA and cell transfection

siRNAs against TSP-1 (siTSP-1, 100 nM; GenePharma, China) (sense 5'-3': CCAACAAACAGGUGUGCAATT; antisense 5'-3': UUGCACACCUGUUUGUUGGTT), along with its negative control siRNA (siNC), were synthesized and procured from GenePharma (China). Following the protocols provided by the manufacturer, a Lipofectamine 3000 kit (Invitrogen, USA) was used to transfect target cells (HUVECs) at a confluency of 60%–70%.

5-Ethynyl-20-deoxyuridine assay

The proliferation ability of the cells was evaluated using the BeyoClick™ EdU Cell Proliferation kit with Alexa Fluor 488 (Beyotime, China). HUVECs from the HG, NG, HG + siNC, and HG + siTSP-1 groups were seeded in 24-well culture plates at ~20 000 cells per well, and each group had three replicates. The same methodology was utilized for HUVECs from the NG, HG, HG + miNC, and HG + miR-221-3p groups. For the HUVECs from the NG, HG, HG + NC-sEVs, and HG + miR-221^{OE}-sEVs groups, the particle concentration of the NC-sEVs or miR-221^{OE}-sEVs was 2×10^{10} particles/ml. After 24 h of culture, a solution containing 10 μM EdU was added to each well and incubated for 2 h. The cells were subsequently fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.3% Triton for 10 min, subjected to click reaction mixture staining for another 30 min, and finally stained with 4',6-diamidino-2-phenylindole (DAPI) for an additional 5 min, after which fluorescence microscopy was used to obtain representative images. The cell proliferation rate was calculated as the ratio of EdU-positive cells (green) to DAPI-positive cells (blue).

Cell counting kit-8 assay

Cell proliferation capacity was evaluated using the Cell counting kit-8 (CCK-8) reagent (Solarbio, China). Complete culture medium was employed to dilute cellular suspensions from different experimental groups to concentrations between 20 000 and 40 000 cells/ml. Subsequently, 100 μl of the cell suspension per well was added to a 96-well plate, and triplicate wells were set up for each group. Following supplementation with 10 μl CCK-8 solution into each well, the plates underwent 2-h incubation at 37°C. Absorbance readings at 450 nm wavelength were subsequently recorded through a microplate reader.

Transwell assay

Cell migration capacities of HUVECs and HMECs were evaluated using transwell migration chambers. Cellular suspensions were loaded into the upper compartments of 24-well plates containing polycarbonate membranes with 8 μm pores. Each experimental group's cell density was diluted to $2-4 \times 10^5$ cells/ml using serum-deprived medium. Aliquots (100 μl) of HUVEC suspensions representing HG, NG, HG + siNC, and HG + siTSP-1 experimental conditions were placed in the upper compartments, while the lower chambers received 500 μl nutrient-rich medium. Parallel experimental setups were implemented for HUVEC groups including

NG, HG, HG + miNC, and HG + miR-221-3p conditions. During sEV-related investigations, HUVECs or HMECs suspended in serum-free medium were introduced into upper chambers, with corresponding lower chambers containing media from NG, HG, HG + NC-sEVs, or HG + miR-221^{OE}-sEVs treatments. Cellular cultures were maintained at 37°C under 5% CO₂ conditions for subsequent development. Following 24-h incubation periods, nonmigratory cells on the membrane's upper surface were carefully eliminated using sterile swabs. Specimens underwent fixation with 4% paraformaldehyde solution for 20-min intervals followed by 10-min staining with crystal violet (Beyotime, China). Finally, five random individual microscope fields were photographed and counted using ImageJ software (Version 1.53e, NIH, USA).

Scratch assay

The cell migration capacities of HUVECs and HMECs were evaluated through a wound healing assay. In this procedure, treated cells from both types were plated in six-well culture dishes. After forming a dense monolayer through cell-cell contact, standardized linear wounds were generated using a 200 μl pipette tip. Following wound creation, all samples underwent continued incubation at 37°C with 5% CO₂. Photographic documentation occurred at baseline (0 h) and 24 h postscratch for HUVECs, while HMEC observations extended to 36 h. Quantitative analysis was performed using ImageJ software, with migration percentage calculated as $[(\text{initial wound area } (M_0) - \text{residual unhealed area } (M_1)) / M_0] \times 100\%$.

Tube formation assay

To evaluate the angiogenic potential of HUVECs and HMECs, an *in vitro* angiogenesis assay was performed. Pre-chilled 96-well plates were layered with 50 μl of Matrigel (Corning, USA) and polymerized at 37°C for 30 min. HUVECs or HMECs ($1-1.5 \times 10^4$ cells/well) from each group were then seeded onto the Matrigel-coated plates. After a 6-h incubation, microscopy was used to acquire images of lumen formation, and parameters such as total lumen fulcrum and lumen length were calculated using ImageJ software.

Western blot analysis

The experimental samples (comprising sEVs, HUVECs, and HMECs) were harvested and homogenized using Radio Immunoprecipitation Assay (RIPA) lysis solution (Solarbio, China) supplemented with protease and phosphatase inhibitors. Protein concentrations were assessed through Bicinchoninic Acid (BCA) protein assay (Solarbio, China). Aliquots containing 20 μg of protein were combined with sample buffer, separated via Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) electrophoresis, and followed by electrophoretic transfer onto polyvinylidene difluoride membranes (Millipore, USA). Following 1–2 h of blocking with 5% skimmed milk at room temperature, membranes were exposed to specific primary antibodies at 4°C overnight. After thorough washing with Tris-buffered saline Tween-20 buffer, membranes were treated with Horseradish Peroxidase (HRP)-conjugated secondary antibodies (1:2000, Zgb-bio, China) targeting mouse or rabbit IgG for 60 min at room temperature. Protein signals were ultimately detected using an Enhanced Chemiluminescence (ECL) detection system (Solarbio, China), with band intensity quantification performed through ImageJ software. Key

antibodies employed in this study targeted TSP-1, β -actin, p-AKT, AKT, p-eNOS, eNOS, calnexin, CD63, TSG101, and CD9 (11 000, CST, USA).

Engineering hucMSCs with lentivirus carrying miR-221-3p

The recombinant shuttle plasmid containing the miR-221-3p coding sequence, along with negative control (NC) and packaging plasmids, was procured from GenePharma (China). Subsequently, 293T cells were transduced using Lipofectamine 3000 transfection reagent to generate lentiviral particles carrying miR-221-3p (Lv-miR-221-3p) and NC (Lv-NC) (Figure S1, see online supplementary material). HucMSCs were transfected with red-stained Lv-miR-221-3p or Lv-NC to obtain miR-221-3p-overexpressing hucMSCs and control hucMSCs, respectively. Puromycin was subsequently used to select successfully transfected hucMSCs. The transfection rate was verified via fluorescence microscopy and flow cytometry.

Acquisition and identification of miR-221-3p-encapsulated mesenchymal stem cell-derived small extracellular vesicles

sEVs were isolated from the culture medium of the miR-221-3p-overexpressing hucMSCs and control hucMSCs via ultracentrifugation, based on protocols used in previous studies [19, 41]. Upon reaching 80%–90% confluency, the conditioned medium underwent initial clarification via centrifugation at 400×g (6 min) and 2100×g (25 min) to eliminate cellular debris [42]. Subsequent filtration through 0.22 μ m membranes removed residual contaminants before processing through two cycles of ultracentrifugation (100 000×g, 75 min, 4°C). The harvested vesicles were either immediately resuspended in Phosphate Buffered Saline (PBS) for experimental use or preserved at –80°C. Vesicle characterization included nanoparticle tracking analyzer (NTA) for size profiling and transmission electron microscopy (TEM) for morphological assessment, complemented by western blot analysis detecting sEV markers (TSG101, CD9, CD63) and excluding calnexin expression as a negative control.

DiI kit for the staining and cellular internalization assay

The DiI labeling kit (YEASEN, China) was employed to stain sEVs, following the manufacturer's guidelines. sEVs were exposed to a 5 μ M DiI solution and maintained at room temperature for 20 min. Following centrifugation at 100 000×g for 75 min, the labeled sEVs were suspended in PBS. HUVECs were cocubated with DiI-stained sEVs for ~6 h. Afterward, the HUVECs were fixed with 4% paraformaldehyde, washed with PBS, and stained with phalloidin (5 μ g/ml; YEASEN, China) for 1 h and Hoechst 33342 (Beyotime, China) for 10 min at room temperature. Cellular images were subsequently obtained using confocal microscopy.

RNA isolation and quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated from NC-sEVs and miR-221^{OE}-sEVs using the SeraMir Exosome RNA Purification system, following the manufacturer's protocols (System Biosciences, USA). Reverse transcription was performed with the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems™, USA). Quantitative real-time PCR analysis was conducted on

the ABI PRISM™ 7900HT platform employing SYBR Premix ExTaq™ II reagents (Takara Biotechnology, Japan). For precise miRNA quantification, stem-loop-based quantitative reverse-transcription PCR methodology was implemented. Experimental data were analyzed through the $2^{-\Delta\Delta CT}$ calculation method, with U6 small nuclear RNA serving as the endogenous control for both EVs and cellular miRNA measurements. All miRNA-specific primers were custom-designed and manufactured by Sangon Biotech (China), with detailed sequences provided in [Supplementary Table S1](#).

RNA protection assay

Based on our previous study [31], miR-221^{OE}-sEVs (1×10^{11} particles) were cocubated with 20 μ l RNase A/T1 solution (Thermo, USA) at 37°C for 30 min with or without 1% Triton X-100. All of the RNAs were extracted via a SeraMir Exosome RNA Purification kit (System Biosciences, USA).

Fabrication and characterization of the GelMA hydrogel and G-miR-221^{OE}-sEVs

The GelMA monomer and lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP) (Engineering for Life, China) were prepared as previously reported [19, 43]. Briefly, the GelMA monomer at a concentration of 15% was blended with the LAP initiator. This homogeneous mixture underwent sterile filtration through 0.22 μ m membranes before ultraviolet (UV)-induced crosslinking (405 nm wavelength, 60-s exposure). Post-crosslinking specimens underwent cryopreservation at –80°C followed by lyophilization. For microscopic analysis, freeze-dried samples were mounted on conductive adhesive substrates, sputter-coated with gold nanoparticles (60-s deposition), and imaged using field-emission scanning electron microscopy (ZEISS SEM, Germany) with secondary electron detection mode.

Tissue adhesion assays

Adhesion assessments under *ex vivo* conditions were conducted utilizing porcine skin specimens to evaluate the binding capacity of G-miR-221^{OE}-sEVs, as previously reported [19, 44]. Freshly obtained porcine tissue was sectioned into rectangular segments (50 × 15 mm) and prepared through three sequential cleansing cycles employing 1 M sodium hydroxide solution, followed by deionized water rinsing to eliminate residual adipose material. A composite formulation containing GelMA precursor polymers, miR-221^{OE}-sEV suspension, and blue food-grade colorant (Sugarman, China) was applied uniformly onto the epidermal surface. Photopolymerization was induced through ultraviolet irradiation to achieve *in situ* hydrogel formation. Adhesion performance was subsequently evaluated through mechanical deformation testing simulating physiological wet environments and dynamic movement patterns.

Animal experiments

All animal studies were performed in compliance with the ethical guidelines established by the PLA General Hospital's Institutional Animal Care Committee. Eight-week-old male diabetic mice (BKS Dock Leprem2Cd479, Db/Db) were purchased from ViewSolid Biotech Co., Ltd, and housed in a standard laboratory animal feeding environment. After achieving anesthesia through intraperitoneal injection of 1% pentobarbital, bilateral full-thickness excisional wounds (1 cm diameter) were surgically created on the dorsal surface. The mice were subsequently randomly divided into five

groups: [1] the PBS group, [2] the GelMA (Gel) group, [3] the NC-sEVs group, [4] the miR-221^{OE}-sEVs group, and [5] the G-miR-221^{OE}-sEVs group. In groups [3] and [4], a solution containing 80 μ l of sEVs or miR-221^{OE}-sEVs (1×10^{11} particles/ml) was injected at four points surrounding the wound site (20 μ l per site). In groups [2] and [5], a solution containing 80 μ l of either GelMA or G-miR-221^{OE}-sEVs was applied to the wounds on the day of surgery and crosslinked via UV radiation for at least 1 min. Wound progression was documented through standardized photography and tissue collection on postoperative Days 0, 4, 8, and 12. Quantitative analysis of epithelialization was performed using ImageJ software, with wound closure percentage determined with the equation: $(W_0 - W_T)/W_0 \times 100\%$, where W_0 represents the initial wound area, and W_T denotes the specific time point at which the wound area was measured. *In vivo* retention of therapeutic agents was monitored using a PerkinElmer IVIS Spectrum imaging system to quantify DiI-labeled miR-221^{OE}-sEVs fluorescence intensity on Days 1, 3, and 5 postadministration. Angiogenesis assessment was conducted on day 12 specimens through stereomicroscopic examination (Leica M205 FA), with neovascular density calculated as the percentage of vascularized area relative to total wound bed area. Comparative analysis of relative vascularization indices was performed across all experimental groups.

Histological analysis and immunohistochemistry

At specified intervals, wound specimens were collected and immersion-fixed in 4% paraformaldehyde solution. The fixed tissues underwent graded ethanol dehydration before being embedded in paraffin blocks and sliced into 5 μ m cross-sectional preparations. Histological evaluation was performed through hematoxylin–eosin (HE) staining and Masson's trichrome protocols, with wound dimensions and collagen deposition levels assessed by applying previously reported methods [19]. Following fixation, CD31 immunohistochemical analysis (Proteintech, China; 1:1000 dilution) was conducted to visualize vascular endothelial cells on postoperative day 8.

In vivo blood perfusion examination

On Day 12, blood perfusion around the wound was assessed using laser speckle contrast imaging (LSCI) following our previously reported method [19]. Images were captured at a consistent distance from the wound with identical scanning site dimensions. Flux images were obtained for each wound using PIMSoft (Moor Instruments Ltd, UK), and the mean perfusion units (MPUs) were quantified by comparing the MPU values detected at the wound site and in its vicinity.

In vivo biodegradability assay

A biodegradability assay of the GelMA hydrogel was conducted as previously reported [45]. Db/db mice were randomly assigned to two groups: GelMA control and G-miR-221^{OE}-sEVs treatment cohorts, and treated as previously reported in the [Animal Experiments](#) section. During surgical intervention, wounds received 50 μ l of fluorescently labeled hydrogel complexes containing Alexa Fluor 750 CS-maleimide (Invitrogen, A30459), with subsequent UV irradiation applied for a minimum of 60 s to crosslink the composite. To examine the biodegradability of the hydrogel, a Perkin Elmer IVIS Spectrum system was used to detect the fluorescence intensity on Days 0, 4, 8, 12, 16, and 17.

Biocompatibility assay *in vivo*

The long-term stability of the GelMA hydrogel *in vivo* was evaluated as previously reported [46, 47]. Experimental Db/db mice cohorts were allocated randomly into three treatment arms: PBS control, GelMA, and G-miR-221^{OE}-sEVs composite with administration methods as previously reported in the [Animal Experiments](#) section. After 32 days, comprehensive biological evaluations were conducted through systematic blood collection for complete hematological profiling and serum chemistry analysis. Concurrently, vital organs including cardiac, hepatic, splenic, pulmonary, and renal tissues were surgically excised for histopathological examination. Excised specimens underwent fixation in paraformaldehyde solution before sequential processing through ethanol dehydration and paraffin embedding. Tissue blocks were microtomed into 5 μ m sections for subsequent histopathological examination via H&E staining.

Statistical analysis

Experimental results are expressed as mean values $\pm$ standard deviations (SDs) derived from three or more independent trials. GraphPad Prism 10.0 software was employed for conducting statistical computations and generating visual data representations. Comparative analyses between two experimental groups utilized two-tailed Student's *t*-tests, while multi-group comparisons involved either one-way or two-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test. A probability threshold of $P < .05$ served as the benchmark for determining statistical significance, with specific sample sizes (*n*) detailed in respective figure captions. The graphical annotation system employs the following conventions: ns indicates nonsignificant findings ($P \geq .05$), single asterisk (*) represents *P*-values between 0.01 and 0.05, double asterisks (**) correspond to 0.001–0.01, triple asterisks (***) denote 0.0001–0.001, and quadruple asterisks (****) signify *P*-values below .0001.

Results

Inhibition of TSP-1 expression improves the proliferation, migration, and tube formation ability of human umbilical vein endothelial cells in high-glucose conditions

Earlier research indicated that HG conditions led to increased TSP-1 expression in vascular endothelial cells [48, 49]. Western blot analysis was conducted to explore how HG affects TSP-1 expression in HUVECs. To assess whether modulating TSP-1 could enhance HUVEC functions under HG conditions, we used TSP-1 siRNA (SiTSP-1) to suppress its expression. [Figure 1a](#) and [b](#) demonstrated that HG conditions significantly elevated TSP-1 expression in HUVECs, while SiTSP-1 treatment effectively reversed this effect, suggesting its role in reducing TSP-1 levels under such conditions.

Following this, we examined how TSP-1 inhibition impacts HUVEC function under HG conditions *in vitro*. The impact of TSP-1 knockdown on the proliferation capacity of HUVECs was evaluated through EdU and CCK-8 assays. The EdU assay results revealed that the HG group had a reduced percentage of EdU-positive cells (green fluorescence), but this increased when SiTSP-1 was added to suppress TSP-1 expression ([Figure 1c](#) and [d](#)). [Figure 1e](#) shows that the Optical Density(OD) 450 nm value in the HG group was markedly

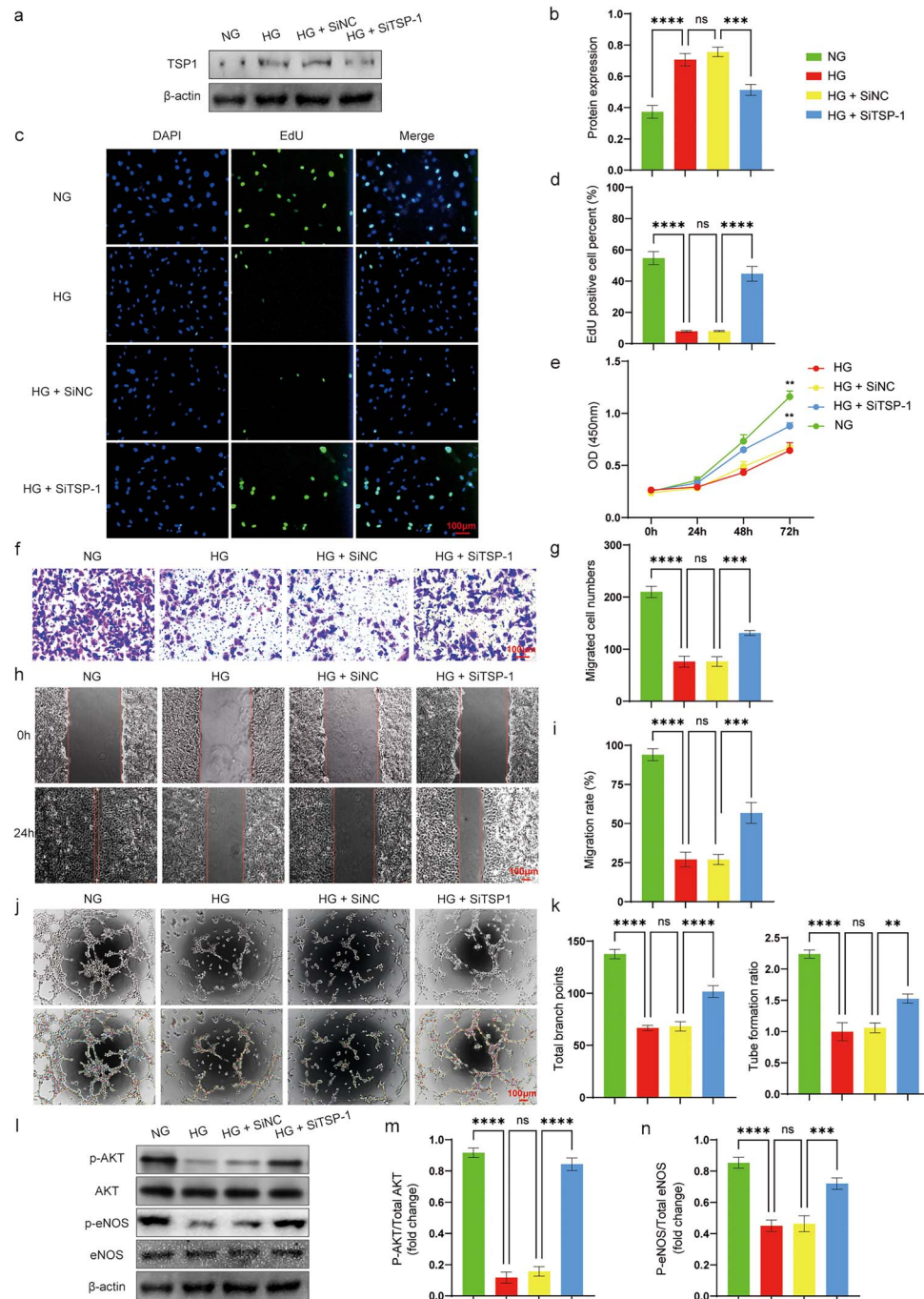


Figure 1. Inhibition of TSP-1 expression improves the proliferation, migration, and tube formation ability of HUVECs under HG conditions. **(a, b)** Western blot analysis **(a)** of the protein expression and corresponding quantification results **(b)** of TSP-1 inside HUVECs treated with NG, HG, HG + SiNC, and HG + SiTSP-1. Protein expression levels were normalized to β -actin levels ($n=3$ per group). **(c)** Fluorescence images showing proliferating HUVECs in different treatment groups (green: EdU staining; blue: DAPI staining) ($n=3$ per group, scale bar = 100 μ m). **(d)** Statistical analysis of a portion of EdU-positive cells (%) in different treatment groups. **(e)** CCK-8 assay revealing the proliferation ability of HUVECs in different treatment groups ($n=3$ per group). **(f)** Representative images of transwell migration assay of HUVECs in different groups ($n=3$ per group, scale bar = 100 μ m). **(g)** Statistical analysis of a migrated number of HUVECs in the transwell assay. **(h)** Representative images of the wound closure of HUVECs in different groups by cell scratch assay at 24 h ($n=3$ per group, scale bar = 100 μ m). **(i)** Statistical analysis of migration rate (%) in scratch assay. **(j)** Tube formation of HUVECs with different treatments ($n=3$ per group, scale bar = 100 μ m). **(k)** Statistical analysis of total branch points and tube formation ratio representing tube formation ability. **(l–n)** Western blot analysis **(l)** of the protein expression and corresponding quantification results **(m, n)** of p-AKT, AKT, p-eNOS, and eNOS inside HUVECs treated with NG, HG, HG + SiNC, and HG + SiTSP-1. Protein expression levels were normalized to β -actin levels ($n=3$ per group). The data are presented as the mean $\pm$ SD. Differences among the groups were examined with one or two-way ANOVA with Tukey's posttest. *** $P < 0.001$, **** $P < 0.0001$, ns, not significant versus the indicated group. NG normal glucose, HG high glucose, TSP-1 thrombospondin-1, AKT protein kinase B, eNOS endothelial nitric oxide synthase, p-AKT phosphorylated protein kinase B, p-eNOS phosphorylated endothelial nitric oxide synthase, DAPI 4',6-diamidino-2-phenylindole, EdU 5-ethynyl-20-deoxyuridine

lower than in the NG group after 72 h, but it increased again following TSP-1 inhibition. The findings indicated that suppressing TSP-1 expression might enhance the proliferation capacity of HUVECs under HG conditions.

Then, the effect of TSP-1 inhibition on HUVEC migration ability under HG conditions was investigated *in vitro* using transwell and scratch assays. Figure 1f and g reveals substantially fewer purple-stained migratory cells (purple stained) under HG conditions relative to normoglycemic controls, which was reversed upon addition of SiTSP-1 to inhibit TSP-1 expression. In line with that, the scratch test revealed that the HG group exhibited marked impairment in wound closure rates during a 24-h culture period compared to the NG group—a deficit substantially mitigated through genetic silencing of TSP-1 expression (Figure 1h and i). These findings suggested that inhibiting TSP-1 expression can enhance HUVEC migration ability under HG conditions *in vitro*. Consistently, Vascular network formation assays further corroborated these findings, with hyperglycemic conditions producing the most compromised tube formation capacity that was substantially improved through TSP-1 suppression, as evidenced by increased branch point quantification and enhanced tubular network density measurements (Figure 1j and k). These results confirmed that targeted TSP-1 inhibition exerted a restorative effect on endothelial angiogenic potential under hyperglycemic stress.

To elucidate the mechanism underlying the functional improvements in HUVECs under HG conditions following TSP-1 inhibition, we focused on the TSP-1/AKT/eNOS signaling pathway. Prior studies have established that TSP-1 suppresses AKT phosphorylation via CD47 binding, thereby indirectly inhibiting eNOS activation—a critical step in endothelial nitric oxide production and angiogenesis [50, 51]. Consistent with this pathway, our western blot data revealed that HG conditions significantly reduced p-AKT/AKT and p-eNOS/eNOS ratios (Figure 1l–n), reflecting impaired AKT/eNOS signaling. Genetic silencing of TSP-1 (siTSP-1) reversed these effects, restoring p-AKT and p-eNOS levels remarkably. These findings aligned with our hypothesis that TSP-1 acted as an upstream suppressor of AKT activity under HG, and its removal relieved this inhibition, enabling AKT-dependent phosphorylation of eNOS.

Targeting TSP-1 by miR-221-3p activates AKT/eNOS pathway and ameliorates the impaired functions of human umbilical vein endothelial cells in high-glucose conditions

MiRNAs are naturally occurring noncoding RNA molecules in the body, which could negatively regulate target genes by direct combination with them [52]. We employed TargetScanHuman8.0 (https://www.targetscan.org/vert_80/) and the miRDB database (<https://mirdb.org/>) to predict possible upstream miRNAs of TSP-1 and found that miR-221-3p could be the candidate miRNA to silence the gene expression (Figure 2a and b) [53, 54]. Previous studies have verified that miR-221-3p could directly bind with TSP-1 in kidney failure-induced cardiac fibrosis [55] and corpus luteum regression [56]. Hence, we fabricated miR-221-3p to examine the effects on TSP-1 in HUVECs while miR-NC is in control. Western blot analysis conducted 48 h post-transfection demonstrated significant downregulation of TSP-1 protein level in miR-221-3p-treated cells compared to the HG group. Concurrently,

we observed enhanced phosphorylation ratios of both AKT (p-AKT/AKT) and eNOS (p-eNOS/eNOS) in the intervention group, indicating activation of this crucial signaling cascade (Figure 2c–f). These experimental outcomes mirror the effects previously observed with siTSP-1 treatment, suggesting that miR-221-3p exerts its regulatory influence through analogous molecular mechanisms targeting TSP-1 expression.

Subsequent investigations focused on evaluating miR-221-3p's influence on HUVEC functionality under hyperglycemic environments. Quantitative analysis revealed a notable enhancement in EdU-positive cell proportions after miR-221-3p administration during HG exposure (Figure 2g and h). Scratch assays in Figure 2i and j showed a markedly higher migration rate following the administration of miR-221-3p. Angiogenic potential evaluation showed improved vascular network formation metrics, including increased total branching points and elevated tube formation ratio in the HG + miR-221-3p cohort (Figure 2k and l). These observations collectively indicate miR-221-3p's therapeutic potential in counteracting HG-induced endothelial dysfunction through TSP-1 modulation. Conclusively, the sequential cascade—TSP-1 downregulation → p-AKT upregulation → p-eNOS activation—validates the proposed mechanistic cascade (Figure 2c–f) and corroborates the known role of AKT/eNOS signaling in endothelial functional recovery [57].

miR-221^{OE}-sEVs are engineered and characterized

Given the inherent vulnerability of miRNAs to enzymatic breakdown and chemical instability *in vivo* [58], we employed sEVs derived from MSCs as a protective coating to maintain the bioactivity of miR-221-3p. Lentiviral vectors containing miR-221-3p or miR-NC sequences were transfected into 293T cells to generate a substantial quantity of lentivirus particles, which were subsequently transfected into MSCs. Following a designated time period, the supernatant was collected for the isolation of engineered and miR-221^{OE}-sEVs and NC-sEVs (Figure 3a). The representative images of transfecting MSCs in different treatment groups are shown in Figure 3b, and the flow cytometry demonstrated a relatively high transfection rate (89.3% for MSC^{221OE} and 82.7% for MSC^{NC}, Figure 3c). The quantitative real-time polymerase chain reaction (qRT-PCR) revealed a 55-fold elevation in miR-221-3p expression within MSC^{221OE} compared to MSC^{NC} (Figure 3d). The morphological characteristics and size distributions of NC-sEVs and miR-221^{OE}-sEVs were assessed using TEM and NTA techniques. No significant differences were observed in the morphology of the two types, which both exhibited a saucer-like shape and a double membrane structure (Figure 3e). NTA results indicated no significant differences in average particle size, main particle size peak, or final concentration between NC-sEVs and miR-221^{OE}-sEVs. The average particle sizes of NC-sEVs and miR-221^{OE}-sEVs were measured to be 119.8 and 116.6, nm respectively. The main particle size peaks corresponded to sizes of ~96.6 and 97.7 nm, respectively. The final concentrations were determined as follows: for sEVs is 4.5×10^{11} particles/ml and for miR-221^{OE}-sEVs is 2.5×10^{11} particles/ml (Figure 3f). Western blot analysis confirmed the conserved exosomal markers (CD9, CD63, TSG101) in NC-sEVs and miR-221^{OE}-sEVs, whereas calnexin was exclusively expressed in MSCs but not detected in sEVs (Figure 3g).

The qRT-PCR was performed to assess miR-221-3p levels in both NC-sEVs and engineered miR-221^{OE}-sEVs. The

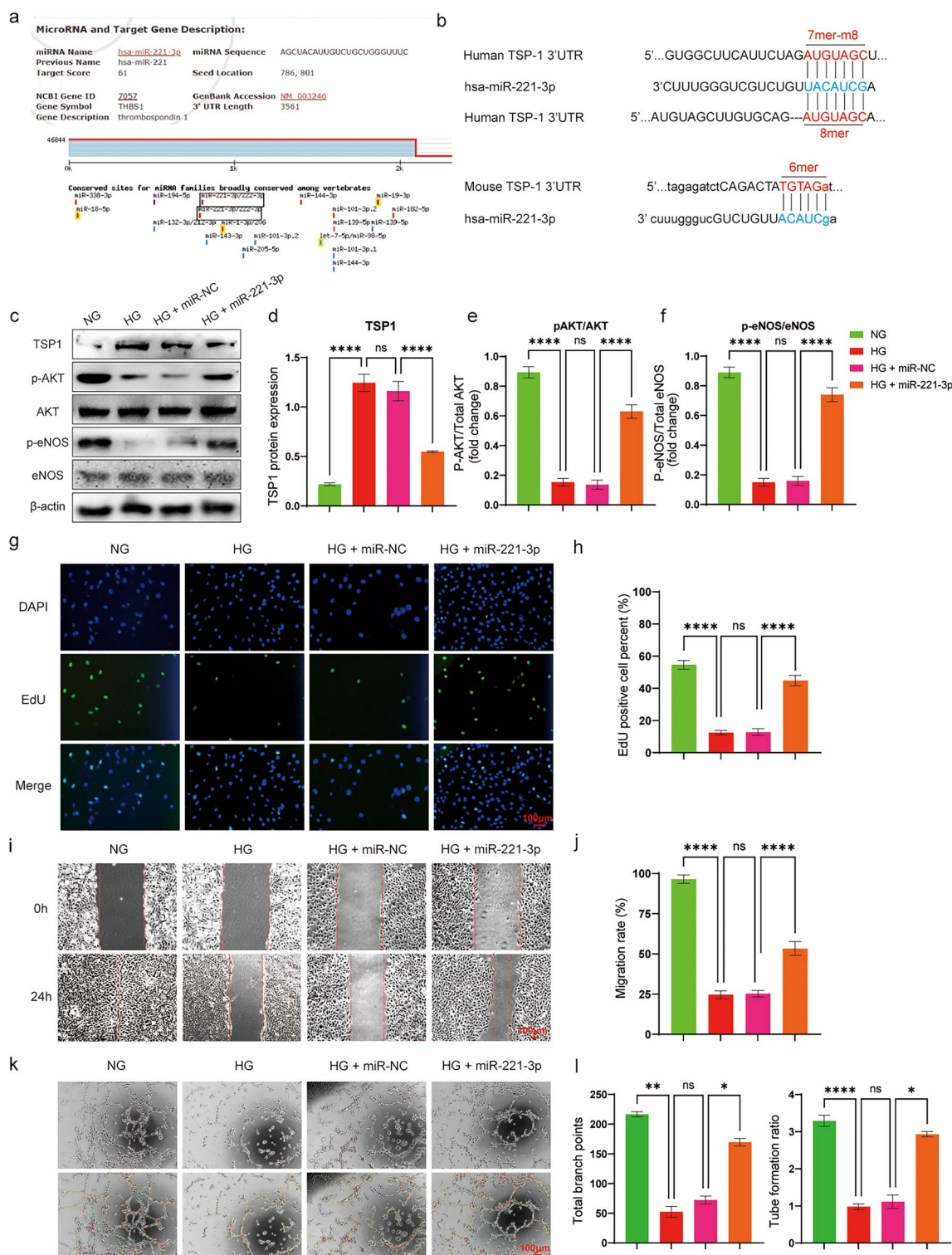


Figure 2. Targeting TSP-1 by miR-221-3p activates AKT/eNOS pathway and ameliorates the impaired functions of HUVECs in HG conditions. **(a)** Confirmation that miR-221-3p could silence the expression of human and mouse TSP-1 by binding their mRNA. **(b)** The binding sites of miR-221-3p on human and mouse TSP-1 mRNA. **(c-f)** Western blot analysis (c) and the corresponding quantification results (d-f) of the TSP-1, p-AKT, AKT, p-eNOS, and eNOS inside HUVECs treated with NG, HG, HG + miR-NC, and HG + miR-221-3p. Protein expression levels were normalized to β -actin levels ($n = 3$ per group). **(g)** Fluorescence images showing proliferating HUVECs treated with NG, HG, HG + miR-NC, and HG + miR-221-3p (green: EdU staining; blue: DAPI staining) ($n = 3$ per group, scale bar = $100 \mu\text{m}$). **(h)** Statistical analysis of a portion of EdU-positive cells (%) in different treatment groups. **(i)** Representative images of the wound closure of HUVECs in different groups by cell scratch assay at 24 h ($n = 3$ per group, scale bar = $100 \mu\text{m}$). **(j)** Statistical analysis of migration rate (%) in scratch assay. **(k)** Tube formation of HUVECs with different treatments ($n = 3$ per group, scale bar = $100 \mu\text{m}$). **(l)** Statistical analysis of total branch points and tube formation ratio representing tube formation ability. The data are presented as the mean $\pm$ SD. Differences among the groups were examined with one-way ANOVA with Tukey's posttest. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant versus the indicated group. NG normal glucose, HG high glucose, TSP-1 thrombospondin-1, AKT protein kinase B, eNOS endothelial nitric oxide synthase, p-AKT phosphorylated protein kinase B, p-eNOS phosphorylated endothelial nitric oxide synthase, DAPI 4',6-diamidino-2-phenylindole, EdU 5-ethynyl-20-deoxyuridine

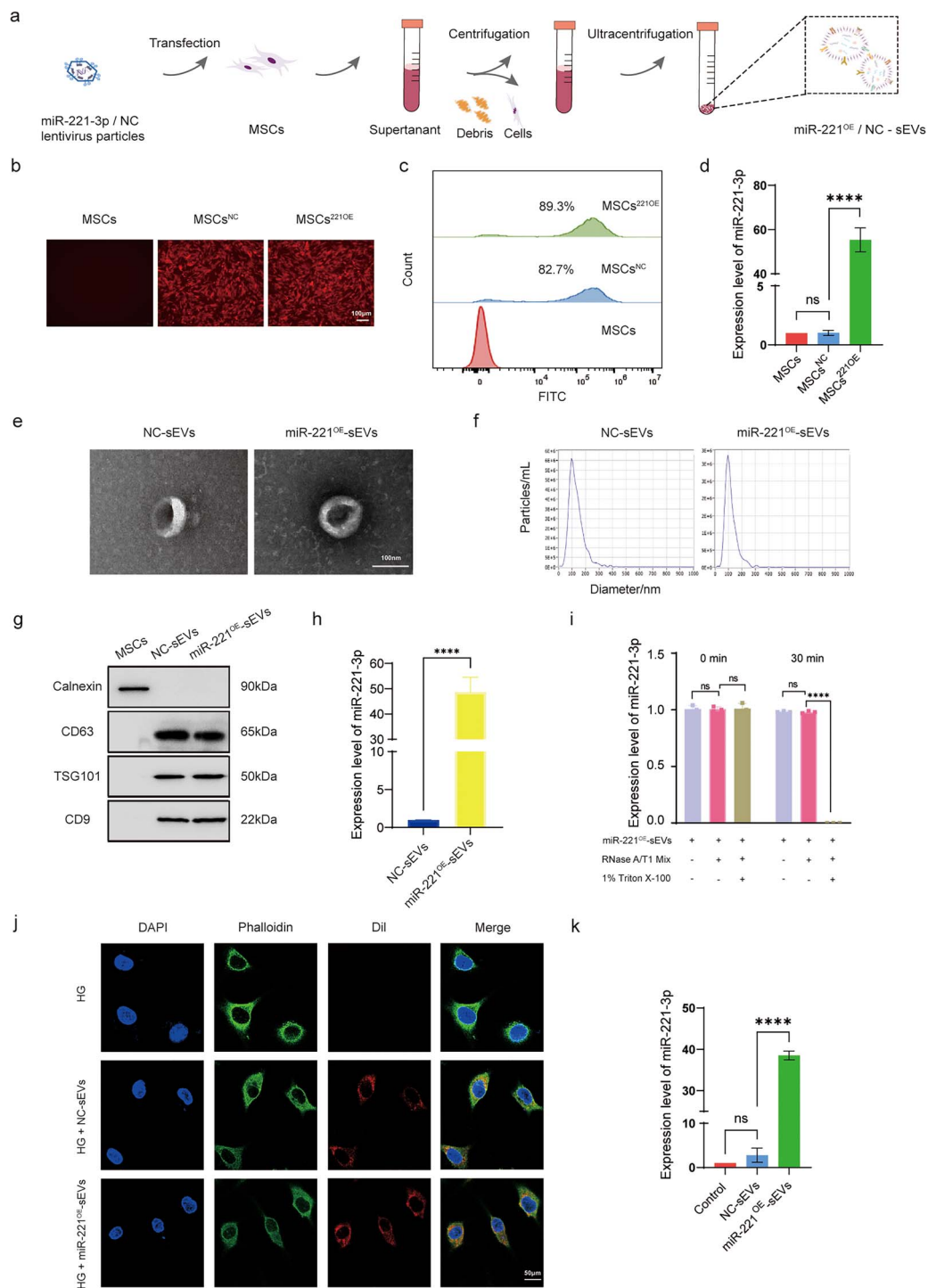


Figure 3. miR-221^{OE}-sEVs are engineered and characterized. **(a)** Schematic diagram illustrating the preparation process of NC-sEVs and miR-221^{OE}-sEVs. **(b)** Representative images of lentivirus transfection of hucMSCs in different treatment groups ($n=3$ per group, scale bar = 100 μ m). **(c)** Flow cytometry analysis of the transfection rate in hucMSCs ($n=3$ per group). **(d)** qRT-PCR analysis showing the relative expression level of miR-221-3p in MSC, MSC^{NC}, and MSC^{221OE} groups ($n=3$ per group). **(e)** Typical TEM images of NC-sEVs and miR-221^{OE}-sEVs (scale bar = 100 nm). **(f)** Particle size distribution of NC-sEVs and miR-221^{OE}-sEVs detected by NTA. **(g)** Western blot analysis showing protein expressions of TSG101, HSP70, CD9, and calnexin in MSCs, NC-sEVs, and miR-221^{OE}-sEVs. **(h)** qRT-PCR analysis showing the relative expression level of miR-221-3p in NC-sEVs and miR-221^{OE}-sEVs ($n=3$ per group). **(i)** qRT-PCR analysis of miR-221-3p abundance in miR-221^{OE}-sEVs after treatments with RNase A/T1 Mix and 1% Triton X-100 for 30 min ($n=3$ per group). **(j)** Respective internalization of NC-sEVs and miR-221^{OE}-sEVs by HUVECs under HG conditions, HG group as control (scale bar = 50 μ m). **(k)** qRT-PCR analysis showing the relative expression level of miR-221-3p in HUVECs from control, NC-sEV cocultured, and miR-221^{OE}-sEV cocultured groups, respectively ($n=3$ per group). The data are presented as the mean $\pm$ SD. Differences among the groups were examined with one-way ANOVA via Tukey's posttest. **** $P < 0.0001$, **** $P < 0.0001$, ns, not significant versus the indicated group. MSCs mesenchymal stem cells, MSCs^{NC} negative control mesenchymal stem cells, MSCs^{221OE} mesenchymal stem cells overexpressing miR-221-3p, miR-221^{OE}-sEVs small extracellular vesicles loaded with miR-221-3p, NC-sEVs negative control small extracellular vesicles, DAPI 4',6-diamidino-2-phenylindole, TSG101 Tumor susceptibility gene 101, CD63 lysosome-associated membrane glycoprotein 3, CD9 tetraspanin 29

experimental data revealed a striking 28-fold elevation in miR-221-3p concentration within miR-221^{OE}-sEVs relative to control vesicles, demonstrating effective incorporation of the miR-221-3p into sEVs (Figure 3h). Moreover, treatment of miR-221^{OE}-sEVs with the RNase A/T1 mixture alone proved ineffective in reducing the level of miR-221-3p, whereas supplementation with 1% Triton X-100 to disrupt vesicular membranes resulted in a marked reduction in miR-221-3p content within sEVs after 30 min. This verified the protective role of sEVs bilayer structure against their cargo (Figure 3i). All these above results indicated the successful construction of engineered miR-221-3p-enriched sEVs.

Internalization by recipient cells is one of the prerequisites for sEVs to exert their biological effects. As soon as they reach target cells, sEVs could be internalized via immediate fusion, receptor-mediated endocytosis, macropinocytosis, and phagocytosis [59–61]. The sEVs labeled with Dil (red fluorescence) were incubated for 5–6 h with the DAPI-stained nuclei (blue fluorescence) of HUVECs. Then, sEVs (red) were observed closely surrounding the nucleus (Figure 3j). The results of qRT-PCR analysis further confirmed substantial elevation of miR-221-3p expression levels in HUVECs exposed to miR-221^{OE}-sEVs, indicating successful intracellular transfer of miR-221-3p into HUVECs (Figure 3k).

miR-221^{OE}-sEVs optimize the proliferation, migration, and angiogenic abilities of human umbilical vein endothelial cells under high-glucose conditions

We have verified that miR-221-3p could abrogate TSP-1 expression, hence exerting proregenerative effects in HUVECs under HG conditions. Subsequent investigations focused on evaluating miR-221^{OE}-sEVs' influence on cellular proliferation, migratory capacity, and angiogenic potential in both HUVECs and HMECs following successful vesicle preparation. Quantitative analysis revealed significantly higher EdU-positive cell percentages in NC-sEV- and miR-221^{OE}-sEV-treated groups compared to HG controls, with miR-221^{OE}-sEVs demonstrating superior enhancement (Figure 4a and b). Parallel CCK-8 assay results demonstrated increased OD450nm values at 72-h incubation in both vesicle-treated groups relative to HG conditions, particularly accentuated in miR-221^{OE}-sEV samples (Figures 4c and S2a, see online supplementary material). Altogether, these findings strongly indicated that both NC-sEVs and miR-221^{OE}-sEVs possessed proliferative-enhancing properties for HUVECs and HMECs, with a superior efficacy demonstrated by miR-221^{OE}-sEVs.

To evaluate the effects of miR-221^{OE}-sEVs on HUVEC and HMEC migration, transwell chambers and scratch wound assays were performed. Both NC-sEVs and miR-221^{OE}-sEVs treatments notably increased migratory cell counts compared to the HG group, with miR-221^{OE}-sEV group demonstrating superior enhancement (Figures 4d and e and S2b and c, see online supplementary material). Moreover, migration rates were higher in both the NC-sEV and miR-221^{OE}-sEV groups than that of the HG group, with a more notable enhancement observed in the miR-221^{OE}-sEV group (Figures 4f and g and S2d and e, see online supplementary material). Hence, both NC-sEVs and miR-221^{OE}-sEVs exhibited an ability to enhance HUVEC and HMEC migration, with a more significant effect observed in miR-221^{OE}-sEVs. The results of

tube formation assay corroborated these findings by revealing maximal total branch points and tube formation ratio in the miR-221^{OE}-sEVs-treated cells, followed sequentially by the NC-sEV and HG groups (Figures 4h and i and S2f–h, see online supplementary material). Collectively, it can be concluded that miR-221^{OE}-sEVs substantially improved HUVECs' and HMECs' proliferation, migration, and angiogenic ability *in vitro*.

Western blot analysis was conducted to evaluate TSP-1 expression dynamics and AKT/eNOS pathway modulation in HUVECs and HMECs under various treatments (Figures 4j–m and S2i–l, see online supplementary material). Relative to the HG group, NC-sEV and miR-221^{OE}-sEV treatments demonstrated substantial suppression of TSP-1 protein expression. Concurrently, these interventions enhanced AKT (p-AKT/AKT) and eNOS (p-eNOS/eNOS) phosphorylation levels, with maximal activation observed in miR-221^{OE}-sEV-treated cells. These findings indicated that miR-221^{OE}-sEVs suppressed TSP-1 expression most and robustly activated the AKT/eNOS pathway. Consistently, this inverse relationship between TSP-1 downregulation and AKT/eNOS activation aligns with our prior mechanistic work, further supporting the role of miR-221-3p in targeting TSP-1 to modulate endothelial signaling under HG conditions.

G-miR-221^{OE}-sEVs are fabricated, characterized, and administrated to accelerate diabetic wound healing in mice

To prolong retention at wound sites, miR-221^{OE}-sEVs were encapsulated within the biocompatible GelMA hydrogel using UV-induced photopolymerization (Figure 5a). The microscopic morphology of the GelMA hydrogel under SEM is shown in Figure 5b, the pore size was ~20.67 μm, similar with our previous studies [19]. As the laser scanning confocal microscopy (CLSM) image demonstrated in Figure 5c, successful encapsulation of miR-221^{OE}-sEVs (red) into GelMA hydrogel exhibited a homogenous and uniform distribution, validating the potential application in wound regeneration. Based on previous studies of our team, we chose GelMA hydrogel of a 15% monomer concentration as the optimal delivery platform of miR-221^{OE}-sEVs to fulfil the following assays on account of suitable pore size, mechanical tension, swelling ratio, and degradation time [19, 43]. Furthermore, miR-221^{OE}-sEVs released from GelMA after 3 days still had an intact morphology with no visibly detectable differences compared with the free miR-221^{OE}-sEVs (Figure 5d). The tissue-adhesive properties of the hydrogel were validated through rigorous mechanical testing on porcine skin substrates, demonstrating maintained adhesion under various stressors including torsion, elongation, and aqueous exposure (Figure S3, see online supplementary material). This bioadhesive characteristic creates a protective microenvironment that prevents microbial invasion and minimizes exudate leakage [62]. G-miR-221^{OE}-sEVs were proved to adhere well to porcine skins after stretching, twisting, bending, and water washing. To further investigate the controlled release of miR-221^{OE}-sEVs, we created two full-thickness wounds ~1 cm in diameter on either side of the dorsal region in one Db/Db mouse. One site was administered with G-miR-221^{OE}-sEVs, while the other received a four-point injection of free miR-221^{OE}-sEVs solution of an equivalent amount.

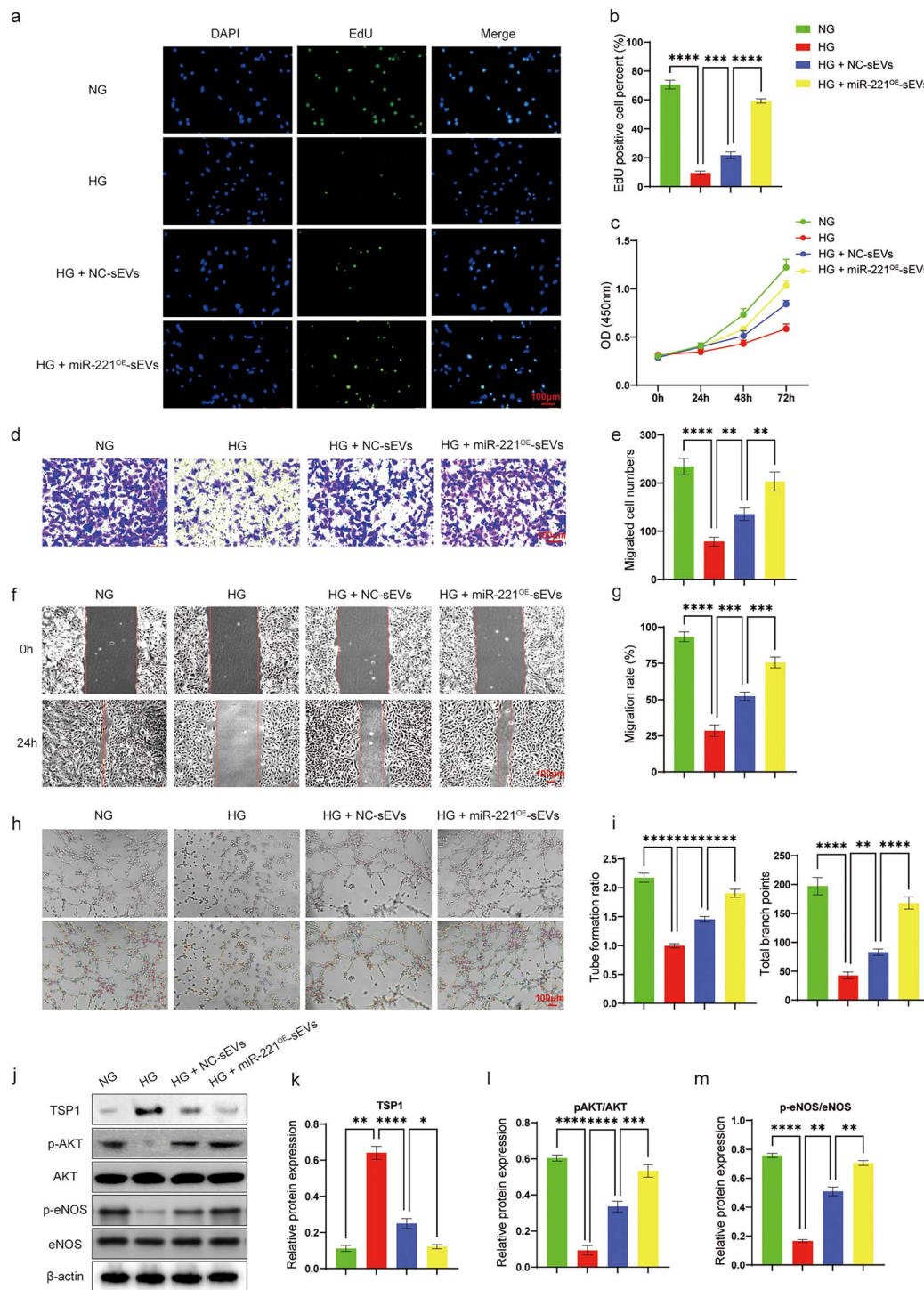


Figure 4. miR-221^{OE}-sEVs improve the proliferation, migration, and angiogenic capacity of HUVECs in HG conditions. **(a)** Fluorescence images showing proliferating HUVECs treated with NG, HG, HG + NC-sEVs, and HG + miR-221^{OE}-sEVs (green: EdU staining; blue: DAPI staining) ($n = 3$ per group, scale bar = 100 μm). **(b)** Statistical analysis of a portion of EdU-positive cells (%) in different treatment groups. **(c)** CCK-8 assay revealing the proliferation ability of HUVECs in different treatment groups ($n = 3$ per group). **(d)** Representative images of transwell migration assay of HUVECs in different groups ($n = 3$ per group, scale bar = 100 μm). **(e)** Statistical analysis of a migrated number of HUVECs in the transwell assay. **(f)** Representative images of the wound closure of HUVECs in different groups by cell scratch assay at 24 h ($n = 3$ per group, scale bar = 100 μm). **(g)** Statistical analysis of migration rate (%) in scratch assay. **(h)** Tube formation of HUVECs with different treatments ($n = 3$ per group, scale bar = 100 μm). **(i)** Statistical analysis of total branch points and tube formation ratio representing tube formation ability. **(j–m)** Western blot analysis (**j**) and the corresponding quantification results (**k–m**) of the TSP1, p-AKT, AKT, p-eNOS, and eNOS inside HUVECs treated with NG, HG, HG + NC-sEVs, and HG + miR-221^{OE}-sEVs. Protein expression levels were normalized to β-actin levels ($n = 3$ per group). The data are presented as the mean ± SD. Differences among the groups were examined with one or two-way ANOVA with Tukey's posttest. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant versus the indicated group. NG normal glucose, HG high glucose, TSP-1 thrombospondin-1, AKT protein kinase B, eNOS endothelial nitric oxide synthase, p-AKT phosphorylated protein kinase B, p-eNOS phosphorylated endothelial nitric oxide synthase, miR-221^{OE}-sEVs small extracellular vesicles loaded with miR-221-3p, NC-sEVs negative control small extracellular vesicles, DAPI 4',6-diamidino-2-phenylindole, EdU 5-ethynyl-20-deoxyuridine

Figure 5e and f illustrates that free miR-221^{OE}-sEVs dispersed randomly around the wound and were left a little after 5 days, while the fluorescent intensity of miR-221^{OE}-sEVs delivered from G-miR-221^{OE}-sEVs was significantly higher on Day 5, denoting a sustained localized retention in hydrogel-mediated delivery. The hydrogel biodegradability *in vivo* was evaluated by assessing the relative fluorescence intensity of the AlexaFluor750 C5-maleimide-dyed hydrogel from the Gel group and the G-miR-221^{OE}-sEV group (Figure S4a, see online supplementary material). The degradation rates of the two groups were approximately equivalent, with almost complete degradation by Day 17 (Figure S4b, see online supplementary material). Additionally, the CCK-8 assay demonstrated unaltered skin cell viability when exposed to each monomer or prepolymer solution of GelMA (Figure S5a–c, see online supplementary material). This indicated that no cytotoxicity was detected in skin cells, likely due to the rapid cross-linking process that limits the exposure time of LAP, as well as the minimal leachables after polymerization [63]. Furthermore, comprehensive hematological profiling and serum biochemistry analysis revealed no significant differences among treatment groups on Day 32 (Figure S6a–k, see online supplementary material). Meanwhile, H&E staining of major organs including the heart, liver, spleen, lung, and kidney of mice administered with G-miR-221^{OE}-sEVs showed no morphological abnormalities or inflammatory cell infiltration compared with those in the gel and control groups (Figure S6l, see online supplementary material). The evidence above confirms that G-miR-221^{OE}-sEVs have excellent biosafety, which is a necessary prerequisite for application.

Given the superiority in bioactivities of miR-221^{OE}-sEVs *in vitro* and the desirable controlled release of G-miR-221^{OE}-sEVs, we subsequently appraised the pro-healing effects in diabetic mice. Two full-thickness skin wounds (1 cm diameter approximately) were surgically established on murine dorsums. About an 80- μ l prepolymer solution of miR-221^{OE}-sEVs, GelMA monomers, and LAP was supplemented to the wound region and cross-linked under UV 405 nm light for a minute or so. On Days 0, 4, 8, and 12 post-treatment, representative macroscopic images of full-thickness wounds under different treatment conditions were obtained. (Figure 5g). The G-miR-221^{OE}-sEVs group exhibited the most rapid wound closure relative to other interventions, reaching ~90% healing rate on Day 12. It was followed by groups treated with miR-221^{OE}-sEVs, sEVs, and Gel, while the control group demonstrated the slowest healing response (Figure 5h–j). H&E staining revealed that the wounds in miR-221^{OE}-sEV and G-miR-221^{OE}-sEV groups exhibited regenerative skin appendages with nascent, well-aligned epidermis, as well as complete re-epithelialization. These two groups had the thickest epidermis, followed by the sEVs, Gel, and control groups (Figure 5k and l). Collagen deposition analysis via Masson's trichrome staining [31] showed enhanced extracellular matrix remodeling in both miR-221^{OE}-sEV groups, with G-miR-221^{OE}-sEVs achieving optimal collagen density (Figure 5m and n). Moreover, the G-miR-221^{OE}-sEV group demonstrated a more organized distribution of collagen and a more mature morphological structure with appendages similar to the normal skin.

G-miR-221^{OE}-sEVs accelerate diabetic wound healing via promoting angiogenesis

The neovascularization of the wound was detected by immunohistochemistry of CD31. The *de novo* vessel area

demonstrated remarkably higher CD31 expression in G-miR-221^{OE}-sEVs group, displaying the most pronounced vascularization degree, followed by the miR-221^{OE}-sEVs, sEVs, Gel, and control groups (Figure 6a and b). Subsequently, on Day 8, we assessed blood perfusion levels in wounds of Db/Db mice from various treatment groups using the LSCI system and found that the G-miR-221^{OE}-sEV group had the highest MPU ratio (Figure 6c and d), followed by the miR-221^{OE}-sEVs, sEV, Gel, and control groups, implying that the G-miR-221^{OE}-sEVs exerted a prominent pro-angiogenic effect. Additionally, neovascularization of wounds was observed through stereoscopic microscopy and revealed that the G-miR-221^{OE}-sEV group exhibited a larger vascularization area (Figure 6e and f), consistent with previous experimental findings. Therefore, it could be concluded that G-miR-221^{OE}-sEV treatment enhanced vascular regeneration mechanisms, thereby accelerating the healing process in diabetic wound pathology through optimized angiogenic activity (Figure 7).

Discussion

Our investigation revealed that hyperglycemia-induced endothelial dysfunction in HUVECs exhibited direct correlation with elevated TSP-1 levels. Targeted suppression of TSP-1 expression was shown to stimulate the AKT/eNOS signaling axis, subsequently improving vascular network formation capacity in these cells. We subsequently confirmed that miR-221-3p exerted similar proregenerative effects on HUVECs by suppressing TSP-1 expression. We then successfully generated engineered MSC-sEVs enriched with miR-221-3p and validated their proangiogenic effects on HUVECs under HG conditions. Finally, we incorporated miR-221^{OE}-sEVs into GelMA hydrogels and achieved remarkable efficacy in diabetic wound healing, with the composite biomaterial G-miR-221^{OE}-sEVs significantly accelerating wound closure through robust neovascularization.

Functional vascular endothelial cells are indispensable for physiological skin wound healing. The hyperglycemic microenvironment-induced impairment of endothelial cell activity represents a key contributor to impaired healing dynamics in diabetic wounds. The overexpression of TSP-1 in endothelial cells under HG conditions inhibits the AKT/eNOS pathway, which regulates cell proliferation and migration, whereas the inhibition of TSP-1 promotes endothelial cell growth and migration [50, 51]. Therefore, these findings provide new avenues and therapeutic targets for treating diabetic wounds. Current research indicates that multiple miRNAs participate in post-transcriptional regulation of TSP-1 expression through sequence-specific mRNA targeting and degradation. Experimental evidence demonstrates that miR-221 ameliorates renal failure-associated cardiac fibrosis by suppressing TSP-1-mediated latent TGF- β 1 activation in cardiac fibroblasts [55]. Moreover, miR-18a normalizes brain endothelial cell function and phenotype by targeting TSP-1, thus reversing brain arteriovenous malformations [64]. In addition, there are other methods available for regulating the expression of target genes, which include transcriptional control, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system, exogenous overexpression, epigenetic regulation, and translational control [65–67]. Here, our findings establish that TSP-1 suppression in endothelial cells activates AKT/eNOS signaling to enhance cellular proliferation and migration. Furthermore,

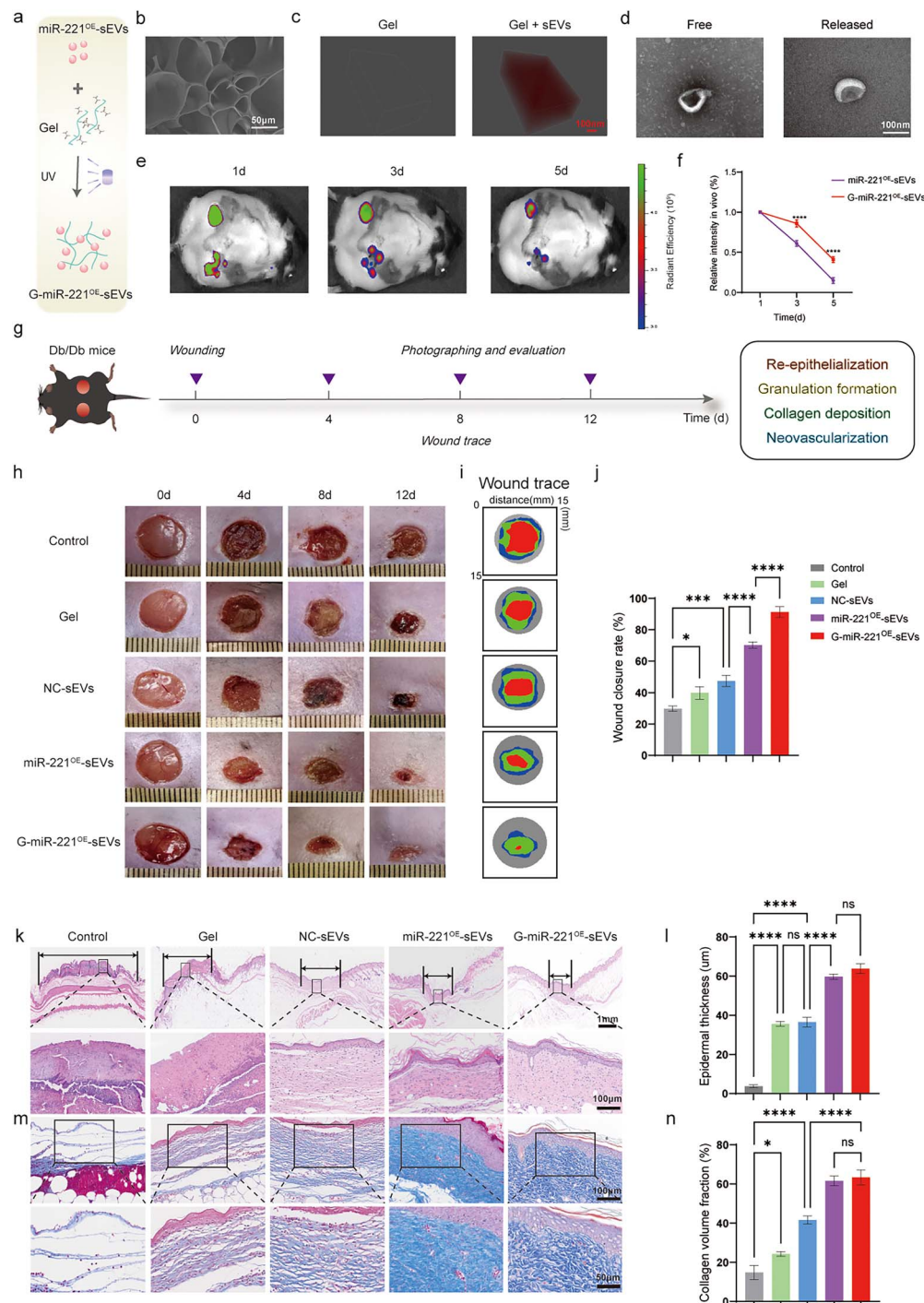


Figure 5. G-miR-221^{OE}-sEVs are fabricated and characterized. Application of G-miR-221^{OE}-sEVs accelerates diabetic wound healing in mice. **(a)** Schematic diagram depicting the preparation process of G-miR-221^{OE}-sEVs. **(b)** Representative cross-section SEM image of GelMA hydrogel (scale bar = 50 μ m). **(c)** CLSM images revealing the distribution of miR-221^{OE}-sEVs in GelMA hydrogel (scale bar = 100 nm). **(d)** Electron microscopic morphology of free miR-221^{OE}-sEVs and miR-221^{OE}-sEVs released from GelMA hydrogel (scale bar = 100 nm). **(e)** Typical *in vivo* imaging pictures reflecting retention of Dil-stained miR-221^{OE}-sEVs in free form and GelMA encapsulation form on wounds at the designed time point. **(f)** Quantitative results of released miR-221^{OE}-sEVs intensity *in vivo* of (e) ($n = 3$ per group). **(g)** Schematic diagram demonstrating the experimental procedure of full-thickness excisional wound healing study on diabetic mice. **(h)** Representative macroscopic images of wound healing in mice treated with control (PBS), GelMA hydrogel, NC-sEVs, miR-221^{OE}-sEVs, and G-miR-221^{OE}-sEVs. **(i)** Healing trace of wounds treated with different groups. **(j)** Quantification of respective wound areas on Day 4, Day 8, and Day 12 in mice with the above-mentioned treatments in relation to their corresponding initial size on Day 0 ($n = 6$ per group). **(k)** H&E staining evaluation of wound regeneration with relevant treatments on Day 12 showing morphometric analysis of newly formed epithelium and wound width ($n = 6$ per group, scale bar: 1 mm and 100 μ m for enlarged images). **(l)** Statistics of epidermis thickness in wounds with the above-mentioned treatments on Day 12. **(m, n)** Representative Masson's trichrome staining images of the wound region with the above-mentioned treatments and quantitative analysis of collagen volume fraction on Day 12 ($n = 6$ per group, scale bar: 100 and 50 μ m for enlarged images). The data are presented as the mean $\pm$ SD. Differences among the groups were examined with one- or two-way ANOVA with Tukey's posttest. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant versus the indicated group. UV ultraviolet, G-miR-221^{OE}-sEVs miR-221^{OE}-sEVs encapsulated GelMA hydrogel, miR-221^{OE}-sEVs small extracellular vesicles loaded with miR-221-3p, NC-sEVs negative control small extracellular vesicles, Gel gelatin methacryloyl.

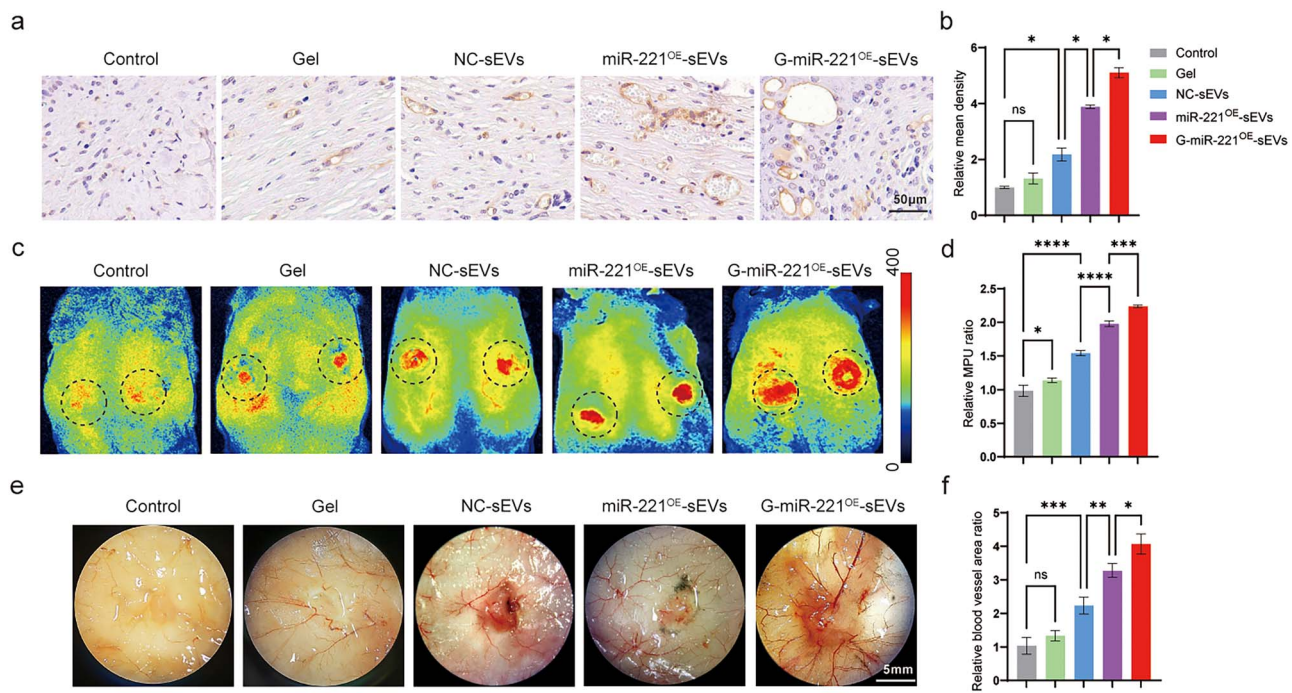


Figure 6. G-miR-221^{OE}-sEVs promote angiogenesis in the diabetic wound healing process. (a, b) Immunohistochemical images and the statistical results of CD31 at wound sites on Day 8 treated with PBS (control), GelMA hydrogel, NC-sEVs, miR-221^{OE}-sEVs, and G-miR-221^{OE}-sEVs to detect the vascular regeneration capacity of endothelial cells ($n=6$ per group, scale bar = 50 μm). (c) Blood flow perfusion of the wound area with a laser speckle contrast imaging (LSCI) system. (d) Statistical results of (c), $n=6$ per group. (e) The new blood vessels around the wound on Day 12 by the stereo microscope (scale bar = 5 mm). (f) The statistical results of (e), $n=6$ per group. The data are presented as the mean $\pm$ SD. Differences among the groups were examined with one-way ANOVA with Tukey's posttest. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant versus the indicated group. MPUs mean perfusion units, G-miR-221^{OE}-sEVs miR-221^{OE}-sEVs encapsulated GelMA hydrogel, miR-221^{OE}-sEVs small extracellular vesicles loaded with miR-221-3p, NC-sEVs negative control small extracellular vesicles, Gel gelatin methacryloyl

miR-221-3p exhibits potent TSP-1 inhibitory effects that stimulate pro-angiogenic signaling through AKT/eNOS pathway activation. However, miRNAs are inherently labile and prone to enzymatic degradation within the wound microenvironment. Hence, it is imperative to identify stable and safe vectors for the efficient delivery of miRNAs.

Overall, sEVs are considered to be a desirable choice for miRNA transportation because of their high stability, biocompatibility, and capacity to traverse diverse biological barriers [68–70]. Reportedly, miRNAs encapsulated in sEVs can remain stable and maintain >90% of their original levels even after 48 h of encapsulation, in contrast with free miRNAs [71]. This preservation capability ensures sufficient bioactive cargo reaches target tissues to modulate gene expression effectively. Additionally, sEVs derived from MSCs exhibit a distinctive proregenerative effect at the wound site via the regulation of inflammation, the facilitation of growth, and the promotion of re-epithelialization via the encapsulation of therapeutic miRNAs [31]. To optimize the inherent limitations of natural vesicle performance, we developed engineered MSC-sEVs through genetic modification of parent hucMSCs. Our methodology successfully generated miR-221-3p-enriched vesicles while preserving native sEV characteristics including structural integrity, size distribution, surface biomarkers, and cellular uptake efficiency. We subsequently evaluated the impact of miR-221^{OE}-sEV administration on HUVEC function. As anticipated, treatment with miR-221^{OE}-sEVs significantly augmented the proliferative, migratory, and angiogenic potential of HUVECs *in vitro*,

aligning with our therapeutic objectives for diabetic wound management.

To further increase the local administration time of miR-221^{OE}-sEVs, we incorporated these sEVs into a GelMA hydrogel, which exhibits promising potential in wound management applications because of its extracellular matrix-mimetic properties, favorable biocompatibility, adjustable morphology, and mechanical strength, as well as its ability to maintain a clean and moist wound bed [72–76]. The utilization of the GelMA hydrogel as a carrier for various drugs, cytokines, and engineered sEVs has demonstrated remarkable efficacy in the fields of diabetic wound healing [47], bone and cartilage regeneration [77, 78], and nerve tissue repair [79]. Building on our previous work, GelMA encapsulation extended the retention of miR-17-5p-enriched and VH298-loaded sEVs beyond 20 and 15 days respectively, thus accelerating the healing of diabetic wounds [19, 43]. The pore size, mechanical strength, swelling, and degradation rate characteristics of GelMA are principally determined by monomer concentration and methacrylation degree [73, 80]. Therefore, to extend the treatment duration of miR-221^{OE}-sEVs and based on our previous experiences, we encapsulated these sEVs within a GelMA hydrogel (15% monomer) to prepare uniformly embedded miR-221^{OE}-sEVs in GelMA. We verified that the incorporation of miR-221^{OE}-sEVs into GelMA achieved desirable sustained release behavior. Moreover, comparative therapeutic evaluation against PBS, blank GelMA, native sEVs, and free miR-221^{OE}-sEVs demonstrated that G-miR-221^{OE}-sEVs accelerated wound healing by promoting neovascularization. These findings

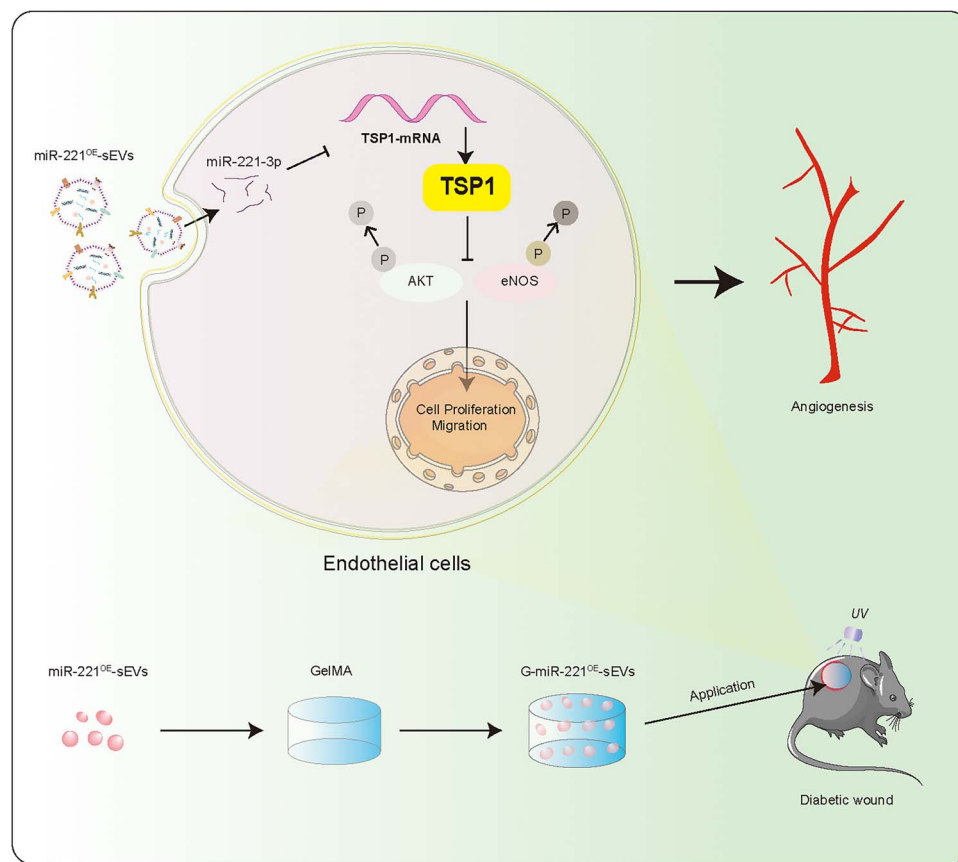


Figure 7. Schematic illustration showing the preparation process of GelMA hydrogel encapsulating engineered miR-221^{OE}-sEVs as well as the regulatory mechanism involving TSP-1-mediated angiogenesis to accelerate skin wound healing in diabetic mice. *G-miR-221^{OE}-sEVs* miR-221^{OE}-sEVs encapsulated GelMA hydrogel: *miR-221^{OE}-sEVs* small extracellular vesicles loaded with miR-221-3p, *GelMA* gelatin methacryloyl; UV ultraviolet, *TSP-1* thrombospondin-1, *AKT* protein kinase B, *eNOS* endothelial nitric oxide synthase

suggest that G-miR-221^{OE}-sEVs have the potential to serve as novel bioactive dressings, thereby suggesting a promising approach for diabetic wound management.

However, the clinical translation of engineered sEVs and GelMA systems still demonstrates numerous challenges. Large-scale production remains a significant challenge because of the need for high yield, consistent quality, and maintenance of material properties. Recent studies have explored innovative approaches to increase sEV production, such as the modulation of the circadian rhythm of adipose-derived stem cells [81] and the combination of superabsorbent polymers in a dialysis membrane with size exclusion chromatography [82]. Moreover, the scaling up of the production of GelMA hydrogels while maintaining their material properties is a challenge. Recent research has demonstrated the importance of optimizing the molecular network density to tailor GelMA hydrogels for specific applications. This optimization is crucial for large-scale production to ensure consistent performance in clinical settings [83]. Moreover, the clinical translation of the system is also hindered by regulatory hurdles. Ensuring the safety, efficacy, and consistency of this system is a prerequisite for regulatory approval. Current clinical trials administering natural sEVs have provided a foundation for achieving these goals [84, 85]; however, the clinical application of engineered sEVs demands further optimization and standardization in terms of fabrication and characterization, as well as desirable biocompatibility, mechanical properties, and degradation

rates of GelMA hydrogels [86]. Additionally, the extraction and utilization of sEVs elicit ethical and privacy concerns, particularly when these sEVs are derived from human cells, thus necessitating strict adherence to legal and regulatory standards throughout the entire process.

Despite these numerous challenges, the use of sEVs and hydrogel systems represents an innovative strategy in biomedical engineering, with broad application prospects and development potential being demonstrated. Future research should focus on translating the findings regarding research on engineered sEVs and GelMA into human clinical settings. Specifically, rigorous clinical trials should be systematically conducted to assess both therapeutic effectiveness and biosafety parameters of these engineered systems. Additionally, the consideration of immune response challenges will be crucial in future research. Paramount attention must be directed toward immunological compatibility challenges in forthcoming studies, particularly elucidating the intricate interplay between engineered sEVs, GelMA matrices, and host immune defenses to prevent undesirable immunological consequences. Moreover, further research should explore the optimization of sEV engineering to increase their stability and targeting capabilities, as well as establishing universal manufacturing guidelines and rigorous quality assurance measures for these therapeutic biomaterials. We believe that these future directions will not only lead to revolutionary advancements in this field but also provide valuable insights for the clinical application of engineered systems.

Conclusions

In summary, the results of this study illustrated that engineered hucMSCs-sEV-mediated transportation of miR-221-3p silenced the expression of TSP-1 and activated p-AKT/AKT and p-eNOS/eNOS pathway in HUVECs under HG conditions, which enhanced proliferation, migration, and tube formation ability, thus accelerating diabetic wound repair. Collectively, this study establishes a novel therapeutic strategy for diabetic wound management through TSP-1 silencing and AKT/eNOS pathway activation.

Author contributions

Y.G., W.Q., and S.C. contributed to conceptualization, design, revision, supervision, and funding; C.Y., M.S., and X.X.Y. performed the experiments, analyzed the data, and wrote the manuscript. C.Y., L.Y., Z.Y., L.W., and Z.L. performed the experiments and revised the manuscript. Yan Cong (Data curation, Investigation, Methodology, Project administration, Writing—original draft, Writing—review & editing [equal]), Sheng Meng (Data curation, Investigation, Methodology, Writing—original draft, Writing—review & editing [equal]), Xiaoye Xie (Data curation, Investigation, Methodology, Writing—original draft, Writing—review & editing [equal]), Yiqi Chen (Methodology, Writing—review & editing [equal]), Yucong Li (Methodology, Writing—review & editing [equal]), Yingqian Zhou (Methodology, Writing—review & editing [equal]), Wandu Li (Methodology, Writing—review & editing [equal]), Lipeng Zhang (Methodology, Writing—review & editing [equal]), Guoqing Yang (Conceptualization, Supervision [equal]), Qian Wei (Conceptualization, Project administration, Supervision, Writing—review & editing [equal]), Funding acquisition [lead]), and Chuan'an Shen (Conceptualization, Funding acquisition, Project administration, Supervision, Writing—review & editing [equal])

Supplementary data

Supplementary data are available at *Burns & Trauma Journal* online.

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Data availability

All data needed to support the conclusions are present in the paper and/or the supplementary materials. Additional data related to this paper may be requested from the authors.

Ethics approval and consent to participate

All animal procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Research Committee of PLA General Hospital in Beijing, China and Viewsolid Biotech CO. Ltd (VS2126A01295).

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