# Impact of chemically defined culture media formulations on extracellular vesicle production by amniotic epithelial cells

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## Abstract

The therapeutic properties of cell derived extracellular vesicles (EVs) make them promising cell-free alternative to regenerative medicine. However, clinical translation of this technology relies on the ability to manufacture EVs in a scalable, reproducible, and cGMP-compliant manner. To generate EVs in sufficient quantity, a critical step is the selection and development of culture media, where differences in formulation may influence the EV manufacturing process. In this study, we used human amniotic epithelial cells (hAECs) as a model system to explore the effect of different formulations of chemically defined, commercially sourced media on EV production. Here, we determined that cell viability and proliferation rate are not reliable quality indicators for EV manufacturing. The levels of tetraspanins and epitope makers of EVs were significantly impacted by culture media formulations. Mass spectrometry-based proteomic profiling revealed proteome composition of hAEC-EVs and the influence of media formulations on composition of EV proteome. This study has revealed critical aspects including cell viability and proliferation rate, EV yield, and tetraspanins, surface epitopes and proteome composition of EVs influenced by media formulations, and further insight into standardised EV production culture media that should be considered in clinical-grade scalable EV manufacture for generation of therapeutic EVs.

## Significance of the Study

The development of cell therapy strategies has gained traction as the interest for more personalized and novel therapeutics heightens. As a cell-free delivery approach, extracellular vesicle (EV)-based therapies are currently in development for clinical translation. However, given the impact of cell source and culture conditions on EV composition, the choice of cell type and culture media are critical factors to be considered and optimised for EV manufacturing. Substitution of serum and non-human growth factors, and other clinically incompatible reagents is requisite for controlling product quality in a therapeutic cell manufacturing process. This study provides an insight into how EV composition and potency is influenced by chemically defined culture media and supplements for production. These are significant considerations in process optimization methods for the generation of therapeutic EV.

# 1 Introduction

The concept of a cell-free alternative to regenerative medicine has gained exponential traction in recent years. The International Society of Extracellular Vesicles (ISEV), the Food and drug Administration (FDA), the International Council for Harmonization (ICH) of Technical Requirements for Pharmaceuticals for Human Use, and the European Medicines Agency (EMA) provide extensive guidance for the development and generation of novel biological medicines with regard to donor/patient care, product safety, and quality (Ancans, 2012; Giannakou et al., 2020; Halme & Kessler, 2006; Harron, 2013; Lener et al., 2015; Mateescu et al., 2017; Reiner et al., 2017; Théry et al., 2018). There are currently over 50 registered clinical trials investigating the safety and efficacy of extracellular vesicle (EV)-based therapeutics in the context of immune modulation, neurological diseases, metabolic diseases, and respiratory diseases (https://clinicaltrials.gov). EVs are released by all cell types and play critical roles in intercellular communication (Xu, Greening, Zhu, Takahashi, & Simpson, 2016; Xu et al., 2018). They package a complex combination of nucleic acids, protein/peptides and lipoproteins, and have similar biological effects as their cells of origin *in vitro* and *in vivo* (Lener et al., 2015; Raposo & Stoorvogel, 2013; Xu et al., 2018). EVs represent an important mode of intercellular communication in many different contexts and pathologies (Kalluri & LeBleu, 2020; Xu et al., 2018). Importantly, EVs traffic their complex molecular cargo in a bidirectional manner, direct their cargoes to specific cell types, and upon uptake, the capacity to induce phenotypic change (Al-Nedawi et al., 2008; Mulcahy, Pink, & Carter, 2014; Schillaci et al., 2017). There is now growing awareness that predominant EV subtypes can be further stratified into distinct subtypes, however specific approaches to purification, and markers for their discrimination are lacking (Webber & Clayton, 2013). While several strategies have been used for EV isolation and purification, such is dependent on the application of EVs, where extensive purification and fractionation to dissect EV subtype heterogeneity may advance understanding of EV characterisation, biology, formation, and function (Gardiner et al., 2016; Huang, Lin, Zhu, Duan, & Jin, 2020; Jeppesen et al., 2019; Willms, Cabanas, Mager, Wood, & Vader, 2018; Xu, Simpson, & Greening, 2017), such requirements may not be key in application of EV-based therapeutics (Lener et al., 2015; Webber & Clayton, 2013; Wiklander, Brennan, Lötvall, Breakefield, & Andaloussi, 2019). In the context of EV-based therapeutics, pivotal advantages include their rapid generation and underpinned by the notion that they avoid the need for

complex cold chain logistics, and specialised requirements for formulation prior to clinical use (Rohde, Pachler, & Gimona, 2019; Wiklander et al., 2019).

While preclinical studies of using EV-based therapeutics are promising(Wiklander et al., 2019), the clinical translation of this technology relies heavily on the ability to manufacture EVs in a scalable, reproducible, and cGMP-compliant manner (Roura & Bayes-Genis, 2019). The choice of cell culture media is a critical component for EV manufacturing. Chemically defined serum-free media have been proposed for clinical-grade EV manufacturing(Gimona et al., 2021; Witwer et al., 2019) and must be evaluated thoroughly. Previous studies show that switching to serum-free media or EV-depleted serum-containing media can alter cell growth and EV content (Eitan, Zhang, Witwer, & Mattson, 2015; Li et al., 2015). However, there is a scarcity of information comparing commercially available chemically defined media on EV production. In this study, we used human stem cell-like amniotic epithelial cells (hAECs) as a cell model system to explore the impact of different chemically defined media formulations and supplement additives on EV production and content.

The hAECs are a population of perinatal stem-like cells derived from the amniotic membrane (Andrewartha & Yeoh, 2019; Evans et al., 2018; Zhang & Lai, 2020). They are immunomodulatory, are capable of multi-lineage differentiation, and have anti-fibrotic and pro-reparative properties (Zhu, Wallace, & Lim, 2014). Moreover, the safety and efficacy of hAECs have been reported and currently assessed in several clinical trials including bronchopulmonary dysplasia (Baker et al., 2019; Lim et al., 2018) (ACTRN12618000920291), ischaemic stroke (Phan, Ma, Lim, Sobey, & Wallace, 2018) (ACTRN12618000076279), liver cirrhosis (ACTRN12616000437460) and Crohn's related perianal fistulas (ACTRN12618001883202). Notably, hAEC-EVs have also been shown to have therapeutic potential in preclinical models of disease including lung injury (Tan et al., 2018), chronic liver disease (Alhomrani et al., 2017), brain injury (Broughton, Tran, Lim, Wallace, & Kemp-Harper, 2018) and wound healing (Zhao et al., 2017). Currently, accumulating evidence has demonstrated that hAECs can provide a beneficial microenvironment for cell survival and activate endogenous mechanisms of tissue regeneration by secreting EVs. This is supported by evidence that the injection of the conditioned media of hAECs could achieve a positive outcome similar to that of cell transplantation (Uchida et al., 2003), representing an acceptable alternative for stem cell-free biotherapy. hAEC-derived EVs have been shown to exert recovery in transplantation (Zhang et al., 2019) and anti-fibrosis function via modulating collagen synthesis and macrophage

polarization in chronic liver fibrosis (Alhomrani et al., 2017). Such studies indicate that hAEC-EV-based therapy may offer important potential therapeutic options. However, chemically defined culture media have not been evaluated for scalable production of hAEC-EVs. Here, we sourced ten commercially available chemically defined, serum-free media to compare their relative efficiency in promoting cell growth and EV yield, as well as their impact on EV surface epitopes including tetraspanins, and EV proteome composition. We describe insights into how EV composition and potency can be influenced by different formulations of chemically defined culture media and supplements.

# 2 Materials and methods

## 2.1 Culture media

Ten different serum-free basal culture media were assessed (**Table 1**) and compared against a control media previously used for hAEC-EV research (i.e. Ultraculture, Lonza) (Alhomrani et al., 2017; Tan et al., 2018). Media 10 was assessed through nine combinations of media and supplements as detailed in **Table 2**. These are listed as Media 10.1 to 10.9. The supplements used include Cell Boost 6 (SH30866, Cytiva) and AdvanceSTEM serum replacement (SH30874, Cytiva). GlutaMAX (35050061, Thermo Fisher Scientific) was added to all media formulations prior to use.

## 2.2 Human amniotic epithelial cell isolation and characterisation

hAECs were isolated from amniotic membranes collected from healthy term pregnancies, delivered by elective caesarean sections, with hAEC isolation performed as previously described[16]. Informed consent was obtained from Monash Health Human Research Ethics Committee (Approval No. 01067B). The mean gestational age of donors was 38 weeks ± 2 days. Briefly, freshly stripped amnions were subjected to trypsin digestion to release hAECs from the amniotic membrane. Cell viability was determined by Trypan blue exclusion (>80% viability was required). Samples of hAECs were tested for contamination by culturing for 7 days without antibiotics. The purity of hAEC preparations were confirmed when preparations were found to be >85% $EpCAM^+$, and <1% $CD90^+$, $CD45^+$ and $CD31^+$ (n=3, representative images in **Figure S1**).

## 2.3 Isolation of human amniotic epithelial cell derived extracellular vesicles

Human amniotic epithelial cell lines (n=3) were assessed in each media formulation compared to control media. Term hAECs ($1 \times 10^6$) were plated on a T175 flask containing 25 mL different/control culture media (**Table 1**). After 4 days of culture at 37°C, 95% humidified air and 5% $CO_2$, cell viability was assessed by Trypan blue exclusion assay and the number of live cells (viable cell number) calculated, normalised to the mean of the control group. Data from all experimental groups were expressed as a % change from the mean of the control group. hAEC conditioned media were also collected after 4 days of culture and EVs were prepared using serial ultracentrifugation as previously described (Rai et al., 2021). The conditioned media were collected in three batches and the data was normalised to the control of each batch. EVs were isolated directly after conditioned media collection by centrifuging at 10,000*g*, 4ºC for 30 min to remove cell debris/apoptotic cells/bodies, and the supernatants were subjected to ultracentrifugation in a new tube at 100,000 *g*, 4ºC for 120 min to pellet EVs. hAEC-EV pellets were washed (100,000 *g*, 4ºC for 120 min) and the pellet resuspended with PBS, aliquoted, and stored at -80ºC until required. A single freeze-thaw cycle was used in this study.

### 2.4 Protein quantitation

The protein concentration of hAEC-EVs were determined with BCA Protein Assay Kit (23225, Thermo Scientific) as described by manufacturer.

### 2.5 Transmission electron microscopy analysis

Isolated hAEC-EVs were resuspended 1:2 with 4% (w/v) paraformaldehyde (PFA). Five microlitres of the EV suspension was placed on a Formvar-carbon coated electron microscope grid and left to dry for 20 min before fixation in 1% (w/v) glutaraldehyde for 5 min followed by 6 washes in distilled water for 1 min each. The grids were placed on 50 μL of uranyl-oxalate solution for 5 min and transferred to 50 μL of methylose cellulose for 5 min at 4ºC. The grid was then lifted using a stainless-steel loop and excess methylose cellulose removed. The grid was air dried for 20 min and images taken using a H7500 Transmission Electron Microscope (Hitachi, Japan) at 70kV. Image J was used for assessment of EV/particle morphology.

### 2.6 Single particle analysis

Particle distribution and concentration were obtained using ExoView R100 (Nanoview Biosciences) analysis. Briefly, binding of EVs to the microarray chip (EV-TETRA-C) coated with different antibodies, including anti-CD9, anti-CD81, and anti-CD63, ensures measured

particles express specific surface components. Nanoview has high sensitivity and can accurately to measure single EV particles ~50 nm diameter using single-particle interferometric reflectance imaging. Briefly, 2.5ug EVs were diluted in 500 μL incubation solution and 40 μL placed on a chip to incubate overnight. Following 3 washes with incubation solution, the chips were then incubated with antibodies (CD9, CD81, and CD63, 1:1200 dilution) at room temperature for 1 hr followed by one wash in incubation solution and 3 washes with wash solution. Chips were rinsed and then analysed (ExoView R100). Chips coated with each antibody were prepared in triplicate and the whole chip was scanned for particle analysis.

**2.7 MACSPlex analysis**

MACSPlex Exosome kit (130-108-813, Miltenyi Biotec) was used to determine 37 surface epitopes. Briefly, 10 μg EVs were mixed with 15 μL of capture beads and incubated overnight at room temperature on an orbital shaker (450 rpm). After washing with MACSPlex buffer, EVs were mixed with 5 μL of CD9, CD63 and CD81 detection reagent and incubated for 1 hr with shaking (450 rpm). EVs were washed with MACSPlex buffer and analyzed for surface epitopes by Canto II flow cytometry following calibration.

**2.8 Proteomics: Sample preparation**

Samples of hAEC-EV were solubilised in 1% (v/v) sodium dodecyl sulphate (SDS), 50 mM HEPES pH 8.0, and quantified by microBCA (Thermo Fisher Scientific). For mass spectrometry-based proteomics, samples (5 μg in 50 μL) were normalized and reduced with 10 mM dithiothreitol (DTT) for 45 min at 25°C followed by alkylation with 20 mM iodoacetamide for 30 min at 25°C in the dark. The reaction was quenched to a final concentration of 20 mM DTT. Magnetic beads were prepared by mixing Sera-Mag Speed Beads A and B at 1:1 (v:v) ratio and washing twice with 200 μL MS-water as described (Hughes et al., 2019; Kompa et al., 2021). Magnetic beads were reconstituted to a final concentration of 100 μg/μL, added to the samples at 10:1 beads-to-protein ratio and ethanol added for a final concentration of 50% (v/v). Protein-bound magnetics beads were washed three times with 200 μL of 80% ethanol and reconstituted in 50 mM TEAB pH 8 and digested with trypsin (Promega, V5111) at a 1:50 enzyme-to-substrate ratio for 16 h at 37 °C at 1000 rpm. The peptide mixture was acidified to a final concentration of 2% formic acid, and centrifuged at 20,000g for 1 min. The peptide digests were collected from the supernatant and kept frozen at -80°C and dried by vacuum

centrifugation, reconstituted in 0.07% trifluoroacetic acid, and quantified by Fluorometric Peptide Assay (Thermo Scientific, 23290).

### 2.9 Proteomic liquid chromatography–tandem mass spectrometry

Peptides were analysed on a Dionex UltiMate NCS-3500RS nanoUHPLC coupled to a Q-Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer equipped with nanospray ion source in data dependent acquisition analysis and positive mode as described (Greening et al., 2019; Kompa et al., 2021). Peptides (250 ng) were loaded (Acclaim PepMap100 C18 3 μm beads with 100 Å pore-size, Thermo Fisher Scientific) and separated (1.9-μm particle size C18, 0.075 × 250 mm, Nikkyo Technos Co. Ltd) with a gradient of 2–28% acetonitrile containing 0.1% formic acid over 95 mins followed by 28-80% from 95-98 mins at 300 nL min-1 at 55°C (butterfly portfolio heater, Phoenix S&T). An MS1 scan was acquired from 350–1,650 m/z (60,000 resolution, $3 \times 10^6$ automatic gain control (AGC), 128 msec injection time) followed by MS/MS data-dependent acquisition (top 25) with collision-induced dissociation and detection in the ion trap (30,000 resolution, $1 \times 10^5$ AGC, 60 msec injection time, 28% normalized collision energy, 1.3 m/z quadrupole isolation width). Unassigned precursor ions charge states and slightly charged species (were rejected and peptide match disabled. Selected sequenced ions were dynamically excluded for 30 sec.). Data was acquired using Xcalibur software v4.0 (Thermo Fisher Scientific). The MS-based proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository and are available via ProteomeXchange with identifier PXD024733.

### 2.10 Data processing and bioinformatics pipeline

MS raw files were analyzed using the MaxQuant (v1.6.14.0)(Tyanova, Temu, & Cox, 2016) software and peptide lists were searched against the human Uniprot FASTA database with the Andromeda search engine as previously described (Cox et al., 2011; Evans, Hutchison, Salamonsen, & Greening, 2020). Tandem mass spectra were searched as a single batch against *Homo sapiens* (human) reference proteome (75,777 entries, downloaded Sep-2020) with a contaminants database employed. Cysteine carbamidomethylation was set as a fixed modification and N-terminal acetylation and methionine oxidations as variable modifications. False discovery rate (FDR) was 0.05 for protein and 0.01 for peptide level with a minimum length of 6 amino acids for peptides and this FDR was determined by searching a reverse sequence database. Enzyme specificity was set as C-terminal to arginine and lysine as expected

using trypsin protease, and a maximum of two missed cleavages were allowed. Peptides were identified with an initial precursor mass deviation of up to 7 ppm and a fragment mass deviation of 20 ppm. Protein identification required at least one unique or razor peptide per protein group. Contaminants, and reverse identification were excluded from further data analysis. 'Match between run algorithm' in MaxQuant(Nagaraj et al., 2012) and label-free protein quantitation (LFQ)was performed. All proteins and peptides matching to the reversed database were filtered out. Data analysed based on inclusion/exclusion EV markers(Jürgen Cox et al., 2014; Jeppesen et al., 2019) in each sample, and intra-sample positive versus negative EV markers comparisons performed using Welch's t-test with reported log2 fold change, p-value and q-values. All data points are visualized using R (ggPlot2) with combined dot plot and boxplot.

### 2.11 Statistical analysis

All data were normalised to the mean of the control group and expressed as mean ± standard deviation (SD) with the exception of data collected using single particle interferometric resonance imaging sensing (SP-IRIS) and proteomics data. Media 1-9 were compared to the control group using an unpaired t-test. Media 10.1-10.9 were compared using a one-way ANOVA followed by Dunnett's multiple comparisons. Statistical significance was accorded when $p<0.05$.

## 3 Results

### 3.1 Cell viability and viable cell number of hAECs

Given that hAECs are not highly proliferative cells, both cell viability and total viable cells were determined – where the latter was reported in lieu of proliferation rate. The cell numbers and viability of hAECs cultured in different media formulations, relative to controls are summarised in **Figure 1**. We observed that only Media 3 had a significant impact on cell viability compared to control (**Figure 1A**, $p<0.05$). Conversely, Media 2 significantly increased total viable cells compared to control (**Figure 1B**, $p<0.05$).

When hAECs were cultured in Media 10, cell viability and total viable cells were comparable to control (**Figure 1C & 1D**), however, the EV protein yield was significantly lower (Figure 2C, $p<0.05$). We employed different culture supplements to optimise cell growth conditions. As detailed in **Table 2**, Cell Boost 6 supplement and/or AdvanceSTEM serum replacement were added to Media 10 to improve hAEC viability and EV yield (**Figure 1F & 1G**). There was

no significant difference in hAEC viability across these groups (**Figure 1F**). The addition of Cell Boost 6 supplement (Media 10.1-10.3), and the combination of Cell Boost 6 supplement and AdvanceSTEM serum replacement (Media 10.7-10.9) did not improve hAEC numbers (**Figure 1G**). While the addition of AdvanceSTEM serum replacement improved hAEC numbers in a dose-dependent manner (Media 10.4-10.6), this remained lower than the control group when supplemented at 5% (Media 10.4, **Figure 1G**; $p<0.05$). Live cell numbers, indicative of cell survival and proliferation, were comparable to control when Media 10 was supplemented with AdvanceSTEM at 10% (Media 10.5). Significant improvement was only observed when Media 10 was supplemented with 15% AdvanceSTEM (Media 10.6, **Figure 1G**; $p<0.05$). As such, media formulation does impact cell viability and cell culture supplements can improve the cell growth in a dose dependent manner.

### 3.2 Protein yield of hAEC-EVs

To gain insights into how different media types influenced the protein yield of EVs, we employed total protein quantification assay. The protein yield of hAEC-EV from culture using Media 3, 6, 7 and 8 were significantly lower than the control group (**Figure 2A**; $p<0.05$), while the hAEC-EV yield from Media 1, 2, 4, 5 and 9 were comparable to control. Notably, the average hAEC-EV protein yield from Media 1 was 1.5-fold greater than controls. However, there was no significant difference in hAEC-EV protein yield regardless of the combination of supplements with Media 10 (**Figure 2B**). Notably, the average hAEC-EV protein yield from Media 10 supplemented with 5% and 15% AdvanceSTEM serum replacement (Media 10.4 and 10.6) were 1.8-fold and 2-fold of the control respectively. This indicates that different media formulation could influence the EV protein yield significantly, and cell culture supplements could increase the EV protein yield.

### 3.3 Characterisation of hAEC-EVs reveals media formulation influencing amount of EVs

Transmission electron microscopy revealed that EVs were intact, spherical in morphology (**Figure S2**). Single particle analysis revealed that all EV samples were positive for CD9, CD81 and CD63 markers, consistent with previous reports(Ahn et al., 2013; Greening, Xu, Hong, Tauro, & Simpson, 2015; Rai et al., 2019). All of the $CD9^+$, $CD81^+$, and $CD63^+$ hAEC-EVs were 50-150 nm in size (**Figure S3, Tables S1 and** S2). Moreover, single particle analysis revealed that following incubation with equal numbers of EVs from all the groups, the anti-CD9 captured more hAEC-EVs across all groups, with 30,000-65,000 particles per μg EVs.

The numbers of $CD63^+$ or $CD81^+$ hAEC-EVs varied between groups with 1,000-10,000 $CD63^+$ particles and 3,000-30,000 $CD81^+$particles per μg hAEC-EVs. The number of hAEC-EV particles from Media 1-9 are detailed in **Table 3**. The number of $CD9^+$ particles were significantly lower in hAEC-EV from Media 1, 5, 6 and 9 compared to the control ($p<0.0001$, $p<0.01$, $p<0.05$, and $p<0.001$ respectively). The number of $CD81^+$ particles were significantly lower in hAEC-EV from Media 1, 5, 7 and 9 ($p<0.0001$, $p<0.001$, $p<0.05$, and $p<0.0001$ respectively), and significantly higher in hAEC-EV from Media 3,6 and 8 ($p=0.001$, $p<0.05$ and $p<0.001$ respectively). The number of $CD63^+$ particles were significantly lower in hAEC-EV from Media 1, 4 and 9 ($p<0.05$, all groups), and significantly higher in hAEC-EV from Media 2, 3, 6 and 8 ($p<0.05$, $p<0.01$, $p<0.0001$, and $p<0.05$ respectively). The particle numbers of hAEC-EV from Media 10 formulations are shown in **Table 4**. The number of $CD9^+$ particles were significantly higher in hAEC-EV from Media 10.1, 10.2, 10.7 and 10.9 ($p=0.0001$, $p<0.05$, $p<0.001$, and $p=0.0001$ respectively). The number of $CD81^+$ particles and $CD63^+$ particles were significantly higher in hAEC-EV from Media 10.5 ($p<0.05$ and $p<0.001$, respectively). Overall, media formulation and culture supplements did not change the size distribution of EVs or the predominant tetraspanin type associated with hAEC-EV, although did influence the particle numbers of $CD9^+$, $CD81^+$, and $CD63^+$ hAEC-EVs.

### 3.4 Media formulation type influence surface epitopes of EVs

Surface epitopes of hAEC-EVs were also determined using flow cytometry and mean fluorescence intensity is shown in **Figures 3 & 4 and Figures S3 & S4**. All common EV tetraspanin markers were expressed by hAEC-EV as shown at the single particle level (**Figure S3**). Tetraspanins are associated with various endocytic membranes including the plasma membrane and highly enriched in EVs (tetraspanin-enriched microdomains), important in formation and function of EVs (Andreu & Yáñez-Mó, 2014; David W Greening, Xu, Gopal, Rai, & Simpson, 2017; Kowal et al., 2016). hAEC-EV displayed soluble endoglin CD105, epithelial marker CD326, and other surface epitopes including CD29, CD40, CD41b, CD133 and CD142. Among the EV common tetraspanins of CD9, CD81 and CD63, there was no difference in mean fluorescence intensity (MFI) of CD9 across all the groups (**Figure S4A & S5A**). However, MFI of CD63 decreased in hAEC-EV isolated from several media types, including Media 3, 4, 6, and 10.4-10.9 (**Figure 3A & 4A**; $p<0.05$, $p<0.01$and $p<0.001$). Further, MFI of CD81 decreased in EVs generated from 8/18 media types, including Media 1, 3, 4, 6, 10.4-10.6, and 10.8 (**Figure 3B & 4B**; $p<0.05$ and $p<0.01$). The MFI of epithelial cells marker

CD326 also decreased in hAEC-EV isolated from 6 of the 18 media formulations (i.e. Media 4, 5, 9, 10.4, 10.5 and 10.6, **Figure 3C & 4C**; $p<0.05$). The MFI of CD41b decreased in EVs from Media 1, 5, 6, 7 and 8, and increased in EVs from Media 10.3 (**Figure 3D & 4D**; $p<0.05$). The MFI of CD133 and CD142 on hAEC-EVs were decreased in Media 3 and 4 (**Figure 3E-F**, $p<0.05$). The MFI of surface epitopes that did not change across the groups are detailed in **Figures S4 and S5**. Overall, all the types of surface epitopes detected were consistent across all the media groups. The FACS data revealed that media formulation and culture supplements had an impact on the MFI of tetraspanins, CD63 and CD81. Media formulation also influenced the MFI of CD326 (EpCAM) which is a surface marker of hAECs, as well as other surface epitopes such as CD41b, CD133 and CD142.

### 3.5 Media formulation type influence proteome composition of EVs

Using cell-derived EVs in distinct media formulations, we wanted to determine the proteome composition of EVs and understand whether inclusion (positive) and exclusion (non-vesicular/negative) markers (as reported by Journal of Extracellular Vesicles and international guidelines on EV research (Théry et al., 2018)) were differentially abundant based on differences in media type used for cell culture. We employed quantitative proteomic profiling of EVs based on label-free quantitation and maxLFQ normalisation (Evans et al., 2019; Greening et al., 2019; Rai et al., 2021). We compared protein identifications and normalized precursor ion intensities of positive and negative EV markers in each sample and performed comparisons for all media formulations (**Figure 5, Table S3**). Inclusion (positive) and exclusion (negative, contaminants/non-vesicular) protein EV marker components are included **Table S4**. We demonstrate that in all media types, derived EVs (total 1737 proteins, average protein identifications for each media type 410 proteins) (**Tables S5-8**), contained positive EV marker proteins (e.g., CD59, CD9, GAPDH, and ANXA1/2/3/4) and increased in expression and quantification (abundance) of positive EV markers compared to exclusion markers of EVs (**Figure 5**. Media types 5-9, and 10.3/10.4/10.8 were found to be significantly enriched in positive versus exclusion EV markers ($p<0.05$) (**Table S3**). These findings support the enrichment strategy of EV in this study.

To understand differences in media type based on total EV proteome, we performed global proteomic analysis of EV proteome for each sample. Here, we normalised LFQ distribution for total EV proteome for each media type (**Figure S6**). We show that protein identifications are influenced by media type (**Figure S7**). Importantly, the level of protein contaminants influences

the total EV proteome, contributed by media type (and additives within, non-human) (**Figure S7, Table S7-8**). Here, we report not only differences in total EV proteome from different media types, but also differences in abundant protein detection based on total MS/MS spectra, including albumin and other additives in media formations and supplements which may influence the EV proteome (**Table S7-8**). Such variables should be considered for future experiments in cell products derived from specific media formulations and a proteomic strategy for detection and analysis. Here, we show that using a proteomic profiling strategy allows assessment of upstream experimental factors, including media formulation, on the composition of EVs and specifically, inclusion/exclusion markers of EVs themselves.

# 4 Discussion

The cell culture conditions used for EV production can vary significantly, even when well-established cell lines are used. The yield and cargo of EV can be drastically altered by culture conditions used for donor cells. Numerous approaches have been employed in upstream manufacturing to increase EV yield. These include cell culture density using rocking platform bioreactors (Glazyrina et al., 2010) and stirred tank systems (Lawson et al., 2017), modulating cell attachment surface area using multi-layer flasks, extracellular matrix scaffolds (Huleihel et al., 2016), microcarriers (Petry et al., 2016) and hollow fiber bioreactor (Curcio et al., 2012) for adherent cell types. Modification of culture conditions such as hypoxia at low oxygen (King, Michael, & Gleadle, 2012; Ludwig, Razzo, Yerneni, & Whiteside, 2019), decreased pH levels (Ban, Lee, Im, & Kim, 2015; Brownlee, Lynn, Thorpe, & Schroit, 2014), addition of supplements such as $Ca^{+}$ ionophores (Savina, Furlán, Vidal, & Colombo, 2003) and liposomes (Emam et al., 2018) have also been tested. Indeed, the medium used for cell culture and EV recovery from supernatants emerges as a crucial factor in EV production. Culture media is a fundamental material for EV production as it impacts cell viability and growth, and therefore, EV yield and composition. To gain direct insights into how different media formulations influence EV yield, biophysical characteristics, and proteome composition, we sourced different commercially available chemically defined serum-free media types, suitable for GMP manufacturing of EVs and evaluated their impact on hAEC proliferation and viability, hAEC-EV yield, hAEC-EV surface epitopes, and hAEC-EV proteome composition. Since the EV isolation from conditioned media containing serum (and other serum-containing media types)

can lead to the co-isolation of exogenous EVs, and other regulatory effects on EV release, non-serum containing media types were employed for this comparison.

Higher cell viability and greater number of viable cells offer the possibility for efficient and consistent EV generation. Consequently, it was unsurprisingly that cells cultured in Media 3 resulting in significantly lower cell viability, also yielded less hAEC-EV. When hAECs were cultured in Media 1, 4, 5, and 9, cell viability and viable cell numbers were similar to control. This resulted in comparable EV yield to the control media group. However, we noted that significantly lower EV yield was observed in hAECs cultured in Media 6, 7 and 8 despite a similar viability and cell number to the control. This indicates that while cell viability and viable cell number are important parameters for cell culture, but they are not reliable quality indicators of EV manufacturing. This observation is in contrast to previous reports that higher yields of MSC-EV were obtained at lower cell seeding density (Patel et al., 2017), and more rapid cell proliferation (Sekiya et al., 2002). The relationship between proliferation and EV yield may thus vary on the cell type.

Adding supplements to a basal media is a common way to improve cell culture conditions. Given that Media 10 generated a significantly lower yield of hAEC-EVs, the supplementation with Cell Boost 6 and/or AdvanceSTEM serum replacement, was evaluated. While Cell Boost 6 failed to improve cell viability or cell numbers, AdvanceSTEM serum replacement increased viable cell numbers in a dose-dependent manner, while concurrently increasing EV yield. This suggests that titration studies of media supplements are justified when looking to achieve the highest EV yield without changing bioactivity. However, further addition of Cell Boost 6 did not significantly improve culture outcomes or EV yield. Others have previously shown that MSC-EV production could be improved by adding FDA approved drugs such as methyldopamine, norepinephire, and forskolin. Moreover, adding forskolin to norepinephire has been shown to further enhance EV production while adding forskolin to methyldopamine did not(Wang, Bonacquisti, Brown, & Nguyen, 2020). This suggests that different cell culture supplements may have variable effects on EV production depending on donor cell type, and it may be difficult to predict the effect of combining culture supplements on EV generation.

The size, concentration, and consistency in EVs is an important part of characterising each EV preparation. Methods such as electron microscopy, single nanoparticle analysis technologies, and resistive pulse sensing and flow cytometry may be used, depending on the application,

instrument access, and throughput. Here, we employed a combination of all methodologies to assess EV characterisation. Single particle interferometric reflectance imaging revealed that $CD9^+$ EVs were the predominant population in all hAEC-EV preparations. While CD9, CD81, CD63 are common tetraspanins in EVs (Andreu & Yáñez-Mó, 2014) due to the membrane content and origin of EVs from cell source (combination of endosomal-derived and plasma membrane-derived (Fordjour, Daaboul, & Gould, 2019)), their relative abundance varies across EVs from different cell sources (David W Greening et al., 2017; Kowal et al., 2016). For example, EV preparations from the B cell lymphoma cell line, SUDHL-6, have relatively high abundance of $CD81^+$ EVs but low levels of $CD63^+$ EVs (Oksvold et al., 2014). In contrast, EV preparations from colon cancer cells, were found to be enriched in $CD63^+$ EVs relative to $CD81^+$ EVs[38]. As such, cell source is a critical consideration is dealing with EV generation. Here, we observed that Media 1 resulted in significantly lower numbers of $CD9^+$, $CD63^+$ and $CD81^+$ hAEC-EVs, and Media 10.5 resulted in significantly higher numbers of $CD63^+$ and $CD81^+$ hAEC-EVs compared to control media. These observations were despite similar effects on hAEC viability, cell number and EV yield. This suggests that different culture media formulations can impact EV generation even when all other culture parameters are maintained, and EV yields are comparable. This may be attributed to the differences in glucose contents, amino acid composition and general nutrient formulation, which may impact cell proliferation or EV yield (Witwer et al., 2019). Such differences may be supported by the observed changes to tetraspanins levels (surface) and total proteome (and the distribution of total proteins identified across each media type), despite normalised to EV yield. Indeed, other researchers found that the metabolites varied between culture media formulations and they affected the cell growth as well as the metabolic profiles of cell secretomes (Daskalaki, Pillon, Krook, Wheelock, & Checa, 2018; Zang et al., 2011).

With the emerging aspects of EV therapeutics towards clinical translation, there has become urgent need to characterize and validate the methods available for production and understanding of EV composition. In this study we employed quantitative proteomics to understand how different media formulations influence the proteome of EVs from amniotic epithelial cells, in addition to monitoring inclusion/exclusion markers of EVs within. Mass spectrometry is known to be the main reference method involved in reference measurement procedures and meets the requirements of *in vitro* diagnostic (IVD) regulations and standards for identification and quantification (Hirtz et al., 2018). We show successful isolation and enrichment of EVs in this study for several media types analysed.

Proteomic profiling revealed that total proteome of EVs was highest in coverage/identification/quantification using media formulations 5-9, and 10.1-10.3, while media types 5-9 and 10.3/10.4/10.8 were found to be significantly enriched in positive versus exclusion markers for EVs. Given that EVs were isolated from the same cell source using a consistent isolation and enrichment strategy, we suggest that variance in total EV proteome may be in part due to contaminating factors within the media formulations or introduced during the manufacturer and generation of isolated EVs and their proteome. This is indeed an important consideration in monitoring EV generation using a proteomic approach. Importantly, one should also consider the data-dependent acquisition mode used in this mass spectrometry-based proteomic approach, a strategy known to have inherent dependence on highly abundant peptides to be assessed by mass spectrometry, and therefore limit detection of low abundant EV-derived factors. Other data-independent strategies may offer advantages in profiling EVs in abundant proteinaceous background (Hallal et al., 2020). Findings from this study allow assessment of upstream experimental factors, including media formulation, on the composition of EVs and specifically, inclusion/exclusion markers for EVs. This has important regulatory implications and understanding functional and therapeutic potential of EVs, cell-derived products, or even donor cells themselves.

When the mean fluorescence intensity (MFI) levels of 35 surface epitopes were analysed concurrently, we observed that differential expression of several tetraspanins including increase in CD81 and CD63, while no change in CD9 and CD105. We also noted that the level of CD326 (EpCAM), an epithelial marker that is typically highly expressed by hAECs, was decreased across several media types. The levels of other surface epitopes that were significantly decreased included CD41b, CD133 and CD142, while the levels of CD40 and CD29 remained unchanged. hAEC-EVs were obtained from Media 2 and 4 with comparable yield and $CD9^{+}$ particles to the control group. However, the MFI levels of CD133 and CD142 were significantly lower than control. This again suggests that comparable EV yields do not necessarily correspond with consistency in EV composition. Tetraspanins are usually post-translationally modified and are generally accepted to organise each other, distinct transmembrane and cytosolic proteins (such as integrins and proteases) to be the multimolecular membrane network called tetraspanin web (Charrin et al., 2009; Hemler, 2005; Stipp, Kolesnikova, & Hemler, 2003). Moreover, many studies have showed that tetraspanin has a role in EV cargo selection and it may influence EV targeting and update (Andreu & Yáñez-Mó, 2014; Mazurov,

Barbashova, & Filatov, 2013; Perez-Hernandez et al., 2013; Rana, Yue, Stadel, & Zöller, 2012; Van Niel et al., 2011). Changes of MFI levels of tetraspanins reflects the changes in EV biogenesis and thus points to potential functional changes.

We observed that hAEC-EVs display CD29, CD41b, CD40, CD133 and CD142 on their surface. Integrins are transmembrane receptors that play important roles in the initiation of signalling cascades and facilitating cell-extracellular matrix adhesion (Horton et al., 2016). Various integrins also play important roles in the cell/tissue recognition for EVs(Hoshino et al., 2015; Nguyen et al., 2021). CD29 (ITGB1) plays an important role in pro-angiogenesis, cell differentiation and cell migration (Mettouchi & Meneguzzi, 2006; Pasqualini & Hemler, 1994). CD41 (ITGA2B) has been shown to played an important role in migration of MSC (Awan et al., 2018). Our proteome data also reflected differences in integrin expression profile, where ITGA2, ITGAV, ITGB4, ITGB6 (increased in Media 5-9, Media 10.1-10.3), ITGA3 (increased in Media 6, 8-9, Media 10.1-10.3), and ITGB1 (increased in Media 1-3, 5-6, 8-9, Media 10.1-10.3) (**Figure S8**). In contrast, other integrins were low in abundance relative to control media types, including ITGB8 (control compared to Media 10.1-10.9) and ITGB1/B6 (Media 2-3). Integrins are part of the main tetraspanin protein partners (Yáñez-Mó, Barreiro, Gordon-Alonso, Sala-Valdés, & Sánchez-Madrid, 2009). They can affect EV biogenesis and regulate EV uptake (Fuentes et al., 2020; Lőrincz et al., 2020; Morelli et al., 2004). Similar to tetraspanins, these collective differences in integrins in hAEC-EVs also reflects the differences in EV biogenesis, which is resulted from the only difference in our EV generation and isolation process-culture media formulation.

CD40 is essential in mediating a broad variety of immune and inflammatory response including macrophage activation and T cell activation(Elgueta et al., 2009). $CD133^{+}$ cells that isolated from bone marrow, cord blood, and peripheral blood have been shown to promote angiogenesis and cell growth, and have been used in clinical trials (Adler et al., 2011; Z. Li, 2013; Nikeghbalian et al., 2011). Further, $CD142^{+}$ microvesicles from various cell sources could promote endothelial cell proliferation or induce apoptosis (Madkhali et al., 2019). These relatively abundant surface epitopes may account for the biological activity of hAEC-EVs, given the overlap with our previous findings on their immunomodulatory effect on macrophage subpopulations and T cell activation (Alhomrani et al., 2017; Tan, Chan, Wallace, & Lim, 2013; Tan et al., 2015; Tan et al., 2018). Furthermore, hAECs also promote angiogenesis (Zhu et al., 2016), and induce lung stem cell proliferation (Zhu et al., 2017). Therefore, the potential

bioactivity of EV epitopes should be considered when developing quality control measures for scalable manufacturing.

We have showed that the different levels of protein contaminants influence the total EV proteomes. Studies have showed that media formulations influenced the EV composition (Daskalaki et al., 2018) as well as generated contaminants in EVs (Auber, Fröhlich, Drechsel, Karaulanov, & Krämer-Albers, 2019). Apart from the media formulations, different EV isolation and purification methods will result in differences in EV yield, purity and homogeneity (Ludwig, Whiteside, & Reichert, 2019; Stranska et al., 2018). For example, EVs isolated with ultracentrifugation have co-isolation of proteins, lipoproteins and other contaminants, with low RNA yield and potential EV damages (Gardiner et al.; Konoshenko, Lekchnov, Vlassov, & Laktionov, 2018; Takov, Yellon, & Davidson, 2019), immunoaffinity isolation targets specific membrane proteins to enrich for specific EV subpopulations (Taylor & Shah, 2015), size exclusion chromatography (SEC) separates particles according to their physical size and can result in a relatively purer EV population compared to other methods (Monguió-Tortajada, Gálvez-Montón, Bayes-Genis, Roura, & Borràs, 2019). However, the limited loading volume is a drawback SEC, thus it is usually employed in combination with ultrafiltration which removes some contaminants and reduces loading volume (Guerreiro et al., 2018). However, ultrafiltration can result in EV loss (Konoshenko et al., 2018). It is also important to note that unique miRNA profiles were observed when different EV purification methods were used (Ding et al., 2018; Rekker et al., 2014), and the levels of tetraspanins such as CD63, CD9, and CD8 also varied between different isolation methods (Serrano-Pertierra et al., 2019) and reflect the variation in EV purity and subpopulations. To date there is no single universally accepted separation method, thus, the approach to EV isolation and purification is dependent on the application (Théry et al., 2018). Taken together, these reports indicate that potential contaminants, as well as changes to EV yield and composition should be investigated as part of any given process development activity.

# 5 Conclusion

This study has uncovered critical aspects to consider in scalable cell-based EV manufacture. In particular, we provide evidence that cell viability and proliferation rate are not reliable quality indicators of EV manufacturing. Subtle changes in culture media formulation have the potential

to affect aspects of EV biogenesis, and consequently bioactivity. The changes to levels of EV surface epitope and functional assays, could be used as quality control criteria for scalable EV production. Such functional assays will be the subject of future studies to verify the functional relevance of the changes to these surface epitopes. Future studies will necessarily include analysis of EV markers consistent in the cell type and media formulation used (e.g. CD59, CD9, ALIX, or specific annexins, integrins for example) as well as using proteomic screen to monitor EV inclusion markers as a signature – an approach which may offer significant regulatory advantage in multi protein detection and quantification across different media types and different cell source or other upstream variables (other culture conditions, approaches in generation, isolation, etc). Furthermore, all cell culture experiments described here were conducted under static conditions to allow variable of media type in this case, to be analysed. Future studies should incorporate the considerations around dynamic culture conditions typically used for scalable manufacturing, such as rocking platform bioreactor and hollow fiber bioreactors. In summary, key findings from this study indicate that EV purity, composition and potencies must be investigated when assessing the suitability of critical raw materials for EV manufacturing and reliance on cell culture parameters are unlikely to be reflective of EV yield and/or quality.

## Author Contribution

DZ, GK, RS, MB, SC, HM, HF, DG and RL performed research, collected and analysed data presented in this manuscript. DZ, DG and RL wrote the manuscript. DZ, EMW, DG and RL conceived and designed the research.


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Media 1-4 were provided by Thermo Fisher for evaluation. Media 10, Cell Boost 6 supplement and AdvanceSTEM serum replacement used for groups 10.1-10.9 were provided by Cytiva.


## Competing Interests

The authors have no conflicts to declare.

**Data and Software Availability:** The MS-based proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository and are available via ProteomeXchange with identifier PXD024733.


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## Figure legends

**Figure 1: Impact of media formulations on viability and total viable hAECs.** (A) Among Media 1-9, hAECs cultured in Media 3 had significant lower viability compared to control media (*p<0.05). (B) hAECs cultured in Media 2 had significant higher number of viable cells (*p<0.05) suggesting of increased proliferation in a cell type that is not highly proliferative (C-D). There was no significant difference in cell viability and viable cell number between Media 10 and control. (E) EV yield from Media 10 was significant lower compared to the control media. (F) Cell viability remain unchanged across groups 10.1-10.9. (G) Viable cell numbers were similarly unchanged in the groups with only supplemented with Cell Boost 6 (Media 10.1-10.3), or in combination with AdvanceSTEM serum replacement (Media 10.7-10.9). However, there was a dose effect on the number of viable cells in the three groups supplemented with AdvanceSTEM (Media 10.4-10.6) (*p<0.05).

**Figure 2: Impact of media formulations on hAEC-EV yield.** (A) The protein yield of isolated EVs from Media 3, 6, 7 and 8 were significantly lower than the control group (*p<0.05). (B) There was no significant difference in EV protein yield between Media 10.1-10.9 groups and control group. However, the average EV protein yield from Media 10.4 and 10.6 was 1.8-fold and 2-fold of the control level respectively. (*p<0.05)

**Figure 3: Impact of media formulations on surface epitope abundance on hAEC-EV.** (A) Media 3, 4 and 6 reduced the abundance of CD63 on the surface of hAEC-EV. (B) Media 1, 3, 4 and 6 reduced abundance of CD81. (C) Media 4, 5 and 9 reduced abundance of CD326. (D) Media 1, 5, 6, 7 and 8 reduced abundance of CD41b. (E) Media 3, 4 and 7 reduced abundance of CD133. (F) Media 3 and 4 reduced abundance of CD142. (*p<0.05 and **p<0.01). *MFI: mean fluorescence intensity.*

**Figure 4: Impact of media additives on surface epitope abundance of hAEC-EV**. (A) Media 10.4-10.9 decreased the abundance of CD63 on the surface of hAEC-EV. (B) Media 10.4-10.6 and 10.8 decreased abundance of CD81. (C) Media 10.4-10.6 decreased abundance of CD326. (D) Media 10.3 decreased abundance of CD41b (*p<0.05, **p<0.01and ***p<0.001).

**Figure 5: Intrasample comparison of EV proteomic analysis reveals differences associated with media formulations.** Proteome analysis of EVs for each specific media type (Media 1-9, A&B; Media 10.1-10.9, C), with inclusion (positive) and exclusion markers of EVs (Table S4) indicated for each media type based on LFQ intensity (log2). Specific EV (positive/negative) proteins for each media type are reported in Table S3(Théry et al., 2018). Intra-sample positive and negative EV marker comparisons performed using Welch's t-test (Table S3). All data visualized using R (ggPlot2) with combined dot plot and boxplot.

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