Sapienza University of Rome

“Foetal bovine serum-derived exosomes affect yield and phenotype of human cardiac progenitor cell culture”

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1. INTRODUCTION

1.1 Exosome overview

Exosomes are bi-lipid membrane vesicles that belong to the extracellular vesicles (EV) class, together with microvesicles and apoptotic bodies. Among these, exosomes are the only ones with endocytic origin and homogeneous shape and size (40-100nm). Their biogenesis starts from the inward budding of the membranes of late endosomes, also called multi-vesicular bodies (MVB), resulting in the formation of intraluminal vesicles (ILVs) [1]. During ILVs formation, transmembrane proteins are incorporated into the folding membrane while the cytosolic components are engulfed within the vesicles. The MVBs move up to the cell surface, fuse with the plasma membrane and, finally, release ILVs outside the cell. Exosomes are released *in vitro* by several cell types, and have been isolated *in vivo* from a variety of body fluids (such as urine, saliva and plasma). Even if their protein composition varies depending on the cell type of origin, a conserved set of membrane markers has been identified, such as Tetraspanins (CD63, CD81 and CD9). Molecules like Alix, TSG101 (Tumor susceptibility gene 101) and clathrin are highly associated with exosomes. One class of cytosolic proteins, the largest small GTPase family, the

Rabs, interact with proteins involved in vesicular transport and fusion, regulating exosome docking and membrane fusion, supported by the Annexins' family. Concerning their content, exosomes are enriched with a wide variety of proteins, such as: heat-shock proteins, metabolic enzymes, ribosomal proteins, signal transduction proteins, adhesion molecules, ATPases, cytoskeletal proteins and ubiquitin molecules [2]. Exosomes content is also enriched with specific nucleic acids, in particular RNAs and miRNAs, which exist within protein complexes. Exosomes have been originally attributed the function of protein excess removal. They are attracting increasing scientific interest, though, since they are no longer considered as simple containers for cell waste, but as cellular structures involved in cell-cell communication. Once in the extracellular space, they are able to interact with target cells inducing, according to the delivered molecules, a modulation of the phenotype towards a differentiated or activated state. They have been extensively studied in cancer research and immunology, because they can affect cancer stem cell niches and tumour progression [3], mediate antigen presentation, responses to infections and autoimmunity. These features suggested novel approaches involving them as biomarkers or immunotherapeutic agents [4, 5]. More recently, exosomes have entered the scenario

of the neurodegenerative disorders [6] and cardiovascular diseases [7] pathophysiology, potentially playing both diagnostic and therapeutic roles.

1.2 Exosomes and stem cells: the paradigm of cardiovascular regenerative medicine.

The fields of stem cells and regenerative medicine are paying increasing attention to the effects mediated by exosomes, particularly in the cardiovascular system. In fact, exosomes derived from mesenchymal, cardiac and embryonic stem cells [8-16] have been shown to exert angiogenic and cardioprotective effects in ischaemic heart failure models, mediating survival and cell-cycle re-entry of cardiomyocytes as well as activation of endogenous cardiac repair by resident progenitor cells. Mesenchymal stem cell-derived exosomes have been shown to produce vascular remodelling and tissue protective effects in a stroke model, as well [17]. Such strong paracrine regenerative approach may overcome several hurdles occurring in the clinical translation of heterotopic stem cell therapy protocols, such as those related to immunologic (i.e. allogeneic mesenchymal cell therapy) or oncologic (i.e. embryonic stem cell-derived teratomas) concerns, since such approach would include only a non-cellular regenerative product, that is EVs. Concerning autologous orthotopic cell products for cell therapies, such as resident Cardiac

Progenitor Cells (CPCs) [18-20] where cardiovascular commitment is intrinsic, paracrine effects are nonetheless important co-factors in the overall therapeutic outcome, together with direct regeneration. In fact, cell sources are needed with unequivocal cardiomyogenic commitment to achieve successful regeneration, and resident CPCs, which have been tested in many animal models and recently in clinical settings, seem to have a very promising potential [21, 22]. Initially, the idea was that transplanted cells, once injected in the infarcted tissue, could directly regenerate new cardiomyocytes. However, pre-clinical studies in different animal models have shown that many of the transplanted cells are lost within few hours after injection, so that only about 5-10% of them can be detected after 1 day [23, 24]. Furthermore, a large number of cells, although initially retained, die because of the unsuitable microenvironment of the damaged tissue. Moreover, the cells that still survive in the heart, only partially differentiate into new cardiomyocytes or vessels [25-27]. However based on the evidence that cell injection has a positive outcome on heart function, even without significant long-term cell engraftment, and that even concentrated stem cell-conditioned media could sustain regenerative effects [28], a new idea has been formulated: the paracrine hypothesis. The rationale of this idea is based on the increasing evidence showing that the observed therapeutic effects, even with cardiovascular-committed

resident CPCs, are partly, but significantly, mediated by secretion of humoral factors [26, 29]. For example, it has been shown that mesenchymal stem cell-conditioned medium (MSC-CM) enhances cardiomyocytes survival after hypoxia induced injury, promotes angiogenesis and reduces infarct size [30, 31]. Paracrine effects have been shown to be responsible also for intercellular communications among different stem cell types, mediating for example the enhancement of cardiovascular commitment of MSCs by CPCs [32]. Moreover a study from Arslan *et al.* [10], demonstrated that exosomes isolated from MSC-CM are able to increase ATP levels, decrease oxidative stress, activate survival pathways, reduce infarct size and prevent adverse remodelling after myocardial ischemic injury (**figure 1**). These evidences allowed exosomes to enter, for the first time, the scenario of paracrine mechanisms of cardiac regeneration. Thereafter several studies have demonstrated that exosomes, independently from the cell type of origin, exert an *in vitro* pro-survival action in hypoxia conditions (as a post HF damage simulation), protect from oxidative stress, promote proliferation, migration and formation of tubes in HUVEC [10-16, 33, 34]. It has also been demonstrated that cardiomyocyte progenitor cell (CMPC)-derived exosomes are able to stimulate endothelial cells migration *in vitro*, increasing capillary density, through mechanisms involving matrix metalloproteinases

(MMP) and extracellular MMP inducers [35] (**figure 1**). CPCs themselves release a wide panel of humoral factors or vesicles (growth factors, cytokines, chemokines, regulatory binding proteins), defining a specific functional "secretome", which exerts proangiogenic and anti-apoptotic effects [26, 36], and mediates the activation of endogenous repair mechanisms *in vivo* [27]. Furthermore, it has been demonstrated that CPCs are able to release exosomes [37], whose regenerative effect has been investigated in two recent papers [13, 38]. The results evidenced that CPC-derived exosomes are able to stimulate angiogenesis *in vitro*, promote cardiomyocytes proliferation and inhibit apoptosis. Similar beneficial outcomes have been observed *in vivo* using a myocardial infarction (MI) mouse model with injection of CPCs conditioned medium (CPCs-CM). In fact, the direct injection of the injured tissue with the exosomes fraction isolated from CPCs-CM, inhibited cardiomyocyte programmed cell death, enhanced angiogenesis, and improved hearth functionality. These effects were similar to what observed with CPCs transplantation in the CADUCEUS clinical trial [21]. Through a qualitative analysis of the CPCs secreted exosomes content, these studies identified several microRNAs (miRs) as new and important mediators of the beneficial effects of regenerative therapy approaches. In general, miR-210 and miR-132 inhibit apoptosis in cardiomyocytes and enhance tube formation in

endothelial cells, respectively, by the ephrin A3, PTP1b and RasGAP-p120 down-regulation [13]. Furthermore miR-146a, in synergy with miR-22 and miR-24, interfering with Toll-like receptor and pro-fibrotic TGF-beta signalling pathways, modulates cardiac fibrosis decreasing scar formation and increasing, at the same time, the viable tissue portion [38].

Overall, these results underline the hypothesis of considering CPC-derived exosomes as a potential therapeutic agents or, at least, as important mediators of regenerative mechanisms for cardiac cell therapy (**figure 1**).

1.3 Exosomes: targets or contaminants.

EVs are present in the mammalian plasma, which means that standard Foetal Bovine Serum (FBS), used as cell culture supplement, contains abundant exogenous EVs. Thus, exosome-free media recipes are necessarily required for the *in vitro* collection of cell-type specific exosomes released in the supernatant, for both their isolation and use as therapeutic tools, as well as for characterization purposes. Nevertheless, bovine exosomes represent an active biological component of FBS, contributing to the overall trophic and proliferative stimuli it grants to cell culture media. A major emerging issue, in any exosome-related research field, is the conflict

between the desired cell-specific purity of any exosome isolation protocol, and the not negligible technical requirements for efficient FBS biological activity on cell cultures. Two recent papers [39, 40] have underlined how the effects of bovine exosomes on cell culture yields and outcomes are important factors to take into account. It has been demonstrated that, the main effect of bovine exosome-depleted media is indeed a reduction of cell growth and proliferation rate. This aspect could highlight the need to analyse, in a critical way, the *in vitro* results obtained until today, in order to understand if and how much the exosomes present in serum might have influenced them. Furthermore, it has been shown that exosomes yield, characteristics and subsequently their effects on cell cultures, are influenced by the isolation protocol used. For this reason, it is important to choose an exosome isolation method according to the requirements needed for downstream applications.

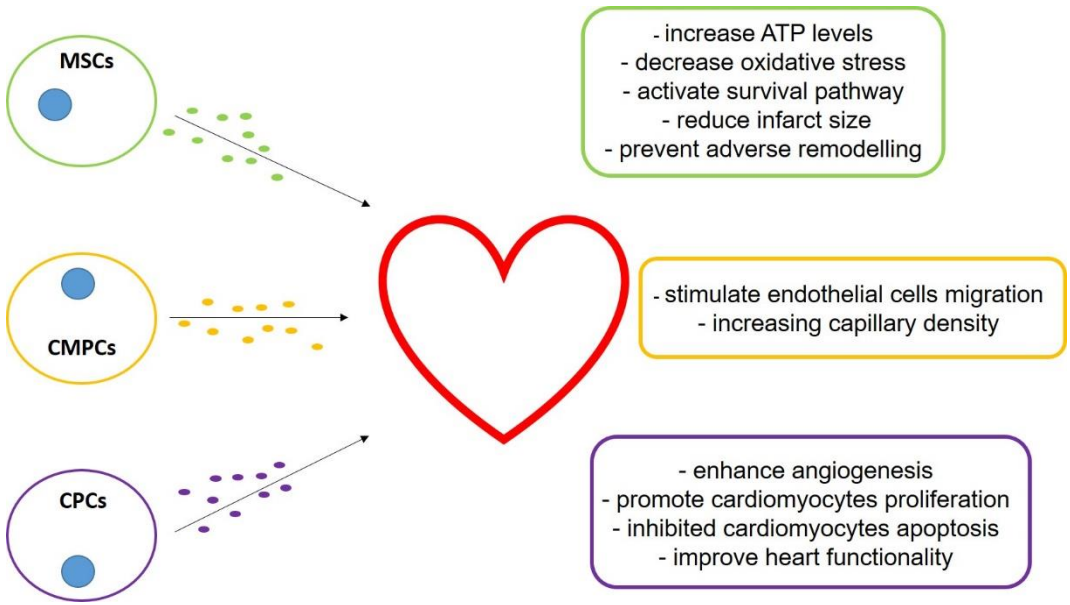

Figure 1. Exosomes derived from MSCs, MPCs and CPCs therapeutical effects based on references [10,13,35,38].

1.4 Exosome isolation protocols

In the last decade, the role of exosomes as one of the key factor for tissue regeneration has emerged, considering the tremendous impetus given to their biotechnological and clinical translation. Therefore, for reliable research data collection, underlying any possible future clinical perspective, many isolation methods will have to be developed to reach a high degree of exosome recovery and specificity, to reduce procedure time and to use easily accessible equipment (**Table 1**). Up to date, every method presents pros and cons that, in different ways, block a possible scalability for a future clinical translation. Starting from the significant effects of FBS exosome-depletion from cell culture media, it could be important, at least in some cell culture protocols, to consider the best suitable isolation method while planning a characterization of cell specific exosomes, and to verify how much the depletion of bovine exosomes from FBS could modify the specific cell phenotype under investigation. The acquired experience may therefore improve the results quality and reliability, making them real facts and not artefacts.

1.4.1 *Ultracentrifugation*

The most commonly used protocol for exosome purification involves several centrifugation and ultracentrifugation (UC) steps [41]. Briefly, the first three steps are designed to eliminate cells (300 rcf for 10 minutes), large dead cells (2,000 rcf at 4°C for 10 minutes) and large cell debris (10,000 rcf at 4°C for 30 minutes). After each step, pellets are discarded and the supernatant is kept for the next. At the end, the supernatant is ultracentrifugated two times at 100,000 rcf at 4°C for 70 minutes to pellet exosomes and discard contaminating proteins. Starting from this protocol, in 2014 Cvjetkovic *et al.* [42] analysed if different rotors, such as fixed angle (FA) or swinging bucket (SW) rotors, and variation in length of ultracentrifugation were able to affect exosomes yield and purity. Both rotor types could be used to isolate vesicles with similar characteristics, in terms of exosomal features, but the composition of the pellet generated by each rotor is slightly different. In particular, the ratio between the protein and RNA fraction within the pellet is higher in the SW than the FA rotor. These results suggest that the choice of the rotor has an impact on the quality of the isolated material and, as the authors suggest, it would be advisable and important to report in any protocol the relative centrifugal force (RCF) as well as the *k*-factor, to ensure accurate replication of any exosomal isolation procedure.

Concerning the centrifugation time, they demonstrated that by extending it, a higher vesicle yield could be obtained. Therefore, the common 70 minutes ultracentrifugation protocol would not be sufficient for the isolation of exosomes, but, on the other side, prolonged time is to be avoided to reduce soluble proteins contamination. The authors then suggest a 4 hours centrifugation time, underlying that longer centrifugation programs could be good for exosome isolation only when followed by purification steps. For that reason, in most of the cases, the exosome pellet, including contaminants, normally undergoes further centrifugation procedures, such as gradient of sucrose or sucrose cushions to separate vesicles and particles based on their density. However, as Jeppesen *et al*. [43] suggest, the use of cushions and gradients increases the time required for purification, and results in loss of sample material. Furthermore, it is also still unclear if the floatation in gradients may affect the biological and functional characteristics of exosomes. The authors examined the impact of differential ultracentrifugation *g*-forces (ranging from 33,000 to 200,000 rcf) on the exosome isolation outcome from two different cell types. A higher purity of the samples was found in both cell lines after different ultracentrifugation rates. Furthermore, rising centrifugal *g*-force leads to increased quantity of contaminating proteins in pellets, while there is a tendency to reach plateau for the number of exosomes recovered. Based

on their results, it seemed that contamination from mitochondria and ER (microsome) could efficiently be eliminated by pre-clearing centrifugation steps at 2,000 rcf and 33,000 rcf, respectively, even if these additional steps reduced the final yield of the isolation process. They also observed, especially in the higher *g*-force fractions, different expression patterns for two exosomal markers (TSG101 and syntenin). Their conclusion was that, probably, there were some sub-populations of exosomes expressing different markers and with different sedimentation profiles.

1.4.2 *Commercial exosome precipitation solutions*

In the last years, several polymer-based exosome isolation kits have become commercially available. Concerning the most used in the literature, the Exo-Quick TC from System-Bio is useful for a quantitative isolation of exosomes from low sample volumes, it is compatible with any bio-fluid, and is an effective and proven alternative to ultracentrifugation. According to the literature and the instruction manual, the culture medium is centrifuged at 3,000 rcf for 15 minutes and the supernatant sieved through 0.22 um filtering units. The appropriate volume of precipitation solution (according to the manufacture's suggestions) is added to the medium,

mixed by inverting and placed at 4°C, ranging from 30 minutes to overnight for serum and other bio-fluids, or culture media, respectively. After refrigeration, the mixture is centrifuged at 1,500 rcf for 30 minutes to remove all traces of fluids, and the exosome pellet is ready to be used. With these solutions, high quality exosomes can be quickly and easily isolated from most biofluids, using a protocol that can be easily performed on multiple samples and requires very low volumes of input. Furthermore, isolated exosomes retain biological activity and can be used in functional assays.

1.4.3 *Heparin affinity purification*

In 2015 Balaj *et al*. [44] have shown that a three day heparin-based affinity chromatography protocol can be used to purify intact EVs to study their functional activities, or to simply use them as isolated biomarkers from bio-fluids. Briefly, the 24-hour conditioned media (DMEM with 5% overnight-UC EV-depleted FBS) was first centrifuged at 300 rcf for 10 minutes to remove cells, then the supernatant was centrifuged at 2,000 rcf for 15 minutes to remove other debris, and finally filtered. At this point, media was concentrated by centrifuging at 1,000 rcf for 10 minutes using a 100 kDa Molecular Weight Cutoff (MWCO) ultra-filtration device. For

heparin purification, the concentrated conditioned media was added to heparin coated beads, incubated overnight on a tube rotator at +4°C to allow binding of EVs to the beads. Heparin beads were spun at 500 rcf for 5 minutes and the unbound fraction (supernatant) was collected. Heparin-coated beads were washed several times with PBS, each wash supernatant was saved, and a solution of NaCl in PBS was added to the beads and incubated overnight at +4°C on a tube rotator. Finally, heparin-coated beads were centrifuged at 500 rcf for 5 minutes, and the supernatant, corresponding to the eluate, was collected and stored at −80°C. In the same work, they also compared, in terms of yield and scalability, the chromatography protocol with ultracentrifugation and commercial kit. They found that, even if starting from the same input volume of media, the yield of RNA was similar in all methods, and the affinity purification is a more scalable method than centrifugation-based purification.

1.4.4 *Sequential filtration*

As discussed in section 3.1 UC protocols are incompatible with future high-throughput automation of exosome isolation and characterization processes, and the development of clinically implementable diagnostics or therapies. Starting from these considerations, in 2014

Heinemann *et al*. [45] proposed sequential filtration as a simplified, clinically applicable method for robust and specific exosomes isolation from biofluids. Their protocol derived from a previous method proposed by Lamparski *et al*. [46], based on tangential flow filtration (TFF) and ultracentrifugation on a deuterium-sucrose cushion, but in their case, they tried to avoid the second part. Sequential filtration consists of three steps: 1) Dead-End filtration, 2) Tangential Flow filtration, and 3) Track-etch filtration. In the first part, the 48-hour conditioned media (with 0.2% of TFF exosome-depleted FBS) was filtered at 22°C using a 0.1 um membrane to eliminate large and rigid media components. In the second step, the filtrate was continuously aspirated from a conical bottle, pumped through a fiber system, with a very low trans-membrane pressure (1.5 and 2.5 PSI), and recycled into the conical bottle. With this mechanism, large molecules, such as free proteins, were discarded. The filtrate went through five rounds of dia-filtration to deplete the sample from contaminants smaller than 500-kDa. Finally, the sample was loaded into a syringe pump and attached to a disposable pressure transducer with a 100 nm filter. The filtration took place at 22°C with a pressure below 3.5 PSI. Compared to a sequential ultracentrifugation pellet, in which they obtained 11% more particles, sequential filtration yielded a more exosome-enriched sample with >80% of the particles in the exosomes size range.

1.4.5 *Size-exclusion chromatography*

Size-exclusion chromatography (SEC) is commonly used to isolate platelets from platelet-rich plasma, and recently Böing *et al*. [47] have investigated the efficacy of single-step SEC for isolation of extracellular vesicles from human platelet-free supernatant. Platelets were isolated with 3 cycles of centrifugation for 20 minutes at 1,550 rcf and 20°C. According to the authors, SEC has several major advantages compared to the most used protocols for vesicles isolation, such as UC. For example, there is no risk of protein complex formation and vesicle aggregation, and the high viscosity of plasma does not affect the recovery of vesicles. The chromatographic column consists of Sepharose CL-2B in a 10mL plastic syringe with a diameter of 1.6 cm and height of 6.2 cm. The supernatant was loaded on the column, followed by elution with PBS/ 0.32% citrate (pH 7.4). Briefly, they showed that vesicles of a diameter larger than 75nm can be isolated from complex body fluids, such as plasma, by single-step SEC. Compared to ultracentrifugation, SEC results in a good recovery of vesicles (43% vs 2-80%) with almost complete removal of contaminants, taking less than 20 minutes (much less time, if compared to 2-96 hours for ultracentrifugation). Thus, in general, isolation of vesicles by SEC is quick, easy and cheap. However, for Welton *et al.* [48] "homemade" columns create problems related to

reproducibility, such as variations from column to column, and the time needed to allow columns to settle without the formation of bubbles. In this perspective, they analysed the potential utility of a commercially available size-exclusion chromatography column for rapid purification of vesicle, exhibiting exosome characteristics. Seven days CM (10% 18-hour UC exosome-depleted FBS) was centrifuged (400 rcf for 10 min and 2000 rcf for 15 min) and filtered through a 0.22 um membrane to remove cell debris. Conditioned medium was added to the commercial column prior to elution with EDTA-PBS buffer. However, even if serial fractions revealed a peak for typical exosomal proteins (such as CD9, CD81), and the columns showed good reproducibility with the exosome-relevant material being collected in less than 10 minutes, nonetheless the post-column vesicle concentration steps lead to low nanoparticles recovery (a loss of over 94%). In conclusion, even if this commercially available column provides a convenient, reproducible and highly effective means of eliminating approximately 95% of non-vesicular proteins from biological fluid samples, several optimizations are certainly still required to minimize vesicle loss.

Table 1. Comparison of different exosome isolation methods based on analysed papers [41-48]

	Time	Exosome recovery	Exosome specificity	Scalability	Pros and cons
Ultracentrifugation[41,42,43]	2-96h	2-80%	YES/NO	NOT YET (too much variability)	Standard gold method but very high variability depending on physical parameters and equipments.
Commercial exosome precipitation kit[44]	2-20h	~80%	YES	YES	Easily accessible, does not require expensive equipments or protocol optimization.
Heparin affinity purification[44]	~33h	~80%	YES	YES	More scalable than ultracentrifugation but requires multiple steps based on different methods.
Sequential filtration[45,46]	Dependent on filtering rate	>80%	YES	YES	Highest exosomes recovery but requires accurate monitoring of flux and pressure parameters .
SEC[47,48]	~10'-20'	43% (without the concentration step)	NO	YES/NO (depending on the column)	Still needs accurate optimization for contaminants removal.

2. AIM OF THE THESIS

Myocardial infarction is still the leading cause for mortality in the Western World. Up to date, the only conclusive therapeutic strategy is heart transplantation that is limited by organs availability and immunological issues. In the last decade, research has focused its attention on cardiac cell therapy as a potential alternative tool to repair a damaged heart and restore, at least partially, its function after injury. Resident CPCs have been tested in multiple animal models, and in few clinical trials, and they seem to hold a very promising potential [20-22]. However, it has been demonstrated that many of the injected cells are lost within few hours after injection, so that only about 5-10% of them can be detected after 1 day [23]. Moreover, the minority of cells that survive in the unsuitable ischemic microenvironment of the damaged heart tissue have not been shown to directly differentiate into new cardiomyocytes with high efficiency [25, 49]. However, based on the evidence that cell injection has a positive outcome on heart function even without long-term engraftment, it has been suggested a paracrine hypothesis. The rationale of this idea is based on the increasing evidence showing that the observed therapeutic effects, even with cardiovascular-committed resident CPCs, are significantly mediated by stem cell secretion

of humoral factors [26, 29]. CPCs release a wide panel of humoral factors and vesicles defining a specific functional "secretome", which exerts proangiogenic, anti-apoptotic and commitment effects [26, 32, 36, 38, 50], and which mediates *in vivo* the activation of endogenous repair mechanisms [27]. Furthermore, recently it has been shown that CPCs secrete exosomes with proliferative, angiogenic and anti-apoptotic properties both *in vitro* and *in vivo* [13, 37, 38]. These evidences support the idea that these vesicles mediate positive paracrine functional effects crucial for cardio-protection. Currently, based on their potential, the principal scientific interest is the characterization of exosomes content, derived from different cell types. However, only few studies have analysed the possible *in vitro* effects of bovine serum-derived exosomes on cell proliferation or differentiation [39, 40]. Our group has recently demonstrated how CPC culture is largely influenced by serum origin and preparation [51], and it has been shown how different media ingredients, signalling molecules and the pathways they activate can significantly affect CPC biology [52-54]. In addition, it is well known that a large amount of exosomes is normally present in FBS. Based on these evidences, the aim of this study was to analyse the *in vitro* effects of exosome-depleted FBS on the proliferation and differentiation properties of CPCs, as an important tool to optimize and scale up CPC production *in vitro*.

3. MATERIAL AND METHODS

3.1 Cell cultures

After informed consent, under an Institutional Review Board approved protocol, surgical auricola biopsies were collected from different patients. Biopsies were cultured as explants, and CPCs were isolated with the Cardiospheres (CSps) protocol, as previously described [55, 56]. Briefly, isolated myocardial tissue was cut into 1- to 2-mm^3 pieces, washed with Ca^{2+}-Mg^{2+}– free phosphate-buffered solution (PBS) (Invitrogen), and digested at 37°C with 0,2% trypsin (Invitrogen) for 15 minutes. Tissue fragments were washed with complete explant medium (CEM) (Iscove's Modified Dulbecco's Medium [IMDM] supplemented with 20% FBS (Gibco), 100 U/mL penicillin G, 100 µg/mL streptomycin, 2 mmol/L L-glutamine, and 0,1 mmol/L 2-mercaptoethanol) and were cultured as explants in CEM at 37°C in normoxic conditions (5% CO_2 and 21%O_2), on Fibronectin coated plates (BD Biosciences). After 3 weeks of culture, Explant derived cells (EDCs) were collected by pooling two washes with Ca^{2+}-Mg^{2+}– free PBS, one wash with 0,53 mmol/L EDTA (Versene, Invitrogen), and one wash with 0,2% trypsin and 0,53 mmol/L EDTA (Invitrogen) at room temperature under visual control. The cells obtained

were seeded at $0,4x10^5$cells/mL in poly-D-lysine-coated 12-multiwell plates (BD Bioscences) in CSps medium (CSM) (35% complete IMDM supplemented with 10% FBS [CSM NORM] or 10% ultracentrifuged FBS (Gibco) [CSM UCF] or 10% exosome-depleted FBS (System-Bio) [CSM DEPL], 65% DMEM–Ham F-12 mix containing 2% B27, 0,1 mmol/L 2-mercaptoethanol, 10 ng/mL epidermal growth factor [EGF], 20 ng/mL basic fibroblast growth factor [bFGF], 40 nmol/L cardiotrophin-1 (all Peprotech), 40 nmol/L thrombin (Sigma), antibiotics and L-Glutamine, as in CEM). After 5 days of culture, CSps from the three different culture conditions were formed and collected. The EDCs that were not used to obtain CSps were collected and grown on a Fibronectin-coated 6-multiwell plates, $0,1x10^5$ cells/well, in presence of IMDM supplemented with 20% FBS [IMDM NORM] or 20% ultracentrifuged FBS [IMDM UCF] or 20% exosome-depleted FBS [IMDM DEPL], for 10 days.

3.2 Exosome depleted FBS

FBS, purchased from Gibco, was filtered (0,2 μm) before adding to the NORM media. To obtain the UCF media, the same serum from Gibco, was ultracentrifuged for 18 hours at 165000 rcf

(SW 41 Ti rotor, Beckman Coulter). The exosome-depleted FBS was purchased directly from System-Bio and filtered (0,2 µm) before adding to the DEPL media.

3.3 ELISA assay

The three different FBS (NORM, DEPL and UCF) underwent an ELISA assay (System Biosciences) to measure their exosomes content (number per mL of serum). Exosomes precipitation was performed using ExoQuick precipitation solution (System Biosciences) that allows pelletting exosomes directly with a simple centrifugation protocol. Once obtained the exosomes from each serum, the ELISA protocol was perfomed following the manufacturer's instructions, using a specific primary antibody against the exosomes membrane protein CD63, and a horseradish peroxidase enzyme-linked secondary antibody (goat anti-rabbit). After the addition of a Super-sensitive TMB ELISA substrate and a Stop Buffer to provide a fixed endpoint, the spectrophotometric lecture was performed at 450 nm for absorbance, using a 96 well plate reader (Robonick). Measurements of exosome numbers were interpolated based on an exosome protein standard curve previously prepared.

3.4 WST-assay

Proliferation was measured by WST-8 assay (Alexis Bioch.) according to the manufacturer's instructions. For each condition, 1000 cells/well in triplicate for 3 time points were plated in 96 multiwell plates. After 2 hours of incubation with WST-8 reagent, absorbance was read at 450nm on a 96-well plate reader (Robonick).

3.5 PCR analyses

RNA from EDCs and CSps was extracted with column-based kits (Qiagen) and quantified by QUANTUS fluorometer (Promega). Reverse transcription was performed on 250 ng starting RNA (Qiagen) in a 20 µL reaction, and 1 µL of cDNA product was then subjected to realtime PCR with Sybr Green Supermix in a MiniOpticon instrument equipped with CFX software (Biorad) for 40 thermal cycles (95 °C for 10 s, 56/58 °C for 10 s, 72 °C for 30 s; see **Table 2** for primers sequence and annealing temperatures). All primers sets were previously tested for optimal efficiency and all reactions were analysed by melting curves at the end to confirm specificity. Each reaction was performed in triplicate on at least 3 biological replicates. The ΔΔCt method was used for relative quantification using GAPDH as the housekeeping gene, and

the expression levels of EDCs and CSps in NORM media culture conditions as the reference to normalize the data.

3.6 Immunostaining and confocal analyses

EDCs and CSps were fixed for 10 minutes with 4% paraformaldehyde at 4°C. For immunofluorescence, cells were permeabilized with 0,1% Triton X-100 (Sigma) in PBS with 1% BSA. Nonspecific antibody binding sites were blocked with 10% goat serum (Sigma-Aldrich) before overnight incubation at 4°C with primary antibodies: Ki67 (Rabbit, AB833-500 Abcam) and Fibronectin (Rabbit, AB2413-500 Abcam). After thorough washing, slides were incubated for 2 hours at room temperature with the appropriate secondary antibodies (Goat Anti-Rabbit IgG, Alexa Fluor 568 Invitrogen) and Topro3 nuclear dye (Invitrogen). Slides were mounted in Vectashield medium (Vector Laboratories) and confocal fluorescence imaging was performed on an inverted microscope (Olympus) equipped with a spectral confocal microscopy system (Olympus Fluoview 1000), using a PlanApo 60x/1,42 NA immersion lens.

3.7 Statistical method

Data are presented as mean value ± standard error of the mean. Two-sided Student's t-test was used to evaluate the statistical significance between any two datasets. Significance threshold was set at $P<0,05$. All experiments were performed on at least three cell lines from different donors.

Table 2. Primers sequence and annealing temperatures

Target	Sequence (5'-3')	T annealing
GAPDH rv GAPDH fw	GCCCAATACGACCAAATCC ACAGTCAGCCGCATCTTC	58°
GATA-4 rv GATA-4 fw	AACGACGGCAACAACGATAAT GTTTTTTCCCCTTTGATTTTTGATC	58°
SMA rv SMA fw	ATGAAGATCCTGACTGAGCG GCAGTGGCCATCTCATTTTC	58°
KDR rv KDR fw	CGGTAGAAGCACTTGTAGGC AAAGGGTGGAGGTGACTGAG	58°
TnI rv TnI fw	AGGGTGGGCCGCTTAAACT GGACAAGGTGGATGAAGAGA	58°
C-KIT rv C-KIT fw	GGGATTTTCTCTGCGTTCTG GATGGATGGATGGTGGAGAC	57°
THY-1 rv THY-1 fw	CGTTAGGCTGGTCACCTTCT CAGCGGAAGACCCCAGT	58°
VIM rv VIM fw	GGTCATCGTGATGCTGAGAA ACCCACTCAAAAAGGACACTTC	56°
HSP90 rv HSP90 fw	CAATGACATCAACTGGGCAA CTGTGCCGTTGGTCCTGT	58°
CX43 rv CX43 fw	GAGTTTGCCTAAGGCGCTC AGGAGTTCAATCACTTGGCG	56°
Ki-67 rv Ki-67 fw	TGACTTCCTTCCATTCTGAAGAC TGGGTCTGTTATTGATGAGC	60°
ITGA1 rv ITGA1 fw	CCAAACATGTCTTCCACCG CTGCTGCTGGCTCCTCAC	60°
LAMB1 rv LAMB1 fw	CAACGCAGACACACTGGC GAACTCTTCTGGGGAGACCC	60°
TLN1 rv TLN1 fw	ACTGTGTGGGCTCCACTAGC AAGGCACTTTGTGGCTTCAC	59°
PXN rv PXN fw	TGTGGGAGGTGGTAGACTCC AGCTAGCGCGACCCTGA	59°
COL1A1 rv COL1A1 fw	CACACGTCTCGGTCATGGTA AAGAGGAAGGCCAAGTCGAG	60°
COL1A2 rv COL1A2 fw	CAGGTCCTTGGAAACCTTGA TGCTGCTCAGTATGATGGAAA	60°
COL3A1 rv COL3A1 fw	CATGCCCTACTGGTCCTCAG ATAGCCTGCGAGTCCTCCTA	60°
FN rv FN fw	CACTCATCTCCAACGGCATAATG AAGACCAGCAGAGGCATAAGG	60°
VCL rv VCL fw	AACTCTTCATCCTTTTCCTCTGG ACCTTGAACAACTCCGACTAAC	60°

4. RESULTS

4.1 FBS-derived exosomes affect EDCs culture

In order to analyse the effects of bovine exosomes on the CSps isolation protocol, human heart biopsies were cultured in three different conditions, using IMDM NORM, DEPL or UCF. After 20 days of culture (**figure 2A-C**) a significant reduction of EDCs yield (containing CSp-forming cells) in UCF media and a complete absence of cells from the explant in DEPL media were observed (**figure 2G**). As no EDCs outgrowth was obtained in the DEPL media (**figure 2B**), explants were cultured firstly in IMDM NORM and then in the three different conditions. Once explant-outgrowing cells reached 80% confluence, EDCs were collected and cultured 10,000 cells/well per conditions in a Fibronectin-coated 6-multiwell plate to analyse proliferation. Interestingly, after 10 days, cells number indicative of cell proliferation displayed the same trend as explant outgrowth (**figure 2D-F**). In fact, the DEPL and the UCF media had a significant reduction of cell number compared to the NORM media (**figure 2H**). These data showed that DEPL and UCF media affected cell proliferation. In order to verify if the reduction of proliferation rate was directly dependent on bovine exosomes, a WST assay was performed with

different bovine exosome concentrations added to the DEPL media, which has been selected for the lower proliferation rate shown by the cells cultured in the medium supplemented with it. Bovine exosomes were obtained from the ultracentrifugated FBS and were quantified with an ELISA assay against the CD63 exosome-specific membrane protein (**figure 3A**). The isolated exosomes were added to the DEPL media at concentrations corresponding either to 1-fold (1x) or 5-fold (5x) that of the normal exosome amount present in the NORM medium. This test allowed to determinate not only whether the exosomes had a direct effect on cell proliferation, but also if this effect was dose dependent. A 4 days assay was performed starting with 1000 cells/condition using the NORM and the DEPL media as positive and negative control, respectively, plus the 1x and 5x media. The WST results demonstrated that the addition of bovine exosomes to the DEPL media rescued cell proliferation and that this effect was dose dependent (**figure 3B-F**). As expected from the ELISA quantification data, the 1x and the NORM media presented a similar curve trend (**figure 3B**). The immunofluorescence analyses for the proliferation marker Ki-67 confirmed the previous results. In fact, in the NORM media there was a higher number of Ki-67^{+} cells with a brighter nuclear localization than the other two conditions (**figure 4**). The morphological analyses did not identify remarkable differences in

the shape of the EDCs, cultured in the three different media (**figure 2D-F**). Next the gene expression profile was analysed for markers involved in cell proliferation and migration, such as Ki-67, Talin-1 (TLN-1), Paxillin (PXL) and Vinculin (VCL), for the mesenchymal related gene THY-1 and for a heat-shock protein gene (HSP90) (figure 5). In DEPL and UCF IMDM, Ki-67 expression was significantly lower than in NORM media, confirming the above-mentioned immunofluorescence data. Furthermore, THY-1 presented a significantly lower expression level compared to the NORM IMDM. In the DEPL IMDM, TLN-1, PXL and VLC were down-regulated, consistently with the reduced proliferative rate and defective explant outgrowth. Nevertheless, even if the TLN-1 and VLC were also down-regulated in UCF media, PXL resulted over-expressed, meaning that several mechanisms could be involved in cell spreading processes. Finally, the down-regulation of HSP90 suggested that bovine exosome-depleted media did not increase cell stress.

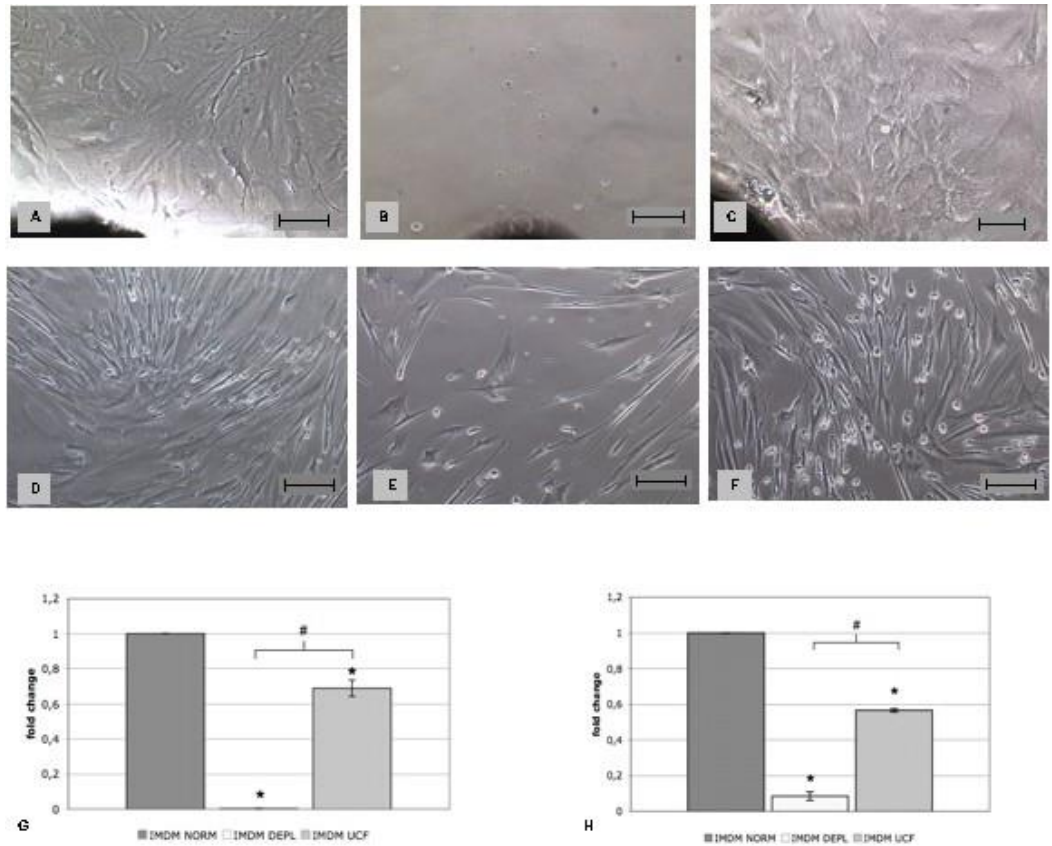

Figure 2 Representative images of heart biopsies explant grown in different media after 30 days of culture [IMDM NORM (**A**), IMDM DEPL (**B**), IMDM UCF (**C**)] and their normalized EDCs yield (**G**) based on the average numbers of collected cells from each condition (n=3). Representative images of EDCs grown in different media [IMDM NORM (**D**), IMDM DEPL (**E**), IMDM UCF (**F**)] after 10 days of culture, starting from 10000 cells/condition, and their normalized proliferation rate (**H**) based on the average final cell numbers from each condition (n=3).

* P<0,05 vs IMDM NORM, # P<0,05 vs IMDM DEPL. Scale bar = 100 μm.

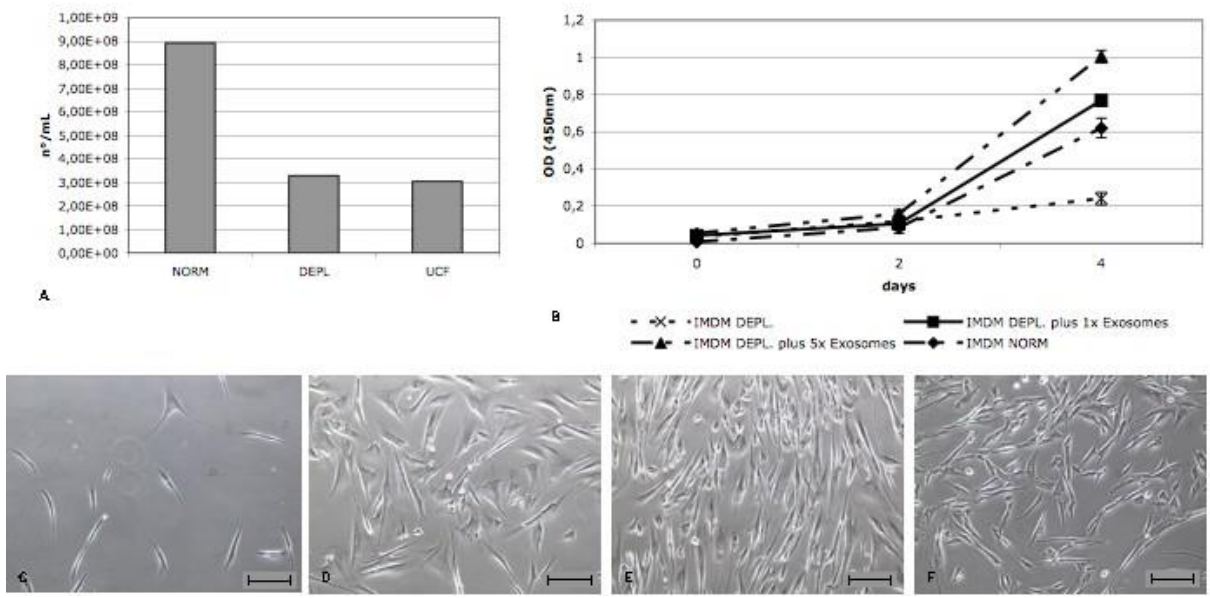

Figure 3 **A**) Quantification of exosomes concentration (number/mL) in each serum obtained with an ELISA assay against exosome membrane protein CD63. **B**) Representative WST assay of EDCs growth for 4 days in presence of different concentrations of bovine exosomes in the media. **C-F**) Representative images of EDCs grown at different bovine exosomes concentration: IMDM DEPL (**C**), IMDM DEPL with 1x Exosomes (**D**), IMDM DEPL with 5x Exosomes (**E**), IMDM NORM (**F**). Scale bar = 100μm.

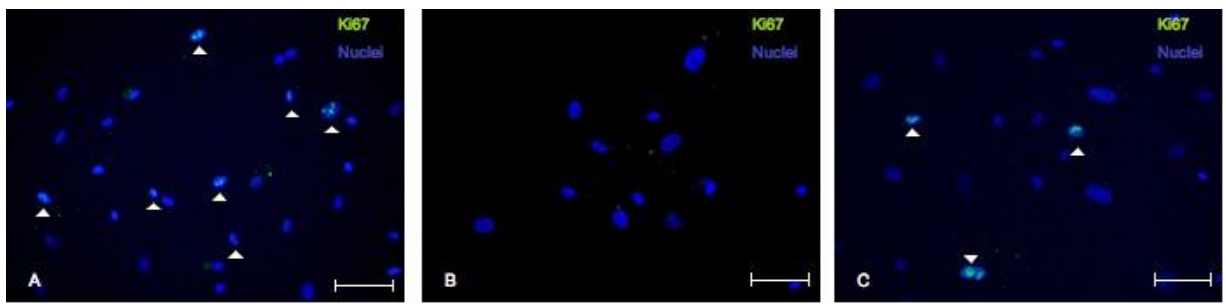

Figure 4 Representative confocal microscope images of immunofluorescence against Ki-67 in EDCs in different media: IMDM NORM (**A**), IMDM DEPL (**B**) and IMDM UCF (**C**). Scale bar = 100μm.

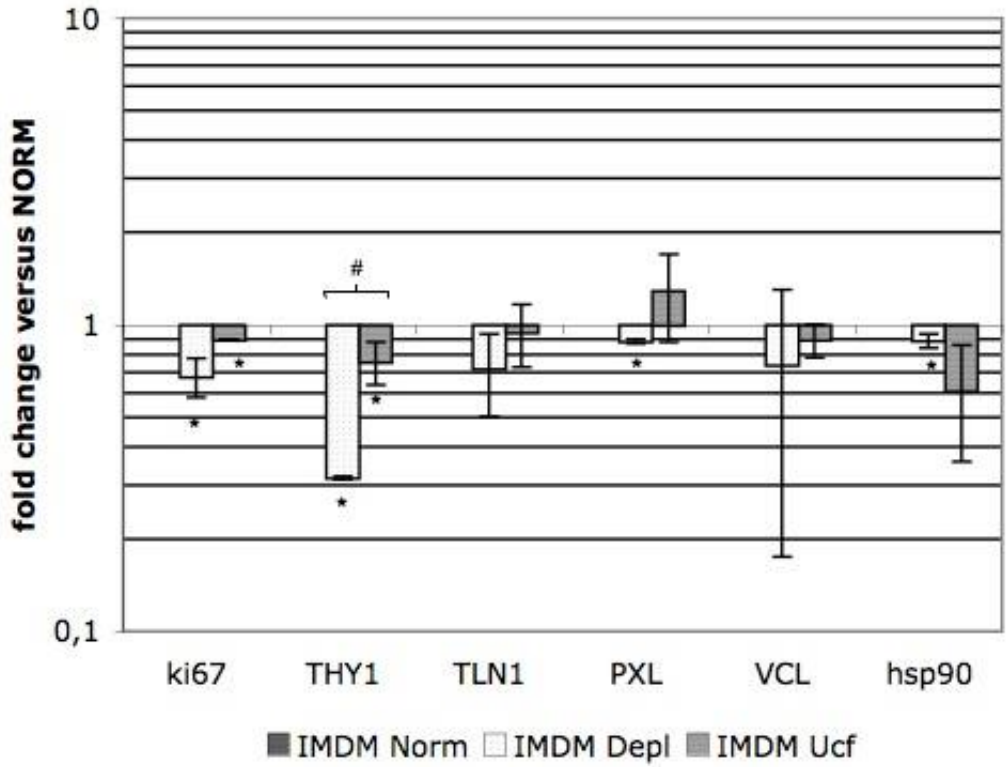

Figure 5 Relative gene expression levels of EDCs cultured in DEPL and UCF, normalized versus IMDM NORM (n=3).

* P<0,05 vs IMDM NORM, # P<0,05 vs IMDM DEPL.

4.2 FBS-derived exosomes modulate CSps formation

Next, the exosomes effects on the three-dimensional cell culture of CSps were analysed. EDCs, previously grown in IMDM NORM, were cultured in the three different CSM media on Poly-D-Lysine coating to form CSps. After 5 days of culture, the collected CSps were counted and their diameter was measured (**figure 6A-C**). There were no differences between the DEPL and UCF CSM in terms of CSps yield and size (**figure 6D,E**). Compared to the NORM CSM, in DEPL and UCF media the number of CSps was significantly higher (1,63±0,29 fold), while on the contrary, the diameter was significantly smaller (61,0±1,5 µm vs 80±6 µm) (**figure 6D,E**). To verify whether there was a correlation between the reduced CSps dimension and the number of cells of each CSp, after 5 days, 1000 CSps from each condition were dissociated and cell number counted. As expected, in UCF media, the number of cells/CSp was lower than the NORM CSM (48,0±6,4 cells/CSp vs 87,0±12,7) (**figure 6F**). Interestingly, despite similar dimensions, in the DEPL media the number of cells per CSp was significantly higher compared to the UCF (81,0±2,1 cells/CSp vs 48,0±6,4) (**figure 6F**), and comparable to the NORM condition (81,0±2,1 cells/CSp vs 87,0±12,7) (**figure 6F**). These results suggested that the bovine exosome depletion influences CSps formation, in terms of yield, as well as cell size and

proliferation. To verify if the bovine exosome depletion was able to affect the gene expression profile of CSps, a real time PCR analysis for a representative CSp-gene panel was performed [57]. Due to the heterogeneous niche-like nature of the CSps [18, 54, 58] we considered genes related to stemness (C-KIT), cardiac progenitors (GATA4), cardiac lineage (TnI, MHC, Cx43), vascular lineage (SMA, KDR), mesenchymal lineage (THY-1, VIM) and proliferation (Ki-67). Results (**figure 7**) suggested that the absence of bovine exosomes in the media, regardless of the depletion method, is associated to a significant down-regulation of the analysed genes, except for those vascular and mesenchymal. The Ki-67 expression level is also in agreement with the previous results, concerning the proliferation of CSp cells. Furthermore, considering the different CSp size and the average number of cells per CSp obtained in the three different media, some ECM related genes expression, such as Intergrin alpha 1 (ITGA1), Laminin beta 1 (LAMB1), Collagen type I (COL1A1, COL1A2), Collagen type 3 (COL3A1) and Fibronectin (FN) was analysed (**figure 8**). Interestingly, we found that in the DEPL CSM, in which CSps were smaller than the NORM media, but with a similar number of cells per CSp, the ECM gene expressions were down-regulated. On the contrary, in the UCF, where CSps were smaller and the number of cells per CSp was lower, the expression of ECM genes was up-regulated.

According also to the FN patterns shown by confocal analyses on CSps (**figure 9**), these results suggested that the absence of bovine exosomes could affect the production of extracellular matrix from the cells.

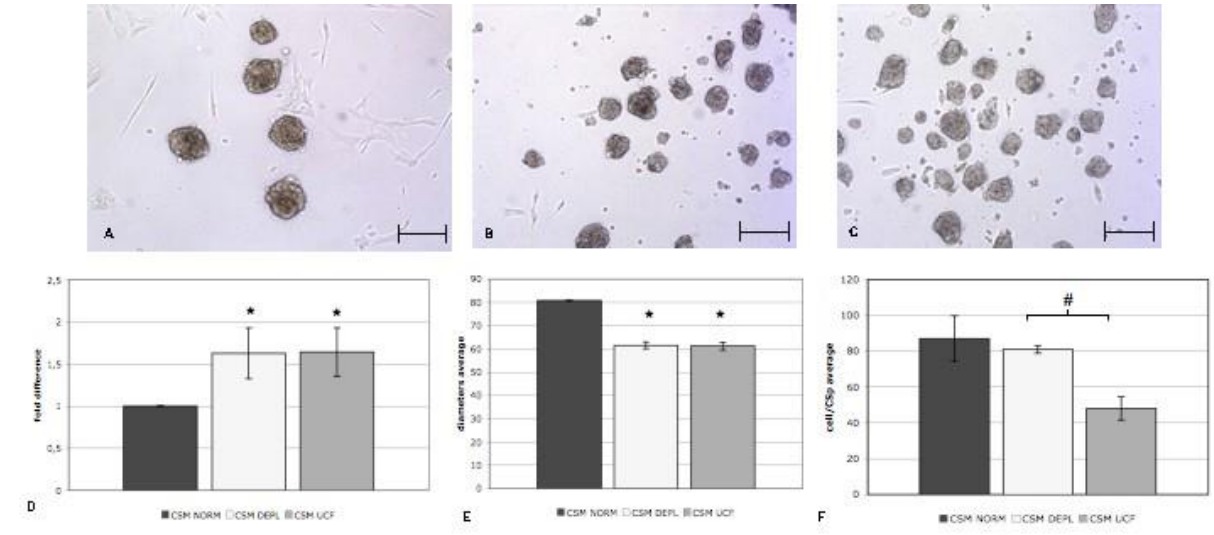

Figure 6 Representative cell culture images of CSps after 5 days of culture in three different media: CSM NORM (**A**), CSM DEPL (**B**), CMS UCF (**C**). Average CSp numbers (**D**) and diameters (**E**) of CSps collected from each condition (n=3). F) Average of the numbers of cell obtained from dissociated CSps from each condition (n=3). Scale bar = 100 μm.

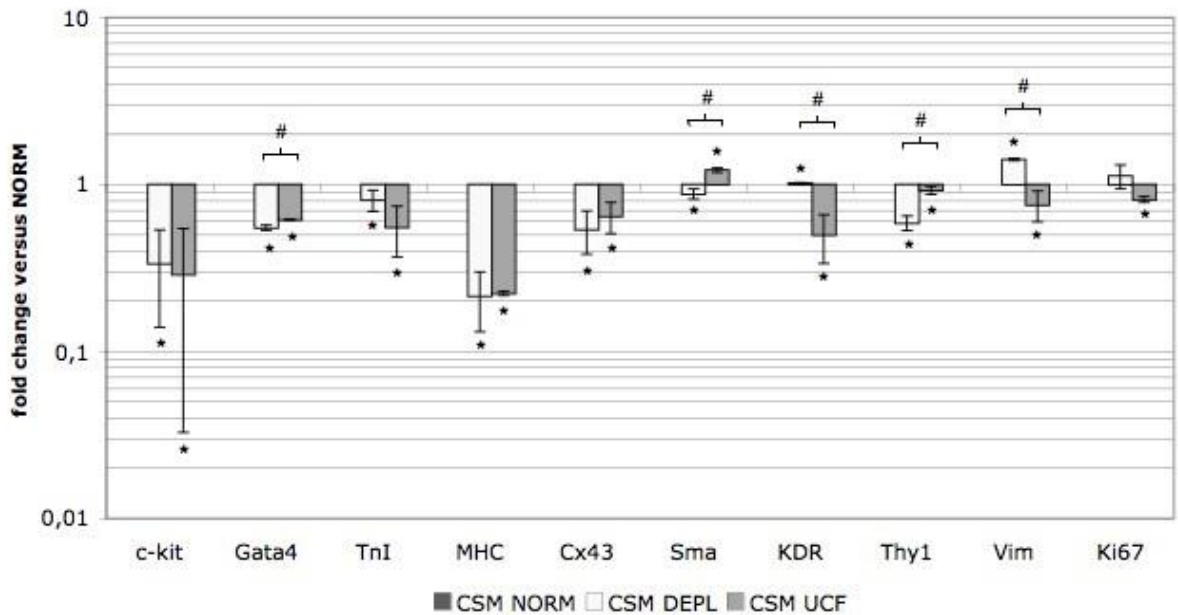

Figure 7 Relative gene expression levels of CSps cultured in DEPL and UCF, versus IMDM NORM (n=3).

* P<0,05 vs IMDM NORM, # P<0,05 vs IMDM DEPL.

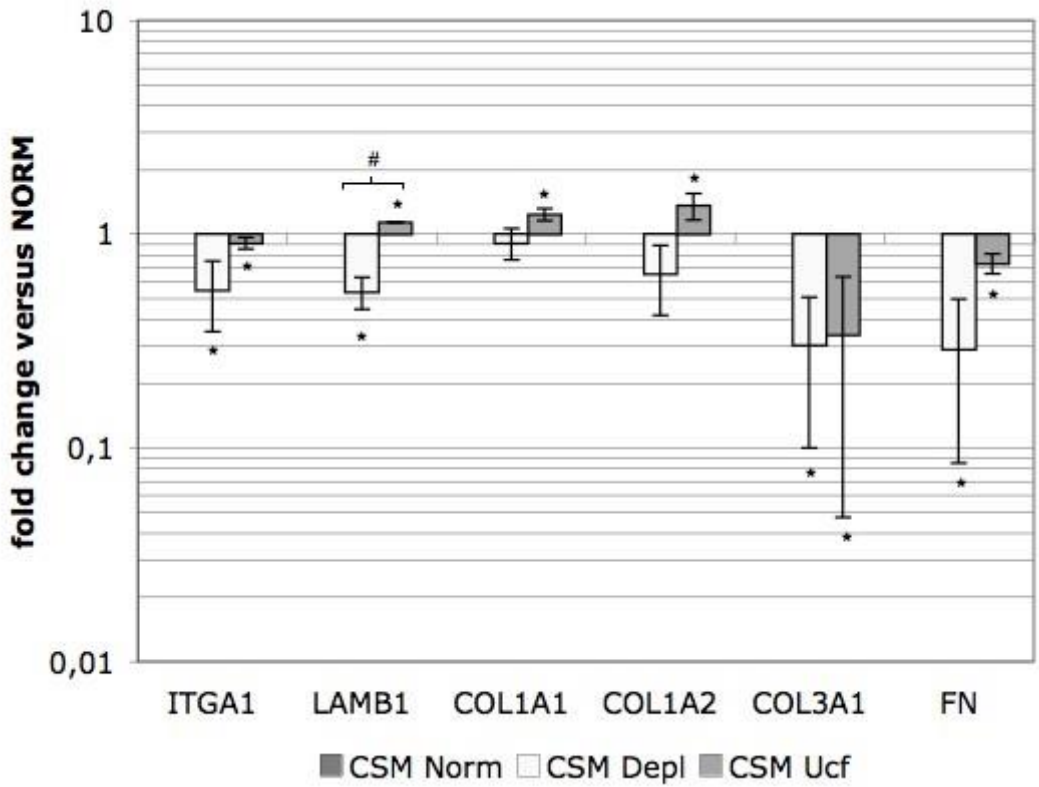

Figure 8 Relative ECM gene expression levels in CSps cultured in DEPL and UCF, versus IMDM NORM (n=3).

* P<0,05 vs IMDM NORM, # P<0,05 vs IMDM DEPL.

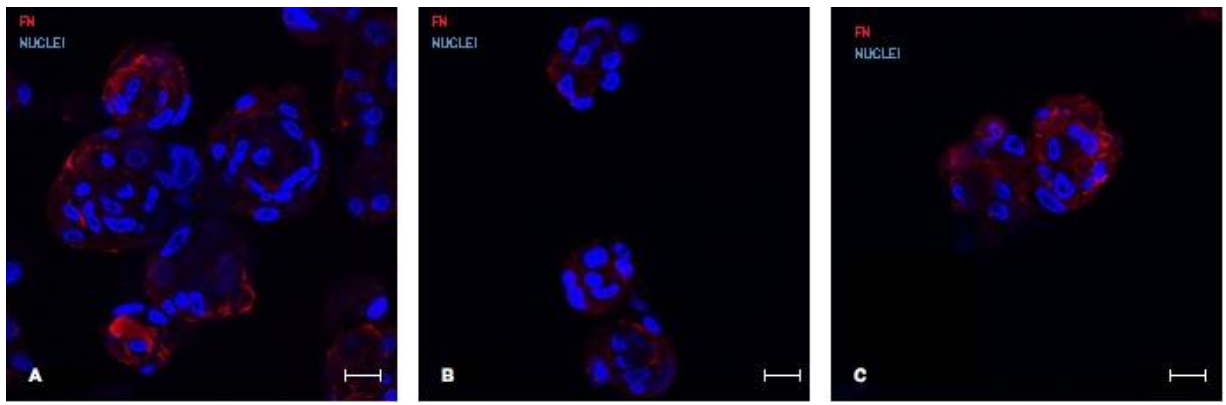

Figure 9 Representative confocal images of fibronectin stainings (red fluorescence) of CSps in different media: CSM NORM (**A**), CSM DEPL (**B**) and CSM (**C**). Scale bar = 20µm. FN= fibronectin.

5. DISCUSSION

Exosomes are present in most mammalian body fluids, such as plasma, urine and saliva, which means that standard culture supplement FBS contains abundant exosomal vesicles. Bovine serum is commonly used for *in vitro* cell cultures to provide hormones, growth factors and other proteins able to support cell survival and proliferation. Besides, bovine exosomes represent another serum active biological component that takes part in the FBS trophic stimuli. Two recent studies [39, 40] have underlined that the effects of FBS exosomes content on cell cultures are important factors to be considered. In fact, they demonstrated, using human tumoral cell lines, that depletion of bovine exosomes in the media decreased cell proliferation rate. Here, we analysed for the first time the effects of serum exosome-depleted media on the isolation procedure of primary human CPCs from explant-derived cells to three-dimensional spheroids (CSps). We have already demonstrated that EDCs and CSps culture yield and phenotype are significantly affected by different serum supplements [51]. We have selected two different exosome-depleted FBSs: one obtained through ultracentrifugation of our commonly used FBS and the other one commercially available. This latter (Exo-FBS™ from System-Bio) is obtained

with a patented method different from ultracentrifugation, involving precipitation with a specific polymer able to bind exosomes. EDCs are the first cell population obtained by explant culture of heart tissue, as they include CSp-forming cells. They have been shown, though, to exert some beneficial effects with possible therapeutic potential [59]. Exosome-depleted media exerted a negative effect on EDCs outgrowth and proliferation. We demonstrated that in DEPL and UCF IMDM, after 10 days of culture, cell number was significantly lower than in the NORM media, consistently with decreased expression of the proliferation marker Ki-67 (**figure 2**, **figure 5**). Furthermore, addiction to the DEPL media of different concentrations of bovine exosomes, isolated from commonly used FBS by ultracentrifugation, was able to restore the proliferative rate in a concentration-dependent manner (**figure 3**). Overall, these results highlight a direct role of FBS exosomes in cell culture proliferation. Based on the literature and on the evidence that, in our experimental conditions, there was a reduction of EDCs proliferation as well as spreading, the expression of integrin-related genes was investigated. TLN-1 and VCL are two key structural proteins of integrin adhesion complexes that regulate the affinity of integrins for ECM ligands, and are required by the actin cytoskeleton to catalyse focal adhesion-dependent pathways [60, 61]. Talin, in particular, is the first component to be recruited to integrin/fibronectin adhesion sites

for the linkage with the cytoskeleton [62], suggesting a scaffolding function to bind adhesion complex adaptors and enzymes [63]. Wang *et al*. attested, in fact, that talin-deficient cells, regardless of a normal actin cytoskeleton, showed defects in adhesion, spreading and proliferation due to the lack in the recruitment of vinculin, paxillin, focal adhesion kinase (FAK) and integrin-linked kinase [64]. Consistently, we observed that talin expression was associated to a reduced proliferation rate. In fact, in the DEPL media, that presented the lowest proliferation rate, TLN, PXL and VCL expressions were reduced versus the other two conditions (**figure 5**). On the contrary, in the UCF IMDM, talin and vinculin expression levels were slightly lower than the NORM condition, suggesting that this down-regulation could partially impair cell proliferation, but not at the same level as in the DEPL media (**figure 5**). It was not surprising to have different results between DEPL and UCF IMDM; it has been already demonstrated, in fact, that the bovine exosomes depletion protocol from FBS could affect its biological outcome on cell cultures [39, 40]. As mentioned above, EDCs contain CSp-forming cells. Our group was the first to describe the culture method to obtain spontaneous scaffold-free spheroids, called CSps, containing human resident adult CPCs [55]. CSps mimic *in vitro* many aspects of a niche microenvironment. In fact, their architecture consists of a central core of undifferentiated cardiac

stem cells and interlinked by extra-cellular matrix. Compared to CPCs from monolayer cultures, cells of the CSp have a lower proliferation rate, but the expression of stem cell transcription factors (such as Oct4 and Nkx2.5) is up-regulated, as well as other factors involved in maintaining a stemness state and managing re-entry into the cell cycle (Gata4 and c-kit) [58], displaying overall a distinctive transcriptomic profile [65]. Due to their composition, resident CPCs grown as CSps have a strong regenerative and paracrine potential *in vitro* and *in vivo* [26, 27, 66]. CSp-derived cells (CDCs) have recently and successfully been tested in the CADUCEUS clinical trial [21]. In the perspective of clinical scalability, it is important to analyse all the possible factors that could affect the culture method and, in the best case, improve them. Since CSps are the selective/inductive stage of the culture protocol [67], in this study we have analysed for the first time the effects of FBS exosomes on CSps culture. Currently it is not possible to obtain directly from EDCs a sufficient amount of primary CSps. In fact, it is necessary to expand CSps as a CDCs monolayer in order to have cell numbers suitable for the *in vivo* applications or to obtain a higher number of secondary CSps [54, 68]. Here we demonstrated that in presence of a low amount of FBS exosomes, there is a significant increase of CSps number together with a significant decrease of spheroids diameter (**figure 6**). Based on these evidences, we could

hypothesize that the use of an exosome-depleted serum, could be an optimization for the *in vitro* culture conditions. We also investigated if exosome depletion affected the gene expression pattern of CSps. RTqPCR analyses were directed against a list of genes that are normally expressed in CSps due to their heterogeneous structure, including genes related to stemness, cardiac and vascular lineages (**figure 7**). In terms of cardiac and stemness genes, we observed overall a significant down-regulation in exosome-depleted conditions. These results evidenced an important influence of bovine exosomes on cardiac commitment of CSps, besides maintenance of their stem potential. Based on the results concerning proliferation and Ki-67 expression, CSps from each condition were dissociated and the ratio cells number/CSp was analysed. Interestingly, despite similar size, the cell number per CSp in the UCF CSM was significantly lower than the DEPL media (**figure 6F**). Furthermore, this latter presented a cell number/CSp comparable to the NORM CSM that together with higher CSp yield suggested higher cell numbers overall, supporting the results on proliferation. We could hypothesize, concerning the DEPL CSM results, a sort of "paradox effect" of bovine exosomes depletion that stimulates proliferation in the 3D structure, but inhibits the same in the monolayer. DEPL and UCF media CSps presented comparable size but different number of cells per CSp. To

understand at least in part these differences, we analysed the expression of ECM-related genes that characterize CSps [58] (**figure 8**). In the DEPL CSM, gene expression levels were overall down-regulated. On the contrary, in the UCF condition, except for ITGA-1 and FN, all the ECM related markers were significantly up-regulated. These results were confirmed also by the confocal analyses of fibronectin expression on CSps from each condition (**figure 9**). We could hypothesize that FBS exosomes were able to modulate ECM production, but it is still unclear, and need a deeper analyses, if this effect is due directly to exosomes depletion or not. It has been demonstrated that various depletion methods are able to differentially affect cell cultures *in vitro* [39]. Here we demonstrated that by using two exosome-depleted sera, produced with different methods, we obtained distinctive results in terms of proliferation and gene expression profiles. For example, concerning the mesenchymal component normally present in CPCs culture, even if we observed in both EDCs and CSps a significant down-regulation of the related gene THY-1 (**figure 5** and **figure 7**), the amount of such effect was different between the two exosome-depleted FBSs. These results confirm that the depletion method can affect exosome quantity, as well as quality, and possibly their content, exerting in the end different stimuli on the same cell

type [69, 70]. Further analyses, that are not the aims of this work, will be necessary to verify this hypothesis.

6. CONCLUSION

In conclusion, we verified that FBS exosome-depletion reduced EDCs proliferation, and the expression of adhesion molecules, and that adding exosomes to depleted media it is possible to restore proliferation rate in a dose-dependent manner. In 3D CPCs culture, exosome-depleted FBSs affected CSps yield, dimension and ECM production. In addition, we observed that CSps displayed a more undifferentiated phenotype. Future efforts may evaluate their effects in terms of functional benefits to understand if the selective and/or timely use of an exosome-depleted FBS in the media could be an improvement or adjuvant of the culture method for a clinical scalability.

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