



Microparticle-Embedded Stem Cell Spheroid Composite Enables High-Yield, Effective, and Homogeneous Production of Extracellular Vesicles

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Abstract

Extracellular vesicles (EVs), inherently nanoscale vesicles, hold promise for regenerative applications. However, their broader utility is limited by low production efficiency and heterogeneity. Here, we introduce a three-dimensional (3D) stem cell spheroid platform incorporating transforming growth factor- β 1 (TGF- β 1)-loaded microparticles to engineer a spatially modulated microenvironment that enhances cellular viability and homogeneity. This engineering system markedly increased EV production efficiency and enhanced compositional consistency compared with conventional spheroids. EVs produced by this system demonstrated strong chondrogenic stimulation mediated by microRNAs in vitro and exhibited regenerative efficacy in a rat osteoarthritis model, validating the performance of the system. Together, these findings present a microparticle-embedded 3D spheroid system that improves both the production efficiency and compositional consistency of EVs, providing a potential strategy for high-yield EV production in regenerative medicine.

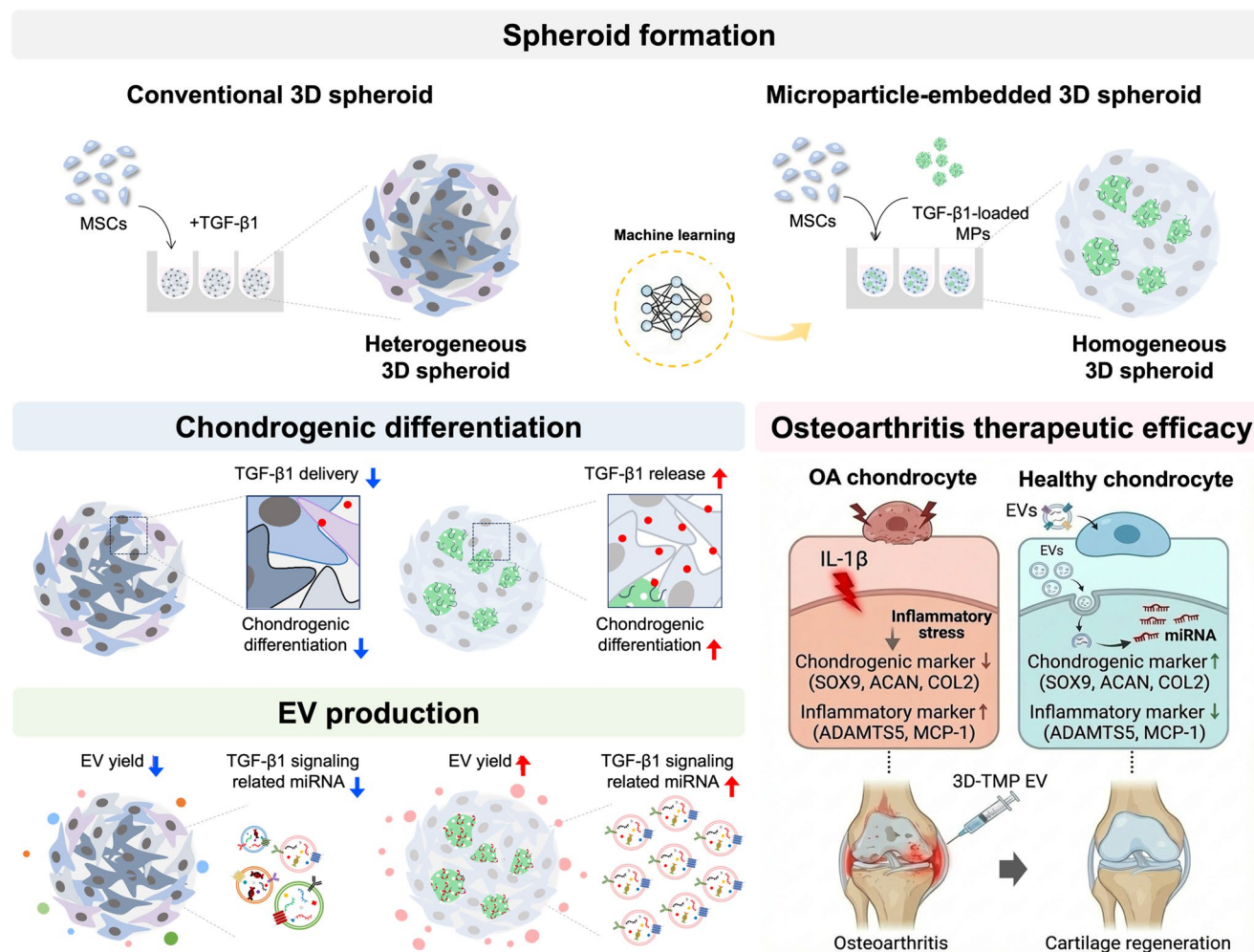
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Graphical abstract



Keywords Microparticles · 3D spheroids · TGF-β1 delivery · Extracellular vesicles · High-yield EV production · Uniform EV production · Osteoarthritis therapy

1 Introduction

Therapies employing mesenchymal stem cell-extracellular vesicles (MSC-EVs) have garnered considerable attention for their potential in regenerative medicine [1]. MSC-EVs mediate many of the therapeutic effects of MSCs through paracrine signaling by delivering bioactive cargo to target cells [2]. Notably, the potential of MSC-EVs in therapeutic applications has gained substantial attention due to their regenerative properties and ability to modulate the cellular microenvironment [3]. MSC-EVs are valuable resources for cell-free therapeutics without the inherent risks of cell transplantation [4]. However, two major challenges remain for MSC-EV-based therapies: the large-scale production of EVs in quantities sufficient for clinical application and the generation of uniform EV populations to ensure consistent

therapeutic efficacy [5, 6]. This heterogeneity arises from variations in size, composition, and origin of the EVs, underscoring the necessity for the development of cell culture technologies capable of producing EVs in large quantities with high uniformity and functional consistency [7, 8].

To improve EV production, there are various strategies, including chemical, mechanical stimulation, and structural modulation approaches [9]. For instance, chemical modulation strategies such as nutrient deprivation, pH regulation, and pharmacological preconditioning using small molecules or growth factors have been shown to enhance EV secretion by altering intracellular signaling pathways and metabolic states. Mechanical stimulation approaches, including shear stress, irradiation, and ultrasound, have also been reported to promote EV release through mechanotransduction and stress-responsive pathways. In addition, structural

modulation using three-dimensional (3D) culture systems has emerged as a promising strategy to enhance EV yield by providing a more physiologically relevant microenvironment and enabling high-density cell culture [10]. However, the simplest 3D spheroid cultures face challenges that lead to unequal exposure to oxygen, nutrients, and signaling molecules, which causes regional differences in cell viability, gene expression, and phenotypic commitment [11]. Additionally, inner core cells may experience hypoxia-induced stress or necrosis, while peripheral cells are more responsive to external stimuli [12]. This spatial imbalance exacerbates the heterogeneity of cells constituting the spheroid, impairing coordinated cellular response for consistent EV biogenesis and functionality.

To address these challenges, we propose introducing regenerative factor-loaded microparticles (MPs) into the 3D spheroid culture system. The incorporation of MPs provides spatial interstices within the spheroid core, enabling uniform and consistent delivery of oxygen and nutrients to the central region, thereby improving the overall cellular microenvironment. In particular, loading transforming growth factor-beta 1 (TGF- β 1), a key regulator of cartilage regeneration and a critical stimulator of chondrogenic differentiation in MSCs, into MPs is expected to facilitate the generation of spheroids with enhanced cartilage properties [13–15]. Importantly, the MP-mediated delivery system allows TGF- β 1 to be evenly distributed throughout the spheroid, including into the central region, thereby promoting homogeneous cellular differentiation and potentially amplifying regenerative efficiency compared to conventional culture systems [16–18]. Meanwhile, we utilized poly(lactide-co-glycolide) (PLGA), a widely used polymer, to fabricate the MPs due to the biocompatibility and biodegradability of PLGA [19]. Notably, PLGA is approved by the U.S. Food and Drug Administration (FDA) for various biomedical applications [20]. Growth factor release from PLGA MPs is governed by diffusion and polymer degradation, with the release profile adjustable through modifications to polymer characteristics such as molecular weight, copolymer composition, and crystallinity [21]. Therefore, by optimizing these formulation parameters, the release kinetics of proteins can be customized to meet the specific demands of a given application.

Building on these concepts, this study aimed to establish an MP-incorporated 3D spheroid system that enables the formation of homogeneous spheroids, which enhances the production of high-quality EVs. We further hypothesized that engineering the microscale presentation of biochemical cues would not only enhance spheroid uniformity but also modulate EV molecular composition in a controlled manner. To establish optimal MP-incorporated 3D spheroid conditions, we combined experimental optimization

with machine learning analysis to support the interpretation of parameter-outcome relationships related to spheroid homogeneity and functional performance. The therapeutic potential of this system was then evaluated through comprehensive analyses of the spheroids and EVs produced under these optimized conditions. Furthermore, single-cell mRNA sequencing was performed to elucidate the underlying molecular mechanisms, highlighting the advantages of our proposed strategy over traditional methods. In addition, the therapeutic efficacy of this system was evaluated in the context of cartilage regeneration.

2 Methods

2.1 Two-dimensional cell cultures

The human MSCs (hMSCs) were obtained from human adipose tissue as previously described [22]. hMSCs were cultured in low-glucose Dulbecco's Modified Eagle's Medium (DMEM; Cytiva, USA) at 37 °C under 5% CO₂. The medium containing 10% (v/v) fetal bovine serum (FBS; Cytiva, USA) and 1% (v/v) antibiotic–antimycotic solution (AA; Cytiva, USA). Use of hMSCs was approved by the Institutional Review Board (IRB) of Dongguk University (Approval Nos. DUIRB-202210-18 and DUIRB-202301-14).

2.2 Fabrication of agarose microwell arrays

Agarose microwell arrays were fabricated using a polydimethylsiloxane (PDMS) mold, which was replicated from a commercially available silicone microwell array (StemFIT 3D[®], H389600L, South Korea) consisting of 389 wells, each 600 μ m in diameter. A sterile agarose solution (2%, w/v in DMEM; Sigma-Aldrich, USA) was prepared by autoclaving at 120 °C for 20 min. The molten solution (60–80 °C) was transferred into PDMS molds for solidification at room temperature (RT). After complete gelation, the agarose structure was carefully removed from the mold, yielding agarose microwell arrays suitable for 3D cell culture.

2.3 Three-dimensional spheroid cultures using agarose microwell

To form 3D spheroids, hMSCs (5×10^5 cells per microwell array) were seeded onto the agarose microwell arrays in 1 mL of culture medium, with or without 0.5 mg of PLGA MPs –either control MPs (CMPs) or TGF- β 1-loaded MPs (TMPs). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 h to allow spheroid formation. After 24 h, the generated spheroids were carefully transferred to a 100 mm culture dish pretreated with

anti-adhesion solution (incubated for 30 min) to prevent spheroid attachment. To remove unaggregated cells and free MPs, the suspension was filtered through a 40 µm cell strainer. The collected spheroids were then cultured in chondrogenic induction medium composed of DMEM, 10% (v/v) FBS, 1% (v/v) penicillin–streptomycin (P/S; Cytiva, USA), 1X insulin–transferrin–selenium (ITS; Thermo Fisher Scientific, USA), 50 µg/mL ascorbic acid (Sigma-Aldrich, USA), 100 nM dexamethasone (Sigma-Aldrich, USA), and 10 ng/mL TGF-β1 (PeproTech, USA). For spheroids containing TMP, the chondrogenic medium did not include exogenous TGF-β1, as the MPs served as the localized source of the growth factor.

2.4 Preparation and characterization of PLGA MPs

To prepare PLGA MPs, an aqueous solution containing 0.72 g of sucrose (Sigma, USA) in 0.6 mL of distilled water was first prepared. Separately, the organic phase comprised 50 mg PLGA (Sigma-Aldrich, USA) in dichloromethane (DCM; Sigma-Aldrich, USA). The two solutions were then mixed and emulsified using a homogenizer at 14,500 rpm for 3 min. The resulting emulsion was added dropwise to 50 mL of 1.25% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich, USA) solution under continuous stirring at 500 rpm for 12 h to allow for solvent evaporation.

After complete evaporation of DCM, PLGA MPs were collected by first removing particles larger than 100 µm using a 100 µm cell strainer. The remaining suspension was centrifuged at 3000 rpm for 10 min to collect the MPs. The collected particles were washed three times by centrifugation (3000 rpm, 10 min) using distilled water. Finally, the purified PLGA MPs were lyophilized and stored for further use. The PLGA MP sizes were determined by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK), and their morphologies were analyzed via SEM.

2.5 Loading of TGF-β1 into PLGA MPs

To facilitate ionic binding between gelatin and TGF-β1, 25 µL of a gelatin (Sigma-Aldrich, USA) solution (20 mg/mL in distilled water) was mixed with 25 µL of a TGF-β1

solution (16 µg in 25 µL distilled water). The mixture was allowed to react at RT for 30 min, resulting in a 50 µL solution containing ionically bonded gelatin and TGF-β1.

Subsequently, 10 mg of lyophilized PLGA MPs was mixed with the 50 µL gelatin–TGF-β1 solution. The mixture was subjected to sonication at RT for 30 min to promote TGF-β1 loading into the MPs by absorption. After incubation, the particles were collected by centrifugation at 3000 rpm for 10 min and washed three times with 50 mL of distilled water. The TGF-β1-loaded PLGA MPs were then lyophilized and stored for further use.

2.6 Characterization of TGF-β1 release from TGF-β1-loaded PLGA MPs

To analyze the release profile of TGF-β1, the resuspended TMPs (2 mg/mL) in PBS were placed on a shaker (100 rpm, 37 °C). At predetermined time points, the supernatant was harvested. The concentration of TGF-β1 in the harvested solution was quantified using the Human TGF-β1 DuoSet (R&D Systems, USA).

2.7 Reverse transcription quantitative PCR (RT-qPCR) for mRNA expression analysis

To extract total RNA from hMSCs, TRIzol reagent (Invitrogen, USA) was used according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from the isolated RNA using the PrimeScript™ 1st strand cDNA Synthesis kit (TAKARA, Japan). Quantitative PCR (qPCR) was conducted on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA) in combination with Power SYBR Green PCR Master Mix (Applied Biosystems, USA). Primer sequences are provided in Table 1. Relative mRNA levels were normalized to *Ribosomal protein S18 (18s)* expression.

2.8 Live/dead assay of 3D spheroid

To evaluate cell viability within 3D spheroids, a live/dead assay was performed using LIVE/DEAD™ Viability/Cytotoxicity kit (Thermo Fisher Scientific, USA). Spheroids were incubated with 2 µM Calcein AM and 4 µM ethidium homodimer-1 (EthD-1) in PBS at 37 °C for 30 min in the dark. Following incubation, the spheroids were washed gently with PBS and imaged using a microplate reader (Cytation 3; BioTek, USA). Live cells were visualized as green, and dead cells were visualized as red. Fluorescence images were analyzed using ImageJ software to quantify the ratio of live and dead cells.

Table 1 Primers used for RT-qPCR analysis

Genes	Forward	Reverse
<i>h 18s</i>	5'-GTA ACC CGT TGA ACC CCA TT-3'	5'-CCA TCC AAT CGG TAG TAG CG-3'
<i>h SOX9</i>	5'-GTA CCC GCA CTT GCA CAA C-3'	5'-TCT CGC TCT CGT TCA GAA GTC-3'
<i>h ACAN</i>	5'-GCC TGC GCT CCA ATG ACT-3'	5'-ATG GAA CAC GAT GCC TTT CAC-3'
<i>h COL2A1</i>	5'-CAC GTA CAC TGC CCT GAA GGA-3'	5'-CGA TAA CAG TCT TGC CCC ACT T-3'

2.9 Machine learning based validation of 3D spheroid optimization

To analyze the relationship between experimental parameters and chondrogenic differentiation efficiency, multiple machine learning models, including support vector machines (SVM), gradient boosting, decision trees, AdaBoost, and random forest regression, were evaluated using Orange software (University of Ljubljana). The dataset includes combinations of cell number (625, 1250, 2,500, 5,000), differentiation day (3,7,14), and cells-to-MPs ratio (140:1, 70:1, 35:1) as input variables, and SOX9 mRNA expression as the output variable. Data from 625, 2,500 and 5,000 cells were used for training and validation, while 1,250 cells were reserved as an external test set. The random forest regression model was configured with 10,000 trees, a maximum depth of 10, and a minimum of 5 samples required to split subsets, with three attributes considered at each split. Model performance across the validation and test datasets was quantified using the coefficient of determination (R^2), root mean square error (RMSE), mean square error (MSE), and mean absolute error (MAE). In addition, the distribution of predicted scores under different experimental parameters was analyzed to interpret the relative influence of each variable and to identify optimal spheroid conditions.

2.10 Immunofluorescence-based spatial analysis of TGF- β 1 expression in 3D hMSC spheroids

Immunofluorescence staining was performed to visualize the expression and localization of TGF- β 1 in 3D spheroids. Three groups of spheroids (3D/T, 3D-CMP/T, and 3D-TMP) were analyzed following standard immunofluorescence protocols. Briefly, spheroids were collected and washed 2–3 times with $1\times$ PBS and subsequently fixed in 4% paraformaldehyde (PFA) at 4 °C overnight. The fixed spheroids were embedded in a HistoGel™ solution and processed using a conventional paraffin embedding procedure. Samples were dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin blocks. Paraffin-embedded spheroids were sectioned at a thickness of 5 μ m using a microtome.

For immunostaining, the sections were rehydrated through a descending ethanol series and washed with PBS. After rehydration, non-specific binding was blocked by incubating the sections with 5% bovine serum albumin (BSA) in PBS at RT for 30 min. The sections were then incubated with a primary anti-TGF- β 1 antibody (1:500 dilution; Santa Cruz Biotechnology, USA) at 4 °C overnight, followed by an incubation with a secondary Alexa Fluor 488-conjugated anti-rabbit antibody (Thermo Fisher Scientific, USA) at RT for 2 h. Nuclei were counterstained with

0.5 $\times$ DAPI for 30 min. Fluorescence images were obtained using a fluorescence microscope.

To evaluate the spatial distribution of TGF- β 1 expression within the spheroids quantitatively, the fluorescent intensity was analyzed in concentric zones sectioned from the center to the periphery of the spheroid using the Clock Scan and Radial Profile plug-ins in the ImageJ software (NIH, USA).

2.11 Single-cell RNA-sequencing

Single cells isolated from spheroids were washed with 0.04% (w/v) BSA in PBS. Cell numbers were counted using an automated cell counter (Countess II, Thermo Fisher Scientific, USA). According to the manufacturer's protocol, the Chromium Next GEM single Cell 3' Reagent Kit v3.1 (10x Genomics, Pleasanton, CA) was employed for preparing libraries. Cell suspensions were loaded onto a Chromium Next GEM Chip G targeting a recovery of about 10,000 cells per sample. Sequencing was performed in multiplex on a NovaSeq X Plus system (Illumina, San Diego, CA) with an average depth of at least 50,000 reads per cell.

2.12 Single-cell RNA-sequencing data analysis

Cell Ranger version 9.0.0 (10X Genomics, CA) was employed to process sequencing reads with the Human reference transcriptome GRCh38 from Ensembl. Downstream analyses were conducted in R v4.2.2, following the generation of gene expression matrices. Data processing and cluster identification were performed using the WinSeurat program (Ebiogen Inc, Korea) based on Seurat v4.2.2. R package [23]. A total of 12,981 and 13,671 cells were initially detected in the 3D/T and 3D-TMP groups. Cells with low transcript complexity (< 500 UMIs and < 300 genes) and those exhibiting high mitochondrial content ($> 10\%$ UMIs) were excluded before downstream analysis. After quality control filtering, 12,598 and 13,367 cells were retained for downstream analysis in the 3D/T and 3D-TMP groups. Data normalization was performed using the scTransform method, and gene expression values were scaled while regressing out total UMI counts and mitochondrial gene percentages per cell. Visualization and differential gene expression testing were subsequently carried out with customized modifications to the standard workflow. Dimensionality reduction was achieved through principal component analysis, and the top 20 components were retained to define clusters at a resolution of 0.8. Cluster structures were subsequently visualized using uniform manifold approximation and projection (UMAP).

2.13 Western blot analysis

2D and all 3D condition cells were washed with PBS three times and was sonicated using an Ultrasonic Homogenizer (10 s at 20% amplitude) in radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich, USA) containing phosphatase inhibitor (Sigma-Aldrich, USA) and protease inhibitor (Merck, USA) to ensure complete lysis. The lysates were centrifuged (13,000 rpm at 4 °C for 30 min), and the supernatant was collected. Total Protein concentration was determined and equalized by the Pierce BCA protein assay kit (Thermo Scientific, USA). 20 µg of protein samples were separated by gel electrophoresis with 10% sodium dodecyl sulfate-polyacrylamide gel. The transferred nitrocellulose (NC) membranes (Bio-Rad, USA) were blocked in Tris-buffered saline (TBS) solution with 0.05% (v/v) Tween-20 (TBS-T) containing 5% (w/v) skimmed milk (BD Difco™, USA) for one hour. The following three times washed with TBS-T, incubated with primary antibodies in 5% (w/v) BSA solution in TBS-T overnight at 4 °C. The following primary antibodies were used: anti-SOX9 (Abcam, ab185966, 1:1000), anti-COL2A1 (Sigma, MAB1330, 1:2000), anti-ACAN (Abcam, ab3778, 1:1000), anti-TGF-β1 (Abcam, ab82630, 1:1000), anti-pSMAD2 (Cell Signaling Technology, #3108, 1:1000), anti-SMAD2/3 (Cell Signaling Technology, #3102, 1:1000), and anti-β-actin (Applied Biological Materials Inc., G043, 1:5000). The membranes were washed three times with TBS-T and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Anti-rabbit IgG, Cell Signaling Technology, #7074; Anti-mouse IgG, Cell Signaling Technology, #7076, 1:5000) in TBS-T containing 5% (w/v) skim milk for 2 h. For the detection and imaging of immunoreactive protein bands, Amersham ECL Select detection reagent (Cytiva, USA) and ChemiDoc™ XRS+ System (Bio-Rad, USA) were used.

2.14 Isolation and characterization of EVs

For EV isolation, cells were cultured in serum-free low-glucose DMEM for 24 h. The conditioned medium was collected and centrifuged at 1300 rpm for 3 min to eliminate cellular debris. The collected supernatant was passed through a 0.2 µm syringe filter and subsequently purified using a tangential flow filtration (TFF) system (TFF-Easy, HansaBioMed Life Sciences LTD). The filtered solution was washed three times with Dulbecco's phosphate-buffered saline (DPBS) and concentrated using an Amicon® Ultra-0.5 Centrifugal Filter Unit with a 10 kDa molecular weight cut-off (Millipore, USA) at 13,000 rpm and 4 °C for 20 min. The final EV fraction was collected from the concentrate retained above the filter membrane. Nanoparticle

tracking analysis (NTA) was conducted using a NanoSight LM10 system (Malvern Panalytical, UK) to measure the isolated EVs size distribution and concentration. The morphology of EVs was visualized by TEM using a Tecnai F20 G2 microscope (FEI, Philips).

2.15 STORM-based super-resolution imaging of EVs

Super-resolution imaging and subsequent classification of EVs were performed using a Nanoimager S (Oxford Nano-imaging, ONI Ltd, Oxford, UK), a dSTORM super-resolution fluorescence microscope. Imaging was evaluated with the built-in quantification software CODI (ONI) using the EV profiling application. EV samples were prepared according to the manufacturer's instructions for the EV Profiler Kit (EV-MAN-1.0, ONI, 210917-01, UK). EVs were immobilized on the chip surface by affinity capture and labeled with fluorescein-conjugated antibodies against the tetraspanin proteins CD9, CD63, and CD81. Labeled EVs were visualized with 488,560 and 640 nm laser excitation on the Nano-imager S system, and cluster sub-types were identified and quantified by the CODI EV profiling algorithm.

2.16 Interleukin-1β (IL-1β)-treated chondrocyte model and EV treatment

IL-1β-treated human chondrocytes were employed to establish an in vitro OA model. Human chondrocytes at passage 3 were seeded in 100 mm cell culture dishes (TPP, Switzerland) at a density of 5×10^5 cells per dish. After incubating overnight, the cells were serum-starved in low-glucose DMEM (Cytiva, USA) supplemented with 0.5% FBS for 12 h.

Following starvation, chondrocytes were treated with recombinant human IL-1β (R&D Systems, MN, USA) at final concentration of 10 ng/mL and incubated for an additional 24 h to induce an inflammatory OA-like phenotype. After IL-1β priming, the chondrocytes were harvested and reseeded into 12-well plates (Falcon, USA) in low-glucose DMEM containing 10% FBS at an appropriate cell density. After a 24-hour recovery period, 2D/T EVs, 3D/T EVs, 3D-CMP/T EVs, and 3D-TMP EVs (1×10^6 particles per well) were added to the culture medium and re-administered every 2 days for a total of 7 days. The therapeutic efficacy of EVs on IL-1β-primed chondrocytes was subsequently evaluated using gene and protein expression analyses.

2.17 Alcian blue staining

Alcian blue staining was performed to evaluate glycosaminoglycan (GAG) production in IL-1β-induced chondrocytes treated with EVs. After EV treatment, the IL-1β-induced

chondrocytes were rinsed three times with DPBS and fixed in 4% PFA (Sigma-Aldrich, USA). Following fixation and additional DPBS washes, samples were incubated with 1% (w/v) Alcian blue 8GX (Sigma-Aldrich, USA) for 1 h. The cells were gently rinsed three times with PBS and GAG deposition was observed under an optical microscope (CKX41; Olympus, Japan). The dye absorbance (590 nm) was quantified after extraction in 4 M guanidine hydrochloride (guanidine-HCl; Sigma-Aldrich, USA) using a Cytation 3 (BioTek Instruments, USA).

2.18 Establishment of the destabilization of the medial meniscus (DMM) rat model and intra-articular injection of EVs.

Female seven-week-old Sprague-Dawley rats (*Rattus norvegicus*, SD rat; Orent Bio, Inc., South Korea) were maintained under standard conditions. Ethical approval was obtained from the Institutional Animal Care and Use Committee of Dongguk University (IACUC-2022-030-1, IACUC-2024-036-1). Following a one-week acclimatization period, OA was induced in rats using the DMM model. Rats were anesthetized through an intraperitoneal injection of Zoletil (50 mg/kg; Virbac, France) combined with Rompun (10 mg/kg; Bayer, South Korea). The right knee underwent DMM surgery to serve the medial meniscus under aseptic conditions. Following DMM surgery, rats were induced for 4 weeks without treatment to allow for OA development.

After 4 weeks, animals were allocated into two independent experimental groups: one for cell-based therapy with osteochondral defect induction, and the other for intra-articular injection of EVs without additional surgery.

2.19 Intra-articular injection of EVs

Beginning in the fifth week after DMM surgery, 50 μ L of PBS or EVs derived from various cell culture conditions, 2D/T EV, 3D/T EV, 3D-CMP/T EV, 3D-TMP EV, were administered via intra-articular injection into the right knee joint (1×10^9 particles/50 μ L) to evaluate their effect on cartilage regeneration. Injections were administered every week for 4 weeks, and knee joint tissues were harvested at 4 weeks following the initial injection for further histological analysis.

2.20 Gait analysis using the ink footprint method

Gait analysis assessed locomotor function and joint impairment at 4 weeks after the first intra-articular injection, corresponding to 8 weeks after DMM surgery. After coating the hind paws with non-toxic ink, rats were guided to walk in a

straight line across a white paper walkway (120 cm $\times$ 8 cm) placed on a transparent platform. After the walk, the footprint patterns were scanned at 600 dpi and analyzed using ImageJ software.

The following gait parameters were measured: stride length (cm), the distance between two consecutive prints of the right foot, step length (cm), the distance between the left and right hind paw prints in a single step, step width (cm), the lateral distance between the left and right hind paw prints, and toe-out angle ($^\circ$), the angle between the direction of movement and the longitudinal axis of the right paw print.

2.21 Safranin O staining and OARSI scoring

Right knee tissues were harvested and fixed in 4% PFA. After decalcification in 10% ethylenediaminetetraacetic acid (EDTA, pH 7.4) with gentle agitation for 3 weeks, the samples were dehydrated through a 70–100% graded ethanol series and embedded in paraffin. and sectioned sagittal Sect. (5 μ m) were prepared, followed by deparaffinization and rehydration. Cartilage matrix content was stained with safranin O and Fast Green (Sigma-Aldrich, USA) according to standard protocols. The stained sections were then dehydrated, cleared, and mounted.

Cartilage degeneration was evaluated using the OARSI scoring system. The severity of osteoarthritic lesions was graded on a scale of 0 to 6, with higher scores indicating more severe cartilage degradation, based on structural changes and loss of safranin O staining intensity.

2.22 IHC staining

Paraffin-embedded tissue specimens were deparaffinized and rehydrated. The sections were subjected to antigen retrieval using a pepsin solution (Sigma-Aldrich, USA), following the manufacturer's instructions. After rinsing with PBS, the sections were incubated in a blocking solution at RT for 1 h to minimize non-specific binding. After blocking, the sections were incubated with a primary antibody at 4 $^\circ$ C overnight. The following primary antibodies were used: anti-COL2 (Sigma, MAB1330; 1:200), anti-MCP-1 (Abcam, ab25124; 1:200), and anti-iNOS (Abcam, ab178945; 1:200). Following three times PBS washes, the sections were incubated with a secondary antibody (GBI Labs, USA) at RT for 1 h. The sections were incubated with a streptavidin-horseradish peroxidase (HRP) conjugate and visualized using a 3,3'-diaminobenzidine (DAB) substrate. All IHC procedures were conducted using a commercial IHC detection kit (GBI kit; GBI Labs, USA) in accordance with the manufacturer's protocol. For IHC, stained

sections were counterstained with hematoxylin, dehydrated, mounted, and imaged using a light microscope.

2.23 Statistical analysis

All data are shown as the mean $\pm$ standard error of the mean (S.E.M.). Statistical analyses were performed using GraphPad Prism (v.10.3.1, GraphPad Software, USA). The two experimental groups were compared using Two-tailed Student's *t*-tests, and comparisons among multiple groups were evaluated by One-way analysis of variance (ANOVA) with Tukey's multiple comparison post hoc test. Statistical significance was defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.000$, and not significant (ns).

3 Results and Discussion

3.1 Preliminary optimization of MPs-embedded hMSC spheroid conditions supporting therapeutic EV potential

To compare the chondrogenic potential of hMSCs under different culture configurations, we evaluated hMSCs cultured with TGF- β 1 in either a conventional 2D monolayer (2D/T) or a 3D spheroid (3D/T). As shown in the schematic (Supplementary Fig. 1a), hMSC spheroids were formed in microwell arrays and stimulated with TGF- β 1 to promote chondrogenesis. A morphological assessment confirmed the formation of compact 3D spheroids, in contrast to the flat morphology observed in 2D cultures (Supplementary Fig. 1b). A qRT-PCR analysis revealed that the expression levels of key chondrogenic markers, *SRY-box transcription factor 9 (SOX9)*, *aggrecan (ACAN)*, and *collagen type II alpha 1 chain (COL2A1)*, were significantly upregulated in the 3D/T model compared to 2D/T (Supplementary Fig. 1c), indicating enhanced transcriptional activation of cartilage-specific genes. Furthermore, Gene Ontology (GO) enrichment analysis of upregulated genes in the 3D/T model relative to 2D/T highlighted biological processes associated with cartilage formation, including extracellular matrix organization, mesenchymal cell differentiation, and cartilage development (Supplementary Fig. 1d). Collectively, these findings demonstrate that 3D culture conditions markedly improve the chondrogenic differentiation capacity of hMSCs in the presence of TGF- β 1.

While 3D spheroid culturing significantly enhanced chondrogenic gene expression compared to 2D monolayers, conventional spheroids remain limited by structural heterogeneity, diffusion gradients, and poor spatial control of biochemical cues. Therefore, to address these limitations and optimize the chondrogenic potential of hMSC spheroids

further, we next developed a MP-embedded spheroid system designed to provide localized and sustained delivery of differentiation factors within the 3D microenvironment.

The porous PLGA MPs were fabricated using a double emulsion method, with gelatin incorporated to facilitate TGF- β 1 loading and promote interactions between MPs and cells (Supplementary Fig. 2a). Successful production of spherical porous MPs was confirmed through scanning electron microscopy (SEM), revealing an average diameter of $19.33 \pm 3.49 \mu\text{m}$ (Supplementary Fig. 2b–d). TGF- β 1 was loaded at an efficiency of $55 \pm 6.25\%$ (Supplementary Fig. 2e). The presence of TGF- β 1 was further confirmed through fluorescence imaging (Supplementary Fig. 2f). Analysis of the TGF- β 1 release profile demonstrated that approximately $54.13 \pm 4.73\%$ of the loaded TGF- β 1 was released within the first 24 h, with a cumulative release of $60.93 \pm 4.26\%$ over 21 days (Supplementary Fig. 2g). Spheroids were successfully formed by mixing the TGF- β 1-loaded MPs (TMPs) with cells.

To optimize the incorporation of MPs into 3D spheroids, control MPs (CMPs) at cells-to-MPs ratios of 140:1, 70:1, or 35:1 (corresponding to approximately 3,500, 7,000, or 14,000 MPs per microwell, respectively) were co-seeded with 500,000 hMSCs per microwell platform. Subsequently, spheroid formation was assessed after 24 h using bright-field microscopy, and the number of CMPs embedded within individual spheroids was quantified (Supplementary Fig. 3a–b). The quantitative analysis showed a significant increase in embedded CMPs in the 70:1 and 35:1 groups compared to the 140:1 group (Supplementary Fig. 3c). However, there was no significant difference between the 70:1 and 35:1 conditions, indicating a saturation of loading capacity beyond the 70:1 threshold (Supplementary Fig. 3d). Based on these findings, 70:1 was selected as the optimal MPs dose, as this dosage achieved efficient and uniform incorporation of MPs into spheroids without excessive loading.

In the standard 3D culture system, spheroid size increased proportionally with cell number (Supplementary Fig. 3e). Spheroids formed with 625 cells had an average diameter of $73.72 \pm 8.85 \mu\text{m}$; those with 1250 cells reached $100.01 \pm 4.39 \mu\text{m}$; spheroids with 2500 cells measured $114.28 \pm 8.23 \mu\text{m}$, and spheroids containing 5000 cells had a diameter of $151.33 \pm 10.78 \mu\text{m}$. This size increase between spheroids demonstrated a direct correlation between the spheroid diameter and cell count. When MPs with an average diameter of $20 \mu\text{m}$ were incorporated, the 3D-CMP spheroids increased in size for each cell count. Meanwhile, the spheroids formed in the 3D-CMP system with 625 cells had a diameter of $114.71 \pm 13.08 \mu\text{m}$; those with 1250 cells measured $144.71 \pm 13.08 \mu\text{m}$; spheroids with 2500 cells

reached $180.06 \pm 18.14 \mu\text{m}$, and those with 5000 cells had an average diameter of $243.26 \pm 14.01 \mu\text{m}$.

To determine the optimal cell number for the 3D-CMP system, cell viability was assessed across different spheroid sizes using a live/dead assay (Fig. 1a–c). Spheroids composed solely of 1250 cells exhibited a dead cell ratio of $46.44 \pm 2.79\%$, with a necrotic core observed at the spheroid center. In contrast, the 3D-CMP spheroids (1250 cells) showed a significantly reduced dead cell ratio of approximately $13.84 \pm 2.98\%$, with no visible necrotic zone. These findings indicate that incorporating MPs effectively enhances cell viability in spheroids with 1250 cells. However, the reduction in dead cell ratio compared to the corresponding 3D spheroids without MPs was not statistically significant for 3D-CMP spheroids with 625, 2500, and 5000 cells. Thus, the optimal cell number for forming 3D-CMP spheroids with improved cell viability was determined to be 1250 cells; these MPs contributed most effectively to spheroid health and structure.

To optimize the culture period and determine the most appropriate time point for EV collection, the temporal mRNA expression patterns of representative chondrogenic markers were analyzed in 3D-TMP spheroids (Supplementary Fig. 3f). *SOX9* expression increased about 33-fold by day 3, peaked at about 80-fold on day 7, and returned to 20-fold by day 14. Comparatively, *ACAN* remained low through day 3 but rose to 12-fold on day 7 and 14-fold on day 14. *Matrix metalloproteinase 13 (MMP13)* remained suppressed until day 7 and then increased sharply over 7 days to about 5-fold on day 14. These results indicate a distinct temporal expression profile, characterized by early upregulation of *SOX9*, subsequent induction of *ACAN*, while *MMP13* remains low until the later culture stages. Overall, the initiation of matrix synthesis and suppression of hypertrophic signaling support day 7 as the optimal culture period for harvesting therapeutically potent EVs from 3D-TMP spheroids.

Experimental optimization is effective for identifying optimal conditions, but it has limitations in systematically capturing interactions among multiple variables and overall trends. In this context, machine learning can be used as a complementary analytical tool to interpret experimental results and explore parameter and outcome relationships.

Next, a machine learning approach was applied to explore the relationship between experimental parameters and chondrogenic differentiation efficiency, using *SOX9* mRNA expression as the output variable in the experimental optimization of MP-embedded spheroid conditions (Fig. 1d). Prior to model selection, multiple machine learning models, including SVM, gradient boosting, tree, AdaBoost, and random forest (Supplementary Tables 1 and Supplementary Fig. 4). Among these, the random forest regression model demonstrated relatively stable and consistent performance

across the dataset and was therefore selected for further analysis.

The model was trained using 36 data points representing all combinations of cell number (625, 1250, 2500, 5000), differentiation days (3, 7, 14) and MPs ratios (140:1, 70:1, 35:1). The model was trained using data from three cell numbers (625, 2500, and 5000), while samples with 1250 cells were reserved for testing (Fig. 1e). The trained model yielded R^2 values of 0.495 (validation) and 0.518 (test), indicating a moderate but meaningful predictive capability (Fig. 1f–g). The model demonstrated moderate accuracy ($R^2 > 0.5$), which is meaningful given the inherent variability of in vitro systems. Nonetheless, despite the biological noise, the model explained over 50% of the variance in *SOX9* expression based on experimental inputs alone, indicating reliable predictive performance. These machine learning predictions support a rationale for optimizing 3D-CMP system fabrication conditions to enhance therapeutic EV production. Analysis of individual variables was consistent with experimental observations, revealing that *SOX9* expression peaked at day 7 and declined at day 14. Among the MPs ratios, the 70:1 condition yielded the most stable predictions (Fig. 1h). Importantly, the model predicted the highest *SOX9* expression at a cell number range of 1200–1700, supporting the selection of 1250 cells as an optimal input condition (Fig. 1i). Based on this combined experimental and computational evidence, we standardized fabrication using 1250 cells, a 70:1 cells-to-MPs ratio, and day 7 as the optimal harvest point, as these structural integrity, viability, and chondrogenic activity in a manner conducive to producing potent therapeutic EVs in the 3D-TMP system. Although the model was developed using adipose-derived MSCs, the overall optimization framework may be applicable to other MSC sources with appropriate adjustment of the input parameters. Cell-based therapies using autologous hMSCs and chondrocytes, which minimize the risk of immune rejection, have shown promising results for cartilage regeneration, making these therapies a suitable candidate for personalized treatments [24–26]. However, the clinical application of these cell-based therapies is constrained by the need for in vivo chondrogenic differentiation after transplantation, as well as challenges associated with harvesting healthy chondrocytes from OA patients and maintaining cell quality during extended in vitro expansion [27–29]. Meanwhile, inducing chondrogenic differentiation of hMSCs in vitro offers functional advantages but requires optimized culture systems to limit extended culture, contamination risks, and costs [30].

The 3D culture system better mimics the in vivo environment and promotes chondrogenic differentiation by enabling more complex cell–cell interactions compared to conventional 2D culture conditions [31, 32]. Consistent with this,

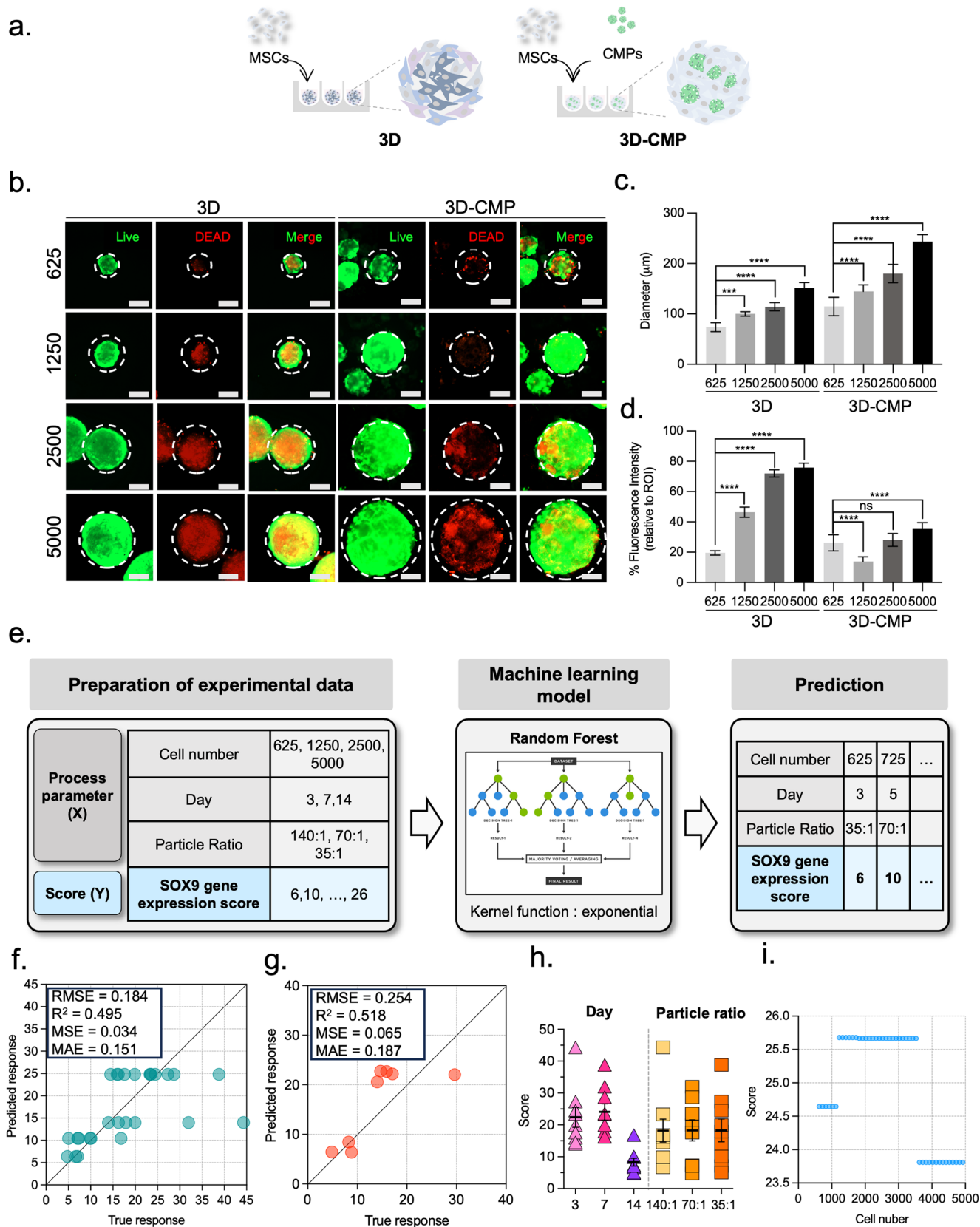


Fig. 1 Optimization of the formation of microparticle-incorporated 3D spheroids. **a** Schematic illustration of 3D spheroid formation with and without MPs. **b** Representative live (green)/dead (red) fluorescence images of 3D and 3D-CMP spheroids formed using different initial cell numbers (625, 1250, 2500, 5000). Scale bar, 100 μ m. **c** Quantification of spheroid diameter for each condition. **d** Percentage of live cells based on fluorescence intensity. **e** The workflow of machine learning model development using random forest. Experimental parameters (cell number, culture day, particle ratio) and *SOX9* gene expression score were used for model training and prediction. **f** Model validation with unseen data from cell numbers 625, 2500, and 5000. **g** Model testing with independent data from the application of 1250 cells. **h** *SOX9* gene expression scores stratified by culture day and particle ratio. **i** Score distribution according to cell number. Data represent mean $\pm$ S.E.M. ($n=3$). One-way ANOVA was used for statistical comparison (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, and not significant (ns))

mRNA sequencing analysis revealed higher expression of cartilage-related genes and ECM synthesis-related genes in the 3D system compared to the 2D system (Supplemental Fig. 1d). However, conventional 3D spheroid cultures are often hindered by necrotic core formation and heterogeneous cell distribution across the spheroid, distinguishing regions as the core, middle, and outer, which can limit therapeutic outcomes [33, 34]. Furthermore, the formation of necrotic zones within spheroids, caused by insufficient oxygen and nutrient supply, can induce extensive cell death, which increases the risk of adverse effects such as inflammatory responses and tissue damage, while making the selective isolation of viable cells from these spheroids for therapeutic use challenging. Moreover, cellular heterogeneity is further exacerbated by difficulties in controlling the differentiation status of constituent cells, combined with the limited penetration of nutrients and signaling molecules from the culture medium into the spheroid core. This heterogeneous cellular composition within the spheroid compromises the consistency and reproducibility of therapeutic outcomes [35]. To address these limitations, we developed a method to generate spheroids composed of homogeneously differentiated cells, thereby maximizing the advantages of 3D culture systems.

3.2 TGF- β 1-loaded MPs enhance TGF- β 1 delivery, cell distribution homogeneity, and chondrogenic differentiation of 3D hMSC spheroids

Next, three experimental groups were established as standard 3D spheroids (3D/T), spheroids containing CMPs (3D-CMP/T), and spheroids incorporating TMPs (3D-TMP) to evaluate the TGF- β 1 delivery efficiency of the TMPs. TGF- β 1 is an essential growth factor for cartilage formation and is well known to promote the chondrogenic differentiation of hMSCs. TGF- β 1 was added to the culture medium for the 3D/T and 3D-CMP/T groups, while the 3D-TMP group was cultured without TGF- β 1

supplementation in the medium. The spheroids were sectioned and analyzed through immunofluorescence (IF) staining to evaluate TGF- β 1 expression (Fig. 2a). Comparison of overall TGF- β 1 expression revealed a statistically significant increase in the 3D-CMP/T group compared to the 3D/T group ($p<0.0001$) (Fig. 2b). Notably, the highest TGF- β 1 expression was observed in the 3D-TMP group, suggesting that the inclusion of the TMPs enhanced TGF- β 1 delivery efficiency in the spheroid system. Thus, to investigate the spatial distribution of TGF- β 1 further, we divided the spheroids into core, middle, and outer regions and analyzed TGF- β 1 expression in each area (Fig. 2c–e). The results show that TGF- β 1 expression in the 3D group was primarily concentrated in the outer region, with 83.1% in the outer section, and only 6.2% in the core and 10.7% in the middle. Similarly, the 3D-CMP/T group exhibited 78.2% in the outer region, 8.9% expression in the core, and 12.9% in the middle. In contrast, the 3D-TMP group displayed an extensively increased homogeneous TGF- β 1 distribution across the spheroid, with 26.3% in the core, 36.9% in the middle, and 36.8% in the outer region. This homogeneous expression tendency suggests that the inclusion of TMPs significantly improved delivery throughout the spheroid. These findings indicate that while TGF- β 1 delivery in the 3D/T and 3D-CMP/T groups was restricted to the outer regions, the 3D-TMP system successfully achieved an even TGF- β 1 distribution across all regions. To further validate whether this improved distribution was associated with enhanced molecular transport, we performed a diffusion assay using FITC-labeled BSA. Spheroids with or without MP incorporation were cultured for 7 days and subsequently treated with BSA-FITC. MP-incorporated spheroids exhibited markedly enhanced penetration of BSA-FITC into the core region compared to conventional spheroids. Line-scan analysis across the spheroid diameter further confirmed increased fluorescence intensity within the central region in the 3D-CMP group (Supplementary Fig. 5). Together, these results suggest that MP incorporation enhances interstitial spacing within spheroids, thereby improving mass transport and facilitating molecular diffusion. This is supported by both enhanced penetration of externally applied molecules (BSA-FITC) and more uniform distribution of internally delivered factors (TGF- β 1).

Next, we evaluated the chondrogenic differentiation efficiency at the cellular level. After 7 days of chondrogenic induction, we compared the expression of chondrogenic markers among the 2D/T, 3D/T, 3D-CMP/T, and 3D-TMP groups. All 3D spheroid systems (3D/T, 3D-CMP/T, and 3D-TMP) demonstrated significantly higher *SOX9*, *COL2A1*, and *ACAN* expressions compared to the 2D/T group (Fig. 2g). Notably, the 3D-TMP group exhibited the highest expression of these chondrogenic marker genes

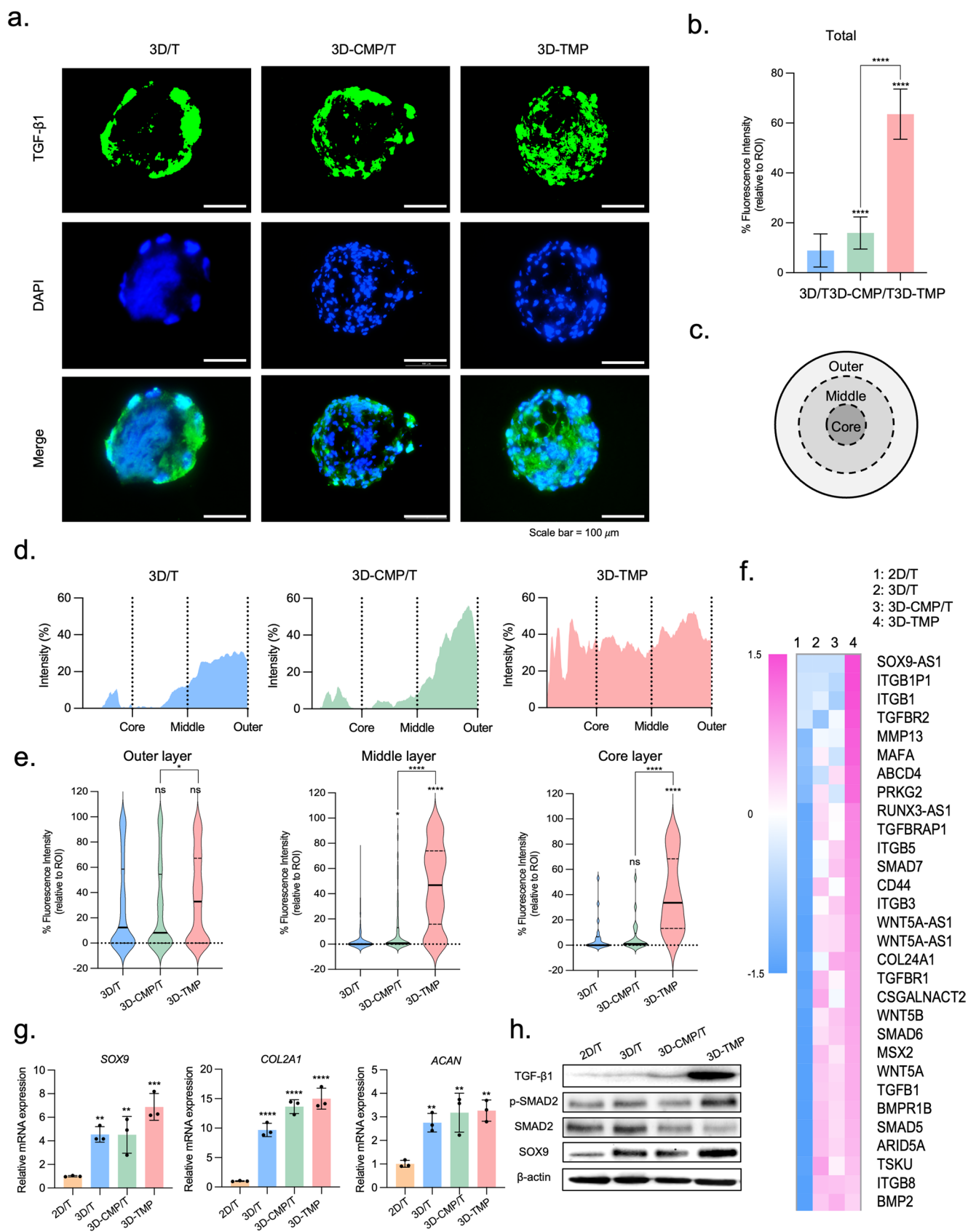


Fig. 2 TGF- β 1 distribution and chondrogenic differentiation within MP-embedded hMSC spheroids. **a** Immunofluorescence images of sectioned 3D spheroids with TGF- β 1 (green) distribution under three conditions. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). **b** Radial intensity profiles of TGF- β 1 IF across spheroid zones (core, middle, outer) for each group. **c** Quantification of total TGF- β 1 fluorescence intensity in entire spheroids. **d** Violin plots of the regional TGF- β 1 intensity across the core, middle, and outer layers. **e** Heatmap of cartilage development-related genes in spheroids based on transcriptomic profiling. **f** Relative mRNA expression of chondrogenic markers (*SOX9*, *COL2A1*, *ACAN*) in each group was measured by qRT-PCR. **g** Western blot analysis of TGF- β signaling proteins (TGF- β 1, p-SMAD2, SMAD2) and SOX9. Data represent mean $\pm$ S.E.M. ($n=3$). One-way ANOVA was used for statistical comparison (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, and not significant (ns))

($p<0.0001$). To verify whether this enhanced chondrogenic differentiation was due to improved TGF- β 1 delivery, we further analyzed the TGF- β /SMAD family member 2 (SMAD2)/SOX9 signaling pathway (Fig. 2h). Here, the TGF- β 1 expression was highest in the 3D-TMP group, which was consistent with our previous immunofluorescence (IF) staining results. This was accompanied by an increase in phospho-SMAD2 (p-SMAD2) and SOX9 protein levels, confirming the activation of the TGF- β signaling pathway.

Furthermore, to investigate how the microenvironment established by TMPs influences the cellular composition of hMSC spheroids during chondrogenic differentiation, we performed single-cell RNA sequencing on hMSC spheroids cultured under 3D/T and 3D-TMP conditions. Unsupervised Clustering and Uniform Manifold Approximation and Projection (UMAP) visualization revealed three transcriptionally distinct cell subpopulations within the spheroids (Fig. 3a). Based on transcriptional profiles, clusters were annotated as progenitor chondrocyte (PrC), regulatory chondrocytes (RegC), and stress-related chondrocyte (SrC) populations for interpretative purposes (Fig. 3b). Meanwhile, a quantitative comparison of cell type distributions showed that 3D-TMP spheroids exhibited a marked enrichment of RegCs (80%), while reducing the proportion of SrCs (16%) and PrCs (3.8%) (Fig. 3c and Supplementary Fig. 6). In contrast, 3D/T spheroids displayed a more heterogeneous distribution, with a substantial presence of SrCs (44%) and a lower percentage of RegCs (48%). These findings suggest that localized and sustained TGF- β 1 delivery via MPs promotes a more homogeneous cellular composition, potentially contributing to the enrichment of regulatory chondrocytes and reduction stress-related cell populations. These subpopulations were defined based on cluster-specific differentially expressed genes (DEGs) identified by comparing each cluster to all others (Fig. 3d) [36–38]. The PrC population was characterized by the expression of proliferation-related genes (*CDC20*, *UBE2C*, *CENPF*, and

BIRC5) (Fig. 3e). The RegC population was characterized by the expression of genes related to extracellular matrix organization and regulatory processes (*TGFBI*, *COL11A1*, *STC2*, and *CCND1*) (Fig. 3f). The SrC population was characterized by the expression of genes related to oxidative stress and cellular stress responses (*SOD3*, *MGP*, *MTIS*, and *WISP2*) (Fig. 3g).

In addition to these compositional changes, 3D-TMP spheroids demonstrated upregulation of key chondrogenic genes such as *TGFI* [39], *COL11A1* [40], and *HAS2* [41] while stress-related markers, including *WISP2* [42], *MGP* [43], and *SFRP4* [44], were reduced (Supplementary Fig. 6). The enrichment of regulatory chondrocytes may reflect a shift toward a more differentiation and functionally relevant cellular state.

Taken together, these results demonstrate that the 3D-TMP system more effectively generates homogeneous, chondrogenically committed spheroids by enriching regulatory chondrocytes and reducing stress-associated populations, underscoring the enhanced TGF- β 1 delivery and regenerative potential of this platform.

3.3 Enhanced EVs production in MP-incorporated 3D spheroid systems

EVs were isolated from each group and confirmed through transmission electron microscopy (TEM) imaging, which revealed spherical membrane-bound structures (Fig. 4a and Supplementary Fig. 7a). Western blot analysis confirmed the presence of positive EV markers CD63 and CD9, while the absence of the cell lysate marker calnexin indicated that the isolated EVs were purified (Fig. 4b) [45]. The size, distribution, and quantity of the EVs were analyzed using nanoparticle tracking analysis (NTA) (Fig. 4c and Supplementary Fig. 7b). Here, EV production was evaluated under identical initial cell numbers to enable direct comparison of total EV yield per unit input. Remarkably, the yield of the EVs in the 3D/T EV group was approximately 3-fold higher than that in the 2D/T EV group. Production increased further in the 3D-CMP/T EV group, resulting in a 5-fold increase, and reached the highest level in the 3D-TMP EV group, where a 7.5-fold increase was observed. The significant increase in EV production with 3D-CMP/T EV ($p<0.0001$) suggests that the incorporation of MPs in 3D spheroids promotes EV generation. PLGA is a biodegradable polymer that generates acidic degradation products [46]. We evaluated whether they could affect production in our system. To validate this, hMSCs were cultured under 2D conditions for 7 days with various CMP input ratios (no CMPs, 140:1, 70:1, and 35:1 cells-to-MPs), and EV yields were compared (Supplementary Fig. 8a). No statistically significant differences were observed in the EV concentrations among the

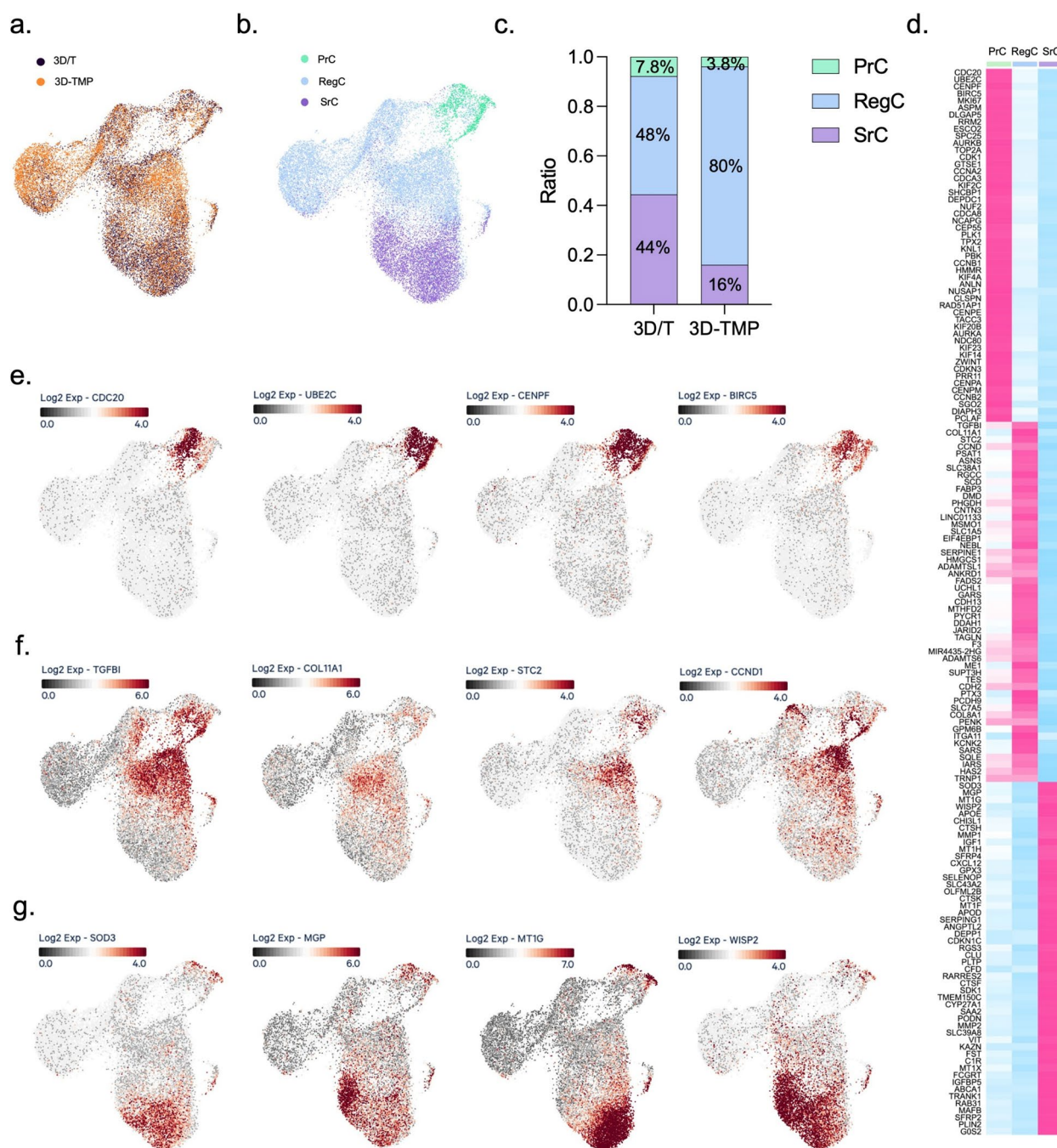


Fig. 3 Single-cell RNA sequencing 3D/T and 3D-TMP. **a** UMAP visualization of single-cell RNA sequencing data in two groups: 3D/T and 3D-TMP **b** UMAP visualization of single-cell RNA sequencing data reveals three cell subpopulations: progenitor chondrocytes (PrCs, green), regulatory chondrocytes (RegCs, blue), and stress-related chondrocytes (SrCs, purple). **c** Bar graph of the proportion of each

subpopulation in the 3D+T and 3D-TMP groups. **d** Heatmap of scaled expression (Z-score) for top 50 DEGs per cluster. **e–f** Feature plots display representative gene expression levels (log2) of proliferation-associated (*CDC20*, *UBE2C*, *CENPF*, *BIRC5*), chondrogenic (*TGFBR1*, *COL11A1*, *STC2*, *CCND1*), and stress-associated (*SOD3*, *MGP*, *MT1G*, *WISP2*) markers across clusters

groups (Supplementary Fig. 8b). These findings suggest that the degradation byproducts by CMPs do not interfere with the EV production under our culture conditions during the 7-day incubation period.

To evaluate the mechanisms contributing to enhanced EV production, we examined the roles of TGF- β 1 and necrotic core formation. TGF- β 1 exhibited a more uniform distribution throughout the 3D-TMP spheroids compared to conventional spheroids, indicating improved spatial delivery

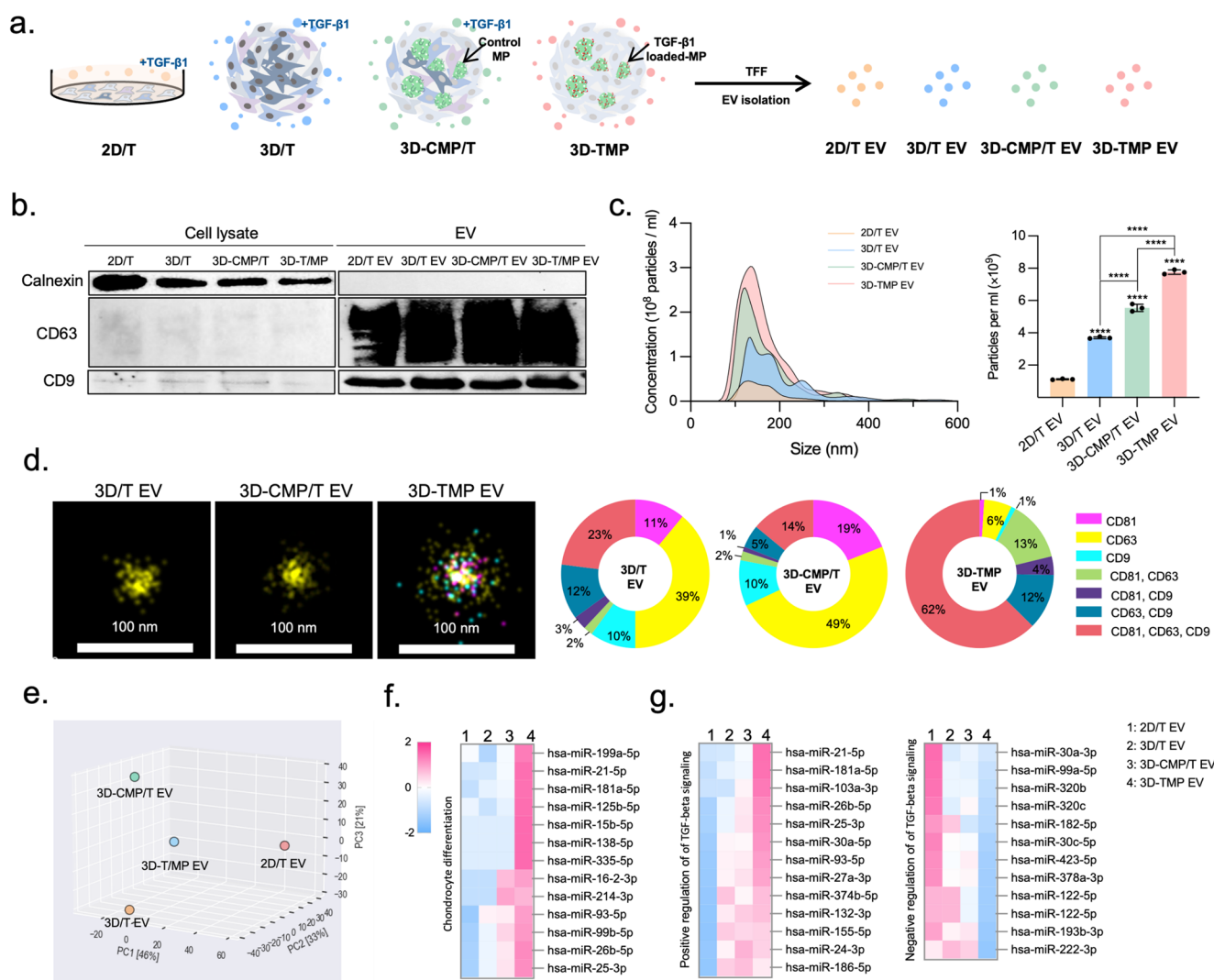


Fig. 4 Characterization of EVs derived from homogeneous 3D cartilaginous spheroid systems. **a** Schematic illustration of EV isolation from hMSCs cultured under four conditions. **b** Western blot analysis of EV markers CD63 and CD9, negative marker Calnexin in cell lysates and EVs. **c** Nanoparticle tracking analysis (NTA) of the size distribution and EV concentrations. **d** Super-resolution imaging was performed using direct stochastic optical reconstruction microscopy (dSTORM) to visualize the surface distribution of CD9, CD63, and CD81 on EVs derived from each spheroid condition. Scale bar, 100 nm.

e Principal component analysis (PCA) of microRNA (miRNA) profiles of EVs with distinct clustering by culture condition. **f** Heatmaps of miRNAs related to chondrocyte differentiation and **g** TGF-β signaling regulation categorized by positive or negative regulation. (1) 2D/T EV; (2) 3D/T EV; (3) 3D-CMP/T EV; (4) 3D-TMP EV. Data represent mean ± S.E.M. ($n=3$). One-way ANOVA was used for statistical comparison ($*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$, and not significant (ns))

of biochemical signals (Fig. 2a). In parallel, TGF-β1 treatment significantly increased EV production, suggesting that TGF-β1 directly enhances EV secretion (Supplementary Fig. 9a-b). Therefore, the uniform distribution of TGF-β1 is likely to facilitate increased EV production across the entire spheroid by enabling more widespread biochemical stimulation. In addition, to assess the effect of necrotic core formation, we established a necrotic core-mimicking condition using CoCl₂ (300 μM, 48 h) to induce cell death. Under this condition, total EV production per mL was significantly reduced compared to control conditions (Supplementary

Fig. 9c). Notably, when normalized to cell number, EV secretion per cell remained largely unchanged, indicating that the decrease in EV yield was primarily attributable to reduced cell viability rather than altered secretion capacity (Supplementary Fig. 9d). These findings suggest that cell death within necrotic regions negatively impacts overall EV production and support the notion that reduced necrotic core formation contributes to enhanced EV yield in the 3D-TMP system.

The substantial increase in EV yields observed in the 3D-TMP system is likely driven by multiple contributing

factors. Cell viability is known to significantly influence the secretion of EVs [47, 48]. In our study, spheroids containing an optimal amount of MPs structure demonstrated a notable improvement in cell viability by effectively reducing necrotic core formation, which is a common issue in conventional 3D spheroids (Fig. 1b, d). This optimized MPs incorporation created a supportive microenvironment that minimized cell death while maintaining structural integrity. Consistent with this, hypoxia reduced total EV production without significantly affecting EV secretion per cell, indicating that overall EV yield is largely dependent on the number of viable cells (Supplementary Fig. 9). In addition to cell survival, MP incorporation is also expected to modulate the internal architecture of spheroids by generating intercellular spaces that drug delivery. Although direct quantification of EV release dynamics within spheroids remains challenging, improved diffusion and TGF- β 1 uptake observed in this system support this interpretation (Fig. 2 and Supplementary Fig. 5). Furthermore, the highest yield of EVs was observed in the 3D-TMP EV group, indicating that TGF- β 1 delivery plays a key role in enhancing EV production. Previous studies have reported that TGF- β 1 can promote EV secretion, stimulating EV biogenesis [49, 50], and our finding further support this by demonstrating that TGF- β 1 enhances EV secretion at the cellular level. Taken together, these results suggest that EV yield is governed by both the number of viable cells and their intrinsic secretion capacity. The enhanced EV production in the 3D-TMP system is therefore best explained by the combined effects of improved cell survival, enhanced TGF- β 1 delivery, and biochemical stimulation. These findings highlight the synergistic interplay between structural and signaling factors within the engineered spheroid microenvironment.

Moreover, to investigate the MP-incorporated system on the distribution and heterogeneity of EVs in 3D spheroid systems, super-resolution imaging and subsequent classification of EVs revealed notable differences in EV composition (Fig. 4d). The EVs in the 3D/T EV group exhibited a relatively heterogeneous profile. The largest EV population consisted of CD63 single-positive EVs (39%), followed by triple-positive EVs (CD81⁺/CD63⁺/CD9⁺, 23%), CD63/CD9 double-positive EVs (12%), and smaller proportions of other subtypes. Representative super-resolution images confirmed a broad distribution pattern with CD63-positive EVs being the most prevalent subtype. A modest enhancement in EV homogeneity was observed. The proportion of CD63 single-positive EVs increased to 49%, while CD81 single-positive EVs and triple-positive EVs accounted for 19% and 14%, respectively. These results suggest a tendency toward a more dominant EV subtype, potentially influenced by the 3D structural environment and interactions with MPs. Notably, the homogeneity of the EVs in the

3D-TMP EV group was markedly enhanced. Triple-positive EVs (CD81⁺/CD63⁺/CD9⁺) accounted for approximately 62% of the total EV population, representing a substantial increase compared to the other two conditions. Super-resolution imaging revealed a more centralized and cohesive clustering of EVs, thereby visually supporting the quantitative data. These results suggest that the incorporation of MPs into the 3D spheroids promotes a selective EV production or stabilization mechanism, favoring a particular subtype and enhancing uniformity of the overall EV population. As the quality control (QC) of EV achieving molecularly consistent EV populations remains a major challenge because EV characteristics can vary depending on isolation methods and the condition of the cells from which they are derived [51]. In this context, our system demonstrated that EVs produced from homogeneous 3D spheroid systems exhibited highly consistent properties, including uniform particle size distribution, surface marker expression.

To further characterize the bioactive cargo of EVs by each culture condition, microRNA (miRNA) sequencing was performed. Principal component analysis (PCA) revealed distinct miRNA profiles in the 3D systems (3D/T EV, 3D-CMP/T EV, 3D-TMP EV) compared to the 2D system (Fig. 4e). Across all 3D systems, 46 miRNAs were upregulated and 61 were downregulated relative to 2D/T EV. These reflect a broad remodeling of EV cargo under 3D culture conditions. Interestingly, 3D-TMP EV also contained a higher abundance of cartilage-related and TGF- β signaling-associated miRNAs compared to other groups (Fig. 4f-g). 3D-TMP EV exhibited 67 upregulated and 12 downregulated miRNAs compared to 2D/T EV. Heatmap analysis through a z-score measurement confirmed the increased expression of 23 miRNAs associated with chondrogenic differentiation (e.g., hsa-miR-140-5p [52], hsa-miR-181a-5p [53]) in 3D-TMP EV. Furthermore, 15 miRNAs linked to the positive regulation of TGF- β signaling (e.g., hsa-miR-21-5p [54], hsa-miR-19a-3p [55]) were upregulated in the 3D-TMP EV group, while 10 miRNAs related to negative regulation (e.g., hsa-miR-29a-3p [56], hsa-miR-101-3p [57]) were downregulated. These molecular signatures indicate that EVs secreted from the 3D-TMP spheroid system are tailored to support cartilage regeneration through both the modulation of the TGF- β signaling pathway and matrix synthesis.

Collectively, the 3D-TMP platform significantly increases EV yield and improves compositional uniformity and functional characteristics. These features enable the simultaneous achievement of quantitative expansion and qualitative control, demonstrating that the approach overcomes key limitations of both conventional 2D culture and 3D spheroid-based systems. Notably, it promotes the enrichment of specific EV subpopulations and modulates

cargo composition, including miRNAs associated with chondrogenesis and TGF- β signaling. These results highlight its potential as a strategy for generating high-quality, functionally enhanced EVs.

Nevertheless, this study has several limitations. First, systematic evaluation under large-scale culture conditions was not performed. Although structurally compatible with such systems, its performance under bioreactor-based conditions remains to be validated. Future studies should determine whether the efficiency observed at the microscale can be maintained or further improved in dynamic culture platforms. In addition, comprehensive profiling of EV cargo, such as proteomic or lipidomic analysis, was not performed. This is due to the nanoscale size and inherent heterogeneity of EVs, which still present challenges for accurately resolving protein and lipid composition at the single-EV level with current analytical technologies [58]. As EV functional properties are influenced by the complex interactions of multiple molecular components, future studies should incorporate advanced analytical techniques, and multi-omics approaches to more systematically characterize these properties.

3.4 Chondrogenic effect of EVs derived from the homogeneous 3D cartilaginous spheroid system on the IL-1 β -induced chondrocyte.

To establish an in vitro OA-like model, primary chondrocytes were treated with IL-1 β (10 ng/mL) for 24 h (Supplementary Fig. 10a) [59]. Alcian blue staining revealed a substantial decrease in glycosaminoglycan (GAG) content following IL-1 β treatment (Supplementary Fig. 10b), which was further confirmed by a significant reduction in soluble GAG (sGAG) levels in the culture medium (Supplementary Fig. 10c). In addition, qRT-PCR analysis showed markedly decreased expression of chondrocyte-specific genes (Supplementary Fig. 10d).

Therefore, to investigate the regenerative potential of EVs, IL-1 β -induced chondrocytes were treated with EVs from each experimental group for 7 days (Fig. 5a). The qRT-PCR analysis showed that chondrogenic markers were upregulated in the 3D/T EV, 3D-CMP/T EV, and 3D-TMP EV groups compared with the 2D/T EV group, with the 3D-TMP EV group showing the highest expression (Fig. 5b). To further evaluate the functional consistency of EVs, dose-response EV activity assays were performed by treating IL-1 β -induced chondrocytes with EVs at increasing concentrations (1-, 5-, and 10-). The 3D-TMP EV group exhibited a clear and reproducible dose-response increase in chondrogenic marker expression (*SOX9*, *COL2A1*, and *ACAN*) (Supplementary Fig. 11). These results support the functional consistency and reproducibility of EVs generated

from the 3D-TMP system. In addition, Western blot analysis confirmed increased protein expression of *SOX9*, *ACAN*, and *COL2A1* in the 3D-CMP/T and 3D-TMP EV groups (Fig. 5c). Notably, the catabolic enzyme ADAM metalloproteinase with the thrombospondin type 1 motif 5 (ADAMTS5) was significantly reduced in the 3D-CMP/T EV and 3D-TMP EV groups compared to the 3D/T EV group. These results indicate that EVs from a 3D culture environment enhance extracellular matrix (ECM) formation and cartilage marker expression in damaged chondrocytes, suggesting improved cartilage repair functionality over 2D/T EV. In addition, sGAG quantification showed a significant increase only in the 3D-TMP EV group compared with the other EV-treated groups, indicating enhanced cartilage matrix synthesis mediated by 3D-TMP EVs (Fig. 5d). Additionally, Alcian blue staining demonstrated enhanced GAG deposition in the 3D-CMP/T EV and 3D-TMP EV groups (Fig. 5e, f). Meanwhile, IF staining showed similar trends in *SOX9*, *ACAN*, and *ADAMTS5* expression (Fig. 5e, g–i), supporting the superior chondrogenic potential of 3D-TMP EV.

3.5 EVs from homogeneous 3D cartilaginous spheroids show therapeutic potential for OA

Next, a destabilization of the medial meniscus (DMM) model was established to determine the therapeutic efficacy of EVs from our spheroid system in OA treatment [60]. Four weeks after model induction, equal amounts of EVs from the 2D/T EV, 3D/T EV, 3D-CMP/T EV, and 3D-TMP EV groups were locally injected into the cartilage once weekly for 4 weeks in the DMM rat model (Supplementary Fig. 12a). The rat knee joints were harvested after 4 weeks of EV treatments in the DMM-induced OA model. Gross images of the excised knee joints illustrate the progressive reduction in periarticular swelling across the treatment groups (Supplementary Fig. 12b–c). The PBS control showed circumferential thickening and slight hyperemia, consistent with severe synovitis. Swelling remained evident in the 2D/T EV group, although the joint capsule was visibly less distended compared with PBS controls. A further decrease in swelling was observed in the 3D/T EV group. Meanwhile, the 3D-CMP/T EV and 3D-TMP EV groups exhibited a smooth joint contour closely resembling the sham group. To evaluate the anti-inflammatory efficacy of the EV formulations at the synovium, hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) staining for inducible nitric oxide synthase (iNOS) were performed on knee joint sections (Supplementary Fig. 12d) [61]. H&E staining of synovial tissue from the sham group showed a thin lining with low cellularity and a loose connective-tissue subintima, prominent fibrin deposition, and dense

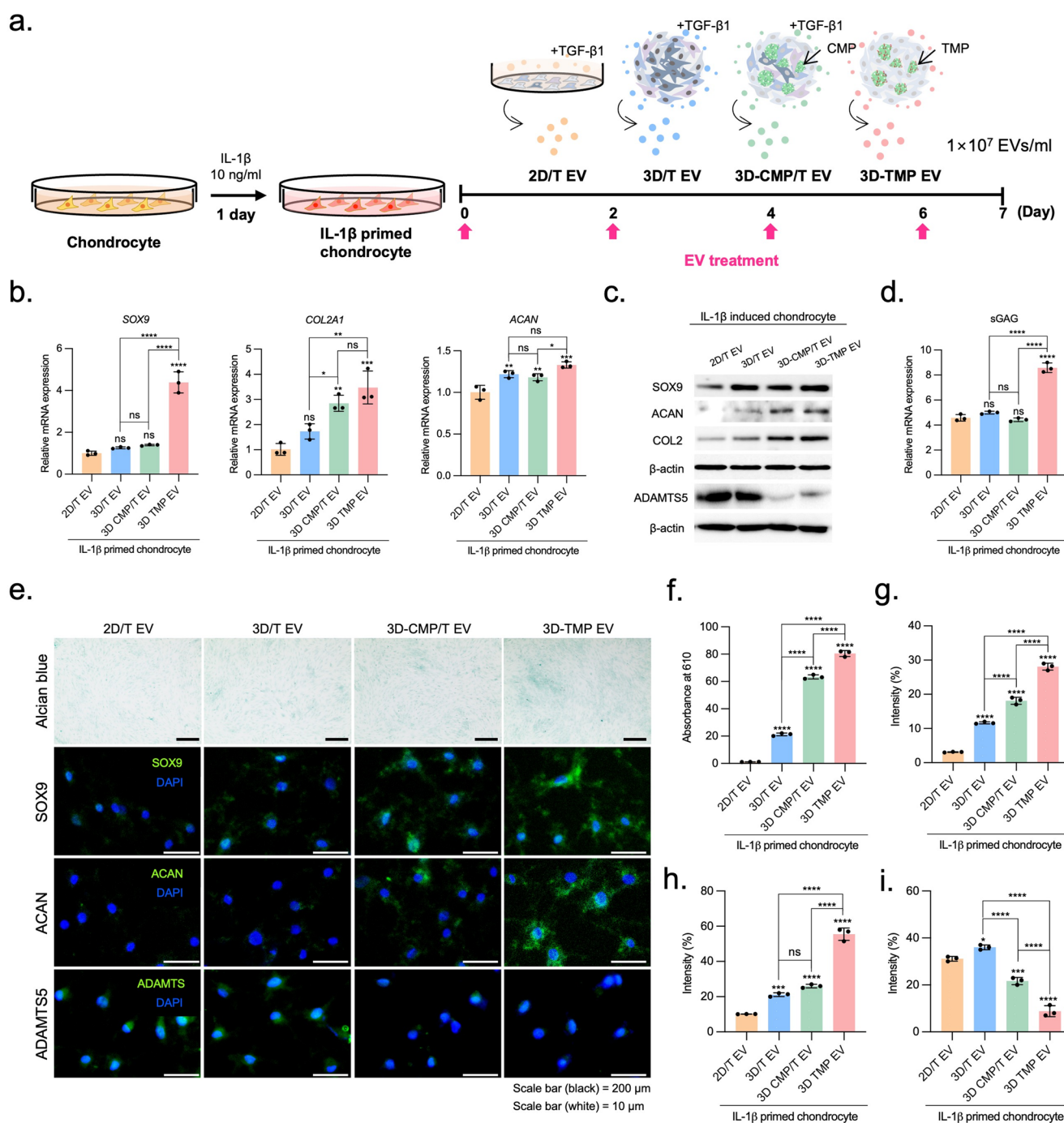


Fig. 5 Chondrogenic effects of EVs derived from homogeneous 3D cartilaginous spheroid systems on IL-1 β -induced chondrocytes. **a** Schematic overview of the experimental design. Chondrocytes were pretreated with IL-1 β (10 ng/mL) for 24 h to induce inflammation, followed by treatment with EVs derived from different hMSC culture systems. **b** Relative mRNA expression of *SOX9*, *COL2A1*, and *ACAN* in IL-1 β -induced chondrocytes after EVs treatment. **c** Western blot analysis of *SOX9*, *COL2A1*, *ACAN*, and *ADAMTS5* expression in EV-treated IL-1 β -induced chondrocytes. **d** Quantification of sGAG production in IL-1 β -induced chondrocytes after EV treatment.

e Histological staining of EV-treated IL-1 β -induced chondrocytes. Alcian blue staining of chondrocytes to visualize GAG and IF staining of *SOX9*, *ACAN*, *ADAMTS5* (green), and nuclei (DAPI, blue) for chondrogenic marker expression. Scale bar = 200 μ m. **f** Quantification of Alcian blue-stained area. **g** Quantification of *SOX9* intensity. **h** Quantification of *ACAN* intensity. **i** Quantification of *ADAMTS5* intensity. Data represent mean $\pm$ S.E.M. ($n=3$). One-way ANOVA was used for statistical comparison (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, and not significant (ns))

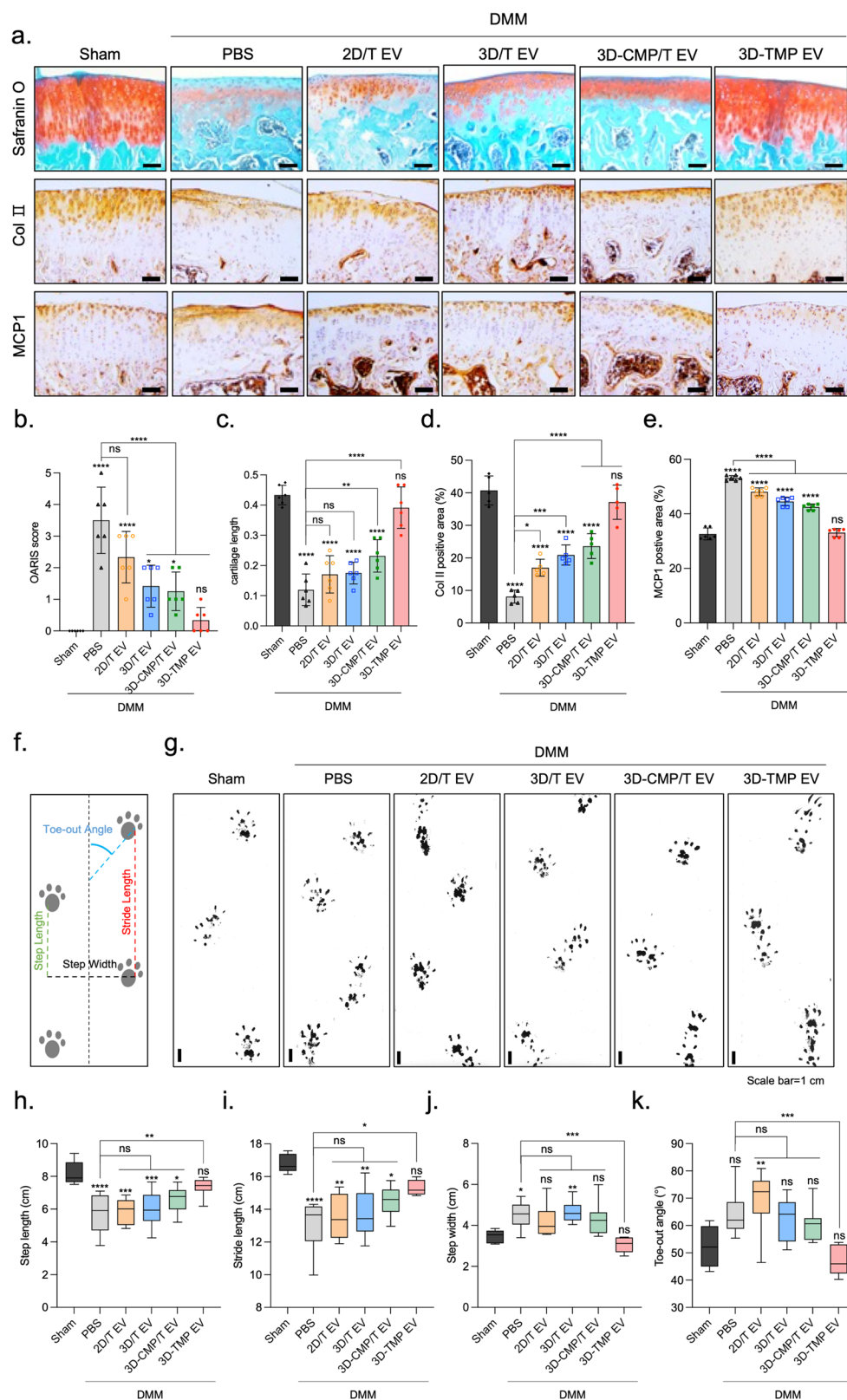
inflammatory-cell infiltration, consistent with active synovitis [62]. In the 2D/T EV and 3D/T EV groups, both fibrin deposition and synovial-lining thickening were noticeably reduced compared with the PBS group. These pathological features were further attenuated in the 3D-CMP/T EV and 3D-TMP EV groups, with the latter displaying a thin synovial lining. Notably, the 3D-TMP EV group exhibited a low cellular density that closely resembled normal synovial tissue. The iNOS staining pattern paralleled the histological observations, whereby strong iNOS positivity was detected throughout the synovium of PBS-treated joints, particularly in endothelial cells and macrophage-rich regions. The signal intensity declined progressively in the 2D/T and 3D/T EV groups, was further diminished in the 3D-CMP/T EV group and was barely detectable in the 3D-TMP EV group. Collectively, these findings indicate that 3D-TMP EVs most effectively suppress synovial inflammation.

Cartilage regeneration was evaluated through safranin O staining (Fig. 6a). Here, cartilage damage was noticeably evident in the sham and PBS groups, verifying successful establishment of the DMM model. The group treated with 2D/T EV exhibited moderate cartilage tissue regeneration compared to the PBS group. Notably, the 3D/T EV and 3D-CMP/T EV groups demonstrated significantly enhanced cartilage regeneration. Meanwhile, cartilage tissue in the 3D-TMP EV group exhibited a thickness and structure closely resembling that in the sham group, thereby indicating high efficacy in OA cartilage regeneration. Furthermore, the Osteoarthritis Research Society International (OARSI) score analysis [63] revealed that cartilage regeneration in the 3D-TMP EV group was statistically comparable to the sham group, underscoring its superior therapeutic potential (Fig. 6b). The PBS group displayed the most severe cartilage damage (3.83 ± 0.7), whereas the score in the 2D/T EV group was lowered to 2.5 ± 0.8 . The 3D/T EV and 3D-CMP/T EV groups presented scores of 1.5 ± 0.05 and 1.33 ± 0.5 , respectively. Notably, the 3D-TMP EV group achieved an OARSI score of just 0.33 ± 0.5 , which was statistically indistinguishable from the sham group yet significantly lower than all other groups. Quantitative analysis of articular cartilage thickness showed that the 3D-TMP EV group restored cartilage thickness to a level comparable to that of the sham group, while the other #V-treated groups showed partial improvement (Fig. 6c). These results suggest that 3D-TMP EVs exhibit improved regenerative potential compared with other groups, with outcomes approaching those of healthy controls. To further validate these histological findings at the molecular level, IHC staining was performed to assess COL2 and monocyte chemoattractant protein-1 (MCP-1) (Fig. 6d–e). Interestingly, COL2A1 expression was decreased in the PBS group. In contrast, the 3D-TMP EV group exhibited a pronounced increase in

COL2A1 expression. These results indicate that 3D-TMP EVs not only restore cartilage morphology but also modulate the molecular microenvironment toward ECM synthetic regeneration. Furthermore, MCP-1, a proinflammatory chemokine associated with OA progression [64], was highly expressed in the PBS and 2D/T EV group. In contrast, markedly reduced MCP-1 staining was observed in the 3D-CMP/T and 3D-TMP EV-treated groups, with the 3D-TMP group showing MCP-1 levels comparable to the sham group.

To analyze joint pain and motor function associated with OA, gait analysis was performed using step length, stride length, step width, and toe-out angle as evaluation parameters (Fig. 6f–j) [65]. Compared to the sham group, the DMM-induced group treated with PBS for 4 weeks demonstrated significant alterations in this gait metrics indicative of OA-associated pain [66]. Specifically, step length was significantly reduced from 8.18 cm (sham) to 5.77 cm, while stride length decreased from 16.82 cm to 13.25 cm. In contrast, step width significantly increased from 3.46 cm to 4.54 cm, and the toe-out angle improved from 52.3° to 64.4° , although this change did not reach statistical significance. These findings align with previous reports indicating that OA-associated pain leads to shortened step and stride lengths, along with increased step width and toe-out angle [67]. Thus, the PBS group retained characteristic gait patterns reflective of joint pain and functional impairment. Although 2D/T EV, 3D/T EV, and 3D-CMP/T EV treated groups showed slight improvement of the mean values of the evaluated gait parameters compared to the PBS group. However, no statistically significant differences were observed among these groups, suggesting that these EVs did not produce sufficient analgesic or functional effects. In contrast, the group administered 3D-TMP EVs exhibited marked improvements in all gait metrics: Step length and stride length increased to 7.33 cm and 15.31 cm, respectively, while step width decreased to 3.07 cm. The toe-out angle was also significantly reduced to 47.41° , indicating an improved gait pattern. Notably, none of the gait parameters in the 3D-TMP EV group showed statistically significant differences from the sham group, suggesting that 3D-TMP EVs effectively alleviated OA-associated pain and restored normal locomotor function. These outcomes highlight that the enhanced molecular composition and homogeneity of 3D-TMP EVs translate into measurable improvements in joint biomechanics, positioning them as a highly promising acellular therapy for OA. Beyond OA treatment, the developed method for generating uniform 3D spheroids suggests that the advantages for tissue engineering. By embedding differentiation cues within MPs, this platform enables precise control over spheroid composition and EV phenotype, allowing the production of vesicles tailored to

Fig. 6 Therapeutic effects of EVs on cartilage regeneration in a DMM-induced OA rat model. **a** Representative histological and immunohistochemical images of knee joint sections. **b** OARSI histological scores quantified from safranin O-stained sections. **c–e** Quantification of articular cartilage properties, including articular cartilage thickness **c**, Col II-positive area **d**, and MCP-1-positive area **e** in articular cartilage. **f** Schematic of the gait analysis parameters. **g** Representative footprint patterns obtained from gait analysis. Scale bar = 1 cm. **h–k** Quantification of gait parameters, including step length **h**, stride length **i**, step width **j**, and toe-out angle **k**. Data represent mean $\pm$ S.E.M. ($n=6$). One-way ANOVA was used for statistical comparison (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, and not significant (ns))



specific therapeutic needs. This strategy may be extended to diverse applications, including organoid engineering, disease modeling, and cancer drug screening, where controlling spheroid uniformity and cellular state is critical. Thus, the 3D-TMP platform represents a versatile approach for producing functional EVs and specialized spheroid constructs with high EV yield for regenerative medicine and biomedical research.

4 Conclusion

Overall, this study successfully developed a 3D-TMP-based spheroid culture system that significantly improved both spheroid homogeneity and chondrogenic differentiation efficiency compared to conventional 3D systems. This engineered microenvironment enabled spatially controlled and sustained TGF- β 1 delivery, resulting in more homogeneous cellular differentiation and improved matrix formation. Importantly, the system significantly increased the yield, molecular uniformity, and therapeutic potency of EVs, producing highly uniform EV populations enriched with pro-chondrogenic and TGF- β -regulating miRNAs. 3D-TMP-derived EVs promoted cartilage regeneration by simultaneously enhancing anabolic matrix synthesis and attenuating inflammatory degradation, leading to functional recovery in both in vitro OA-like models and in vivo DMM models. This system demonstrates the 3D-TMP spheroid platform as a technically advantageous strategy for producing high-quality therapeutic EVs through controlled spheroid engineering. This approach holds strong potential for translation in osteoarthritis therapy and may be broadly applicable to other fields, including regenerative medicine, organoid engineering, and disease modeling.

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Author contributions H.P., Y.A. and S.-H.L. conceptualized the study and experiments. H.P. performed the majority of the experiments and analyzed the data. I.B., M.K., W.J.C., Y.K., K.-Y.C., and J.K. assisted with experiments and data collection. H.-D.J. performed machine-learning analysis. H.P. wrote the original draft. H.P., H.-D.J., Y.A., and S.-H.L. contributed to manuscript editing. Y.A. and S.-H.L. supervised the research, provided resources and funding, administered the project, and contributed to manuscript revision. All authors approved the paper.

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Data availability No datasets were generated or analysed during the current study.

Declarations

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

Competing interests The authors declare no competing interests.

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