European Journal of Cell Biology 101 (2022) 151270

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

Effect of cell culture media on extracellular vesicle secretion from mesenchymal stromal cells and neurons

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ARTICLE INFO

Keywords:
Extracellular vesicles
Mesenchymal stromal cells
Cell culture media
HPSC neurons

ABSTRACT

Background: Extracellular vesicles (EVs) secreted by neuronal cells *in vitro* have promising therapeutic potential for brain diseases. Optimization of cell culture conditions and methodologies for high-yield isolation of EVs for preclinical and clinical applications, however, remains a challenge.
Objective: To probe the cell culture conditions required for optimal EV secretion by human-derived neuronal cells.
Methodology: First, we optimized the EV purification protocol using human mesenchymal stromal cell (MSC) cultures. Next, we compared the effects of different variables in human pluripotent stem cell (hPSC)-derived neuronal cultures on EV secretion. EVs were isolated from cell conditioned media (CCM) and control media with no cells (NCC) using ultrafiltration combined with size-exclusion chromatography (SEC). The hPSC neurons were cultured in 2 different media from which EVs were collected at 2 maturation time-points (days 46 and 60). Stimulation with 25 mM KCl was also evaluated as an activator of EV secretion by neurons. The collected SEC fractions were analyzed by nanoparticle tracking analysis (NTA), protein concentration assay, and blinded transmission electron microscopy (TEM).
Results: A peak in cup-shaped particles was observed in SEC fractions 7–10 of MSC samples, but not corresponding media controls, indicating successful isolation of EVs. Culture medium had no significant effect on EV yield. The EV yield of the samples did not differ significantly according to the culture media used or the cell maturation time-points. Stimulation of neurons with KCl for 3 h reduced rather than increased the EV yield.
Conclusions: We demonstrated successful EV isolation from MSC and neuronal cells using an ultrafiltration-SEC method. The EV yield from MSC and neuronal cultures exhibited a large batch effect, apparently related to the culture media used, highlighting the importance of including NCC as a negative control in all cell culture experiments.

1. Introduction

Extracellular vesicles (EVs) are membrane-enclosed vesicles involved in intercellular communication (Maas et al., 2017; Raposo and Stoorvogel, 2013). Because the delivered EV cargo molecules, including mRNA and microRNA, affect target cell gene expression, function, and phenotype (Yáñez-Mó et al., 2015), EVs hold great promise as therapeutic agents for different diseases. Therapeutic effects of EVs secreted by mesenchymal stromal cells (MSCs) are reported for kidney, cardiovascular, and neurologic diseases (Keshtkar et al., 2018; Yin et al., 2019). Compared with therapeutics based on viable cells such as cell transplantations, EV-based therapeutics are considered easier to manufacture, store, and transport (Reiner et al., 2017).

Studies evaluating the therapeutic effects of EVs have provided promising results, but EV isolation from cell cultured media remains challenging. Accumulating data indicate that fetal bovine serum (FBS), which is commonly used as supplement for cell culture media, includes vesicles that can contaminate the purification of cell-secreted vesicles

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https://doi.org/10.1016/j.ejcb.2022.151270
Received 31 January 2022; Received in revised form 11 August 2022; Accepted 11 August 2022
Available online 13 August 2022

(Lehrich et al., 2021). For example, bovine micro RNAs not identified in the mouse genome have been found in EV preparations made from mouse cell lines (Wei et al., 2016). Omitting FBS from the media is one option to overcome this issue, but serum-free culture conditions affect the quantity and protein composition of EVs, *e.g.*, neuroblastoma-derived EVs (Li et al., 2015). Depletion of EVs from FBS is also challenging (Kornilov et al., 2018), and is reported to affect the growth of rat primary astrocytes (Lehrich et al., 2018). Furthermore, serum-free media supplements also contain contaminating micro RNAs (Auber et al., 2019; Mannerström et al., 2019).

In 2006, Fauré et al. were the first to isolate EVs from rodent primary cortical cultured neurons (Fauré et al., 2006). Neuronal EVs have an important function in cell-to-cell communication in the central nervous system (Holm et al., 2018). EVs secreted by neurons are taken up by other neurons and astrocytes, and modify the functions of the recipient cells (Chivet et al., 2014; Men et al., 2019; Morel et al., 2013). EVs have been isolated from human induced pluripotent stem cell (hiPSC)-derived neuronal cultures (Candelario et al., 2020; Guix et al., 2018; Podvin et al., 2020; Reilly et al., 2017, 2020). These studies mainly focused on studying the disease-specific properties of EVs, their RNA, or their protein cargo, and differences in neuronal culture conditions are sparsely studied. Further, several of the studies used ultracentrifugation or precipitation-based EV isolation methods that result in suboptimal purification (Karttunen et al., 2018; Lee et al., 2019; Van Deun et al., 2014). The EV purification method influences the yield and purity of the EV preparation and can therefore have a major effect on the outcome. Ultrafiltration combined with size exclusion chromatography (UF-SEC) results in a high yield of relatively pure vesicles from cell culture supernatant compared with the commonly used ultracentrifugation method (Benedikter et al., 2017; Lee et al., 2019). In general, SEC results in intact and biologically functional vesicles and it is considered easily adaptable and suitable EV isolation method for many downstream purposes (Clos-Sansalvador et al., 2022). A recent study showed that adding an SEC step to the ultracentrifugation purification increases the therapeutic effects of MSC-derived EVs, highlighting the importance of the EV purification efficiency (Forteza-Genestra et al., 2020).

Here, we describe the isolation of EVs from cell culture media of MSCs with UF-SEC. We used commercial exosome-depleted FBS (Exo-FBS) and performed the EV isolation and analysis in unconditioned media in parallel with conditioned media. Next, different cell culture conditions for hiPSC-neurons were probed to evaluate their effects on EV secretion. Comparisons were made between 2 cell culture media, 2 neuron maturation phases, and with/without stimulation by 25 mM KCl. The secreted EV number was evaluated using nanoparticle tracking analysis (NTA) and blinded transmission electron microscopy analysis (TEM).

2. Materials and methods

2.1. Cell lines, media, and cell cultures

2.1.1. Ethical approval

This study was approved by the regional ethics committee of Pirkanmaa Hospital District for the derivation, culture, and differentiation of hiPSCs (R08070) and to use generated cell lines in neuronal research (R05116) and for MSCs (R15161).

2.1.2. MSC cultures

Human MSCs (line HFSC 9/15, passages 2–3) were isolated from an adipose tissue sample obtained from a surgical procedure conducted in the Department of Plastic Surgery, Tampere University Hospital (Tampere, Finland). The donor provided permission for utilization of the sample in research.

MSCs were isolated and cultured, and the cells were banked according to previously described protocols (Lindroos et al., 2009). The cells were identified as mesenchymal based on their plastic adherence, differentiation potential towards adipogenic and osteogenic lineages, and the surface marker expression pattern conveying the criteria given by the International Society for Cellular Therapy (Dominici et al., 2006; Hyväri et al., 2020). For EV isolation, frozen MSCs were thawed in a 1:4 vol of culture medium at passage 2 in T75 flasks and cultured for 7 days to reach 60–80 % confluency. Then, either EV collection was started (round 1) or the cells were passaged again at a 1:4 vol of culture medium and cultured for an additional 7 days for EV collection (rounds 2 and 3). Before EV collection, the MSCs were cultured in Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12 1:1 mixture (DMEM/F12; Thermo Fisher Scientific Inc., Carlsbad, CA) supplemented with 5 % human serum (GE Healthcare, Buckinghamshire, U.K.), 1 % GlutaMAX (Thermo Fisher Scientific), and 1 % PEN/STREP (100 U/ml penicillin and 0.1 mg/ml streptomycin; Thermo Fisher Scientific).

2.1.3. Collection of EVs from MSCs

EV collection was started when the cultures reached 60–80% confluency. Cells were washed once with phosphate-buffered saline (PBS) and the medium was changed to EV collection medium. The medium used was either (1) DMEM/F12 (Gibco 21331) supplemented with 10 % exosome depleted FBS (Exo-FBS, Gibco Life Technologies, ref. A25904DG), 1% GlutaMAX (Gibco 35050), and 1 % PEN-STREP (Lonza DE17–602E) or (2) OPTIMEM reduced serum medium (Gibco Life Technologies, ref. 31985–047) supplemented with 10 % Exo-FBS, 1 % GlutaMAX, and 1 % PEN-STREP. A detailed description of the Exo-FBS lots used is provided in Supplementary Table 1. The cells were cultured in EV collection media for 48 h prior to collection. After media collection, the number of cells was counted in the individual flasks used for incubation for both types of media. The volume of media used in the EV isolation was 20 ml (round 1) or 40 ml (rounds 2 and 3). For comparisons, EV numbers and protein concentrations were normalized by the volume of media used in each round.

2.1.4. Neuronal cultures

Human neuronal cells were differentiated from hPSCs using an hiPSC line 10212. EURCCs available at the Faculty of Medicine and Health Technology, Tampere University, Finland (Kiamehr et al., 2019). The hiPSC maintenance in feeder-free culture conditions and neuronal differentiation protocols were described previously (Hongisto et al., 2017; Hyvärinen et al., 2019). Briefly, hiPSCs and differentiating neurons were cultured on top of a coating comprising 100 μg/ml poly-L-ornithine (MilliporeSigma) and 15 μg/ml recombinant human laminin-521 (Biolamina, Sweden). Neuronal maturation medium (NMM) comprising 1:1 DMEM/F12:Neurobasal, 0.5 % N2, 1 % B27 with retinoic acid, 0.5 mM GlutaMAX, 0.5 % non-essential amino acids, 50 μM 2-mercaptoethanol (all from Thermo Fisher Scientific), 2.5 μg/ml insulin (MilliporeSigma), and 0.1% PEN-STREP (Thermo Fisher Scientific) was used. During days 1–26 of differentiation, the NMM was supplemented with 100 nM LDN193189 and 10 431542 (both from MilliporeSigma) and 20 ng/ml fibroblast growth factor-2 (Thermo Fisher Scientific). After day 26, NMM was supplemented with 20 ng/ml brain-derived neurotrophic factor (BDNF, R&D Systems), 10 ng/ml glial-derived neurotrophic factor (GDNF, R&D Systems), 500 μM dibutyryl-cyclicAMP (db-cAMP, MilliporeSigma), and 200 μM ascorbic acid (MilliporeSigma).

2.1.5. EV collection from hPSC-derived neurons

On day 32, neurons were plated onto 6-well cell culture plates (50,000 cells/cm^2 and 2–3 ml media/well) on top of the poly-L-ornithine and recombinant human laminin-521 coating. The culture medium was replaced with fresh EV collection medium 48 h prior to the final EV collection on day 46 or day 60. The EV collection medium was either (1) NMM or (2) OPTIMEM media supplemented with BDNF, GDNF, db-cAMP, and ascorbic acid at similar levels as in NMM. At the 46-day and 60-day time-points, half of the neuronal samples from each media was used in the stimulation experiment. To ensure similar culture conditions, the cells were cultured for 45 h in the EV collection media.

Then, the whole medium was changed and the cells were stimulated with 25 mM KCl for 3 h prior to the final EV collection. Culture medium was collected for EV isolation both before stimulation at 45 h, and then at 48 h (*i.e.,* after 3 h of KCl stimulation). EVs collected at 45 h were included as replicate samples for the EVs collected after 48 h. After the media collection, the cell number was calculated from 1 OptiMEM well.

2.2. EV isolation with size-exclusion chromatography

All the cell culture media samples from MSCs and hPSC-derived neurons were handled similarly. Altogether, 4 types of media samples were used for isolation (Table 1). The cell culture media were centrifuged for 5 min at 200 g to remove the cell debris and stored at + 4 °C or − 70 °C, depending on when SEC was performed (same day or later). Before purification, a 100-µl aliquot of cell culture medium was stored in − 70 ° for later NTA and protein concentration analysis. The rest of the media was centrifuged (16,500 g, 20 min, +4 °C). The supernatant was concentrated to ~200 µl using Amicon Ultra centrifugal filters (100 K, #UFC810024, Merck Millipore) and diluted to 400 µl with citrate buffer (PBS + 0.32 % sodium citrate). The 400-µl sample was loaded onto a 10-ml sepharose column (Karttunen et al., 2018) and 25 fractions (500 µl each) were collected. With this protocol, EVs elute in fractions 7–10 (Böing et al., 2014; Karttunen et al., 2018). The fractions were stored at − 70 °C. The protein concentrations of the cell culture media and the fractions were measured using a Pierce BCA protein assay kit according to the manufacturer's instructions (#23225, Thermo Fisher Scientific).

2.3. Nanoparticle tracking analysis

The relative concentration and size distribution of the particles in the SEC fractions were measured using NS300 NanoSight (Malvern, Worcestershire, UK). For recordings, the camera level was adjusted to 13 (range 1–16) and the remaining settings were set to automatic. The sample was injected into the sample chamber at a constant flow rate using the Malvern NanoSight syringe pump system. Three 30-s captures per sample were recorded. For analysis, the detection threshold was set to 5 and other settings were set to automatic. Each sample was diluted 1:50 or more to produce a concentration between 5×10^7 and 2×10^9 particles/ml.

2.4. Transmission electron microscopy

For both MSC and neuronal sample preparations, 400-ul aliquots of successive SEC fractions (7–10 or 13–15) were pooled and concentrated to a final volume of approximately 150 µl using Amicon® Ultra-4 Centrifugal Filters (#UFC801024, Merck Millipore). TEM samples were prepared from the concentrated fractions as previously described (Karttunen et al., 2018). Imaging was performed using a JEOL JEM-2100F electron microscope (Jeol Ltd, Tokyo, Japan) at 200 kV.

In the blinded TEM analysis, the grids were imaged and evaluated with the investigator blinded to the sample identity. Acquisition of images for the analysis was performed by scrolling through the grid, stopping at 10 random spots where an image was taken with 10 000× magnification. If stopped on the edge of the grid, the image was taken at the nearest spot inside the grid so that no grid edges were included in the image. After obtaining the images, the sample was inspected in more detail and additional images were taken at different magnifications. Because of the large variation in sample quality due to technical factors in the TEM sample preparation (*e.g.,* differences in coating thickness), which affected the quality of the random images, the evaluation of EV quantity was based both on the random images and on the subjective overall assessment of the sample. The number of cup-shaped vesicles in the samples was rated according to a 4-step scale where "-" corresponds to "none", and "+++ " to "a large number" of vesicles (for scoring details, see Supplementary Figure 1).

Table 1
Composition of cell culture media used in the EV collection.

Media	Composition
MSC Exo-FBS	DMEM/F12 (Gibco 21331) supplemented with 10 % exosome-depleted FBS (Exo-FBS, Gibco Life Technologies, ref.A25904DG), 1 % GlutaMAX (Gibco 35050), 1 % PEN-STREP (Lonza DE17–602E)
MSC OptiMEM	OPTIMEM reduced serum medium (Gibco Life Technologies, #31985–047) supplemented with 10% Exo-FBS, 1% GlutaMAX, 1 % PEN-STREP.
Neurons: NMM	1:1 DMEM/F12:Neurobasal, 0.5 % N2, 1 % B27 with retinoic acid, 0.5 mM GlutaMAX, 0.5 % -non-essential amino acids, 50 µM 2-mercaptoethanol (all from Thermo Fisher Scientific), 2.5 µg/ml insulin (MilliporeSigma), and 0.1 % PEN-STREP (Thermo Fisher Scientific) with 20 ng/ml brain-derived neurotrophic factor (BDNF, R&D Systems), 10 ng/ml glial-derived neurotrophic factor (GDNF, R&D Systems), 500 µM dibutyryl-cyclicAMP (db-cAMP, MilliporeSigma), and 200 µM ascorbic acid (MilliporeSigma).
Neurons: OptiMEM	OPTIMEM media supplemented with BDNF, GDNF, db-cAMP, and ascorbic acid, similar to the NMM

2.5. Statistical analysis

Statistical analyses were performed using Prism 8 for macOS, version 8.2.1 (279). Differences in the particle numbers between the 2 MSC culture media and the different conditions in hiPSC-derived neuron cultures were compared using the non-parametric Kruskal–Wallis analysis of variance test followed by *post hoc* analysis with the Mann–Whitney U test. P-values < 0.05 were considered statistically significant. The blinded TEM analysis was semi-quantitative and the difference between groups was not statistically evaluated.

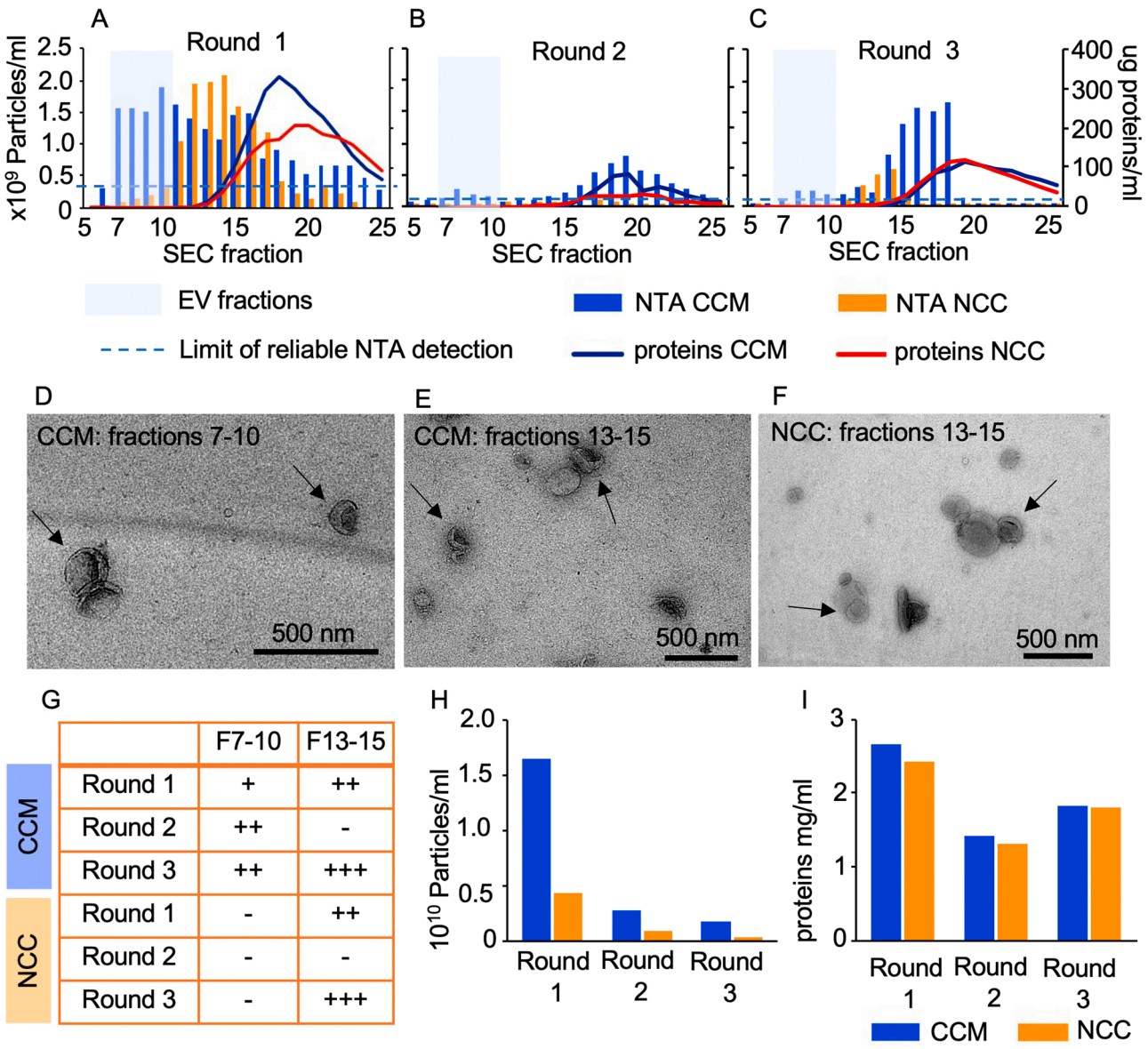
3. Results

3.1. Size-exclusion chromatography isolates EVs from MSC cell culture media

The secretion of MSC-derived EVs was evaluated with SEC purification in 3 separate purification rounds. The EV collection media compositions were similar in all rounds, but the commercial batches of the media supplements (*e.g.,* Exo-FBS) differed between rounds (Table 1, Supplementary Table 1). For all rounds, each type of medium used was purified similarly to cell cultured media as a negative control. The MSCs from 1 flask were imaged before changing the EV collection media and before collecting the EVs. The different compositions of the EV collection media did not affect cell viability or morphology (Supplementary Figure 2).

Protein concentrations and particle numbers were measured from SEC fractions 5–25. As the volume of the media used varied between rounds, the final particle numbers and protein concentrations are presented per milliliter of media used for SEC purification. In all 3 rounds, the peak in the particle concentration in cell culture media (CCM) samples collected from Exo-FBS was observed in EV-containing fractions 7–10, whereas particle concentrations collected in NCC samples did not reach a reliable detection limit (Fig. 1A-C). The concentration of EVs per milliliter of the starting media varied between the 3 rounds and the cell numbers calculated from individual flasks varied. Therefore, as the cell numbers were not calculated from all used flasks, the EV concentration/cell number ratio could not be reliably assessed (Table 2). As the protein concentrations of fractions 7–10 were below the BCA assay detection limit (Fig. 1A-C), there was not enough material for Western blot analysis (data not shown). In all rounds, fractions 17–22 contained the highest peak of non-EV related proteins, indicating good separation of EVs from proteins during SEC.

To confirm the presence of EVs, fractions 7–10 were combined and concentrated for TEM analysis. According to a blinded TEM analysis, cup-shaped EVs were visible in fractions 7–10 in the CCM samples, but



		F7-10	F13-15
CCM	Round 1	+	++
	Round 2	++	-
	Round 3	++	+++
NCC	Round 1	-	++
	Round 2	-	-
	Round 3	-	+++

Fig. 1. *EV isolation from MSC cultures.* (A-C) Protein and particle profiles of size-exclusion chromatography fractions from 3 culture rounds with MSC cell line using exosome-depleted fetal bovine serum-based media. Values are calculated per milliliter of cell culture media used in the EV isolation. Representative transmission electron microscopy (TEM) images show cup-shaped particles (black arrows) in (D) cell culture media fractions 7–10, (E) cell culture media fractions 13–15 and (F) no cell control fractions 13–15 from the round 3 samples. (G) Blinded TEM analysis of the EV-containing fractions and middle fractions from each culture round. (H) Particle and (I) protein concentrations of the cell culture media of each round before the concentrating step. *Abbreviations*: CCM: cell culture media, NCC: no cell control, NTA: nanoparticle tracking analysis.

Table 2
Cell numbers were calculated from 1 flask in each round. EV number per cell was calculated based on the cell number evaluation and nanoparticle tracking analysis measurement.

	Cells/ Flask	EVs/Cell
MSC round 1: edFBS	5.54×10^5	1.29×10^5
MSC round 2: edFBS	5.45×10^5	1.60×10^4
MSC round 2: OptiMEM	4.08×10^5	2.05×10^4
MSC round 3: edFBS	1.28×10^5	5.20×10^4
MSC round 3: OptiMEM	1.33×10^5	8.01×10^4
Neurons round 1: OptiMEM day 46	1.30×10^6	1.64×10^5
Neurons round 1: OptiMEM day 60	2.40×10^6	1.91×10^5
Neurons round 2: OptiMEM day 46	9.80×10^5	1.20×10^5
Neurons round 2: OptiMEM day 60	1.10×10^6	2.74×10^5

Abbreviations: edFBS: exosome-depleted fetal bovine serum-based media MSC: mesenchymal stromal cells.

absent in the NCC samples (Fig. 1D and G).

3.2. Cell culture media includes a population of cup-shaped vesicles

In addition to particles in fractions 7–10, NTA analysis showed an even higher particle concentration in mid-fractions 11–15 in rounds 1 and 3 in both CCM and NCC samples (Fig. 1A and C). In round 2, NCC samples included no peaks in the particle concentrations whereas CCM samples had a second peak from fraction 15 onwards (Fig. 1B). TEM imaging from pooled mid-fractions indicated that both the NCC and CCM samples contained cup-shaped vesicles and also round particles that resembled lipoproteins (Fig. 1E and F). The presence of vesicles with a cup-shaped morphology was further confirmed with a blinded TEM analysis (Fig. 1G).

To investigate differences between the 3 media batches in more detail and exclude any effects of the SEC purification, the particle and

protein concentrations were measured from the collected media (CCM and NCC samples) before the initial concentrating step. In accordance with the SEC results, the media used in the first round contained the highest number of particles with 1.7×10^{10} particles/ml in CCM and 4.4×10^{9} particles/ml in NCC (Fig. 1H). The CCM samples in the second and third rounds had lower particle numbers than the NCC samples in the first round (2.9×10^{9} and 1.8×10^{9} particles/ml *vs* 4.4×10^{9} particles/ml, respectively). In all 3 rounds, the particle number was higher in the CCM samples than in the NCC samples, confirming the secretion of vesicles. The protein concentration was highest in the media sampled in the first round (CCM 2700 mg/ml and NCC 2400 mg/ml). Unlike the particle number, however, the protein concentrations of the media were lower in the second round than in the third round (CCM 1400 *vs* 1800 mg/ml and NCC 1300 *vs* 1800 mg/ml, respectively) (Fig. 1I). The results show that the particle numbers and protein concentrations prior to the EV purification correlated with the amounts gained after purification.

3.3. Comparison of the 2 MSC culture media

Next, we assessed the effect of the 2 types of EV collection media on MSC-derived EV yields in rounds 2 and 3. The EV collection media used was either Exo-FBS or OptiMEM, both containing EV-depleted FBS. The CCM and NCC samples were analyzed with NTA and blinded TEM analysis. The Exo-FBS media yielded a mean of 8.3×10^{8} particles/ml and the OptiMEM media yielded a mean of 9.6×10^{8} particles/ml when the yields from fractions 7–10 were pooled together (Fig. 2A). The numbers of EVs produced did not differ significantly between the 2 types of media. The TEM analysis results were consistent with the NTA results (Fig. 2B).

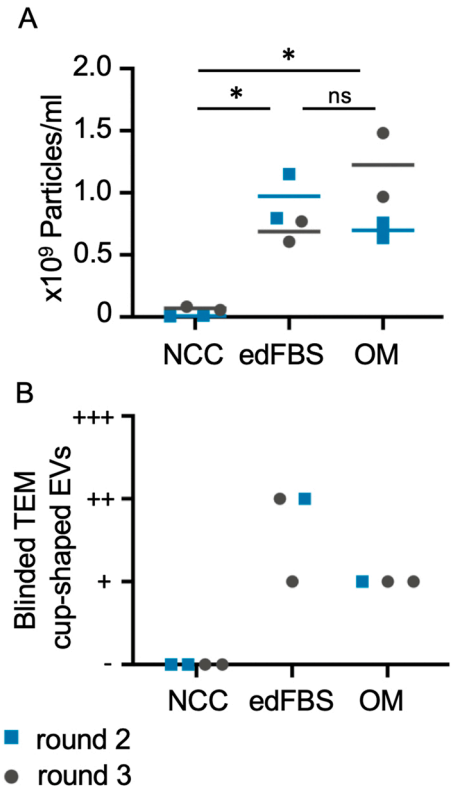


Fig. 2. : Comparison of EV production by MSCs. EVs collected from 2 cell culture media with (A) nanoparticle tracking analysis and (B) blinded transmission electron microscopy (TEM) analysis from EV-containing size-exclusion chromatography fractions 7–10. Data presented are per milliliter of cell culture media used in EV isolation. In round 2, TEM was performed only for 1 replicate sample. * $p < 0.05$. *Abbreviations*: edFBS: exosome-depleted fetal bovine serum-based media, NCC: no cell control, OM: Optimem-based media, TEM: transmission electron microscopy. Statistical significance: *$p < 0.05$ (Mann–Whitney U).

From round 3, an additional RNA analysis was performed to SEC fractions from cell cultured supernatants and to uncultured cull culture media without SEC purification (Supplementary figure 3). Two miRNAs were analysed using droplet digital PCR. miR-142–3p, previously been shown to peak at EV fractions in plasma (Karttunen et al., 2018), peaked in EV-containing SEC fractions from cell cultured media samples and was found in high level from non-conditioned media samples. miR-146a-5p had peaks in both, EV and non-vesicular protein fractions in cell cultured samples. Only a low amount was found from non-cultured media samples. When two culture media were compared, miR-142–3p levels were similar with both media, whereas miR-146a-5p level correlated with the particle concentration measured with NTA. This indicated that miR-142–3p originates at least partly from cell culture media whereas miR-146a-5p was mainly secreted by the cells.

3.4. Effect of culture conditions on EV yield from hiPSC neurons

After confirming that SEC is a reliable method for EV isolation from MSCs, we next assessed EV secretion from hiPSC-derived neurons under different conditions. For comparison, we used 2 types of cell culture media (OptiMEM and NMM without FBS supplement) and 2 maturation phases (day 46 and day 60) (Table 1). In addition, we assessed the efficacy of 3-h stimulation with 25 mM KCl on the induction of EV secretion in both media and at both maturation time-points. The experimental design is summarized in Fig. 3A and was repeated twice. EV samples collected at 45 h before the 3-h KCl stimulation were used as a replicate of the non-stimulated sample collected at 48 h. As the individual SEC-fractions did not reach a high enough particle concentration to be analyzed with NTA, SEC fractions 7–10 were pooled, and the final particle number was calculated per milliliter of media used for the SEC purification. It should be noted that according to our previous data, this culture can contain smaller population of endogenous astrocytes which amount is relatively low, 0–4 % at day 46 of differentiation, and reaches 15–26 % at day 74 (Hyvärinen et al., 2019) according to analysis of GFAP protein with immunocytochemistry.

To evaluate the EV secretion, samples from both culture media and both time-points were pooled together, excluding the stimulated samples. Within individual rounds, the NTA analysis revealed higher particle concentrations in non-stimulated CCM than in NCC samples (mean for round 1: 2.1×10^{11} *vs.* 5.4×10^{10} particles/ml, $p < 0.01$; round 2: 7.9×10^{10} *vs.* 5.2×10^{9} particles/ml, $p < 0.01$), indicating successful secretion and isolation of EVs (Fig. 3B). The particle concentration was significantly higher in round 1 compared with round 2 in both the CCM ($p < 0.01$) and NCC ($p < 0.05$) samples (Fig. 3B). Blinded TEM analyses results were consistent with the NTA results (Fig. 3C) and TEM imaging confirmed the presence of cup-shaped vesicles in SEC fractions 7–10 (Fig. 3D). The cell numbers calculated from individual flasks varied between time-points, and as they were not calculated from each flask used for EV collection, a direct association between the cell numbers and EV counts could not be calculated. (Table 2).

We then assessed the difference in EV secretion in the 2 different types of EV collection media. Because the EV numbers differed between individual rounds, comparisons were performed for each round separately. The samples from both maturation points were combined for comparison. Within each round, vesicle numbers were comparable between the OptiMEM and DMEM media (mean for round 1: 2.0×10^{11} *vs.* 2.2×10^{11} particles/ml, $p > 0.05$; round 2: 6.7×10^{10} *vs.* 9.2×10^{10} particles/ml, $p > 0.05$) (Fig. 3E). The blinded TEM analysis results were consistent with the NTA results (Fig. 3H).

Next, we compared the EV secretion on day 46 and day 60 of neuronal cell maturation. The comparison was performed separately for both rounds. This time, the samples from both types of cell culture media were combined for the analysis. Within the individual rounds, vesicle

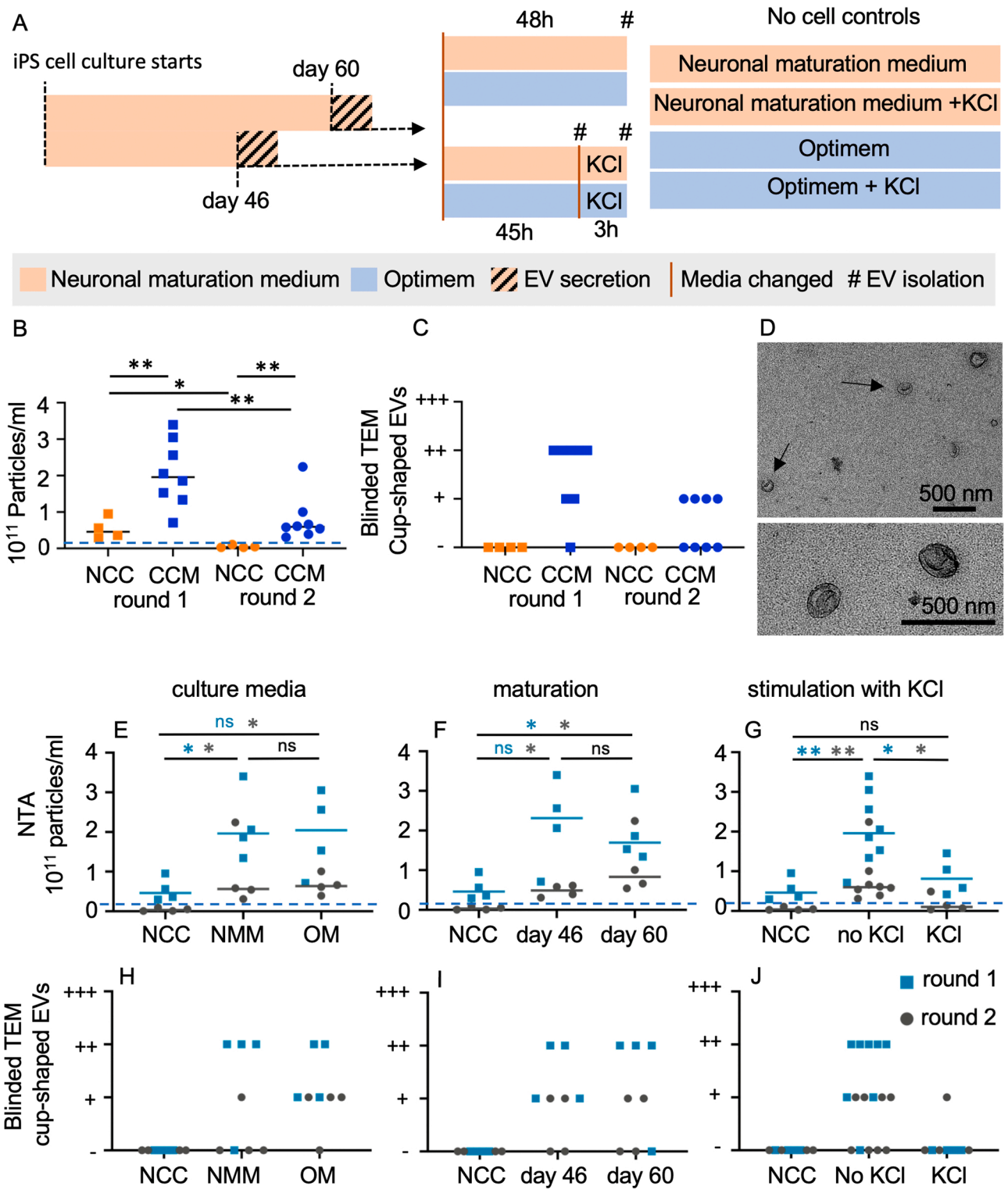


Fig. 3. : *Comparison of EV secretion of iPS neuronal cell lines.* (A) Schematic presentation of the cell culture experiment that was repeated 2 times. (B) NTA and (C) blinded TEM comparison of the 2 rounds shows EV secretion in CCM samples compared with NCC. (D) Representative TEM images of individual sample from neuronal maturation medium show the presence of cup-shaped vesicles. NTA and blinded TEM analysis showed no difference in EV secretion regardless of (E, H) cell culture media or (F, I) maturation time. (G, J) Stimulation with KCl did not enhance EV secretion. *Abbreviations*: CCM: cell culture media, NCC: no cell control, NMM: Neuronal maturation medium, NTA: Nanoparticle tracking analysis, OM: OptiMem-based media, TEM: transmission electron microscopy. Statistical significance: *p < 0.05, * *p < 0.01, ns: not significant, (Mann–Whitney U).

numbers did not differ in the samples collected on day 46 or on day 60 (Fig. 3F and I)

Finally, we attempted to increase the EV yield by stimulating with 25 mM KCl for 3 h. Two cell culture rounds were compared separately, and the data from the 2 types of culture media and 2 maturation phases were combined. Stimulation with 25 mM KCl did not increase the EV secretion as the vesicle numbers measured with NTA in KCl-stimulated cells were similar to those in unconditioned media samples (mean for round 1: 8.7×10^{10} *vs.* 5.4×10^{10} particles/ml, $p > 0.05$; round 2: 1.8×10^{10} *vs.* 5.2×10^{9} particles/ml, $p > 0.05$) and lower than that in cell-cultured media samples without stimulation (mean for round 1: 2.1×10^{11}, $p < 0.05$; round 2: 7.9×10^{10} particles/ml, $p < 0.05$) (Fig. 3G). The blinded TEM analysis results were consistent with the NTA results, and cup-shaped vesicles were found only in 1 KCl-stimulated cell culture sample (Fig. 3J).

4. Discussion

Our objective was to evaluate the effect of different cell culture media on the EV yield in human MSC- and hiPSC-derived neurons. We confirmed that the production and isolation of MSC-derived EVs was successful using the UF-SEC method. With the hiPSC-derived neurons, we evaluated different culture conditions for optimal EV secretion and found the highest variability between 2 culture rounds.

4.1. Size exclusion chromatography resulted in the successful isolation of MSC-secreted vesicles

To evaluate the SEC-based EV isolation method, we compared the EV numbers between cell cultured media and unconditioned media samples prepared from the MSC cell line in 3 replicate rounds. Using NTA and blinded TEM, vesicles were detected in the SEC fractions 7–10 of the cell cultured media samples in each round, thereby confirming the previous findings by Böing et al. (2014) and data obtained with similarly sized commercial SEC columns (Hicks et al., 2020). Fractions 7–10 did not contain measurable numbers of vesicles in unconditioned media samples, confirming that the vesicles were secreted by the cells. We were not able to perform western blot analysis with neither positive nor negative EV markers due to the low protein and vesicle concentrations, so contamination of intracellular vesicles was not evaluated. Minor contamination of vesicles from unconditioned media could also be detected with more sensitive methods. Previously, for example, RNA sequencing demonstrated cell culture medium contamination (Driedonks et al., 2019; Mannerström et al., 2019). Also, our preliminary ddPCR analysis found at least miR-142–3p present in the unconditioned media samples. In general, SEC is more gentle EV isolation method compared to ultracentrifugation-based methods that uses high centrifugal forces or precipitation where the membrane structure is disrupted. Still, it is possible that a minor part of the vesicles was disrupted during the purification.

4.2. MSC culture media included a contaminating subgroup of vesicles

The commonly used cell culture medium supplement, FBS, is well known to contain vesicles that can contaminate purified EV preparations. Different approaches have been developed to deplete EVs from FBS, but total depletion is difficult to achieve (Kornilov et al., 2018; Lehrich et al., 2018; Shelke et al., 2014). The EV depletion can also negatively affect the growth, viability, and phenotype of particular cell types such as primary astrocytes and muscle cells (Aswad et al., 2016). We observed no distinct negative effect of FBS on EV depletion in adipose tissue derived MSCs, consistent with a previous study by Kornilov et al. (2018).

Unexpectedly, the mid-SEC fractions of unconditioned media used in MSC cultures contained a high number of particles on 2 of the 3 rounds. This was detected by both the NTA and blinded TEM analysis. The corresponding fractions also included vesicles in the cell cultured media samples and it is unclear whether these particles originated from the media only or were partially secreted by the cells. In the remaining round, the particle peak in the mid-SEC fractions in the cell cultured media samples was detected with NTA but not with a blinded TEM. The mid-SEC fractions are generally considered to contain lipoproteins (Böing et al., 2014; Karttunen et al., 2018). In our preparations, the vesicles detected in the mid-fractions by blinded-TEM analysis had a cup-shaped morphology. In TEM, EVs are usually described as cup-shaped particles due to the collapse of the water-including interior during sample preparation whereas lipoproteins do not encapsulate water and are described to have a rounder morphology (Böing et al., 2014; Lobb et al., 2015; Rikkert et al., 2019; Yuana et al., 2014). In our preparations, SEC was able to separate these "vesicles", but whether it applies to other EV isolation methods remains to be explored.

The EV yield and the presence of particles in mid-fractions varied between the 3 culture rounds. To eliminate possible effects of the concentrating step prior SEC, we also analyzed the media before EV purification and confirmed batch-to-batch differences induced by the media supplements. Thus, our results indicate that even if the media composition remains the same, different commercial batches can affect the results.

4.3. Neither culture media nor neuron maturation time affects neuronal EV yield

After confirming that our protocol was successful for separating EVs from the cell culture media, we investigated the EV secretion of human neurons and tested whether different culturing conditions alter the EV secretion. Under all the conditions tested, the EV particle levels detected were at the lower limit of reliable detection. Pooling EV-containing SEC fractions 7–10 resulted in detection of individual EVs with both NTA and TEM analysis methods.

The protocols used to collect media from hiPSC-derived neurons for EV isolation vary remarkably between studies. For example, Podvin et al. collected media every 3–4 days for several weeks for proteomics analysis of the EVs, whereas Aulston et al. collected media every 2–3 days during weeks 3–5 after the initiation of neuronal differentiation (Aulston et al., 2019; Podvin et al., 2020). These studies did not report the final volume from which the EVs were isolated. Other articles reported using 2 ml of cell cultured media for EV purification after 3 days in culture (Reilly et al., 2017; Winston et al., 2019). Most previous studies used a precipitation-based EV isolation method (Aulston et al., 2019; Podvin et al., 2020; Reilly et al., 2017; Winston et al., 2019) or ultracentrifugation (Guix et al., 2018). As we used UF-SEC, comparison with previous studies is difficult. Our protocol is similar, however, to that used by Hicks et al. (2020), in which hiPSC-derived neurons were cultured in DMEM/F12:neurobasal medium, the volume of media collected for EV isolation was 50 ml, and it was concentrated to 0.5 ml before SEC (Hicks et al., 2020). Here, we collected 18 ml media for EV isolation and concentrated it before SEC. Importantly, previous studies did not include unconditioned media as a negative control. Apart from Hicks et al. (2020), previous studies also did not confirm the presence of vesicles in the EV isolate by TEM imaging.

Comparison of the 2 types of cell culture media revealed no difference in the EV yield. The yields from the cell cultured media were significantly higher than that in the unconditioned media, however, further verifying the isolation of cell-secreted EVs from human neuronal cultures. Importantly, none of the neuronal EV-collection media assessed contained FBS.

Maturation of hiPSC-derived neuronal cultures takes several weeks, especially when considering their functional maturation (Hyvärinen et al., 2019). Next, we compared whether a longer maturation time would result in a higher yield of EVs. Neuronal networks form a robust synchrony across the network around day 53 after differentiation, which is considered the stage of *in vitro* maturation (Hyvärinen et al., 2019).

Comparison of EV yield of the same cells after a 46- or 60-day maturation period, *i.e.*, before and after achieving network synchrony, revealed no difference. To our knowledge, this is the first study comparing EV secretion between different maturation time-points of individual hiPSC-derived neuronal cultures. We compared only the number of particles and more detailed analysis with RNA sequencing or proteomics would be an interesting next step.

4.4. Stimulation did not improve neuronal EV secretion

Neuronal EV secretion can be upregulated by various means, including fast depolarization of rat cortical neurons by 3-h exposure to KCl (Fauré et al., 2006; Gong et al., 2016; Lachenal et al., 2011; Wang et al., 2017), by increasing spontaneous synaptic glutamatergic activity after blockade of inhibitory $GABA_A$ receptors (Chivet et al., 2014; Lachenal et al., 2011), and by increasing neuronal activity with basic fibroblast growth factor 2 (Kumar et al., 2020). Here we attempted to increase EV secretion in human cells by exposing them for 3 h to 25 mM KCl. Unexpectedly, this did not result in any increase in the EVs compared with that in the unconditioned media. The low particle number is partly explained by the timing of the sample collection for EV isolation. The non-stimulated samples were collected after 45–48 h in culture, whereas the stimulated samples were collected already after 3 h in culture. It should be noted that in previous studies the increased EV secretion was determined based on mainly on semi-quantitative analysis by Western blotting rather than on NTA and TEM analysis.

4.5. Conclusions and next steps

Our study demonstrates that setting up EV isolation from cell cultures is not straightforward. Importantly, we observed some similarities in EV yield when comparing the data from MSC and neuronal cells, which are important to acknowledge. First, there was large variability in the EV number between the cell culture rounds, even when the same cell line was used. Second, the overall yield of EVs in MSC -and hiPSC -derived neuronal cells was low even after the development of spontaneous network activity or the use of 25 mM KCl to induce neuronal activity. We detected EVs with NTA and TEM, but the protein amount was too low for Western blot analysis. The low and variable yield is a well-recognized problem in the EV field. One way to overcome the low EV-secretion level is to use 3-dimensional cell cultures in bioreactors, which has been shown to increase the EV yield by 20–100-fold compared with 2-dimensional culture (Haraszti et al., 2018; Palviainen et al., 2019). Our results outline the need for more basic studies of the effect of cell culture conditions in EV yield and profile. Importantly, unconditioned media should be used as a basic negative control in all experiments instead of checking its effect only occasionally.

Funding

This study was supported by the European Union's Seventh Framework Program (FP7/2007–2013) under grant agreement no. 602102 (EPITARGET, AP), the Academy of Finland (AP: Grants 272249, 273909 and 2285733–9; SN: grant number 336665; SM: grant number 326588), and the Sigrid Jusélius Foundation (AP).

CRediT authorship contribution statement

JK: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Roles/Writing - original draft, Writing - review & editing; **MH:** Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Roles/Writing - original draft, Writing - review & editing; **TJ:** Investigation, Visualization, Roles/Writing - original draft; **AH:** Conceptualization, Investigation, Writing - review & editing; **VNF:** Conceptualization, Formal analysis, Investigation, Methodology; **SM:** Methodology, Resources, Writing - review & editing; **SN:** Conceptualization, Methodology, Resources, Supervision, Roles/Writing - original draft, Writing - review & editing; **AP**: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Roles/Writing - original draft, Writing - review & editing.

Acknowledgments

We acknowledge SIB Labs for technical assistance in TEM imaging. The work was supported by the Imaging Facility and iPS Cells Facility (Faculty of Medicine and Health Technology, Tampere University). The authors also thank Biocenter Finland for the support of the imaging and iPS cell facilities.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcb.2022.151270.

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