

Towards Clinical-grade Stem Cell-derived Extracellular Vesicles

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Stem Cell Community Day 2023
Leiden (NL)
November 21, 2023



Das Forschungszentrum in Kooperation mit der AUVA



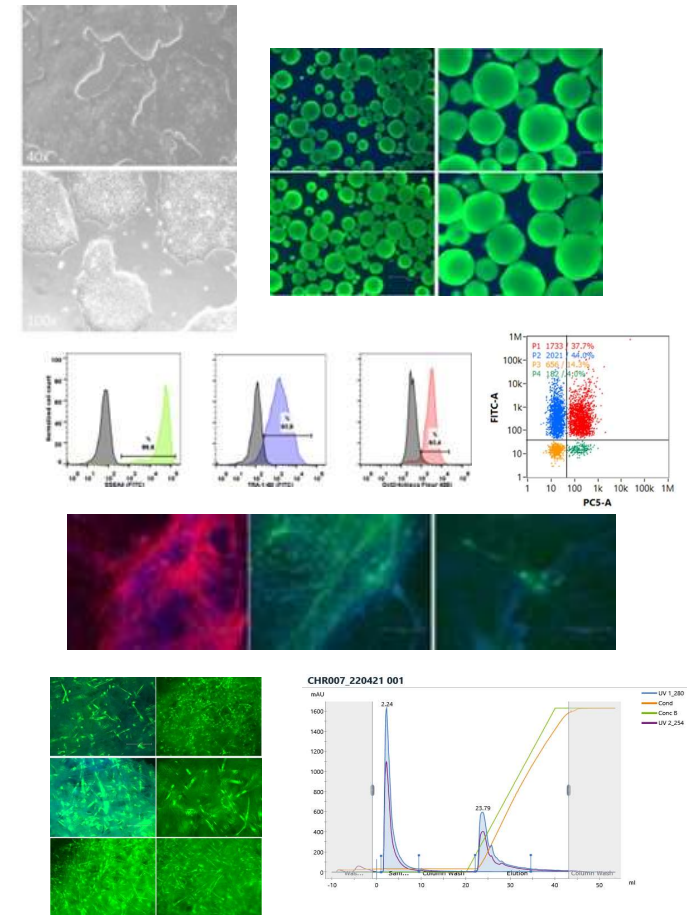
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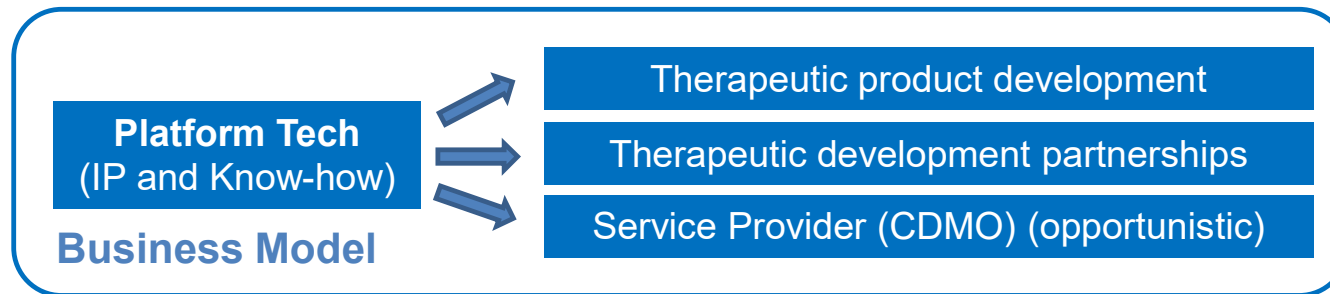
At a Glance



- Phoenestra was founded to bridge the gap between science and clinical translation
- We develop platform technologies for
 - Urine cell-derived iPS cell lines and cell banking
 - Bioreactor-based stem cell expansion (iPSC, MSC/TERT)
 - Cells and cell-derived vesicles as the product
 - Productive and scalable isolation and purification processes (DSP)
 - Analytical characterization and control methods
- EV-related Products and Services
 - EV deliveries from 5×10^9 to over 1×10^{13} EV from one batch
 - From MSC/TERT cell lines or iPSC
 - Process development and optimization
 - Analytical characterization network
- In-house GMP resources in preparation



Our strategy towards harvesting the promises of Cell-based Therapies



- iPS cell line generation and engineering
- Cell banking
- Stem Cell expansion (scalable bioreactor systems)
- EV manufacturing (scalable, consistent)
- Process development and analytics
- GMP services from 2024

Our Next-generation EV Manufacturing Approach

From R&D to GMP



Stable MSC lines

- Compliant sourcing
- Immortalized (virus-free) and stable
- Xeno-free

Scalable Processing

- Cell Banking
- Cell expansion
- (Continuous) EV harvesting
- EV purification and formulation
- GMP supplies

Quality Control Characterization

- Biophysical
- Biochemical
- Biological (mode-of-action)

Composition – Function Relationships

Biomarkers

Cell lines / Vesicles

- Cell line engineering
- EV engineering
- Cargo



Source: www.eppendorf.com



Extracellular Vesicles (EV) from Different Telomerized MSC Lines



Stable, telomerized MSC lines (MSC/TERT)* – fully documented - GMP ready – Cell banking

*licensed from Evercyte

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

Cryovial

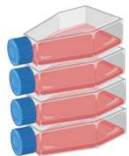
Seed train

Fully controlled bioreactor-based expansion

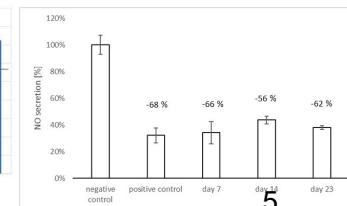
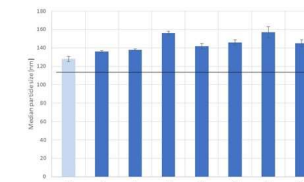
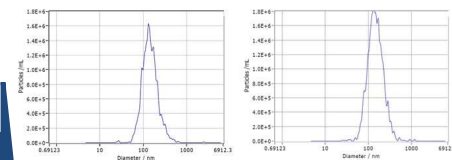
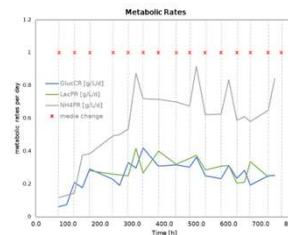
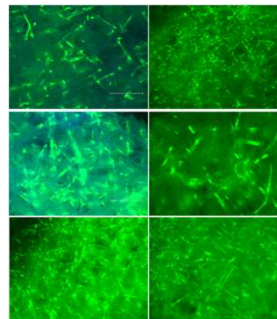
High yield of functional EV



© Nalgene Thermo
Fisher Scientific



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Platform Processes for Consistent and Scalable MSC-EV Supplies (Available: NOW)



Telomerized MSC lines*

*licensed from Evercyte



Inoculum preparation
Seed train

Bioreactor
cultivation
(Perfusion)

Exosome
Harvest

TFF

Further DSP

From R&D Bulk via Process Development to GMP Supplies*

*In-house GMP cell banking and manufacturing from 2024

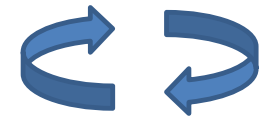
R&D

cGMP

Bulk EV quantities
up to 1×10^{13} EV
from one 250 mL
batch

Consistent pre-clinical
and clinical supplies

Process definition and
transfer to GMP
manufacturing



Product definition and
development of scalable
processes

Stable GMP-grade cell
lines (MSC/TERT)

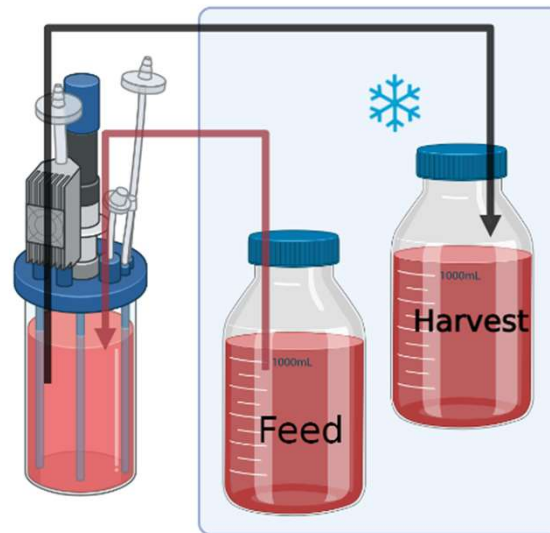
QbD CMC Development Concept

MSC/TERT Expansion in a Proven, Proprietary, Well-controlled bioreactor setup

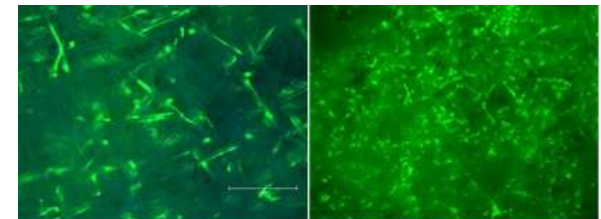
- ✓ Packed-bed repeated batch or perfusion mode, proprietary setup (patent pending)
- ✓ MSC or EV/exosomes as the product
- ✓ Easy transition into GMP manufacturing



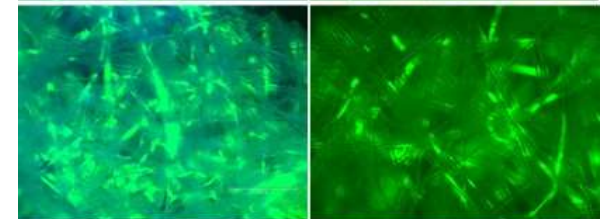
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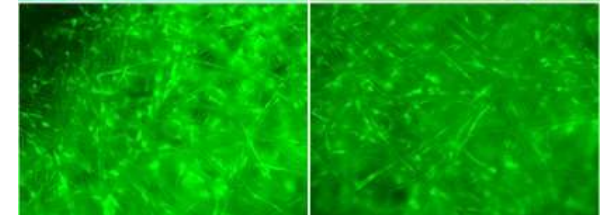
day 2



day 4

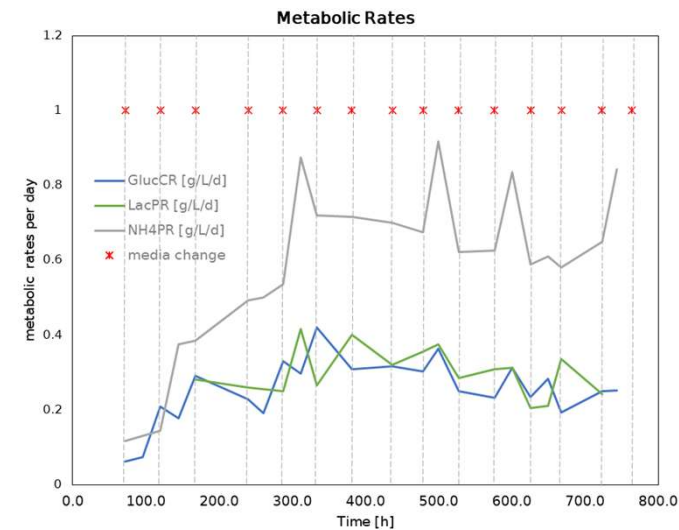
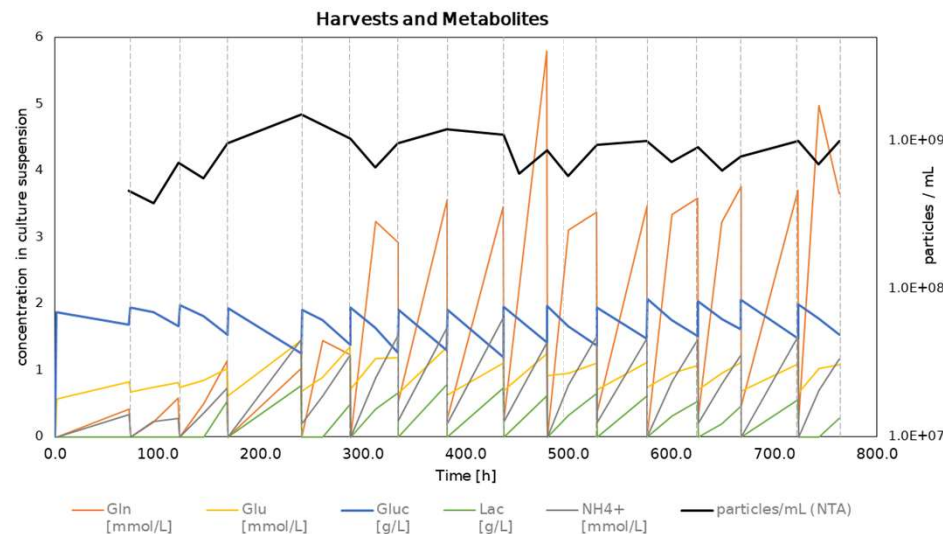


day 7



Live cells (green) and dead cells (blue) on cell carrier

Case Study 1: Long-term Bioreactor Cultivation of WJ*-MSC/TERT and Harvest of EV from Conditioned Media

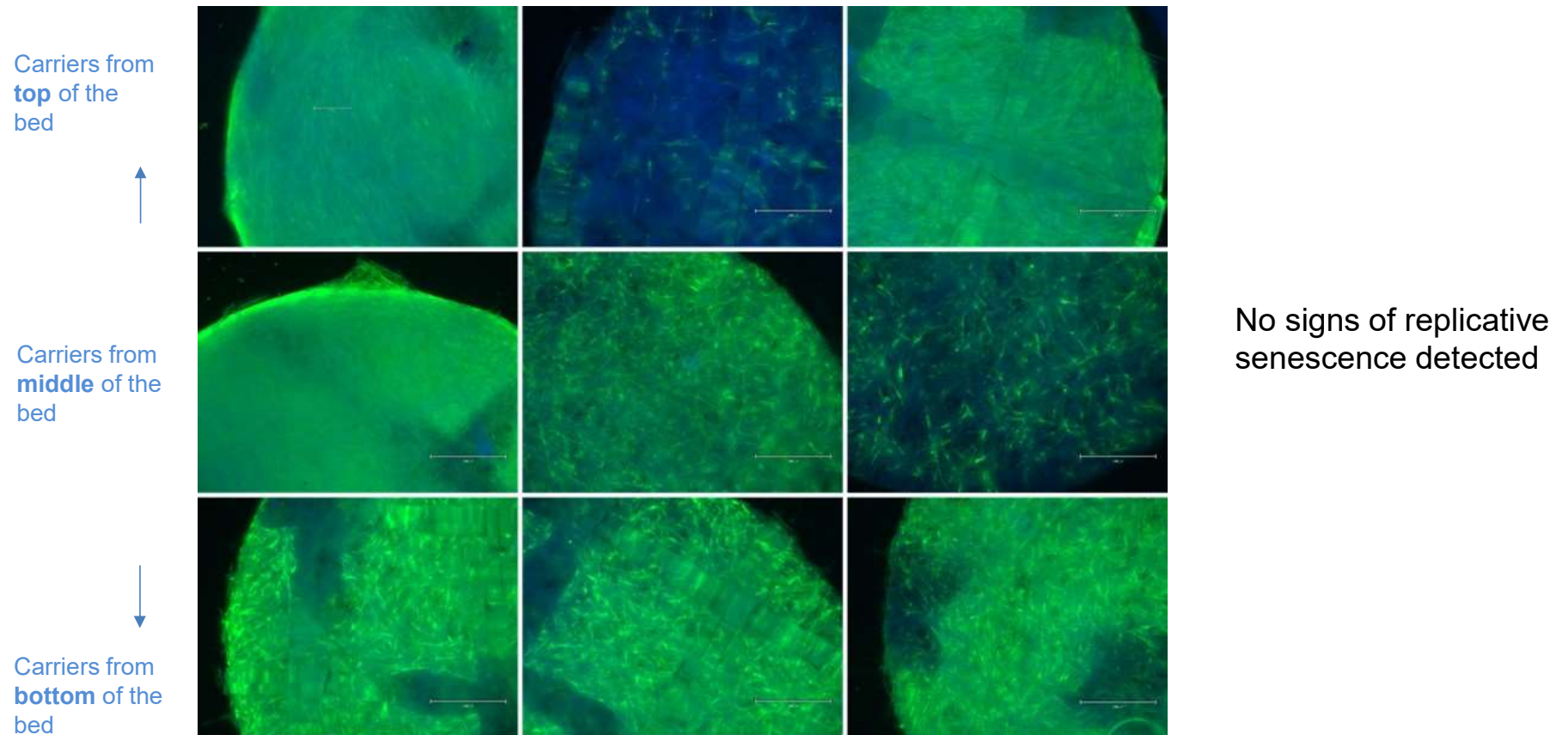


* Wharton's Jelly

CR...consumption rate
PR...production rate

- 15 harvests (repeated-batch) over **32 day-run time**
- Particle numbers by NTA
- Stable process controls, high cell viability until the end of production
- MSC marker profile for seed and at the end of cultivation (d 32) confirmed
- Very consistent performance of WJ-MSC/TERT over 32 d in our repeated-batch setup

Microscopic Evaluation of the Packed Bed at the End of Cultivation (WJ-MSC/TERT)



Live cell staining (Calcein) of carriers from different positions inside the packed bed **after 32 days** of cultivation. WJ-MSC are well attached and highly viable. Cell density differences are most likely generated during seeding, optimizations ongoing.

Harvest Data for a Bioreactor-controlled WJ-MSC/TERT-EV Manufacturing Run



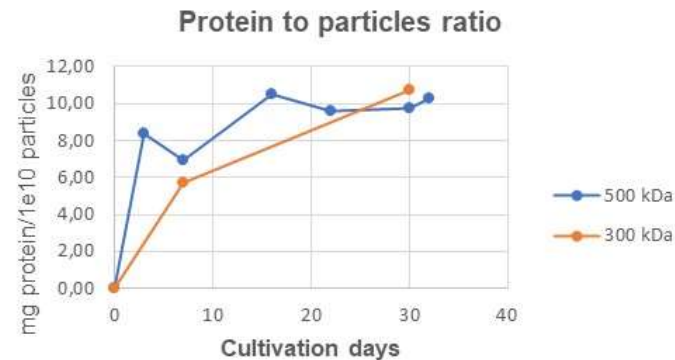
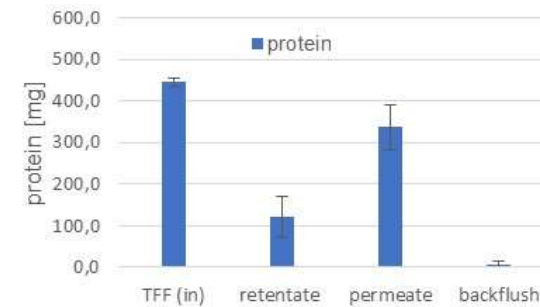
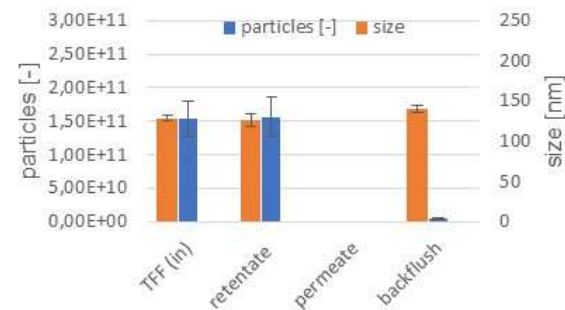
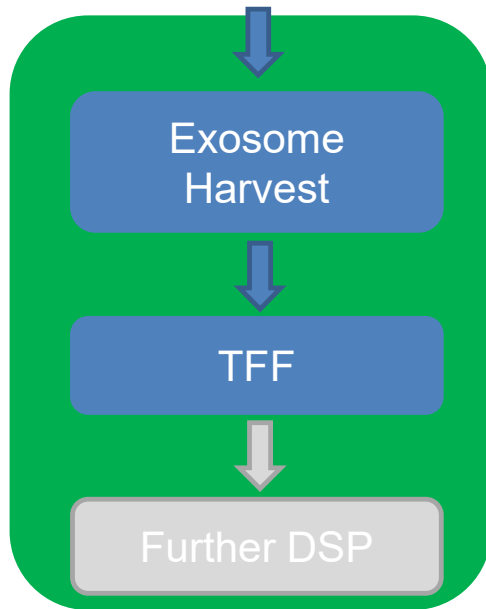
Harvest	Time [h]	Particles/mL	Volume [mL]	Total Particles
USP23-0040	71.75	4.60E+08	195	8.97E+10
USP23-0042	120.0833	7.07E+08	177	1.25E+11
USP23-0044	167.75	9.67E+08	184	1.78E+11
USP23-0045	239.75	1.50E+09	190	2.85E+11
USP23-0046	287.25	1.03E+09	180	1.86E+11
USP23-0048	333.6667	9.57E+08	180	1.72E+11
USP23-0049	381.5	1.20E+09	185	2.22E+11
USP23-0050	436.5833	1.10E+09	190	2.09E+11
USP23-0052	478.75	8.60E+08	178	1.53E+11
USP23-0054	527.25	9.40E+08	182	1.71E+11
USP23-0055	576.0833	1.00E+09	194	1.94E+11
USP23-0058	625.8333	9.07E+08	182	1.65E+11
USP23-0060	667.5833	7.87E+08	175	1.38E+11
USP23-0061	723.3333	9.97E+08	187	1.86E+11
USP23-0063	763.5833	1.00E+09	167	1.67E+11
		<i>estimated</i>	Total:	<u>2.64E+12</u>

**15 harvests from 32 process days
(repeated batch mode)**

2746 mL harvest volume in total from a 250 mL bioreactor

which resulted in **2.6 · 10¹² particles in total** (measured by NTA)

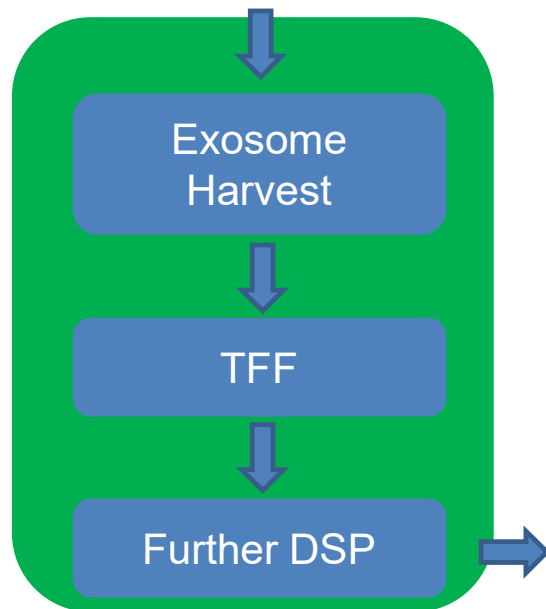
Tangential Flow Filtration is a Proven Method for Purifying and Concentrating Extracellular Vesicles (EV)



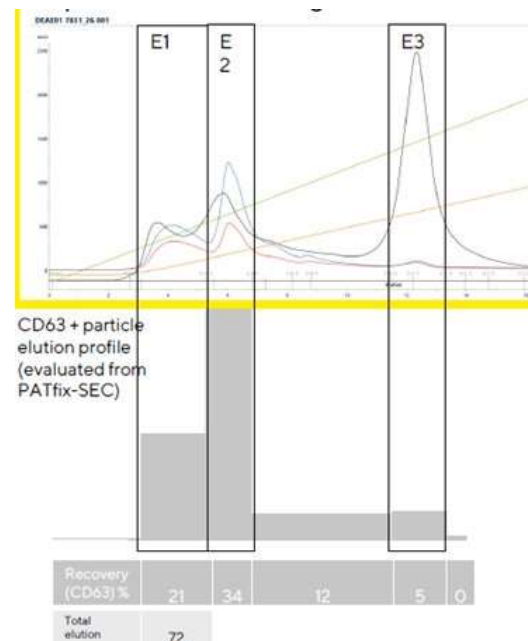
- TFF removes proteins, nucleic acids and media components (membrane cut-off dependent)
- The protein-to-particle ratio increases in the first two weeks of cultivation and remains constant afterwards
- Processing step yields are good to excellent

Step	Protein Yield [%]	Protein Recovery [%]	Particle Yield [%]	Particle Recovery [%]
Filtration	98	102	78	83
TFF	27	105	102	105

Different Chromatographic Methods to Further Separate and Characterize EV Preparations are under Evaluation*



- Screening of different separation principles
- Analysis of composition(s) displaying biological activities
- Options for further definition and purification of EV preparations as useful and needed



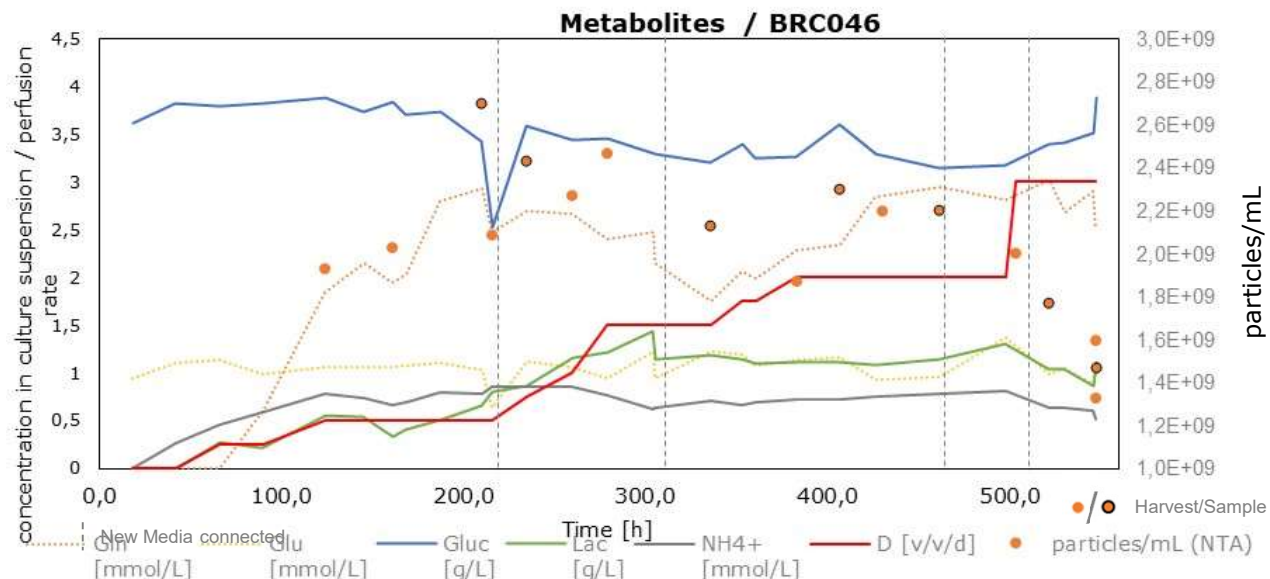
Example Anion-Exchange Chromatography: Preparative separation is followed by UV (280/260), fluorescence (FLD) and Light Scattering (MALS, particle conc and size distribution)

Analytical immune-fluorescence chromatography is used to quantify EV marker presence in different fractions or fraction pools (CD63 shown in this example)

* In collaboration with Sartorius BIA Separations

Case Study 2: Long-term Bioreactor Cultivation of CP*-MSC/TERT and Harvest of EV from Conditioned Media

* Chorionic Plate (Placenta)

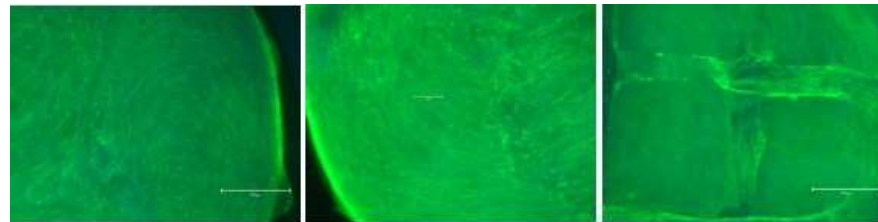


Perfusion rate adapted to keep steady
glucose and lactate levels over time

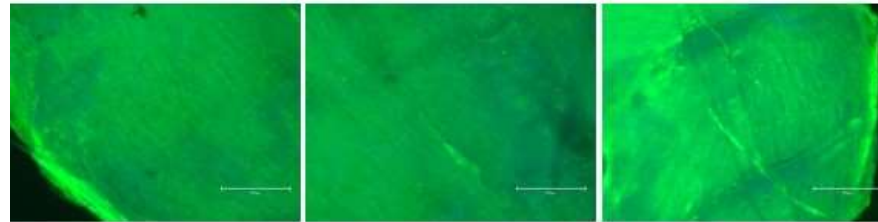
- Particle concentrations (culture supernatant) in $1.5 - 2.5 \times 10^9/\text{mL}$ range
- Concentration increase in first phase
- Decrease of particle numbers by high perfusion rate towards the end
- Excellent performance of proprietary perfusion setup

Microscopic Evaluation of the Packed Bed at the End of Cultivation (CP-MSC/TERT)

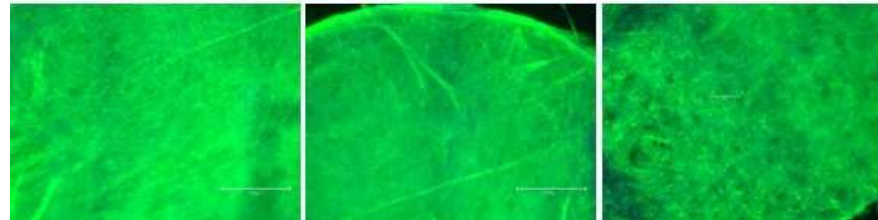
Carriers from
top section
of the bed



Carriers from
middle section
of the bed



Carriers from
bottom section
of the bed



No signs of replicative
senescence detected

Live/Dead cell staining (Calcein/DAPI) of carriers from different positions inside the packed bed **after 23 days** of cultivation. CP-MSC/TERT are well attached and highly viable

Harvest Data for a Bioreactor-controlled CP-MSC/TERT-EV Manufacturing Run



Timepoint	Harvest	Volume	particles/mL	total particles
BRC046.1.05-124h	harvest 1 (day 5)	150 mL	1.93E+09	2.90E+11
BRC046.1.07-161h	harvest 2 (day 7)	210 mL	2.03E+09	4.26E+11
BRC046.1.11-216h	harvest 3 (day 9)	230 mL	2.09E+09	4.81E+11
BRC046.1.12-235h	harvest 4 (day 11)	330 mL	2.27E+09	7.49E+11
BRC046.1.14-279h	harvest 5 (day 12)	188 mL	2.47E+09	4.64E+11
BRC046.1.15-304h	harvest 6 (day 13)	380 mL	1.93E+09	7.33E+11
BRC046.1.17-335h	harvest 7 (day 14)	480 mL	1.70E+09	8.16E+11
BRC046.1.20-383h	harvest 8 (day 16)	820 mL	1.87E+09	1.53E+12
BRC046.1.23-430h	harvest 9 (day 18)	1000 mL	2.20E+09	2.20E+12
BRC046.1.26-503h	harvest 10 (day 21)	1485 mL	2.00E+09	2.97E+12
BRC046.1.30-548h	harvest 11 (day 23)	1400 mL	1.60E+09	2.24E+12
BRC046.1.30-548h	harvest 11 from BR (day 23)	200 mL	1.33E+09	2.66E+11
		6873 mL		1.3E+13

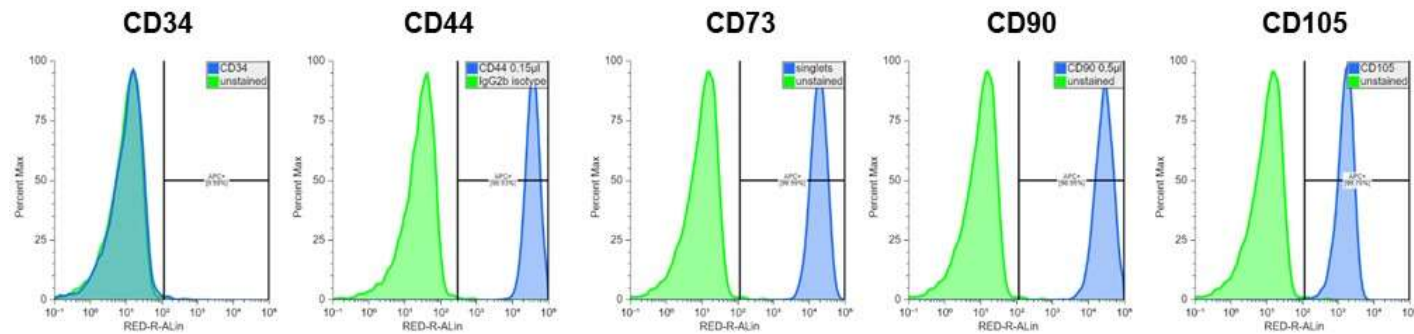
**11 harvests from 23 process days
(perfusion mode)**

**6.8 L harvest volume in total from a 250 mL
bioreactor**

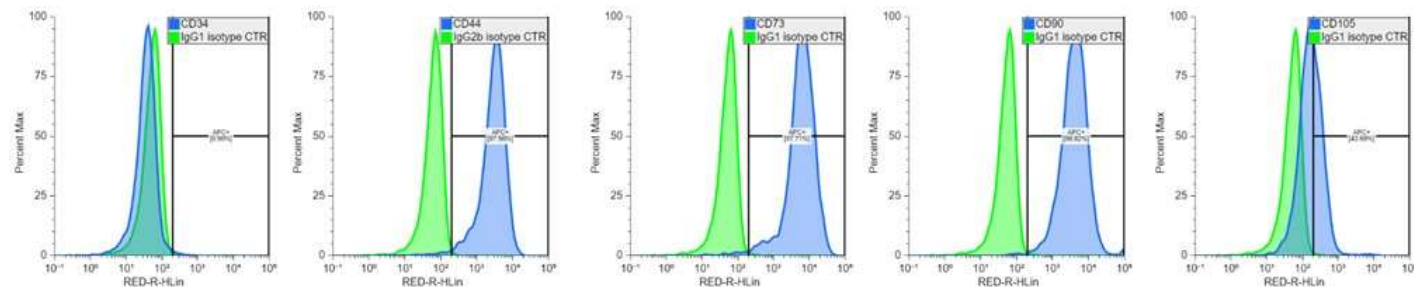
which resulted in **$1.3 \cdot 10^{13}$ particles in total**
(measured by NTA)

Expression of MSC Biomarkers is Monitored Over the Cultivation Period

Top
MSC marker expression at
seed / start of bioreactor

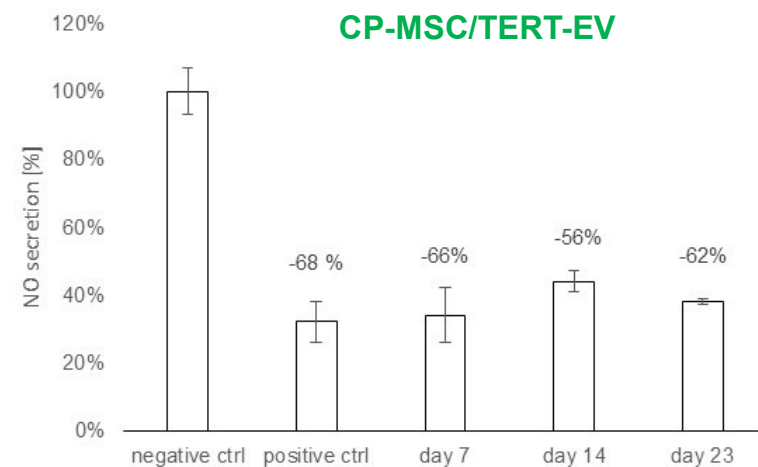
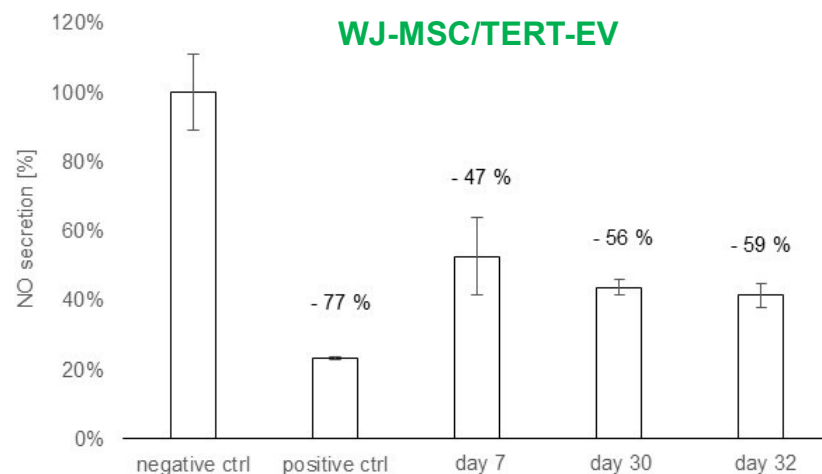


Bottom
MSC marker expression at
harvest of bioreactor after
23 days



Biological Activity of Bioreactor-produced MSC/TERT-EV Processed by TFF

Extracellular Vesicles harvested over the course of a 32-day or 23-day process, respectively, display anti-inflammatory activity in a cell-based bioassay* * In collaboration with Evercyte



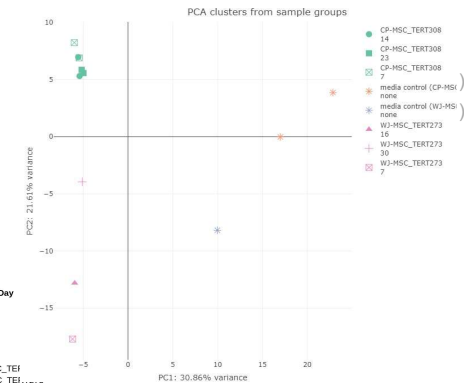
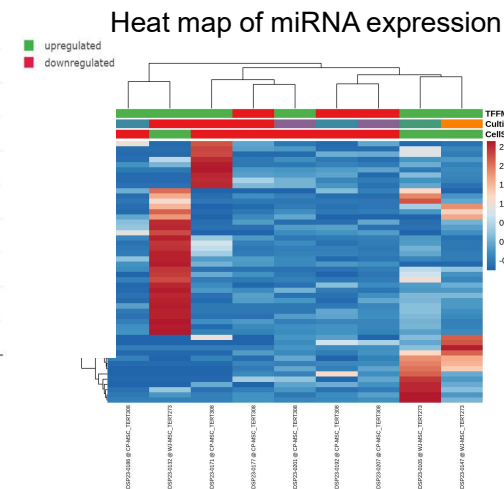
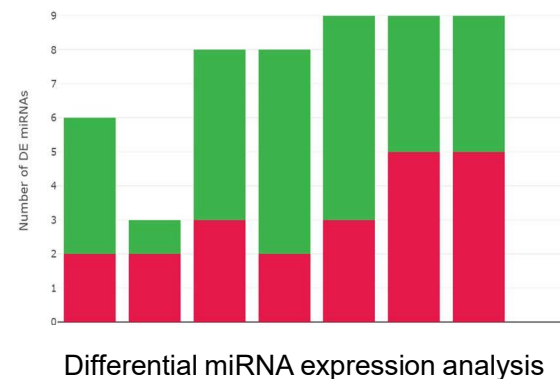
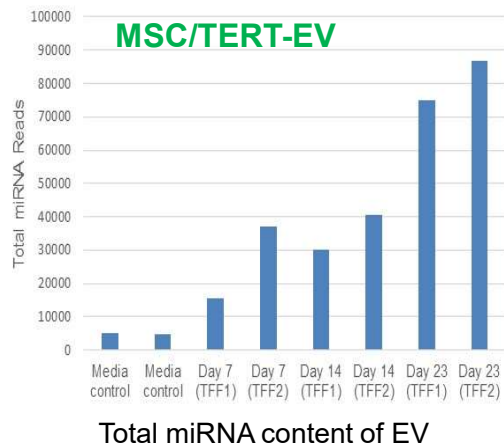
Anti-inflammatory Activity: the alteration of NO secretion of inflammation-triggered (LPS) macrophages when exposed to Dexamethasone (positive control) and respective EV preparations (TFF retentates) of different harvests throughout a 32-day-cultivation (left) and a 23-day cultivation is shown. The negative control is TFF buffer solution.

RNA Profiling by NGS Delivers an Important Edge for EV Characterization

A small RNA preps are being performed from selected EV samples and are analyzed by Next Generation Sequencing (NGS)*

* In collaboration with TAmiRNA

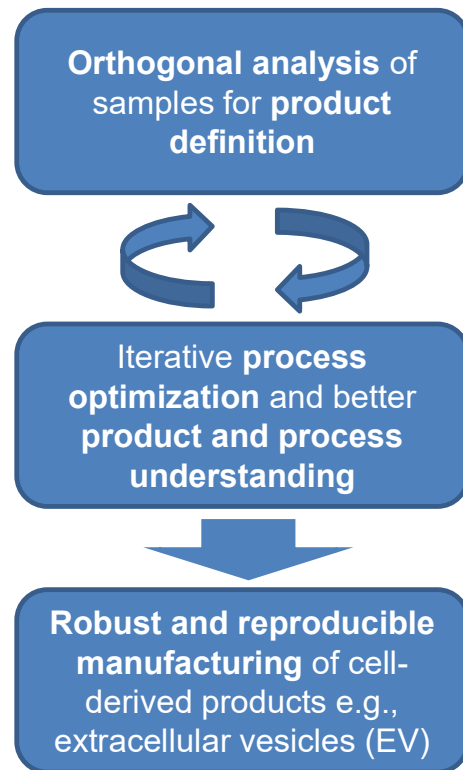
- Total number of reads per small RNA (several hundred miRNAs detected, strong increase over time)
- Detailed analysis ongoing e.g., correlations on a single miR level with functional bioassay data (anti-inflammatory, anti-fibrotic, pro-angiogenic, and others)



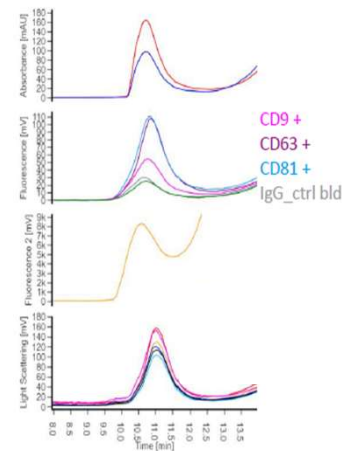
Principal component analysis (PCA)

Orthogonal Analytical Methods are ...

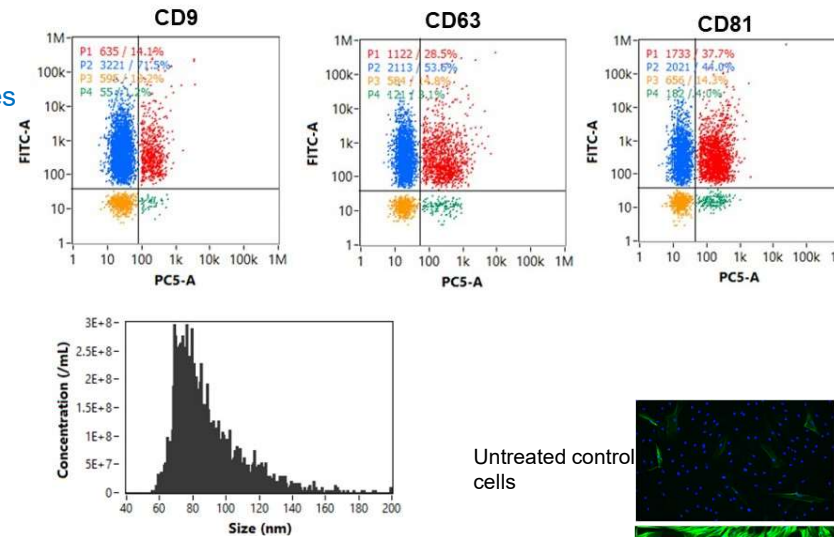
... key for EV-based product definition and translation into clinical successes



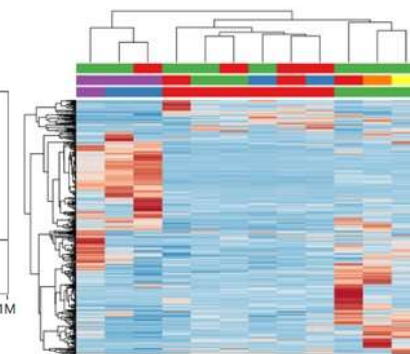
Chromatographic separation of stem cell-derived exosomes with multiple detectors



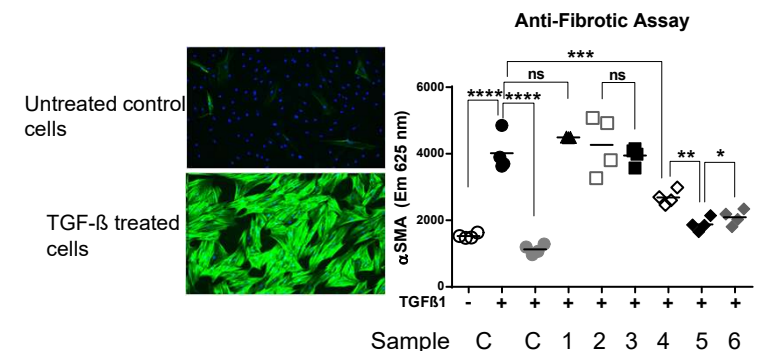
In collaboration with Sartorius BIA Separations



EV analysis by flow cytometry (NanoFCM) (particle number and size distribution (above) as well as EV marker distribution (top row))
In collaboration with NanoFCM and JKU (Linz, AT) core facilities



In collaboration with TAmiRNA



Exosome preparations produced by Phoenestra reduce fibrosis marker expression *in vitro*

In collaboration with Evercyte GmbH

Conclusions



- Phoenestra has developed an EV manufacturing setup which overcomes many of the shortcomings of other EV manufacturing systems based on
 - Stable, telomerized cell lines (MSC/TERT)
 - Cell banking for consistent starting materials
 - Well-controlled perfusion bioreactor processes for consistent and scalable EV supplies
 - Arsenal of orthogonal analytical methods to better define biological activity
 - Downstream processing methods as needed
- Clinically meaningful exosome quantities can be produced from small bioreactor sizes in one bioreactor run within weeks!

Hypothetical calculation of clinical doses from a real CP-MSC/TERT run

Particles	1.30E+13
60% EVs	7.80E+12
50% DSP recovery	3.90E+12
Clinical doses (1x10¹⁰ EV/dose)	390
Clinical doses (5x10¹¹ EV/dose)*	26

* ExoFlo™ dosing in successful Ph II ARDS trial

Direct Biologics (Press Release: [Direct Biologics Announces Publication of Significant Survival Benefit with ExoFlo™ in its Phase 2 Randomized Controlled Clinical Trial in the Journal CHEST | Direct Biologics](#))