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

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The Case for Extracellular Vesicles (EV)

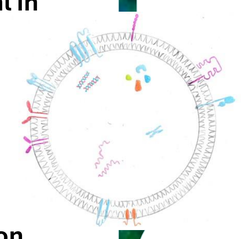
EV are natural and universal 'cargo ships' between cells in all organisms and tissues

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 consistent manufacturing of MSC/TERT EV from different tissues. In a next step, preparative and analytical chromatographic separation is used to further study determinants of biological functionalities.

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 in preparation

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:

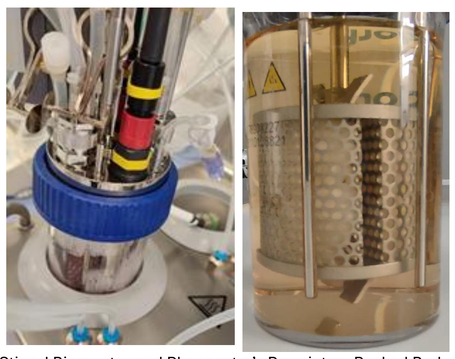
- Stable phenotype and growth
- Consistent quality of cells and EV
- Proven biological activities
- Tiered cell banking concept possible

** In collaboration with Evercyte GmbH*

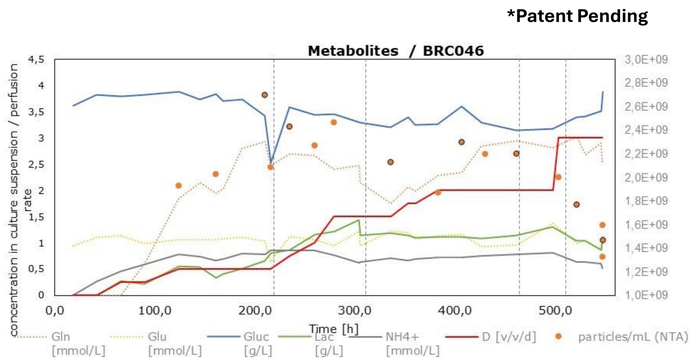
Code	Tissue Source	GMP ready
ASC/TERT	Adipose Tissue	Yes
BM-MSC/TERT	Bone Marrow	Yes
WJ-MSC/TERT	Wharton´s Jelly	Yes
P-MSC/TERT	Placenta	Yes
CP-MSC/TERT	Chorionic Plate	Yes
DP-MSC/TERT	Dental Pulp	Yes
More in preparation	Several	In prep

Upstream - Agitated Packed Bed Bioreactor System*

***Patent Pending**



Stirred Bioreactor and Phoenestra's Proprietary Packed Bed Perfusion System



We have developed a scalable perfusion process setup including an agitated packed-bed filled with cell carrier material which allows for serum- and platelet lysate-free MSC/TERT expansion and continuous EV/exosome harvest for several weeks. Process performance and EV yield for an example process are summarized below. Theoretical clinical doses have been calculated based on assumptions:

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 - $1.1 \cdot 10^6$ ** (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 (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).

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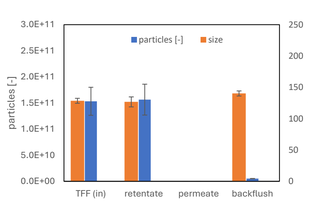

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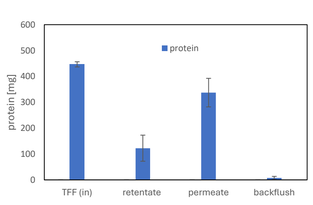

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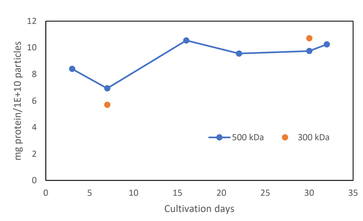

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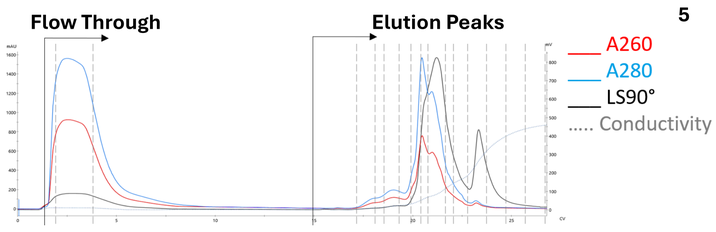

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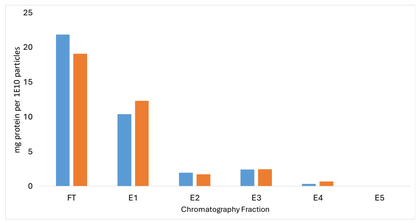
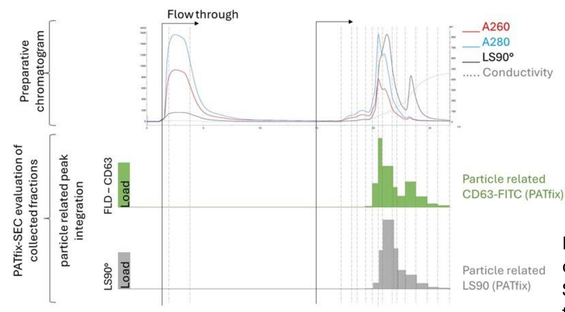
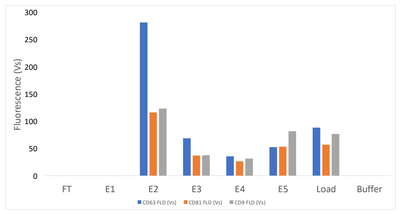


1 Particle yields and median particle sizes (both by NTA) of a representative TFF operation; **2** Protein content of TFF process samples from harvests of an MSC/TERT-bioreactor perfusion run; **3** Protein to particle ratio of retentates processed with two different TFF cutoffs; **4** Example chromatogram of an Anion-exchange chromatography separation (CIMmultus QA™, BIA Separations d.o.o.) of an MSC/TERT-EV TFF retentate; **5** Protein-to-particle ratio of the flow-through (FT) and fractions E1-E5 from 2 chromatographic separations.

Analysis of chromatographic fractions with the PATfix® HPLC platform





In collaboration with BIA separations / SARTORIUS

Chromatograms kindly provided by Darja Božič (Sartorius BIA Separations)

PATfix® enables analytical separation (size-exclusion shown) and online detection of A260/280, Fluorescence (FLD) and Light Scattering (MALS) for deeper insights into processed EV samples: the tetraspanin marker distribution in chromatography fractions is shown (graph to the right).

Towards Rational Selection of EV Preparations for Further Use

EV preparations (TFF retentates and chromatographic fractions) are characterized with a panel of orthogonal methods to assess differences in EV composition and/or biological activities as well as correlations which could help understand components of biological activities better.

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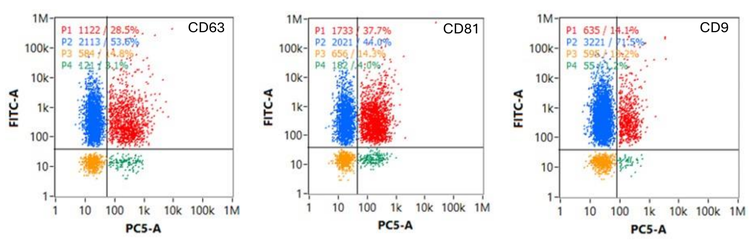

6 NanoFCM analysis of the EV tetraspanins CD63, CD81 and CD9 (red dots) in a representative TFF retentate sample. Non-stained non-lipid particles (yellow), stained non-lipid particles (green) and unstained lipid particles (blue) are shown.

Anti-marker Abs: APC (PC5) labelled
Lipid dye: MemGlow488 (FITC) labelled

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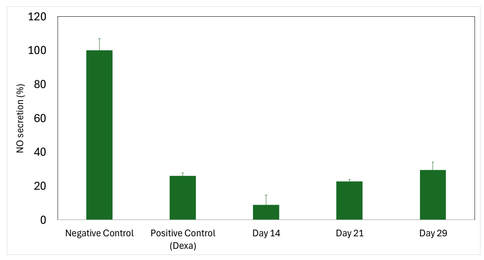

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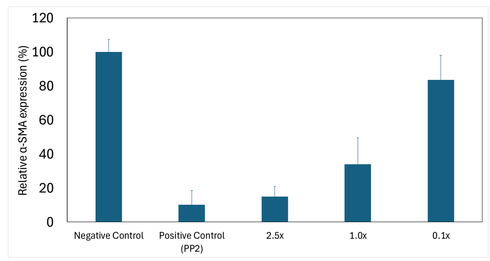

7 Anti-inflammatory activity of EV preparations (TFF retentates) from a P-MSC/TERT perfusion run over 28 days show reduction in NO secretion in the range of the positive control (10 µM Dexamethasone, LPS trigger of mouse macrophages); **8** Dose-dependent **anti-fibrotic activity** of a TFF retentate from a CP-MSC/TERT perfusion run at day 23. Fibroblast cells are triggered with TGF-β to induce α-SMA (α-smooth muscle actin) expression, the Src-kinase inhibitor PP2 is used as a positive control.

In collaboration with EVERCYTE

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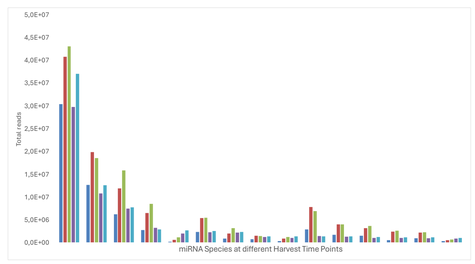

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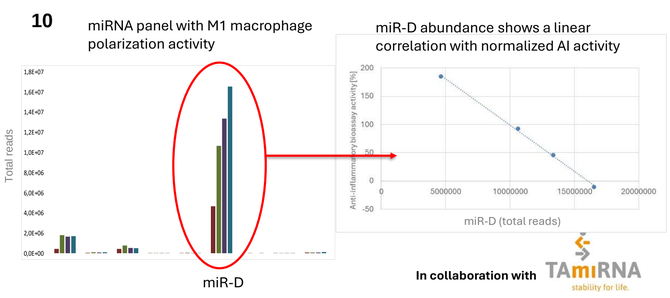

In collaboration with TAmiRNA

9 Small RNA sequencing gives distinct miRNA patterns for each MSC/TERT cell line. The Top 15 miRNAs from a CP-MSC/TERT-EV preparation (TFF retentate) are shown. **10** Correlations between miRNA patterns and marker expression or bioactivities are investigated. An example of a linear, inverse correlation between cell-based activity (anti-inflammatory) and the expression of a specific miRNA (miR-D) with known M1 macrophage polarization activity in an ASC/TERT-EV preparation (TFF retentate) is shown. These correlations will be investigated further.

Chromatographic fractions generated by quantitative CIMmultus QA separation of a TFF retentate have been tested for anti-fibrotic activity. Also, small RNA sequencing of the chromatographic fractions addressed potential changes in miRNA profiles in the collected fractions.

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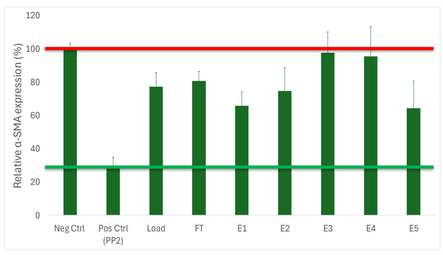

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Load	E1	E2	E3
miR-B	miR-B	miR-B	miR-B
miR-D	miR-D	miR-D	miR-D
miR-F	miR-M	miR-F	miR-F
miR-H	miR-F	miR-M	miR-M
miR-M	miR-H	miR-H	miR-H

Ranking of miRNAs based on total reads

E4	E5
miR-A	miR-A
miR-C	miR-B
miR-E	miR-D
miR-B	miR-F
miR-D	miR-H

11 Fractions E1, E2 and E5 showed significant anti-fibrotic activity. The detailed composition of these fractions is currently under investigation **12** Also miRNA patterns (Top 5 per fraction shown) change over the CIM QA elution peak, correlations with bioassay data etc., are under investigation.

Conclusions

- EVscale™ sets the stage for efficient and scalable EV manufacturing from different cell lines (MSC/TERT) which will be straight forward to translate into GMP manufacturing
- Already from 250 mL bioreactors we can produce EV quantities meaningful for clinical studies
- These MSC/TERT EV display functional biological activities in cell-based bioassays
- Preparative and analytical chromatographic separation delivers fractions with different protein-to-particle ratio, different EV marker protein and miRNA patterns as well as biological activities
- With EVscale™ we present an excellent platform approach for producing clinical-grade EV and the approach for defining therapeutic EV product candidates for various conditions

Thanks to our collaboration partners and funding agencies