ReNeuron

PROTEOMIC PROFILING OF STEM CELL-DERIVED EXTRACELLULAR VESICLES AND THEIR BIOLOGICAL FUNCTION

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Vice President of Research

SCIENCE & INNOVATION – State of the Art in EV (Exosome) Research: Rigor and Function
Thursday 5th May 2022, 08:45 Plenary hall

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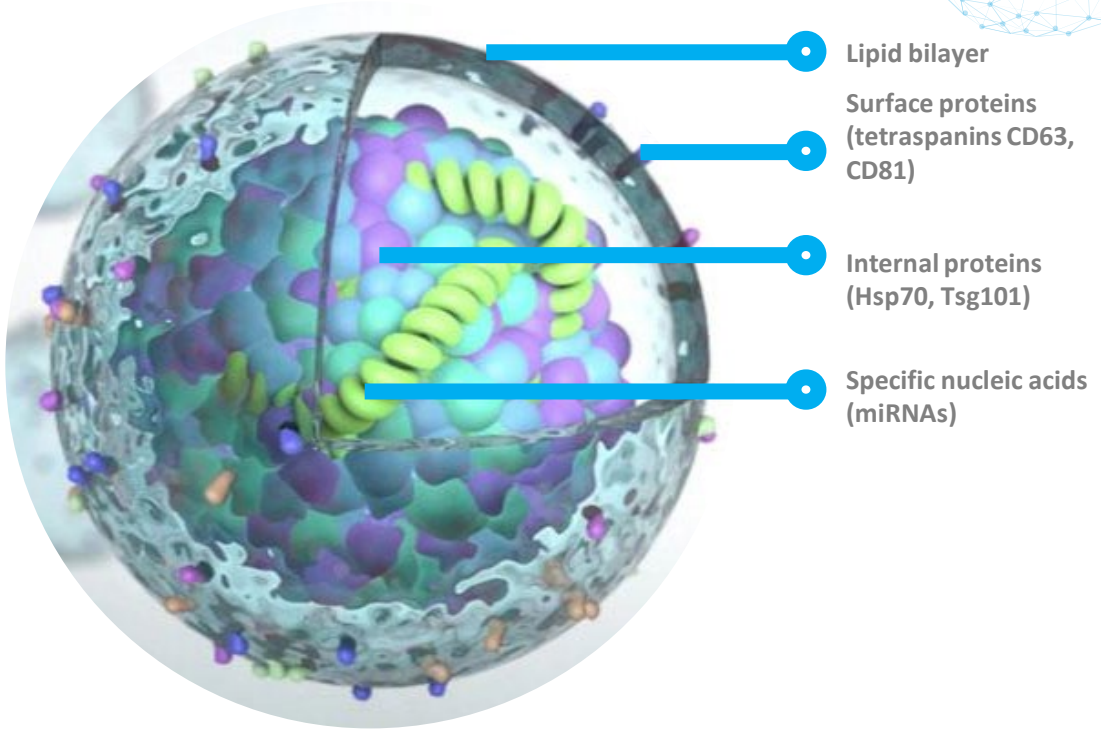
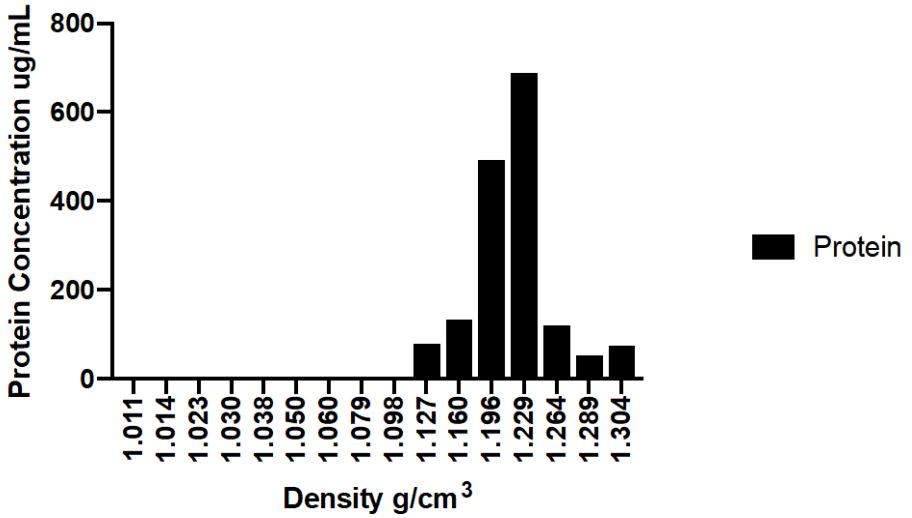
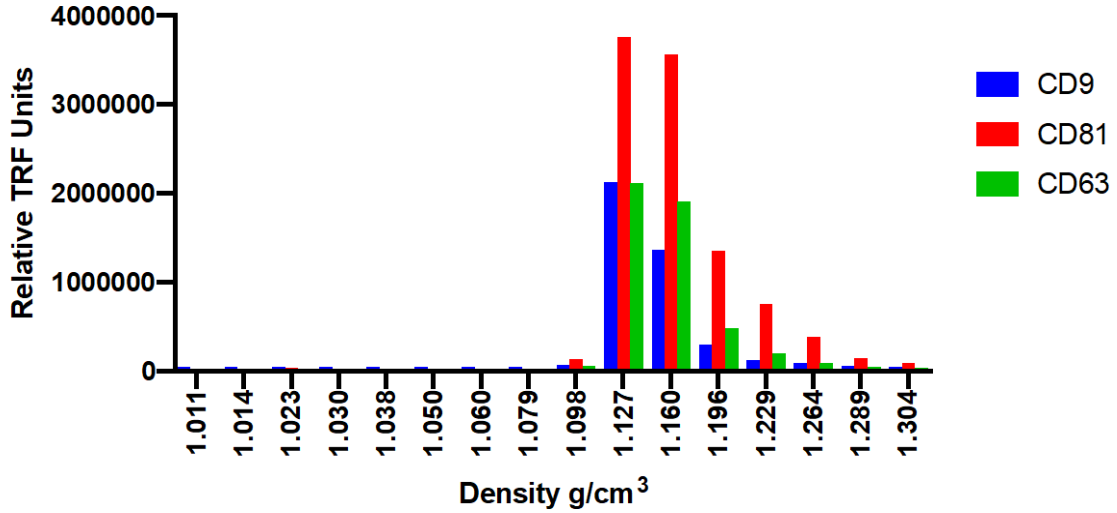
EXTRACELLULAR VESICLES: A TARGETED DELIVERY PLATFORM

- Naturally occurring, nanoparticles released by all cell types in a functionally relevant manner as a means of intercellular communication
- Proven ability to carry a range of biologically active cargos including nucleic acids and proteins
- Target recipient cells via specific surface proteins that are determined by their cell of origin
- Increasing interest across the industry in extracellular vesicles as biomarkers, standalone therapeutics and as delivery vectors for complex drug modalities



ISOLATION BY ULTRACENTRIFUGATION – GOLD STANDARD

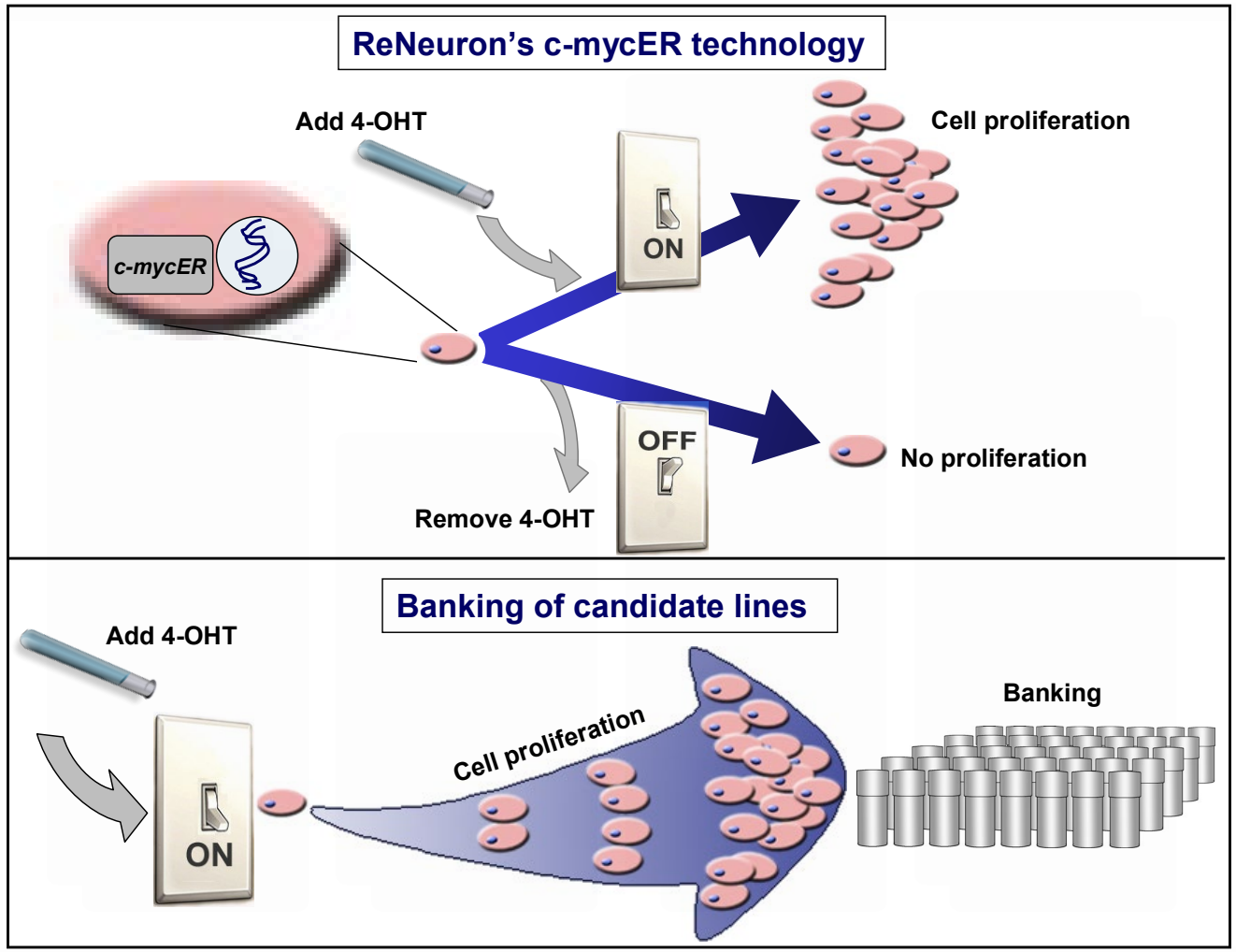




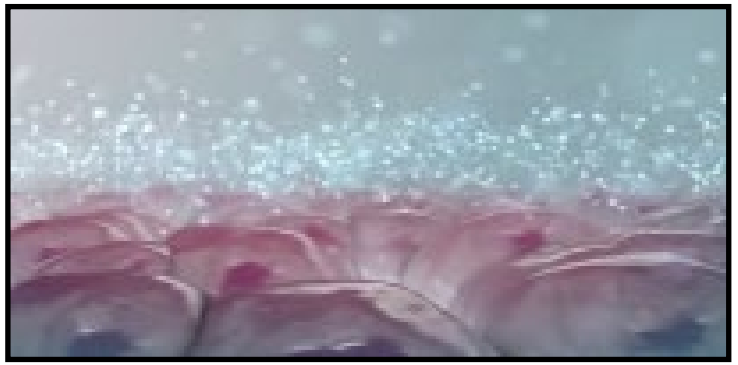
Density gradient separation

- EVs were separated along a continuous sucrose gradient as previously described (Raposo et al., 1996).
- ultracentrifuged at 8,000x*g* for 1 hr, 67,000x*g* for 1 hr, 200.000x*g* for 16hr, 67,000x*g* for 1hr, 8,000x*g* for
- 16 fractions were carefully taken from the top of the gradient until no liquid remained in the tube.
- Fractions were labelled 1-16 with fraction 1 being the first fraction taken and therefore the lightest.
- The refractive index of collected fractions was measured at 20°C and from this the density was calculated based upon known data.
- Washed at 200,000x*g* in a Beckman TLA-100 rotor in an Optima-Max ultracentrifuge for 1hr and resuspended in 30uL PBS

CONSISTENT AND SCALABLE EV PRODUCTION: CONDITIONAL IMMORTALISATION



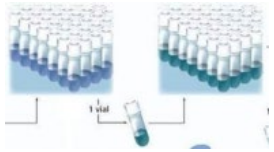
- Stable producer cell line - Consistent phenotype maintained over multiple passages
- Fully qualified xeno-free GMP process – tightly controlled USP with strict release criteria
- Scalability - produced to a commercially relevant scale in multi-tier tissue culture flasks or bioreactors
- Stable exosome product at 4'C, -80'C
- Safe: No c-MycERTAM within exosomes



EVs harvested from CTX producer cells

4-OHT = 4-hydroxy tamoxifen

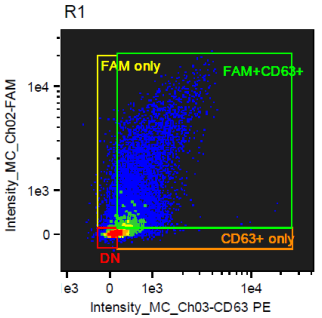
SCALABLE MANUFACTURING PROCESS



MCB / WCB

Conditioned media

Tangential Flow Filtration (TFF)



UPSTREAM PROCESSING

DOWNSTREAM PROCESSING

CELL BANKING

- Native CTX / Engineered
- Cryopreservation & cryostorage
- Master Cell Bank (MCB)
- Working Cell Bank (WCB)

EXOSOME PRODUCTION

- 2D adherent
- Flask based
- Collection of conditioned media

EXOSOME PURIFICATION & CONCENTRATION

- Centrifugation
- Tangential flow filtration (TFF)
- Buffer exchange
- Size exclusion
- IEX
- Affinity

FILL/FINISH

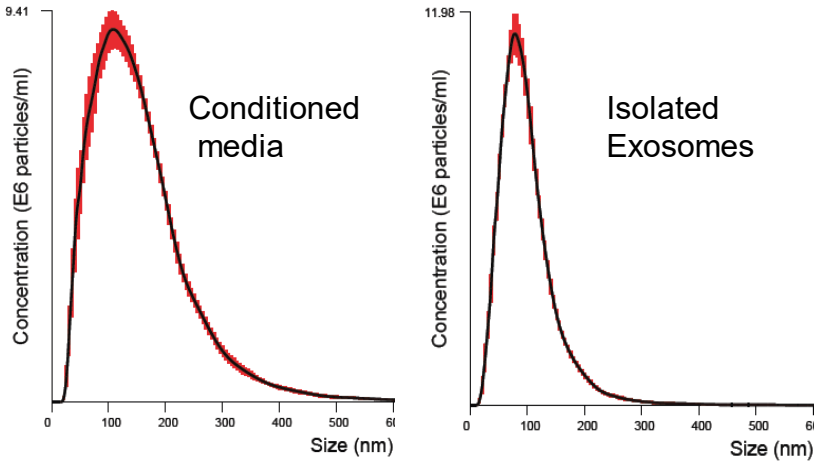
- Sterile dispense

ANALYTICS

- Protein concentration
- Particle concentration
- Sterility
- Mycoplasma
- Endotoxin
- HCP
- **Advanced analytics**

STABLE AND CONSISTENT PRODUCT

Nanoparticle Tracking Analysis (NTA):

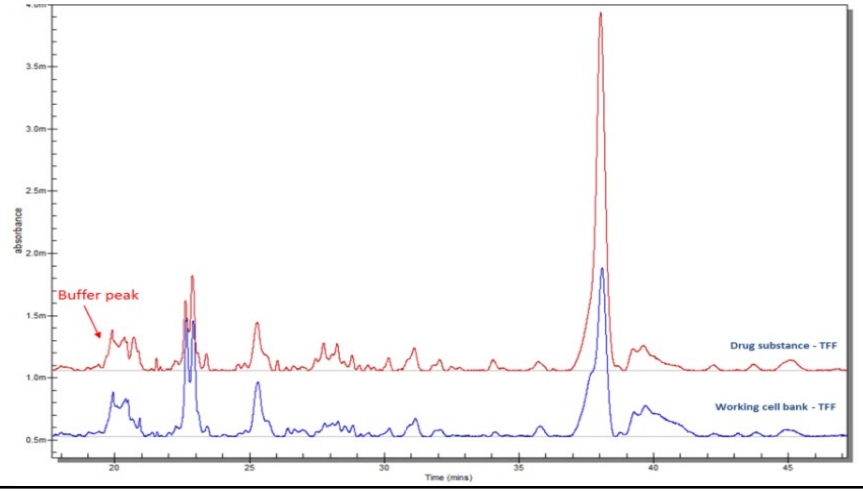


Next-Generation miRNA Sequencing (NGS):

	Batch 1	Batch 2	Batch 3	Batch 4
hsa-miR-A	1	2	1	1
hsa-miR-B	2	1	3	3
hsa-miR-C	3	3	4	4
hsa-miR-D	4	5	2	2
hsa-miR-E	5	7	6	7
hsa-miR-F	6	6	5	5
hsa-miR-G	7	12	9	10
hsa-miR-H	8	8	8	8
hsa-miR-I	9	11	12	15

Characteristic	Assay	Test	Specification
Purity			
Vesicle no. and Size distribution	**Established**	NTA (30-200nm)	Mode particle size 100±25nm
Protein content	**Established**	A280	10^8 vesicles/µg protein
Identity			
Surface markers	**Established**	ELISA (CD63, 81, 9)	CD81>CD9>CD63 Other specific surface markers
miRNA profile	NGS (**Established**) QPCR modification (in development)	PCR	Presence of specific miRNA
Proteomic fingerprint	**Established**	Capillary Electrophoresis (AUC)	Peak 1 – 10 ± 2% Peak 2 – 10 ± 2% Peak 5 - 19 ± 4%
FIO			
Visualisation	**Established**	TEM	Particle size 20-250nm

Proteomic Fingerprint - Capillary Electrophoresis (CE):



ReNeuron

BIOLOGICAL FUNCTION

EV FUNCTION ACQUIRED FROM THEIR SPECIFIC CARGO

Physiological

- Immune response (Raposo et al., 1996)
- Proliferation and migration (Hakulinen et al., 2008)
- Cell-to-cell communication (Qin et al., 2016)

Pathophysiological

- Cancer metastasis and disease progression (Skog et al., 2008)
  - Growth factors, Wnt signalling proteins and cytokines
- Neurodegeneration (Rajendran et al., 2006)
  - Transfer of neuropathological proteins
- Cardiovascular disease (Yu et al., 2012)
  - Cytokine release and cell dysfunction

RNA CARGO – miRNA IN LOW ABUNDANCE

PNAS

Quantitative and stoichiometric analysis of the microRNA content of exosomes

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- miRNA molecules per EV number
- 5 sources
- Less than one molecule per EV for most abundant

PLOS GENETICS

- EVs with and without viral miRNA
- Small fraction of EVs carried miRNA
- Minimal fusion – little/ no delivery

RESEARCH ARTICLE

MicroRNAs are minor constituents of extracellular vesicles that are rarely delivered to target cells

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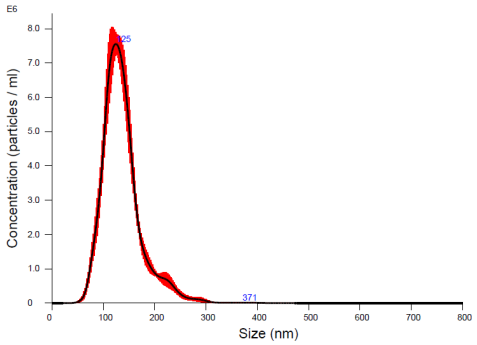
Check for updates

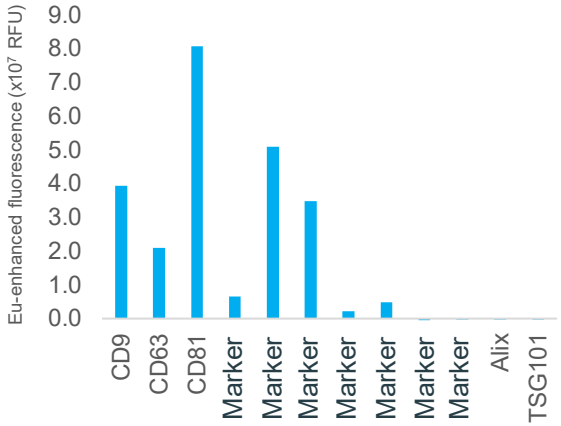
FOCUS ON PROTEOMICS - DISCRETE MEMBRANE PROTEINS

4 different stem cell line – 4 different surface marker profiles

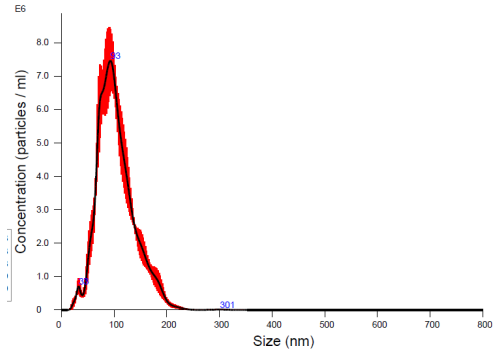
Nanotracking Analysis



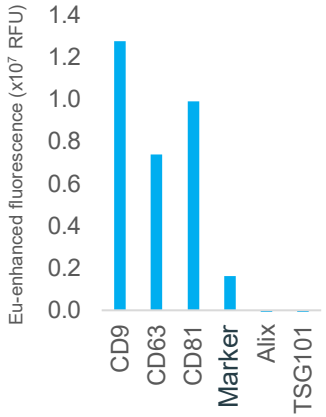
ELISA surface marker profile

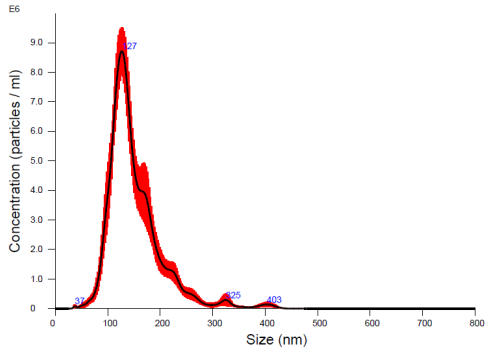


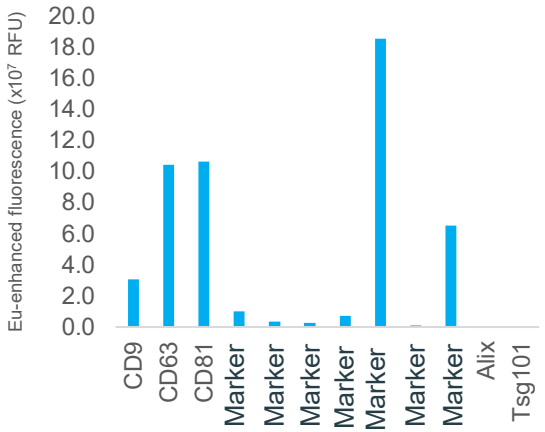
Nanotracking Analysis

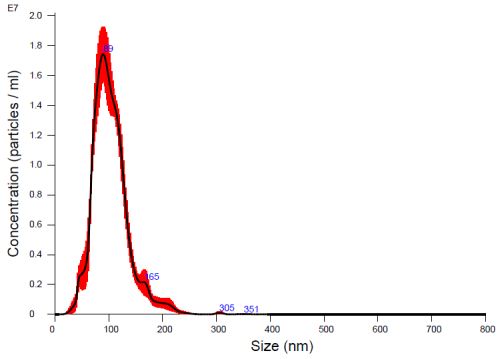


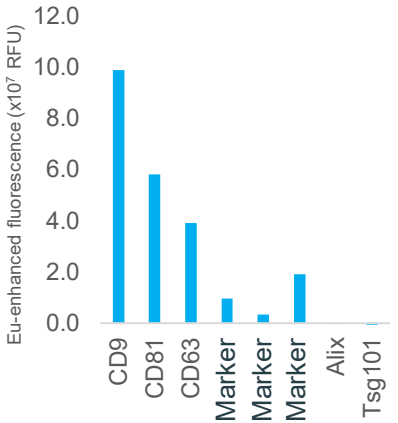
ELISA surface marker profile



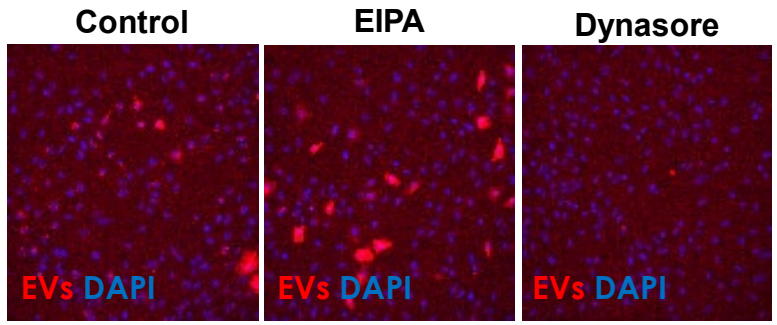


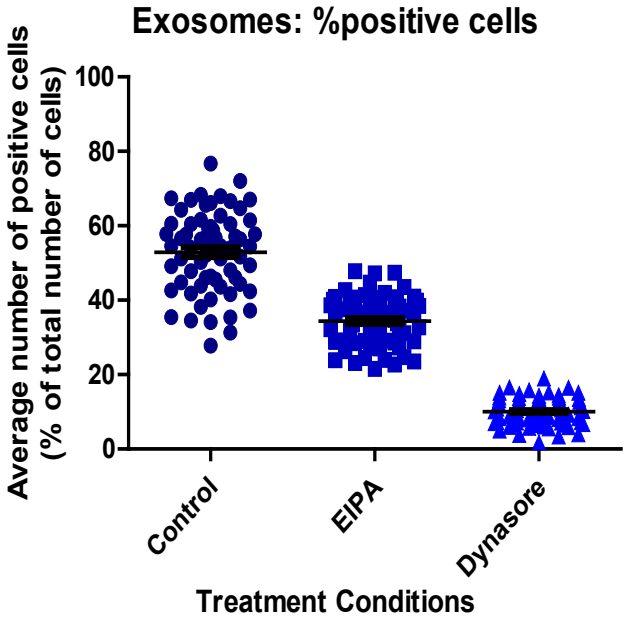


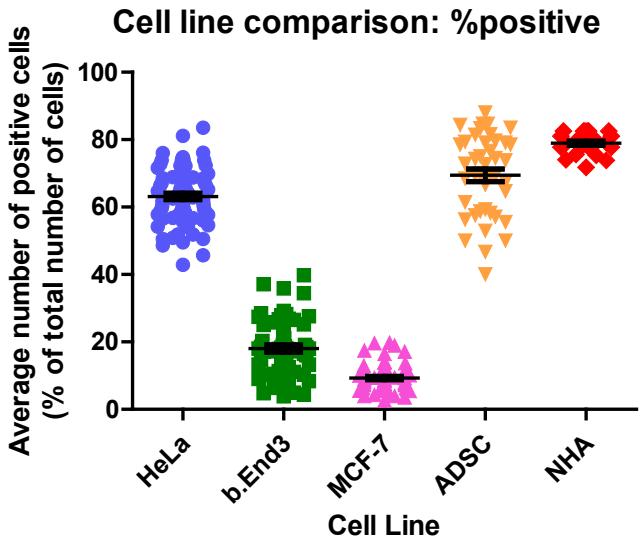


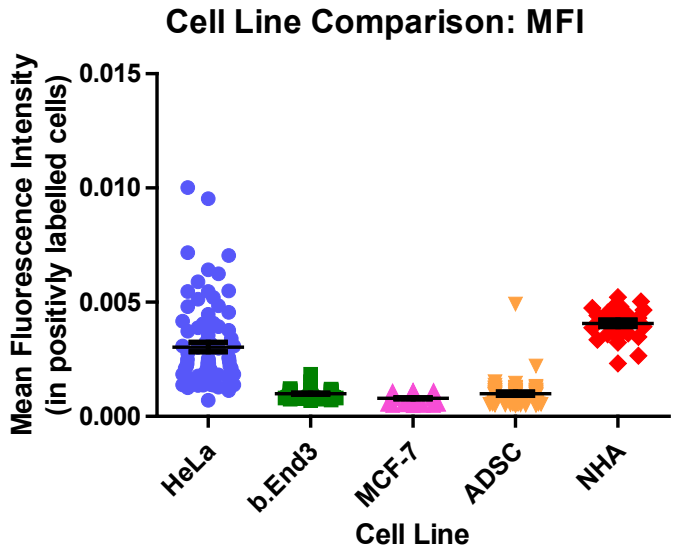


CTX DERIVED EVs ARE PREDOMINANTLY TAKEN UP BY CLATHRIN MEDIATED ENDOCYTOSIS BY SPECIFIC CELL TYPES





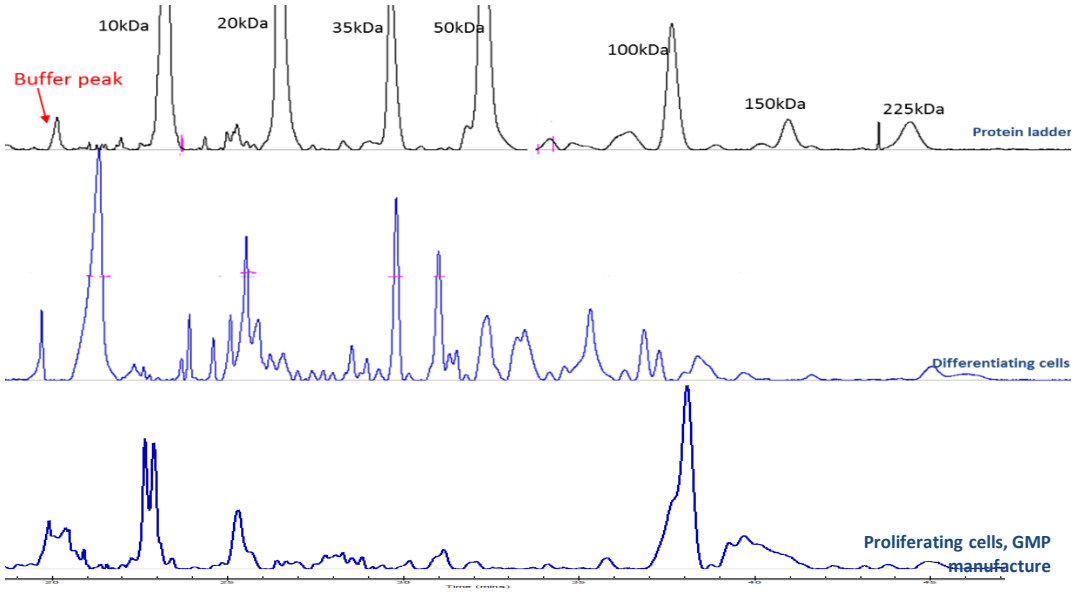


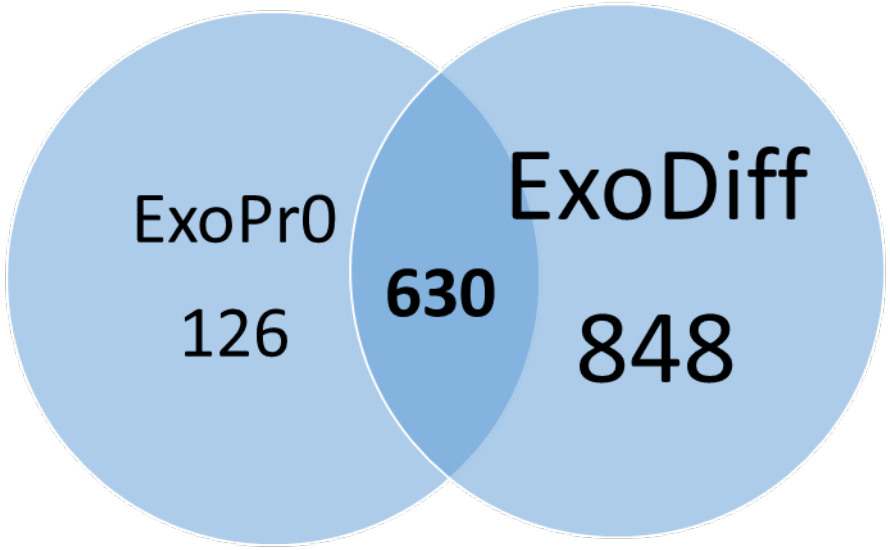


Aim: To determine whether CTX-derived EVs are preferentially taken up by specific cell types.

From a panel of 5 different cell types, CTX-derived EVs are is taken up by the cells in the following order; Normal human astrocytes (NHA; ~85%), Adipose-derived stem cells (ADSC; ~75%), HeLa (~65%), b.End3 (endothelial cells; 18%) and MCF-7 (breast cancer; ~10%).

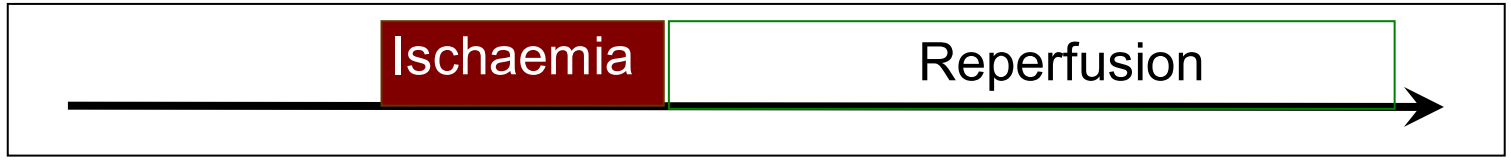
CHANGES IN UPSTREAM PROCESSING DRAMATICALLY ALTERS THE PROTEOMIC PROFILE OF EVS - PRODUCT BY PROCESS





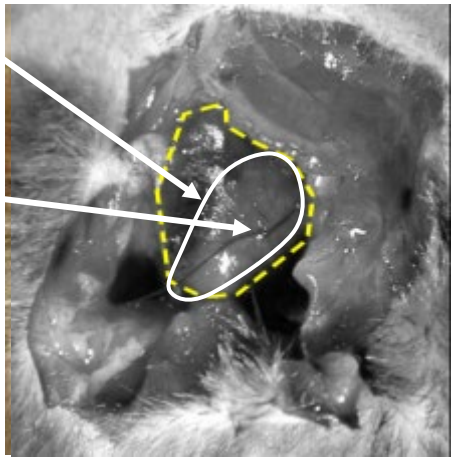
- EV cargo changes during cell differentiation
- Proteomic finger printing important characterisation
- Individual proteins can be set and monitored for comparability

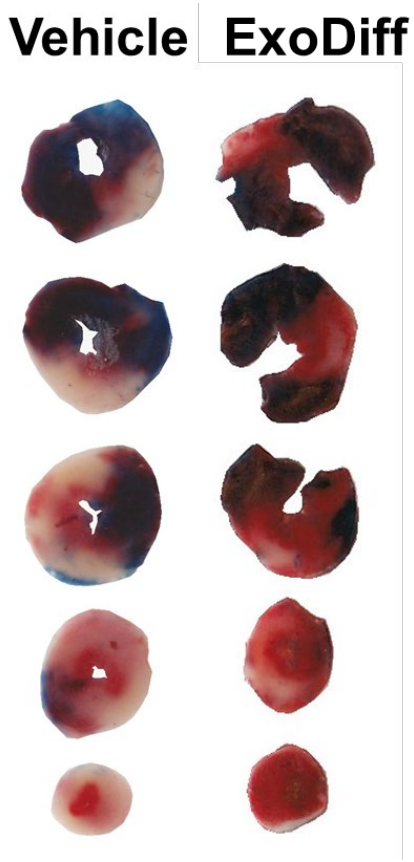
DIFFERENTIATED NEURAL STEM CELL DERIVED EVs PROTECT AGAINST ISCHAEMIA AND REPERFUSION INJURY



Left Ventricle

Coronary Artery Ligation





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ORIGINAL ARTICLE

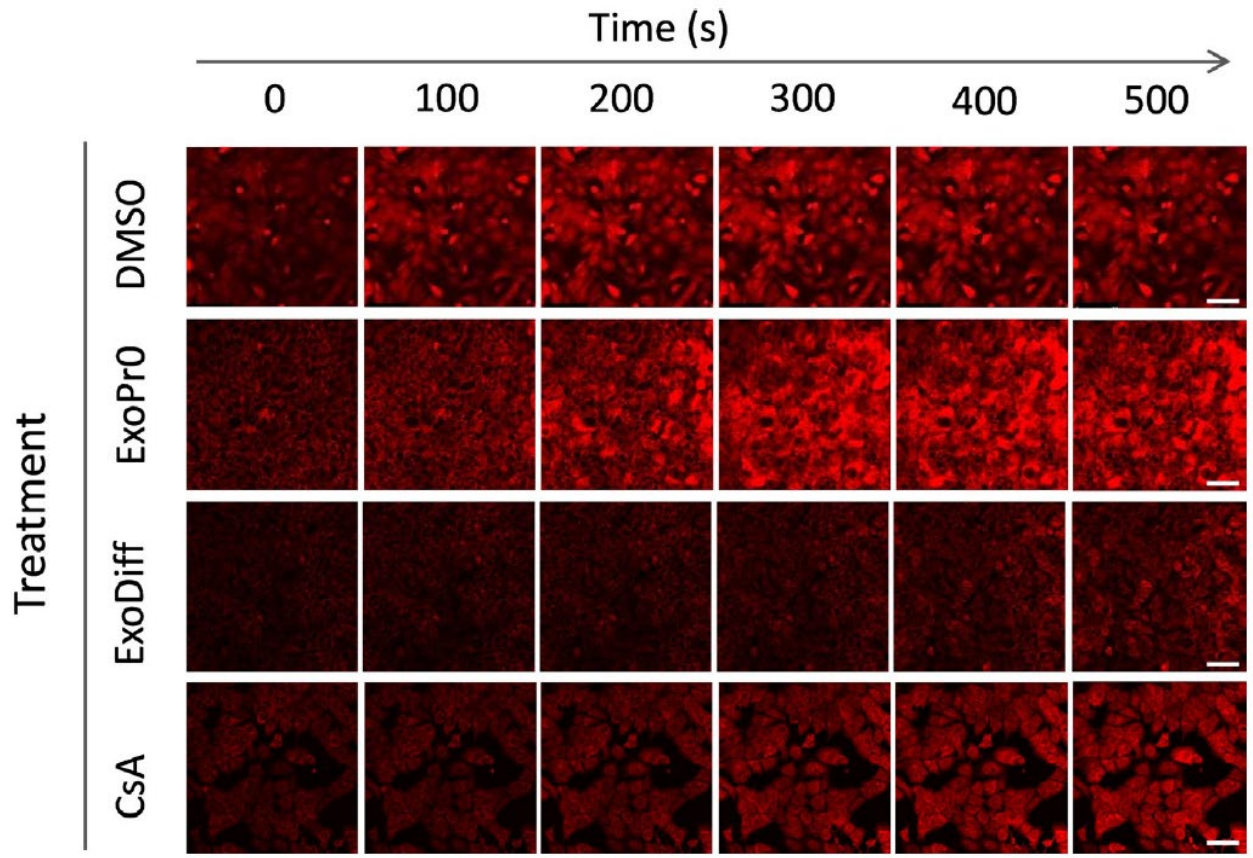
WILEY

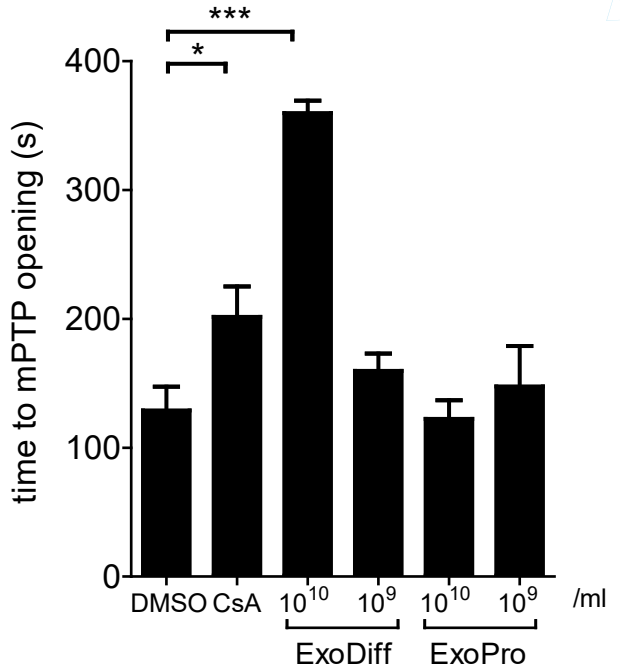
Exosomes from neuronal stem cells may protect the heart from ischaemia/reperfusion injury via JAK1/2 and gp130

Miroslava Katsur[1] | Zhenhe He[1] | Vladimir Vinokur[2] | Randolph Corteling[3] | Derek M Yellon[1] | Sean M Davidson[1]

- Exosomes injected i.v. via jugular vein,
- 5 min before
- 40 min ischaemia
- 2 h reperfusion

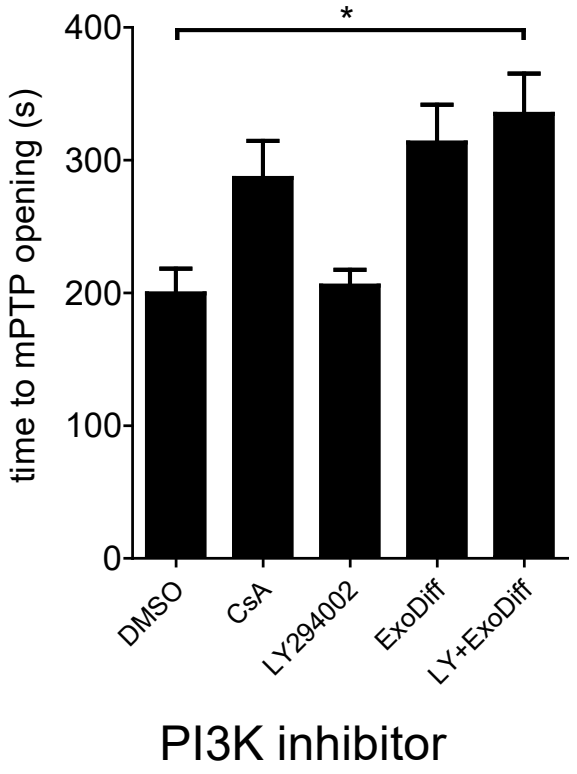
DIFFERENTIATED NEURAL STEM CELL DERIVED EVs DELAY mPTP OPENING IN HL-1 CARDIOMYOCYTES



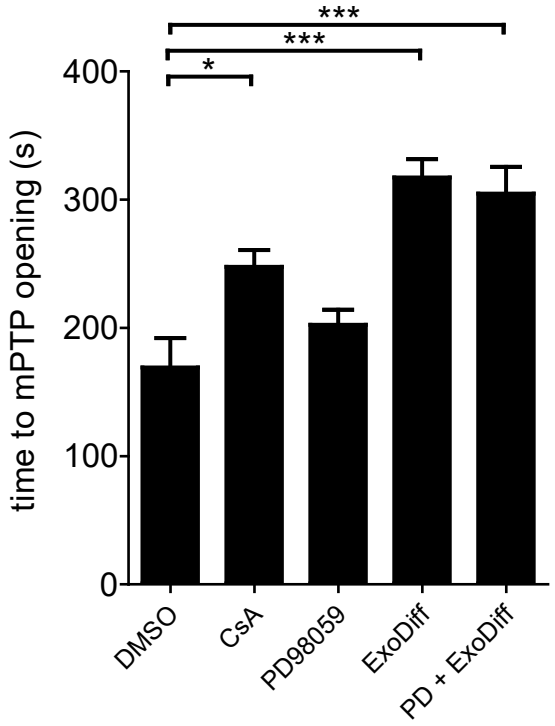


HL-1 cells plated o/n at 100K/well.
15 min pre-treatment with DMSO, 0.2 uM CsA or indicated concentration of exosomes during loading with 3 uM TMRM then replaced with imaging buff and drug/exosome, 3 fields imaged continuously at 50% 543nm laser then $t_{1/2max}$ calculated.
P<0.0001 by 1way ANOVA
N=6,6,3,3,3,2
Post test comparison to DMSO with Bonferonni correction
*<0.05 ***<0.001

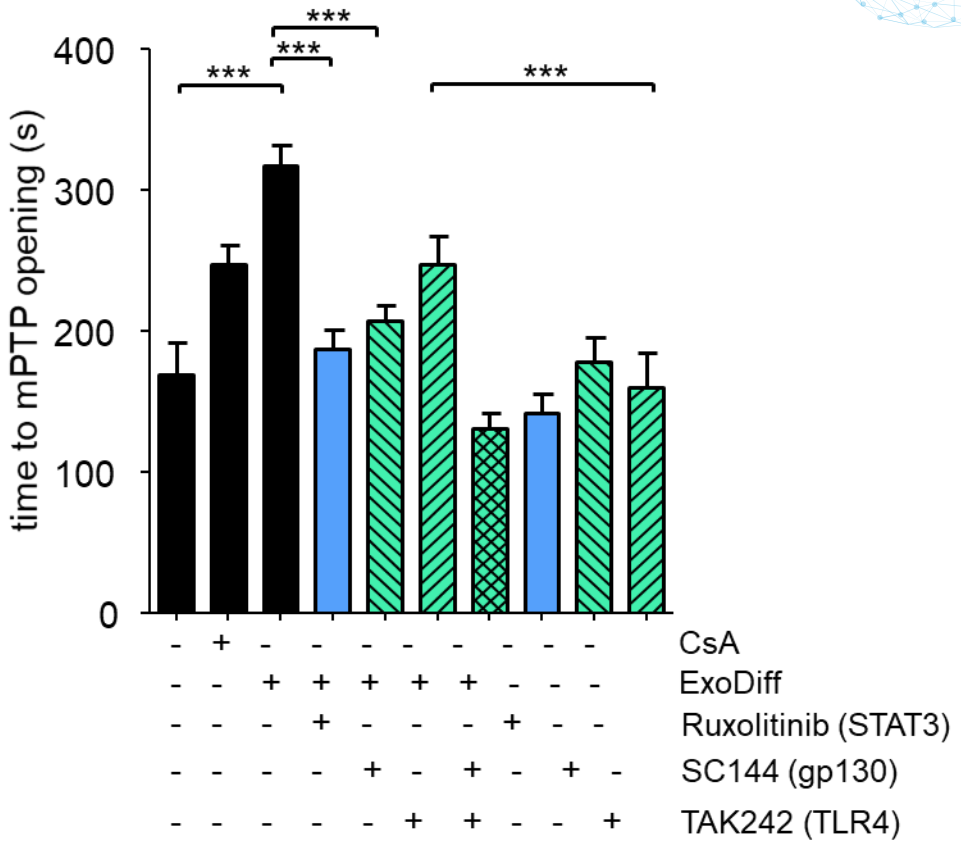
DIFFERENTIATED NEURAL STEM CELL DERIVED EVs PROTECT CARDIOMYOCYTES VIA GP130, TLR4 AND STAT3 SIGNALLING



PI3K inhibitor



MAPK inhibitor



SUMMARY

- Membrane bound vesicles involved in a range of biological processes
- The accuracy and reproducibility of the EV research depends on good EV isolation and purification methods
- EV function is dependent upon surface proteins (tropism) and cargo
- Cargo is dependent on the state of the parental cell (differentiation)
- Differentiated neural stem cell derived EVs protect against ischemia/ reperfusion injury
- Cardiomyocyte protection requires gp130, TLR4 and STAT3 signalling through the delivery of cytokines by differentiated neural stem cell derived EVs

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- Pete Watson
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- Rob Knight

University College London

- Dan Bracewell
- Ben Barnes
- Derek Yellon
- Sean Davidson

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