Placenta stem/stromal cell-derived extracellular vesicles for potential use in lung repair

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List of Abbreviations

AFM, atomic force microscopy; AFM-IR, atomic force microscope infrared-spectroscopy; CMSC29, chorionic mesenchymal stromal cell line; COPD, chronic obstructive pulmonary disease; DMSC23, decidual mesenchymal stromal cell line; EVs, extracellular vesicles; HBSS(-), Hanks' Balanced Salt Solution; LPS, lipopolysaccharide; MSC, mesenchymal stem/stromal cell; NTA, nanoparticle tracking analysis; SEM, scanning electron microscopy; SFM, serum-free media; TEM, transmission electron microscopy; TRPS, tunable resistive pulse sensing.

Keywords: Extracellular vesicles, placenta mesenchymal stromal cells, regenerative potential, lung diseases

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Abstract

Many acute and chronic lung injuries are incurable and rank as the fourth leading cause of death globally. While stem cell treatment for lung injuries is a promising approach, there is growing evidence that the therapeutic efficacy of stem cells originates from secreted extracellular vesicles (EVs). Consequently, EVs are emerging as next-generation therapeutics. While EVs are extensively researched for diagnostic applications, their therapeutic potential to promote tissue repair is not fully elucidated. By housing and delivering tissue-repairing cargo, EVs refine the cellular microenvironment, modulate inflammation, and ultimately repair injury. Here, we present the potential use of EVs derived from two placental mesenchymal stem/stromal cell (MSC) lines; a chorionic MSC line (CMSC29) and a decidual MSC cell line (DMSC23) for applications in lung diseases. Functional analyses using *in vitro* models of injury demonstrated that these EVs have a role

in ameliorating injuries caused to lung cells. We also showed that EVs promote repair of lung epithelial cells. This study is fundamental to advancing the field of EVs and to unlock the full potential of EVs in regenerative medicine.

Statement of significance of the study

Current studies provide strong evidence that the therapeutic benefit of stem cells is mainly through the paracrine actions of extracellular vesicles (EVs). Here we demonstrate that EVs derived from placental mesenchymal stem/stromal cell lines ameliorate inflammation and stimulate tissue repair. Direct delivery of EVs to lungs is a promising therapeutic approach to drive lung tissue regeneration after injury. In this study, we present the use of single vesicle characterisation and microRNA profiling for identifying types of EVs that contain the appropriate molecular composition for use in lung injuries. We provided an extensive approach to EV characterisation using our highly novel resonance enhanced atomic force microscopy method, which exceeds recommended minimum reporting requirements for EVs, and which enables single vesicle structural and molecular analysis. The use of our approach allows for unequivocal analysis of vesicle heterogeneity and identification of nanoscale differences in EV composition. Both parameters are pivotal for identifying and isolating - EV subtypes that have potential use in ameliorating diseases. Together with microRNA profiling, these data are of fundamental importance when designing rational approaches that employ EVs for lung tissue repair.

1. Introduction

Acute and chronic respiratory diseases remain incurable and new treatment strategies are urgently required ^[1-3]. Each year, four million people die prematurely due to respiratory diseases ^[3]. Acute respiratory distress syndrome, pulmonary fibrosis and chronic obstructive pulmonary disorder (COPD) are amongst the most frequent causes of deaths globally, despite

ongoing advances in supportive care ^[1, 4]. Current strategies for enhancing tissue regeneration are only supportive and do not actively promote tissue repair.

Stem cell-based therapy for tissue regeneration emerged as a promising option not only for controlling symptoms of lung diseases but also for its potential as a curative treatment regimen ^[5, 6]. The efficacy of mesenchymal stem cells (MSCs) in alleviating inflammation, a major hallmark of treatment for many lung diseases, is confirmed in numerous studies ^[6, 7]. Moreover, cell-free regenerative therapy using MSC-derived extracellular vesicles (EVs) is emerging as a more promising alternative due to the lower risk of an immunogenic response, preservation of biochemical activity upon storage ^[8] and higher stability in hostile environments compared with MSCs ^[9]. MSCs respond to their changing microenvironment by selectively packaging EVs with heterogeneous compositions of proteins, lipids, and nucleic acids ^[10]. The transfer of specific protein and microRNA (miRNA) cargo to the injured cell is widely accepted as an essential driver of the regenerative process, through its impact on multiple downstream biological pathways, and its ability to regulate the host immune response ^[11].

We investigated the potential of placenta-derived mesenchymal stem/stromal cell-derived EVs for regenerative medicine. Human placenta is an abundant source of stem cells, yet it is usually discarded at birth. Placenta-derived stem cells grow fast and more robustly compared to stem cells from other tissues while having the lowest osteogenic potential ^[12]. Our focus was on developing therapeutic EVs for direct delivery to the lung to yield enhanced therapeutic effects. The EVs secreted by the chorionic MSC line (CMSC29) and decidual MSC line (DMSC23) were analysed using a combination of conventional microscopy, nanoparticle tracking analysis, tunable resistive pulse sensing, Nano Flow Analyzer and biological analyses. We also used resonance enhanced atomic force microscope-infrared spectroscopy (AFM-IR) for the characterisation of EV chemical composition and Nano Flow

Analyzer for the determination of total nucleic acid content in EVs. Our recently published study shows AFM-IR enables the nanoscale study of structural and molecular differences of individual EVs^[13]. In this study we include functional analyses of *in vitro* models of injury to demonstrate the potential of EVs derived from the DMSC23 cell line in reducing cellular stress, which support progress into pre-clinical studies. Our work is fundamental in advancing the use of placental cell line-derived EVs for future application in regenerative medicine.

2. Materials and Methods

2.1 Cell culture and maintenance

Primary MSCs from the fetal chorion and maternal decidual components of the term human placenta were transduced with telomerase reverse transcriptase (hTERT) to create the CMSC29 and DMSC23 cell lines respectively^[14]. These two cell lines have extended life span while maintaining stemness, whereas primary MSCs have limited life spans and their stemness are subject to patient-to-patient variation. CMSC29 was cultured in AmnioMAXTM (InvitrogenTM, Thermo Fisher Scientific, North Ryde, NSW, Australia) and DMSC23 was cultured using the MesencultTM proliferation kit (STEMCELL Technologies, Tullamarine, VIC, Australia).

The immortalised human bronchial epithelial cell line (BEAS-2B) and the human monocyte cell line (THP-1) were cultured in Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute medium (RPMI 1640) respectively (Sigma-Aldrich, Castle Hill, NSW, Australia). Both these lines were supplemented with 10% foetal bovine serum (FBS; French origin, Scientifix, Cheltenham, VIC, Australia) and 1% penicillin-streptomycin 5000 U/ml (PenStrep[®], Thermo Fisher Scientific). Hanks' Balanced Salt Solution (HBSS(-), Sigma-Aldrich) was used for washing DMSC23 and CMSC29, while phosphate buffer saline without Ca²⁺ and Mg²⁺ (PBS; Lonza, Bella Vista, NSW, Australia) was used for BEAS-2B and THP-1. TrypLETM Select Enzyme (Thermo Fisher Scientific) was used as the

dissociation reagent. DMSC23 and CMSC29 were used at passages P23-28, while BEAS-2B and THP-1 cells were used at P12-16 and P14-20 respectively. All cells were maintained at 37°C and 5% CO₂ in incubators.

2.2 Isolation of EVs by ultracentrifugation

DMSC23 and CMSC29 were cultured until 80% confluence in normal maintenance culture media then cells were washed twice with HBSS(-). Cells were then cultured in EV isolation media (serum-free media containing 0.5% bovine serum albumin and 1% PenStrep) for 48 h. EV-containing media were collected and centrifuged at 500×g for 5 min, then the supernatant was further centrifuged at 2,000×g for 10 min to remove cells and debris. The supernatant was centrifuged at 31,200 rpm (100,000×g) for 60 min at 4°C using a Ti-70 rotor in an Optima LE-80K Ultra Centrifuge (Beckman Coulter, Lane Cove West, NSW, Australia). The supernatant was discarded and the EV pellet was resuspended in 1 ml RNase-free PBS (Lonza). The ultracentrifugation was repeated at 31,200 rpm (100,000×g) for 60 min at 4°C and the supernatant was discarded. The EV pellet was resuspended in 200 µl RNase-free water, transferred to RNase-free microcentrifuge tubes and stored at -80°C if not used immediately.

2.3 Measurement of size and concentration of EVs

2.3.1 Nanoparticle tracking analysis (NTA)

EV samples were diluted 1:40 with nuclease-free water, vortexed at a moderate speed for 30 secs and then measured using a NanoSight NS300 (Malvern Instruments, ATA Scientific, Australia). Diluted samples (1 ml) were infused using a syringe pump with temperature control set at 25°C and the particles were imaged with an auto-focus camera for 60 secs, and this process was repeated three times per sample. Data were processed using the NTA software using camera level 11 and a detection threshold of 4, which allowed the calculation and comparison of mean particle diameter, mode, D50 values and particle concentration.

2.3.2 Tunable Resistive Pulse Sensing (TRPS)

For the measurement of particle size and concentration with higher precision and accuracy, TRPS was used using a qNano (IZON Science, Christchurch, New Zealand). Particles were resuspended in electrolytes and passed through an engineered pore (NP100), which provided a direct measurement of size and concentration. All reagents were freshly prepared and filtered (0.22 µm).

2.3.3 Protein quantification using Bradford assay

The total protein in each EV sample was quantified using the Pierce™ Detergent Compatible Bradford Assay Kit (Thermo Fisher). EV samples were lysed by incubating a 10 µl sample with 290 µl of radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher) in each well of a 96-well plate. After thorough mixing by pipetting, 10 µl from each well was transferred into a new 96-well plate. To each well, 300 µl of Bradford Assay Reagent was added and mixed by pipetting. BSA 2.5 – 25 µg/ml was used to prepare a standard curve. After a 10 min incubation at room temperature in the dark, the absorbance at 600 nm was measured using a plate reader (VICTOR X, Perkin Elmer, Melbourne, VIC, Australia).

2.4 Analysis of size and morphology of EVs

2.4.1 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to observe the changes in morphology and particle size. Samples were gold sputter-coated using a K550X sputter coater (Quorum Emitech, Kent, UK). Images were obtained using a Zeiss Sigma high definition field emission gun scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany).

2.4.2 Transmission electron microscopy

The size and morphology of the particles were analysed using transmission electron microscopy (TEM). Samples were mounted on carbon and Formvar-coated copper grids for 30 minutes and fixed with 2.5% glutaraldehyde for 30 min. Following three washes in sterile PBS, they were negative stained with 2% (w/v) ammonium molybdate in water for 5 min.

Excess stain was removed by blotting and the samples were air-dried further, before imaging in JEOL JEM-1400 TEM (Tokyo, JPN).

2.4.3 Atomic force microscopy

For observing particle size and 3D morphology, EV samples were placed onto freshly cleaved mica and dried for atomic force microscopy (AFM) on the nanoIRTM AFM-IR instrument (Anasys Instruments, USA). Images were obtained at a scan rate of 0.5 – 0.8 Hz using a tapping-mode with an EX-T125 probe (resonance frequency 200-400 kHz and spring constant 13-77 N/m, Anasys Instruments).

2.5 Analysis of EV molecular composition using atomic force microscope-infrared spectroscopy (AFM-IR) and Nano Flow Analyzer

Our protocol for using AFM-IR (nanoIRTM, Anasys Instruments) for EV characterisation [13] was followed. Each EV sample (5 μ l) was placed on a zinc selenide prism and dried overnight. After optimisation of the laser signal, AFM-IR spectra were collected from 1000 to 1800 cm^{-1} at 1 cm^{-1} intervals with a scan rate of 0.1 Hz and with a cocoverage of 16. A silicon nitride cantilever (EXC450 tips, AppNano, CA, USA) with a nominal spring constant of 0.5 Nm^{-1} was used for all measurements. The scan sizes were 10 $\times$ 10 μm for probing one point per EV and 1 $\times$ 1 μm for probing multiple points on individual EVs. Analysis StudioTM software was used for data analyses. Smoothing of the spectra using the ‘Savitzky-Golay’ function was achieved by using the polynomial function of 2, and 15 numbers of points.

The size and nucleic acid content of EVs were measured using Nano Flow Analyser (nanoFCM, China). The standard 200 nm polystyrene spheres (100 μ l) were used to align the laser. After the laser optimization, the size of silica nanospheres was measured and used as the size standards, followed by the construction of a calibration curve. The size measurement of each EV sample (diluted in 100 μ l RNase-free PBS) was acquired directly using the same instrument settings as the silica nanoparticles reference standards. To quantify total nucleic acid content, each EV sample was diluted in 50 μ l RNase-free PBS and SYTO RNASelect

green fluorescent cell stain 5 mM stock solution in DMSO (Thermofisher) was added to make the final concentration 10 μ M. The mixture was incubated at 37°C for 30 minutes. Subsequently, the samples were loaded on the exosome spin column MW 3000 (Thermofisher) to remove all unbound dyes and measured on Nano Flow Analyzer. The control samples were PBS and basal medium without EVs using the same RNA staining process.

2.6 RNA isolation from EVs

Total RNA was isolated from EVs using the TRIzolTM reagent (Invitrogen), following the manufacturer's protocol with minor modifications. Briefly, 300 μ l TRIzol was added directly to the EV pellet following ultracentrifugation (see 2.2 above) and then this was transferred to an RNase-free microcentrifuge tube. An additional 200 μ l TRIzol was added to the ultracentrifuge tube to ensure all the residual pellet was collected, and then samples were stored immediately at -80°C.

2.7 OpenArray[®] for miRNA profiling of EVs

For interrogating the miRNA content in EVs, OpenArray[®] was conducted, with a modified protocol for low sample input. RNA samples were diluted to 10 ng/ μ l. Reverse Transcriptase (RT) mastermix was prepared using the manufacturer's protocol. Mastermix (4.5 μ l) was added to each sample, inverted to mix, centrifuged and incubated on ice for 5 min. For cDNA synthesis, the thermocycler program was: 40 cycles (16°C for 2 min, 42°C for 1 min, 50°C for 1 sec), 85°C for 5 min, and then held at 4°C. To generate pre-amplified cDNA, PreAmp master mix (TaqMan PreAmp Mastermix) and PreAmp primers (Thermo Fisher Scientific, diluted in nuclease-free water), were added and the thermocycler program was: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min, 16 cycles (95°C for 15 sec, 60°C for 4 min), 99°C for 10 min, and then held at 4°C. Preamplified cDNA was diluted 1:20 in 0.1 $\times$ TE buffer. The diluted preamplified cDNA (5 μ l) was combined with TaqMan OpenArray real-time PCR

master mix (5 μ l). The plate was sealed and vortexed to mix. After transferring 5 μ l of each sample to each well of the 384-well sample plate, the plate was centrifuged at 490 $\times$ g for 1 min at 4°C. QuantStudio 12K Flex software was initialised and the qPCR was run. All run files were uploaded to Thermo cloud software for data normalization, which was performed using global normalisation method. Normalised data were exported, analysed and visualised as heatmap, volcano plots and bar graphs. The RStudio software (version 1.0.153) was used for generating heatmaps and Prism software was used for Volcano plots and bar graphs. Reproducibility of data were confirmed from four replicate samples for CMSC29 and DMSC23 cells and five replicate samples for CMSC29 EVs and DMSC23 EVs.

2.8 Assessment of cell migration and invasion upon EV treatment

2.8.1 Cell wound repopulation assay

Epithelial cell migration in the presence of DMSC23 EVs or CMSC29 EVs was assessed by comparing the extent of closure of a two-dimensional scratch wound on a monolayer of cells. BEAS-2B cells were seeded at 1×10^4 cells/well on 96-well plates and were cultured in normal media until 90% confluent. The cells were then incubated in low serum medium (containing 1% FBS) for 24 h prior to scratching a wound on the midline of the culture well using a 96-pin wound making tool (IncuCyte[®] WoundMakerTM). After washing with low serum medium twice to remove cell debris, fresh medium was added. EVs were added at 5×10^5 particles per well. Wound images were taken every 2 h with 10 $\times$ magnification using the IncuCyte[®] live cell imaging system and IncuCyte ZOOM software program (Essen BioScience, Inc. Ann Arbor, MI, USA).

2.8.2 Cell invasion and migration assay using impedance measurement

Cell invasion and migration were monitored using an xCELLigence[®] Real-Time Cell Analyzer Dual Purpose (RTCA DP) instrument using the 16-well electronic cell invasion and migration plate (CIM-Plate[®] 16). Following the manufacturer's instructions, the CIM-Plate[®]

16 was filled with media and equilibrated at 37°C for 1 h, then background impedance values were recorded.

BEAS-2B cells were passaged a day prior to the experiment, kept in serum-free media (SFM) for 24 h. Each plate was seeded with BEAS-2B cells in the upper chamber at 40,000 cells per well in 100 µl serum-free media. DMSC23 EVs and CMSC29 EVs were diluted to 6×10^5 particles in 20 µl SFM and added to cells immediately after seeding. Plates were left at room temperature for 30 min to allow the cells to settle at the bottom of the upper chamber. The plates were then loaded onto the RTCA DP instrument inside a 37°C incubator and the impedance was measured every 10 min for 72 h.

2.9 Analysis of cellular responses to EV treatment after LPS injury

EVs were diluted in DMEM complete media and transferred to a 24-well plate, with each well containing 1×10^5 particles in 100 µl media. The control group was given medium without EVs. Then BEAS-2B were seeded at 2×10^4 cells per well on 24-well plate, to incubate with EVs 30 min prior to exposing cells to lipopolysaccharide (LPS) injury used at final concentration of 100 ng/ml (*E. Coli* O111:B4, Sigma-Aldrich). After 16 h incubation, cells were collected and prepared according to the manufacturer's instructions for the MuseTM Nitric Oxide Assay Kit (Merck Millipore, Frenches Forest, NSW, Australia).

2.10 Measurement of cytokine release in response to LPS injury after EV treatment

THP-1 cells were seeded at 3×10^5 cells per well (180 µl of 1.6×10^6 cells/ml) then treated with LPS (*E. coli* O111:B4, Sigma-Aldrich) at 100 ng/ml final concentration, incubated on a shaker at 37°C, 5% CO₂ for 4 h. EVs were made to 1.5×10^7 or 7.5×10^6 particles/ml and added to THP-1 cells at 5 particles/cell.

The enzyme-linked immunosorbent assay (ELISA) was conducted using IL-6 and TNF-α ELISA kits (BD Biosciences, North Ryde, NSW, Australia) following the manufacturer's protocols. Briefly, Nunc[®] MaxiSorpTM plates (Thermo Fisher Scientific) were coated with

capture antibodies (100 μ l/well; 40 μ l in 10 ml for one 96-well plate to give a final dilution of 1:250) and incubated at 4°C for 16 h (20 h LPS exposure in total). Samples were mixed by pipetting, collected (240 μ l) and centrifuged to remove cells. The supernatant (220 μ l) was transferred to an empty 96-well plate (i.e. the sample plate) and checked using a light microscope to ensure no cells were present. IL-6 and TNF- α standards were prepared in assay diluent. Wash buffer diluted from a 20 $\times$ stock solution was used to wash away unbound capture antibodies, blocked with assay diluent for 1 h and then the manufacturer's protocol was followed for subsequent washing and incubation steps, using the BD OptEIA™ set (BD Biosciences).

2.11 Statistical analysis

A minimum of triplicate, or quadruplicate samples, were used for reliability in each experimental condition being examined. Data were analysed and presented as a mean $\pm$ standard deviation. The differences between the experimental and control groups were analysed using the one-way analysis of variance (ANOVA) test. A p-value of less than 0.05 was considered to be a statistically significant difference.

3. Results and Discussion

3.1 Comparison of EV size and concentration

Analysis of 3D images of EVs showed that EVs were spherical with no morphological differences between EVs isolated from CMSC29 or DMSC23 cells. AFM height images revealed the EVs diameters were in a range of 20-100 nm. EV subpopulations that with larger diameters: 100 to 300 nm were also identified (Fig. 1A).

TEM analyses showed primarily smaller vesicles (less than 100 nm), which were characterised by a typical lipid bilayer, and there was evidence of smaller vesicles within larger vesicles (Fig. 1B).

Fig. 1. **A.** Three-dimensional atomic force microscopy (AFM) image of DMSC23 EVs; **B.** Transmission electron micrographs of DMSC23 EVs showing the lipid bilayer structures, scale bars 100 nm; Scanning electron micrographs of: **C.** DMSC23 EVs; and **D.** CMSC29 EVs (*left*: secondary electrons detector, and *right*: InLens detector, scale bars 200 nm). Comparison of particle size and concentration for DMSC23 EVs and CMSC29 EVs, using: **E.** nanoparticle tracking analysis (NanoSight); tunable resistive pulse sensing (qNano) of: **F.** DMSC23 EVs and **G.** CMSC29 EVs; **H.** protein quantification (Bradford assay); and size distribution using Nano Flow Analyzer of: **I.** DMSC23 EVs and **J.** CMSC29 EVs.

Further analyses of morphological changes of the surface were by SEM. Due to the limitation in resolution, only EV subpopulations with sizes of about 150-300 nm in diameter were analysed (Fig. 1C, D). The use of InLens signal merged with secondary electron detection produced images with higher contrast and detail (Fig. 1C, D (*right*)). The surface of EVs appeared to be relatively smooth with small protruding patches of a secondary structure on the membrane. These patches could be associated with components of secretome, e.g. proteins (Fig. 1C).

Table 1. Summary of averaged particle size and concentration measured using NTA and TRPS methods for CMSC29 EVs and DMSC23 EVs.

Measurement Method	NTA		TRPS			Nano Flow Analyzer
	Mean (nm)	Mode (nm)	Mean (nm)	Mode (nm)	Concentration (particles/ ml)	Mean (nm)
CMSC29 EV	106.9 ± 13.0	65.3 ± 39.5	101.5 ± 6.4	75 ± 2.8	1.3×10^{10}	77.7 ± 16.6
DMSC23 EV	131.9 ± 0.4	114.6 ± 10.1	112.5 ± 3.5	106.0 ± 1.4	2.2×10^9	79.0 ± 18.4

The size distribution and concentration of EVs were analysed using NTA and TRPS. These methods confirmed a relatively broad size distribution for both CMSC29 EVs and DMSC23 EVs (Table 1, Fig. 1E-G). The averaged mean and mode sizes identified from NTA were slightly larger than those measured by TRPS (Table 1, Fig. 1E-G). The mode diameters for EVs measured by NTA and TRPS were 114.6 ± 10.1 nm and 106.0 ± 1.4 nm for DMSC23 EVs respectively, and 65.3 ± 39.5 nm and 75 ± 2.8 nm for CMSC29 EVs respectively. Although NTA and TRPS yielded comparable total particle counts overall, NTA detected more particles with larger diameter (i.e. greater than 110 nm) while TRPS detected more particles in the smaller diameter (less than 110 nm) range. Quantitative assessments using NTA and TRPS were influenced by the type of particle as well as the settings used e.g. camera level and detection threshold for NTA, and nanopore size for TRPS ^{[15],[16]}. As a nanopore NP100 was used in this study, the detectable vesicle size for TRPS was in the range of 50 to 330 nm and was less sensitive for detection of EV particles smaller than 50 nm.

The size distributions of DMSC23 EVs and CMSC29 EVs were also analysed in Nano Flow Analyzer (Table 1, Fig 1I, J), which is able to detect the smaller diameter particles due to the lower detection threshold. The mean sizes of DMSC23 EVs and CMSC29 EVs were $79.0 \pm$

18.4 nm and 77.7 ± 16.6 nm respectively, which obviously indicate the higher sensitivity of Nano Flow Analyzer compared to NTA and TRPS (Table 1).

Protein content analysis

Proteins, which are essential building blocks for tissue repair, were quantified using the Bradford assay. Results of the assay showed the total protein content was higher for DMSC23 EVs (99.7 ± 4.8 $\mu\text{g/ml}$) compared with that of CMSC29 EVs (84.1 ± 9.1 $\mu\text{g/ml}$) (Fig. 1H).

Cumulatively, particles size/concentration and protein analyses demonstrated comparable size distribution, with CMSC29 EVs having a broader size distribution compared to DMSC23 EVs in both analysis methods. CMSC29 EVs appeared to be more aggregated. However, EVs imaged using the AFM did not reveal any aggregates, which could be attributed to differences in sample preparation and the number of particulates that are typically analysed by imaging techniques, when compared to size/concentration analysis using dynamic light scattering or TRPS.

It is important to note that there were some discrepancies in data generated by each of the characterisation methods, which emphasised their individual limitations and reinforced the need to use multiple techniques to increase the accuracy of EV size and concentration measurements. The limitations of these techniques must be taken into account when results are interpreted.

3.2 Analysis of EV molecular composition using AFM-IR and Nano Flow Analyzer

Heterogeneity and molecular composition of individual CMSC29 EVs and DMSC23 EVs was interrogated using resonance enhanced AFM-IR^[13]. For both samples characteristic peaks that corresponded to proteins, nucleic acids, and lipids were identified. There were substantial differences between both groups of EVs (Fig. 2.1). DMSC23 EVs characterised with lower amount of proteins and relatively broad peak with three dominant peaks at 1650,

1590 and 1530 cm^{-1} indicated conformational changes to the protein structure Amide I and Amide II. Additional peak at 1730 cm^{-1} (C=O stretch) confirmed presence of phospholipids. DMSC23 EVs characterised with very strong peaks at 1105 cm^{-1} and 1025 cm^{-1} . Whereas, CMSC29 EVs had a dominating amide I peak at 1600 cm^{-1} and well-defined peaks in the range 1450 and 1350 cm^{-1} which corresponded to the phosphatidylcholine head group and thymine that reveals presence of RNAs in EVs. Overall these peaks, when analysed in relation to protein peaks, were of higher intensity for DMSC23 EVs. Importantly, we observed two dominating peaks in DMSC23 EVs at 1080 and 1030 cm^{-1} ; these peaks are associated with glycogen and nucleic acid: desoxyribose, RNA and ribose. In summary, DMSC23 EVs characterised with relatively higher amount of DNA comparing to amount of protein. Whereas, CMSC29 EVs had very 'well-balanced' composition. Qualitative analysis of the peak intensities showed that CMSC29 EVs samples had much stronger peaks with lower noise-to-signal ratio which suggests greater concentration of the molecular content.

The differences of nucleic acid contents between DMSC23 EVs and CMSC29 EVs were further demonstrated using Nano Flow Analyzer (Fig 2.2). Indeed, the exosomal nucleic acids of DMSC23 (2.6%) are higher (Fig 2.2A) than CMSC29 EVs (1.9%) (Fig 2.2B) (red dots represented for the fluorescence residues). Differences in total RNA and DNA contents were in agreement with quantification using AFM-IR (Fig. 2.1). The control group, which were basal medium and PBS with RNA staining (Fig 2.2C and D), indicated no fluorescence presented in both samples.

Total nucleic acids content of EVs comprises of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It was previously shown that the RNA spectrum has two clear spectral components centered at 1240 and 1220 cm^{-1} , while the ds-DNA band in the same region is sharper and peaked at 1226 cm^{-1} {Zucchiatti, 2016 #1264}. The symmetric stretching band of nucleic acid phosphate is typically centered at $\sim 1086 \text{ cm}^{-1}$. Conversely, the

ribose nature of the phosphate sugar backbone of RNA is defined in spectral band centered at $\sim 1119\text{ cm}^{-1}$ (stretching vibration of the skeletal structure around the C2' -OH group of ribose) (39) and characterise with strong spectral features of the C–O are close to each other for ribose (1059 cm^{-1}) and deoxyribose (1053 cm^{-1}) (24). Analysis of the spectra for EVs isolated from DMSC23 and CMSC29 showed presence of a $\sim 1230\text{ cm}^{-1}$ band which was stronger for DMSC23 EVs, suggesting greater amount of total nucleic acids content. This result agrees with total DNA/RNA content obtained using Nano Flow Analyser. However, CMSC29 EVs showed a band $\sim 1080\text{ cm}^{-1}$ associated with nucleic acid, which for DMSC23 EVs was shifted to $\sim 1105\text{ cm}^{-1}$ which could be associated with phospholipids C–O stretch. CMSC29 EVs characterised with two peaks $\sim 1120\text{ cm}^{-1}$ and $\sim 1045\text{ cm}^{-1}$, which are associated with RNA. Peaks in these positions were identified only for some of the DMSC23 EVs, which suggests that the EVs were heterogenous. AFM-IR does not allow determining miRNA content, therefore the comparison to total miRNA measured using Open Array is not possible. However, AFM-IR identified major differences in the composition of both populations of EVs in terms of RNA, DNA content, protein content and structure and some differences in lipid structure.

Fig. 2.1. Infrared absorption spectra of EV molecular components with corresponding topographical AFM images for: **A.** DMSC23 EVs; and **B.** CMSC29 EVs, where the spectra is generated from probing of individual EV particles.

Fig. 2.2. The real time burst trace of size scattering (blue) and fluorescence FITC (green) channels with corresponding dot-plots in RNA staining of: **A.** DMSC23 EVs, **B.** CMSC29 EVs, **C.** Basal medium DMSC23 and **D.** PBS

3.3 DMSC23 EVs and CMSC29 EVs increase lung epithelial cell migration

Lung epithelial cells (BEAS-2B) treated with DMSC23 and CMSC29 EVs were placed in a chamber with a chemotactic gradient (FBS) to test whether the EVs increase BEAS-2B migration. From the results of the impedance values that measure the translocation of cells as they migrate from the upper chamber to lower chamber (xCELLigence CIM-16), an increased number of cells passed through the chamber when treated with EVs (Fig. 3). Both DMSC23 EVs and CMSC29 EVs increased the migration of BEAS-2B, which suggests that lung epithelial cells with increased mobility may be helpful in the microenvironment during the recovery processes from a lung injury. This was further investigated by the wound closure assay. In a simplified wound model using a scratch wound, DMSC23 EVs increased the speed of cells migrating towards the ‘wound’. Cell migration-enhancing capability is important in wound healing as it is a feature of natural wound repair processes in the epithelium. The progenitor cells near the injured epithelium migrate and spread to cover the denuded surfaces^[17].

Fig. 3. Assessment of cell migration of BEAS-2B in response to EV treatment by: **A.** Impedance measurement sensing cell translocation towards chemoattractant (xCELLigence); **B.** Scratch wound assay with calculated percentage of wound closure from live cell imaging (IncuCyte); **C.** Images from the scratch wound assay with highlighted wound area over 24 h, 10 × magnification.

3.4 DMSC23 EVs reduce cellular stress and the inflammatory response caused by LPS

As is the case with DMSC primary cells, DMSC23 cells are resistant to oxidative stress due to the pre-conditioning of the placenta-derived tissue where the cells are isolated from^[14] We previously showed that DMSC23 are more resistant to hypoxic stress compared to CMSC29^[18]. Since EVs convey similar properties to the cells which they are secreted from^[19], DMSC23 EVs were expected to reduce oxidative stress in an established *in vitro* model of lung injury; LPS-injured BEAS-2B lung epithelial cells.

The measurement of nitric oxide expression allows quantification of cellular stress in response to injury. Nitric oxide is a small molecule generated by the enzyme nitric oxide synthase (NOS) in cells, which participates in many biological functions^[20]. In the context of cellular stress, nitric oxide is produced in large amounts in response to inflammatory stimuli, as part of a defense mechanism in oxidative toxicity^[21]. The intracellular detection of nitric oxide is important for the detection of apoptotic signal cascades as the cell protects itself from pathological processes including inflammation and apoptosis^[22]. The comparison of the nitric oxide profiles after LPS injury demonstrated that the cells treated by EVs had lower nitric oxide expression as shown from the reduction in the average percentage of nitric oxide positive cells from $8.0 \pm 0.4 \%$, to $7.0 \pm 0.6 \%$ and $5.5 \pm 1.3 \%$, respectively for DMSC23 and CMSC29 EVs (Fig. 4. A,B). This suggests that the oxidative stress signalling cascade within cells is modified in response to LPS injury after the uptake of CMSC29 EVs ($p < 0.05$), more obviously than cells treated with DMSC23 EVs.

The immunomodulatory effects of DMSC23 EVs on cytokine release by THP-1 were investigated using ELISA. Although the effects of EVs on IL-6 was minimal, both EV types reduced the release of TNF- α (Fig. 4. C, D), a pro-inflammatory cytokine implicated in many diseases and particularly well characterised in pulmonary diseases including acute lung injuries, asthma, emphysema and pulmonary fibrosis^[23].

Fig. 4. Comparison of cellular responses to CMSC29 EV and DMSC23 EV treatment: **A.** Nitric oxide expression profiles of LPS-injured BEAS-2B cell line, representative of triplicate samples; **B.** Comparison of total nitric oxide-positive cell population; ELISA analyses of pro-inflammatory cytokines released from THP-1 upon treatment with LPS, with or without EVs for: **C.** IL-6, and **D.** TNF-α; * $p < 0.05$; **E.** Unsupervised hierarchical cluster analysis of microRNA profiles in DMSC23 and CMSC29 cells (4 replicates for each type) and EVs (5 replicates for each type) is presented as a heatmap.

3.5 The therapeutic potential of DMSC23 EVs and CMSC29 EVs is attributable to their molecular composition

EVs produced by MSCs reprogram tissue-injured cells by delivering mRNA and/or miRNA to modulate soluble paracrine mediator production, mediate cell de-differentiation and cell-

cycle re-entry and thereby contributing to tissue regeneration ^[24]. Another important rationale for analysing the EV miRNA profile is that the EVs released from the injured cells may mediate phenotypic transfer of surrounding MSCs to acquire certain features by delivering mRNA and/or miRNA to MSCs ^[24]. Thus, comparative analysis of the miRNAs carried in EVs and miRNAs contained in the cells secreting those EVs may reveal important mechanisms by which MSCs and their EVs carry out their therapeutic effects. We interrogated the miRNA content of CMSC29s and DMSC23s and their EVs, using an OpenArrayTM platform, which enabled the analysis of 754 miRNAs of which 405 miRNAs were detected (Fig. 4E). Unsupervised hierarchical clustering demonstrated that miRNA profiles of CMSC29s and DMSC23s were similar than their respective EVs (Fig. 4E) implying differences in packaging of microRNA cargos in EVs from the two cell types. There were more miRNAs packaged in CMSC29 EVs compared to DMSC23 EVs, which was in agreement with the AFM-IR results. Furthermore, the CMSC29 EV miRNA profile was more similar to the miRNA profile of secreting CMSC29 cells, than the miRNA profiles of DMSC23 EVs compared with secreting DMSC23 cells (Fig. 4E). Univariate analysis presented in Supplementary Figure 1A identified several miRNAs that are significantly differentially expressed (Supplementary Figure 1B,C). Since miRNAs are known to be important regulators of cellular repair and tissue regeneration, we assessed our data for the presence of key miRNA in EVs and secreting cells that have been previously reported to be involved in lung repair (Table 2). Amongst the known miRNAs associated with lung repair (Table 2), our study did not find miR-505 to be localized to EVs released from either of the two cell types. Similarly, miR-30a-3p was also consistently undetectable from the EVs in DMSC23 cell-derived EVs. Intriguingly, some miRNAs (miR-548c, miR-548a as well as miR-603) that came up in our discovery analyses were found to be consistently and selectively packaged into EVs assessed from both cell types, whilst remaining to be depleted

from the cells that transcribe them. These may potentially be effector molecules targeting specific cells and could also be biomarkers of early response to injury. On the other hand, miRNA candidates such as let-7a and miR-181a-2#, although present in both cell types were always excluded from EVs (Suppl. Figure 2). The process of selective packaging of molecular components in EVs by cells may be investigated in future studies to gain an insight into modifying the content of specific miRNAs and other biological signals carried inside the EVs for potential therapeutic applications. Our analyses from miRNA profiling studies not only demonstrate differences between miRNA composition based on the secreting cell type, but also identify candidates that could be included in future clinical studies/trials for screening tissue damage and response. These studies also highlight the need for further investigations to interrogate molecular composition of EVs for potential therapeutic applications [25].

Table 2. Selected miRNAs in EVs that may be involved in tissue repair [26].

4. Concluding remarks

A critical component of the mechanism for MSC-driven tissue regeneration is now widely accepted to be the transfer of genetic information between MSCs and injured cells in tissues. The results from this study demonstrate that DMSC23 EVs and CMSC29 EVs were beneficial in promoting migration and reducing oxidative stress and inflammation, suggesting their potential application in reducing lung injury and enhancing cellular repair. This study leads to further *in vivo* studies that will be fundamental in advancing the field of EV therapeutics, in particular to improve the regeneration of the lung after injury. Further

miRNA	Functions
miR-505	Highly involved in targeting relevant gene functions in repair
miR-29a/b	The ‘master regulators of tissue regeneration’
miR-146a	Reduce inflammation, increase proliferation, migration, and angiogenesis of endothelial progenitor cells
miR-30d	Important in the late repair phase
miR-133a	Regulatory roles in airway inflammation, tumour suppressor
miR-30c	Important in the late repair phase
miR-30a-3p	Wound healing
miR-30a-5p	Tumour suppressor, modulate epithelial-mesenchymal transition
miR-483-5p	Protective role in chronic obstructive pulmonary disease
miR-130a	Involved in lung organogenesis

development of the single vesicle characterisation technique using AFM-IR as presented in this study will enable the identification of the most therapeutically relevant EV subtypes.

Taking this innovative approach together with profiling of miRNA and proteins will advance the field of EV-therapies towards clinical translation.

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Conflicts of interest

The authors have declared no conflict of interest.

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