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IN VITRO EVALUATION OF THERAPEUTIC POTENTIAL OF EXOSOMES DELIVERED BY HUMAN CARDIAC PRIMITIVE CELLS IN CARDIAC REGENERATION

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ABBREVIATIONS

αSARC	Alpha Sarcomeric Actin
BM	Bone Marrow
cDNA	Complementary Deoxyribonucleic Acid
CDS	Coding Sequence
CF	Cardiac Fibroblast
CF-P	Cardiac Fibroblasts from Pathological Hearts
CMs	Cardiac Myocytes
CPC	Cardiac Primitive Cells
CPC-N	Cardiac Primitive Cells from Normal Hearts
CPC-P	Cardiac Primitive Cells from Pathological Hearts
Ct	Cycle Threshold
DAPI	4',6-diamidino-2-phenylindole
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F12
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECM	Extracellular Matrix
ECs	Endothelial Cells
EDTA	Ethylendiaminetetraacetic Acid
EGF	Epidermal Growth Factor
FGF-6	Fibroblasts Growth Factors-6
FGF-7	Fibroblasts Growth Factors-7

GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
IGF-1	Insulin Like Growth Factor 1
EGFR	Epidermal Growth Factor Receptor
ES	Embryonic Stem Cells
ESCRT	Endosomal Sorting Complexes Required for Transport
EVs	Extracellular Vesicles
Exo-CPC	Exosomes from Cardiac Primitive Cells
Exo-CPC-N	Exosomes from Cardiac Primitive Cells of Normal Hearts
Exo-CPC-P	Exosomes from Cardiac Primitive Cells of Pathological Hearts
FBS	Foetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
HGF	Hepatocyte Growth Factor
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
iPSC	Induced Pluripotent Stem Cells
LV	Left Ventricle
MHC I	Major Histocompatibility Complex Class I
MI	Myocardial Infarction
miRNA	MicroRNA
mRNA	Messenger RNA
MVB	Multivesicular Bodies
PDGFRα	Platelet-derived Growth Factor Receptor Alpha
PDGFRβ	Platelet-derived Growth Factor Receptor Beta

PCR	Polymerase Chain Reaction
PMSF	Phenylmethanesulfonyl Fluoride
RIPA	Radio Immunoprecipitation Assay
RNA	Ribonucleic Acid
SCF	Stem Cell Factor
SCFR	Stem Cell Factor Receptor
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error Mean
SMA	Smooth Muscle Actin
SMC	Smooth Muscle Cells
TGFβ	Transforming Growth Factor Beta
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VEGFR3	Vascular Endothelial Growth Factor Receptor 3

ABSTRACT

Although, when injected, human Cardiac Primitive Cells (CPC) are not retained by host myocardium, they still improve cardiac function. Emerging evidence supports the hypothesis that exosomes may be responsible for beneficial effects induced by stem cells delivered in the infarcted myocardium. Exosomes are nano-sized vesicles naturally secreted by almost all cells and ubiquitously found in cell culture supernatants and biological fluids. Transporting and transferring peptides, lipids, and nucleic acids, exosomes have the potential to modulate signaling pathways, cell growth, migration, and proliferation of recipient cells. Accordingly, CPC may deliver chemoattractive, pro-survival and differentiating signals to resident cells through exosomes. To test our hypothesis, we isolated exosomes released in culture by CPC isolated from adult human myocardium (Exo-CPC) and analyzed the composition of their cargo and the effects elicited *in vitro* by their administration to resident population of CPC or fibroblasts.

Specifically, we searched for the presence of specific factors known to regulate CPC migration, survival and differentiation. Additionally, we tested *in vitro* the potential of Exo-CPC of either regulating CPC proliferation and programmed cell death, and modulating interstitial fibrosis, extracellular-matrix (ECM) synthesis and deposition. Interestingly, on one hand, signals delivered by Exo-CPC affected proliferation and survival of CPC and, on the other hand, regulated ECM protein production. Therefore, we might speculate that Exo-CPC have potential effect on both resident CPC and fibroblasts when injected in cardiac wall.

1. INTRODUCTION

Within the last two decades the most challenging purpose of cardiovascular regenerative medicine has been the restoration of myocardial integrity and function by cell-based or stem cell-based therapies, small molecules and tissue engineering-based or material-based methods (1).

The major approach in this field, to date, has been the replacement of lost or damaged tissues with new cells or to promote tissue regeneration by stem cell transplantation. Cell based therapy to treat the damaged heart, including injection of stem cells from various sources, have yielded mixed results in several species (2-4). Multiple experimental and several clinical studies have shown that different subsets of bone marrow-derived cells, isolated either from bone marrow (BM) or from peripheral blood, and other adult progenitor cells isolated from the cardiac tissue improved the recovery of heart function (5–11). Although the degree of improvement in cardiac function varies depending on the target population and cell type (8), there is mounting evidence that cell therapy is safe and does have a beneficial effect on left ventricle (LV) function (12) that translates into clinical benefit (10). One of the most important questions concerning cell therapies is to elucidate the mechanism by which stem/progenitor cells achieve functional improvement. A modest number of retained cells in the myocardium appeared to account for marked improvements in cardiac function as emerged from preclinical and clinical studies involving cell-based therapies (13). These observations have raised the possibility that grafted cells may amplify their effects by producing growth factors, cytokines, or other signaling

entities that improve the performance or survival of resident or recruited cells. Several studies have suggested that a paracrine mechanism contributes significantly to myocardial repair triggered by cell-based therapies (14-16). More recently, pluripotent cells derived from embryonic stem cells (ES) or induced pluripotent stem cells (iPSC), obtained by cutting-edge cellular reprogramming technologies, have been considered as an innovative source of modern cell therapies (17). In 2006, Takahashi and Yamanaka discovered that fully differentiated cells, such as skin cells from mouse, could be made pluripotent by artificially activating four specific genes. These cells, called iPSC, could self-renew and differentiate like embryonic stem cells (18). The following year, iPSC were successfully reprogrammed from human skin cells too (19). iPSC provide a way to create genetically-matched pluripotent cells for patients, avoiding immune system incompatibility issues and ethical issues of embryonic stem cells.

1.1 FROM BENCH TO CLINIC

Although we are far from effective therapies, the positive outcomes of the clinical application of stem cell therapies has been remarkable, with thousands of trials currently registered in the National Institutes of Health clinical trials database (www.clinicaltrials.gov). Results from the early phase trials have demonstrated cell therapy as safe, feasible and potentially efficacious, in a wide range of diseases and medical fields, from cardiology (20), ischemic stroke (21) and peripheral ischemia (22), to cancer (23). Despite the enthusiastic results, for many of these therapies, therapeutic benefit cannot be attributed to stem cell

survival and differentiation, as they are not retained by organs (24-26). The beneficial effects of cell implants without cell survival and retention, along with well-researched trophic effects of the cell-conditioned culture medium suggest that secreted paracrine factors may be involved and could be responsible for the observed results (27,28). The paracrine hypothesis has been strengthened by the recent discovery that stem cells release not only soluble factors, like cytokines and chemokines, but also extracellular vesicles (EVs) eliciting similar biological activity to the stem cells themselves (28-31).

1.2 EXOSOMES

EVs are membrane surrounded structures released by cells in an evolutionarily conserved manner. Their diagnostic and therapeutic exploitation is now under intense investigation.

Extracellular vesicles are heterogeneous and a consensus on terminology for their different subtypes is yet to emerge. The major populations include exosomes, microvesicles and apoptotic bodies. Exosomes size ranges between 40 and 100 nm, and they are considered membrane vesicles of endocytic origin secreted by most cell types under normal and pathological conditions. Microvesicles are shed directly from the plasma membrane and can be larger than exosomes (50-1000 nm) (32), (Fig 1 b), while apoptotic bodies originate at the cell membrane as cells undergo apoptosis (Fig 1 c). EVs can interact with target cells by two crucial mechanisms: transmembrane proteins on EVs can interact with receptors on the target cell membrane to initiate a signaling cascade (33, 34), or can directly fuse

with their target cells by endocytosis/transcytosis, with subsequent release of their content into the cytosol of the target cell.

Among EVs, exosomes in particular, had gained immense research interest over the last few years due to their promising diagnostic and therapeutic potential (35) as cell-free approach (36).

They were first described in a study to track the fate of recycling transferrin receptor during maturation of sheep reticulocytes (37). Exosomes have been found in a number of human body fluids, including blood plasma (38), urine (39), breast milk (40), amniotic fluid (41), (Fig 2). In 1940, Chargaff and others noted lipoproteins of a very high particle weight readily forming sediments in a strong centrifugal field but remaining in solution when subjected to weak centrifugal field (42). Despite the limited technology of the time, Chargaff & West (43) accurately predicted an important role of these particles in health and disease. Two decades later, Wolf and associates (44) isolated these particles from the subcellular fraction and resolved them using electron microscopy. Distinct small vesicles ranging from 20 to 50 nm were observed and were then termed platelet dust. The term exosomes was first coined by Johnstone and others to describe vesicles released from cultured maturing sheep reticulocytes (45, 46). These particles had enzymatic (e.g., acetylcholinesterase and transferrin) activity that was lost during reticulocyte-to-erythrocyte maturation. The first hypothesis was that these vesicles were a means to remove factors from the plasma membrane that were no longer needed by the mature erythrocyte (38). With the scientific progress, paracrine mechanisms became better understood and the field

of exosome biology spread out: particles once thought to be involved in waste management are now widely accepted as highly conserved elements in a pathway of short- and long-range communication. All the examined eukaryotic cell types, including hematopoietic cells, epithelial cells, neural cells, stem cells, adipocytes, and cancer cells secrete exosomes in culture (84).

1.2.1 EXOSOMES BIOGENESIS

The formation of exosomes starts from the cell surface, where proteins are internalized and delivered to early endosomes. In early endosomes, proteins can be recycled to the plasma membrane or delivered to late endosomes. By now, intraluminal endosomal vesicles are formed by budding of the endosomal limiting membrane into the lumen, forming multivesicular bodies (MVB). MVB can follow a different fate: they can fuse with lysosome for protein degradation, or merge with the plasma membrane, releasing the content into the extracellular space as exosomes (39, 47-48), (Fig 1 a). This mechanism presents an advantage to EV characterization, as exosomes can be further identified by markers of their biogenesis. Specifically, exosomes contain remnants of the ESCRT (endosomal sorting complexes required for transport) pathway, even if the markers associated with ESCRT are not entirely exclusive: some of the markers conserved in exosomes, such as tetraspanins (CD63, CD81, and CD9), are also present on the plasma membrane, in the cytosol, and in vesicles derived from membrane shedding (49). The wide presence of tetraspanins is likely due to the multiplicity of mechanisms in which these proteins are involved, including cell activation and

proliferation, cell adhesion and motility, and cell differentiation. The endosomal system controls the uptake and processing of various types of macromolecules from the extracellular environment and the plasma membrane into the cell. It consists of different interconnected vesicular organelles, basically the primary endocytic vesicles, the early endosomes, the recycling endosomes, the late endosomes and the lysosomes (50). The process of MVB formation is coordinated by the ESCRT.

The ESCRT complexes are cytoplasmic proteins involved in membrane budding or bending events, such as multivesicular body biogenesis, the abscission step of cell division. This multiproteic structure, consisting of about thirty proteins, is assembled in four soluble complexes called ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III. The ESCRT-0 recognizes and sequesters ubiquitylated proteins in the endosomal membrane, whereas the ESCRT-I and -II appear to be responsible for membrane deformation into buds with sequestered cargo, and ESCRT-III components subsequently drive vesicle scission (51, 52), (Fig 3). The current knowledge of how MVBs are fated for either fuse with lysosomes to degrade their cargo or to fuse with the plasma membrane to release their intraluminal vesicles as exosomes into the extracellular environment is very preliminary. However, exosome production and release seem to depend on the activity of different Rab-GTPases and some of their effectors, which are known to regulate the vesicular transport between specific organelles (53). Rab proteins, reviewed in Stenmark 2009 (54), are essential regulators of intracellular vesicle transport between different compartments. Rabs can be involved in either vesicle budding,

mobility through interaction with the cytoskeleton, or tethering to the membrane of an acceptor compartment. The Rab family is composed of more than 60 GTPases, each of which is preferentially associated with one intracellular compartment.

RAB11 was the first Rab reported to be involved in exosome secretion.

1.2.2 EXOSOMES STRUCTURE AND COMPONENTS

Exosomes differ from each other in their composition, as they directly represent the proteome of the source cells. Analysis of urinary vesicles showed a link between exosomes containing aquaporin-2 and their origin from the urogenital tract (39). Several studies have shown that all mammalian exosomes share some common characteristics, for example the lipid bilayer structure, the size, the density and overall the composition in proteins. Some proteins are located on the surface, other in the lumen and are considered exosomal markers. Notably, these include cytoplasmic proteins such as tubulin, actin, actin-binding proteins, annexins and Rab proteins as well as molecules responsible for signal transduction, such as protein kinases and heterotrimeric G-proteins (55-57). Most exosomes also contain major histocompatibility complex class I (MHC I) molecules (58, 59) and heat-shock proteins such as Hsp70 and Hsp90 (60, 61). The protein family most commonly associated with exosomes is the tetraspanin including CD9, CD63, CD81 and CD82 (62-64). Consistent with their endosomal origin, there are typically no proteins of the nucleus, mitochondria, or endoplasmic reticulum detectable in exosomes (65).

Exosomes are also enriched in certain raft-associated lipids such as cholesterol (primarily B lymphocytes), ceramide (implicated in the differentiation of exosomes from lysosomes) other sphingolipids, and phospoglycerides with long and saturated fatty-acyl chains (66-68). Exosomes also show saccharide groups on their outer surface. This was investigated recently by Batista (69) who found that these were enriched in mannose, polylactosamine, α-2,6 sialic acid, and complex N-linked glycans. Exosomes have been reported to contain significant amounts of miRNA, other non-coding RNAs, as well as mRNA. Valadi et al. reported that, although the RNA appeared to be mostly degraded to less than 200 nt fragments, some full-length molecules must also be present, since the extracted RNA could be used to generate identifiable full-length proteins using an *in vitro* translation system (70). Several papers indicate that the RNA "cargo" of exosomes is significantly different from the parental cell content, i.e. certain RNAs are present at significantly different levels compared to the total cell lysate from the originating cells (71-73).

1.2.3 ROLES OF EXOSOMES

Once exosomes are secreted outside the cells, they can follow one of these three pathways: 1) they can be captured by neighboring cells or by the same cells that have given rise to them; 2) they can be internalized by cells that are within a certain distance; or alternatively, 3) they can enter the systemic circulation and, then, be taken up by different tissues (47). Exosomes, once thought to be only responsible for the removal of unnecessary proteins from the cells, are now

considered the key factor in cell–cell communication (74). In addition, they participate in several different functions and in a large variety of pathways, such as immune response, cardiovascular protection and cancer. Exosomes, first simply considered a garbage cane, are now viewed as specifically secreted vesicles that enable intercellular communication and have become the focus of exponentially growing interest, both to study their functions and to understand ways to use them in the development of a non-invasive diagnostics and therapy.

1.3 MYOCARDIUM SECRETES EXOSOMES

Exosomes are demonstrated to be mediators of extracellular communicators; therefore, it is fitting to propose that they can be important communicators of ischemic signaling and myocardial repair. Reports have suggested that the myocardial tissue secretes exosomes, and exosomes and microvesicles could be an important mechanism involved in heterocellular communication in the adult heart (75, 76), especially exosomes emerging from telocytes in the border zone of myocardial infarction (MI) (76). Barile et al (75) provided ultrastructural evidence for the first time that exosomes and microvesicles are secreted by the progenitor cells in a normal adult mouse heart. They have also demonstrated a possible uptake of exosomes by cardiomyocytes in the adult mouse heart. Recently, Sahoo and Losordo provided ultrastructural evidence of exosome-like vesicles packed in MVBs of CPC from cardiospheres, characterized by a large nucleus and a thin cytoplasm, in a healthy mouse heart, as well as in the cytoplasm of cardiac myocytes (CMs) from the left ventricle of a healthy human

and from a patient with ischemic heart disease (77). Electronic microscopy observations showed that murine and human CPC are able to produce exosome-like structures that are generated in MVBs (75, 77). These exosomes had a size of 30-90 nm and were enriched with proangiogenic miR-132, miR-146a and miR-210 that increased survival in cultured murine cardiomyocytes and induced tube formation in HUVEC cultures. Furthermore, treatment of rats with experimentally-induced IM by Exo-CPC resulted in the improvement of cardiac function, less profound cardiac apoptosis and enhanced intracardiac angiogenesis (78).

1.4 EXOSOMES IN CARDIOVASCULAR DISEASE

Intercellular communication mediated by extracellular vesicles is crucial for preserving vascular integrity and in the development of cardiovascular and other diseases. In cancer, exosomes have been described to possess the ability to pass on malignancy from cell to cell and shape the environment by stimulating blood vessel formation, while concomitantly inhibiting immune response to the tumor (79-81).

In recent years, many published reports suggest that in addition to cancers and neurodegenerative disorders, major cardiovascular and metabolic pathologies like coronary artery disease, myocardial infarction, heart failure, and diabetes are highly influenced by the exosome-directed transfer of molecules. It has been demonstrated that exosomally delivered mRNA and miRNAs are translated and regulate gene expression of acceptor cells influencing their biology. They

contribute to the maintenance of cardiovascular and arterial homeostasis as well as their pathologies and play a functionally significant role in processes such as immune response, tumor progression, proliferation and apoptosis (70, 82-84). At present, several promising strategies for the application of exosomes in cardiovascular therapy are under development. Stem-cell-derived exosomes have a great therapeutic potential for cardiac repair in regenerative medicine. Further, exosomes could be used as nanoparticles for targeted delivery of various bioactive molecules, such as miRNAs. For example, endothelial-specific miR-126 plays a key role in angiogenesis and vessel integrity and, therefore, could be used for vascular repair and regeneration (85).

2. HYPOTHESIS AND AIMS OF THE STUDY

As exosomes are thought to play a crucial role in cell-cell communication and this communication may occur as a result of the transfer of important molecules and genetic material, we hypothesized they have the potential to modulate signaling pathways, cell growth, migration, survival and proliferation of recipient cells and even to modulate the extracellular compartment, by affecting fibroblast synthesis of ECM proteins.

The overall aim of the study was to evaluate, *in vitro*, the effects on CPC and fibroblasts exerted by exosomes released by CPC isolated from adult human hearts.

More specific aims were to:

- isolate and characterize the composition of the cargo of exosomes delivered by CPC from both normal (Exo-CPC-N) and pathological (Exo-CPC-P) hearts in order to compare them with focus on the expression of growth factors and specific genes involved in cardiac development and differentiation.
- determine whether exosomes contain mRNA involved in cardiac development and differentiation, and if so:
- evaluate, *in vitro*, the effects elicited by the administration of Exo-CPC-N on CPC-P, in terms of cell proliferation, migration and survival;
- evaluate, *in vitro*, the effects of Exo-CPC-N on CF-P in terms of extracellular matrix deposition.

3. MATERIALS AND METHODS

3.1 CELL CULTURES

Cardiac tissue samples were obtained from normal and pathological adult human hearts. Samples of atrial appendages from normal hearts (n=11, 6 males and 5 females, mean age 35 ± 12 years) were collected from heart waste fragments of donors whose dead was not due to cardiovascular diseases, i.e. tissue trimmed off the heart while adjusting atrium size and form during organ transplantation. Pathological samples were taken from the corresponding region of explanted hearts of patients with end-stage heart failure due to ischemic cardiomyopathy and undergoing heart transplantation (n=20, 14 males and 6 females, mean age 56 ± 5,5 years, mean ejection fraction 25 ± 1%). Cardiac tissue samples were dissected, minced, and enzymatically disaggregated by incubation in 0.25% trypsin and 0.1% (w/v) collagenase II (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37ºC. The digestion was stopped by adding a double volume of Hank's balanced salt solution (HBSS) supplemented with 10% foetal bovine serum (FBS). This preparation was further disaggregated by pipetting and tissue debris and cardiomyocytes were removed by sequential centrifugation at 100g for 2 minutes, passage through 20μm sieve, and centrifugation at 400g for 5 minutes. Cell population was seeded on culture dishes in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 5% horse serum (Sigma-Aldrich), 10 ng/ml basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA), 0.2

mM glutathione (Sigma-Aldrich), 5U/L erythropoietin, 50 µg/ml porcine gelatine, 10,000 U penicillin and 10 mg/ml streptomycin (Life Technologies, Paisley, UK).

3.1.1 ISOLATION OF CARDIAC PRIMITIVE CELLS

Once the adherent cells were more than 75% confluent, they were detached with 0.25% trypsin-EDTA (Sigma-Aldrich) and cell suspension was used to isolate cardiac primitive cells by immunomagnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). In particular, cell suspension first depleted of fibroblasts, was incubated with anti-human-CD117 MicroBeads to select the positive fraction of CD117(+) CPC. Both CPC-N and CPC-P were plated at a $2x10^4/cm^2$ density and cultured with DMEM-Ham F12 medium (Sigma-Aldrich). To check effectiveness of sorting, cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature, blocked with 10% donkey serum and stained with primary antibody against human stem cell marker CD117 (mouse monoclonal, Merck Millipore, Billerica, Massachusetts, USA) Cells were then incubated with secondary antibodies conjugated with rhodamine (Jackson ImmunoResearch Europe, Newmarket, UK). Nuclei were counterstained with DAPI (Merck Millipore) and stained area of culture dish was mounted in Vectashield (Vector Labs, Burlingame, CA, USA). Microscopic analysis was performed with a Leica DMLB microscope equipped with epifluorescence EL6000 system (Leica Microsystems, Wetzlar, Germany) and connected to a digital camera DFC345FX (Leica Microsystems).

3.1.2 ISOLATION OF CARDIAC FIBROBLASTS

CF-P were isolated by outgrowth. Briefly, small fragments of specimens were placed in culture plates under cover glass and incubated with Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich) and 0.5% penicillin and streptomycin (Life Technologies) in a humidified incubator at 37°C and 5% CO_2 in air. The medium was exchanged every 2-3 days to remove cell debris and maintain a physiological pH.

3.2 ISOLATION OF EXOSOMES FROM CELL CULTURES

Once CPC-N and CPC-P were 80-90% confluent, complete medium was replaced with a serum-free medium, which was collected after 48h and processed in order to isolate exosomes by *ExoQuick-TC Exosome Precipitation Solution kit* (System Biosciences, Mountain View, CA, USA). After a centrifugation at 3000 rpm for 15 minutes at 4°C to eliminate cells and cellular debris, the medium was incubated overnight at 4°C, with a polymeric resin, specific for exosomes isolation, according to the manufacturer's protocol. The pellet, containing exosomes obtained from the two populations of cells (Exo-CPC-N and Exo-CPC-P), was used to perform specific assays to analyze the content of their cargo.

3.3. ANALYSIS OF mRNA BY REAL-TIME PCR

3.3.1 PURIFICATION AND ISOLATION OF RNA FROM EXOSOMES

Pellet was resuspended in 700 µl of *Trizol* (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA); the solution was vortexed and incubated at room temperature for 5 minutes for a complete dissolution of nucleoproteic complexes. A volume of 90 µl of chloroform was added to the resulting lysate and after a vigorous agitation and a centrifugation at 1200 rpm for 15 minutes at 4°C, the aqueous phase, holding the total RNA, was collected. The further steps of the extraction were performed using *RNeasy Micro* kit (Qiagen, Hilden, Germany) with columns equipped with high affinity silicium membranes, according to the manufacturer's protocol. The RNA obtained was resuspended in 14 µl of *RNase-free* water and quantified by spectrophotometric analysis with *Nanodrop2000* (Thermo Scientific).

3.3.2 cDNA SYNTHESIS

RNA extracted as described above, was then retrotranscribed into cDNA with *QuantiTect Reverse Transcription kit* (Qiagen). According to the manufacturer's protocol, 50 ng of each RNA were incubated for 2 minutes at 42°C with the reagent *gDNA Wipeout Buffer,* to eliminate remaining traces of genomic DNA in samples. To each sample was then added a reverse transcriptase enzyme (*Quantiscript Reverse Transcriptase*), deoxynucleotides and a mixture of *primers* containing both oligo (dT) and *random* hexamers; samples were further incubated for 20 minutes at 42°C and finally, for 3 minutes at 95 °C to deactivate

the enzyme.

3.3.3. REAL-TIME PCR

In order to assess the presence and the quantity of specific transcripts, a *Real-Time PCR* was performed using *PrecisionPLUS^{TM} MasterMix* kit (Primerdesign, Southampton, UK) according to manufacturer's protocol.

The detection was performed by measuring the binding of the fluorescent dye SYBR Green I to double-stranded DNA. DNA amplification was carried out using Mastercycler ep realplex^{4S} (Eppendorf, Hamburg, Germany); the thermal cycling conditions included an initial enzyme activation at 95°C for 2 minutes and 40 cycles consisting of a denaturation step at 95°C for 15 seconds and an annealing step at 60°C for 60 seconds. Melt curve analysis was used to assess amplification of non-specific products and uniformity of product and primer dimers formation. Comparative quantification of target genes expression in the samples was based on cycle threshold (Ct) normalized to the housekeeping gene (GAPDH), using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All the primers used in this study, listed in table 1, were designed with Primer3 software (http://frodo.wi.mit.edu) starting from the CDS (*coding sequence*) of mature mRNA available on GeneBank.

The PCR products were size-fractionated by electrophoresis in 2% agarose gel. The DNA bands were visualized and photographed under UV light exposure with FireReader XS D-55 imaging system equipped with 1D software (UVItec Limited, Cambridge, UK). To assess the length of amplicons, each product was

compared with a known molecular weight marker (BenchTop pGEM DNA marker, Promega, Madison, WI, USA).

3.4. EXOSOME LYSATES AND QUANTIFICATION OF PROTEIN CONTENT

Protein extracts were prepared from both Exo-CPC-P and Exo-CPC-N. Pellet obtained as described previously, was resuspended in 200 µl of *RIPA buffer solution* (Tris-HCl pH 7.6 25 mM, NaCl 150 mM NP-40 1%, sodium deoxyicholate 1%, SDS 0.1%) containing prothease inhibitors (1 mM DTT, 2 mM PMSF, 2 µg/ml aprotinin and 10 µg/ml leupeptin), to promote the disruption of exosomes, vortexed for 15 seconds and incubated at room temperature for 5 minutes. Protein concentration was determined by *Bio-Rad Protein Assay* (Bio-Rad Laboratories, Hercules, CA, USA) using as a standard albumin from bovine serum (Sigma-Aldrich).

3.4.1. GROWTH FACTORS ARRAY

In order to determine the content of Growth Factors in exosome cargo, protein array was performed on both Exo-CPC-P and Exo-CPC-N, using Human Growth Factor Antibody Array C1 kit (RayBio®, Norcross, GA, USA), according to the manufacturer's protocol.

The array membranes bounding primary antibodies were blocked and incubated with each sample (100 µg of protein).

After washing, the membranes were incubated with a cocktail of biotin-

conjugated antibodies, followed by an incubation with HRP-conjugated streptavidin in blocking buffer. Signals were detected by chemiluminescence and autoradiography.

3.5 EXOSOME ADMINISTRATION *IN VITRO*

3.5.1 PREPARATION AND ADMINISTRATION OF EXOSOMES CONDITIONED MEDIUM TO CPC-P

Based on results obtained with real-time PCR and growth factor array Exo-CPC-N from cardiac primitive cells isolated from normal hearts were used to prepare a conditioned medium, to test, *in vitro*, effects elicited by exosomes on both CPC-P and CF-P.

Exo-CPC-N were resuspended in specific medium at a concentration of 0,5 mg/ml and added to CPC-P and CF-P in culture. After treatment, morphology of cells was evaluated by phase contrast microscope observation with Nikon Eclipse T*i*-E DS-Qi2 Microscope (Nikon Instruments Europe, Holland).

3.5.2 EVALUATION OF PROLIFERATION AND APOPTOSIS OF CARDIAC PRIMITIVE CELLS

To evaluate proliferation and apoptosis, CPC-P treated with Exo-CPC-N and CPC-P not treated with Exo-CPC-N (control group) were stained with Cell-Clock Cell Cycle Assay (Biocolor, UK) and Cell-ApoPercentage Apoptosis Assay (Biocolor), respectively, according to the manufacturer's protocol. In particular, the first assay is a live-cell detection system employed to monitor the four major phases of the mammalian cell cycle during *in vitro* culture. Cells were

incubated at 37°C for 1 hour with a redox dye, taken up by live cells. Following dye uptake and incubation, a distinct color change occurs within cells, with particular color changes being associated with cells in the G1, S, G2 and M phases of the cycle.

Cell-ApoPercentage Apoptosis Assay, instead, is a detection and measurement system to monitor the occurrence of apoptosis in mammalian, anchorage-dependent cells during *in vitro* culture. CPC-P with or without Exo-CPC-N were first incubated with 3% hydrogen peroxide in the complete medium for 12 hours and stained with the ApoPercentage dye that is selectively imported by cells that are undergoing apoptosis.

Microscopic analysis was performed with a Leica DM2000 LED microscope (Leica Microsystems) equipped with a digital camera Leica ICC50 HD (Leica Microsystems). Data were expressed as mean percentage of cycling cells over total cells $\pm$ SEM for Cell-Clock Cell Cycle Assay, and as mean percentage of stained cells over total cells for Cell-ApoPercentage Apoptosis Assay.

3.5.3 EVALUATION OF MIGRATION OF CARDIAC PRIMITIVE CELLS

To evaluate the speed of migration of CPC-P in the absence (control group) or presence of Exo-CPC-N, scratch wound assay was performed. Briefly, cells were grown to confluence and a thin scratch was produced in straight line on culture plates with a 10 µl pipette tip, leaving a cell-free zone. Plates were placed under Nikon Eclipse T*i*-E DS-Qi2 Microscope (Nikon Instruments Europe) equipped with stage incubator (Okolab, Pozzuoli, Italy) and the migration was documented

acquiring one picture every 10 minutes for 8 hours. Data were analysed by NIS software (Nikon) and expressed as mean speed of migration ± SEM.

3.5.4 EVALUATION OF MATRIX DEPOSITION BY FIBROBLASTS

In order to evaluate effects of Exo-CPC-N on the deposition of ECM, CF-P treated with Exo-CPC-N and CF-P not treated with Exo-CPC-N (control group) were fixed in 4% paraformaldehyde for 20 minutes at room temperature. After blocking with 10% donkey serum, plates were first incubated with primary antibody against human fibronectin (rabbit polyclonal, Sigma-Aldrich), human collagen I (mouse monoclonal, Sigma-Aldrich), collagen III (mouse monoclonal, Sigma-Aldrich), collagen IV (rabbit monoclonal, Sigma-Aldrich) human laminin (mouse monoclonal, Sigma-Aldrich), and tenascin (mouse monoclonal, Sigma-Aldrich), for 1 hour at 37°C and then were incubated for 1 hour at 37°C with secondary antibodies conjugated with fluorescein or rhodamine (Jackson ImmunoResearch Europe). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (Merck Millipore) and stained area of culture dish was mounted in Vectashield (Vector Labs). Microscopic analysis was performed with Nikon Eclipse T*i*-E Microscope DS-Qi2 (Nikon Instruments Europe) by three independent observers. Synthesis and deposition of ECM proteins was compared in treated and untreated using an arbitrary scale ranging from negative (-) in case of immunonegativity, to triple positive (+++), in case of strong immunopositivity.

3.6 STATISTICAL ANALYSIS

All numerical data are presented as mean ± SEM. Statistical differences between groups were evaluated with Student's two-tailed unpaired t-test. A value of $p<0.05$ was considered statistically significant.

4. RESULTS

4.1 CELL CULTURES

Observation by phase contrast microscope provided images of primary cell cultures (Fig 4) obtained from normal (A) and pathological (B) adult human hearts. Phase contrast microscope image of CF-P are shown in Fig 5.

4.1.1 CHARACTERIZATION OF CPC POPULATION

Immunofluorescence for CD117 confirmed the purity of cell population obtained by immunomagnetic cell sorting from primary cardiac cell culture (as described in Materials and Methods section 3.1.1). Purity of sorted cells reached 98% (Fig 6). Phase contrast microscope images show sorted populations of both CPC-N **(**Fig 7 A) and CPC-P **(**Fig 7 B).

4.2 REAL-TIME PCR

The pellet obtained after centrifugation of medium collected from CPC-N and CPC-P in culture, was significant and sufficient to extract RNA.

qPCR analysis of Exo-CPC cargo revealed that Exo-CPC-N and Exo-CPC-P carry specific transcripts for proteins involved in cardiac and mesenchymal differentiation.

Notably, exosomes delivered by both CPC-N and CPC-P transport specific transcripts for cardiac cell progenitors or precursors of cardiomyocyte, as Mef2C, Nkx2.5 and Alpha Sarcomeric Actin (α-SARC), and smooth muscle cells

(SMCs), as GATA6 and Smooth Muscle Actin (SMA). Further, they also carry transcripts for mesenchymal cells, as TM4SF1, SOX9, CD90 and CD106 (Fig 8). No transcripts were found for proteins that prompt endothelial lineage differentiation, like FVIII and Ets-1. Comparing expression levels obtained with the $2^{-\Delta\Delta Ct}$ method for mRNAs from Exo-CPC-N and EXO-CPC-P, statistically significant differences only emerged for Mef2C (fold change 0.32 $\pm$ 0.04) and Nkx2.5 (fold change 0.25 $\pm$ 0.00), mainly carried by Exo-CPC-N, and for SMA (fold change 8.63 $\pm$ 0.8) and TM4SF1 (fold change 3.08 $\pm$ 0.14) mainly carried by Exo-CPC-P (Fig 9).

4.3 GROWTH FACTORS ARRAY

Protein array performed to analyze growth factor cargo of Exo-CPC, revealed differences in the content of Exo-CPC-N and Exo-CPC-P. The comparison of the two membranes clearly showed that Exo-CPC-N carried factors mostly involved in biological processes like cell proliferation and differentiation, as EGF (Epidermal Growth Factor), FGF-6 and FGF-7 (Fibroblasts Growth Factors-6 and 7). Further, they show a considerably higher amount of IGF-1 (Insulin Like Growth Factor) which is a potent modulator of stem cell replication, commitment to the myocyte lineage and differentiation, and SCF (Stem Cell Factor) responsible for preserving stem cells characteristics and guiding them to niches. On the contrary, they did not carry factors involved in tumorigenesis and angiogenesis processes, as TGF (Transforming Growth Factor), HGF (Hepatocyte Growth Factor) nor VEGF (Vascular Endothelial Growth Factor) at

all, clearly present in Exo-CPC-P. Additionally, a higher expression of receptors VEGFR2, VEGFR3 and SCFR in Exo-CPC-P emerged. The assay did not reveal, in Exo-CPC-N, the presence of receptors like SCFR, EGFR, PDGFRα and PDGFRβ, essential for mediation of signals and cell/tissue response (Fig 10).

4.4 PROLIFERATION AND APOPTOSIS OF CPC

After treatment with specific dye, microscopic analysis of CPC-P in culture with or without Exo-CPC-N revealed that although morphology did not differ significantly (Fig 11; A, B; Fig 12; A, B), the presence of Exo-CPC-N clearly affects both proliferation and apoptosis. Notably, CPC-P treated with Exo-CPC-N showed higher proliferation rate compared to control group, as indicated by the ratio of cells in S to M phases (Fig 11; C, D). In the presence of Exo-CPC-N the proportion of proliferating cells in M phase reached a percentage of 20.52 $\pm$ 4.43%, while the control group showed a lower amount of proliferating cells in the same phase, equal to 1.068 $\pm$ 0.79%. The percentage of cells in G2-S phase was 14.19 $\pm$ 0.63% in CPC-P treated with Exo-CPC-N, against a 27.48 $\pm$ 2.13% observed in CPC-P untreated. No statistically significant differences were observed for cells in G1 phase, that showed a percentage of 65.28 $\pm$ 5.04 and 71.45 $\pm$ 2.42% for CPC-P treated with Exo-CPC-N and untreated, respectively (Fig 11 E).

From microscopic analysis also appeared that the presence of Exo-CPC-N prevented CPC-P apoptosis induced by oxidative stress, as demonstrated by the lower amount of nuclei stained with the dye, selective for apoptotic cells (Fig 12;

C, D). In fact, oxidative stress, *in vitro*, induced apoptosis in only 2.25 ± 0.31% in case of CPC-P treated with Exo-CPC-N, while untreated CPC-P resulted more susceptible to apoptosis, showing a higher percentage of apoptotic nuclei, equal to 26.74 ± 8.08% (Fig 12 E).

4.5 MIGRATION OF CPC

The scratch wound assay performed, is a simple, reproducible assay commonly used to measure basic cell migration parameters such as speed, persistence and polarity. Cells at the wound edge polarize and migrate into the wound space. Cell migration was monitored in real time by time-lapse imaging which can also yield valuable cell morphology and localization. Scratched area of cell monolayer was recorded every 10 minutes for 8 hours (Fig 13). Results obtained indicated that migration is not affected by administration of Exo-CPC-N, as the speed of migration appeared almost the same in CPC-P treated with exosomes (11.28 ± 1.34) compared to control group (11.43 ± 1.31) (Fig 13 E). After the scratch, cell migration was observed both in CPC-P untreated and treated with Exo-CPC-N, but full wound healing only occurs after 8 hours for both cell populations (Fig 13; C, D).

4.6 MATRIX DEPOSITION BY FIBROBLASTS

Immunostaining performed on CF-P showed effects exerted by Exo-CPC-N on different extracellular matrix proteins, their deposition and synthesis (Fig 14). In general, when cultured in Exo-CPC-N conditioned medium, CF-P exhibited less

elongated morphology. From microscopic observation CF-P treated with Exo-CPC-N showed a stronger immunopositivity for collagen IV (Fig 14 F), while no significantly differences were found for collagen I and III, all proteins providing tensile strength and structural integrity, but also playing a role in cell adhesion and migration. Fibronectin, that influences cell growth and response to injury, like wound repair, shows a dramatic higher expression and well-arranged pattern in treated CF-P compared to control group, as indicated by the more intense emission of fluorescence (Fig 14; D, H). On the contrary, synthesis of laminin, important for activating specific signaling networks governing adhesion, migration and differentiation, decreased in the presence of Exo-CPC-N (Fig 14; G). Finally, tenascin, associated with cell migration, especially fibroblast migration and epithelial-mesenchymal/mesenchymal-epithelial transition did not show consistent differences in the presence or absence of Exo-CPC-N in CF-P in culture. Differences observed for synthesis and deposition of collagen IV, laminin and fibronectin are summarized in Tab 2.

5. DISCUSSION AND CONCLUSION

Stem cells or progenitor cells are presently our best therapeutic candidate to treat intractable degenerative or genetic diseases. In fact, many of these stem or progenitor cells are being tested in clinical trials to treat many different diseases such as acute myocardial infarction. The hypothesis that stem cells could exert therapeutic activity through their secretions is highly plausible as stem cell secretions are known to include many biologically potent molecules such as growth factors, cytokines, chemokines, and bioactive lipids that could elicit wide-ranging physiological effects (86). Growing evidence suggest that adult stem cells may exert dramatic effects in the repair of various tissues through secreted factors including exosomes and EVs (87). Indeed, up to 80% of the therapeutic activities of adult stem cells in infarcted hearts have been shown to occur through paracrine mediated effects (88), giving rise to the novel notion of stem cell therapy without cells (87). Exosomes are secreted by cardiac and vascular cells and stem cells in culture (89, 90-92). Moreover, exosomes have been shown to mediate communication between endothelial cells (ECs) and SMCs (93), ECs and pericytes (94), CMs and ECs (95) and fibroblasts and cardiac myocytes CMs (96). Recently, Sahoo and Losordo (77) provided ultrastructural evidence of exosome-like vesicles packed in MVBs of a CPC in a healthy mouse heart, as well as in the cytoplasm of CMs from the left ventricle of a healthy human and from a patient with ischemic heart disease. Very few studies, including the present study, have investigated the therapeutic potential of

human Exo-CPC and characterized the content of their cargo. In particular, we determined the presence of specific growth factors in Exo-CPC-N and Exo-CPC-P. Protein array for growth factors revealed remarkable differences between the content of Exo-CPC-N and Exo-CPC-P suggesting that exosomes secreted by CPC-N could be the best candidate to treat cardiac diseases. As a matter of fact, we found that Exo-CPC-N carry growth factors involved in biological processes like cell proliferation, development, differentiation and migration (EGF, FGF-6, FGF-7, IGF-1) (97), and the SCF, exerting a crucial role in maintenance of stem cells compartment. All these factors could potentially boost cardiac repair mainly through endogenous stem cell homing and activation. Exo-CPC-P, instead, transport considerably higher amount of growth factors mainly involved in tumorigenesis and angiogenesis (TGF, HGF, VEGF), strengthening the hypothesis that cardiac disease affect CPC compartment too, and that Exo-CPC-N are preferable candidates for treating cardiovascular diseases. However, CPC-P transport higher amount of receptors like VEGFR2, VEGFR3 and SCFR, stimulating angiogenesis, essential to prevent heart failure through the control of cardiomyocyte hypertrophy and contractility and ensuring promptness to respond to SCF stimulation. In addition to growth factors, exosomes are lipid vesicles, which represent ideal vehicles to deliver genetic materials, such as mRNA, from one cell to another, and fulfill their function. In recent years, many published reports suggest that in addition to cancers and neurodegenerative disorders, major cardiovascular and metabolic pathologies like coronary artery disease, myocardial infarction and heart failure are highly influenced by the exosome-

directed transfer of molecules (98-100). It has been demonstrated that exosomally delivered mRNAs and miRNAs are translated and regulate gene expression of acceptor cells influencing their biology (101). Our findings by Real-time PCR analysis of Exo-CPC cargo revealed the presence of transcripts specific for cardiac or mesenchymal differentiation in Exo-CPC-N as well as in Exo-CPC-P. A primary obstacle to functional recovery of the infarcted or failing human heart is the limited proliferative capacity of cardiomyocytes and their insufficient mechanism for regeneration. The loss of cardiomyocytes after injury, in fact, cannot be compensated and a strategy to replace them could be inducing resident CPC to differentiate to new, healthy cardiomyocytes. Hence, Exo-CPC-N, seem to be suitable candidates for a therapeutic approach, as they show a best potential of delivering signals to prompt differentiation to cardiomyocyte, expressing higher levels of Nkx2.5 and Mef2c, while in Exo-CPC-P we found signals inducing mostly smooth muscle or mesenchymal cell differentiation, as they expressed high levels of SMA and TM4SF1. Additionally, the delivery of CPC-N secreted factors to CPC-P might lead CPC-P ahead of the differentiation block which prevents progenitors to proceed towards precursor state, already documented (102). The diversity of this EV content provides a rationale for the many reported stem cell-based therapeutic outcomes. With increasing evidence that EVs are major mediators of intercellular communication in many cell types (103, 104), it is likely that EVs also perform similar functions for stem cells. As such, EVs are likely to support the hypothesis that stem cells exert therapeutic activity through their secretions by communicating therapeutic signals from stem

cells to recipient cells to initiate repair and regeneration. EVs from CPC have also been reported to be efficacious in cardiovascular disease; in particular, they inhibited cardiomyocyte apoptosis and improved cardiac function after MI in animal models, ameliorated the deleterious consequences of myocardial ischemia, and enhanced cardiogenesis (105-108). Further, exosomes enhanced endothelial migration and were also shown to reduce myoblast apoptosis *in vitro* and decrease myocyte cell death in an animal MI model (106). Here, we tested, *in vitro*, potential effects exerted by Exo-CPC-N on capacity for migration, proliferation and susceptibility to apoptosis of CPC-P. On the basis of results obtained from analysis of Exo-CPC cargo, we considered plausible exclusively administrating Exo-CPC-N to CPC-P and CF-P. A similar speed of migration was observed for CPC-P either in the presence or absence of Exo-CPC-N, probably due to growth factors enhancing migration, found both in Exo-CPC-N (IGF1 and FGF6) and Exo-CPC-P (HGF). We also reported that, even if morphology did not differ significantly, probably due to presence of specific growth factors, CPC-P treated with Exo-CPC-N conditioned medium had an increased proliferation rate, as demonstrated by the ratio of cells in active phases of cell cycle. At the same time, it was apparent that Exo-CPC-N could represent cardioprotective factors for CPC-P, preventing apoptosis induced by oxidative stress.

It is well-researched that the fate of the cell is determined by coordinated and dynamic interactions among a number of factors, acting in a defined microenviroment. In particular, stem cells are highly sensitive to extracellular

signals that play a critical role in maintenance of stem cells characteristics, differentiation, and interplay with somatic cells (109). Cellular cross talk plays a central role both in cardiac homeostasis and in adaptive responses of the heart to stress. Chronic adaptive responses to stress, referred to as cardiac remodeling, include interstitial fibrosis, CMs hypertrophy, and changes in contractility and blood vessel density (110). Post MI, the proliferation of fibroblasts lead to the formation of non-contractile scar tissue (111), which, when combined with the extensive cardiomyocyte death (77), leads to long-term systolic dysfunction. In the damaged heart, fibroblasts are stimulated by cytokines such as TGF-β, which leads to exacerbation of extracellular matrix production (112), and enhanced fibrosis (113). CPC have been shown to exert potentially anti-fibrotic effects by transferring exosomes to fibroblasts and by promoting cardiac myocyte survival *in vitro* (94). In this study, we investigated if Exo-CPC-N produced any changing in the synthesis and deposition of ECM by CF-P, and if any difference occurred analyzing composition of ECM in terms of proteins and comparing them to the control group. ECM account for approximately 24% of myocardial volume and network rearrangement and enlargement is an essential component of cardiac remodeling at various pathological stages (114). The ECM elasticity determines stem cell lineage specification, expansion and differentiation (115) and (116), in the heart, ECM exhibits all the mechanical properties and provides a framework required for differentiation of cardiac stem cells (117). In any necrotic tissue, fibronectin and collagen deposition and disarrangement is responsible for scar formation, which preserves wall integrity and thickness.

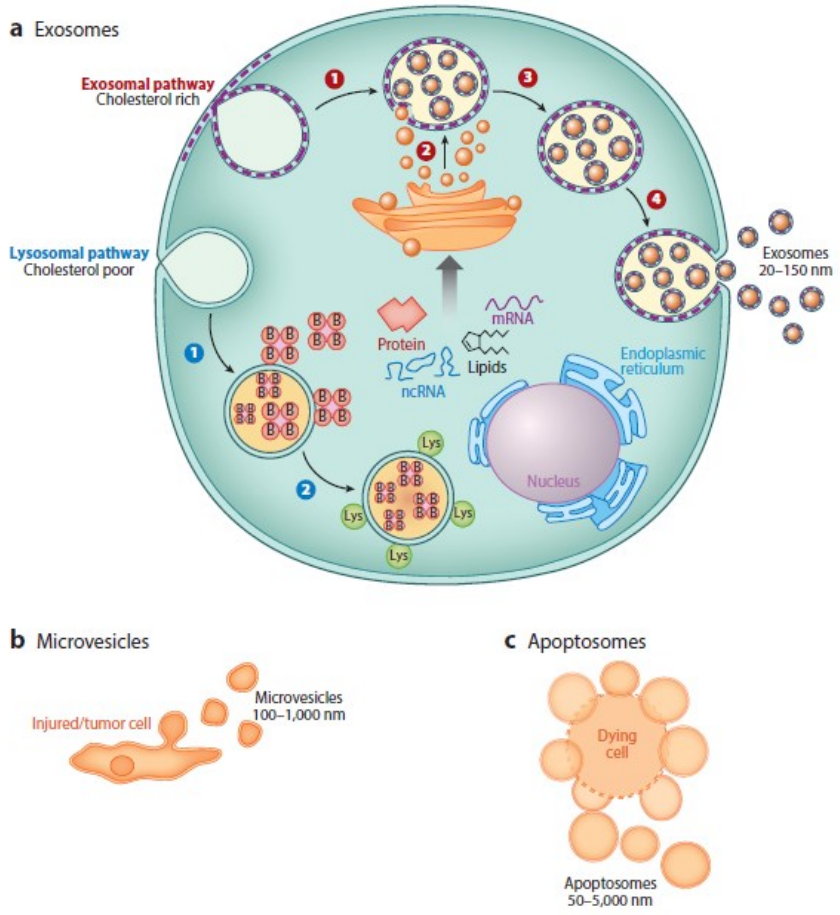
These proteins present unique expression pattern, with high levels during organogenesis, virtual absence in normal adult tissue, and re expression in response to injury and tissue regeneration. In the heart, osteopontin, osteonectin, thrombospondin, tenascin, and CNN family are the matricellular proteins identified so far (118), but laminin-1 also fulfills the requirements (119). Given these properties, extracellular matrix, and matricellular proteins in particular, can drive cardiac tissue regeneration, described in adult human heart in infarction or pressure overload (120). From the observation of CF-P in culture with Exo-CPC-N conditioned medium emerged differences in the deposition of collagen IV and fibronectin, while synthesis of collagen I and collagen III remained almost constant when compared to the control group. Interestingly, synthesis of laminin seemed to slightly decrease in CF-P treated with Exo-CPC-N. Moreover, Exo-CPC-N seem to revert fibronectin disarrangement *in vitro*, conferring to mentioned ECM protein deposit an ordered and organized pattern. These changings could validate our hypothesis that Exo-CPC-N could positively affect the extracellular compartment during the response to injury.

There is now ample evidence of the effective recapitulation of the therapeutic efficacy of stem cells by their secreted EVs. This renders stem cell-derived extracellular vesicles a compelling alternative off-the-shelf, cell-free therapeutic modality that could be effective, safer, and cheaper. However, realizing this promising therapeutic modality of stem cell EVs would require extensive testing to validate their safety and efficacy (121).

The use of stem cell-derived EVs instead of stem cells engraftment might open new perspectives for cardiac regenerative therapies. This would first resolve safety concerns related to uncontrolled dissemination of transplanted cells or aberrant stem cell differentiation. In addition, immunosuppressive effects reported for certain EVs could favor efficient cardiac tissue regeneration (122). Moreover, since exosomes could be easily stored and keep their biological properties over an extended storage period, they can overcome many of the limitations linked to the use of viable cells in regenerative medicine. However, further investigations would be needed to optimize their characterization, quality, and purification in order to accurately control their production in the perspective of clinical uses (123).

Cell-based therapy for cardiovascular disorders experiences only limited success due to cells retention and engraftment issues as well as potential immunogenicity. Exosome-based treatment options, however, provide cell-free therapeutic candidates that offer to provide comparable benefits. One of the most active areas of translational research is to identify the most effective cell type and enviroment for generation of most effective cardio-protective and cardio-regenerative exosomes (89, 124, 125).

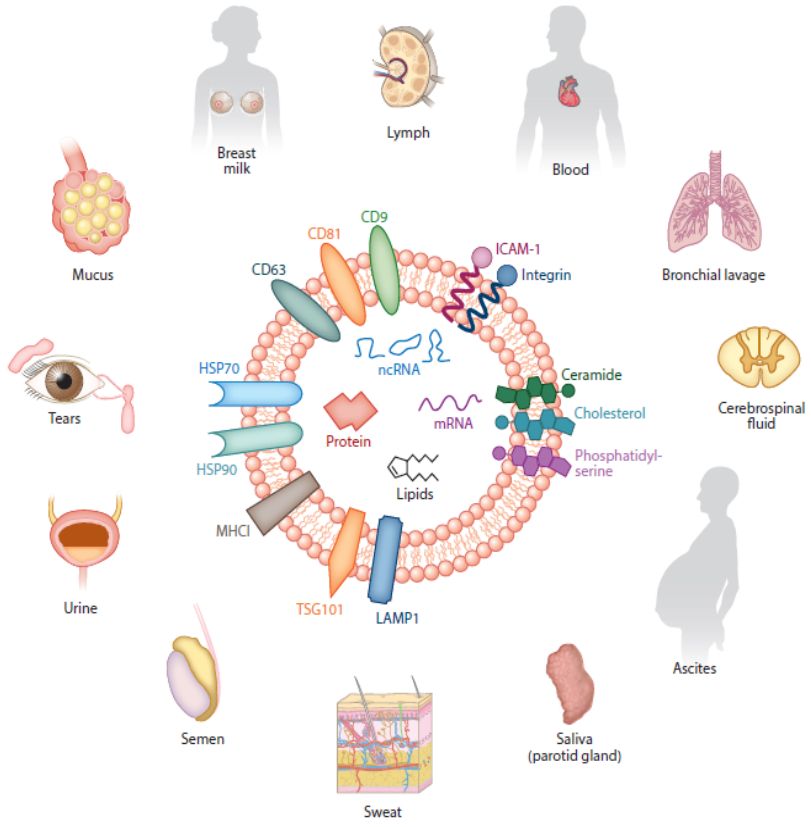
6. ICONOGRAPHY



A. Ibrahim and E. Marban, Annual Review of Physical Chemistry; 2016

Fig. 1

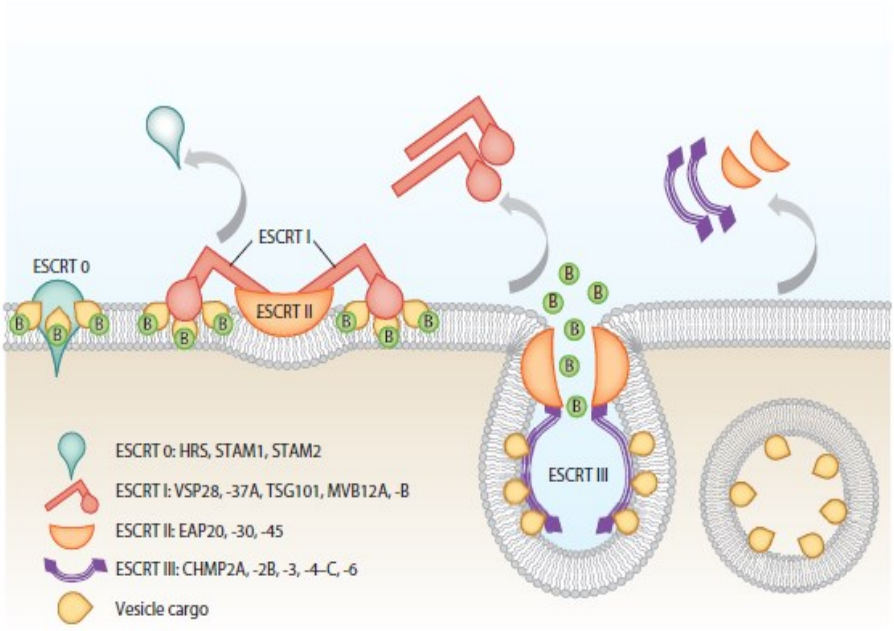
Formation of two different multivesicular bodies (MVBs) through invagination of the plasma membrane (**a**). Steps 1–4 (red): Invagination of the plasma membrane to form a secretory endosome (1), followed by budding of payload into the endosomal membrane to form multivesicular endosomes (2). Maturation of the late endosome through acidification (3) triggers fusion with the plasma membrane and release of exosomes (4). Representation of microvesicles (**b**) andapoptotic bodies (**c**).



A. Ibrahim and E. Marban. Annual Review of Physical Chemistry: 2016

Fig. 2

Exosomes are nanosized particles with a diameter range of 30–100 nm, enriched in various types of lipids, and secreted by all cell types. Markers ubiquitous in most exosomes include tetraspanins (CD9, CD63, and CD81); heat shock proteins; adhesion molecules; and markers of the ESCRT (endosomal sorting complexes required for transport) pathway, including LAMP1 and TSG101.



A. Ibrahim and E. Marban, Annual Review of Physical Chemistry; 2016

Fig. 3

Image representing the four ESCRT complexes. ESCRT 0 complex proteins (B represents a ubiquitin group bound to a protein substrate) recognize ubiquitinated protein and recruit ESCRT I, which in turn recruits ESCRT II and triggers membrane involution. ESCRT III complexes cause further invagination of the membrane and ultimately both membrane abscission and budding of the proteins into the lumen of the endosome and the elimination of ubiquitin outside the endosome.

REAL-TIME PRIMERS		
GENE	**FORWARD PRIMER** **REVERSE PRIMER**	**AMPLICON LENGTH (nt)**
GAPDH	5'-CTCTCTGCTCCTCCTGTTCG-3' 5'-ACGACCAAATCCGTTGACTC-3'	114
Mef2C	5'-AGGCAGCAAGAATACGATGC-3' 5'-TACGGAAACCACTGGGGTAG-3'	88
ACTC1 (α-Sarcomeric Actin)	5'-TCGGGACCTCACTGACTACC-3' 5'-TACGGAAACCACTGGGGTAG-3'	125
NKX2.5	5'-ACTTGAATGCGGTTCAGAGC-3' 5'-GAGTCAGGGAGCTGTTGAGG-3'	137
ETS1	5'-TGGGGACATCTTATGGGAAC-3' 5'-TGGATAGGCTGGGTTGACTC-3'	88
FVIII	5'-GCTCTGGGATTATGGGATGA-3' 5'-TCTTGAACTGAGGGACACTGC-3'	80
GATA6	5'-GTGTGCAATGCTTGTGGACT-3' 5'-TGTTCTTAGGTTTTCGTTTCCTG-3'	103
ACTA2 (Smooth Muscle Actin)	5'-CTGAGCGTGGCTATTCCTTC-3' 5'-CTGAGCGTGGCTATTCCTTC-3	133
CD90	5'-CTAGTGGACCAGAGCCTTCG-3' 5'-GCCCTCACACTTGACCAGTT-3'	198
CD106	5'-AAAATGGAAGATTCTGGGGTT-3' 5'-TTGACACTCTCAGAAGGAAAAGC-3'	134
TM4SF1	5'-ATGCCTCCGAAAACCACCTC-3' 5'-GCACATCGTTTGCCACAGTT-3'	157
SOX9	5'-CCAACGCCATCTTCAAGG-3' 5'-CCAACGCCATCTTCAAGG-3'	141

Tab. 1

List of primer sequences used for Real-time PCR analysis.

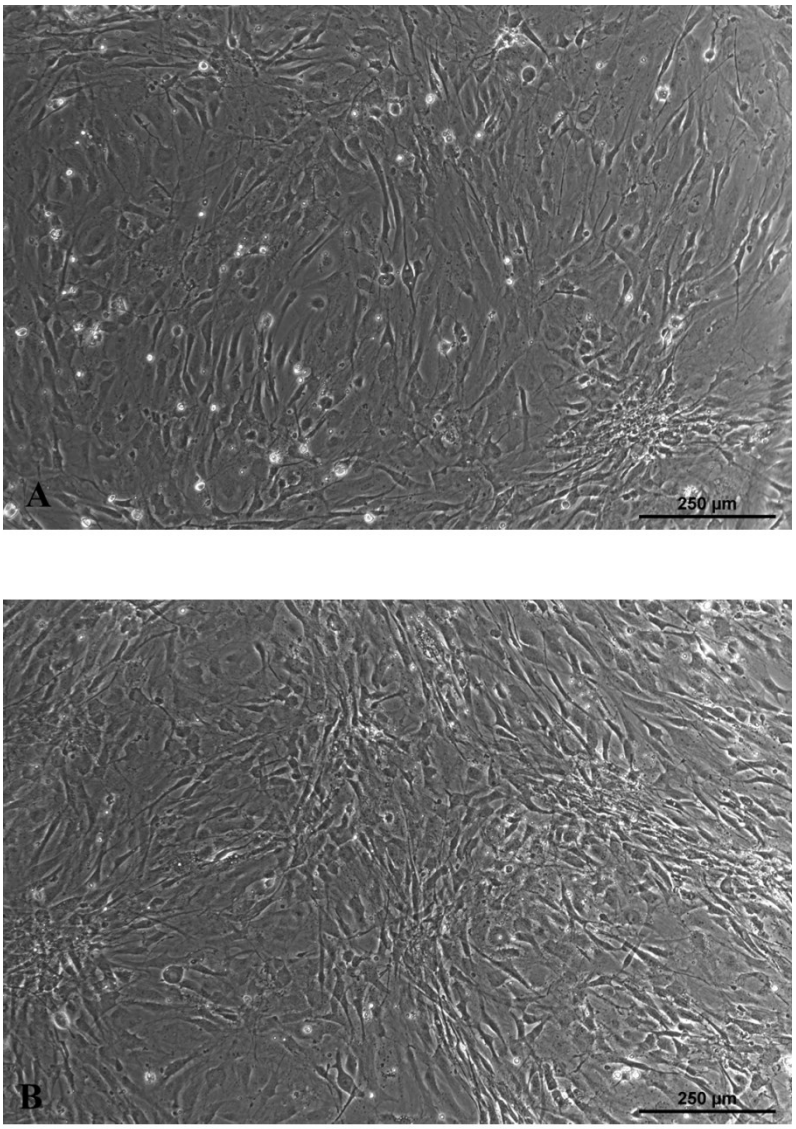


Fig. 4

Phase contrast microscope images of primary cell cultures obtained from normal **(A)** and pathological **(B)** adult human hearts.

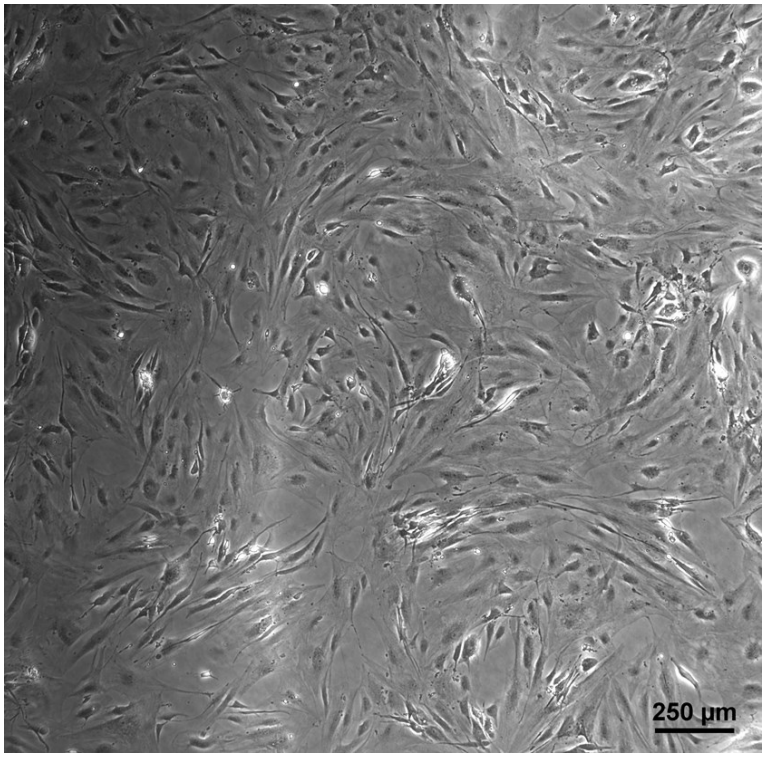


Fig. 5

Phase contrast microscope image of CF-P in culture.

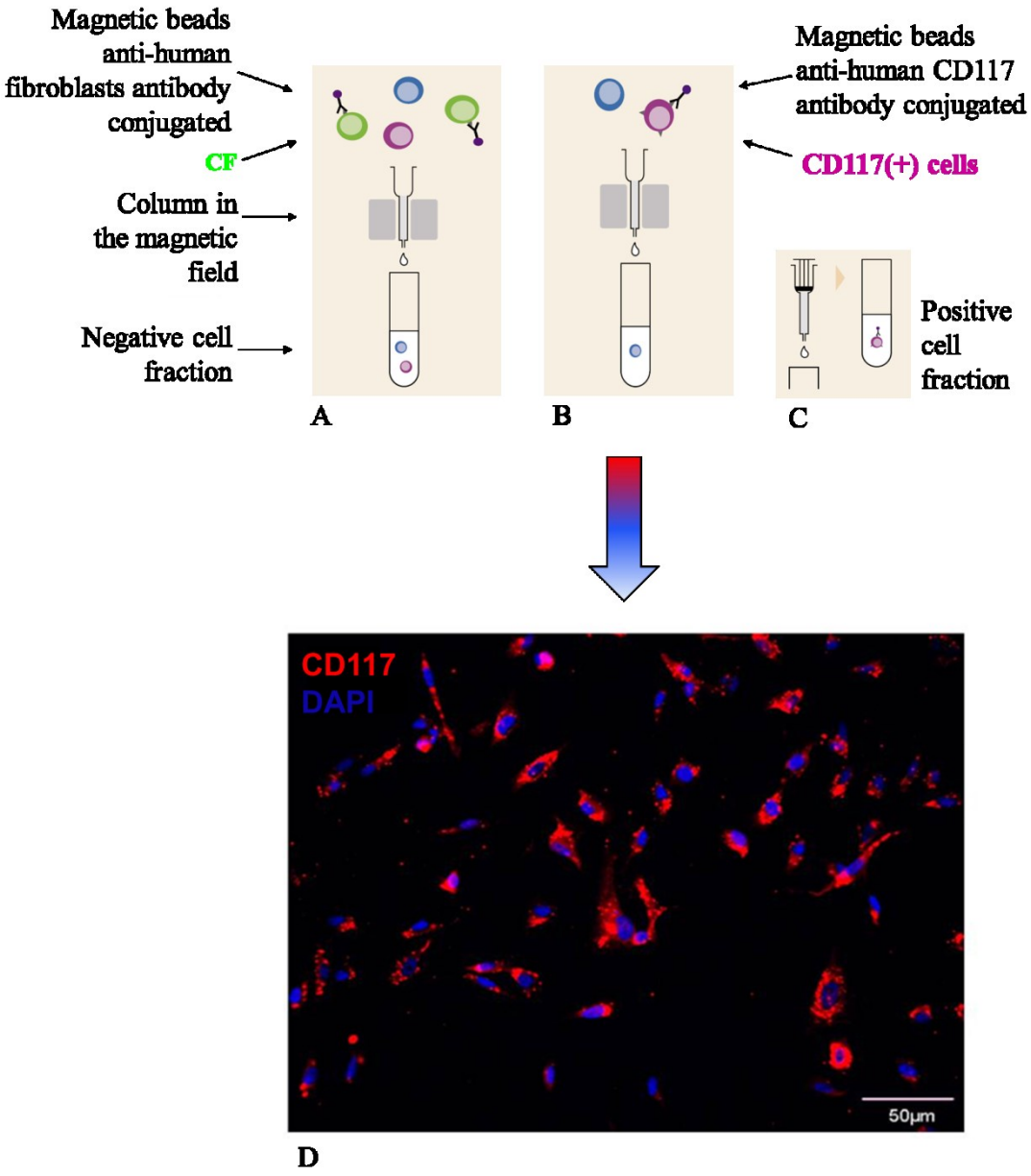


Fig. 6

Immunomagnetic cell sorting: preventive cardiac fibroblasts depletion from primary culture **(A)** and CD117(+) cardiac primitive cells isolation **(B-C).** Fluorescence microscope images of CD117(+) cardiac primitive cells **(D)**.

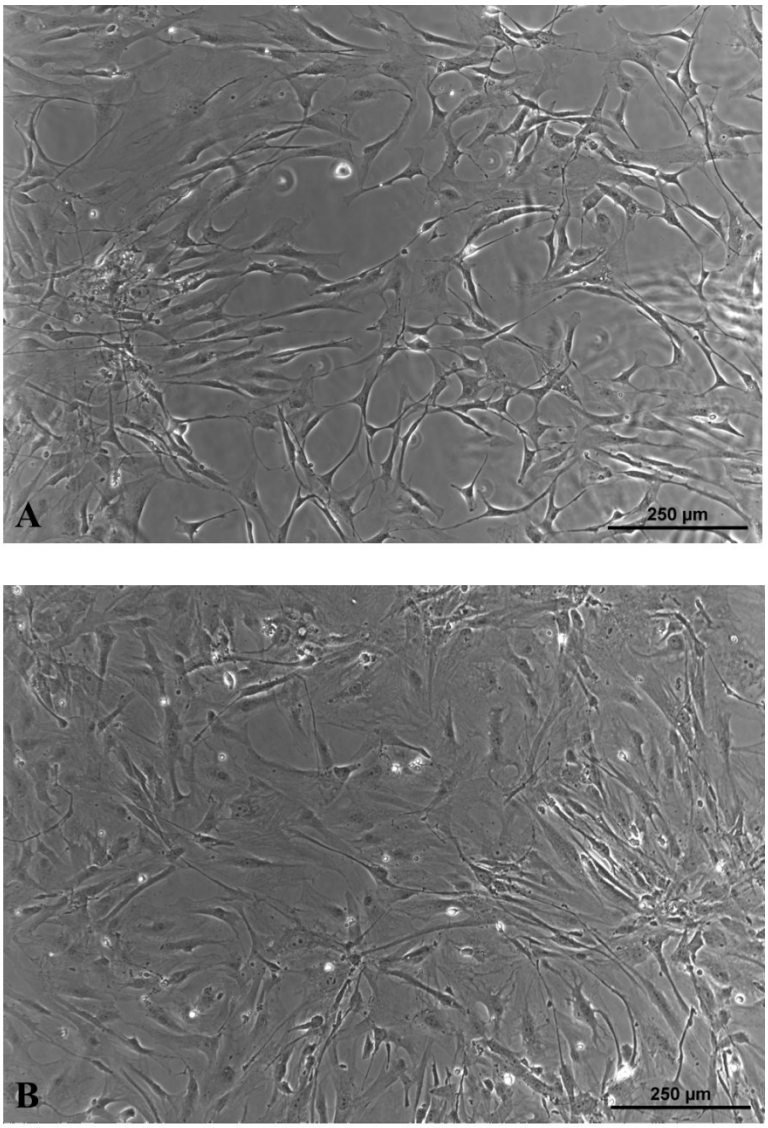


Fig. 7

Phase contrast microscope images of CPC-N **(A)** and CPC-P **(B)** in culture.

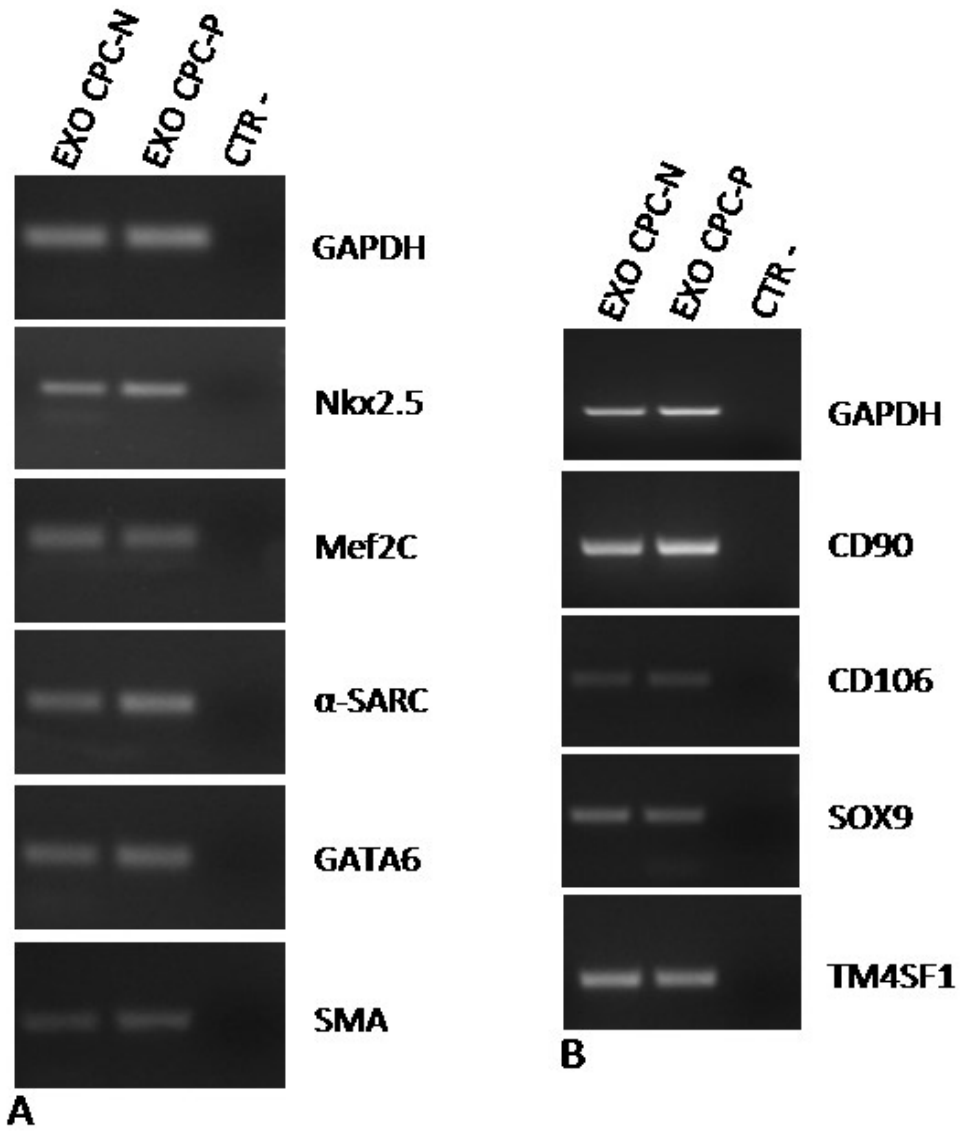


Fig. 8

Agarose (2.5%) gel electrophoresis of PCR products. **(A)** Cardiac differentiation transcripts; **(B)** Mesenchymal differentiation transcripts.

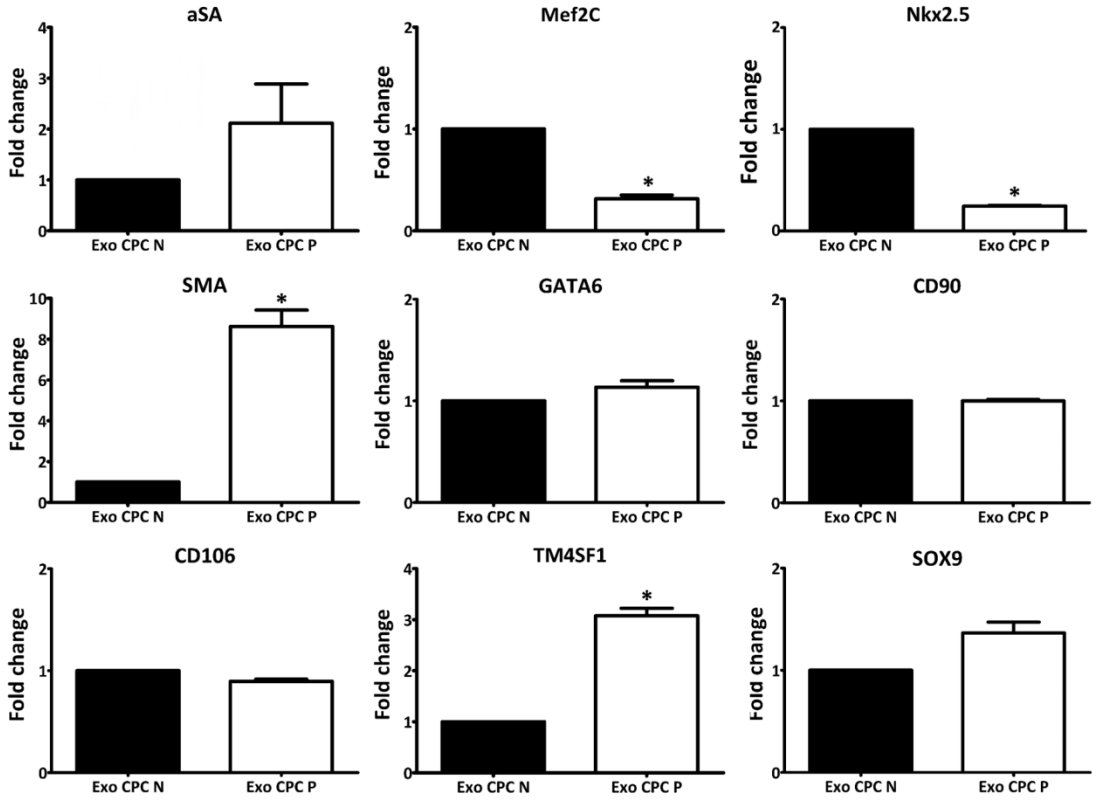


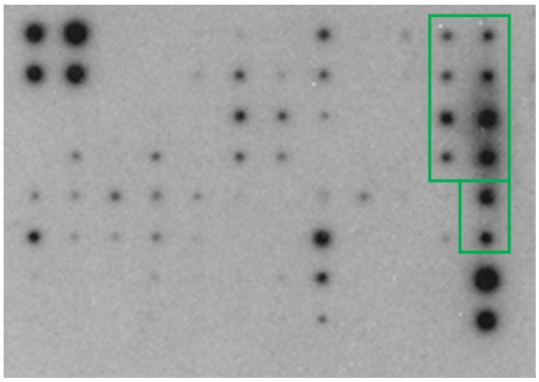
Fig. 9

Expression levels of mRNAs from Exo-CPC-N and Exo-CPC-P. Statistically significant differences, indicated by * ($p<0.05$), only emerged for MEF2C, Nkx2.5, SMA and TM4SF1.

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	AREG	bFGF	b-NGF	EGF	EGFR	FGF-4	FGF-6	FGF-7
2	POS	POS	NEG	NEG	AREG	bFGF	b-NGF	EGF	EGFR	FGF-4	FGF-6	FGF-7
3	G-CSF	GDNF	GM CSF	HB EGF	HGF	IGFBP 1	IGFBP 2	IGFBP 3	IGFBP 4	IGFBP 6	IGF-1	IGF-1 sR
4	G-CSF	GDNF	GM CSF	HB EGF	HGF	IGFBP 1	IGFBP 2	IGFBP 3	IGFBP 4	IGFBP 6	IGF-1	IGF-1 sR
5	IGF-2	M-CSF	M-CSF R	NT-3	NT-4	PDGF R alpha	PDGF R beta	PDGF AA	PDGF AB	PDGF BB	PLGF	SCF
6	IGF-2	M-CSF	M-CSF R	NT-3	NT-4	PDGF R alpha	PDGF R beta	PDGF AA	PDGF AB	PDGF BB	PLGF	SCF
7	SCF R	TGF alpha	TGF beta	TGF beta 2	TGF beta 3	VEGF	VEGF R2	VEGF R3	VEGF D	BLANK	BLANK	POS
8	SCF R	TGF alpha	TGF beta	TGF beta 2	TGF beta 3	VEGF	VEGF R2	VEGF R3	VEGF D	BLANK	BLANK	POS

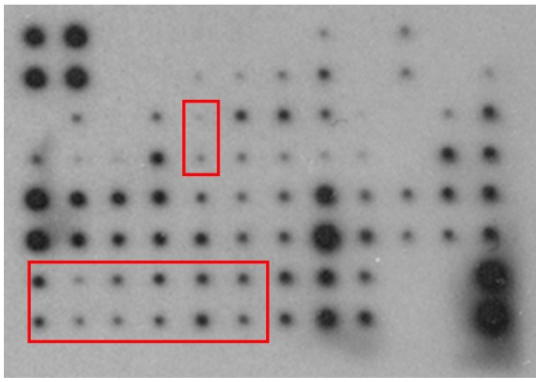
A

Exo-CPC-N



B

Exo-CPC-P



C

Fig. 10

Growth Factor array analysis of Exo-CPC-N and Exo-CPC-P cargo. Table of searched Growth Factors **(A)**; membranes with spots representing Growth Factors carried by Exo-CPC-N **(B)** and Exo-CPC-P **(C)**.

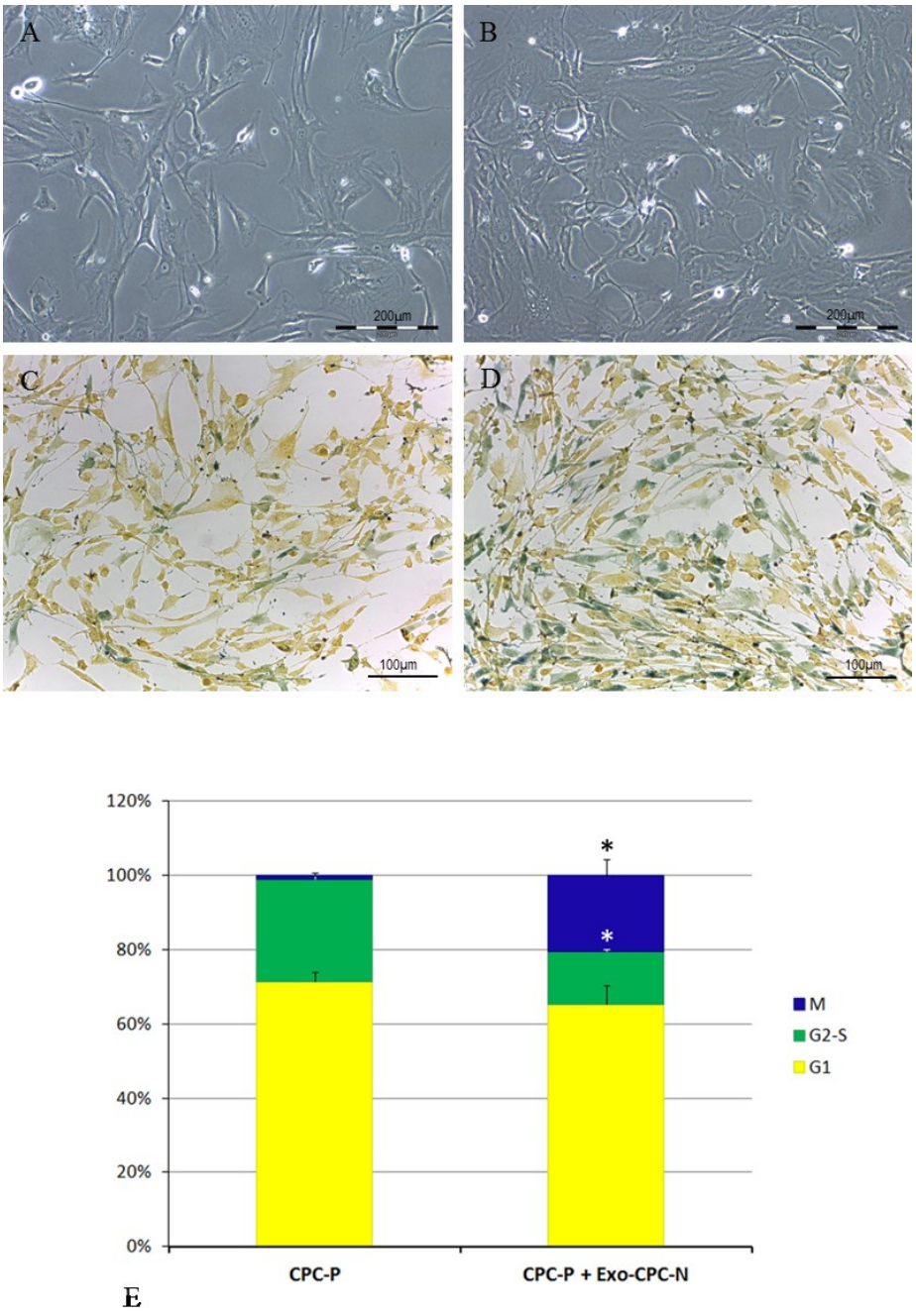


Fig. 11

Microscopic images of vital stainings of CPC-P in culture, either in the absence **(A, C)** or presence **(B, D)** of Exo-CPC-N. Phase contrast images of CPC-P **(A, B)**; cells in G1-S-G2-M phase of cell cycle **(C, D)**. Proliferative **(E)** rates of CPC-P treated with Exo-CPC-N and CPC-P untreated (control group). Statistically significant results are indicated by * ($p<0.05$).

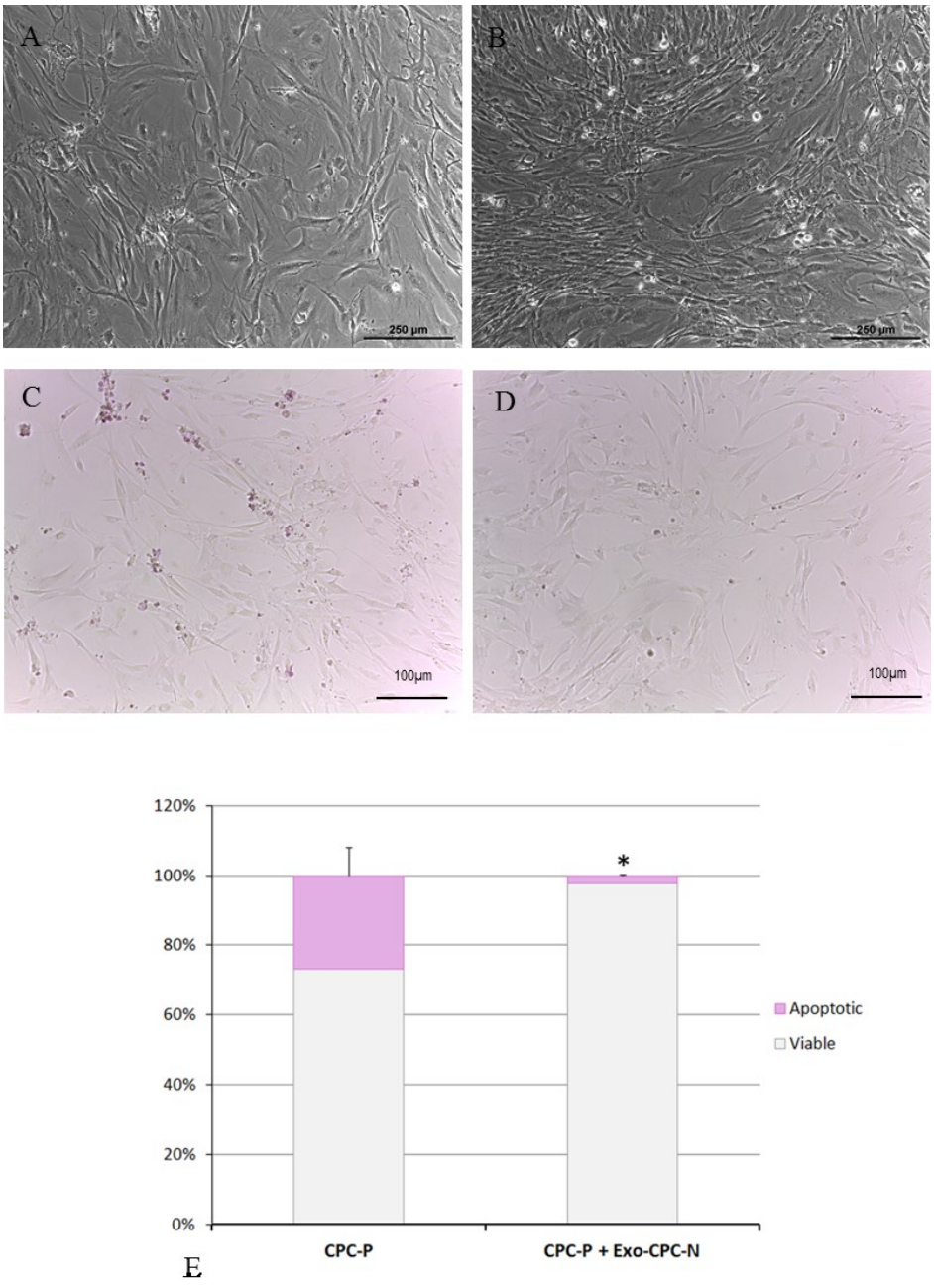
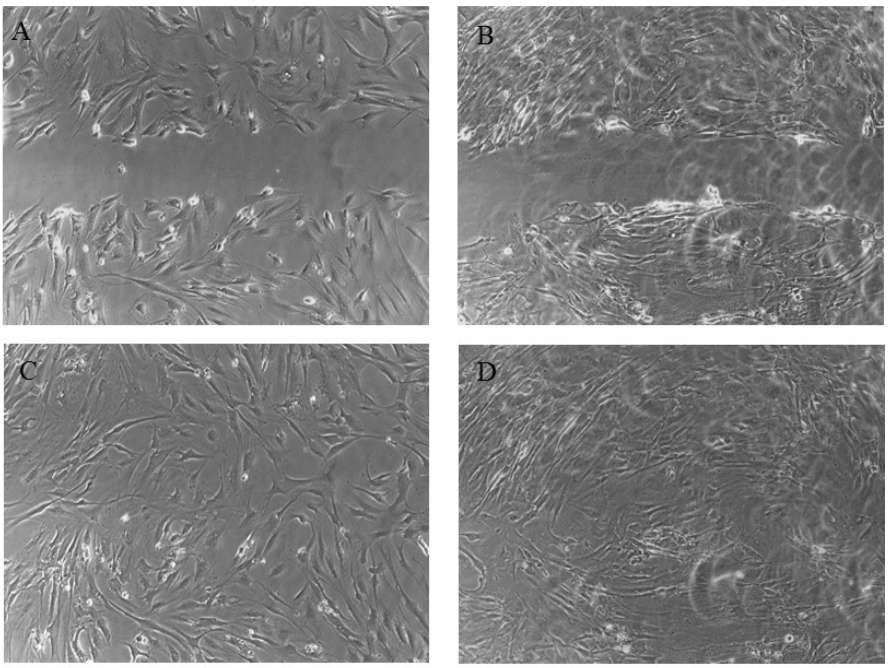
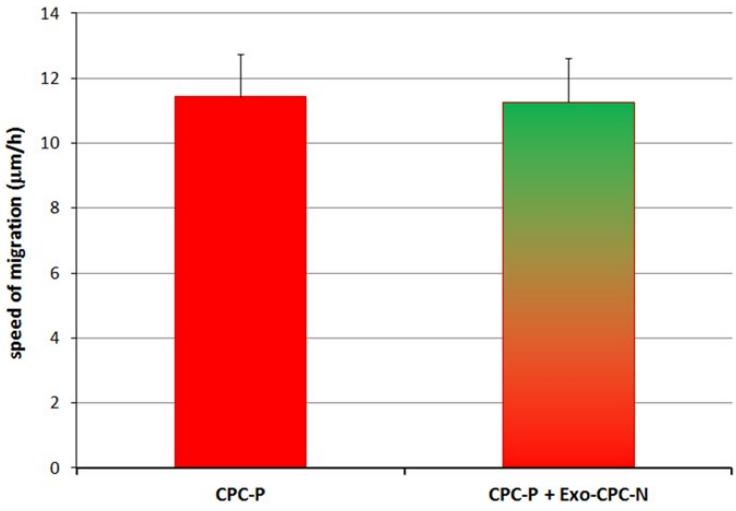


Fig. 12

Phase contrast microscope images of CPC-P in absence **(A)** or presence **(B)** of Exo-CPC-N. Images of apoptotic cells after Cell-ApoPercentage Apoptosis assay **(C, D)**. Apoptotic rates **(E)** of CPC-P treated with Exo-CPC-N and CPC-P untreated (control group). Statistically significant results are indicated by * ($p<0.05$).





E

Fig. 13

Microscopic images of migration of CPC-P, either in the absence **(A, C)** or presence **(B, D)** of Exo-CPC-N. T0 **(A, B)** and after 8 hours **(C, D)**. Speed of migration of CPC-P untreated and treated with Exo-CPC-N **(E)**. No statistically significant differences were observed.

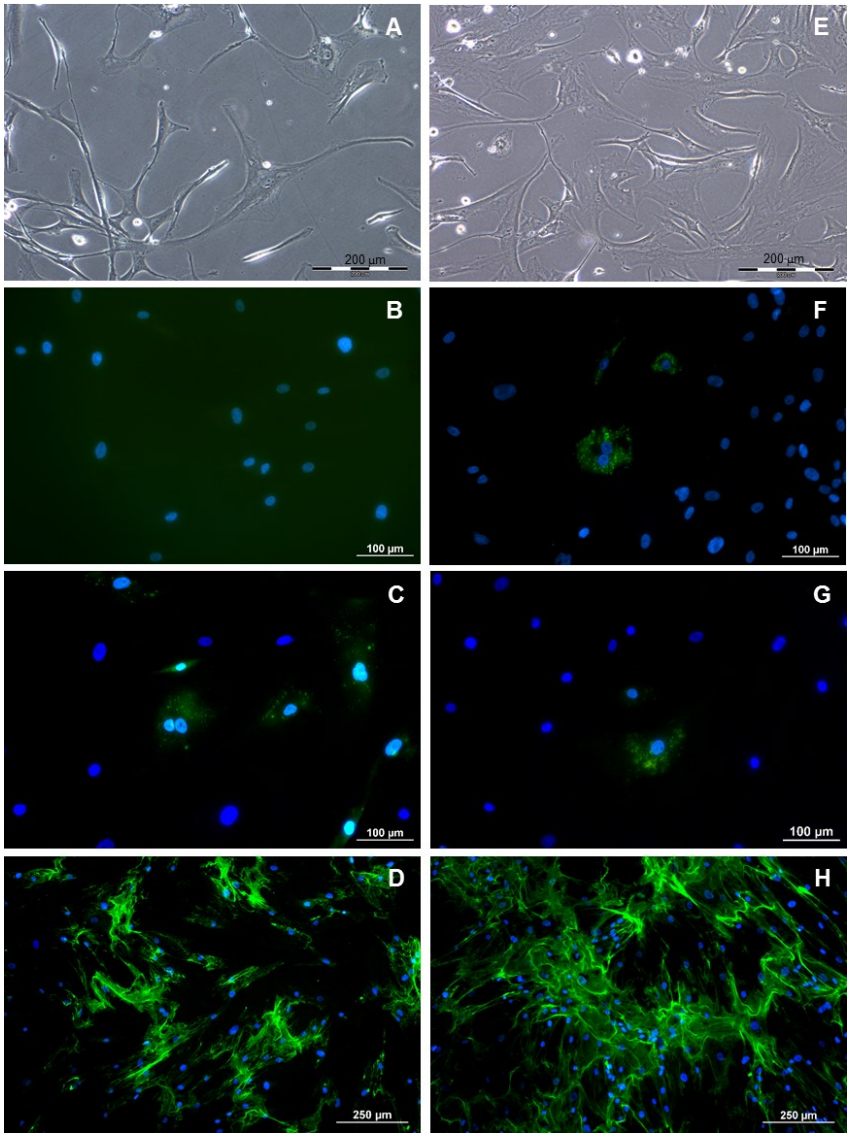


Fig. 14

Phase contrast microscope images of CF-P in culture, either in the absence **(A)** or presence **(E)** of Exo-CPC-N. Fluorescence microscope images of CF-P in culture, either in the absence **(B, C, D)** or presence **(F, G, H)** of Exo-CPC-N. **Collagen IV** and **Nuclei** of cells **(B, F)**; **Laminin** and **Nuclei** of cells **(C, G)**; **Fibronectin** and **Nuclei** of cells **(D, H).**

PROTEIN	CF-P TREATED	CF-P UNTREATED
Collagen IV	++	+
Laminin	+	++
Fibronectin	+++	++

Tab. 2

Framework of results observed for synthesis and deposition of collagen IV, laminin and fibronectin quantified using an arbitrary scale ranging from negative (-) in case of immunonegativity, to triple positive (+++) in case of strong immunopositivity.

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