

Detailed Materials and Methods

This study has been approved by the Institutional Review Board of Tokai University School of Medicine (Isehara, Kanagawa) under the approval number n. 221012. Informed consent for the collection and use of surgical waste products for research purposes was obtained from every patient. All animal experiments have been conducted according to ARRIVE guidelines and in respect of the 3R principle.

Overview of the in vitro study

NPCs isolated from young and healthy or mildly degenerated donors (modified Pfirrmann grades: 1-4, mean age: 28.5 years) were culture-expanded according to a standard or a Tie2-enhancing protocol and subsequently exploited for EV isolation and characterization. EVs were also extracted from a commercial cell line of human BM-MSCs (Poietics™, Lonza, USA) characterized, and used for later experiments in the in vivo part of the study. Then, the effects of NPC-derived EVs were assessed by treating degenerative NPCs (dNPCs) and evaluating cell proliferation and senescence (Fig. 1).

Cell isolation and culture

Human IVD tissue samples were collected from patients undergoing spine surgery for traumatic or degenerative disorders at Tokai University Hospital (Isehara, Japan). Demographics, diagnosis, operated level(s), modified Pfirrmann grade of harvested IVD(s), comorbidities, and allocation to different experiments are depicted in Table 1. NPCs derived from young and healthy or mildly degenerated donors were cultured according to either standard primary non-Tie2-optimized (NPC^{STD}) [1] or Tie2-enhancing culture strategies (NPC^{Tie2+}) [2]. Briefly, upon arrival at the laboratory, surgical specimens were washed with sterile phosphate-buffered saline (PBS) and minced into pieces 3-5 mm in diameter with sterilized scissors and scalpels, carefully separating the NP tissue as previously

described [3]. Minced tissues were transferred to 15-mL tubes and centrifuged at 1200 rpm for 5 min at 4°C. In the NPC^{STD} group, the supernatant was removed after centrifugation, and the tissue was resuspended in 5 mL of 0.25% trypsin-EDTA (Thermo Fisher Scientific, USA) and digested with gentle swirling at 37°C for 30 min. Next, the tissue was further digested in a mixture of 5 mL of 10% (v/v) fetal bovine serum (FBS, Sigma Aldrich, USA), Dulbecco's Modified Eagle Medium High Glucose (DMEM-HG; Wako, Japan), and 0.25 mg/mL collagenase P (Roche, Switzerland), and incubated for 4 h at 37°C. After digestion, the suspension was filtered through an 80 µm-cell strainer (Corning, USA). The resulting cell suspension was seeded on standard polystyrene 100-mm dishes (Corning) or cryopreserved at -196°C in CELLBANKER (Zenoaq Resource, Japan). According to the recommendations of the Orthopaedic Research Society (ORS) Spine community [1], $\sim 1 \times 10^5$ cells/dish were seeded in NPC^{STD} were maintained in DMEM-HG + 10% FBS + 1% penicillin/streptomycin (Gibco, USA) + 50 µg/mL L-ascorbic acid (Sigma Aldrich, USA) at 37°C in 5% CO₂ and 5% O₂ to mimic the hypoxic IVD environment. The same protocol was adopted to culture NPCs harvested from degenerative IVDs.

After washing and mincing, the tissues undergoing the NPC^{Tie2+} culture approach were directly cultured in polystyrene 6-well plates (IWAKI, Japan) with a commercially developed NPC-optimized, blended medium (MEMα:32%, DMEM:48%, FBS:20%; TUNZ Pharma Co. Ltd., Japan) as previously described [2]. Tissue fragments were cultured at 37°C in 5% CO₂ and 5% O₂ for 14 days without media replenishment. Subsequently, cultured tissue fragments were digested as described above, and derived cells were seeded in monolayer at a density of 30,000 cells per 100 mm-dish (545.5 cells/cm²) using the same medium. BM-MSCs were maintained in DMEM HG + 10% FBS + 1% penicillin/streptomycin at 37°C in 5% CO₂ and 21% O₂. Media were refreshed every 3 days and cells up to passage 3 (P3) were used for the experiments.

EV isolation

To remove bovine-derived EVs, FBS ultracentrifugation was performed to obtain EV-free FBS (110,000 g for 17 h) as previously described [4]. Thereafter, the supernatant was filtered through a 0.22- μ m filter and such EV-free FBS were used to culture NPCs and BM-MSCs undergoing EV isolation. After reaching a cell confluency of 50-60%, NPCs^{STD}, NPCs^{Tie2+}, and BM-MSCs were cultured in EV-free media for 2 additional days, and EV isolation was performed as previously described [4]. Briefly, cell culture conditioned media were collected and centrifuged at 1500 g for 15 min and then at 12,000 g for 30 min to remove dead cell material. The solution was then filtered with a 0.22- μ m membrane to further remove cell debris and larger vesicles. Eventually, the media were ultracentrifuged at 110,000 g for 70 minutes at 4°C to obtain an EV pellet, which was resuspended in PBS, ultracentrifuged again at 110,000 g for 70 minutes at 4°C, and then used for further experiments or stored at -80°C.

EV characterization

EV characterization was performed according to the International Society for Extracellular Vesicles (ISEV) guidelines [5]. Transmission electron microscopy (TEM) was used to obtain both close-up and wide-field images of EVs. Furthermore, single-particle analysis to measure EV size and distribution was performed through tunable resistive pulse sensing and carried out using the qNano system (Izon Science Ltd, New Zealand) [6]. The expression of CD63, CD9, Tsg101, and lamin A/C was comparably detected by Western blot in NPCs, BM-MSCs and their EVs as described below. EVs were quantified using the micro bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific) according to the manufacturer's protocol following lysis in RIPA buffer (Thermo Fisher Scientific) and sonication.

EV uptake

Before EV treatment, recipient NPCs were stained with Hoechst 33342 (Lonza) as per manufacturers' protocols. Following isolation, NPC-derived EVs were incubated with PKH26 and Diluent C (Sigma-

Aldrich) for 5 min at room temperature, according to manufacturer's instructions. After being resuspended in DMEM + 10% FBS and then ultracentrifuged for 70 min at 110,000 g, PKH26-labeled EVs were resuspended in PBS and incubated with NPCs for up to 180 min at 37°C and 5% CO₂. Stained cells were observed under a confocal laser scanning microscope (LSM 700, Carl Zeiss, Germany) at 0 and 180 min timepoints to assess for EV uptake.

Cell treatment

Following the isolation of NPC^{STD}- and NPC^{Tie2+}-EVs, endemic NPCs isolated from patients with IDD as described above were exploited to evaluate EV potential regenerative effects by being divided in the following groups: control groups (standard medium or IL-1 β alone), NPC^{Tie2+}-EVs, NPC^{STD}-EVs, IL-1 β + NPC^{Tie2+}-EVs and IL-1 β + NPC^{STD}-EVs. IL-1 β (PeproTech, USA) was employed at a concentration of 10 ng/mL to mimic the inflammatory IDD environment as previously described [7] and evaluate the potential blunting effect of EVs on IL-1 β -induced cell catabolism.

Cell proliferation

To detect the most appropriate EV concentration to treat recipient cells, a dose-response curve with increasing EV concentrations was performed using the CCK-8 assay (Dojindo, USA) to evaluate cell proliferation. Briefly, NPCs were transferred to 96-well plates (5 $\times$ 10³ cells/well) and incubated with either NPC^{STD}- or NPC^{Tie2+}-EVs at different concentrations according to previous studies [8] (25, 50, 75, and 100 μ g/mL), with or without 10 ng/mL IL-1 β , for 3 and 7 days. Subsequently, 10 μ L of CCK-8 solution was added to each well and plates were incubated for 2 h at 37°C. Finally, an auto-microplate reader was used to read the absorbance of each well. Cell proliferation was calculated as percent absorbance change compared to the control group (DMEM only).

Cell senescence

NPCs were plated into 6-well plates (2.5×10^5 cells/well) and treated as explained above for 72 h. Subsequently, cells were washed with PBS, fixed, and incubated overnight with the senescence-associated β -galactosidase (SA- β -gal) staining (Cell Signaling, Danvers, USA) at 37° in a dry environment. Cells were observed under a phase contrast light microscope. At least six random fields per each well were acquired at 10x magnification. Intensely blue-stained cells were considered senescent as per the manufacturer's protocol. The proportion of senescent cells was calculated in a blinded manner as the ratio between positive cells and the total number of observed cells.

Western Blot

EVs and intact NPCs or BM-MSCs were lysed in RIPA buffer (Thermo Fisher Scientific) as per manufacturer's protocol, and protein concentration was measured with the BCA assay (Thermo Fisher Scientific). Protein lysates were resolved by SDS-PAGE (200 V, 22 min) using NuPAGE 4–12% Bis-Tris Gel (Invitrogen), MES Running buffer, and blotted (20 V, 60 min) onto PVDF membranes. Nonspecific signals were blocked with 3% bovine serum albumin. The membranes were incubated with primary antibodies (1:1000) at 4°C overnight and secondary antibodies (1:2000) at room temperature for 1 h. The primary antibodies used were: CD9 (sc13118, Santa Cruz Biotechnology, USA), CD63 (BD556019, BD Biosciences, USA), TSG101 (ab30871, Abcam, United Kingdom), lamin A/C (MAB3211, Sigma-Aldrich). GAPDH (SAB2108668, Sigma-Aldrich) was used as a loading control. Immunoreactive bands were detected using an Amersham Healthcare ECL Prime Western Blotting Detection Reagent (Cytiva, Japan). Protein signals were quantified by scanning densitometry using a CS analyzer (ATTO, Japan).

Overview of the in vivo study

To directly assess the effect of EVs on IVD tissues in vivo, a rat model of IDD. Animals were randomly assigned to four groups: sham (n=4), NPC^{STD}-EVs (n=4), NPC^{Tie2+}-EVs (n=4), and BM-MSC-EVs (n=4). Randomization was performed using a computer based random order generator.

From baseline to sacrifice, pain behavior assessment was performed every other week. Radiographic measurement of disc height was carried out on a monthly basis. Serum analysis of pain biomarkers was performed at baseline and after 4 and 12 weeks. After sacrifice, macroscopic and microscopic evaluation of IVD morphology was performed (Fig. 2).

Animal model

Sixteen Sprague Dawley rats aged between 10 and 12 weeks (MIZUSETSU, Japan) were employed. Upon delivery to the facility, animals were hosted in their cages in pairs and sequentially assigned with a numeric ID (from #1 to #16). Ear punching was performed to distinguish between even (punched) and uneven (not punched) animals in the same cage. Male animals were chosen due to reduced behavioral variability in response to stressors compared to female peers [9, 10]. Following acclimation and acquisition of baseline data, IDD was induced by annular puncture of 3 coccygeal IVDs as previously described [11]. Briefly, under continuous 2.5% isoflurane inhalation and aseptic conditions, coccygeal IVDs from Co5-Co6 to Co7-Co8 were surgically exposed, needle-punctured with a 21G needle, and approximately 10 μ L of NP tissue were aspirated to induce IDD under fluoroscopic guidance. Co4-Co5 and Co8-Co9 were kept as healthy controls. Subsequently, with the use of a Hamilton microliter syringe and a 27G needle, 2 μ L of a solution containing either PBS (sham group), NPC^{STD}-EVs, NPC^{Tie2+}-EVs, or BM-MSC- EVs were injected in the punctured discs according to randomization. Approximately 1.5×10^6 EVs and were injected at each level as per previous studies [12]. During the surgical procedure, three different investigators were involved for each animal. A first investigator, unrelated to the study, performed the surgery. A second investigator, unaware of group allocation, prepared unlabeled syringes with products, while a third investigator, aware of treatment allocation, communicated to the other two which syringe to deliver to each animal. Allocation concealment was guaranteed by the investigator in charge of randomization until data analysis was completed. Eventually, skin was closed with a 3-0 nylon suture. At the end of surgery, animals received a single subcutaneous injection of buprenorphine (0.05 mg/kg) to control

postoperative pain and were closely monitored until recovery from sedation before placing them back into their cages. In addition to animal staff routine care, investigators monitored the animals on a daily basis until 10 days after surgery for postoperative complications.

A priori sample size calculation was performed using the G*Power software. As the primary outcome of the in vivo part of this study was structural restoration of the IVD, effect size was calculated taking into consideration the difference among histological scores of healthy (2.8), moderately (8.7) and severely degenerated (12.1) discs (according to the standardized ORS histopathology scoring system). Calculated effect size was 1.068. With an α of 0.05 and a power of 95%, a total sample size of 16 animals (3 experimental IVDs each) was calculated.

Behavioral assessment

Behavioral tests to evaluate animal sensitivity were performed at baseline and at 2-, 4-, 6-, 8-, 10-, and 12-weeks post-puncture as previously described [13, 14]. Pain-related behaviors were evaluated by placing animals in a 60×60 cm open-field maze and recording them with a camera (GoPro Hero 11, GoPro, USA) for 10 minutes from a bird's eye view. Prior to the behavioral test at each timepoint, animals were placed individually in the open-field enclosure for 10 min on the day before testing to acclimate to the experimental environment. Room lighting and time of day were consistent among trials. Ethovision XT Automated Behavior Recognition software (Noldus Information Technology, The Netherlands) was used to track and analyze 42 parameters of animal motion and stress-related behavior. Among these, 18 outcomes were selected and further processed due to their clinical relevance to LBP as per previous studies [14].

The Von Frey test was performed to evaluate mechanical tissue hypersensitivity using an aesthesiometer (IITC, 2391) with a rigid tip and an outer diameter of 0.8 mm. Three animals at a time were allowed to acclimate for 15 min in clear acrylic chambers placed on a metal grid. A maximum force of 28 g was applied ramping up over 10 s and kept for a maximum of 40 s. Withdraw threshold and time were assessed bilaterally by sequentially and alternatively applying the probe on hind paws,

tail base, and surgical site (corresponding to the portion of the tail ranging from Co5 to Co8) of all animals. This process was repeated at least three times per each site and the average threshold and withdrawal time was calculated. Investigators were blind to cohort allocation of the animals during the behavioral tests and analysis.

Serum biomarkers

Peripheral venous blood samples were obtained at baseline, 4, and 12 weeks postoperatively. Serum was isolated by centrifugation and frozen at -80°C until analyzed. Serum concentration of pain biomarker IL-6 was quantified using ELISA kits (RayBiotech, USA) as per the manufacturer's instructions.

Radiographic assessment

Radiographic assessment was conducted at baseline and 4, 8, and 12 weeks postoperatively. Briefly, images were obtained from the coccygeal region using a fluoroscopic imaging intensifier (DHF-105CX, Hitachi, Japan) after placing the animals in the supine position under continuous 2.5% isoflurane inhalation. In a blinded manner, disc height index (DHI) was calculated as previously described [15] and normalized to pre-transplantation DHI.

Macroscopic, histological and immunohistochemical evaluation

At 12 weeks after treatment, animals were sacrificed by means of cervical dislocation under continuous 2.5% isoflurane inhalation, tails were quickly dissected, and functional spine units (FSUs) were isolated. The Thompson grading system [16] was used to macroscopically assess IDD by two blinded investigators. Then, from each rat, FSUs of interest were collected and placed in 4% paraformaldehyde at 4°C . Subsequently, the specimens were placed in 70, 80, 96, 80, 70% (V/V) ethanol solutions for 12 h, followed by decalcification (WAKO solution A, WAKO) for 18 h, and 5% sodium sulfate for 12 h. Finally, samples were dehydrated by 70, 80, 96, and 100% (V/V)

ethanol and xylene. Samples were placed in paraffin blocks and 8- μ m sections were obtained with a Yamato REM710 microtome (Yamato Kohki Industrial Co. LTD., Japan) for histological assessment. Tissue sections were prepared for histological staining with hematoxylin/eosin, Safranin-O/Fast-Green and Picrosirius-Red. Sections were graded based on the rat-specific ORS Spine histological grading scheme [17] by two blinded investigators.

Statistical analysis

All quantitative data are expressed as means $\pm$ standard deviation (SD). The normal distribution of data was determined with the Wilk-Shapiro test. Statistical analysis of the results was performed using one-way or two-way analysis of variance (ANOVA) with Dunnett's or Tukey's post-tests for multiple comparisons wherever applicable. The Kruskal Wallis test was performed to analyze non-normal data among multiple groups. Statistical significance was set as $p < 0.05$. Formal analysis was performed using Prism 8 (GraphPad, San Diego, CA, USA). Representative data are shown.

References

1. Basatvat S, Bach FC, Barcellona MN, Binch AL, Buckley CT, Bueno B, et al. (2023) Harmonization and standardization of nucleus pulposus cell extraction and culture methods. *Jor Spine*. doi: 10.1002/jsp2.1238
2. Sako K, Sakai D, Nakamura Y, Schol J, Matsushita E, Warita T, et al. (2021) Effect of Whole Tissue Culture and Basic Fibroblast Growth Factor on Maintenance of Tie2 Molecule Expression in Human Nucleus Pulposus Cells. *International Journal of Molecular Sciences* 22. doi: 10.3390/ijms22094723
3. Sakai D, Schol J, Bach FC, Tekari A, Sagawa N, Nakamura Y, et al. (2018) Successful fishing for nucleus pulposus progenitor cells of the intervertebral disc across species. *Jor Spine* 1. doi: 10.1002/jsp2.1018
4. Ito M, Kudo K, Higuchi H, Otsuka H, Tanaka M, Fukunishi N, et al. (2021) Proteomic and phospholipidomic characterization of extracellular vesicles inducing tumor microenvironment in Epstein-Barr virus-associated lymphomas. *The FASEB Journal* 35. doi: 10.1096/fj.202002730R
5. Thery C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. (2018) Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 7:1535750. doi: 10.1080/20013078.2018.1535750
6. Kakizaki M, Yamamoto Y, Otsuka M, Kitamura K, Ito M, Kawai HD, et al. (2020) Extracellular vesicles secreted by HBV-infected cells modulate HBV persistence in hydrodynamic HBV transfection mouse model. *Journal of Biological Chemistry* 295:12449-12460. doi: 10.1074/jbc.RA120.014317

7. Vadalà G, Di Giacomo G, Ambrosio L, Cicione C, Tilotta V, Russo F, et al. (2022) The Effect of Irisin on Human Nucleus Pulposus Cells: New Insights into the Biological Crosstalk between the Muscle and Intervertebral Disc. *Spine* Publish Ahead of Print. doi: 10.1097/brs.0000000000004488
8. DiStefano TJ, Vaso K, Danias G, Chionuma HN, Weiser JR, Iatridis JC (2021) Extracellular Vesicles as an Emerging Treatment Option for Intervertebral Disc Degeneration: Therapeutic Potential, Translational Pathways, and Regulatory Considerations. *Advanced Healthcare Materials* 11. doi: 10.1002/adhm.202100596
9. Vieira JO, Duarte JO, Costa-Ferreira W, Morais-Silva G, Marin MT, Crestani CC (2018) Sex differences in cardiovascular, neuroendocrine and behavioral changes evoked by chronic stressors in rats. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 81:426-437. doi: 10.1016/j.pnpbp.2017.08.014
10. Mosley GE, Wang M, Nasser P, Lai A, Charen DA, Zhang B, et al. (2020) Males and females exhibit distinct relationships between intervertebral disc degeneration and pain in a rat model. *Sci Rep* 10:15120. doi: 10.1038/s41598-020-72081-9
11. Schol J, Sakai D, Warita T, Nukaga T, Sako K, Wangler S, et al. (2022) Homing of vertebral-delivered mesenchymal stromal cells for degenerative intervertebral discs repair – an in vivo proof-of-concept study. *Jor Spine*. doi: 10.1002/jsp2.1228
12. Barcellona MN, McDonnell EE, Samuel S, Buckley CT (2022) Rat tail models for the assessment of injectable nucleus pulposus regeneration strategies. *Jor Spine* 5. doi: 10.1002/jsp2.1216
13. Lillyman DJ, Lee FS, Barnett EC, Miller TJ, Alvaro ML, Drvol HC, et al. (2022) Axial hypersensitivity is associated with aberrant nerve sprouting in a novel model of disc degeneration in female Sprague Dawley rats. *Jor Spine*. doi: 10.1002/jsp2.1212
14. Wawrose RA, Couch BK, Dombrowski M, Chen SR, Oyekan A, Dong Q, et al. (2022) Percutaneous lumbar annular puncture: A rat model to study intervertebral disc degeneration and pain-related behavior. *Jor Spine* 5. doi: 10.1002/jsp2.1202
15. Hiraishi S, Schol J, Sakai D, Nukaga T, Erickson I, Silverman L, et al. (2018) Discogenic cell transplantation directly from a cryopreserved state in an induced intervertebral disc degeneration canine model. *Jor Spine* 1. doi: 10.1002/jsp2.1013
16. Thompson JP, Pearce RH, Schechter MT, Adams ME, Tsang IKY, Bishop PB (1990) Preliminary Evaluation of a Scheme for Grading the Gross Morphology of the Human Intervertebral Disc. *Spine* 15:411-415. doi: 10.1097/00007632-199005000-00012
17. Lai A, Gansau J, Gullbrand SE, Crowley J, Cunha C, Dudli S, et al. (2021) Development of a standardized histopathology scoring system for intervertebral disc degeneration in rat models: An initiative of the ORS spine section. *Jor Spine* 4. doi: 10.1002/jsp2.1150