A Novel Manufacturing System for Standardized Extracellular Vesicles from telomerized human Mesenchymal Stromal Cells - EV*scale*™

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The Case for Extracellular Vesicles (EV)

EV are natural and universal 'cargo ships' between cells in all organisms and tissues

EV display biologically relevant mechanisms of action(MoA) e.g., EV derived from Mesenchymal Stromal Cells (MSC):

- **Anti-inflammation**
- **Anti-fibrosis**
- **Tissue-protection**
- **Pro-angiogenesis**
- **Proliferation**

EV possess Broad Therapeutic potential in Regenerative Medicine e.g., in:

- **Acute Lung Inflammation**
- **Lung / Liver / Kidney Fibrosis**
- **Cardiac Fibrosis**
- **Organ Injury**
- **CNS Inflammation, Neurodegeneration**

Extracellular Vesicles (EV) from different cell sources offer great promise for therapeutic applications in Regenerative Medicine. However, manufacturing of EV in needed quantities and with consistent quality attributes has proven difficult. Therefore, Phoenestra has recently developed a scalable manufacturing setup using stable, telomerized MSC (MSC/TERT) lines which are fully documented and characterized (GMP-grade). With this proprietary setup (EVscale™), we have been assessing the expansion and continuous cultivation of MSC/TERT derived from different tissues as well as the yield and quality profile of naturally secreted EV over the cultivation period and between different runs.

EVscale™ – Extracellular Vesicles *at scale* - In a Nutshell

End-to-end approach

- ✓ Xeno-free, telomerized MSC lines
- ✓ Cell Banking (MCB/WCB approach)
- ✓ Scalable Technology Platforms
- ✓ Systematic Process Development
- ✓ GMP manufacturing resources

Quality, regulatory and cost

- ✓ Compliant sourcing
- ✓ GMP-ready MSC/TERT Lines
- ✓ Quality Control Strategy
- ✓ Product Characterization and Definition
- ✓ Leading Productivity
- ✓ Leading Cost of Manufacturing

Telomerized MSC (MSC/TERT) Library

Phoenestra uses fully documented and characterized MSC/TERT lines*. These stable MSC lines have distinct advantages versus primary MSCs:

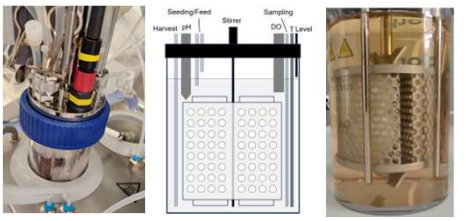
- Stable phenotype and growth
- Consistent quality of cells and EV
- Proven biological activities
- Tiered cell banking concept possible

** In collaboration with Evercyte GmbH*

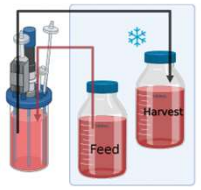
Code	Tissue Source	GMP ready
ASC/TERT	Adipose Tissue	Yes
BM-MSC/TERT	Bone Marrow	Yes
WJ-MSC/TERT	Wharton´s Jelly	Yes
P-MSC/TERT	Placenta	Yes
CP-MSC/TERT	Chorionic Plate	Yes
DP-MSC/TERT	Dental Pulp	Yes
More in preparation	Several	In prep

Upstream - Agitated Packed Bed Bioreactor System*

*Patent Pending



Stirred Tank Bioreactor and Phoenestra's Proprietary Packed Bed System

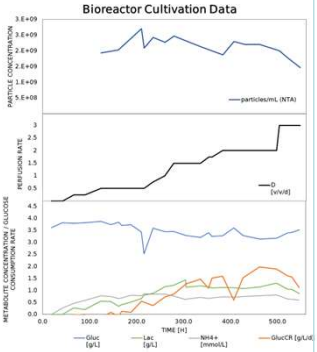


Controlled Bioreactor Cultivation in Perfusion Mode with a constant in- and out-flow of media

Based on these excellent cell lines, Phoenestra has developed a scalable bioprocess setup including an agitated packed-bed filled with carrier material which allows for efficient MSC expansion and EV/exosome production at lab scale (250 mL bioreactor) for up to and beyond 42 days:

Exemplary case	Seed (Day 0)	Harvest (Day 23)
Total Cells	$1.3 \cdot 10^7$	$2.2 \cdot 10^8$
Cells / mL	$5 \cdot 10^4$	$0.9 \cdot 10^6$ * (17-fold) 0.4 - 1.1·10^6 ** (μ = 0.1-0.2 d^{-1})
Cells / cm2	$2 \cdot 10^3$	$3.3 \cdot 10^4$
Cell Proliferation	17-fold, μ = 0.1 - 0.2 d^{-1}	

*Cell Titer Glo Assay, ** Calculation from Glucose Consumption Rates

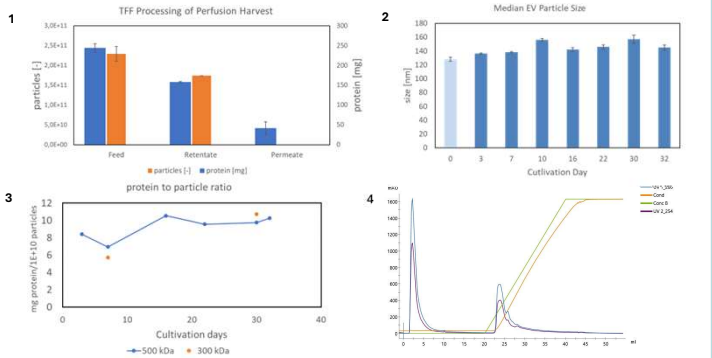


Total Particle Harvest*	**$1.3 \cdot 10^{13}$ particles** (11 harvests, 6.8 L)
EV productivity	**5000 - 10000 particles/cell/d**
Theoretical Clinical Patient Doses**	**30 doses in total** from a 250 mL bioreactor unit

*measured by nanoparticle tracking analysis (NTA),
** Assumptions: 70% lipid particles, 50% downstream recovery, 1.5×10^{11} EV/dose (up to 10^{12} per dose, e.g., NCT05125562)

Isolation and Chromatographic Separation of EV

EV/exosome containing supernatants are at first depth-filtered, then concentrated and buffer-exchanged by Tangential Flow Filtration (TFF). With this downstream setup we achieve particle yields of 50 – 70 % (NTA). The resulting retentates are subject to in-depth analytical characterization and further downstream processing (e.g., fractionation via anion-exchange chromatography).

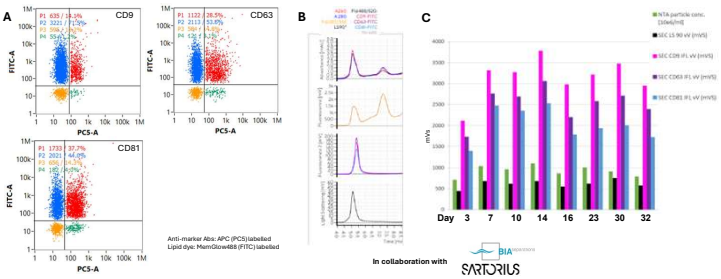


1 Protein (bicinchoninic acid (BCA) assay) and particle (NTA) recoveries of a representative TFF operation; **2** Median particle sizes (NTA) of processed retentate samples from harvests of an MSC/TERT-bioreactor perfusion cultivation over 32 days; **3** Protein to particle ratio of retentates processed with two different TFF cutoffs; **4** Early example of an Anion-exchange chromatography separation (CIMmultus QA™, BIAseparations d.o.o.) of an EV TFF retentate.

Thanks to our collaboration partners and funding agencies

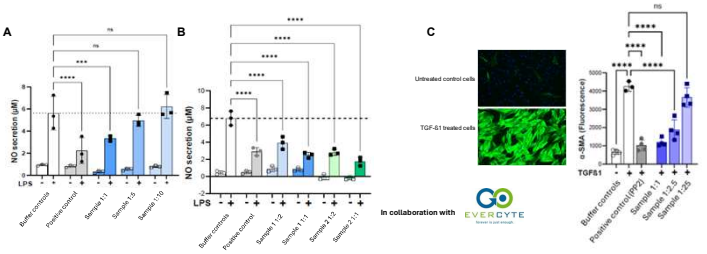
Characterization of Extracellular Vesicles

EV preparations are characterized with a panel of orthogonal methods to assess differences in EV composition and/or biological activities. Parameters are MSC/TERT lines from different human tissues, harvest time points, cultivation parameters (e.g., DO, pH, T, D (perfusion rate)), and isolation/purification methods.



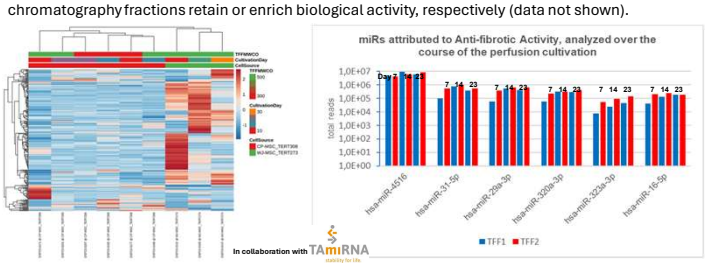
EV marker distribution, ratio of lipid particles vs. non-lipid particles, size distribution by, **A** NanoFCM and, **B** an example analytical immunofluorescence chromatogram (PATfix™, BIA Separations d.o.o.), **C** the EV marker distribution of harvest samples of a 32-day perfusion bioreactor run. Data shown are from first experiments, method optimization ongoing.

Between 60 to 80% of particles are usually lipid particles which contain at least one of the EV markers CD9, CD63 and CD81. The PATfix™ chromatography system allows for online detection of UV, fluorescence and Light Scattering (particle size and quantification) in parallel. The EV marker pattern remains fairly consistent over the course of the 32-day perfusion cultivation shown.



Anti-inflammatory (AI) Activity (above, A and B): the alteration of NO secretion of inflammation-triggered (LPS) macrophages was tested when exposed to Dexamethasone (positive control) and respective EV preparations (TFF retentates, dose-dependent) of different harvests throughout a perfusion cultivation. The negative control is TFF buffer solution.
Anti-fibrotic (AF) Activity (above, C): the inhibition of smooth-muscle actin expression is tested as a surrogate to assess the anti-fibrotic activity of relevant samples in a dose-dependent manner.

Extracellular Vesicles harvested over the course of a perfusion process display dose-dependent **anti-inflammatory and anti-fibrotic activities** in developed cell-based bioassays. However, the extent varies between different cell lines derived from different tissues. Preliminary results also indicate that chromatography fractions retain or enrich biological activity, respectively (data not shown).



RNA sequencing is used to further characterize different EV samples. EV from two different cell lines (WJ- and CP-MSC/TERT) show marked differences in their miRNA patterns (heat map above left), while selected miRs seem to correlate with biological function, results for selected miRNAs isolated from different harvest time points and attributed in publications to anti-fibrotic activity are shown (above right).