EXOSOMES FOR CARDIOVASCULAR THERAPEUTIC APPLICATIONS: GMP-GRADE LARGE SCALE MANUFACTURING METHOD, PRODUCT CHARACTERIZATION AND STABILITY

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INTRODUCTION

Human cardiac progenitor cells (CPC) release exosomes (Exo) that prevent early decline in cardiac function in rat models of myocardial infarction (MI) *(Barile L et al, Cardiovasc Res 2014)*. In view of the future clinical translation, a Good Manufacturing Practice (GMP)-compliant method was set-up for large-scale culture of CPC and production of Exo; a comprehensive Quality Control (QC) strategy was also developed *(Andriolo G et al, Front Physiol 2018)*.

MATERIAL AND METHODS

CPC were isolated from cardiac atrial appendage specimens and cultured in xeno-free conditions; a Master Cell Bank (MCB) was frozen at passage 2 (P2) and a Post Production Cell Bank (PPCB) at P6. MCB aliquots were thawed, CPC expanded up to P4 (2.5 m^2 culture surface, about $8x10^8$cells) and induced to release Exo; 8 L of Exo-containing conditioned medium (CM) were collected. Exo were isolated through a closed system, encompassing CM clarification, concentration and diafiltration by tangential flow filtration, and final sterilizing filtration. The final solution containing Exo in a clinical grade vehicle was aliquoted and frozen at -80°C. Three GMP Exo lots were produced. QC tests were performed to evaluate identity, potency and safety of both CPC as cell source and Exo as final product. Stability study is on going for two Exo lots. Exo activity was evaluated *in vitro* and in *vivo*, in small (rat) and large (pig) animal acute myocardial infarction (MI) models.

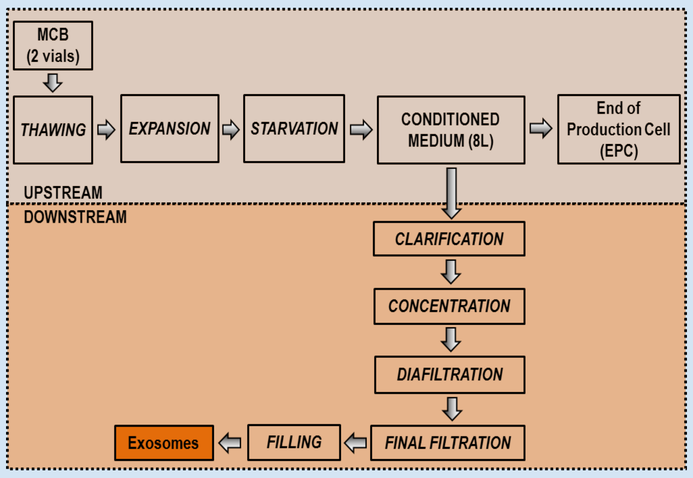
RESULTS

QC strategy

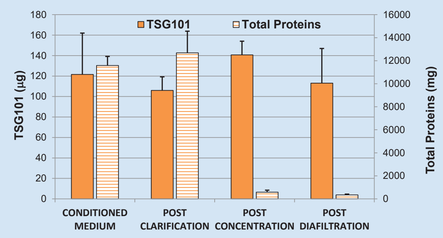
Test category	Parameter/Test	MCB	PPCB	EPC	EXO
IDENTITY/POTENCY	Cell concentration	x	x	x	
	Cell viability	x	x	x	
	Cell immunophenotype: CD90/CD105/CD73	x	x	x	
	GATA4/TBX5/TBX18/MESP-1 expression (RT-PCR)	x	x	x	
	Exo size & concentration (Nanoparticle Tracking Analysis)				x
	TSG101 content (ELISA)				x
	Apoptosis inhibition assay				x
	Angiogenesis assay				x
	Exosome immunophenotype: CD9/CD63/CD81/…				[x]
	miRNA profile				[x]
PURITY	Total protein content				x
	Total DNA content				x
SAFETY	Sterility (EP 2.6.27)	x	x	x	x
	Endotoxin (LAL test EP 2.6.14)	x	x	x	x
	Mycoplasma (PCR)	x	x	x	
	Mycoplasma (culture assay) #	x	x	[x]	
	Adventitious Viruses #	x	x	[x]	
	Karyotype #	x	x	[x]	
	Cell Senescence	x	x	[x]	
	Tumorigenicity	x	x	[x]	

[x] = validation phase only # = outsourced assay

Large-scale GMP Exo production



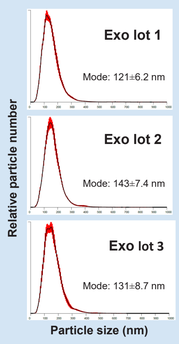
- *Xeno-free process*
- *Pilot scale: 8 L*
- *2.5 m^2 cell culture surface*
- *≈$8x10^8$ total cells*
- *≈$3x10^{13}$ particles/lot, high purity*
- *Overall TSG101 yield: 94% ± 8%*
- *Overall Total Protein removal: 97%± 1%*
- ***Further scalable***
- ***Applicable to other cell sources***



Quality control of Exo lots and EPC

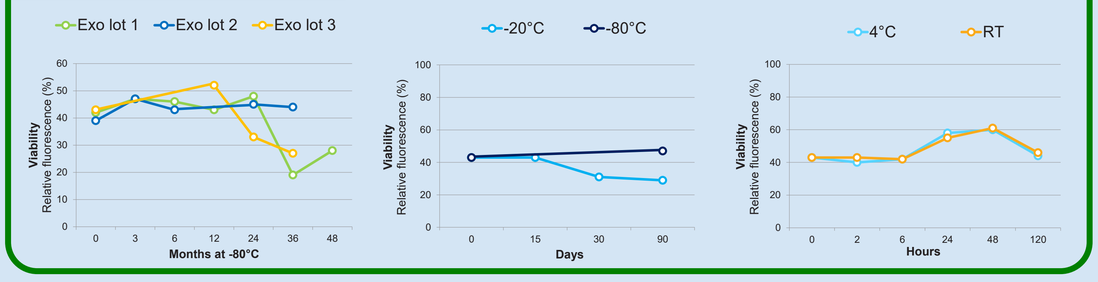
Exo			Results		
Parameter	Method	Specification	Exo lot 1	Exo lot 2	Exo lot 3
Sterility	Microbiological control for cellular products	Absence of growth	Absence of growth	Absence of growth	Absence of growth
Bacterial Endotoxin (EU/mL)	Quantitative LAL test	< 5.0	< 5.0	< 5.0	< 5.0
TSG101 (ng/ml)	ELISA	For Information Only	285	272	203
Total Proteins (mg/mL)	BCA assay	For Information Only	1008	840	1094
Particle concentration (p/mL)	Nanoparticle Tracking Analysis	For Information Only	$8.26x10^{10}$	$1.12x10^{11}$	$1.02x10^{11}$
Particle size (nm)		50-150	121	143	131
CD9 expression	Flow cytometry	Positive	Positive	Positive	Positive
CD63 expression		Positive	Positive	Positive	Positive
CD81 expression		Positive	Positive	Positive	Positive

EPC			Results		
Parameter	Method	Specification	EPC lot 1	EPC lot 2	EPC lot 3
Sterility	Microbiological control for cellular products	Absence of growth	Absence of growth	Absence of growth	Absence of growth
Bacterial Endotoxin (EU/mL)	Quantitative LAL test	< 5.0	< 5.0	< 5.0	< 5.0
Mycoplasma	PCR	Negative	Negative	Negative	Negative
Cell concentration (cells/ml)	Trypan blue, automated cell counting	> $2x10^6$	$2.24x10^6$	$2.37x10^6$	$3.09x10^6$
Cell Viability (%)		≥ 70	81	87	81
CD90 (% positive cells)	Flow cytometry	≥ 90.0	97.3	94.6	98.8
CD105 (% positive cells)		≥ 90.0	98.8	96.6	98.3
CD73 (% positive cells)		≥ 90.0	99.7	99.0	99.5
CD14/20/34/45-DR (% positive cells)		< 10.0	6.7	2.9	9.8

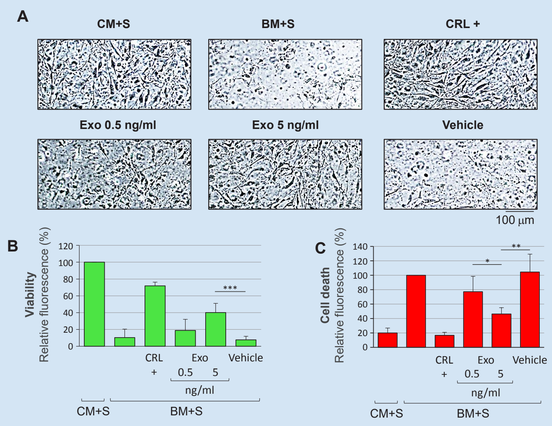


Nanoparticle Tracking Analysis profiles (Nanosight LM10, Malvern).

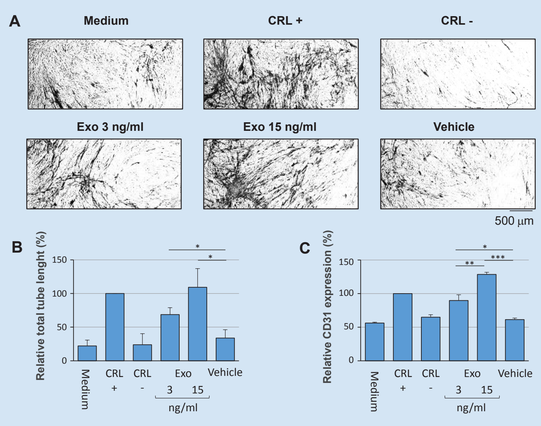
Exo stability: anti-apoptotic activity



Exo *in vitro* functional activity

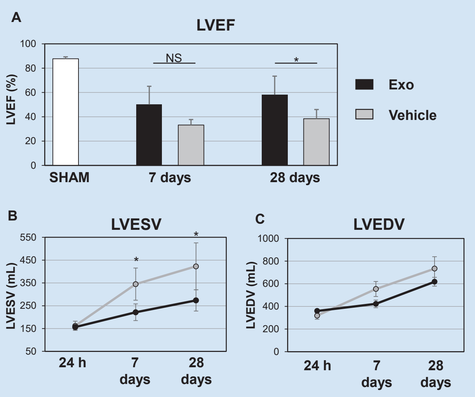


Anti-apoptotic activity. CPC were stained with calcein and PI and fluorescence was measured as representative for viability and apoptosis, respectively. Staurospine was used as apoptosis inducer. **A**) Representative images at of cells (10x) after treatments. **B-C**) Results from calcein and PI staining (n=5). Complete medium + staurosporine (CM+S) is the 100% survival reference for calcein, while basal medium + staurosporine (BM+S) is the 100% apoptosis reference for PI. CRL+ = 10% FBS.

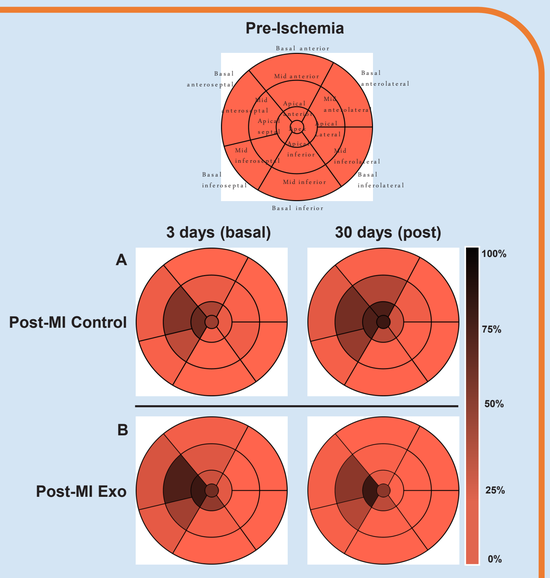


Pro-angiogenic activity. Tubule formation (Huvec cells co-cultured with fibroblasts, V2a Kit ,Cellworks), was visualized by microscopy (CD31 staining). Total tube length and CD31 expression were also determined. **A**) Representative images (4x) of cells after treatments. CD31-stained tubules in black. **B-C**) Results (n=3). CRL + = VEGF; CRL - = Suramin; Vehicle = Plasma-Lyte A®. **B**) Total tube length (AngioSys 2.0 Image Analysis Software). **C**) CD31 expression detected by ELISA.

Exo *in vivo* functional activity



Rat model **A**) LVEF in sham-operated rats at day 28 after MI (SHAM, n=5), in Exo injected rats (black bars, n=10) and vehicle injected rats (grey bars, n=5) 7 and 28 days after MI. Bars: mean + SD. Measurements of LVESV **B**) and LVEDV **C**) in Exo injected rats (black line) and vehicle injected rats (grey line) 24 h, 7 and 28 days after MI. Mean±SD is shown. Vehicle = PlasmaLyte A®. *p<0.05, Student's t-test.



Pig model, preliminary results.
Scar Transmurality Area, measured after 3 and 30 days after the acute MI in control pigs (**A**, n=5) and Exo-treated pigs (**B**, intracoronaric injection of about $6x10^{12}$ particles, n=4).

CONCLUSIONS

Three Exo-CPC lots produced so far resulted sterile and negative for bacterial endotoxins; they contained typical exosomes (121-143 nm diameter), expressing CD9/CD63/CD81 and CPC markers (CD44/CD105) and showing anti-apoptotic and pro-angiogenic activity *in vitro* and therapeutic potential *in vivo* in rat and pig models of acute myocardial infarction. The stability study indicated no loss of functional activity in the storage conditions for at least 24 months. In essence, our standardized, large-scale Exo-CPC production method guarantees high process yield (up to total $3x10^{13}$ EV particles/run, $1x10^{11}$ particles/ml in the final product) and efficient contaminant removal (>97% proteins). Our standardized GMP Exo production method opens new perspectives for reliable human therapeutic applications for acute MI and can be easily applied to other cell sources for different therapeutic areas.

NEXT STEPS

The established manufacturing method will be up-graded in the context of the MARVEL collaborative project by integrating an affinity step in the current workflow to improve product purity by minimizing the co-isolation of contaminants.

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