

RESEARCH ARTICLE

Distinct molecular properties and functions of small EV subpopulations isolated from human umbilical cord MSCs using tangential flow filtration combined with size exclusion chromatography

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Abstract

As functional derivatives of mesenchymal stem cells (MSCs), small extracellular vesicles (sEVs) have garnered significant attention and application in regenerative medicine. However, the technical limitations for large-scale isolation of sEVs and their heterogeneous nature have added complexity to their applications. It remains unclear if the heterogeneous sEVs represent different aspects of MSCs functions. Here, we provide a method for the large-scale production of sEVs subpopulations derived from human umbilical cord mesenchymal stem cells (HucMSCs), utilizing tangential flow filtration combined with size exclusion chromatography. The resulting subpopulations, S1-sEVs and S2-sEVs, exhibited stable variations in size, membrane-marked proteins, and carrying cargos, thereby displaying distinct functions both in vitro and in animal disease models. S1-sEVs, that highly expressed CD9, HRS and GPC1, demonstrated a greater immunomodulatory impact, while S2-sEVs with enriched expression of CD63 and FLOT1/2 possessed enhanced capacities in promoting cell proliferation and angiogenesis. These discrepancies are attributed to the specific proteins and miRNAs they contain. Further investigation revealed that the two distinct sEVs subpopulations corresponded to different biological processes: the ESCRT pathway (S1-sEVs) and the ESCRT-independent pathway represented by lipid

Wei Liu, Xinyu Wang, and Yating Chen contributed equally to this study.

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rafts (S2-sEVs). Therefore, we propose the potential for large-scale isolation and purification of sEVs subpopulations from HucMSCs with distinct functions. This approach may provide advantages for targeted therapeutic interventions in various MSC indications.

KEYWORDS

mesenchymal stem cells, sEVs, size exclusion chromatography, tangential flow filtration, targeted therapies

1 | INTRODUCTION

Given the properties of self-proliferation and differentiation into multi-lineage cell types, mesenchymal stem cells (MSCs) have become attractive candidates in regenerative medicine (Hoang et al., 2022; Zhang et al., 2022). However, despite all the proposed promising effects, such as their immunomodulatory and regenerative functions and low immunogenicity, the clinical utility of MSCs is still challenged due to their low in vivo survival, inaccurate delivery to inflamed lesions, inconvenience of transportation or storage and even ethical considerations (Lee et al., 2021; Liu et al., 2019). Although direct and indirect mechanism such as cellular engraftment, response to injury site or produced cytokines and growth factors have been proposed, increasing evidence shows that the beneficial effects of MSCs may be attributed to their paracrine action via extracellular vesicles (EVs) (Asami et al., 2013; Zhang et al., 2017). EVs are heterogeneous lipid bilayer-surrounded vesicles secreted by all cell types, from prokaryotes to eukaryote, not limited to MSCs. Their primary function is to facilitate intercellular communication by transmitting biological signals (Barile & Vassalli, 2017; Isaac et al., 2021). Compared to the MSCs, MSCs derived EVs not only offer convenience in terms of extraction, storage and transportation but also exhibit lower immunogenicity and higher biology stability (Patel et al., 2017; Tang et al., 2021; Zhao et al., 2020). Moreover, they preserve the therapeutic effects of MSCs in various diseases, such as immune-related diseases, cardiovascular disorders, diabetes or neurological diseases, and so forth (Chen et al., 2023; Seo et al., 2019; Turano et al., 2023). Thus, MSC-EVs, as a cell-free therapy, may be more suitable for clinical application than MSCs (Jafarinia et al., 2020; Shi et al., 2021).

According to different biogenesis pathways, EVs can be categorized into three classes: microvesicles (MVs), sEVs and apoptotic bodies. MVs, size ranges from 50 to 1000 nm, are released by direct outward budding of the plasma membrane. Apoptotic bodies have a broader size range from 50 nm to 5000 nm and originate from fragmentation of the cell membrane of apoptotic cells. Compared to the other types of EVs, sEVs seem more 'homogeneous' in size, typically ranging from 40 to 160 nm (Jia et al., 2022; Kalluri & LeBleu, 2020; Rufino-Ramos et al., 2017). However, they are of particular interest due to distinct intracellular regulatory mechanisms through the endo-lysosomal pathway (Hur et al., 2020). Different mechanisms have been proposed for the biogenesis of sEVs which include the endosomal sorting complex required for transport (ESCRT)-dependent and ESCRT-independent pathways (Babst, 2011; Ferreira et al., 2022). The ESCRT family consists of four members: ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III (Henne et al., 2011; Vietri et al., 2020). These components act together with auxiliary proteins such as vesicle trafficking 1 (VTA-1), ALG-2 interacting protein X (ALIX), and Vacuolar protein sorting 4 (VPS4) in the stepwise formation of multivesicle bodies (MVBs) (Hurley, 2010; Lee et al., 2023). Nevertheless, both CD63 and CD81 have been reported to sort a series of ligands into sEVs through ESCRT-independent mechanisms (Berditchevski & Odintsova, 2007; Edgar et al., 2014; Larios et al., 2020; Perez-Hernandez et al., 2013; van Niel et al., 2011). Secretion of sEVs into the extracellular matrix depends on the soluble NSF attachment protein receptor (SNARE) protein mediates MVBs translocation and fusion of sEVs with lysosomes or cell membranes (Dingjan et al., 2018; Fader et al., 2009; Hessvik et al., 2023; Wang et al., 2017). In addition, the MVB anchoring mechanism, including the substrates of the GTPases RAB27A, RAB27B, RAB35 and RAS like proto-oncogene A (RALA) also affects the release of sEVs into extracellular fluid (Pfeffer, 2007; Song et al., 2019; Ungermann & Kümmel, 2019).

Despite the perceived 'homogeneity', sEVs have been identified as the major contributor in mediating a diverse array of effects on recipient cells. Considering that sEVs biogenesis is a highly selective process, the heterogeneous characters of sEVs can be reflected by their size, content, functional impact on recipient cells, and cellular origins (Kalluri & LeBleu, 2020). Thus, it is conceivable that there exists subpopulations of sEVs with defined molecular compositions and biological properties. However, the progress of such studies has been impeded by technical limitations in effectively isolating and separating them. By using density gradient centrifugation, one study isolated sEVs into two distinct subpopulations that exhibited different molecular compositions and elicited differential gene expressions upon uptake by recipient cells (Willms et al., 2016). Another study discovered at least two distinct subpopulations of sEVs using the traditional ultracentrifugation method after inhibiting the Rab27a-mediated endosome pathway (Ostrowski et al., 2010). Compared to ultracentrifugation, size exclusion chromatography better preserves the integrity of sEVs structures by reducing sEVs aggregates and maintaining the bilayer membrane without damage. Additionally, it is more user-friendly and time saving than ultracentrifugation (Hong et al., 2016; Sidhom et al., 2020). To date, the SEC method has been successfully used for the isolation and enrichment of sEVs from a wide range of biological fluids, including

urine, saliva, serum and others (Wang et al., 2024). When applied in separating urine sEVs, four main fractions were obtained which sub-proteomes showed great difference in constitution (Guan et al., 2019). The state-of-the-art asymmetric flow field-flow fractionation (AF4) technology has also been used to separate out different sized groups of sEVs, including non-membranous nanoparticles termed 'exomeres' (~35 nm). However, the limited loading capabilities and possible contamination on the membrane due to sEVs adsorption hinders its application in large-scale production sEVs. (Mudalige et al., 2015; Zhang et al., 2018). Since most well-known single sEVs enrichment methods have their own limitations, combining two or more methods would overcome the shortcomings of each technique and enhance the overall efficiency and specificity of sEVs isolation. For example, centrifuge-based ultrafiltration in combination with size exclusion chromatography is a highly efficient method to improve the purity and yield of sEVs (Benedikter et al., 2017). Additionally, the combination of tangential flow filtration (TFF) and size exclusion chromatography (SEC) has also been proposed as a primary isolation method suitable for large-scale applications based on its high yield, purity, quality, and specificity (Visan et al., 2022). Therefore, by implementing a multimodal approach to sEVs isolation, we can not only produce high-quality, contaminant-free sEVs for industrial applications, but also characterize their subpopulations, specific cargos, and distinct biological functions.

Until now, only a limited number of human clinical trials have been conducted in MSC-sEVs (Fusco et al., 2024). There are many challenges that need to be solved, for example, whether the heterogeneous population of sEVs is beneficial, or some specific sub-populations represents specific functions of MSCs on target tissues or cells; how to standardize the sEVs isolation and purification workflow for its clinical utility with high yield, low cost and characterized contents. The aim of the study is to set up a protocol suitable for large-scale production of MSC sEVs based on the combination of TFF and SEC. The protocol has been optimized to separate subpopulations of sEVs (40–160 nm). According to the fraction peaks, we divided isolated sEVs as two subpopulations (S1-sEVs and S2-sEVs). The difference of the two subpopulations was manifested by differentially expressed sEVs markers, subpopulation proteomics or miRNAs. Most importantly, we found they represented different aspects of MSCs functions in various animal models. Our study provides a solution for industrial-scale production of subpopulations of MSCs sEVs, which is crucial for enabling targeted treatment of different MSCs indications.

2 | MATERIALS AND METHODS

2.1 | Isolation and culture of HucMSCs

Fresh umbilical cords were collected from consenting mothers at Sir Run Run Hospital, Nanjing Medical University. The study was approved by the Ethics Committee of Nanjing Medical University (approval number: (2018) 371). Human umbilical cord mesenchymal stem cells (HucMSCs) were isolated as previously described (Yang et al., 2020). The phenotype of HucMSCs was evaluated by flow cytometry using antibodies against positive markers CD73, CD90 and CD105, and negative markers CD11b, CD19, CD14, CD34, CD45, F4/80, CD11c, CD206 and HLA-DR. The differentiation of HucMSCs was assessed at passage 7 by using osteogenic (HUXUC-90021, Cyagen, USA), adipogenic (HUXUC-90031, Cyagen, USA), or chondrogenic differentiation media (HUXUC-9004, Cyagen, USA), with differentiated cells identified by alizarin red, oil red O, and Alcian blue staining, respectively.

2.2 | Isolation of sEVs by tangential flow system and size exclusion chromatography

HucMSCs were cultured in serum-free medium (NC0103+NC0105.S, Yacon, Beijing, China) and passaged every 2–3 days, depending on the cell growth rate. Cell supernatant for sEVs isolation was collected from HucMSCs at passages 5 to 7. After removing cell debris through deep filtration (Sartopore 2 XLG 8 µm, 0.45 µm; Sartorius, German), clarified cell culture conditioned media (500 mL to 1 L) was concentrated by a TFF system (ÄKTA flux S, Cytiva, USA) according to the instruction manual. In brief, the supernatant was initially processed through the TFF system using a MaxCell hollow fiber cartridges (750,000 NMWC, Cytiva, USA) to collect the filtrate. This filtrate was then further filtered through a 300,000 NMWC hollow fiber cartridges and the concentrate was collected with a final volume of 15 to 30 mL. Subsequently, the concentrate was injected into a size exclusion column (Superdex 200 HiLoad 26/60, Cytiva, USA) for sEVs isolation using the ÄKTA pure chromatography system (Cytiva, USA). The UV-visible detector was set at 280 nm and the temperature of column was set at 6°C. The flow rate was set to 0.5 mL/min using PBS buffer as the solvent, and 4 mL of each fraction was collected. The procedure was repeated until all the concentrate was processed. The fractions corresponding to each peak were then pooled and concentrated 20-fold using TFF. The concentrate after TFF and the separated and pooled fractions were stored at –80°C for future use. The columns were washed with one column volume of 500 mM NaOH between runs, followed by two column volumes of 30% ethanol, before being stored at 4°C. Prior to applying the samples, the column was equilibrated with at least two column volumes of sterile-filtered PBS.

2.3 | Lipophilic fluorescent dye labelling

The PKH67 (Sigma-Aldrich) or DiR (Thermo Fisher Scientific) labelling was performed following the manufacturer's recommended protocol. Briefly, freshly isolated S1-sEVs or S2-sEVs were incubated with PKH67 or DiR at 4°C overnight, protected from light. Subsequently, the sEVs samples were diluted to a final volume of 38.6 mL with double-filtered (0.22 µm) PBS and centrifuged at 120,000 × g at 4°C for 60 min to remove free dye. The labelled sEVs pellets were suspended in PBS and either used directly for experiments or stored in aliquots at −80°C.

2.4 | NTA measurement with Zetaview (Particle Metrix, Germany)

The particle size distribution and concentration of the samples were analysed using the ZetaView nanoparticle tracking analyser (model: Zetaview-PMX120-Z, software version: 8.05.14 SP7) manufactured by Particle Metrix GmbH, Germany. Prior to measurement, the samples were diluted with 1X PBS buffer to an appropriate concentration aiming for an ideal particle count of 140–200 particles per frame. The measurements were conducted at a constant temperature of 25°C. For each sample, three cycles of measurements were performed, scanning 11 cell positions per cycle, and capturing 60 high-definition frames per position. Subsequently, the captured videos were analysed using the ZetaView software with specific analysis parameters: Maximum particle size: 1000, Minimum particle size: 10, Minimum brightness: 30, Maximum Brightness: 255 (Bachurski et al., 2019; Gardiner et al., 2013).

2.5 | Flow cytometry analysis

MSCs/BMDMs/RAW264.7 cells were collected for immunophenotypic analysis. Phycoerythrin (PE)-labelled anti-CD34, CD45, CD19, CD11b, CD31, CD73, F4/80 and IgG isotype control, allophycocyanin (APC)-labelled anti-HLA-DR, CD105, CD206 and IgG isotype control, fluorescein isothiocyanate (FITC)-labelled anti-CD90, CD11c and IgG isotype control were incubated with respective cells for 30 min at 4°C. After washing in PBS for three times, cells were analysed by flow cytometry (BD Biosciences). F4/80, CD11c, CD206 antibodies were purchased from Biolegend (San Diego, CA, USA); the remaining antibodies were purchased from BD Biosciences (Franklin Lakes, New Jersey, USA).

2.6 | Nanoflow analysis

Took 30 µL of sEVs diluent (the dilution factor of the sample is approximately 30) and incubated it with 20 µL FITC-labelled Mouse Anti-Human CD9 and CD63 (BD, Franklin Lakes, New Jersey, USA), as well as FITC-labelled Mouse IgG (BioLegend, San Diego, USA), at 37°C for 30 min in the dark. Then, 1 mL of pre-cooled PBS was added to the mixture and centrifuged at 110,000 g for 70 min. The supernatant was carefully removed, and the pellet was resuspended in 50 µL of pre-cooled PBS. The protein index was detected using a NanoFCM instrument (NanoFCM, XiaMen, China).

2.7 | Cell proliferation, glucose and lactate concentration assays

Mouse 3T3 cells were seeded into 96-well plates and treated with sEVs subpopulations at different concentrations: 1×10^9 (L), 1×10^{10} (M) and 1×10^{11} (H) particles/mL, respectively. In a separate experiment, cells were incubated with TFF concentrate, S1-sEVs and S2-sEVs at a same concentration of 1×10^{10} particles/mL for 48 h. Cells were also pretreated with cisplatin (CIS) for 12 h and then cultured in the presence of S1-sEVs or S2-sEVs at 1×10^{10} particles/mL for 48 h to evaluate cell proliferation. The serum was removed when adding sEVs or the control PBS. The cells were then collected at different time points (0 h, 6 h, 12 h, 24 h, 48 h) for Cell Counting Kit-8 (CCK-8) cell proliferation analysis. CCK-8 working solution (Vazyme, Nanjing, China) was added at each time point and the cells were further cultured for 2 h at 37°C before the cell proliferation was assessed by measuring the absorbance value at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA). To evaluate the glycolysis levels after treatment with sEVs subpopulations, glucose and lactate concentration of cell culture supernatant was determined according to the manufacturer's protocol (Abbkine, Wuhan, China). For glucose: the absolute values of the samples were measured at an absorbance of 630 nm, and the concentration was expressed as mg/dL. For lactate: the absolute values of the samples were measured at an absorbance of 450 nm, and the concentration was expressed as mM. The 3T3 cell line was gifted by other laboratories.

2.8 | Macrophage polarization induction experiment

Bone marrow was extracted from the femur and tibia using a 10 mL syringe and needles. The flushed cells were then cultured in RPMI 1640 medium (Bio-sharp, China) for 7 days, supplemented with 10% FBS (10099141, Gibco), penicillin/streptomycin (100 µg/mL), and 10% (vol/vol) conditional medium of L929 mouse fibroblasts, to obtain primary mouse BMDM cells. Mouse BMDMs and macrophage cell line RAW264.7 was cultured in six-well plates and treated with LPS 100 ng/mL for 24 h to induce its polarization to M1 macrophages. TFF concentrate and the two subpopulations of sEVs (S1-sEVs and S2-sEVs) at 1×10^{10} particles/mL were then added into the cells for the treatment of another 24 h. The transition of macrophage polarization states from M1(CD11c+; CD206−) to M2 (CD11c−; CD206+) macrophages were then detected by flow cytometry and the expression levels of M1 macrophage markers in different treatment groups were examined by qRT-PCR. The purity of BMDMs and RAW264.7 cells was assessed by flow cytometry, which showed negative expression of MSC markers CD73, CD90 and CD105 (Figure S3C).

2.9 | Experimental animals

Mice and rats were obtained from the Medical Laboratory Animal Centre of Nanjing Medical University (Nanjing, Jiangsu, China) and housed in the animal facility of Nanjing Medical University. Mice and rats were maintained on a 12-h light: 12-h darkness cycle and were provided with food and water ad libitum. Except for the rats that had just been scalded, all other mice were housed in groups, with a maximum of five mice per cage. Adult male C57BL/6 mice aged 8–12 weeks were selected to establish hindlimb ischemia and colitis models. Adult female Sprague-Dawley rats (200 ± 20 g) were selected to establish a skin scald model. All experiments requiring the use of animals were approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University (approval numbers: IACUC-2204058 & IACUC-2206057).

2.10 | Establishment of a mouse hindlimb ischemia model

Ten-week-old male C57BL/6 mice were chosen and divided into four groups: the sham group, the control group, the S1 group and the S2 group. After the intraperitoneal injection of an appropriate amount of the anaesthetic tribromoethanol (Aibei Biotechnology, Nanjing, China), an incision was carefully made on the skin of the medial left thigh of the mice using ophthalmic scissors. Subcutaneous and muscular tissues were then carefully separated with microvascular forceps and ophthalmic forceps until the left iliofemoral artery was exposed. Next, the left iliac artery and surrounding tissues were separated, and the free arteries were ligated on both sides using 4-0 sutures. The arteries were then cut between the ligation points to establish a unilateral lower limb ischemia model in mice. In the sham group, the same steps were followed until the left iliofemoral artery was exposed. The left iliac artery and surrounding tissues were separated but not ligated or clipped. On postoperative day 1, 200 µL PBS was injected into the quadriceps muscle on the operated side of the mice in the sham and the control groups. In the S1 group and the S2 group, 200 µL S1-sEVs and S2-sEVs containing 1×10^{10} particles, respectively, were injected into the quadriceps muscle on the operated side of the mice (4 injection sites per quadriceps). The lower limb blood flow was measured in all mice on day 21 after surgery to assess the recovery of blood flow in the affected limb, using the blood flow instrument. The quadriceps muscle on the operated side of each group was collected for further experiments and analysis.

2.11 | The application of blood flow instrument

In a dark environment at room temperature, the mice were carefully anesthetized with isoflurane gas and secured on a plate, ensuring minimal movement and exposing their hind limbs. Turn on the blood flow instrument (moor FLPI-2, Moor Instruments, UK). The USB data interface on the scanning head was then connected to a computer, and the scanning head was fine-tuned to be perpendicular to the testing surface. The distance between the scanning window and the testing surface was maintained within the range of 10–38 cm. Once the scanning head was powered on, the measurement process could begin by launching the Moor-O2FLO measurement V1.0 software and accessing the measurement. The acquired data were further analysed using mFLPI2 V2.0.002 software.

2.12 | Rat skin scald model

200 ± 20 g male SD rats were anesthetized by intraperitoneal injection. After removal of back hair, the skin was scalded in a circular area of 2 cm² with 90°C hot water for 10 s, followed by immediate blotting with sterile gauze. Rats in different treatment

groups received a subcutaneous injection of different subpopulations of MSC-sEVs at the burn site on day 0, with a dose of 200 μL containing a particle number of 1×10^{10} . Rats in the control group received a subcutaneous injection of the same dose of PBS. The wound healing of the rats was observed and recorded for 14 consecutive days. After euthanizing the rats on the 14th day, the skin tissues in the wounds were analysed for morphology and molecular biology. The histologic scoring criteria are elaborated in Table S1 (Sen et al., 2020).

2.13 | Dextran sulphate sodium induced colitis mouse model

Colitis was induced by administering 2.5% dextran sulphate sodium (DSS) (Merck, Germany) through drinking water ad libitum for 10 days. 1.0×10^{10} particles of S1-sEVs and S2-sEVs were infused intravenously into mice 1, 4 and 7 days after the beginning of DSS treatment. Mice received an equal amount of PBS were served as the control group. Body weight and the disease activity index (DAI) were monitored daily. The following parameters were used for the calculation of DAI: (a) weight loss (0 points: none; 0.5 points: 1%–5% weight loss; 1 point: 5%–10% weight loss; 2 points: 10%–15% weight loss; 3 points: more than 15% weight loss); (b) bleeding/diarrhoea (0 points: pellet faeces, no bleeding; 1 point: shapeless stools, slight bleeding; 2 points: loose stools, bleeding; 3 points: gross bleeding, watery diarrhoea); (c) fur/body condition (0 points: smooth fur; 1 point: mild scruffiness; 2 points: hunchback, inactivity; 3 points: curled up and still). The DAI was calculated as the sum of these scores, resulting in a total DAI score ranging from 0 (normal) to 9 (severe colitis). All mice were sacrificed on day 10 after being fed with DSS water. Colon length was recorded and the tissues were processed for normal and Swiss-roll sections. The following parameters were used for the estimation of histological activity index (HAI): (a) epithelial damage (0 points: none; 1 point: minimal loss of goblet cells; 2 points: extensive loss of goblet cells; 3 points: minimal loss of crypts and extensive loss of goblet cells; 4 points: extensive loss of crypts); (b) infiltration (0 points: none; 1 point: inflammatory cell infiltration around crypt bases; 2 points: infiltration in the muscularis mucosa; 3 points: extensive infiltration in the muscularis mucosa with edema; 4 points: infiltration of the submucosa). HAI was calculated as the sum of the epithelial damage and infiltration scores, resulting in a total HAI score ranging from 0 (normal) to 8 (severe colitis).

2.14 | Construction and analysis of the stable knockout HEK293T cell lines

The homoHGS-sgRNAs were inserted into the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 vector LentiCRISPRV2 (Puro) to obtain the LentiCRISPRV2-homoHGS-sgRNA plasmids. The construct was confirmed by Sanger sequencing. HEK293T cells were transfected with Lenti-HGS-sgRNA, using Lipofectamine 3000. After 24 h, the antibiotics (puromycin) were added to the cells for screening. Single-cell amplification was conducted when the cell count was lower than 10% of the control group. The successfully HGS knocked-out monoclonal 293T cell line was identified through protein blotting experiment. The HEK293T cell line was gifted by other laboratories.

2.15 | EdU cell proliferation assay in vivo

The mice received an intraperitoneal injection of EdU at a dose of 5 mg/kg of body weight. After 24 h, the quadriceps muscles were harvested, embedded in OCT, and frozen sectioned. Staining of the EdU-labelled quadriceps muscles was performed using the Cell-Light Apollo Stain Kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. The sections were counterstained with Hoechst 33342 and viewed under the LSM 800.

2.16 | Immunoelectron microscopy

Cells were fixed with 4% PFA/0.1% glutaraldehyde/0.1 M phosphate buffer at pH 7.4, infused with 2.3 M sucrose, and supported in 12% gelatine gelatin. Approximately 70 nm sections were cut using a Leica Reichert ultracut S EM-FCS cryo-ultramicrotome (Leica, Germany) at -120°C . The sections were picked up using a 1:1 mixture of methylcellulose and 2.3 M sucrose and dropped onto formvar and carbon-coated hexagonal 100-mesh copper grids (Agar Scientific, UK), then stored at 4°C . For labelling with antibodies to CD63 and EGFR, the primary antibody was followed by a bridging antibody. Sections were subsequently labelled using protein-A gold at 37°C . Finally, prepared samples were observed and imaged with an electron microscope (FEI, 80 kV). The antibodies used are listed in Table S2.

2.17 | Statistical analysis

GraphPad Prism 8.0 were used to perform the Student's *t*-test or two-way ANOVA to compare the statistical differences between experimental groups. ImageJ (NIH, USA) was used to analyse western blot protein bands and confocal images. All data are presented as mean $\pm$ SEM.

2.18 | Data and materials availability

Raw data and extracted text files for quantitative proteomics have been deposited into PRIDE, under accession nos. PXD050064. Fastq data and the processed data file for transcriptome sequencing have been deposited into the Deposited in GEO under accession nos. GSE256371.

3 | RESULTS

3.1 | Separation of sEVs subpopulations from HucMSCs by combining TFF with SEC system

In order to enable large-scale production of MSC-sEVs suitable for clinical application, we employed a method combining TFF with SEC to separate and purify sEVs from conditional media of HucMSCs (Figure 1a). The characteristics of HucMSCs are detailed in Figure S1. After gel filtration, three peaks (Peak 1, 36–60 mL; Peak 2, 60–84 mL and Peak 3, 84–108 mL) of filtrates were collected for western blots and NTA analysis. The particle concentrations obtained were 1.14×10^{10} particles/mL, 7.04×10^9 particles/mL, and 1.26×10^9 particles/mL in the filtrates from Peaks 1, 2 and 3, respectively. Corresponding to these, the protein concentrations were 39.44 μ g/mL, 61.28 μ g/mL and 251.68 μ g/mL in the filtrates from Peaks 1, 2 and 3. Compared to the protein concentration of 382.59 μ g/mL in the TFF concentrate before SEC, we found that most of the proteins were separated and enriched in Peak 3. Western blots also showed a significant enrichment of Ferritin proteins in Peak 3 fractions, which is a common contaminant during sEVs isolation (Figure S3A) (Watson et al., 2018). Therefore, after TFF and SEC, protein contaminants in medium has been removed effectively. Peak 1 and Peak 2 fractions may represent sEVs subpopulations with high purity (particle number per unit mass of sEVs: 2.90×10^8 particles/ μ g in Peak 1 and 1.18×10^8 particles/ μ g in peak 2) (Figure 1B). Next, we collected a total of 12 fractions from Peak 1 and Peak 2 for further analysis of sEVs properties. Each fraction contains 4 mL of elutes: Peak 1 fractions were FC1–FC6, and Peak 2 fractions were FC7–FC12. TEM images showed typical sEVs structure and a gradually decreased size from FC2 to FC12 (Figure 1c). Western blots also demonstrated distinct expression patterns of sEVs markers between the two peaks. Specifically, CD9 and ALIX expression levels decreased gradually from FC1–FC6 of Peak 1, while CD63 protein levels increased gradually from FC7–FC12 of Peak 2. HGS expression was highest in FC3 and FC4 of Peak 1 (Figure 1d). NTA and TEM analyses showed the difference on particle size between Peak 1 and Peak 2 particles (Figure 1e,f). The particle size ranges from 70 to 200 nm with a peak at 113.3 nm in Peak 1, which was larger than the size of Peak 2 at 88.3 nm, which ranging in size from 50 to 160 nm (Figure 1e). Therefore, Peak 1 sEVs are characterized by the high expression levels of CD9, ALIX and HGS, while CD63 are highly expressed in Peak 2 sEVs (Figure 1g). The differential expressions of CD9 and CD63 were also clarified in the two peaks of sEVs by Nanoflow cytometry (Figure 1h). Despite the presence of heterogeneity within each peak, considering the size difference and the distinct expression of sEVs markers between the two peaks, we can conclude that the isolated two peaks likely represent two distinct subpopulations of sEVs. We named the subpopulations of sEVs corresponding to these two peaks as S1-sEVs and S2-sEVs, respectively.

3.2 | Characterization of MSC-sEVs subpopulations

The differential expression of sEVs markers means they may have different contents or functions. To characterize the heterogeneity of MSC-sEVs subpopulations, proteomic profiling was first conducted using 4D-label-free mass spectrometry. Differential enrichment of sEVs proteins in the two subpopulations was verified by 3D-principal component analysis (PCA) analysis (Figure S2A). Totally, 1326 and 1246 proteins were identified in S1- and S2-sEVs subpopulations, respectively (Figure S2B), among which 447 proteins were highly expressed in S1-sEVs and 127 proteins were enriched in S2-sEVs ($FC > 2$, $p < 0.05$) (Figure 2a, Table S5). Although most of proteins were identified expressed in both groups, we still observed 205 and 125 proteins specifically expressed in S1-sEVs and S2-sEVs (Figure S2C). Gene Ontology (GO) analysis then revealed the terms of antigen processing and presentation via major histocompatibility complex (MHC) class I, interleukin 1 (IL1) mediated signalling pathway and proteasome-mediated ubiquitin-dependent protein catabolic process in S1-sEVs differential proteins and glycolytic process, gluconeogenesis, and negative regulation of apoptotic process in S2-sEVs subpopulation (Figure 2b). Cellular component analysis

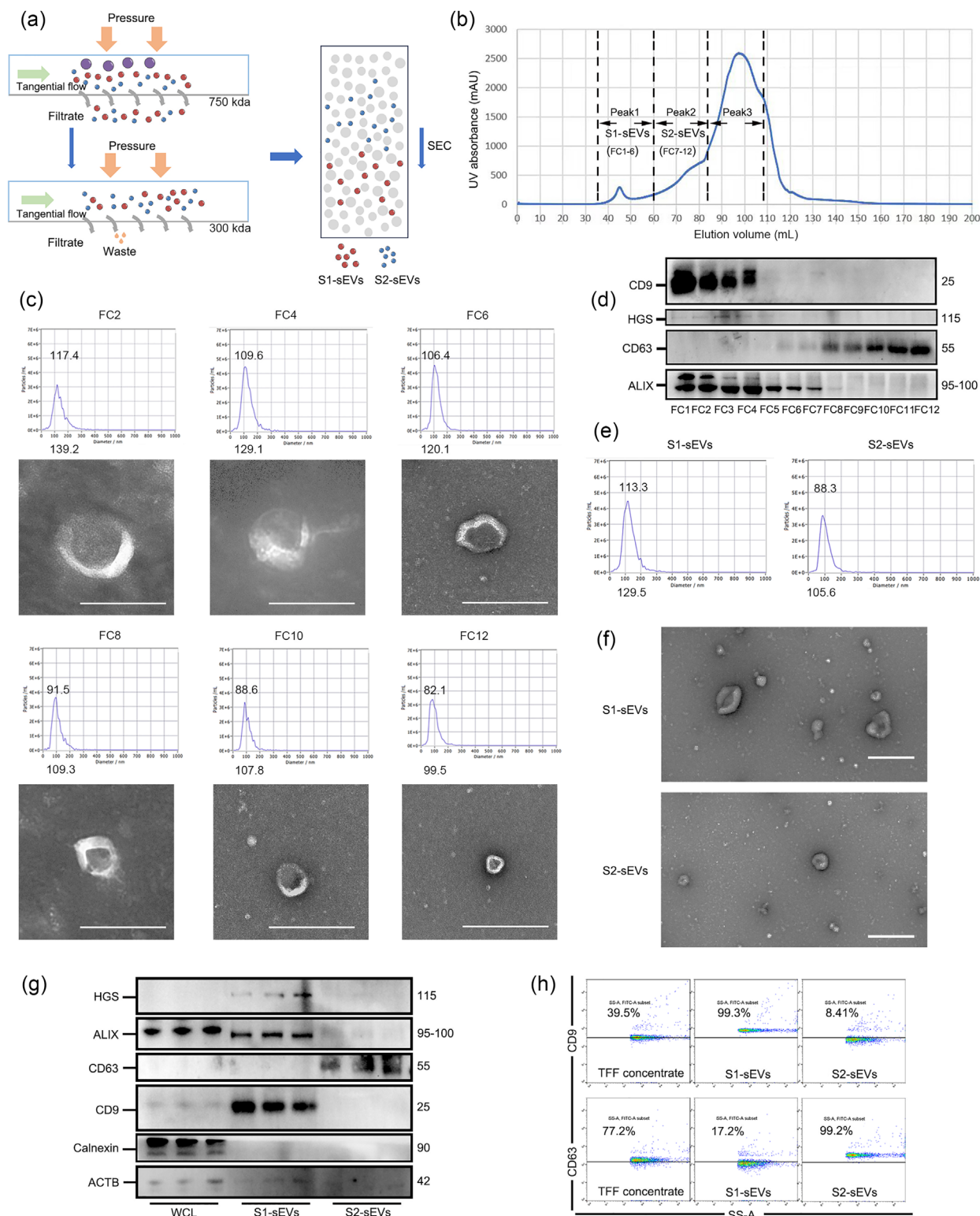


FIGURE 1 Separation and characterization of sEVs subpopulations from HucMSCs by TFF combined with SEC system. (a) Schematic diagram illustrating the process of sorting HucMSCs-derived sEVs subpopulations (S1-sEVs and S2-sEVs) using TFF combined with size SEC. (b) Chromatogram of HucMSCs derived sEVs separated by SEC. (c) NTA and electron microscopy of 12 fractions collected according to the peaks of SEC. Total 12 fractions were collected and 6 fractions were selected for characterization. (d) Western blots on the collected fractions using known sEVs markers. (e), (f) NTA and electron microscopic characterization of the two sEVs subpopulations, S1-sEVs and S2-sEVs. The fractions were pooled according to the differential expression of sEVs markers with FC1-FC6 for S1-sEVs and FC7-FC12 for S2-sEVs. (g) Differential expression of sEVs markers between S1-sEVs and S2-sEVs. The expression of ACTB was used as the internal control. (h) Differential distribution of sEVs markers CD9 and CD63 in S1-sEVs and S2-sEVs detected by Nanoflow cytometry. The experiments were repeated at least three times. All bars = 200 nm. HucMSCs, human umbilical cord mesenchymal stem cells; SEC, size exclusion chromatography; sEVs, small extracellular vesicles; TFF, tangential flow filtration.

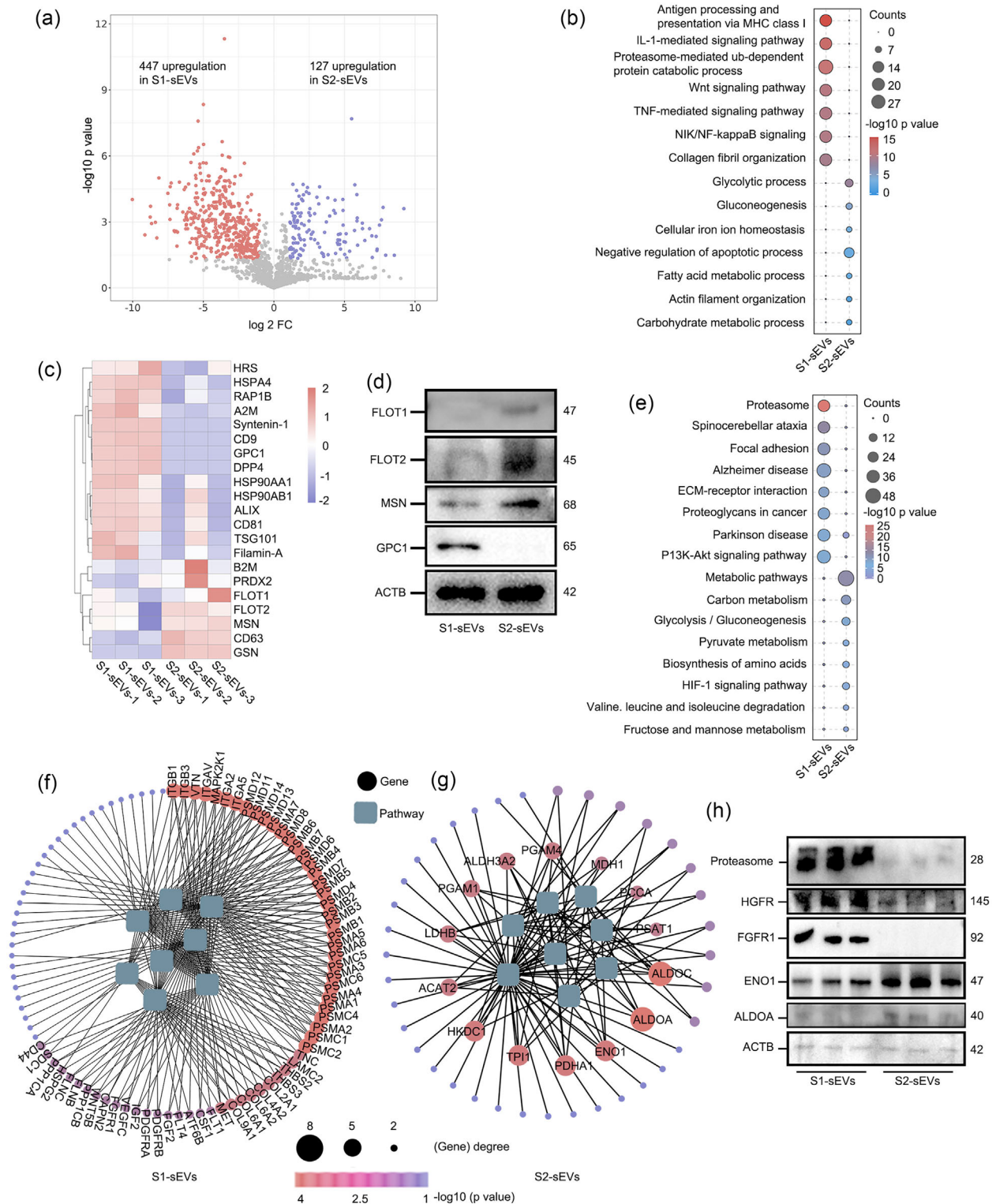


FIGURE 2 Proteomics analysis of the two sEVs subpopulations. (a) A volcano plot of protein profiles carried by S1-sEVs and S2-sEVs (red dots: upregulated in S1-sEVs; blue dots: upregulated in S2-sEVs). (b) GO enrichment analysis of differentially expressed proteins between S1-sEVs and S2-sEVs. (c) Heatmap of known EVs and sEVs markers enriched in S1-sEVs and S2-sEVs. (d) Differential expression of sEVs markers between S1-sEVs and S2-sEVs by western blotting. ACTB was used as the internal control. (e) KEGG pathway enrichment analysis of differentially expressed proteins between S1-sEVs and S2-sEVs. (f) An advanced network plot of identified node proteins of KEGG-enriched pathways in S1-sEVs. (g) An advanced network plot of identified node proteins of KEGG-enriched pathways in S2-sEVs. (h) Western blots of representative node proteins identified in (f) and (g). ACTB was used as the internal control. The experiments were repeated for three times. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; sEVs, small extracellular vesicles.

also revealed a high degree of variability between S1-sEVs and S2-sEVs (Figure S2D). Especially, we identified over 100 membrane proteins in S1-sEVs enriched proteins. Besides the differential distribution of known sEVs membrane markers between the two subpopulations (Figure 2c), we also identified a series of specific membrane markers in either S1-sEVs or S2-sEVs (Figure S2E). Figure 2d manifested the specific expression of glypican 1 (GPC1) on S1-sEVs and moesin (MSN), flotillin 1 (FLOT1), FLOT2 in S2-sEVs. Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis demonstrated proteasome pathway as the most enriched pathway in S1-sEVs enriched proteins and metabolic pathway displayed the highest level of enrichment in S2-sEVs (Figure 2e). The representative pathways in S1-sEVs and S2-sEVs by using Gene Set Enrichment Analysis (GSEA) were plotted in Figure S2F. The core node sEVs proteins of the two subpopulations were then demonstrated by KEGG advanced network diagram (Figure 2f,g). In S1-sEVs, we detected all seven α (PSMA1-7) and seven β (PSMB1-7) chains of the 20S core proteasome together with other regulatory subunits of 26S proteasome. This means S1-sEVs may carry possibly functional 20S proteasome to degrade misfolded proteins in target cells. Additionally, we also observed an enrichment of growth factor receptors in S1-sEVs, such as epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR), fibroblast growth factor receptor 1 (FGFR1) et al. However, in S2-sEVs, hub proteins were closely associated with metabolism, glycolysis, and the hypoxia-inducible factor 1 (HIF-1) signalling pathway. Western blot demonstrated the differential expressions of some core node proteins Proteasome α 1-7, HGFR and FGFR1 in S1-sEVs and glycolytic enzymes alpha-Enolase 1 (ENO1) and aldolase A (ALDOA) in S2-sEVs (Figure 2h). The presence of distinct containers in S1-sEVs and S2-sEVs implies that they might have different roles in the regulation of target cell activities.

To explore other cargos in sEVs subpopulations, we performed miRNA sequencing and identified 173 and 79 miRNAs in S1-sEVs and S2-sEVs, respectively. The differentially expressed miRNAs between the two sEVs subpopulations were shown in Figure 3b ($FC > 2$, $p < 0.05$) in which 113 unique miRNAs were found in S1-sEVs as compared to only 19 miRNAs in S2-sEVs (Figure 3a, Table S6). Representative miRNAs enriched in S1-sEVs or S2-sEVs, including *let-7b-5p*, *mir-155-5p*, *let-7c-5p*, *mir-9-5p*, and *mir-30d-5p*, were validated through qRT-PCR (Figure 3c–g). The target genes of differentially expressed miRNAs in each sEVs subpopulations were then analysed using miRNet 2.0. Senior network diagrams of differential miRNAs and target genes revealed core node target genes in each group with a degree cutoff 1.0 (Figure 3h,i). In S1-sEVs, the core node target genes included tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ), BTB domain and CNC homolog 1 (*BACH1*), plexin D1 (*PLXND1*), high mobility group AT-hook 1 (*HMGAI*), SMAD family member 2 (*SMAD2*), *IL6*, *IL6R* and transforming growth factor beta receptor 1 (*TGFBRI*). In S2-sEVs, the core node target genes were identified as PR/SET domain 1 (*PRDM1*), cyclin dependent kinase inhibitor 1A (*CDKN1A*), fibrillin 2 (*FBN2*), notch receptor 1 (*NOTCH1*), KLF transcription factor 6 (*KLF6*) and beclin 1 (*BECN1*). GO analysis showed enriched biological processes related with intracellular protein transport, chromatin or histone modification in target genes of S1-sEVs group, while negative regulation of cell proliferation or metabolism-related biological processes were enriched in target genes of S2-sEVs group (Figure 3j). Since miRNAs target genes to exert negative regulation, miRNAs enriched in S2-sEVs may play a role in upregulating cell proliferation and metabolic pathways, consistent with the results of proteomic analysis. By KEGG pathway analysis, we found that miRNAs target genes of S1-sEVs were more enriched in signalling pathways related with RNA transport or adherent junction, etc., while miRNAs target genes in S2-sEVs were more enriched in mitogen-activated protein kinases (MAPK) and Wingless/Integrated (WNT) signal pathways (Figure 3k).

3.3 | The differential regulation of S1-sEVs and S2-sEVs on cell proliferation and macrophage polarization

Based on the distinct cargo compositions of the two sEVs subpopulations, we next sought to determine if S1-sEVs and S2-sEVs exhibited differing effects on the rate of cell growth and division. 3T3 cells were cultured with three different doses (Low: 1×10^9 particles/mL, Medium: 5×10^9 particles/mL, High: 1×10^{10} particles/mL) of S1-sEVs or S2-sEVs, respectively. Although the dose- and time-dependent effects on cell proliferation were observed in both S1-sEVs and S2-sEVs treated cells, there was a significant and consistent enhancement in cellular growth among cells treated with S2-sEVs at all tested concentrations when compared to those groups treated with S1-sEVs (Figure 4A). The high dose (1×10^{10} particles/mL) of S1-sEVs and S2-sEVs were then used to examine their activity on 3T3 cell proliferation in comparison to the treatment with TFF concentrate at the same dose. Interestingly, we found the bioactivity of TFF concentrate fell between S1-sEVs and S2-sEVs groups, further validating the differential impacts of S1-sEVs and S2-sEVs on cell proliferation (Figure 4b). We then investigated the anti-apoptotic effects of sEVs subpopulations by co-treating 3T3 cells with the chemotherapy drug cisplatin at a concentration of 20 μ M (Figure S3B). Cisplatin treatment induced cellular growth arrest and apoptosis as shown by CCK-8 cell proliferation assay (Figure 4c), terminal uridine nucleotide end labelling (TUNEL) staining (Figure 4d,e) and increased expression of cleaved poly-ADP-ribose polymerase (PARP) and cysteinyl aspartate specific proteinase (Caspase) 3 (Figure 4f–h). Once again, S2-sEVs had a more pronounced protective effect against cisplatin-induced cell toxicity compared to the S1-sEVs subpopulation. Since S2-sEVs carried a lot of enzymes related with glycolysis, we next measured glucose and lactate contents in cell-supernatant at different time points. The results showed a significant consumption of glucose in S2-sEVs cultured cells, accompanied by an increase in lactate production

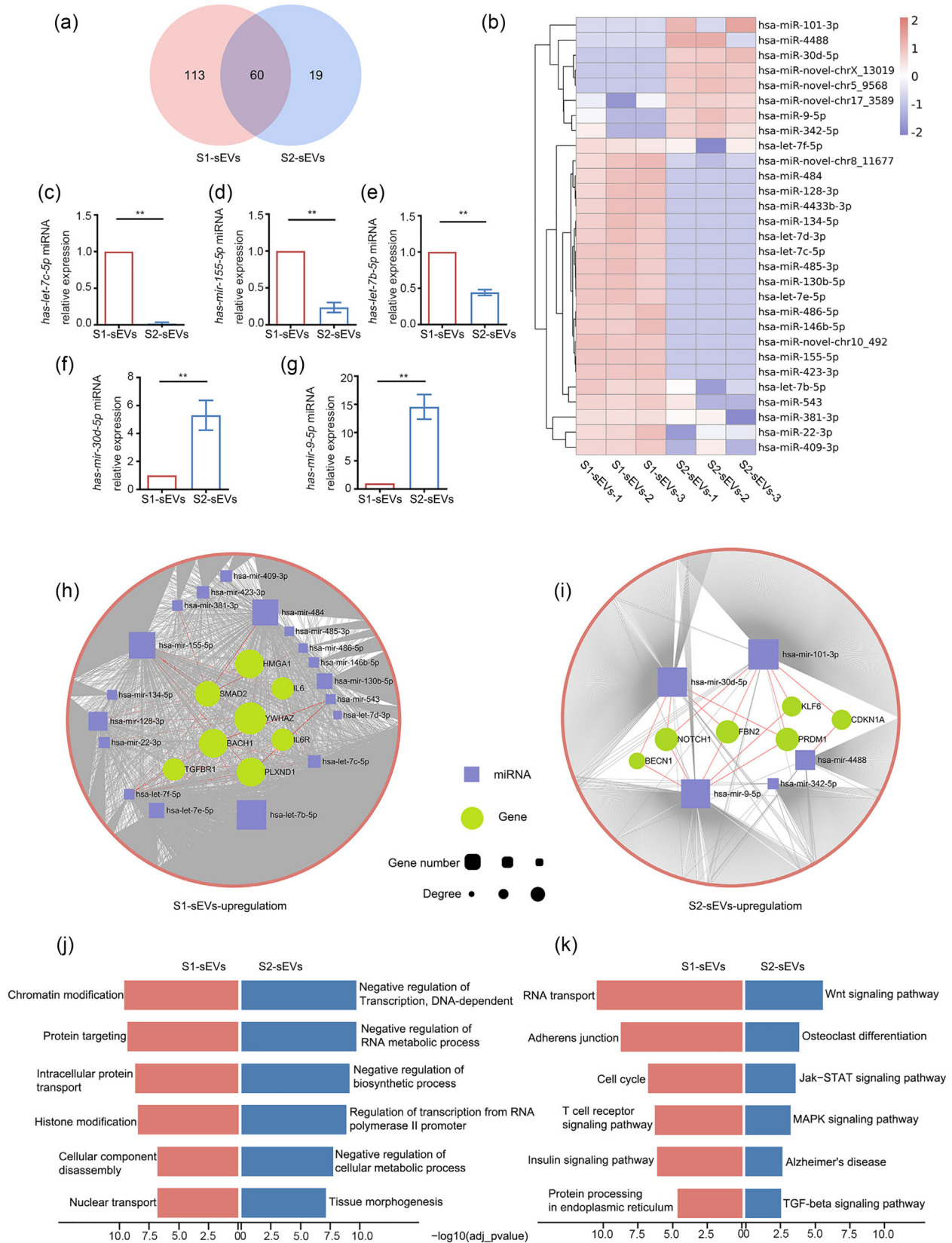


FIGURE 3 miRNA profiles of the two sEVs subpopulations. (a) Venn plots of miRNAs carried by S1-sEVs and S2-sEVs. (b) Clustering analysis of statistically different miRNAs between S1-sEVs and S2-sEVs. (c)–(g) qRT-PCR validation of highly enriched miRNAs identified in S1-sEVs and S2-sEVs. The levels of all tested miRNAs in S1-sEVs group were set to 1. (h) Network analysis of differential miRNAs enriched in S1-sEVs with target proteins. (i) Network analysis of differential miRNAs enriched in S2-sEVs with target proteins. (j) GO analysis of target genes in differentially expressed miRNAs enriched in S1-sEVs and S2-sEVs. (k) KEGG analysis of target genes in differentially expressed miRNAs enriched in S1-sEVs and S2-sEVs. The experiments were repeated for three times. Data are expressed as mean $\pm$ SEM. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; sEVs, small extracellular vesicles.

** $p < 0.01$.

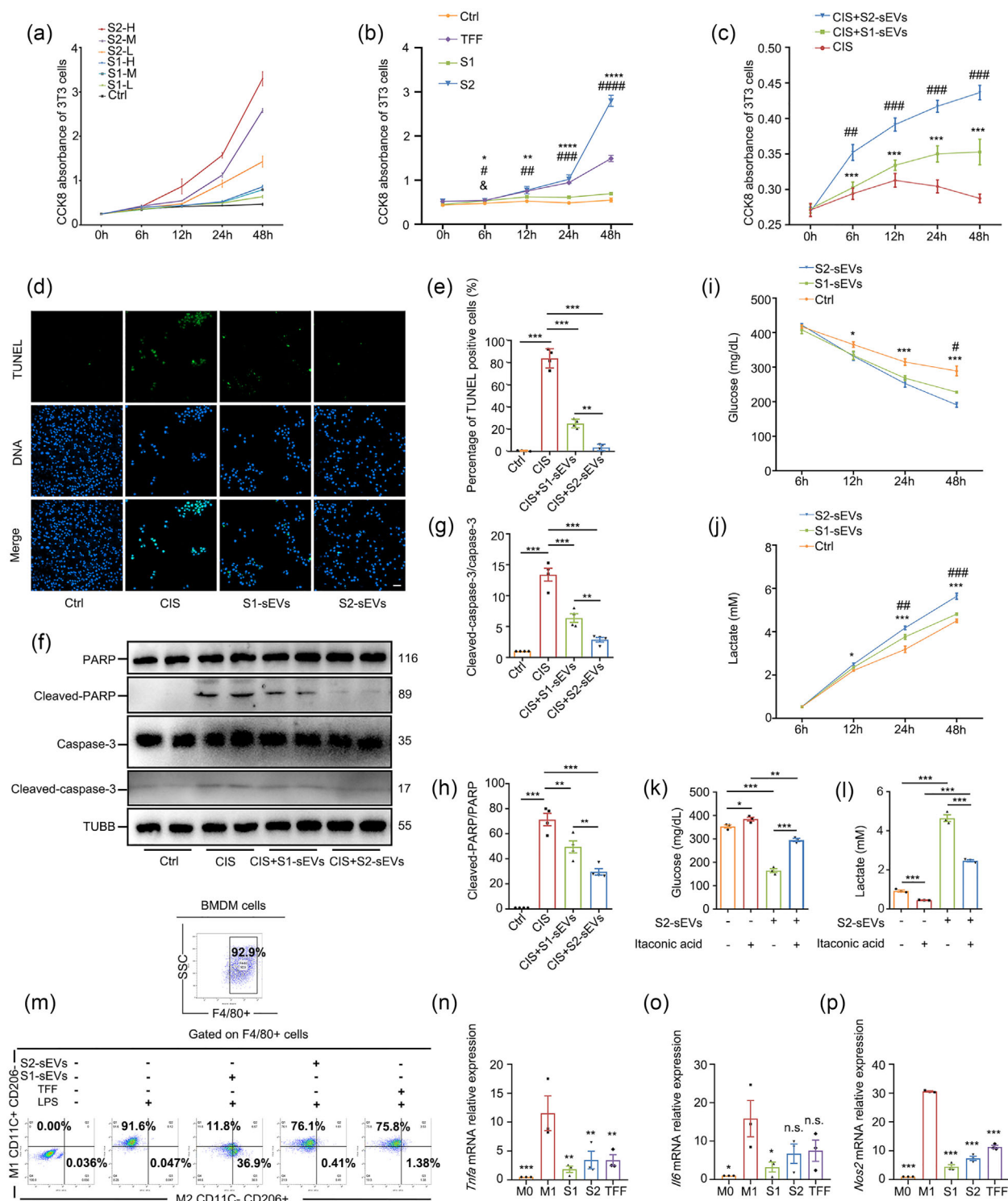


FIGURE 4 The differential regulation of S1-sEVs and S2-sEVs on cell proliferation and macrophage polarization. (a) Dose-dependent and time-dependent effects of S1-sEVs and S2-sEVs on cell proliferation. Mouse 3T3 cells were treated with different doses of S1-sEVs and S2-sEVs: 1×10^9 particles/mL (l); 5×10^9 particles/mL (m); 1×10^{10} particles/mL (h). Cells were collected at 6, 12, 24 and 48 h of treatment for CCK-8 analysis. ($n = 3/\text{group}$). (b) Time-dependent effects of S1-sEVs, S2-sEVs, and TFF concentrate (TFF) on cell proliferation. (c) Anti-apoptotic effects of S1-sEVs and S2-sEVs. 3T3 cells were pretreated with cisplatin (CIS) for 12 h and then cultured in the presence of S1-sEVs or S2-sEVs at 1×10^{10} particles/mL for 48 h. Cells were collected at 6, 12, 24 and 48 h for CCK-8 analysis ($n = 3/\text{group}$). (d), (e) TUNEL fluorescence staining (d) and apoptotic cell counting (e) in each group ($n = 4/\text{group}$). (f)–(h) Western blots of apoptosis-related proteins in each group after 48 h of treatment (f). Densitometry of cleaved caspase-3 (g) and cleaved-PARP (h) in western blots. The expression of TUBB, caspase-3 and PARP was used as internal controls ($n = 4/\text{group}$). (i), (j) Glucose and lactate concentrations in the culture medium after 3T3 cells were treated with S1-sEVs and S2-sEVs for 48 h. Culture medium samples were collected at 6, 12, 24, and 48 h for glucose and lactate assays ($n = 3/\text{group}$). (k), (l) The effects of S2-sEVs on glycolysis could be blocked by glycolysis inhibitor itaconic acid. After a 24 h-pretreatment of the glycolysis inhibitor itaconic acid, the S2-sEVs were added into the 3T3 cells. Following a 48-h incubation, the levels of glucose (k) and lactate (l) were measured in the culture medium. S2-sEVs without itaconic acid pretreatment were utilized as the positive control, while normally cultivated cells served as the negative

(Continues)

FIGURE 4 (Continued)

control ($n = 3/\text{group}$). (m) Differential regulation of S1-sEVs and S2-sEVs on macrophage polarization. Mouse BMDMs were polarized to M1 state after 24 h of LPS treatment. After that, S1-sEVs, S2-sEVs and TFF concentrate at 1×10^{10} particles/mL were added for an additional 24 h. M1 macrophage, CD11c+, CD206-; M2 macrophage, CD11c-, CD206+. (n)–(p) qRT-PCR showed the expression of proinflammatory factors *Tnfa*, *Il6* and *Nos2* mRNAs in each group. The levels of all tested mRNAs in the control group were set to 1 ($n = 3/\text{group}$). Data are shown as mean $\pm$ SEM. n.s. > 0.05 , * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. In the line graph (b), data are shown as mean $\pm$ SEM. The statistical difference between S1 group versus Ctrl group is indicated: * $p < 0.05$. The statistical difference between TFF group versus Ctrl group is indicated: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$. The statistical difference between S2 group versus Ctrl group is indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. In the line graphs (c), (i), (j), data are shown as mean $\pm$ SEM. The statistical difference between S2 group versus Cisplatin group is indicated: * $p < 0.05$, *** $p < 0.001$. The statistical difference between S2 group versus S1 group is indicated: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. All bars = 50 μm . sEVs, small extracellular vesicles.

(Figure 4I, J). The substantial elevation in glycolytic levels after S2-sEVs treatment aligns with the accelerated cell proliferation observed in the group. Moreover, when S2-sEVs were incubated with a glycolytic inhibitor, itaconic acid, the stimulating effect on glycolysis was almost completely blocked (Figure 4K, L). These findings suggest that S2-sEVs can directly regulate the glycolytic process in target cells through the active enzymes they contain.

MSC-sEVs have been widely studied for their ability to modulate macrophage polarization. In particular, they have been found to exhibit inhibitory effects on the M1 phenotype and stimulate the polarization of M2 macrophages (Zhang et al., 2018). To explore the capability of S1-sEVs and S2-sEVs on macrophage polarization, we treated mouse bone marrow-derived macrophages (BMDMs) with lipopolysaccharide (LPS) for 24 h. After polarization, S1-sEVs, S2-sEVs and TFF concentrate were added for another 24 h with the particle concentration as 1×10^{10} particles/mL. As shown in Figure 4M, LPS treatment led to the polarization of 91.6% of macrophages towards M1 state. S1-sEVs treatment caused a significant decrease in M1 macrophages to 11.8%, a reduction far more pronounced than the decreases observed in S2-sEVs and TFF concentrate treated groups, which were 76.1% and 75.8%, respectively. In addition, S1-sEVs effectively enhanced the polarization of M1 macrophages towards the M2 phenotype, achieving a remarkable conversion rate of 36.9%, which was significantly higher than the 0.41% observed with S2-sEVs and the 1.38% observed with TFF concentrate. This was further supported by qRT-PCR results which revealed the most significant decrease of M1 macrophage markers, tumour necrosis factor (*Tnfa*), nitric oxide synthase 2 (*Nos2*) and *Il6* in S1-sEVs treated group compared to LPS, S2-sEVs subpopulation and TFF group (Figure 4n–p). We also replicated the experiment on mouse macrophage cell line RAW264.7 and the result was consistent with what we observed on BMDMs (Figure S3D). Again, the immunomodulatory activity of TFF concentrate is intermediate between the S1-sEVs and S2-sEVs treated groups. Therefore, compared to both the TFF concentrate and S2-sEVs, S1-sEVs demonstrate a greater immunomodulation capacity.

3.4 | The differential regulation of S1-sEVs and S2-sEVs in hindlimb ischemia

MSCs and its derived sEVs have been widely reported in hindlimb ischemia treatment by promoting the formation of new blood vessels (Hu et al., 2015; Zhang et al., 2020; Zhang et al., 2018). To see the effects of S1-sEVs and S2-sEVs on angiogenesis and the regeneration of ischemia tissues, mouse hindlimb ischemia model was established with unilateral femoral artery ligation (Figure 5a). On the first postoperative day, same doses of S1-sEVs and S2-sEVs (1×10^{10} particles) were injected into the quadriceps area of the affected limb and bilateral lower limb flowmetry were analysed in all groups after 21 days of surgery (Figure 5b). Compared to the control group, both S1-sEVs and S2-sEVs treated mice showed some extent of blood flow recovery in the operated lateral hindlimb. However, upon analysing the ratio of ischemic to non-ischemic signal densities, only S2-sEVs treatment exhibited a significant increase of blood flow in the ligated hindlimb (Figure 5c). The bilateral quadriceps of all mouse groups were then collected, and we observed a reduction in the size of the operated lateral quadriceps in both the control and S1-sEVs treated mice compared to the non-operated lateral quadriceps (Figure 5d). However, in the mice treated with S2-sEVs, the weight of the ischemic lateral quadriceps even showed a slight increase, providing evidence for the complete recovery after tissue injury (Figure 5e). Hematoxylin-eosin (H&E) staining further revealed well organized muscle fibres in S2-sEVs treated quadriceps, whereas in control and S1-sEVs groups, the fragmented muscle tissues were still evident (Figure 5f). Although we observed decreased TUNEL signals in both S1-sEVs and S2-sEVs treated quadriceps, 5-ethynyl-2'-deoxyuridine (EdU) labelling only showed stronger proliferating signals in the S2-sEVs group (Figure 5g–j). Consistent with EdU results, Ki67 and CD31 double staining showed the capillary regeneration in S2-sEVs treated quadriceps but not in control and S1-sEVs groups (Figure 5k). Moreover, after vessel counting, S2-sEVs treated quadriceps demonstrated an even higher capillary numbers compared to sham quadriceps (Figure 5l). As is well known, the induction of HIF-1 α is essential for neovascularization in an ischemic and hypoxic environment (Shen et al., 2013; Zhou et al., 2016). Again, we observed an increase of HIF-1 α expression in S2-sEVs treated quadriceps, when compared with S1 group (Figure 5m,n). Additionally, protein blotting experiments on muscle tissues from each group revealed that the expression levels of both HIF-1 α and p-HIF-1 α were significantly higher in the S2-sEVs treatment group compared to all other groups, including the S1-sEVs treatment group (Figure 5o). Therefore, in contrast to S1-sEVs, S2-

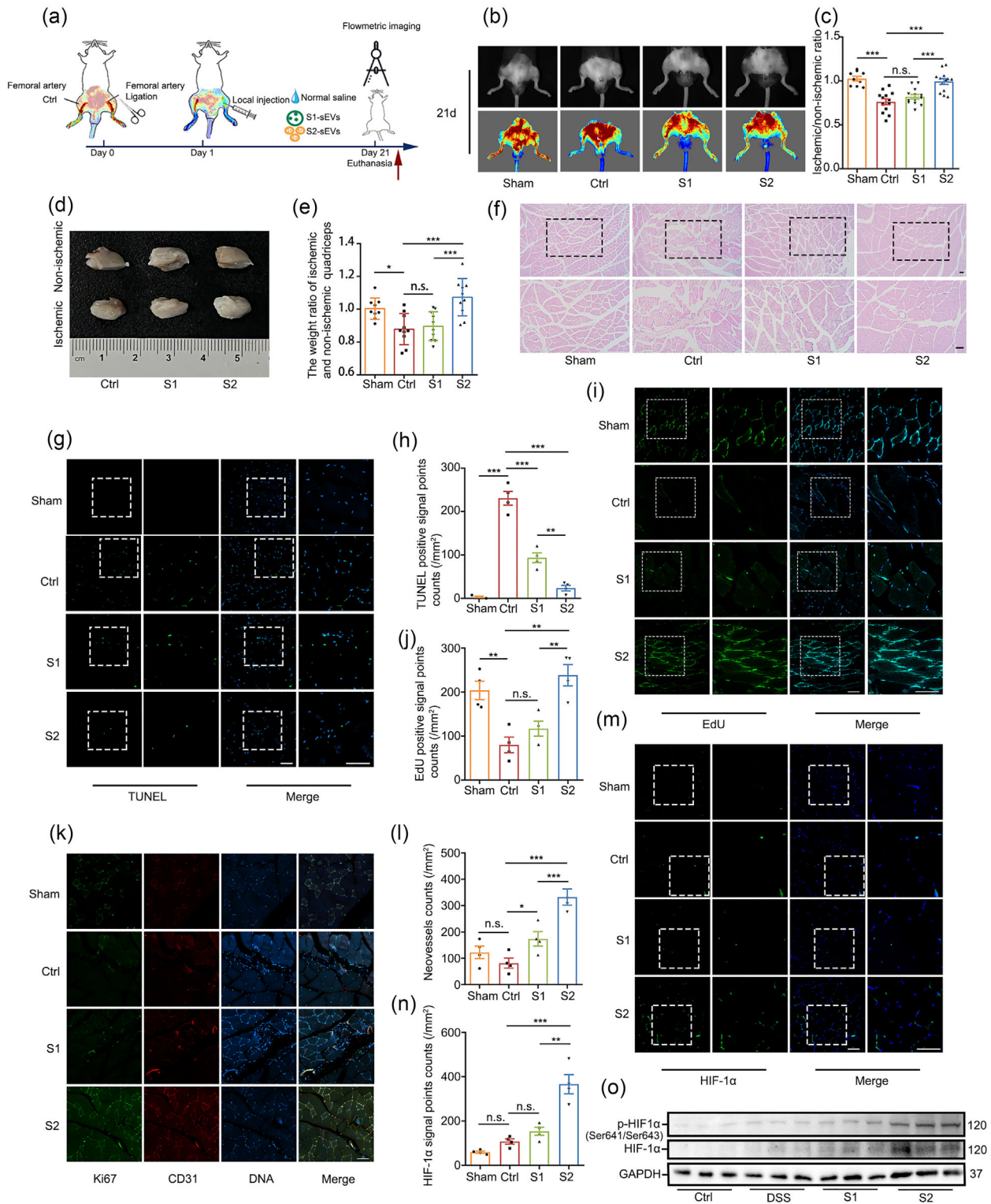


FIGURE 5 The differential therapeutic effects of S1-sEVs and S2-sEVs in hindlimb ischemia. (a) A flowchart outlining the use of different sEVs subpopulations for the treatment of a mouse model of lower limb ischemia. The operated lateral quadriceps were given a single injection of PBS (Ctrl) or 1×10^{10} particles of S1-sEVs (S1) or S2-sEVs (S2). (b), (c) Hemodynamic imaging (b) and blood flow statistics (c) in each group ($n = 9$ in sham group, $n = 13$ in every other group). (d) The size of the quadriceps muscle in each group of mice. (e) The weight ratio of operated lateral quadriceps (ischemic) to sham lateral quadriceps (non-ischemic) ($n = 9$ in sham group, $n = 10$ in every other group). (f) H&E staining of operated lateral quadriceps in each group. Morphology of quadriceps from sham mice were used for comparison ($n = 4$ /group). (g), (h) TUNEL staining (g) and apoptotic cell counts (h) in each group ($n = 4$ /group). Green: TUNEL signals; blue: DNA. EdU staining (i) and proliferating cell counting (j) in each group ($n = 4$ /group). Evaluation of angiogenesis in each group by immunofluorescence staining of Ki67 and CD31 (k) and neovessel counting (l) ($n = 4$ /group). Immunostaining of HIF-1 α (m) and HIF-1 α positive cell counts (n) in each group ($n = 4$ /group). (o) Western blots of HIF-1 α and p-HIF-1 α (Ser641/Ser643) in quadriceps muscle of each group. GAPDH was used as the internal control ($n = 3$ /group). Data are expressed as mean $\pm$ SEM. n.s. > 0.05 , * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All bars = 50 μ m. H&E, Hematoxylin-eosin; sEVs, small extracellular vesicles.

sEVs exhibit superior pro-angiogenic properties, resulting in rapid and complete hindlimb repair and regeneration following ischemia-induced injury.

3.5 | Roles of S1-sEVs and S2-sEVs in skin wound healing

MSCs and their derived sEVs have been extensively studied for their roles in skin wound healing caused by surgical, trauma, burns, or diabetic diseases (Bakhtyar et al., 2018; Bian et al., 2022; Zhang et al., 2015). To see the effects of the isolated sEVs subpopulations in skin wound healing, we established a rat model with deep second-degree burn injury. On day 0 after scalding, S1-sEVs, S2-sEVs or PBS were injected subcutaneously at multiple sites around the wound with a total particle size 1×10^{10} . The trauma area was measured in each group and the trauma closure rate was recorded. The results showed the noticeably accelerated wound contraction occurred in S2-sEVs treated rats after day 7 of injury (Figure 6a–c). The skin tissues of the burn site were collected at day 14 after scalding, and the wound healing was evaluated and scored using H&E staining (Sen et al., 2020). As shown in Figure 6e, the wounds in the model rats were still devoid of epidermis and only partial re-epithelialization was observed in S1-sEVs treated group. However, in S2-sEVs treated rats, we found reconstructed stratified epidermis and well-formed dermal structures with typical secondary features such as sebaceous glands and hair follicles. Undoubtedly, wound scoring demonstrated the highest score of the S2-sEVs group (Figure 6d). When compared to the model skin, TUNEL staining showed a significant decrease in apoptosis in wounds treated with both S1-sEVs and S2-sEVs (Figure 6f,j). Proliferating cell nuclear antigen (PCNA) and alpha smooth muscle actin (α SMA) staining also revealed increased fibroblast cell proliferation in both treated groups, however, the S2-sEVs group exhibited an exceptionally higher number of proliferating cells (Figure 6g,h,k). Masson staining revealed large amounts of scar-forming myofibroblast in the wound area of the model rats. Shattered and scanty collagen fibres were found to be dispersed within the myofibroblast. Although a slight improvement of collagen production was observed in S1-sEVs treated wounds, the distribution of fibroblast was still disorganized. In contrast to these two groups, we found the best collagen deposition with well-organized fibroblast distributed in the wound of S2-treated rats (Figure 6i). Consistently, qRT-PCR results demonstrated the significantly increased expression of transforming growth factor beta 1 (*Tgfb*), actin alpha 2 (*Acta2*) and collagen family members, collagen I (*Col1*) and collagen III (*Col3*) in S2-sEVs treated wounds (Figure 6l–o). The increased protein level of α SMA was then manifested in S2-sEVs group. Meanwhile, it showed a significant reduction on the expression of the pro-fibrotic factor Vimentin (Figure 6p). The activation of glycolysis has been reported to be essential for restoring the structure of injured tissues in wounds (Kim et al., 2023). Given that S2-sEVs has been characterized for their cargos related with glycolysis, and that the activation of glycolytic pathway is also associated with their stimulating effect on 3T3 cell proliferation, we next assessed the glycolytic levels in injured tissues of different groups by examining the expression of glycolytic enzymes ALDOA and ENO1. These two proteins have been identified as node proteins in S2-sEVs and their reduced expression has also been linked to diabetic wound healing (Liu et al., 2023; Lu et al., 2023). As shown in Figure 6p, the result demonstrated the significant elevation of protein levels of ALDOA and ENO1 in S2-sEVs treated tissues. Based on our findings, it can be concluded that S2-sEVs exhibits highly beneficial effects on cutaneous wound healing through its positive impact on glycolysis.

3.6 | The distinct therapeutic effect of S1-sEVs and S2-sEVs in DSS-induced colitis

Inflammatory bowel diseases (IBD), mainly including ulcerative colitis and Crohn's disease, are characterized by intestinal chronic inflammation. Due to the well-known immune regulatory functions of MSCs or MSC-derived sEVs, numerous studies have proposed their potential benefits in both experimental and clinical studies (Huldani et al., 2022). Following our discovery of the differential regulation of macrophage polarity by S1-sEVs and S2-sEVs, we proceeded to evaluate their immune regulatory functions in a mouse colitis model. In the study, mice were injected intravenously with same doses of S1-sEVs, S2-sEVs (1×10^{10} particles) or PBS at day 1, day 4 and day 7 after starting the DSS treatment (Figure 7a). Firstly, both DiR labelling of S1-sEVs and S2-sEVs were found robustly accumulated in the intestines 12 h after injection (Figure 7b,c). After DSS induction, body weight decreased dramatically over time, particularly after 5 days of treatment. S2-sEVs injection has no rescuing effect on this body loss. However, we found a significant improvement of body weight in S1-sEVs treated mice (Figure 7d). We then scored the disease in mice of all groups based on blood in the stool, weight decrease and physical posture (Guo et al., 2022). The mice in the control group was scored as 0 and we found a remarkable rescuing effect after 6 days of S1-sEVs treatment (Figure 7e). On day 10 after DSS administration, we collected the intact colon to measure their length. Both the morphology and the statistical graph revealed the significant increase of colon length in S1-sEVs treated mice compared to PBS and S2-sEVs groups (Figure 7f,g). Moreover, Swiss roll section of colon histological analysis revealed that the destruction of villus structure, loss of crypt, and inflammatory cell infiltration in PBS and S2-sEVs treated colon. However, in the S1-sEVs treated colon, a complete villus structure was observed, indicating that the mucosal damage caused by DSS has been effectively restored. The colon histological scores also supported the rescuing effect of S1-sEVs treatment (Figure 7h,i). Immunofluorescence of F4/80 was then performed to label macrophages in affected colon tissues. In S1-sEVs treated mice, seldom macrophages were detected in the colon villi, however,

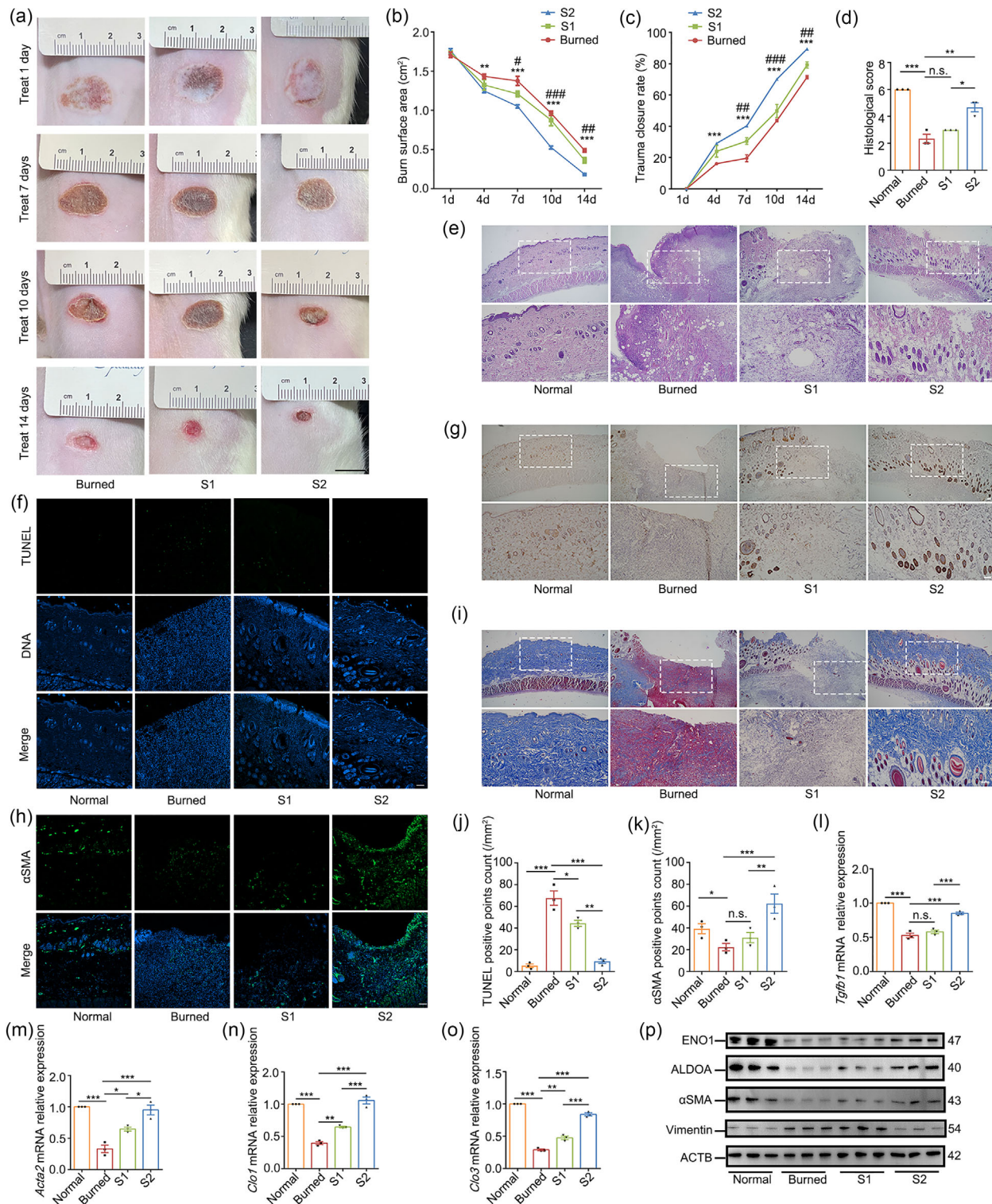


FIGURE 6 The differential effects of S1-sEVs and S2-sEVs on skin wound healing. Model rats were subcutaneously injected with PBS or 1×10^{10} particles of S1-sEVs and S2-sEVs on days 1, 5 and 10 after scalding. Wound closure in each group was then followed every day until day 14 after scalding. (a) Skin appearance in different treatment groups on days 1, 7, 10 and 14 after burns ($n = 3/\text{group}$). Measurement of burn surface area (b) and wound closure rate (c) in each group ($n = 3/\text{group}$). H&E staining of skin wound and histopathological scores in each group ($n = 3/\text{group}$). TUNEL staining (f) and apoptotic cell counts (j) in skin wound of each group ($n = 3/\text{group}$). Green: TUNEL signals; blue: DNA. (g) Cell proliferation shown by PCNA staining in skin wound of each group ($n = 3/\text{group}$). Fibroblast labelled by α -SMA (h) and Fibroblast cell counts (k) in skin wound of each group ($n = 3/\text{group}$). Green: α -SMA; blue: DNA. (i) Masson staining in skin wound of each group ($n = 3/\text{group}$). Blue: collagen; red: fibrosis. Relative expression of *Tgfb1*, *Acta2*, *clo1*, *clo3* mRNAs in skin wound of each group ($n = 3/\text{group}$). The levels of all tested mRNAs in the control group were set to 1. (p) Western blots of ENO1, ALDOA, α -SMA, and Vimentin in wound skin of each group ($n = 3/\text{group}$). Data are expressed as mean $\pm$ SEM. n.s. > 0.05, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. In the line graphs (B, C), the statistical difference between S2 group versus Burned group is indicated: ** $p < 0.01$, *** $p < 0.001$. The statistical difference between S2 group versus S1 group is indicated: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. Bar in (a) = 1 cm; Bars in others = 100 μm . H&E, Hematoxylin-eosin; sEVs, small extracellular vesicles.

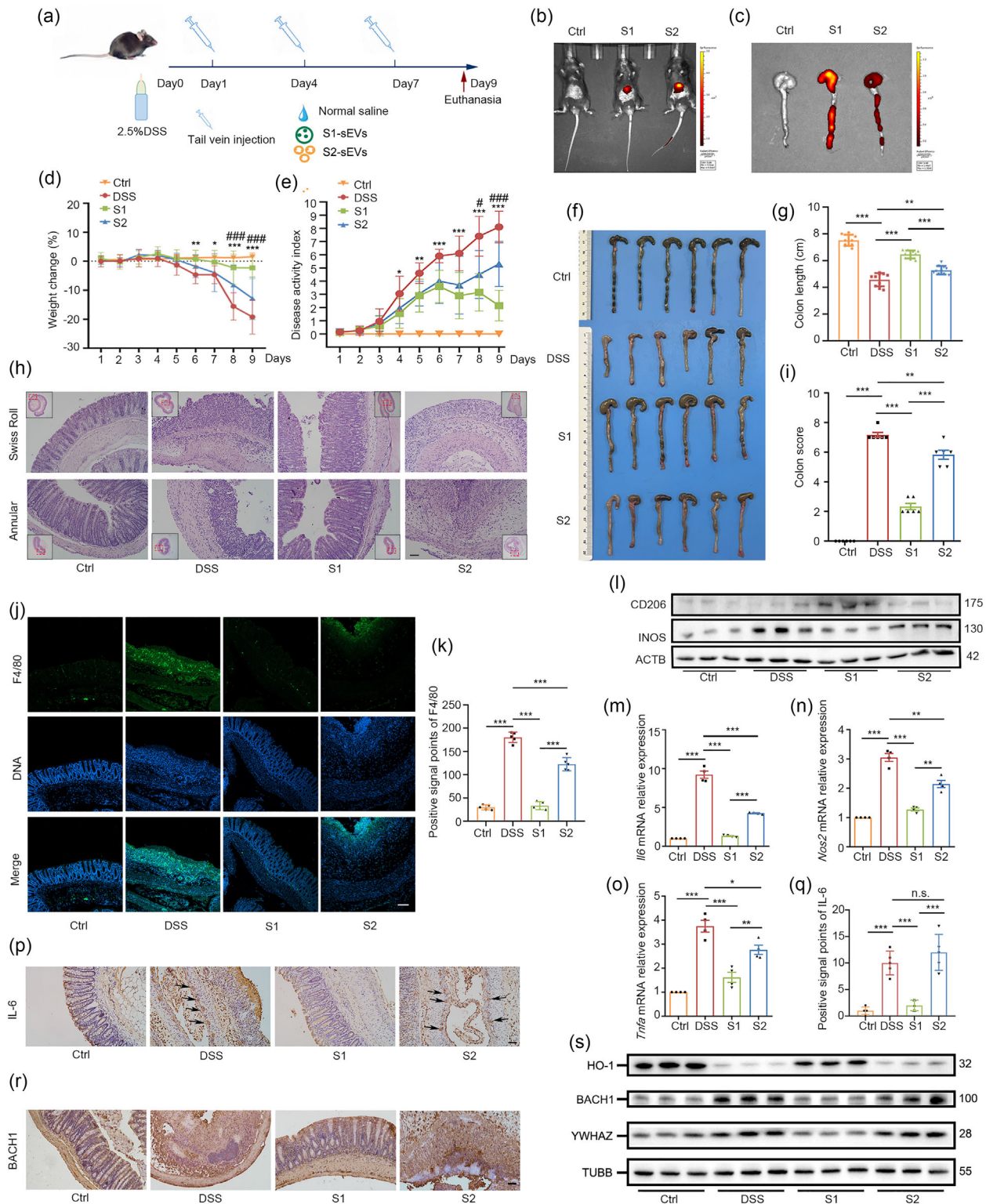


FIGURE 7 The distinct therapeutic effects of S1-sEVs and S2-sEVs in DSS-induced colitis. (a) Schematic diagram demonstrated the model of DSS-induced colitis in mouse. Mice were intravenously injected with saline, 1×10^{10} particles of S1-sEVs, and S2-sEVs at day 1, day 4 and day 7 of DSS treatment ($n = 10/\text{group}$). In vivo fluorescence images of DiR-labelled S1-sEVs and S2-sEVs (b) and the distribution of sEVs in the colon of each group (c). Saline or DiR-labelled S1-sEVs and S2-sEVs were administrated to mice via intravenous injection. After 12 h, mice in each group were subjected to in vivo fluorescence imaging. Evaluation of weight change (d) and DAI in each group of mice ($n = 10/\text{group}$). Appearance of the colon (f) and measurement of the colon length (g) in each group of mice ($n = 10/\text{group}$). H&E staining (h) and histopathological score (i) of the colon tissue in each group of mice ($n = 6/\text{group}$). Macrophage infiltration by F4/80 immunostaining (j) and macrophage cell counting in the colon tissue of each group (l) ($n = 5/\text{group}$). Green: F4/80; blue: DNA. (l) Western blots of NOS2 and CD206 expressions in colon tissue of each group. ACTB was used as the internal control ($n = 3/\text{group}$). (m)–(o) Relative expression levels of *Il6*, *Nos2*, *Tnfα* mRNAs in colon tissues of each group. The levels of all tested mRNAs in the control group were set to 1 ($n = 4/\text{group}$). Immunostaining of IL-6 (p) and IL-6 positive cell counting (q) in colon tissue of each group ($n = 5/\text{group}$). (r) Immunohistochemically staining of BACH1 in

(Continues)

FIGURE 7 (Continued)

colon tissue sections of each group. (s) Western blots of BACH1, YWHAZ and HO-1 expressions in colon tissue of each group. TUBB was used as the internal control ($n = 3/\text{group}$). Data are expressed as mean $\pm$ SEM. n.s. > 0.05 , * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. In the line graphs (d), (e), the statistical difference between S1 group versus DSS group is indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The statistical difference between S1 group versus S2 group is indicated: # $p < 0.05$, ## $p < 0.001$. All bars = 50 μm . DAI, disease activity index; H&E, Hematoxylin-eosin; sEVs, small extracellular vesicles.

PBS and S2-sEVs treated mice showed large amounts of positive signals in the necrotic tissues (Figure 7j,k). Immunofluorescence staining with NOS2 and CD206 was then performed to label the distribution of different macrophage subtypes in the colon tissues. As shown in Figure S3E, macrophages in the colon tissues of mice treated with PBS or S2-sEVs were predominantly of the M1 type, while those in the colon tissues of mice treated with S1-sEVs were relatively fewer and predominantly of the M2 type. The Western blot experiments for NOS2 and CD206 in the colon tissues of mice from each group also supported this result (Figure 7l). Consistently, when we examined the mRNA expression levels of pro-inflammatory cytokines (*Tnfa*, *Il6*, *Nos2*), the colon treated with S1-sEVs exhibited the lowest expression of these factors compared to the DSS and S2-sEVs groups. In fact, the mRNA levels of these inflammatory factors in the S1-sEVs treated colon were comparable to those observed in the normal control group (Figure 7m-o). IL-6 immunostaining then revealed the stronger staining signals in necrotic tissues, especially in the macrophages line on the basal layer of DSS and S2-sEVs treated colon, however, in S1-sEVs treated colon, the infiltration of macrophage was seldom observed and the numbers of IL-6 positive cells decreased significantly (Figure 7p,q). According to what we have reported, the miRNA profiles of S1 and S2 subpopulation sEVs were quite different. When analysing the network of differential miRNAs and target genes in S1-sEVs subpopulation, the hub genes related with inflammatory regulations, included *BACH1*, *YWHAZ*, *IL6*, and *IL6R*, were identified (Figure 3h). As a transcriptional inhibitor of heme oxygenase 1 (HO-1), deficiency of BACH1 has been reported to ameliorate 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis through increased expression of HO-1 (Harusato et al., 2013). YWHAZ, an adapter protein, has also been implicated in the immune response of bone marrow-derived dendritic cells (BMDCs) in DSS-induced colitis (Feng et al., 2022). BACH1 immunostaining were then performed and the results showed the lower staining signals in S1-sEVs groups as compared to its staining in necrotic tissues of DSS and S2-sEVs treated colons (Figure 7r). Figure 7s showed the protein levels of HO-1, BACH1, and YWHAZ in each group. We observed a dramatic reduction of BACH1 and YWHAZ proteins along with a significant increase in HO-1 in colons treated with S1-sEVs. Thus, our results revealed the specificity of S1-sEVs in the treatment of DSS-induced colitis.

3.7 | The differential regulation of biogenesis in the two subpopulations of sEVs

Both the ESCRT-dependent pathway and ESCRT-independent pathway have been reported to direct the biogenesis of sEVs. In the study, we found the differential expression of sEVs markers in S1-sEVs and S2-sEVs. For example, HGS, also known as a subunit of ESCRT-0, was highly expressed in S1-sEVs, while CD63, the marker of the ESCRT-independent pathway, was enriched in S2-sEVs (Figure 1g). This means the subpopulations of sEVs may be generated through different intracellular pathways. To see if such differences in sEVs characteristics between S1-sEVs and S2-sEVs are consistent in other cells, we applied the same isolation method to separate sEVs from the culture media of 293T cells. After performing western blot analysis on the collected fractions, similar to what we observed in MSC-derived S1-sEVs and S2-sEVs, differential expressions of HGS, CD9, ALIX and CD63 were also found in the subpopulations of sEVs derived from 293T cells (Figure S4A,B). The findings suggest the existence of common mechanisms involved in regulating the biogenesis of different sEVs subpopulations. Recent reports suggest that HGS is capable of recognizing and directing ubiquitinated cargos from the early endosome to the MVBs through its multiple ubiquitin-interacting motifs (UIMs) (Edgar et al., 2014; Katzmann et al., 2003; Shields et al., 2009). To further verify the biogenesis of S1-sEVs is through the ESCRT-dependent pathway, we explored the trafficking of EGFR, which is an established HGS substrate in the canonical endocytosis pathway, into two subpopulations of sEVs derived from MSCs (Flores-Rodriguez et al., 2015; Henne et al., 2011). The ubiquitinated EGFR was first examined by immunoprecipitation in S1-sEVs and S2-sEVs. Although EGF proteins could be observed in both S1-sEVs and S2-sEVs, the level of EGFR ubiquitination was much higher in the S1-sEVs (Figure 8a). Actually, we observed increased ubiquitination levels in S1-sEVs overall when using a pan-ubiquitin antibody for protein blotting (Figure 8d). We then introduced exogenous Flag-tagged ubiquitin in MSCs and the results demonstrated the increased overall ubiquitination levels as well as ubiquitinated EGFR in S1-sEVs (Figure 8c-e). Recent research has revealed that PTP1B-mediated dephosphorylation of EGFR appears to be important for the interactions of ubiquitinated EGFR with HGS (Wong et al., 2018). We then knocked down *PTP1B* in MSCs with specific siRNAs and meanwhile, co-transfected with CD63-Myc and HGS-HA to check the distribution of EGFR in intra-luminal vesicles (ILVs). The knockdown efficiency of *PTP1B*-siRNA was verified by protein blotting (Figure 8f). Figure 8g showed the decreased ubiquitination levels of EGFR after knockdown of *PTP1B* in MSC-sEVs. In control cells, the colocalization of EGFR, HGS and CD63 suggested the correct trafficking of EGFR in ILVs (Figure 8h,i). However, in *PTP1B* knockdown cells, EGFR failed to localize in ILVs (Figure 8h,j). Immunoprecipitation for both proteins further revealed that the interactions between EGFR and HGS were almost completely blocked in *PTP1B* knockdown cells (Figure 8k,l).

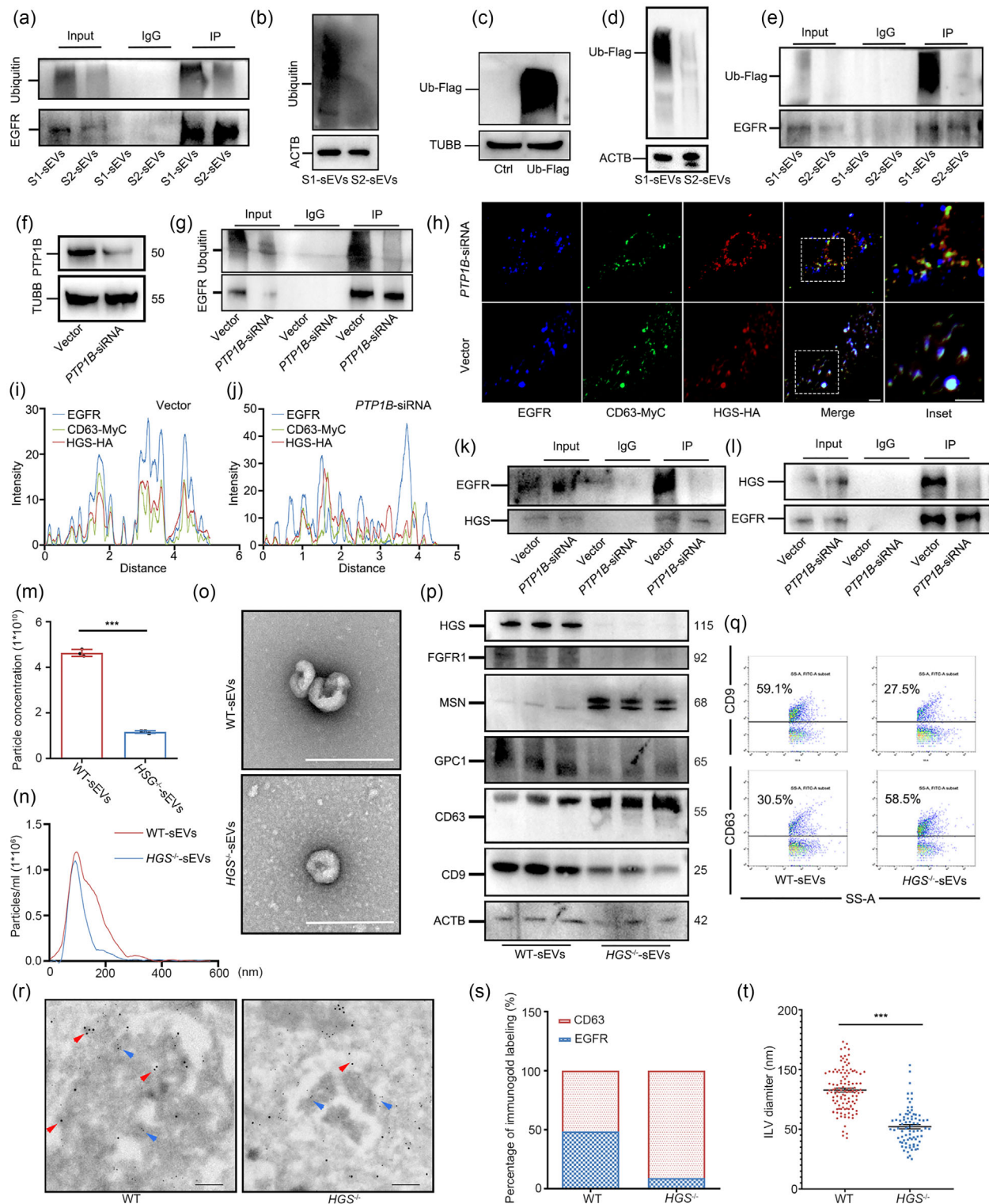


FIGURE 8 Differential regulation of biogenesis between the two subpopulations of sEVs. (a) The immunoprecipitation analysis of EGFR ubiquitination in S1-sEVs and S2-sEVs. (b) Western blots of ubiquitin levels in S1-sEVs and S2-sEVs. (c)–(e) Distribution of ubiquitinated EGFR in S1-sEVs. HucMSCs were transfected with Ubiquitin-Flag (Ub-Flag) plasmid and the expression of Ub-Flag was detected in HucMSCs (c), S1-sEVs and S2-sEVs (d). Immunoprecipitation of EGFR with exogenous Ub-Flag in S1-sEVs and S2-sEVs (e). (f) Knockdown of *PTP1B* in HucMSCs. HucMSCs were transfected with *PTP1B* siRNA for 48 h and cells were collected for western blot. (g) Immunoprecipitation analysis of EGFR ubiquitination after knockdown of *PTP1B* in sEVs isolated from HucMSCs. (h) Immunofluorescence of EGFR, CD63 and HGS in MVBs of HucMSCs. CD63-Myc and HGS-HA were co-transfected in control and *PTP1B* knockdown HucMSCs to label MVBs. Blue: EGFR; green: CD63-Myc; red: HGS-HA. MVBs in the inset of each group were magnified. (i), (j) Fluorescence co-localization analysis of EGFR, CD63 and HGS in control (i) and *PTP1B* knockdown HucMSCs (j). Fluorescence co-localization analysis range: signals inside the white rectangular box. (k), (l) Protein interactions between EGFR and HGS by immunoprecipitation in normal and *PTP1B* knockdown MSCs. (m), (n) NTA analysis of particle concentrations (m) and particle distributions (n) after HGS being knockout in 293T cells. (o) TEM of representative sEVs derived in wild type (WT-sEVs) and HGS knockout 293T cells (HGS^{-/-}-sEVs). (p) Western blots of known and newly identified sEVs markers in

(Continues)

FIGURE 8 (Continued)

WT-sEVs and *HGS*^{-/-}-sEVs. (q) NanoFlow cytometry analysis of sEVs markers CD9 and CD63 in WT-sEVs and *HGS*^{-/-}-sEVs. (r) Immunogold labelling of ILVs by CD63 (small dots, blue arrow) and EGFR (large dots, red arrow) in wild type and *HGS*^{-/-} 293T cells. (s) Distribution of CD63 and EGFR positive signals in a single MVB of WT and *HGS*^{-/-} 293T cells. (t) Scatterplot of ILV diameters in WT and *HGS*^{-/-} 293T cells. All experiments were repeated for at least 3 times. Data are expressed as mean $\pm$ SEM. ****p* < 0.001. Bars in (h) = 10 μ m; Bars in others = 200 nm. EGFR, epidermal growth factor receptor; HucMSCs, human umbilical cord mesenchymal stem cells; ILVs, intra-luminal vesicle; sEVs, small extracellular vesicles.

Since other growth factor receptors were also enriched in S1-sEVs, to see if ubiquitination represents a common mechanism for the target selection of these growth factor receptors in sEVs, we performed immunoprecipitation assays with endogenous or exogenous ubiquitin to detect FGFR1, both the results demonstrated the specifically enriched ubiquitinated FGFR1 in S1-sEVs (Figure S4C,D). However, in *PTPIB* knockdown cells, the interactions between FGFR1 and HGS and the trafficking of FGFR1 in ILVs was not affected (Figure S4E-I). Consequently, these findings suggest the critical role of HGS in directing the ubiquitinated cargos into S1-sEVs, although different regulatory mechanisms may be at play regarding the ubiquitination of the specific cargos.

Next, to investigate the necessity of HGS in the biogenesis of sEVs subpopulations, we created a *HGS* knockout cell line by CRISPR-Cas9 technology in 293T cells. The deletion of HGS was first manifested by protein blotting (Figure S3J). We then collected culture media from wild type (WT) and *HGS* knockout 293T cells for sEVs isolation. After NTA analysis, we found a significant decrease of sEVs production from *HGS*-knockout cells (from 4.64×10^{10} /mL to 1.17×10^{10} /mL), along with identifiable differences in particle size distributions (average particle size from 121.29 nm to 74.88 nm) (Figure 8m,n). The representative sEVs derived from WT and *HGS* knockout cell line in TEM was shown in Figure 8o. Western blot demonstrated a significant decrease in the expression of FGFR1, GPC1 and CD9, which are known to be enriched in S1-sEVs, in the *HGS*^{-/-} sEVs. Conversely, the expression of CD63 and MSN, markers typically associated with S2-sEVs, exhibited a significant increase in the *HGS*^{-/-} sEVs (Figure 8p). Nano-flow detection was employed to assess the expression levels of CD9 and CD63 in both WT and *HGS*^{-/-} sEVs. In comparison to the 59.1% CD9 and 30.5% CD63 expression observed in WT sEVs, a decrease to 27.5% CD9 and an increase to 58.5% CD63 expression were observed in the *HGS*^{-/-} sEVs (Figure 8q). Thus, the above results suggest that deletion of *HGS* induces a significant reduction on the biogenesis of S1-sEVs, but it had little effect on the production of S2-sEVs. Finally, we used immunogold labelling electron microscopy to detect the distributions of EGFR (large) and CD63 (small) particles in MVBs of WT and *HGS*^{-/-} cells (Figure 8r). In WT cells, both EGFR and CD63 particles were found to localize on ILVs of MVBs. However, in *HGS*^{-/-} cells, most of EGFR particles were found to distribute outside of MVBs and the percentage of EGF particles on ILVs decreased from 48.54% to 9.16%. Meanwhile, dramatically decreased MVB size and ILV diameters were found in *HGS*^{-/-} cells which the average ILV diameter in *HGS*^{-/-} cells decreased from 82.75 to 52.13 nm (Figure 8s,t). Our findings suggest that the HGS-dependent ESCRT pathway directs the biogenesis of S1-sEVs. Recently, RAB31-FLOTs machinery has been reported to sort CD63 into sEVs by passing the ALIX-ESCRT-III pathway. Due to the enrichment of CD63 and lipid raft protein FLOT1 and FLOT2 in S2-sEVs (Figure 2c,d), our results demonstrate that the S1-sEVs and S2-sEVs are secreted through different regulatory mechanisms, which are referred to as the ESCRT-dependent and the ESCRT-independent pathways, respectively.

4 | DISCUSSION

Nanosized sEVs are increasingly recognized as MSC substitutes due to their comparable therapeutic features. Their low immunogenic and tumorigenic properties, especially their ability to cross the blood-brain barrier and deliver their cargo to damaged brain tissue, may endow them greater clinical potential compared to their parent cells in various therapeutic applications (Phinney & Pittenger, 2017; Rani et al., 2015; Yari et al., 2022). However, the highly heterogeneous nature of sEVs, including variations in size, carried cargos, and intracellular biogenesis pathways, poses challenges for their clinical progress (Kimiz-Gebologlu & Oncel, 2022). Until now, no single method for isolating EVs is perfect (Chen et al., 2021; Li et al., 2017). A common practice is to combine two or more isolation methods to overcome the deficiencies of individual techniques, while simultaneously improving the overall purity and productivity of the isolated vesicles (Veerman et al., 2021). TFF is a fast-processing technique in which the sample flow is directed in parallel to a semipermeable membrane (Visan et al., 2022). This setup allows for concentration of the sample while simultaneously removing non-EV components (Busatto et al., 2018). SEC is another ideal methodology for EVs isolation due to its gentle nature and the ability to eliminate contaminating materials (Monguió-Tortajada et al., 2019). When combined with TFF, the inherent limitations of column loading volume in SEC can be addressed through the concentration of the conditioned media by TFF. Therefore, TFF-SEC workflow has been proposed as a reliable and robust EV isolation approach for scalable production of sEVs with high purity. (Visan et al., 2022) In the study, by using TFF-SEC workflow, we could obtain $1-2 \times 10^{13}$ particles/L conditional media. Compared to only $1-2 \times 10^{11}$ particles produced from 1 L conditioned media by ultracentrifugation method, TFF-SEC actually achieved a 100-fold increase in sEVs yield (Takov et al., 2019). This ratio is similar as what was observed by other comparative studies (Visan et al., 2022; Watson et al., 2018). Moreover, after SEC purification, we obtained two MSC-sEVs subpopulations, S1-sEVs and S2-sEVs. The two subpopulations of sEVs exhibited significant heterogeneity in terms of size, membrane markers, cargo contents, as well as biological functions. With commercially available TFF

systems capable of filtering more than 1000 L and industrial-grade SEC columns that can be packed on-site for large-scale applications (Subramanian et al., 2005), our study presents an appealing workflow for the production of industrial- and Current Good Manufacture Practices (cGMP)-grade MSC sEVs subpopulations with functional specificity for the treatment of clinical diseases.

It's now well acknowledged that sEVs exhibit heterogeneity not only in origin, density, and size, but also in the composition and functions (Mathieu et al., 2021; Willms et al., 2016). However, because various isolation methods were used, it's hard for proper classification and nomenclature (Ma et al., 2024). For example, a novel kind of EVs smaller than sEVs (peak around 80.9 nm) were isolated when higher centrifugation speeds were applied (Lee et al., 2019). These small EVs expressed most of sEVs markers (CD63, tumor susceptibility 101 (TSG 101), heat shock protein 70 (Hsp70)) but are negative for CD81 expression. Through a combination of polymer-based precipitation and size exclusion chromatography (Pre-SEC), two subpopulations of EVs (148.9 ± 0.45 nm vs. 124.0 ± 0.95 nm) were isolated from a breast cancer cell line MDA-MB-468 with differential proteomics (Martínez-Greene et al., 2021). In our study, the two sEVs subpopulations we identified also exhibited distinct particle sizes. Moreover, in the collected continuous fractions, which correspond to the two sEVs subpopulations (S1-sEVs and S2-sEVs), we observed gradual density changes of sEVs markers with decreased expression of CD9, ALIX and HGS in S1-sEVs fractions (FC1–FC6), and increased expression of CD63 (FC7–F12) in S2-sEVs fractions. Although most of differentially expressed proteins are contained in both subpopulations, unique proteins were still identified in S1-sEVs and S2-sEVs, respectively. We found the enriched membrane proteins in S1-sEVs and identified with a series of specific markers representative of each subpopulation. These markers are good candidates that can be used for immune-isolation of specific sEVs subpopulations in future research and clinical applications. In the study, our results showed that S2-sEVs significantly enhanced cellular proliferation more than S1-sEVs when the same particle number of both was applied to 3T3 cells. Additionally, S2-sEVs showed stronger abilities of angiogenesis and tissue regeneration in mouse hindlimb ischemia and skin wound healing models. From GO and KEGG pathway analysis, the terms of metabolic pathway and glycolysis/gluconeogenesis were specifically enriched in S2-sEVs. These are consistent with what we observed about the increased expression of HIF-1 in mouse hindlimb ischemia model and the elevated protein levels of glycolytic enzymes, ENO1 and ALDOA in wounds after S2-sEVs treatment. Both ALDOA and ENO1 are hub proteins identified in S2-sEVs. Preincubation of glycolysis inhibitor itaconic acid with S2-sEVs significantly decreased the glycolytic levels in 3T3 cells. Previous studies have demonstrated significantly decreased expressions of ALDOA and ENO1 in diabetic wound healing (Liu et al., 2023; Lu et al., 2023). Therefore, the significant increase in ALDOA and ENO1 protein levels in S2-sEVs group indicates that active glycolysis occurred, which accelerated wound repair following S2-sEVs treatment. Unlike the cell type-dependent distribution patterns of sEVs markers in Exo-S (60–80 nm) and Exo-L (90–120 nm) isolated by AF-4 (Zhang et al., 2018), our results revealed an identical expression pattern of specific sEVs markers, including CD9, HGS, ALIX and CD63 in the two sEVs subpopulations isolated from both MSCs and 293T cells. Relying solely on particle size without considering the isolation methods has led to a confusing functional annotation of different sEVs subpopulations. Based on the repeatability of TFF combined with SEC to generate S1-sEVs and S2-sEVs from MSCs with distinct cargos, sEVs markers and functions, our study provides a valuable avenue to explore potential applications with the two sEVs subpopulations.

While MSC-based therapies have been extensively tested for the treatment of a variety of diseases, it is true that MSCs are not retained in organs for more than a few hours. Although many paracrine growth factors and cytokines secreted by MSCs have been identified, none of them has been proven sufficient to mediate the effect of MSCs (Deng et al., 2018). Therefore, sEVs derived from MSCs, which are able to transport a large cargo of proteins, lipids, and nucleic acids and serve as intercellular messengers, has been proposed and tested for producing therapeutic benefits similar to those of MSCs (Rani et al., 2015; Weng et al., 2021). However, it's hard to tell if the heterogeneous sEVs as a whole, or specific sub-populations, are responsible for the therapeutic benefits observed. As we know, the mechanisms underlying the therapeutic effects of sEVs derived from MSCs can be explained by their pro-angiogenic, anti-apoptotic, anti-inflammatory, anti-hypoxic or anti-fibrotic properties, depending on the specific type of disease being treated (Lai et al., 2015; Yin et al., 2019). In our study, we have demonstrated that S2-sEVs exhibit stronger pro-angiogenic, anti-apoptotic and regenerative abilities when compared to S1-sEVs. In contrast, S1-sEVs exhibit a unique immunomodulatory property on macrophage polarity and DSS-induced colitis, which is not observed within S2-sEVs. Numerous experimental studies have demonstrated the therapeutic potential of MSC-sEVs in various degenerative and inflammatory disorders, such as Crohn's disease, graft-versus-host disease, diabetic nephropathy and organ fibrosis (Ebrahim et al., 2018; Heidari et al., 2021; Lin et al., 2022; Shi et al., 2018; Zhang et al., 2018). Both local and systemic administration of MSCs derived sEVs reveal an efficient suppression on detrimental immune response in inflamed tissues. Although the mechanisms underlying these therapeutic effects are multifaceted, in general, they can be attributed to the ability of sEVs to regulate damaged tissues, enabling them to form a balanced inflammatory and regenerative microenvironment in the presence of vigorous inflammation. Macrophages have been identified as the most important cells driving colon inflammation (Morales et al., 2014; Zhang et al., 2020). In the study, S1-sEVs showed distinct immunosuppression on M1 polarity in LPS treated mouse BMDMs and RAW264.7 macrophages. In DSS-induced mouse colitis, after 9 days of DSS treatment, S1-sEVs showed the rescuing effect in terms of body weight, disease index, colon length, colon morphology and decreased expression of inflammatory factors. On the contrary, no beneficial effects were observed in mice treated with S2-sEVs. This is related with the differential miRNAs contained between the two sEVs subpopulations. In S1-sEVs, we enriched more specific miRNAs compared to S2-sEVs. Moreover, our results also revealed the decrease of the target hub genes of these miRNAs, such as BACH1, YWHAZ, IL6 and IL6R in

S1-sEVs treated mouse colitis. Thus, our study suggests the presence of a specific anti-inflammatory mechanism in one of the subpopulations of MSC-sEVs. Further research is needed to determine if S1-sEVs treatment shows this specificity in other inflammatory disorders. Nevertheless, this finding provides a new insight into implementing disease-specific therapy using specific sEVs subpopulations from MSCs.

sEVs contain ubiquitinated proteins (Bissig & Gruenberg, 2014; Katzmann et al., 2001). Both HGS and STAM1, which are components of the ESCRT-0 complex, are responsible for identifying ubiquitinated cargoes (Bache et al., 2003). HGS then recruits TSG101, a component of the ESCRT-I complex, which helps to recruit the ESCRT-III complex, either via ESCRT-II or ALIX, for membrane deformation and abscission during ILV biogenesis (Christ et al., 2016; Larios et al., 2020; Teo et al., 2006). In our study, we observed a notable enrichment of HGS and ALIX, along with significantly increased ubiquitin levels, in S1-sEVs. Specially, when using EGFR as a representative, we found the enrichment of ubiquitinated EGFR in S1-sEVs. In the study, more than 100 membrane proteins were identified in S1-sEVs which include several receptor tyrosine kinases, such as FGFR1, platelet derived growth factor receptor alpha (PDGFRA), platelet derived growth factor receptor beta (PDGFRB), HGFR or insulin like growth factor 2 receptor (IGF2R) et al. HGS plays a pivotal role in regulating the endocytic sorting of ubiquitinated receptor tyrosine kinases for subsequent degradation in lysosomes (Belleudi et al., 2009). Besides EGFR, we also observed the HGS-dependent sorting of ubiquitinated FGFR1 in S1-sEVs. In addition to protein degradation, ubiquitination is involved in various biological processes such as endocytosis, DNA modification, and signal transduction (Ageta & Tsuchida, 2019). Thus, our results implicate the HGS-dependent secretion of ubiquitinated receptor tyrosine kinases in S1-sEVs. This finding suggests a potential new mechanism by which MSCs regulate the functions of target cells. Interestingly, in S1-sEVs, we also identified the presence of all components of 20S core proteasome. Previous study reported the presence of functional 20S proteasomes in MSC sEVs (Lai et al., 2012). These proteasomes work synergistically with other cargo molecules within the sEVs to mitigate tissue damage by reducing the levels of oligomerized proteins in a mouse model of myocardial infarction. In line with the study, our findings further validated the mechanism underlining cellular extrusion and extracellular transport of proteasomes through sEVs. Moreover, based on the enrichment of 20S proteasome in S1-sEVs, we propose an ESCRT-dependent secretion pathway for functional proteasomes into extracellular space. It has been reported circulating proteasomes are functionally active. Extracellular alveolar proteasomes are believed to play a crucial role in alveolar maintenance, lung injury, and repair (Sixt & Peters, 2010). These extracellular proteasomes have also been postulated to be important in degrading soluble peptides to avoid potentially pathogenic protein aggregation, such as Alzheimer's amyloid plaques (Haass & Selkoe, 2007). Future study will explore if the enrichment of proteasomes or ubiquitinated proteins, particularly ubiquitinated tyrosine kinases in S1-sEVs is responsible for its unique immunomodulation in different animal disease models.

Mechanisms controlling the biogenesis of ILVs include three primary pathways: the ESCRT-dependent machinery (Katzmann et al., 2001; Wenzel et al., 2018); tetraspanins cluster membrane-associated molecules at domains prone to ILV formation (Silva et al., 2021); RAB31-FLOTs machinery dependent on lipid raft microdomains (Wei et al., 2021). In our study, the two subpopulations of sEVs are formed through ESCRT-dependent and ESCRT-independent pathway, respectively. When HGS was knocked out in 293T cells, in addition to the decrease in sEVs production and the reduced size of ILVs and sEVs, we found a significant increase of MSN and CD63 in *HGS*^{-/-} sEVs. This is consistent with the previous report that depletion of HGS promotes the formation of a uniformly sized population of small ILVs, whose formation requires CD63 (Edgar et al., 2014). Our results then revealed the enrichment of non-ubiquitinated EGFR in S2-sEVs. Moreover, in *PTPIB* knockdown MSCs, only the interactions between EGFR and HGS was reduced, however, it did not completely affect the transport of EGFR in CD63-positive ILVs. Based on the enrichment of lipid raft pathway-dependent proteins, FLOT1 and FLOT2, in S2-sEVs, our findings indicate RAB31-FLOTs machinery being involved in the biogenesis of S2-sEVs.

5 | CONCLUSION

Here, our study provides an industrial-scale approach using a TFF-SEC system for the scalable production of distinct sEVs subpopulations from HucMSCs. We identified and characterized two distinct sEVs subpopulations, S1-sEVs and S2-sEVs, which differ in their biogenesis, characterization, and cargo content. These differences lead to variations in their biological functions. While MSC-derived sEVs are well-regarded for their clinical potential, our research introduces a novel concept of harnessing these distinct subpopulations of MSC sEVs for targeted therapeutic applications in specific diseases.

AUTHOR CONTRIBUTIONS

Wei Liu: Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); project administration (lead); software (lead); supervision (lead); validation (lead); visualization (lead); writing – original draft (lead). **Xinyu Wang:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); software (equal); validation (equal); visualization (equal). **Yating Chen:** Data curation (equal); methodology (equal); software (equal); validation (equal); visualization (equal). **Jiapei Yuan:** Software (equal); validation (equal); visualization (equal). **Huiyu Zhang:** Validation (equal); visualization (equal). **Xin Jin:** Funding acquisition (supporting); resources (supporting); validation (supporting). **Yuying Jiang:**

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CONFLICT OF INTEREST STATEMENT

T.W. is a member of the board of directors of Kornelis Bio-pharmaceutical Company Limited, Nanjing, China. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Raw data and extracted text files for quantitative proteomics have been deposited into PRIDE, under accession nos. PXD050064. Fastq data and the processed data file for transcriptome sequencing have been deposited into the Deposited in GEO under accession nos. GSE256371.

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