TÉCNICO LISBOA

Investigating the therapeutic potential of extracellular vesicles from hiPSCs and hiPSC-derived cardiac cells for heart regeneration

Marta Rodrigues de Oliveira

Thesis to obtain the Master of Science Degree in

Biomedical Engineering

Supervisor(s): Dr. Maria Margarida de Carvalho Negrão Serra
Prof. Dr. Maria Margarida Fonseca Rodrigues Diogo

Examination Committee

Chairperson: Prof. Dr. Cláudia Alexandra Martins Lobato da Silva
Supervisor: Dr. Maria Margarida de Carvalho Negrão Serra
Member of the Committee: Dr. Hugo Agostinho Machado Fernandes

December 2020

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Declaro que o presente documento é um trabalho original da minha autoria e que cumpre todos os requisitos do Código de Conduta e Boas Práticas da Universidade de Lisboa.

Preface

This work was supported by funding received from EU-funded project BRAV3 (H2020, ID:874827) and by Fundação para a Ciência e Tecnologia (FCT)-funded projects NET-DIAMOND (SAICTPAC/0047/2015), MetaCardio (PTDC/BTMSAL/32566/2017) and iNOVA4Health (ref. UIDP/04462/2020), a program financially supported by Fundação para a Ciência e Tecnologia/Ministério da Educação e Ciência through national funds.

Thesis Publications

(In final preparations for submission)

- Louro AF, **Oliveira M**, Paiva MA, Gomes-Alves P, Alves PM, Serra M. *hiPSC and hiPSC-cardiomyocytes as alternative EV sources for cardiac regeneration.*
- Paiva MA, Louro AF, Christensen RK, **Oliveira M**, Rosholm KR, Wilson S, Larsen NB, Alves PM, Serra M. *Dynamic 3D cultures and metabolic modulation as critical parameters for hPSC-cardiomyocyte maturation in scale-up processes.*

Thesis Communications

- Louro AF, **Oliveira M**, Paiva MA, Gomes-Alves P, Alves PM, Serra M. *hiPSC and hiPSC-cardiomyocytes as alternative EV sources for cardiac regeneration* [Paper Presentation]. GSEV-UKEV Joint Autumn Meeting. Virtual Meeting, 2-3 September; 2020.

Acknowledgments

Em primeiro lugar, gostaria de agradecer à Dr. Paula Alves e Prof. Manuel Carrondo por me terem proporcionado a oportunidade de realizar a tese de mestrado no iBET.

À professora Margarida Diogo, obrigada por todo o apoio e aconselhamento ao longo do meu percurso académico, e pela constante disponibilidade e palavras encorajadoras nos últimos meses. Foi uma verdadeira honra ser sua aluna de mestrado.

À Guida, um grande obrigada por me ter acolhido no grupo e pela confiança que depositou em mim desde o primeiro dia. Agradeço profundamente por esta experiência fantástica que foi trabalhar no iBET, por ter sido uma mentora e conselheira espetacular, e igualmente uma amiga, sempre disponível para me ajudar e guiar nos momentos mais difíceis.

Filipa e Marta Paiva, um obrigada não chega para agradecer todo o apoio, orientação e ajuda incansáveis que me deram durante este período. Este trabalho tem tanto de vosso como tem de meu, e por isso estou-vos eternamente grata. Levo comigo os vossos exemplos de enorme profissionalismo, competência, brio e empenho que me mostraram e ensinaram, assim como uma bela amizade e um pedaço dos minions no meu coração.

A todos no grupo das Stem, Cláudia, Daniela, Joana, Zahara, Henrique, Pedro, Marta Costa, Hélio, Bea, Ana e Inês, obrigada por me terem recebido tão calorosamente, por todos os bons momentos e pela ajuda valiosa ao longo deste ano.

Agradeço a todos os meus amigos, grupeta, crew e triângulo, por estarem sempre lá, desde sempre. Esta é mais uma de muitas etapas que concluo convosco ao meu lado.

Por fim, o maior obrigado de todos vai para a minha família. Aos meus pais, ao Gui e à Fi, os meus melhores amigos deste mundo, por acreditarem sempre em mim. À Kodinha, por me obrigar a sair de casa e me manter sã em momentos de loucura. Aos meus avós, por me mimarem como se não houvesse amanhã. E ao Di, o meu maior apoiante neste e em todos os momentos, obrigada por fazeres de mim uma melhor pessoa.

Resumo

Doenças cardiovasculares são a causa dominante de mortalidade mundial, sendo que as terapias atualmente empregues não abordam os mecanismos fundamentais de regeneração do tecido cardíaco. Estratégias baseadas no transplante de células não conseguiram ainda demonstrar resultados clinicamente significativos, apresentando níveis de eficácia modestos e inconsistentes. Estudos recentes sugerem que os efeitos terapêuticos das células transplantadas são mediados pelos seus fatores secretados e que as vesículas extracelulares (EVs) desempenham um papel importante na estimulação da reparação cardíaca endógena.

Neste trabalho, EVs de células estaminais pluripotentes induzidas humanas (hiPSCs) e seus derivados cardíacos foram isoladas do respetivo meio de cultura por centrifugação diferencial e ultracentrifugação em gradiente de densidade. A caracterização das partículas confirmou uma adequada recuperação e purificação de EVs. Ensaios de bioatividade *in vitro* e análise do conteúdo de microRNA foram utilizados para avaliar as propriedades angiogénicas das amostras de EVs.

As EVs derivadas de hiPSCs (hiPSC-EVs) demonstraram uma indução mais significativa de capacidade migratória em comparação com os restantes grupos cardíacos, assim como a estimulação da formação de estruturas capilares quando utilizadas como tratamento em células endoteliais, sugerindo assim um papel para estas EVs na promoção de angiogénese no coração. A análise dos perfis de expressão de microRNA indicou especificidade no conteúdo das EVs, apoiada pelo enriquecimento dos grupos de EVs de derivados cardíacos em microRNAs envolvidos no desenvolvimento do músculo cardíaco. Adicionalmente, hiPSC-EVs exibiram uma forte sinalização celular de vias relacionadas com pro-sobrevivência e pro-angiogénese. Estudos adicionais serão fundamentais para compreender e validar a bioatividade das EVs cardíacas e desvendar o papel cardioprotetor de hiPSC-EVs.

Palavras-chave: vesículas extracelulares, células estaminais pluripotentes induzidas humanas, diferenciação cardíaca, angiogénese, medicina regenerativa cardiovascular

Abstract

Cardiovascular disease is the dominant cause of morbidity and mortality worldwide, with current therapies still failing to address the fundamental mechanisms of cardiac tissue regeneration. Cell transplantation strategies have failed to demonstrate clinically meaningful results, showing inconsistent and modest efficacy. Mounting evidence suggests that the therapeutic effects of transplanted cells are mediated by secreted factors and that extracellular vesicles (EVs) play a major role in the stimulation of endogenous cardiac repair.

In this work, EVs from human induced pluripotent stem cells (hiPSCs) and hiPSC-derived cardiac cell populations were isolated from conditioned culture medium by differential centrifugation and density gradient ultracentrifugation. Particle characterization confirmed the successful recovery and purification of EVs. *In vitro* bioactivity assays were used to assess wound healing and angiogenic properties of EV samples.

hiPSC-EVs demonstrated more significant wound healing capacity than cardiac-derived groups, and the ability to stimulate the formation of capillary-like structures when used as treatment in endothelial cells, suggesting a role in the promotion of angiogenesis in the heart. MicroRNA expression profiles obtained by RNA-seq indicated specificity of EV cargo, substantiated by the enrichment of cardiac-derived EVs in microRNAs involved in cardiac muscle development. Furthermore, hiPSC-EVs displayed increased targeting of pro-survival and angiogenesis-related pathways. Additional work will be paramount to validate and enhance the bioactivity of cardiac EVs and to further unveil the role of hiPSC-EVs in cardioprotection.

Keywords: extracellular vesicles, human induced pluripotent stem cells, cardiomyocyte differentiation, angiogenesis, cardiac regenerative medicine

Contents

List of Tables

List of Figures

Acronyms

BSA Bovine serum albumin

CCM Conditioned culture medium

CDC Cardiosphere-derived cell

CDE Caveolin-dependent endocytosis

cDNA Complementary DNA

CKD Chronic kidney disease

CM Cardiomyocyte

CME Clathrin-mediated endocytosis

CMi Immature cardiomyocyte

CMm Mature cardiomyocyte

CPC Cardiac progenitor cell

CSC Cardiac stem cell

cTnT Cardiac troponin T

CVD Cardiovascular disease

DC Dendritic cell

DE Differential expression

DPBS Dulbecco's phosphate buffered saline

DYN Dynasore

EC Endothelial cell

ECBM/**ECGM** Endothelial cell basal/growth medium

ECM Extracellular matrix

ESCRT Endosomal sorting complex required for transport

EV Extracellular vesicle

FACS Fluorescence-activated cell sorting

Fb Fibroblast

FBS Fetal bovine serum

FDA Food and Drug Administration

FGF Fibroblast growth factor

FSG Fish skin gelatin

GF Growth factor

GFP Green fluorescent protein

GMP Good Manufacturing Practices

GO Gene ontology

GvHD Graft-versus-host disease

hESC Human embryonic stem cell

hiPSC Human induced pluripotent stem cell

HSP Heat shock protein

HUVEC Human umbilical vein endothelial cell

ILV Intraluminal vesicle

ISEV International Society for Extracellular Vesicles

KEGG Kyoto Encyclopedia of Genes and Genomes

LDL Low-density lipoprotein

MHC Major histocompatibility complex

MI Myocardial infarction

miRNA Micro RNA

MISEV Minimal information for studies of extracellular vesicles

MMP Matrix metalloproteinase

mRNA Messenger RNA

MSC Mesenchymal stem/stromal cell

MVB Multivesicular body

NGS Next Generation Sequencing

NTA Nanoparticle Tracking Analysis

ODG Optiprep density gradient

PCA Principal Component Analysis

rRNA Ribosomal RNA

RT-qPCR Real time quantitative polymerase chain reaction

SC Stem cell

SKM Skeletal myoblast

SMC Smooth muscle cell

TEM Transmission Electron Microscopy

TMM Trimmed mean of M-values

tRNA Transfer RNA

VEGF Vascular endothelial growth factor

WCL Whole cell lysate

Chapter 1

Introduction

1.1 Cardiovascular Disease and Therapeutic Strategies

The global burden of cardiovascular disease (CVD) is a daunting and pressing issue. CVD covers a range of diseases related with the circulatory system, including coronary heart disease, stroke, heart failure, arrhythmias, congenital diseases, among other conditions. CVD has been the number one cause of mortality worldwide for several decades [1], and is one of the leading causes of total years of life lost [2]. Current therapeutic strategies consist in management of symptoms through a lifetime of medication or surgical intervention. For patients with an irreversibly damaged heart, the remaining option is heart transplantation, contingent on the scarce availability of organ donation [3] and possible postoperative complications [4]. The search for alternative therapeutic modalities is deemed urgent and extensive work has been developed in this research field, including the employment of Regenerative Medicine approaches.

The human heart holds many particular traits, one of them being its limited regenerative capacity. The growth of the human heart during prenatal development results from an increase in cardiomyocyte (CM) number, mediated by the differentiation of progenitor cells and proliferation of immature cardiomyocytes [5]. However, once past the perinatal period, the main mechanism responsible for heart growth in healthy individuals is the increase in cardiomyocyte size rather than number [6], with a 3-fold increase in cell diameter from infancy to adulthood. Although the heart is not an entirely postmitotic organ and cardiomyocyte renewal does occur, the turnover of cardiac muscle cells is thought to be of around 1% in adults [7]. Such flat renewal rate is insufficient to counteract the damage caused by cardiac injury. It is estimated that around 1 bil-

lion cardiomyocytes are lost following myocardial infarction [8], making it completely inconceivable to recover heart function after such event.

Different strategies have been studied to rehabilitate injured tissue and restore cardiac function, centered around the regeneration of endogenous cardiomyocytes along with the repair through exogenous cell sources.

1.1.1 Cell-based therapies and tissue engineering: a quest for clinical significance

The first suggestion of cell transplantation as a way to regenerate injured hearts emerged from the findings that transplanted cardiomyocytes could merge with the hearts of healthy mice to form coherent tissue. These pioneering studies were conducted by Loren Field and his team using fetal cardiomyocyte grafts [9]. Once this approach was brought to light, it was a question of finding the ideal cell type for transplantation. Conceptually, the optimal cells should present a set of key characteristics that make them the most fitting type of cell for this purpose: they should be safe for clinical use, including a secure minimally-invasive cell delivery, without any possibility of immunological rejection or harmful adverse effects for the patients; they should bring a clear benefit to the patient and enhancement of cardiac function, through a smooth integration into the native heart; they should fill basic requirements in terms of source availability, ethical principles and appropriate conduct in the manipulation of the cells [10].

The launch of cell-based therapy

The initial attempts used skeletal myoblasts (SKMs), derived from skeletal muscle progenitor cells, as substitutes for cardiomyocytes. SKMs possess many interesting features, such as a contractile phenotype and proliferative response upon tissue damage, while also presenting autologous availability. Furthermore, experimental animal studies showed promising results towards their potential clinical application [11, 12]. However, a randomized, placebo-controlled, double-blind Phase II trial to assess Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) reported no significant improvement in heart function, along with occurrence of arrhythmic events [13]. These outcomes strongly diverted the attention from SKM-based therapy, nonetheless paving way for new approaches.

Alongside the experiments using SKMs, other cell types of noncardiac origin have been tested for transplantation, notably bone marrow mononuclear cells (BMMNCs)

and mesenchymal stem/stromal cells (MSCs). Both therapies have been deemed feasible and safe [14, 15], although more encouraging and predominantly positive results have been reported for bone marrow- and adipose tissue-derived autologous MSCs [16, 17]. More recently, peripheral blood mononuclear cells (PBMCs) [18] and endothelial progenitor cells (EPCs) from bone marrow and peripheral blood origin [19] have also been considered as a possible source of cells for transplantation.

Heart-derived cells

Once it was established that cardiomyocyte turnover occurs in the heart, the concept of enhanced endogenous renewal as a way of reconstituting cardiac tissue was brought to attention. Cardiac stem cells (CSCs) can be distinguished and isolated through characteristic surface markers such as Sca-1 and c-kit, or can be cultured as explants and give rise to cardiospheres (CSs) and cardiosphere-derived cells (CDCs). These three cell types have been studied as potential candidates after showing encouraging results in pre-clinical studies [20–23] and have been taken into clinical trials [24, 25], albeit with modest results .

Pluripotent stem cells

In addition to the aforementioned cell types of cardiac and non-cardiac origin, stem cells have also been investigated as potential sources for cell transplantation in the heart. Stem cells are a category of cells that exist in all multicellular organisms, with unique proliferation, self-renewal and differentiation properties. For the past two decades, their potential has been continuously explored and unveiled, with an ever-increasing amount of funding and publication count dedicated to this subject [26, 27]. In particular, pluripotent stem cells can generate cells of all three germ layers formed in early embryonic development (ectoderm, mesoderm and endoderm), essentially all cells except those of extra-embryonic tissues [28].

The first-time isolation of human embryonic stem cells (hESCs) in 1998 [29] would start an era of Regenerative Medicine and Tissue Engineering as conceivable therapeutic approaches for clinical practice. However, hESC pluripotency was soon considered a deceptive advantage, with promising potential but also an associated risk for use in medical applications. Indeed, *in vivo* transplantation of undifferentiated ESCs may lead to tumor-like formations [30, 31] and faulty control of differentiation into the desired tissue [32]. Amidst this debate, there was a continuous search for the underlying mechanisms of pluripotency in hESCs, and in 2007, Shinya Yamanaka and his

team discovered a way to obtain embryonic-like human stem cells without requiring an embryo, by reprogramming adult cells into a state of pluripotency [33]. This can be achieved through the ectopic expression of key transcription factors Oct3/4, Sox2, Klf4 and c-Myc in somatic cells, usually fibroblasts, blood cells, keratinocytes or urine derived-epithelial cells [34]. The discovery of human induced pluripotent stem cells (hiPSCs) held a tremendous promise to the cell-therapy industry, in the sense that it still allowed the manipulation of pluripotent stem cells without facing ethical issues, furthermore avoiding problems of immune rejection in the case of transplantation, seeing that hiPSCs can be derived from the patients themselves. Nonetheless, hiPSCs still faced the same issue of teratogenic potential [35].

Conceptually, both hESCs and hiPSCs can be converted into any type of cell, by manipulating their differentiation along the desired pathways. The differentiation process is carried out in calculated steps following signal transduction pathways of interest, defined by specific agonist/antagonists. This process of *in vitro* differentiation of SCs simulates orderly and defined phases of embryonic development. To obtain cardiomyocytes from the starting point of a hiPSC, it is necessary to guide the differentiation process into the mesodermal lineage. The main regulators of early-stage cardiogenesis are growth factors belonging to the nodal signalling/bone morphogenetic protein (BMP), wingless/INT protein (WNT) and fibroblast growth factor (FGF) families [36], therefore controlled expression of key growth factors is used in specific temporal contexts to induce mesoderm differentiation.

The differentiation of hiPSCs to CMs can be executed in 2D monolayers or 3D spheroid culture systems. Both present advantages and disadvantages, although cell aggregates can be cultured in suspension using large dynamic bioreactors, easing its scalability to an industrial level and facilitating future clinical applications [36]. Additionally, it is intuitively understood that aggregate-based methods more closely resemble the heart environment, which translates into an enhanced maturation of CMs [37]. This is an important point in favor of 3D culture systems, seeing that lack of CM maturation has been a major issue of *in vitro* development of cardiac tissue since its first reports. Multiple efforts to induce cardiac maturation have been made, although researchers are still to find a method that achieves full cardiac maturation, defined by an adult-like phenotype of cells at all molecular, structural and functional levels.

The panorama of stem cell applications is vast and expanding, including uses in

disease modelling and drug screening, as depicted in Figure 1.1. Upon their establishment within the scientific community, the potential of employing cardiac-committed cells derived from ESCs and iPSCs in cell-based therapy emerged. Both approaches have been widely explored in pre-clinical studies using animal models [38, 39] and, in the case of hESCs, in clinical trials [40, 41], though still requiring further analysis to validate real benefits. Cardiac therapy using hiPSCs is also making its way into the clinical trial domain, with an ongoing study from Yoshiki Sawa's group working on the transplantation of patient-specific hiPSC-derived cardiac muscle cells on degradable sheets [42].

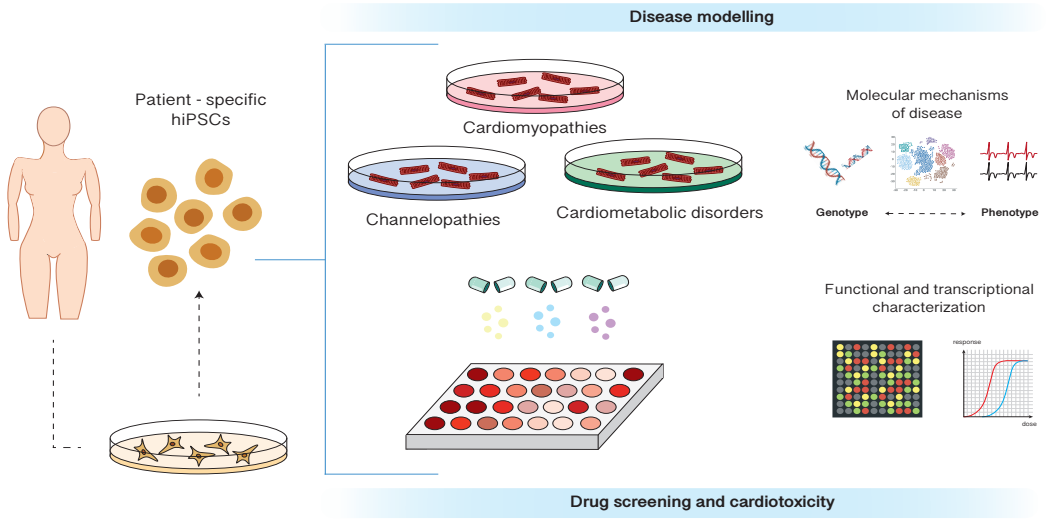


Figure 1.1: **Applications of hiPSC technology in disease modelling and drug screening.** Somatic cells from diseased patients can be reprogrammed into hiPSCs and used to create *in vitro* models of cardiovascular diseases for studies of their molecular mechanisms. These can also be used in high-throughput compound screening for cardiotoxicity studies, avoiding the need to use unethical animal experiments, and for the discovery of new drugs.

Shortfall in clinical translation

The question to pose now is why is cell therapy still not the norm in medical practice. Unfortunately, in spite of the numerous attempts of bringing cell therapy to clinical application through transplantation of the previously described types of cells, a number of issues have stood in the way of achieving this scientific goal (Figure 1.2). The first concern to be weighed in is the cardiogenic potential of the cells in use, which has been a cause of controversy in some cases, such as bone marrow-derived cells [10]. Another primary consideration is the safety of the patients, which once again has been

questioned in several experiments, including the risks of tumor formation, arrhythmias and violent immune reactions.

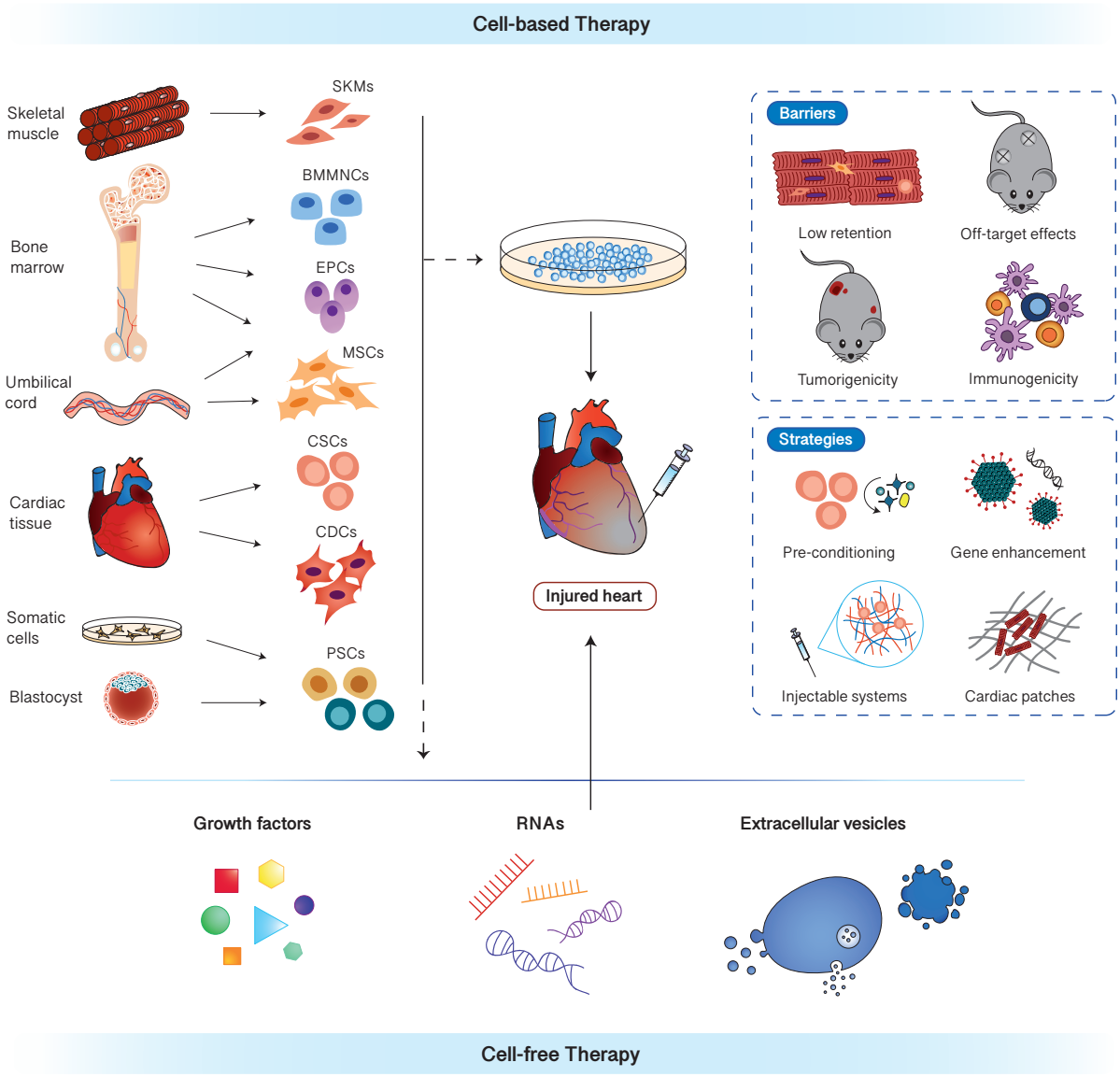


Figure 1.2: **Cell-based therapies for heart failure, barriers for clinical translation and possible solutions.** Isolation, expansion and transplantation of cells from various biological sources as a means to restore cardiac function, by regenerating damaged tissue and activating endogenous mechanisms of cardiac repair. Many hurdles remain in the way of clinical application of such strategies, such as low retention and tumorigenicity. Issues of poor cell engraftment and retention in the heart can be tackled by the use of engineered delivery systems. Cell pre-conditioning works to boost reparative signals and enhance therapeutic effects. Such cues are mediated directly by the transplanted cells and equally by their secretome. The paracrine potential of cells can be explored to advance on cell-free therapy approaches.

Still, a substantial number of studies and trials have succeeded in dealing with these two fundamental issues. However, they have come to face other problems associated with the overall efficiency of the treatment. There are several factors to be considered when overlooking the root of this question, the first being the administration route and subsequent biodistribution within the myocardium, which play a crucial role in the experimental outcome. The most commonly practiced methods are intramyocardial injection, intracoronary delivery and intravenous infusion, although the latter has shown significant off-target homing of the cells and thus is not ideal [43]. Secondly, cell retention and long-term survival of cells are major issues in cell therapy when employing injectable delivery systems, given the hostile ischemic conditions of the injured heart. Survival rates as low as 2% [44] fall short of expectations and are unsatisfactory for any hope of clinical significance. Lastly, appropriate maturation and integration of the cells into the myocardial tissue and successful cell engraftment are serious concerns for researchers.

A number of strategies have been developed to tackle many of these problems and improve the efficacy of cell-based therapy. One of the most common approaches focuses on enhancing the therapeutic potential of the cells with some form of treatment previous to transplantation. Multiple variants of pre-conditioning can be employed, aiming to boost homing and engraftment capacities of cells, making use of small molecules and growth factors to promote these functions [45, 46]. Similarly, pretreatments can also be applied to the host heart tissue in order to optimize cardiac repair. Another successful approach is the use of Tissue Engineering to improve the delivery of the cells to the target tissue, while helping to recapitulate the characteristics and environment of the human heart. Biomaterials can be used to support the transplanted cells and guarantee their proper integration. Injectable vector-like systems have shown to improve attachment, retention, survival and maturation of cells in pre-clinical studies. Furthermore, such biomaterials, of both natural [40, 47] and synthetic [48] origin, can also be used as a platform for delivery of certain factors with ability to trigger bioactive signals and cues to promote therapeutic action. Ultimately, these Tissue Engineering strategies ensure the establishment of a more physiological and favorable environment for the transplanted cells and play a contributing role in overcoming some of the hurdles blocking the way of the clinical application of cell therapy.

One final consideration to be mindful of is the importance of instituting good man-

ufacturing practices (GMP) in the production process of the elements that comprise cell-based therapies, so that its commercialization can become a reality [49]. When it comes to the use of stem cells in clinical applications, regulatory agencies have imposed rigorous standards to ensure the quality and safety of all approved medicinal products, such that previous definition of clinical specifications, product requirements and cost structure are well advised, along with the implications of scalability and reproducibility of the product [50]. All of these factors contribute to the prosperous implementation and commercialization of a cell-based therapy.

1.1.2 The promise of cell-free therapy

In the face of the limited and mostly moderate benefits observed in cell-based attempts at regenerating the heart, noticeable evidence hinted at the contribution of paracrine effects to the positive experimental outcomes [51]. These observations sparked a new interest in the secretome of cells and its possible impact in tissue repair and recovery from injury. This proposition, designated ”the paracrine hypothesis”, expresses the favorable effect of certain factors, such as cytokines and growth factors (GFs), and more recently, microRNAs (miRNAs) and extracellular vesicles (EVs), which can match the level of direct action from the transplanted cells [52].

The paracrine potential of cytokines and growth factors has been deeply studied in preclinical work on animal models, with encouraging results [53–55]. The use of growth factors in cardiac repair has also been tested in clinical trials [56, 57], although studies employing these proteins as single factors in therapy revealed very modest to no significant improvement. However, these outcomes may be closely related to a non-optimized duration of GF expression and mistargeting of intended cells. These issues can be tackled with Tissue Engineering strategies in order to facilitate systemic delivery of the GFs [58], as well as Genetic Engineering approaches to overexpress these factors and extend their limited half-life [59].

MicroRNAs have shown potential as therapeutic targets for cell-free therapy due to their regulatory role in paracrine activity [60]. These small RNAs can be manipulated either by downregulation through their inhibition or upregulation through the delivery of miRNA mimics. Studies focusing on the miR-15 family have revealed promising results [61].

Extracellular vesicles have been increasingly drawing the attention of researchers

due to their extensive therapeutic potential. As the focus of this work, EVs will be deeply explored and discussed further along. These vesicles are abundantly secreted from many of the aforementioned cell types adopted for cell therapy, and play a critical role in the regulation of regenerative processes in wounded tissues by mediating cell-cell communication. Numerous pre-clinical studies and, more recently, clinical trials have indicated the involvement and importance of EVs in biological events, although this research field still requires thorough investigation and extensive groundwork to fully validate its applicability.

1.2 Extracellular Vesicles

The intricate processes of cellular communication are essential for survival, and their mediating agents are the orchestrators of cellular coordination and regulation in all organisms. The secretion of membrane vesicles is an evolutionarily conserved biological phenomenon observed in organisms of all three phylogenetic domains of life, archae, bacteria and eukarya [62]. Extracellular vesicles (EVs) are nanosized particles delimited by a lipid bilayer and without a functional nucleus that transport a cargo of biomolecules between cells, acting as facilitators in intercellular communication.

The first evidence of the existence of these vesicles was found in 1946 in human platelet-derived microparticles with procoagulant activity, obtained from high-speed centrifugation of blood plasma [63]. However, at this time researchers were still unaware of the significance and potential of their breakthroughs. The true discovery of EVs was initiated by Christian de Duve, who acknowledged the existence of the lysosome and fueled the research interest on endocytosis and membrane trafficking. Among these studies were two papers released simultaneously in 1983, focused on the pathways of receptor-mediated endocytosis [64, 65] - more specifically, the transferrin receptor recycling pathway during the maturation of reticulocytes. By monitoring reticulocyte transferrin receptor turnover with colloidal gold-conjugated transferrin (AuTf), Harding found that the gold particles were internalized by receptors but were not found within lysosomes. Instead, they were mainly found within small vesicles inside multivesicular bodies (MVBs), which then seemed to fuse with the plasma membrane and release the AuTf-labeled vesicles to the outside of the cell [65]. These unfamiliar vesicles are now known to be EVs, and these pioneering studies uncovered a new mechanism of exocytosis that would give way to the complex research field of EVs. EVs were

initially seen as ”garbage cans”, and their excretion was thought to be a mechanism of eliminating unwanted material, as an alternative to degradation [66]. This belief was broken by the findings of Graça Raposo and her team in 1996 [67], showing a much deeper functionality for EVs when they demonstrated that exosomes secreted from MVBs of B lymphoblastoid cells can activate T cell responses, suggesting that EVs are involved in antigen presentation.

Since then, this new research field has bloomed with an exponential number of dedicated publications and an ever-increasing interest from researchers worldwide, especially given the recent focus and enthusiasm for the secretome.

1.2.1 Biogenesis, cargo and uptake of EVs

The vast domain of extracellular vesicles encompasses a number of subpopulations. There are three main subtypes of EVs - exosomes, microvesicles/ectosomes and apoptotic bodies. They are distinguished essentially by their size and morphology, intracellular origin and cargo composition. However, due to overlapping of many intrinsic characteristics and absence of clear subtypic markers, these subtypes are still poorly characterized and discriminated [68]. Furthermore, there is still some confusion regarding EV nomenclature, given the initial mention of ”exosomes” as referring to the ensemble of vesicles found in pioneering studies. This terminology is inaccurate, seeing that exosomes and ectosomes are hardly distinguishable through isolation procedures, justifying the designation of EVs as a combination of vesicle subtypes [69].

Biogenesis

One of the main differences between each EV subtype is their intracellular origin (Figure 1.3). Exosomes derive from an internal compartmentalization process within the cell, whereas microvesicles stem from the plasma membrane, and apoptotic bodies, as the name suggests, are an end result of apoptosis. These processes will be further reviewed throughout this section, with particular emphasis on exosomes and microvesicles due to their potential use in therapeutic applications.

Exosomes Exosomes range between 50 and 150 nanometers in size, and are enriched in Alix, TSG101 and tetraspanin (CD63, CD81, CD9) proteins [70]. Their biogenesis stems from endosomal compartments [64].

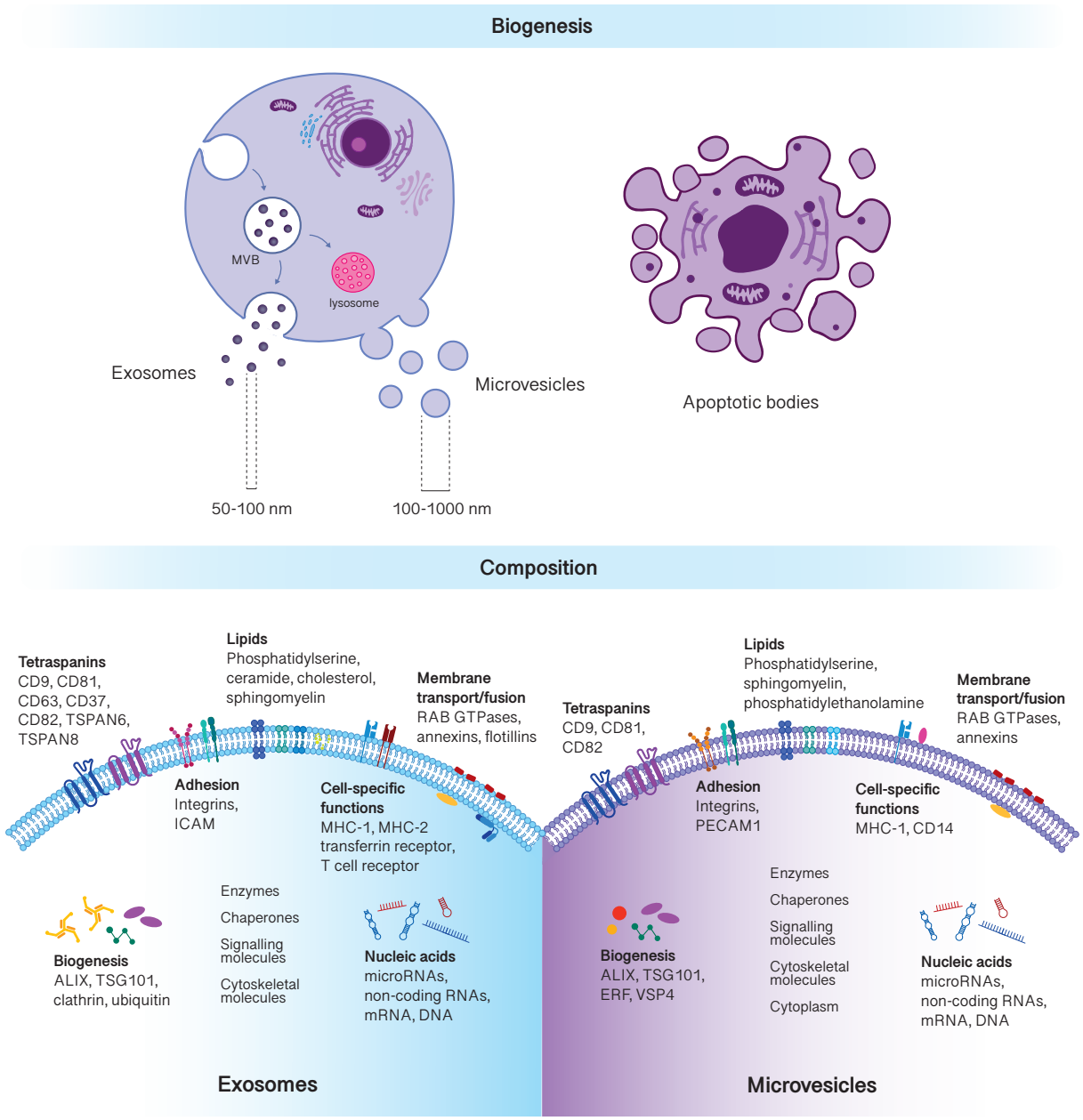


Figure 1.3: **Biogenesis and overall composition of extracellular vesicles.** EV subtypes are defined according to their distinct cellular origins, either from intraluminal vesicles formed within endosomal compartments, direct budding of the plasma membrane or the process of apoptotic cell disassembly. EV cargo contains numerous molecules, including proteins, lipids and nucleic acids, which varies greatly with vesicle subtype, cell source and intended target. Exosomes and microvesicles show a set of subtype-specific molecules, some of which are used as markers for isolation, but still share a number of common features within their composition, including important tetraspanins and lipids that compose their membrane. Adapted from [72].

The endosomal pathway involves the complex and dynamic cellular processes met by various active membrane compartments, involved in the internalization of molecules

and extracellular components and their degradation and/or recycling to the plasma membrane. The route of endocytosis follows the maturation of early endosomes into late endosomes, through which inward budding of the endosomal membrane creates intraluminal vesicles (ILVs) inside of the endosome lumen. These structures are named multivesicular bodies (MVBs) [71]. During this process, cytosolic proteins, lipids and RNAs are sorted to the endosomal membrane, and then engulfed by the ILVs. The MVBs embrace one of two fates: the degradative pathway, which is the primary destination for MVBs, by fusing with the lysosomes for degradation; or the secretory pathway, joining with the plasma membrane and releasing the exosomes. There is evidence that MVB subpopulations within both pathways exist concurrently in cells, given that markers of both early and late endosomes can be found in different MVBs with exocytic fate [72].

The mechanisms that rule IVL formation are closely related to the process of cargo recruitment to the MVB membrane [73], and can be dependent or independent on the endosomal sorting complex required for transport (ESCRT). The ESCRT machinery, composed of four protein complexes, was the first to give insights into the process of ILV formation. ESCRT-0 clusters the ubiquitinated transmembrane proteins on the endosomal delimiting membrane, ESCRT-I and -II facilitate membrane budding and recruit ESCRT-III, responsible for the fission that creates the IVLs [74]. ESCRT-independent mechanisms for IVL formation in MVBs also take place, suggested by the findings that MVB formation also occurs when proteins of all four ESCRT complexes are inactivated [75]. One of these mechanisms is the hydrolysis of sphingomyelin to ceramide by the neutral type II sphingomyelinase enzyme [76], which allows ceramide to generate microdomains with a negative curvature in the MVB membrane. Another ESCRT-independent mechanism involves the participation of CD63 and other tetraspanins in the formation of microdomains, by creating structures with other proteins on the endosomal membrane [77].

Overall, exosome biogenesis is a complex and still not entirely understood subject, given that its mechanisms are influenced by a number of factors such as cargo composition, external signals, physiological conditions or even cell type and stage of maturation. Further studies are needed to understand the interconnection of all these factors.

Microvesicles Microvesicles or ectosomes are larger in size when compared to exo-

somes, ranging between 100 nm and 1000 nm. The processes that drive their biogenesis are broadly different from the ones seen for exosomes. Microvesicles derive from an outward budding of the plasma membrane and subsequent shedding of the vesicles, as the interaction between the cytoskeletal proteins and the plasma membrane is gradually lost [78]. The budding of the membrane is induced by the generation of subdomains with altered lipid and protein composition, controlled by the levels of intracellular Ca^{2+}. Calcium-dependent activity of enzymes such as proteolytic enzyme calpain, which promotes the dismantling of the cytoskeleton, and phospolipid transporters flippase, floppase and scramblase, that induce changes in the lipid bilayer, leads to a reorganization of the membrane that promotes protrusion and microvesicle release [79].

The molecular composition of microvesicles is still not consensual and largely unknown. It is also dependent on the cell type to a great extent. Some suggested ectosomal enriched proteins are matrix metalloproteinases (MMPs), integrins, selectins and glycoproteins GPIb and GPIIb/IIIa [80].

Apoptotic bodies Apoptosis is the process of programmed cell death and the primary destruction mechanism for both normal and cancerous cells. Apoptotic cell death follows a cascade of complex biochemical processes, initiated by caspase-mediated apoptosis induction [81]. Once triggered, these molecular pathways unfold, resulting in membrane blebbing and transfer of the cellular content and organelles of the fragmented cell into membrane-bound vesicles. Because of their specific nature, these vesicles are named apoptotic bodies, and are usually between 500 nm and 5 μm in size, allowing their separation from exosomes and microvesicles.

To some extent, apoptotic bodies are secondary research subjects comparing to other EV subtypes with more prominent biological activities. However, they are considered more than mere debris of cellular destruction, and their potential value is still under study.

Taken together the distinction of EV subtypes and different biogenesis mechanisms described in this section, it is important to consider the substantial heterogeneity within each category, not only regarding origin but many other aspects. The lack of general agreement also extends to nomenclature, with a number of EV subpopulations being described by different research groups, each to his own designation. The scarcity of

scientific consent within this field is a major issue and one of the main priorities currently being tackled by leading organizations of EV research [82], given its consistent presence throughout the numerous topics covered by EV research. As was previously mentioned, ”exosome” is still wrongly used as a generic title for EVs, as is ”microvesicle”. Another greatly debated case in point is the term ”oncosome”, which has been discordantly used in various articles [83]. It is evident that restricting the description of EV biogenesis to three defined subtypes is insufficient to cover all mechanisms engaged in the origin of extracellular vesicles. Given the scope of this work, such mechanisms are only briefly described, with extensive descriptions available in dedicated literature reviews [84].

Cargo

Just as the biogenesis and loading mechanisms of EVs change according to the physiological conditions and stimuli experienced by the cell, so does the composition of its cargo. EVs carry an assortment of proteins, lipids and nucleic acids, depicted in Figure 1.3, that vary from cell to cell and vesicle to vesicle. Extensive research has been conducted to isolate, purify and analyse EV contents, in attempts to understand its intended function and also to find suitable and consistent markers for each EV subtype.

Proteins The evolution of proteomic analysis tools from lowthroughput immuno-based techniques to highthroughput proteome-scale approaches has led the way for advances in the non-biased detection and identification of the protein content of EVs. Still, given the proteomic variability between different-sized vesicles and cell types, as well as unevenness in isolation procedures, the inferences made in this domain are not fully conclusive [72].

Because of the specificity of vesicle formation mechanisms, many of the commonly found proteins are those associated with the biogenesis process, generally proteins from the endosomal pathway (in relation to exosomes), plasma membrane and cytosol, rather than those from the endoplasmic reticulum, Golgi complex and nucleus. Thus, the proteomic profiles described for exosomes are partly distinct from the ones found for microvesicles, hinting at the separate systems involved in their formation process [70]. For exosomes, some familiar proteins include tetraspanins (CD63, CD81, CD9), proteins involved in the ESCRT machinery (Alix, TSG101), proteins of ESCRT-

independent pathways of vesicle formation (RAB GTPases, Flotillins) and other proteins with various functions, such as transmembrane, signal transduction, adhesion and lipid raft-associated molecules [85]. Proteins with cell-specific functions are also found in EVs, such as antigen presentation MHC-I/MHC-II molecules.

Lipids The lipid content of EVs plays a crucial role in numerous processes, not only providing stability for the accommodated cargo, but also acting in biogenesis, release, uptake and several biological functions of the vesicles. The lipid composition of EV membranes presents a number of similarities to the plasma membrane of cells, and additionally shares some of these characteristics with the donor cell.

Some lipids found in EVs include sphingomyelin, phosphatidylserine and cholesterol, particularly enriched in exosomes [86], and also phosphatidylethanolamine, ceramide and gangliosides. Phosphatidylcholine is also found in EVs, albeit in lower ratios when compared to the cells of origin [87]. The lipid composition of microvesicles varies from exosomes, more closely resembling that of the plasma membrane of cells and further hinting at the influence of biogenesis mechanisms on the selectivity of cargo content. Additionally, exosomes present a higher lipid packing density when compared to larger vesicles, suggesting the existence of lipid rafts in their membranes, detergent-resistant subdomains rich in cholesterol and sphingolipids. This is supported by the presence of these lipids and also lipid raft-associated proteins in EVs [88].

Nucleic Acids One of the most important breakthroughs in EV research was the discovery of nucleic acids in exosomes. The findings of Ratajczak and colleagues in 2006 [89] and Lötvall in 2007 [90] showed that messenger RNA (mRNA) and microRNA molecules entrapped within the vesicles were delivered to neighbouring cells, further endorsing the idea of a functional role for EVs mediated by horizontal transfer of cargo. Since these observations, many studies have focused on the genetic material of EVs.

EVs present a diverse frame of genetic material, including both DNA and RNA, but are mostly enriched in small RNAs with less than 200 nucleotides. Multiple RNA species have been identified in EVs, such as mRNA and miRNA, and also rRNA, tRNA, long non-coding RNA, piwi-interacting RNA and small nucleolar RNA. Furthermore, because the RNA molecules are contained within the lipid bilayer membrane of EVs, they are resistant to RNase digestion once released from the cell. This protective feature has led many researchers to analyse the viability of exosomal miRNAs as biomarkers for diagnosis [91]. Indeed, the genetic content of EVs has been shown to depend on

and reflect the physiological state of the cells it originated from, albeit with a fairly different transcriptomic profile [92].

Uptake

Once released into the extracellular space, EVs move towards adjacent or distant cells and transfer their cargo to trigger functional responses and physiological changes, mediating intercellular communication. During this process, vesicles are internalized by recipient cells. Currently, it is believed that EV uptake is an active energy-dependent process that can occur in two ways, by endocytosis or fusion with the plasma membrane of the cell [93, 94], although a growing body of evidence suggests endocytosis as the main mechanism of vesicle internalization [95]. There are different types of endocytic pathways, namely clathrin-mediated endocytosis (CME) and caveolin-dependent endocytosis (CDE), macropinocytosis, phagocytosis and lipid raft-mediated endocytosis (Figure 1.4).

CME is activated through ligand binding to transmembrane receptors on the cell surface, that set in motion the progressive recruitment of clathrin triskelions and adaptor proteins to the inner surface of the membrane. These protein complexes gradually flex the membrane inwards creating invaginations, and eventually form clathrin-coated pits, that are excised at the neck by conformational changes in GTPase Dynamin 2 [96]. The endocytic vesicle is then uncoated and fuses with the endosome, where its contents are released. CME is thought to be involved in EV uptake, supported by studies that show reduced vesicle internalization upon the use of CME inhibitors, such as chlorpromazine [97] and dynasore [98].

CDE is a clathrin-independent mechanism that is also believed to be implicated in EV uptake. As the name suggests, it is mediated by caveolae, small caves or invaginations in the plasma membrane characterized sub-domains of glycolipid rafts rich in cholesterol, sphingolipids and caveolins. Caveolins contain oligomerization domains that allow them to oligomerize and create caveolin-rich raft-like microdomains. When these scaffolding domains attach to the plasma membrane and levels of cholesterol increase, caveolar invaginations expand and form endocytic vesicles [99]. Similarly to CME, Dynamin 2 mediates the parting of the vesicle from the plasma membrane. Still, CDE is not as extensively studied and some of its underlying mechanisms are not yet fully understood. Particularly, caveolin-1 is thought to be an important protein for this

endocytic process, seeing that it is necessary for caveolae formation [100], and that CAV1 knockdown leads to reduced EV uptake [101]. However, studies showing that knockdown of caveolin-1 increased the uptake of glioblastoma-derived EVs by mouse embryonic fibroblasts reveal opposing observations [102], suggesting that caveolin-1 may not be required for this process.

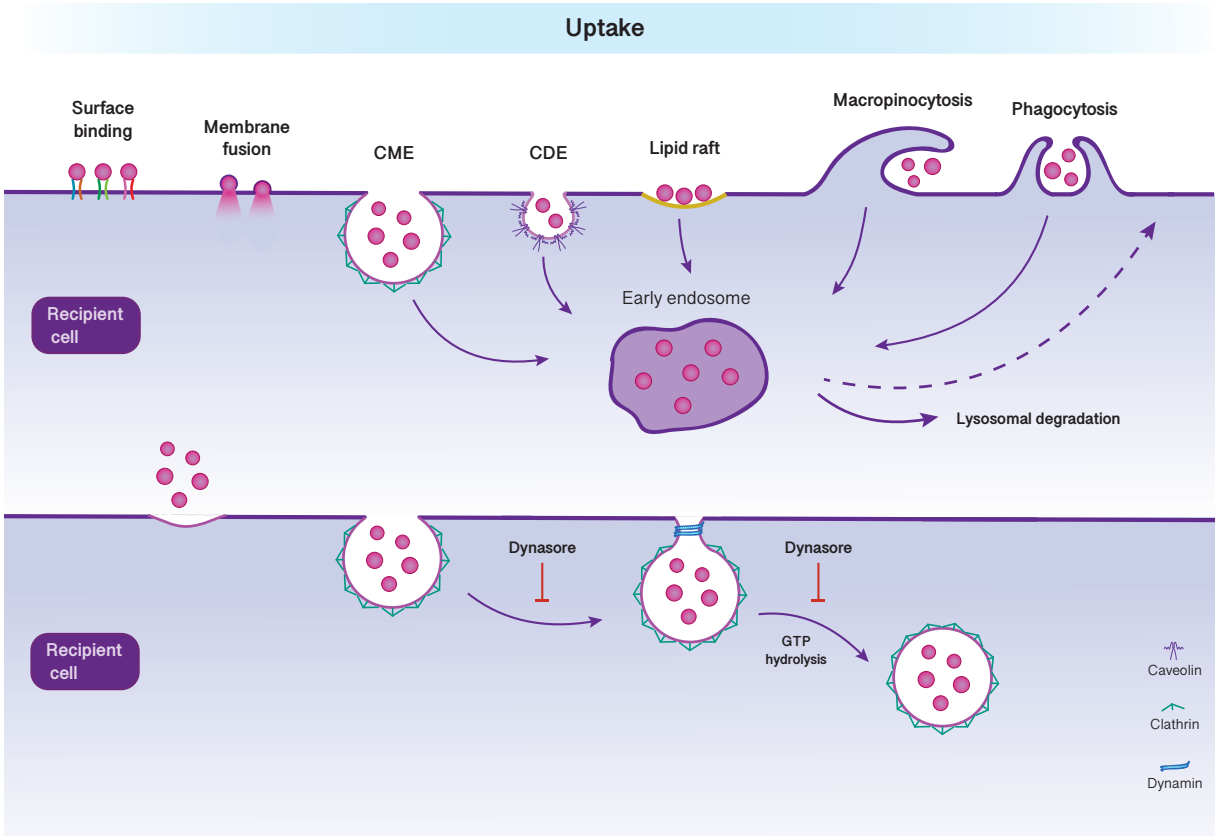


Figure 1.4: **Uptake of extracellular vesicles by recipient cells.** EVs circulate within the body until reaching their target cells and binding to the cell surface. Adhesion of EVs to recipient cells is mediated by exosomal surface proteins and cell membrane receptors. EVs can be internalized by membrane fusion or through different endocytic routes, after which vesicle contents are delivered to the endosomal pathway, and are then sent for lysosomal degradation or recycled to the plasma membrane. Adapted from [77].

Macropinocytosis involves the actin-dependent formation of protrusions in the plasma membrane, termed lamellipodia, that sample the extracellular environment. Activation of GTPase Rac1 promotes actin polymerization and subsequent lamellipodia formation. These membrane ruffles bend to form pocket-like structures, engulfing the extracellular fluid and material. Once closed and pinched off the plasma membrane, the macropinosome vesicles mature and eventually fuse with the lysosome. Studies

suggest macropinocytosis as a minor pathway in EV uptake [101], although some evidence points to a rather cell-specific mechanism of internalization, as seen in microglia [103]. Phagocytosis is a receptor-mediated mechanism, generally opsonins, that calls for membrane invaginations. Contrarily to macropinocytosis, it involves direct contact with the internalized particle and does not require the membrane to extend around it. Phagocytosis is usually characteristic of specialized cell types, such as dendritic cells, macrophages and other white blood cells. Despite being generally employed to internalize larger particles, phagocytosis is thought to be involved in EV uptake [104].

Lipid rafts are also believed to mediate EV internalization, given their role in the uptake of other particles. Moreover, these structures are rich in cholesterol molecules, which largely contribute to clathrin-independent endocytic pathways. This proposition has been further substantiated by studies showing reduced EV uptake following structural perturbation of the raft, through treatment with cholesterol-reducing agents [102].

The understanding of the mechanisms involved in vesicle uptake is critical to narrow the bridge between fundamental studies and clinical translation, and ultimately use EVs as therapeutic agents. The first step in this direction is to monitor EV internalization. This can easily be achieved using fluorescent labelling of either the vesicle membrane, with lipophilic dyes such as PKH26, or the internal EV content using fluorescent proteins. Importantly, some studies have used adequate controls to exclude the possibility of wrongly detecting fluorescence signals from leaching of the dye, rather than actual EV uptake [105], and also to guarantee that the vesicles were internalized and not merely positioned on the cell surface. Recently, modern imaging techniques have been brought into this field of research, allowing EV tracking in vivo (reviewed elsewhere [106]).

Endocytic mechanisms for vesicle internalization have been greatly unravelled in recent years, in spite of the multifold components still to uncover. Even so, many of the key players have been identified and studied and we now know, as previously mentioned, that multiple proteins and lipids are crucial for uptake. One question that remains unanswered is the circumstances under which each pathway is pursued. Multiple factors play a role in this, namely the recipient cell and its physiological state, which raises the question if the mechanisms for EV uptake present clear targeting and tissue specificity. Given the vast heterogeneity of EVs and difficulty in isolating each specific subtype, this issue remains difficult to tackle and reinforces the need for better

defined markers. The use of pharmacological inhibitors may assist with this purpose, although it is still unclear whether such compounds display full specificity to unique endocytic pathways.

1.2.2 The physiological and pathological role of EVs in the heart

As we have seen, extracellular vesicles carry specific information that changes according to the intrinsic and extrinsic conditions experienced by the cells of origin [107]. Their cargo is strongly modulated by the biological and functional state of the parent cell. Cellular crosstalk mediated by EVs control a range of different physiological and pathological events, such as immune responses, metabolic changes, inflammation, infection and tumorigenesis. The mutable character of EV content and its reflection on biological processes once released into the extracellular space has been intensely studied in recent years, with compelling evidence of its physio- and pathological role in countless *in vitro* experiments, and increasingly so in a number of *in vivo* studies.

Since the pioneering discovery of Graça Raposo and colleagues of EV-mediated activation of T cell responses [67], a large focus has been directed towards the role of EVs in immune regulation. Vesicles secreted from different cell types exert both activating and inhibiting effects on various immune cells [108]. Furthermore, concerning tumorigenesis, EVs largely participate in cancer pathology, including cell proliferation, migration and metastasis, and have been proposed as biomarkers for tumor development [109].

The role of extracellular vesicles in cardiac physiology and pathology has emerged in recent years, with extensive evidence alluding to the biological activity of EVs derived from heart cells. The heart contains a variety of cells in addition to cardiomyocytes (CMs), such as endothelial cells (ECs), fibroblasts (Fbs), smooth muscle cells (SMCs) and cardiac progenitor cells (CPCs), which require an intricate balance of cell-cell communication in response to physiological stimuli. To maintain this balance, proteins, growth factors, RNAs and other molecules are exchanged in the form of vesicle secretion, contrarily to outdated beliefs appointing cardiomyocytes as non-secretory cells. Indeed, cardiomyocytes have been shown to secrete EVs [110] and to modulate their cargo depending on a healthy or compromised state. Furthermore, EVs are capable of exerting both cardioprotective and cardiopathogenic effects in distinct conditions.

Cardiac repair and rehabilitation following myocardial infarction (MI) requires a com-

bination of restorative actions from cardiac cells. Cell-cell communication is crucial in this process, seeing that it requires a substantial reorganization of the heart structure. Fibrosis acts to maintain cardiac integrity, but can be detrimental when excessively stimulated. Vesicle-mediated crosstalk between CMs and Fbs in injured hearts has been evidenced by studying co-cultures of the two cell types [111], revealing the cardioprotective action of Fb-EVs through transfer of exosomal miR-423-3p [112]. However, EV-induced pro-fibrotic action can also lead to compromised cardiac function, due to extreme cardiac hypertrophy and excessive fibroblast proliferation upon heart injury [113].

Angiogenesis, the growth of the vascular network, is also essential for cardiac regeneration and healing [114]. Numerous studies suggest CPC-EVs as critical modulators of this process, involved in the promotion of proliferation, migration and remodelling of ECs in the heart. Importantly, CPC-EVs have shown pro-angiogenic activity *in vitro* using human umbilical vein endothelial cells (HUVECs), and anti-apoptotic properties when used as treatment in serum-deprived CMs [115]. Equally positive results have been obtained *in vivo*. Furthermore, mesenchymal stem/stromal cells (MSCs) have been found to secrete exosomes enriched in pro-angiogenic factors under ischemic conditions, and to promote angiogenesis *in vivo* after MI [116].

Indeed, research efforts to uncover the role of EVs in the heart environment and its therapeutical translation have leaned towards the use of primary CPCs and MSCs as cell sources [115–118]. Still, the complex reparative mechanisms mediated by EV-induced cell communication comprise many other cardiac cells, with an increasing number of studies now focusing on cardiomyocytes. In animal models, CM-derived exosomes were found to reduce cardiac fibrosis in diabetic mice hearts through the delivery of exosomal miR-29b and miR-455 during physical exercise [119]. Oppositely, inhibition of angiogenic effects was detected upon exosomal transfer of miR-320 from diabetic rat CM-EVs to ECs, further affirming the dual action of EVs. Several CM-derived exosomal microRNAs are involved in cardiac remodelling following stress conditions, namely miR-30a released by hypoxic CM-EVs regulating autophagic response [120].

Numerous of the above-mentioned studies have identified regulatory roles for an extensive collection of microRNAs, in both healthy and diseased cardiac environments, delivered to cells via vesicular transport. Given this dynamic modulation of EV cargo,

namely on a transcriptomic level, studies have also began to explore the use of exosomal miRNAs as biomarkers for cardiovascular disease. Research has shown that protein and RNA content of EVs is not only reflective of the cell of origin, but also of the cellular stress conditions experienced during heart failure [121, 122], further emphasizing the possible applications of these studies in cardiovascular diagnostics.

Altogether, these findings reinforce the heterogeneity of functions performed by EVs within the heart environment, and further emphasize the impact of both resident physiological conditions and extrinsic stressors on the subsequent paracrine responses.

1.2.3 Therapeutic potential and translation into clinical trials

Many research teams are now focusing on deepening our understanding of the exact physiological functions of EVs. Although this is still largely undeciphered, the notion of EVs as transport vehicles for biological messages is certainly well established. In this regard, the experimental significance witnessed to date suggests their potential to act as therapeutic agents against disease or as representative biomarkers.

Indeed, the shift from a cell-based to a cell-free approach and acceptance of EV-based therapeutics as an attainable reality offers a number of advantages. EVs are significantly more stable than cells, capable of enduring high speed centrifugation for long time periods and multiple freeze-thaw cycles [123]. Regarding biocompatibility, EVs do not induce obvious nor severe adverse effects. Moreover, recent studies have shown that CPC-derived EVs are not only safe for intramyocardial delivery by not inducing allogenic immune responses, but also trigger an anti-inflammatory effect in immunocompetent mouse models of MI [124]. These findings suggests that future EV administration to humans may not require previous immunosuppression. Seeing that EVs are naturally less dynamic than cells, they should also present advantages concerning reproducibility and batch-to-batch variation, facilitating quality control procedures [125]. Combined efforts have been made to tackle standardization in this field, through multiple publications of guidelines, minimal requirements and position papers by the International Society for Extracellular Vesicles (ISEV) [126], and the launching of the EV-TRACK database. Additionally, EVs serve as both transport vehicles and actuators, containing the ability to cross physical barriers within the body and to protect their contents from degradative enzymes. The characteristics of vesicle composition and encapsulation, together with the possibility of adjusting membrane and cargo through

engineering techniques to target specific functions, make EVs excellent candidates for therapy. Furthermore, their inherently longer shelf-life represents a major advantage to the alternative use of cells [127]. Therefore, despite the inability of cellular secretions to replace the loss of CMs and remuscularize the heart post-injury, EV-based therapy can contribute to attenuate this loss, reduce the extent of tissue damage and improve cardiac function by promoting angiogenesis, cell survival and endogenous stem cell activation.

Standing in the way of clinical translation is the need for standardization within the field, through the development of isolation, purification and characterization technologies for EVs uniformly used by research groups. Such reproducibility will be key to establishing GMP standards in future hopes of clinical applications, making the issues of scalability and production efficiency important to address. Furthermore, mechanisms for optimal delivery and targeted internalization of EVs are still being developed and are crucial for the reality of EV therapeutics in the cardiovascular field. In this regard, bioengineering strategies to potentiate cardioprotective action of EVs, increase retention and stability within the targeted tissues and control their release and cellular uptake are determinant for clinical translation [128, 129]. An extensive review of current developed approaches can be found elsewhere [106].

Preliminary pharmaceutical development strongly relies on the significance of results from pre-clinical and clinical studies. The therapeutic potential of EVs has been disclosed in many pre-clinical studies, which has led to its progression into clinical trials (Table 1, pp. 25-26), moving a step closer in the direction of their commercialization as *Advanced Therapy Medicinal Products* [130].

Pre-clinical studies applying dendritic cell-derived EVs (DC-EVs) pulsed with tumor cell peptides to murine tumors showed growth suppression, suggesting a role for EVs in cancer immunotherapy [131]. These findings supported the initiation of two Phase I trials in this domain, both operating in conformity with GMP requirements, employing DC-EVs pulsed with melanoma-associated antigen (MAGE) peptides to treat melanoma [132] and non-small cell lung cancer patients [133]. These studies concluded the feasibility and safety of this therapeutic approach, opening way to a Phase II clinical trial (NCT01159288) targeting non-small cell lung cancer patients that used tumor antigen-loaded DC-EVs to activate the immune system, with prior administration of cyclophosphamide to inhibit Treg activity and further induce T and NK cell effector

functions [134]. Results showed that administered EVs enhanced NK cell functions but did not induce antigen-specific T cell responses.

Pre-clinical work indicating tumor-derived vesicles as a source of tumor-rejection antigens [135] has led to a Phase I clinical trial performed in China that used EVs from the ascites fluid of colorectal cancer patients in combination with an immune-stimulatory adjuvant to trigger anti-tumor immunity [136]. This therapeutic approach was deemed safe and feasible and showed promising results. MSC secretions have also been considered for their immunomodulatory role. Treatment attempts using MSC-derived EVs have been made for graft-versus-host disease (GvHD) [137], diabetes mellitus, chronic kidney disease (CKD) [138] and acute ischemic stroke. Furthermore, an increasing number of clinical trials using MSC-derived EVs have been initiated recently, as seen in Table 1, albeit still in very early stages.

In addition to their utility as potential agents for regenerative therapy, EVs serve as great candidates for drug delivery, given their ability to carry cargo inside their characteristic lipid bilayer. A Phase II clinical trial (NCT01854866) proposed the delivery of chemotherapeutic drugs through EV-mediated transport as a treatment for cancer. The same rationale has been applied in a Phase I trial (NCT01294072), using curcumin from the anti-inflammatory spice turmeric to regress colon cancer growth in newly diagnosed patients.

EVs are now more than ever considered an added benefit to therapeutic strategies against a variety of health conditions. An increasing number of clinical trials continue to be registered to test this potential, namely for diseases related to the cardiovascular system, still in preliminary stages. The use of EVs in the treatment of MI is being investigated in two studies, one interventional clinical trial using drug-loaded EVs and another observational study to investigate the microRNA expression profile of peripheral blood-derived EVs following MI. Another clinical study aims to compare epicardial fat-derived EVs from patients with atrial fibrillation to those of healthy donors, in order to uncover a role for these EVs as biomarkers for arrhythmia.

Regarding cellular secretions from ESCs/iPSCs and derived cells, clinical trials are yet to advance in the direction of using this source of EVs for cell-free therapy. Notwithstanding, an escalation of pre-clinical work demonstrates that this strategy might be of significant clinical interest to tackle loss of cardiac function and heart failure [139–141]. Moreover, the use of hiPSCs as sources of EVs hold the valuable advantage of

providing greater cell availability for large-scale EV production. An increasing amount of research is being developed in this department, working towards the possibility of a future clinical application of this novel approach.

Table 1.1: Summary of past and current clinical trials of EV-based therapies.

Disease	EV source	Phase, Patients	EV modification	Results, Status	Reference
Melanoma	Autologous monocyte-derived DCs	Phase I n = 15	Pulsed with MAGE 3 tumor peptides	Safe and feasible. No major (> Grade II) toxicity observed and maximal tolerated dose not achieved. One minor, one partial, one mixed and two stable responses to therapy.	[132]
Non-small cell lung cancer	Autologous monocyte-derived DCs	Phase I n = 13	Pulsed with MAGE-A3, -A4, -A10 and MAGE-3DPO4 tumor peptides	Safe and feasible. No major (> Grade II) toxicity observed. Only 9 patients completed therapy. DTH reactivity against MAGE peptides detected in 3/9 patients, MAGE-specific T cell responses in 1/3, NK lytic activity increased in 2/4.	[133]
Non-small cell lung cancer	Autologous (IFN)-γ-maturated monocyte-derived DCs	Phase II n = 22	Pulsed with MAGE-A1, -A3, NY-ESO-1, Melan-A/MART1, MAGE-A3-DP04, EBV tumor peptides	One patient exhibited a grade 3 hepatotoxicity. 32% of patients with stable disease for > 4 months, below the primary endpoint of 50%. No T cell responses, but an increase in NKp30-dependent NK cell functions in a fraction of patients with defective NKp30 expression.	NCT01159288 [134]
Colon cancer	Autologous ascites	Phase I n = 40	Unmodified or in combination with GM-CSF	Safe and feasible. Toxicity of Grade I-II observed. Induction of beneficial tumor-specific cytotoxic T lymphocyte response in +GM-CSF group.	[136]
GvHD	Allogenic bone marrow-derived MSCs	Treatment attempt n = 1	Unmodified	Clinical symptoms improved significantly upon the start of therapy and were stable after 4 months.	[137]
Diabetes mellitus type 1	Allogenic umbilical cord blood-derived MSCs	Phase I n = 20	Unmodified	Unknown	NCT02138331
CKD	Allogenic umbilical cord blood-derived MSCs	Phase II/III pilot study n = 40	Unmodified	Safe and well tolerated. No adverse effects observed. Significant improvement of kidney functions and decreased inflammation.	[138]
Malignant ascites and pleural effusion	Tumor cells	Phase II n = 22	Loaded with chemotherapeutic drugs	Unknown	NCT01854866
Acute ischemic stroke	Allogenic MSCs	Phase I/II n = 5	Enriched with miR-124	Completed	NCT03384433

Disease	EV source	Phase, Patients	EV modification	Results, Status	Reference
Colon cancer	Fruit (plant origin)	Phase I n = 35	Loaded with curcumin	Active, not recruiting	NCT01294072
Oral mucositis	Grape (plant origin)	Phase I n = 60	Unmodified	Active, not recruiting	NCT01668849
Malignant pleural effusion	Malignant pleural effusion	Phase II n = 90	Loaded with methotrexate	Recruiting	NCT02657460
Intractable cutaneous ulcers	Autologous plasma	Phase I n = 5	Unmodified	Enrolling by invitation	NCT02565264
Metastatic pancreatic cancer	Allogenic MSCs	Phase I n = 28	Loaded with KrasG12D siRNA	Not yet recruiting	NCT03608631
Macular holes	Allogenic umbilical cord-derived MSCs	Phase I n = 44	Unmodified	Recruiting	NCT03437759
Bronchopulmonary dysplasia	Bone marrow-derived MSCs	Phase I n = 18	Not specified	Recruiting	NCT03857841
Acute myocardial infarction	Not specified	Phase I n = 18	Loaded with PEP drug	Not yet recruiting	NCT04327635
Dystrophic epidermolysis bullosa	Allogenic MSCs	Phase I/II n = 10	Loaded with AGLE-102 drug	Not yet recruiting	NCT04173650
Chronic GvHD	Allogenic umbilical derived-MSCs	Phase I/II n = 27	Unmodified	Recruiting	NCT04213248
Refractory depression and neurodegenerative dementia	Amniotic fluid	N/A n = 300	Unmodified	Enrolling by invitation	NCT04202770
Severe lung disease	Allogenic adipose-derived MSCs	Phase I n = 10	Unmodified	Recruiting	NCT04313647
Novel coronavirus pneumonia	Allogenic adipose-derived MSCs	Phase I n = 30	Unmodified	Not yet recruiting	NCT04276987
Atrial fibrillation	Epicardial fat	N/A n = 35	Unmodified	Recruiting	NCT03478410
Myocardial infarction	Peripheral blood	Observational n = 10	Unmodified	Not yet recruiting	NCT04127591

Motivation and Aim of Studies

The adult human heart holds limited regenerative capacity, with a renewal rate of around 1% that is insufficient to counteract the damage caused by cardiac injury. Considering the global burden of cardiovascular disease and the limitations of current pharmacological/medical interventions, alternative strategies have been studied to restore cardiac function. Despite efforts to translate stem cell research to the clinical setting, the hypothesis of myocardium remuscularization through stem cell transplantation has been gradually replaced by evidence that a larger therapeutic action is mediated by paracrine mechanisms, including that of EVs.

Concerning cardiac regenerative medicine, there is a growing interest in using EVs as cell-mimetic therapeutics for their expected superiority, along with their advantages over cell transplantation, namely the absence of oncologic risk, low immunogenicity and possibility of robust large-scale production. Research efforts have leaned towards the use of adult cells with limited self-renewal capacity, namely CPCs and MSCs, as cellular sources of EVs. Still, the therapeutic potential of EVs from hiPSCs and its derivatives remains largely understudied.

This work focuses on the isolation and characterization of EVs along the hiPSC-CM differentiation and maturation timeline, aiming to identify a potential cell candidate to generate EVs with clinically relevant yields and promising therapeutic potential in the cardiovascular context. Through the implementation of a robust workflow for EV separation from conditioned culture media of hiPSCs, hiPSC-CPCs, immature and mature hiPSC-CMs, along with the extensive characterization of the isolated EV populations regarding particle descriptors, cargo composition and functional potential, we have striven to advance the knowledge on promising EV-producing cells. With this, we aim to deepen the understanding of EV isolation/purification and functionality, in the hope of contributing to the translation of EV research into clinical applications and vindication of EVs as cell-free therapeutics.

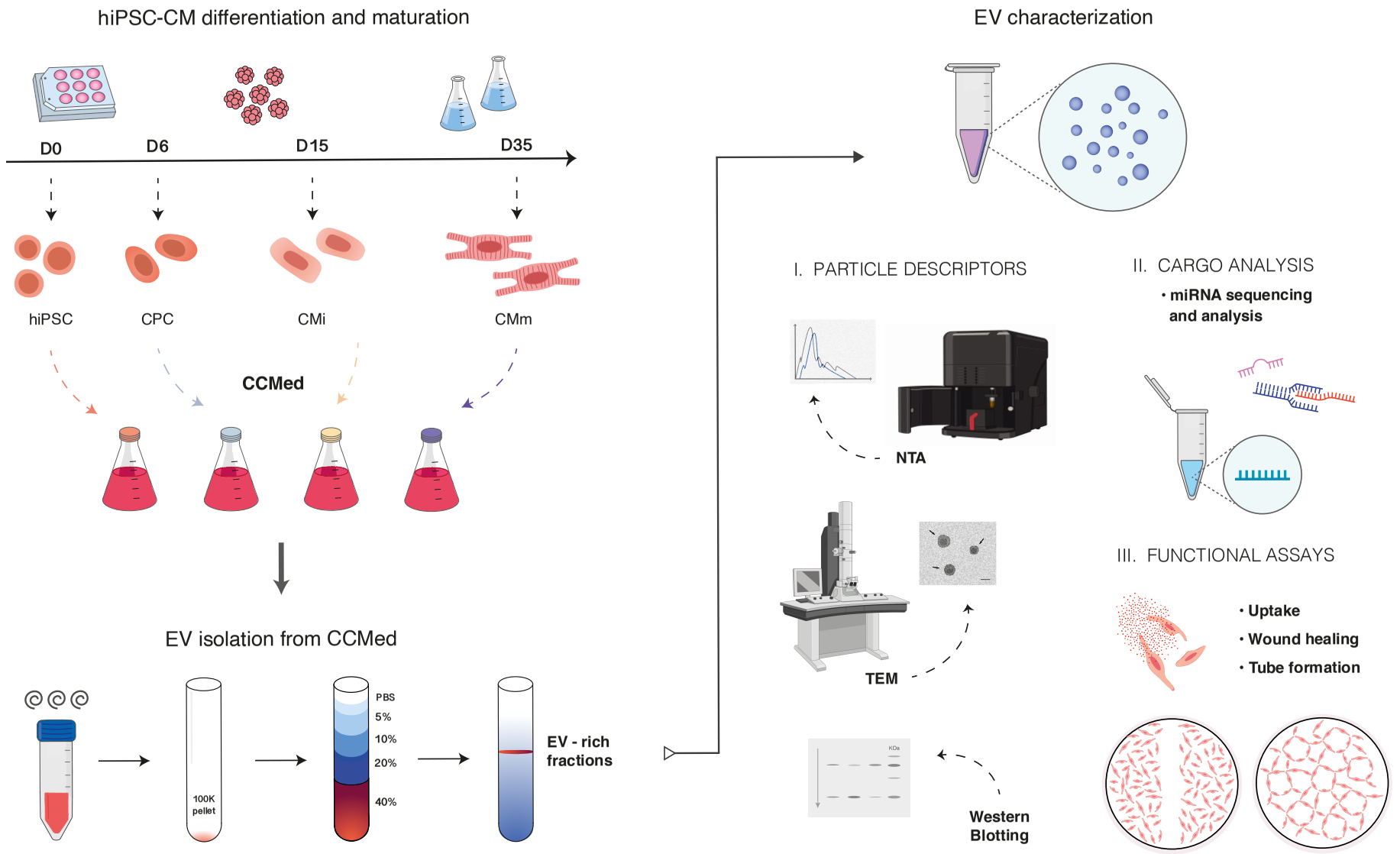


Figure 1.5: **Overview of the experimental workflow for cardiomyocyte differentiation and EV isolation from different cell populations: human induced pluripotent stem cells (hiPSC) and their derivatives including, cardiac progenitor cells (CPC), immature and mature cardiomyocytes (CMi and CMm).** hiPSCs were differentiated into CMs using a 3D differentiation protocol previously developed by iBET. Conditioned culture medium (CCMed) was harvested at days 0 (hiPSC), 6 (CPC), 15 (CMi) and 35 (CMm) of culture and processed by multiple centrifugation steps and filtration through 0.45 µm to remove major contaminants. The cleared CCMed was ultracentrifuged at 110.000 g_{max} for 2h40 min to produce a 100K pellet, which was loaded on the bottom of an OptiPrep™ density gradient and centrifuged for 18h at 110.000 g_{max}. EV-rich density fractions 8-9 were collected and concentrated. Particle characterization was performed to ensure purity of EV samples and functional assays were carried out to assess therapeutic potential of EVs in the cardiac setting. MicroRNA analysis was perfomed to give further insights on target functions and specificity of released EV cargo.

Chapter 2

Materials and Methods

2.1 hiPSC culture

2.1.1 hiPSC expansion

The hiPSC line IMR(90)-4 (WiCell) was used for this purpose. hiPSCs were cultured and routinely expanded on Matrigel coated plates (Matrigel® hESC-Qualified Matrix, Corning®) in serum-free mTeSR™1 medium (StemCell Technologies) at 37°C in a humidified atmosphere of 5% CO_2. The same expansion procedure was followed for hiPSCs to be used for EV isolation (day 0 sample), using feeder-free and animal component-free TeSR-E8™ medium (StemCell Technologies) to ensure complete EV depletion from external sources. Cells tested negative for mycoplasma contamination.

2.1.2 hiPSC differentiation into cardiomyocytes

Differentiation of hiPSCs into CMs was initiated once cells reached 80-90% confluence and performed following a previously described protocol [142], by temporal modulation of the Wnt/β-catenin signalling. Briefly, at day 0 of differentiation, expansion medium was replaced by RPMI 1640 medium (Thermo Fisher Scientific) supplemented with B27 without insulin (Thermo Fisher Scientific)(RPMI+B27-I), and 80 ng/ml Activin A (PeproTech), 12 μM CHIR99021 (Tocris Bioscience) and 50 μg/ml Ascorbic acid (Sigma-Aldrich). At day 1, approximately 24h after differentiation induction, the medium was replaced by RPMI+B27-I supplemented with 5 μM IWR-1 (Selleckchem) and 50 μg/ml Ascorbic acid (Sigma-Aldrich). At day 3, the medium was replaced by RPMI+B27-I supplemented with 5 μM IWR-1. At day 6, the medium was exchanged for RPMI medium supplemented with B27 with insulin (Thermo Fisher Sci-

entific) (RPMI+B27).

Upon reaching 80% of beating cells in monolayer (between days 7 and 8 of differentiation), cells were dissociated by incubation with TrypLE Select (ThermoFisher Scientific) for 8 min and aggregated using AggreWell^{TM}400 (STEMCELL Technologies) at a seeding density of 1.500 cell/microwell, according to a previously described protocol [37]. Approximately 48h after aggregation, CM aggregates were harvested and transferred to an orbital suspension culture system, at an agitation rate of 90 rpm. Medium (RPMI+B27) was replaced every two days thereafter.

At day 15 of culture RPMI+B27 medium was replaced by maturation media, composed of RPMI 1640 without glucose (MP biomedicals, ThermoFisher Scientific) supplemented with B27, 1 mM of glutamine, 10 mM of galactose, 100 μM of oleic acid and 50 μM of palmitic acid, to promote CM metabolic maturation [142]. Maturation medium was changed every other day thereafter. CM aggregates were cultured in these conditions for an additional 20 days, reaching a total of 35 days of culture.

Cells were characterized along the differentiation and maturation timeline as described in Section 3.2. Conditioned culture medium (CCMed) was collected at days 0, 6, 15 and 35 of culture and processed according to Section 3.3.

2.2 Cell characterization

2.2.1 Cell viability

Qualitative cell viability was assessed through cell membrane integrity analysis by incubation of cell samples with enzyme substrate fluorescein diacetate (FDA, Sigma-Aldrich) and DNA-binding dye propidium iodide (PI, Sigma-Aldrich) in DPBS (Gibco®, Thermo Fisher Scientific). Phase contrast and fluorescence images were acquired using an inverted fluorescence microscope (DMI6000, Leica Microsystems GmbH) and analysed with ImageJ open source software. Quantitative cell viability was assessed by trypan blue exclusion assay.

2.2.2 Flow cytometry

hiPSC and CM phenotypes were confirmed by flow cytometry (FC), through the analysis of pluripotent stem cell markers (TRA-1-60 and SSEA4) and early differentiation marker (SSEA1) for cells at day 0 and cardiomyocyte markers SIRPα/β, VCAM and

cardiac Troponin T (cTnT) at days 7, 15 and 35 of differentiation. For this, cells were dissociated using Versene (Gibco®, ThermoFisher Scientific) or TrypLE Select (Gibco®, ThermoFisher Scientific), respectively hiPSCs or CMs and washed twice with FACS buffer composed of 5% (v/v) fetal bovine serum (FBS) in DPBS. For the detection of intracellular epitopes (cTnT), cells were fixed and permeabilized using a commercial kit (BD Cytofix/Cytoperm, BD Biosciences). Cells were resuspended in the respective primary antibody or isotype control (Table 2.1) and incubated for 1h at 4°C. Additional incubation with secondary antibody was performed for samples where primary unconjugated antibodies were used. Samples were analysed on a BD FACS Celesta (BD Biosciences), with a minimum of 20.000 events recorded per sample. Data was acquired using the BD FACS DIVA software and analysed with FlowJo software (Becton, Dickinson & Company).

Table 2.1: List of antibodies used in flow cytometry.

Antibody	Dilution	Reference
Primary Conjugated		
Anti-SSEA4, FITC (IgG3)	1:10	560126 (BD Biosciences)
Isotype control IgG3, FITC	4:10	355578 (BD Biosciences)
Anti-SSEA1, FITC (IgM)	1:10	sc-21702 (Santa Cruz Biotechnology)
Isotype control IgM, FITC	1:400	553474 (BD Biosciences)
Anti-SIRPα/β, PE (IgGκ1)	1:5	323806 (BioLegend)
Anti-VCAM, PE (IgGκ1)	1:5	555647 (BD Biosciences)
Isotype control IgGκ1, PE	1:5	sc-2878 (Santa Cruz Biotechnology)
Primary Unconjugated		
Anti-TRA-1-60 (IgM)	1:10	sc-21705 (Santa Cruz Biotechnology)
Isotype control IgM	1:10	sc-3881 (Santa Cruz Biotechnology)
Anti-Cardiac Troponin T (IgG1)	1:200	MS-295-PI (Thermo Fisher Scientific)
Isotype control IgG1	4:10	sc-3877 (Santa Cruz Biotechnology)
Secondary		
Alexa Fluor 488 anti-mouse IgM	1:200	A21042 (Thermo Fisher Scientific)
AlexaFluor 488 anti-mouse IgG	1:200	A11001 (Thermo Fisher Scientific)

2.2.3 Gene expression

mRNA was extracted from cell pellets using a High Pure RNA isolation Kit (Roche) according to the manufacturer's instructions and quantified with Nanodrop 2000c (Thermo Fisher Scientific). cDNA synthesis was performed on 50 µg of RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Real time quantitative polymerase chain reaction (RT-qPCR) was performed using the LightCycler 480 Instrument II 384-

well block (Roche) in the following cycles: incubation at 95°C for 10 min; 45 cycles of amplification with denaturation at 95°C for 15 sec, and annealing at 60°C for 1 min; extension at 72°C for 5 min. The primers/probes used for this purpose are available in Table 2.2. The Cycle threshold (Ct) was determined using the LightCycler 480 software (Roche). Gene expression data was normalized to housekeeping genes RPLP0 and GADPH and relative changes were analysed using the $2^{\Delta\Delta Ct}$ method (D6 vs. D0, D35 vs. D15). Only data obtained for ISL1, GATA4, TNNI1, TNNI3, MYH6 and MYH7 genes was used for this work.

Table 2.2: List of primers/probes used in RT-qPCR.

Gene	Reference	Gene	Reference
GAPDH	Hs99999905	TNNI3	Hs00165957
RPLP0	Hs99999902	MYH6	Hs01101425
NKX2.5	Hs00231763	MYH7	Hs01110632
GATA4	Hs00171403	SCN5A	Hs00165693
ISL1	Hs00158126	CACNA1C	Hs00167681
NANOG	Hs02387400	GJA1	Hs0748445
POU1F5	Hs00999632	KCND3	Hs05056365
TNNT2	Hs00165960	ATP2A2	Hs0054487
TNNI1	Hs00913333		

2.3 EV isolation and purification

2.3.1 Conditioned culture medium harvest

Conditioned culture medium (CCMed) was harvested at days 0, 6, 15 and 35 of culture, respectively of hiPSCs, CPCs, immature CMs (CMi) and mature CMs (CMm). For each harvest, total cell number and cell viability were estimated by cell counting using trypan blue exclusion dye. The same volume of medium (180 mL) was collected and processed at each time point.

2.3.2 EV separation from conditioned culture medium

EVs were isolated from CCMed using the gold standard combination of differential centrifugation and density gradient ultracentrifugation, which have been shown to outperform other regularly used isolation methods [68, 143].

Briefly, the collected CCMed was processed to ensure depletion from major contam-

inants. For this, two low-speed centrifugation steps (rotor A-4-81, 5810 R centrifuge, Eppendorf) were performed at 4°C immediately after harvest, 1x 300 g for 10 min to remove remaining cells and 1x 2000 g for 10 min to remove dead cells, followed by a 0.45 µM filtration of the resulting supernatant (Nalgene™ Rapid-Flow™, Thermo Fisher Scientific) to deplete the sample of all other debris. The cleared CCMed was then ultracentrifuged in 30 mL conical open-top polyallomer tubes (Beckman Coulter) at 110.000 g_{max} for 2h45 using a XL-100 ultracentrifuge (SW 28 rotor, Beckman-Coulter) to produce an EV pellet, designated as the 100K pellet.

For the density gradient ultracentrifugation, an OptiPrep™ density gradient (ODG, Axis Xield Diagnostics) was prepared as previously described in the literature [143], with minor modifications. An iodixanol working solution (50% (w/v) iodixanol) was made by adding a working solution buffer (60 mM Tris-HCl, 6 mM EDTA, 0.25 M sucrose (pH 7.4)) to a stock solution of OptiPrep™ (60% (w/v) aqueous iodixanol solution). Appropriate amounts of the iodixanol working solution and a homogenization buffer (10 mM Tris-HCl (Tromethamine - Hydrochloric acid), 1 mM EDTA (Ethylenediaminetetraacetic acid) and 0.25 M sucrose (pH 7.4)) were combined in order to prepare 5%, 10%, and 20% (w/v) iodixanol solutions. The 100K pellet was combined with the 50% iodixanol working solution, to create a 40% (w/v) iodixanol EV-containing solution. The discontinuous bottom-up ODG was prepared by loading 4 mL of 40% solution (containing the EV sample) on the bottom of a 16.8 mL open-top polyallomer tube (Beckman Coulter), followed by consecutive layering of 4 mL of 20%, 4 mL of 10%, 3.5 mL of 5% solutions and 1 mL of DPBS. The gradient was centrifuged at 4 °C for 18 h at 110.000 g_{max} using an XL-100 ultracentrifuge (SW 28 rotor, Beckman-Coulter). During centrifugation, EVs migrate upwards by flotation and settle upon reaching their buoyant density (1.08-1.19 g/mL) [144]. After centrifugation, 16 fractions of 1 mL were collected from top (fraction 1) to bottom (fraction 16). Density fractions 8-9, which have been found to contain most of the EV sample of interest [143], were pooled and concentrated to 300 µL using Amicon® Ultra-2 mL 10 KDa filter units (Merck Millipore). Negative and positive control gradients were prepared with blank DPBS and Fetal Bovine Serum (FBS qualified, EU-approved, Gibco®, Thermo Fisher Scientific), respectively.

2.4 EV characterization

2.4.1 Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) was performed using the NanoSight NS300 (Malvern Instruments Ltd., Malvern, UK) equipment, with a 488 nm laser ($<$ 55 mW maximum power) and an automatic syringe pump system. NTA was used to measure size, size distribution and concentration of particles from the pooled Optiprep^{TM} density gradient EV-rich fractions 8-9. Three videos of 60 s were recorded for each individual sample and considered using detection threshold 3, camera level 14 and a syringe pump infusion speed of 40. All videos were analysed by NTA software version 3.3. To achieve optimal measurements, samples were diluted with DPBS to obtain a particle concentration within the ideal range for the NTA software ($3x10^8$ – $1x10^9$ particle/mL). All size distributions determined with NTA correspond to the hydrodynamic diameters of the particles in suspension, as calculated by the Stokes-Einstein equation.

2.4.2 Transmission Electron Microscopy

Transmission Electron Miscroscopy (TEM) was performed on EV-rich fractions 8-9. A drop (5 µL) of sample was deposited on a precoated formvar/carbon 150 mesh Veco copper grid for 2 min. Grids were fixed with 4% (w/v) PFA for 20 min, washed with sterile water, stained with 2% (w/v) uranyl acetate for 2 min and dried at room temperature (20-22°C). Grids were examined using electron microscopy (Hitachi H-7650, 120 KV electron microscope, Hitachi High-Technologies Corporation).

2.4.3 Refractive Index and EV density

A digital refractometer AR200 (Leica Microsystems GmbH) was used to measure the refractive index of EV-rich fractions 8-9. The refractive index was converted to density based on a standard linear regression curve ranging from 10% to 30% (w/v) iodixanol (Supplementary information Figure 4.1). To compute this curve, iodixanol solutions were prepared with concentrations varying from 10% to 30% in working solution buffer, in incremental steps of 2%. Density-equivalent values for each iodixanol solution were provided by the manufacturer. Refractive indexes were measured for each solution and computed into a linear regression curve using GraphPad Prism 7 (GraphPad Software).

2.4.4 Western Blotting

Western blot was performed on EV-rich fractions 8-9 and whole cell lysates for all cell types for EV specific transmembrane protein CD63 and cytosolic protein Flotillin-2 [82], and for non-EV common contaminant AGO2 [148]. EV samples were normalized by volume (20 µL of sample per lane) and whole cell lysates were normalized by protein amount (10 µg of protein was applied per lane). Protein concentrations were determined by BCA (ThermoFisher Scientific).

Samples were diluted in lysis buffer (NuPAGE® LDS sample buffer 4x, Novex®, Life Technologies Europe B.V.) in reducing (Flotillin-2, AGO2) or non-reducing conditions (CD63) and boiled for 5 min at 95 °C. Protein samples were separated by SDS polyacrylamide gel electrophoresis in MES running buffer (Novex®, Life Technologies Europe B.V.) for 50 min and transferred to a nitrocellulose membrane (iBlot® Transfer Stack, nitrocellulose, mini, Novex®, Life Technologies Europe B.V.). Membranes were stained with Ponceau S solution (Sigma Aldrich) immediately after transfer for acquisition of pictures to confirm the success of the transfer, and then washed with distilled water, de-stained with 1 M NaOH solution, and again washed extensively with distilled water. Membranes were blocked with 5% (w/v) skim milk in Tris-Buffered Saline + 0.1% (v/v) Tween 20 (TBST) for 1h at room temperature and then incubated overnight at 4 °C in primary antibodies diluted in 5% skim milk prepared in TBST: CD63 (1:1.000, ab59479, Abcam), Flotillin-2 (1:1.000, 610383, BD) and AGO2 (1:1.000, ab32381, Abcam). The following day, membranes were washed with TBST, incubated with the respective secondary antibody for 1h at room temperature (anti-rabbit HRP, 1:20.000, System Biosciences; anti-mouse ECL, 1:5.000/ 1:10.000, NA931V, GE Lifesciences) and then washed again. For the revelation step, chemiluminescent substrate (Western-Bright Sirius, Advansta) was added to the membranes according to the manufacturer's instructions and imaging was performed using a ChemiDoc imaging system (Bio-Rad Laboratories, Hercules).

2.5 Functional assays

2.5.1 HUVEC culture

HUVECs (catalog No. C2517A, Lonza) were cultured according to the manufacturer's specifications. Briefly, cells were maintained in Endothelial Cell Growth Medium 2

(ECGM-2, Promocell), containing 2% (v/v) of Fetal Calf Serum (FCS, Promocell), in a humidified atmosphere at 37°C in 5% CO_2. Medium was changed every 48h. Cells were subcultured upon reaching 70-85% confluence. HUVECs were only maintained and used for experiments up to passage 5, including.

2.5.2 EV uptake assays

EV labelling was performed using the PKH26 Red Fluorescent Cell Linker Mini Kit for General Cell Membrane Labelling (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, the EV 100K pellet was resuspended in 20 µL of 0.2% (w/v) bovine serum albumin (BSA, Gibco®, Thermo Fisher Scientific) and diluted in 80 µL of diluent C. For every sample, 1.5 µL of PKH26 were diluted in 100 µL of diluent C and EVs were transferred to the diluted PKH26. The mixture was incubated for 3 min at room temperature and the labelling reaction was stopped by adding 100 µL of 0.1% BSA. A negative control was prepared with PBS following the same protocol. Stained vesicles (PKH26-EVs) and the corresponding control (PKH26-PBS) were loaded on the bottom of a density gradient, and ultracentrifuged as previously described, to separate labelled particles from unbound dye.

HUVECs were seeded in 8-well chamber Ibidi® slides (Ibidi GmH) at a density of 8.000 cells/cm^2 or in 12-well plates at a density of 18.000 cells/cm^2, to be used for immunofluorescence and flow cytometry respectively, and treated with PKH26-EVs or PKH26-PBS appropriately diluted in ECGM-2 at a dose of 3.000 particles per cell. For each sample, 1 well was prepared with the addition of dynamin inhibitor Dynasore (Sigma Aldrich) in a concentration of 50 µM, to prevent EV uptake. HUVECs were incubated for 2h30 in a humidified environment (37 °C, 5% CO_2). Cells to be used for flow cytometry were dissociated using Versene (Gibco®, ThermoFisher Scientific), washed twice with FACS buffer and then analysed using a BD FACS Celesta™ cytometer (BD Biosciences). Cells to be used for immunofluorescence were fixed with 4% (w/v) PFA for 20 min at room temperature, and then stained for CD31 marker. For this, fixed cells were washed twice with DPBS, blocked with 0.2% (w/v) FSG for 30 min at room temperature and incubated overnight with primary anti-CD31 antibody diluted in 0.2% FSG (1:50; clone JCF0A, M0823, DAKO Omnis, Agilent Technologies), or the corresponding mouse IgG isotype control (1:50, sc-3877, Santa Cruz Biotechnology) at 4°C. The following day, cells were washed with DPBS and incubated with secondary

antibody diluted in 0.2% FSG (1:200, Alexa 647, donkey anti-mouse IgG, 31571, ThermoFisher Scientific) for 1h at room temperature. Nuclei were counterstained with DAPI (ThermoFisher Scientific) in a 1:1.000 dilution in DPBS. Fluorescence images were acquired using an inverted fluorescence microscope (DMI6000, Leica Microsystems GmbH) and signal intensity was quantified using ImageJ software.

2.5.3 Wound healing assay

HUVECs were seeded at 35.000 cells per well onto 48-well plates coated with 0.1% (w/v) gelatin (Sigma-Aldrich). A sterile 200 µL pipette tip was used to create a scratch in the cell monolayer, after which cells were washed twice with DPBS. EV samples were diluted in appropriate amounts of reduced-supplementation media (with 0.5% (v/v) FCS and without growth factors) and blank gradient. Positive (fully supplemented ECGM-2), untreated negative (Endothelial Cell Basal Medium-2 (ECBM-2, consisting of ECGM-2 without any added supplements) + 0.5% FCS) and FBS-EVs (isolated through the same process as CCMed) controls were prepared. Vehicle controls, consisting of the 8-9 fractions of a blank gradient in volumes consistent with each EV sample, were also prepared for each day 0, 6, 15 and 35. The same procedure was conducted with the addition of Dynasore in concentration of 50 µM (Sigma-Aldrich), which inhibits the uptake of EVs by HUVECs and demonstrates that the wound healing effect observed in the assay is indeed mediated by EVs. 500 µL of corresponding treatment was added per well, normalized to a dose of 3.000 particles per cell. Cells were placed in a humidified environment (37 °C, 5% CO_2) and images were acquired at time-points 0h, 8h and 24h post-scratch using an inverted microscope (DMI6000, Leica Microsystems GmbH). Wound width was analysed with MRI Wound Healing tool of ImageJ software, by calculating the percentage of wound closure relative to time-point 0h.

2.5.4 Cell proliferation assay

Following the wound healing assay, 1 µM of EdU (5-ethynyl-2'-deoxyuridine) was added to each well. HUVECs were incubated for 24h in a humidified incubator (37 °C, 5% CO_2) and then fixed with 4% (w/v) PFA for 20 min at room temperature. The Click-iT® EdU (ThermoFisher Scientific) reaction kit was used according to the manufacturer's instructions. HUVEC nuclei were counterstained with DAPI reagent and HUVECs were further stained for CD31 marker, as previously described. Fluorescence images were

acquired using an inverted fluorescence microscope (DMI6000, Leica Microsystems GmbH). Edu- and DAPI-positive nuclei count was performed using ImageJ software. A minimum of 1.000 DAPI-positive nuclei were considered for each condition. EdU-positive nuclei were measured as a percentage of total DAPI-positive nuclei.

2.5.5 Tube formation assay

HUVECs were seeded at 12.000 cells per well onto 48-well plates coated with 40 µL of basement membrane extract (Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free, Corning®). HUVECs were resuspended in the corresponding EV sample appropriately diluted in ECBM-2, at a dose of 3.000 particles per cell. Positive (ECGM-2), untreated negative (ECBM-2), FBS, PBS and vehicle controls were prepared. For each sample, the same procedure was conducted with the addition of Dynasore in concentration of 50 µM. To validate the efficacy of this assay, Suramin Sodium Salt (TargetMol), an inhibitor of endothelial cell proliferation and migration, was added to positive control cells in concentrations of 5 µM and 10 µM diluted in ECGM-2. The plate was placed in a humidified environment (37 °C, 5% CO_2) for 8 h. Cells were then stained with a 1:500 solution of FDA (Sigma-Aldrich) in DPBS. Fluorescence images were acquired at 5x magnification using an inverted fluorescence microscope (DMI6000, Leica Microsystems GmbH). Pictures were analysed using the Angiogenesis Analyzer plugin of ImageJ software.

2.5.6 microRNA analysis

Total RNA was isolated from EV samples (N=3 replicates for each sample) using Norgen Biotek Exosomal RNA Isolation Kit (Cat.58000). microRNA library preparation was performed by PCR amplification using Norgen Biotek Small RNA Library Prep Kit (Cat. 63600) and library quality control was achieved using Bioanalyzer to estimate library size and concentration. Libraries were denatured and diluted to the required concentration and then applied onto the suitable flowcell and sequenced using Illumina NextSeq 500 sequencing platforms. Data analysis was carried out using an advanced pipeline for processing of raw reads and mapping to the genome and annotated transcriptome. Data was filtered and normalized using the trimmed mean of M-values (TMM) normalization method for differential expression (DE) analysis. DE analysis between each two groups was performed using the EdgeR statistical software package and false discov-

ery rate was adjusted through the Benjamini-Hochberg procedure. For the comparison of hiPSC-EVs with the designated CPC/CMi/CMm-EVs group (the remaining groups considered as a whole), replicates of each biological group were considered as individual replicates of the CPC/CMi/CMm-EVs group. DE was considered significant for log fold change $\geq$ 1 or $\leq$ -1 at p-value and FDR $\leq$ 0.05. Results were further trimmed by considering only miRNAs with log fold change $\geq$ 2 or $\leq$ -2. Putative targets of up- and down-regulated miRNAs between hiPSC-EVs and CPC/CMi/CMm-EVs were predicted, along with KEGG pathways and GO-terms analysis from the selected targets, using the DIANA-miRPath v3 software, considering only results with p-value $\leq$ 0.05.

2.6 Statistical analysis

Statistical analysis was entirely performed using GraphPad Prism 7 (GraphPad Software). Significance was tested using either Student's T test or one-way ANOVA. Statistical significance was considered for p-values $<$ 0.05. All experimental outcomes correspond to a minimum of 3 biological replicates, with the exception of CMm-EVs, with 2 biological replicates and technical duplicates for one of the replicates.

Chapter 3

Results and Discussion

3.1 Cell differentiation and cardiomyocyte maturation

In this work, EVs from four hiPSC derivatives were isolated and characterized. To ensure phenotypic and genotypic identity of cell populations and their validity as the designated EV sources, key cell characterization methodologies were performed throughout the differentiation and maturation timeline. Briefly, hiPSCs were cultured in 2D monolayer and differentiated towards the cardiac lineage [142]. Upon reaching the cardiac progenitor contractile stage, cells were harvested and aggregated in AggreWell^{TM} plates for 48h, after which spheroids were transferred to orbital suspension cultures and maintained until day 35.

Live/Dead staining was performed at all stages and confirmed cell viability throughout the culture timeline, with CPCs showing increased cell death as a result of cells that did not commit to the cardiac lineage (Figure 3.1 a).

Immunophenotyping was performed for hiPSCs and CMs by flow cytometry. Analysis of pluripotency markers SSEA-4 and TRA-1-60 and early differentiation marker SSEA-1 revealed a population of 99% SSEA-4 and 94% TRA-1-60 positive cells, and over 99.9% of cells negative for SSEA-1. CMi and CMm populations were analysed for CM-specific markers SIRPα/β, VCAM-1 and cardiac troponin T, with purity of over 80% for CMi and 75% and CMm populations (Figure 3.1 b).

Maturation of cardiac cells was assessed from changes in gene expression indicative of an adult-like signature. CMs at day 35 of culture showed increased ratios of adult to fetal isoforms of sarcomeric genes when compared to day 15, including MYH7/MYH6 and TNNI3/TNNI1, encoding adult/fetal myosin heavy chain and cardiac/slow skeletal

muscle troponin I, respectively. CPC identity was confirmed by increased expression of genes implicated in cardiac commitment and promotion of cardiogenesis, GATA4 and ISL1 [145, 146]. These results confirm a distinct maturation profile for CMi and CMm cell groups and cardiac progenitor genotype for CPCs, and further demonstrate an enhancement in hiPSC-CM maturation provided by a 3D culture environment [37].

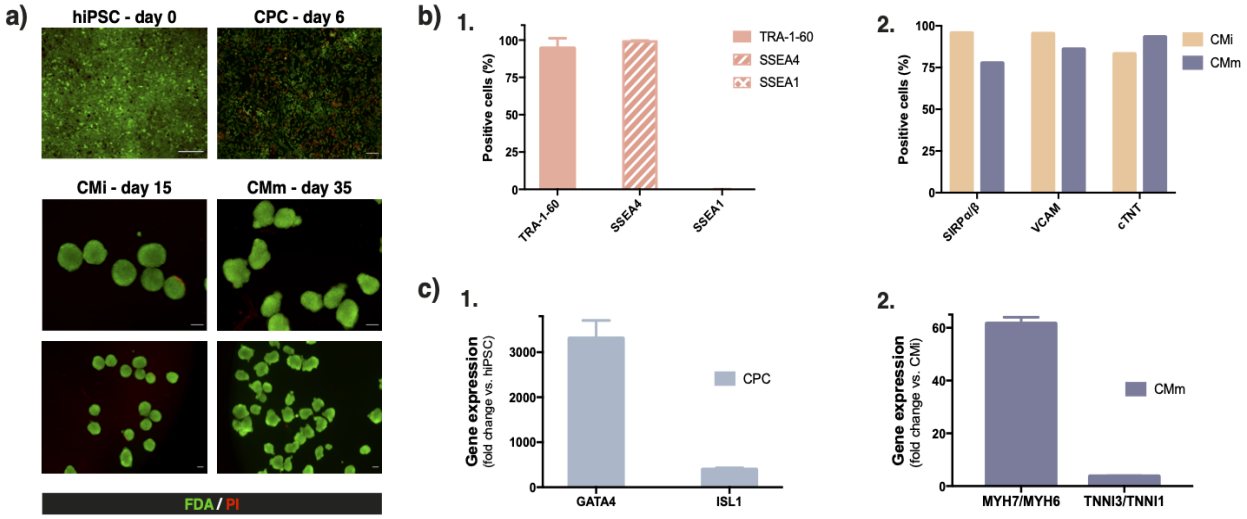


Figure 3.1: **Characterization of cell samples. a)** Fluorescence images of all cell types (hiPSC, CPC, CMi and CMm) throughout the differentiation timeline. Cells were stained with fluorescein diacetate (FDA) and propidium iodide (PI), respectively live (green) and dead (red) cells. Scale bar: 200 µm. **b)** Flow cytometry analysis for validation of 1. pluripotency and 2. cardiomyocyte phenotypes, expressed as percentage of positive cells. Results shown as mean ± SD (1. n=2 and 2. n=1). **c)** Gene expression data of CPC and CMm for characteristic markers of progenitor and mature phenotypes, respectively. Gene expression is presented in fold change relative to 1. hiPSC data and 2. CMi data.

3.2 Establishment of an effective EV isolation and purification method and EV characterization

The need for highly effective and consistent methods for the isolation of extracellular vesicles from culture supernatant and a diversity of other sample populations is pressing, if there is ever a hope for EV-based medicinal products to meet standardization levels required by the pharmaceutical market. In this thesis, the isolation of EVs from CCMed of the previously described cell populations was performed following an ISEV-approved density-based method (see Section 3.3) that complies with the

recommended specifications on EV recovery and purity [82].

Numerous protocols for EV isolation are currently employed across the scientific community, most of which based on specific particle attributes such as size, density and protein affinity. The choice of isolation method is a critical parameter known to significantly impact size, yield and purity of EV samples [147]. OptiPrep^{TM} density gradient, the adopted method for this work, has been shown to outperform other commonly used approaches in terms of enrichment in exosomal markers and absence of contaminants by several independent studies [68, 143].

Standard particle characterization was conducted for fractions 8-9 of each EV group. For this, NTA was performed on EV samples to assess size distribution and particle concentration, a technique that relies on the relation between the brownian motion of spherical particles and their hydrodynamic radius as derived by the Stokes–Einstein equation. Results indicated a typical NTA profile for the size distribution of all samples, ranging between 50 and 500 nm. More specifically, particle concentration peaks occur for particle sizes of 134.5 and 176.5 nm for hiPSC-EVs, 138.5 nm for CPC-EVs, 105.5 nm and 139.5 nm for CMi-EVs and 127.5 nm for CMm-EVs (Figure 3.2), which stand within the reported range for EV size [77]. hiPSC-EVs and CPC-EVs showed the highest particle concentration values, 1.54×10^{8} and 1.59×10^{8} particle/mL, respectively. On the other hand, CMi-EVs and CMm-EVs showed the lowest concentrations of 9.46×10^{7} and 7.74×10^{7} particle/mL. The yield of EVs was estimated, defined as the number of isolated particles secreted per million viable cells per day of production. As shown in Figure 3.2 b)-1, EV yield analysis indicates a distinct profile regarding EV production capacity to that which might be interpreted following NTA results. When considering the variable of cell number, EV yield is higher for CMm cells (6.50×10^{7} particle.10^{6} cell^{-1}.day^{-1}) than would be expected from NTA results, given that the number of CMm cells in culture was significantly lower than the remaining cell types. Still, this value could be overestimated, since cell viability significantly decreased towards the end of the cell culture timeline, creating a large disparity in cell number from CMm to the other cell populations. Contrary to previous findings that reported limited EV release by CMs relative to CPCs [139], when normalization to the number of EV-producing cells is performed, CPCs seem to give the lowest EV yield (8.50×10^{6} particle.10^{6} cell^{-1}.day^{-1}) out of the four considered cell populations, in spite of showing the greatest particle concentration by NTA. Furthermore, EV yield is highly influenced by the choice of isolation

method [144]. Given that the present methodology contains an exhaustive series of centrifugation steps, it may compromise yield to a similar extent in all samples.

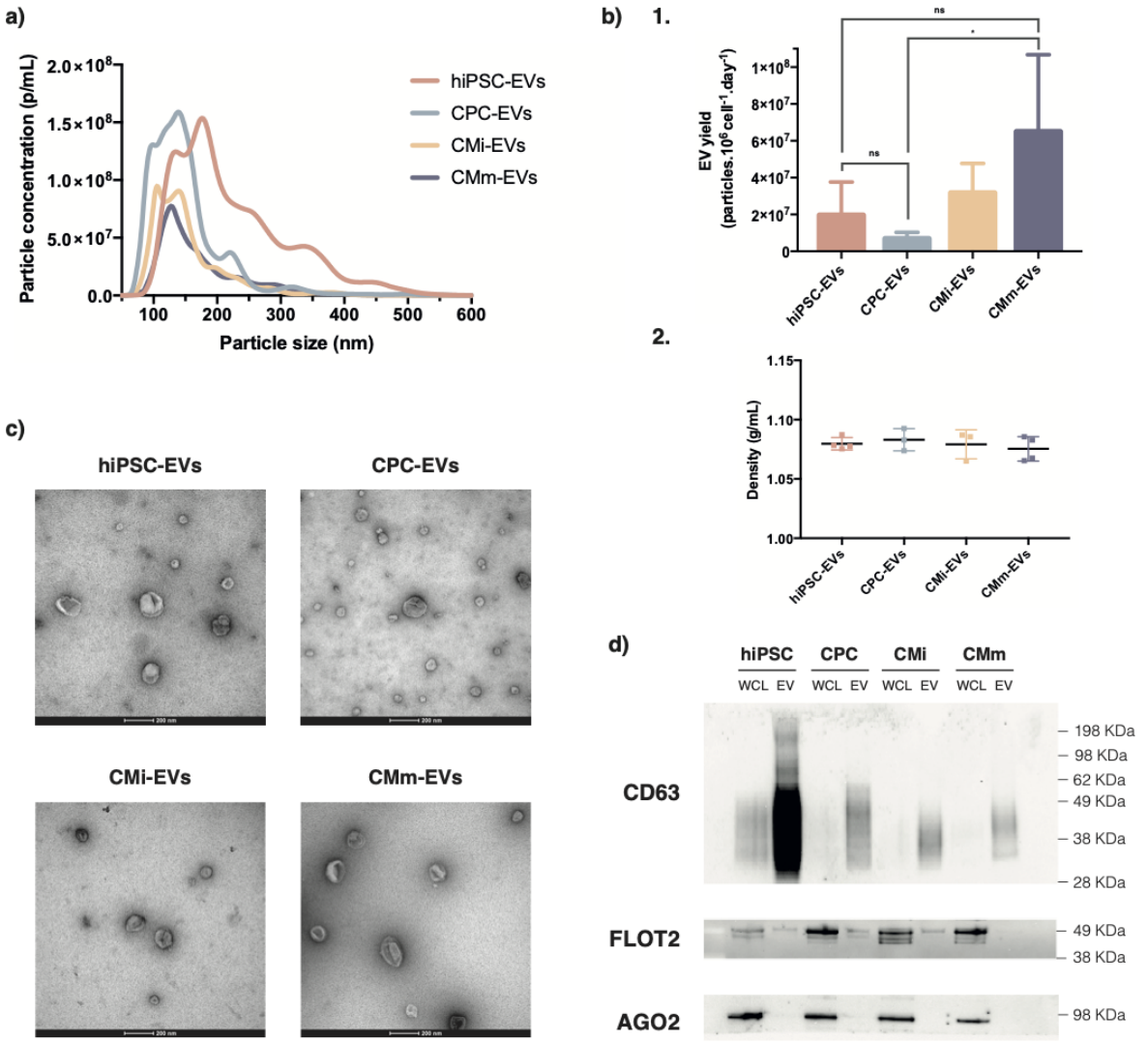


Figure 3.2: **Characterization of EV samples. a)** Size distribution profile of EV samples as analysed by Nanoparticle Tracking Analysis, expressed in particles per mL of sample (n=4). **b) 1.** EV yield, measured as total number of isolated particles per million viable cells per day. **2.** Density of isolated EVs in the pooled 8-9 fractions. Results shown as mean $\pm$ SD ($n \geq 3$). *$p < 0.1$, ns non-significant. **c)** Representative negative staining close-field transmission electron microscopy (TEM) images of EV samples. Scale bar: 200 µm. **d)** Western blot analysis of characteristic EV markers (transmembrane protein CD63 and cytosolic protein Flotillin-2) and co-isolated contaminant (Argonaute 2) of EV samples and respective cell lysate. WCL – whole cell lysate.

Visualization of particles by Transmission Electron Microscopy confirmed typical EV morphology and absence of similar-sized contaminant particles such as low-density

lipoproteins (LDLs) (Figure 3.2 c). To further substantiate the identity of isolated EV samples, Western Blotting was performed for detection of specific markers (Figure 3.2 d). According to the most recently published minimal information for studies of EVs [82], the demonstration of purity in EV preparations through protein-based characterization requires the presence of one transmembrane and one cytosolic protein commonly found in EVs and one non-EV co-isolated protein. In effect, tetraspanin CD63 was detected in all EV samples, especially enriched in hiPSC-EVs, and cytosolic lipid-binding protein Flotillin-2 was found to be present in EV samples and corresponding producing cells, although very faintly in CMm-EVs (Figure 3.2 d). Non-EV contaminant Argonaute 2 was present in cell lysates and absent in EV samples, serving as a negative marker and indicator for the degree of purity of isolated EV populations [148].

3.3 Functional analysis of EV biological activity

EV labelling, cellular internalization and uptake blockage

Labelling of extracellular vesicles is a long-standing technique for the visualization of such small-sized particles. This can be achieved by numerous methods, most commonly making use of fluorescence-based recombinant proteins (e.g. GFP) or organic dyes, such as lipophilic carbocyanines (PKH26, PKH67, DiI, DiR, DiD) and other substances (CFDA-SE, Calcein AM) [149]. In this thesis, PKH26 was used to label lipid-rich EV membranes and detect their internalization by HUVECs.

PKH26 has been reported to increase EV size, with a detectable shift in the size distribution of labelled particles obtained by NTA [150], possibly affecting cellular uptake. Furthermore, studies suggest that non-EV nano-sized structures may be formed from fluorescent dye and be confused for EVs [151]. However, for the purposes of this work, PKH26 was used solely for visualization and analysis of EV internalization and uptake blockage, and not for the validation of functional bioactivity, seeing that functional assays were carried out using non-labelled particles. Additionally, to discard the possibility of non-EV nanoparticle formation from fluorescent dye, a PKH26-labelled PBS control was prepared and subjected to the same isolation process as the EV samples of interest. As seen in Figure 3.3 a), fluorescent signal can be detected in fractions 8 and 9 for all EV samples, given that labelled EVs have migrated to their buoyant density-equivalent fractions, whereas no detectable band is present for the PBS con-

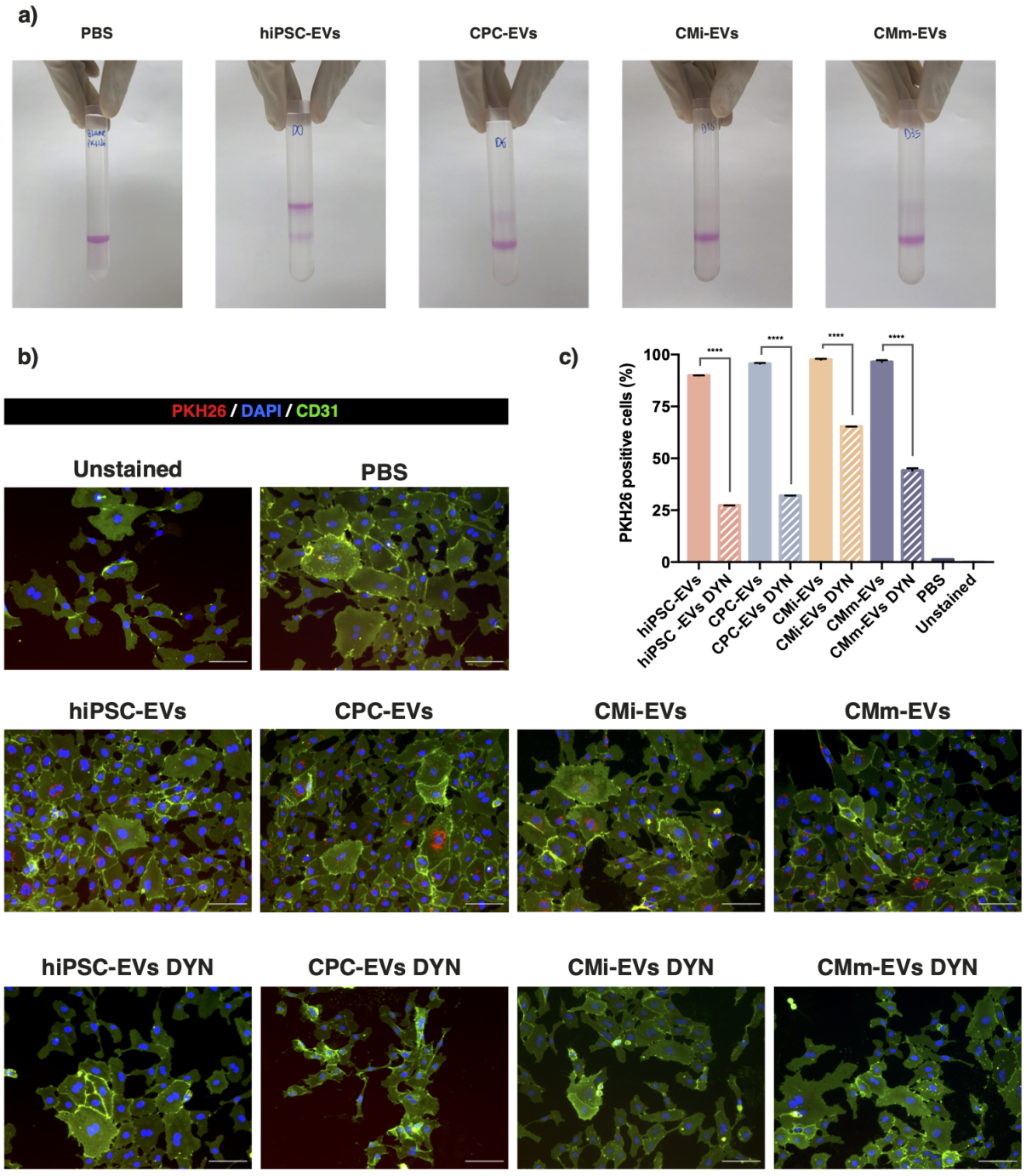


Figure 3.3: **Uptake of PKH26-labelled EVs by HUVECs. a)** Isolation of PKH26-labelled EVs from D0 (hiPSC), D6 (CPC), D15 (CMi) and D35 (CMm) cells by density gradient centrifugation. EV-bound lipophilic dye PKH26 is visible in EV-rich fractions after centrifugation, while unbound dye remains at the bottom of the gradient. **b)** Representative immunofluorescence images of EV uptake and uptake blockage. HUVECs incorporated PKH26-labelled EVs, as seen by the red dots present inside the cells. EV uptake was blocked by the presence of Dynasore (DYN). HUVECs were stained for transmembrane protein CD31 (green) and nuclei were counterstained with DAPI (blue). Scale bar: 100 µm. **c)** Percentage of PKH26-positive cells quantified by flow cytometry. Results shown as mean ± SD. **** $p < 0.0001$.

trol, indicating the absence of non-EV fluorescent structures.

HUVECs were incubated with labelled EVs to analyse cellular uptake. From the resulting immunofluorescence images of cells treated with hiPSC, CPC, CMi and CMm-EV samples (Figure 3.3 b), EVs can be detected in the cell cytoplasm by the visible red fluorescent signal. Uptake blockage was performed with the use of Dynasore, a GTPase inhibitor which acts on Dynamin 2 to prevent detachment of the endocytic vesicle from the cell membrane during CDE/CME and therefore inhibit EV internalization by recipient cells. The results obtained from the addition of Dynasore (Figure 3.3 b); DYN) show a clear reduction in red signal intensity in all conditions, indicating that EVs may be taken up by HUVECs via dynamin-dependent processes.

For a more quantitative analysis of EV internalization, flow cytometry was perfomed to assess the percentage of PKH26-positive cells after treatment with labelled vesicles. Results show no major differences between the number of cells with fluorescent signal, which equates to a near total cell population for all EV samples (Figure 3.3 c). Still, it is worth noting that the present analysis does not provide information on whether a similar number of EVs is being internalized per cell nor if the cargo is being delivered uniformly within each cell population. Interestingly, in the presence of Dynasore, the percentage of PKH26-positive cells is lower but not null compared to the non-DYN counterparts. This indicates that, while most EVs are uptaken by HUVECs via dynamin-dependent processes, a subpopulation of labelled EVs continues to be internalized through alternative dynamin-independent pathways upon dynamin inhibition.

Migration potential and the wound healing assay

Angiogenesis is the intricate process by which new blood vessels are formed from pre-existing ones, consisting in a fundamental step for cardiac tissue rehabilitation following assault [114]. Angiogenesis is mediated by migration, proliferation and remodelling of endothelial cells following complex molecular signalling mechanisms and cellular crosstalk [152]. To study the involvement of EVs in the collective migratory capacity of endothelial cells, the wound healing assay was performed on HUVECs. Reduced-supplementation media was used to ensure that observed effects were not a consequence of growth factor-activated cell migration nor stimulated by other interfering mechanisms, namely cell proliferation.

Results demonstrate that all EV samples exhibit significant capacities to promote

HUVEC migration (Figure 3.4 a-b). This migratory stimulation is observed to a similar extent for all groups, with enhanced significance for hiPSC-EVs. Furthermore, this effect is suppressed with the addition of Dynasore (Figure3.4 c), suggesting that the observed outcome is impacted by EV activity. The following experimental outcome goes in accordance with previously reported studies on EC migration that verify the wound healing effects of CPC- and CM-EVs, possibly due to the matrix degradation and remodelling action of MMPs found in secreted vesicles [153, 154]. In addition, the inability of FBS-EVs to close the wound could advocate for specificity in EV activity, given that this treatment shows a non-significant effect in cell migratory potential.

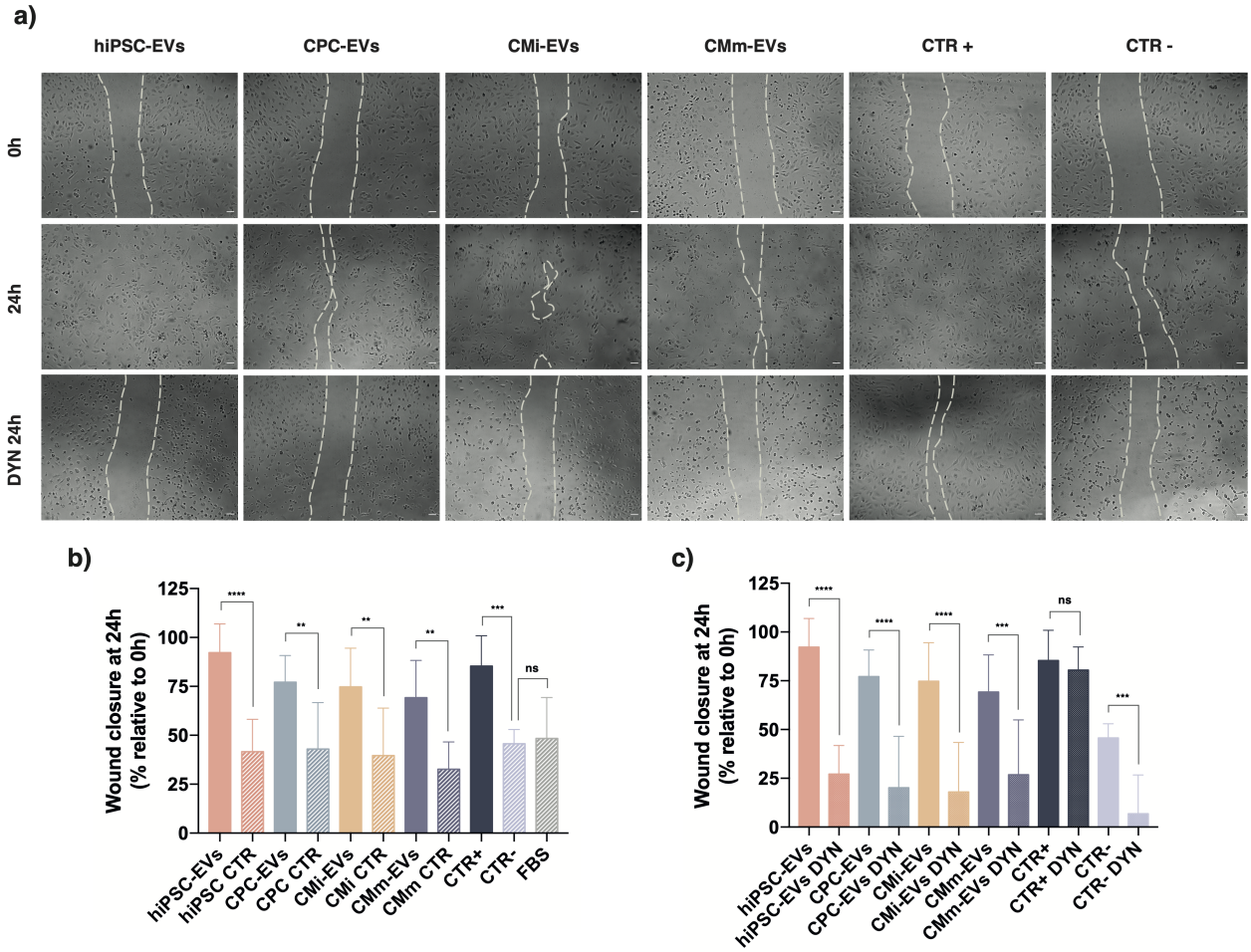


Figure 3.4: **Effect of EVs from hiPSCs and hiPSC-cardiac derivatives on HUVEC migration, quantified by the wound healing assay. a)** Representative images of cell migration at timepoints 0h and 24h post-scratch, in absence or presence of Dynasore. Scale bar: 100 µm. Percentage of wound closure at 24h relative to wound area at 0h, with the respective comparison to **b)** negative or vehicle control, and **c)** treatment in the presence of Dynasore. All data shown as mean ± SD (n=3). **p $<$ 0.01, ***p $<$ 0.001, ****p $<$ 0.0001, ns non-significant.

Control of cell proliferation is needed to ensure that the wound healing effects can be attributed mainly to cell migration [155]. Proliferation can be recorded by the incorporation of a nucleoside analog, namely EdU (5-ethynyl-2'-deoxyuridine), during DNA synthesis in proliferating cells. From this method, images were obtained and quantified (Supplementary information Figure 4.4), from which no significant changes in cell proliferation upon different treatments were detected. Such findings indicate that EV-stimulated HUVEC migration observed in Figure 3.4 is not subject to a confounding effect of cell proliferation.

Angiogenic potential and the tube formation assay

EVs from multiple cell sources have been reported to impact the angiogenic potential of endothelial cells [115, 116, 154]. The tube formation assay is a common method for assessment of angiogenesis, determined by the cell capacity to form capillary-like structures on a Matrigel support, quantified by specific measurements of the resulting network [156]. In this assay, total tube length (sum of all segments) and number of nodes (pixels with 3 neighbors, i.e. intersections of 3 segments) were assessed to determine the tube formation capacity of HUVECs upon EV treatments, with results shown in percentage relative to the negative control, the latter equating to 100% of each given measurement. Additionally, suramin sodium salt, a compound that suppresses endothelial cell migration, proliferation and angiogenesis via growth factor blocking, was used as an inhibitor control to confirm robustness and sensitivity of the assay.

Our results demonstrate that only hiPSC-EVs displayed significant angiogenic potential (Figure 3.5). The remaining EV samples showed no angiogenic properties from both analysed measurements when compared to their vehicle controls, and in similar degree to that of FBS-EVs. Such observations go in line with the highest significance score in migration potential obtained previously for hiPSC-EVs, suggesting an increased capacity for therapeutic stimulation of ECs from this EV sample.

These findings oppose those reported in the literature by several independent groups, that indicate pro-angiogenic activity for EVs from CPCs [115, 117] and CMs [154, 157], along with the established therapeutic properties found for iPSC-EVs [158, 159]. Furthermore, it would be expected that cardiac-derived EVs would be equally or more effective at stimulating cardiac repair mechanisms, namely wound healing and angiogenesis, than EVs from unrelated cell sources [160]. Still, the functional assays chosen

for this work to assess the therapeutic properties of EVs are purely observational, lacking specificity and the ability to quantitatively evaluate EV-directed cues. Additionally, other factors may play a role in the observed effects. Despite normalization to particle number, it is not certain that the same number of particles is being uptaken by HUVECs for each different sample, nor that their cargo is being internalized uniformly, which could be determinant for the experimental outcome.

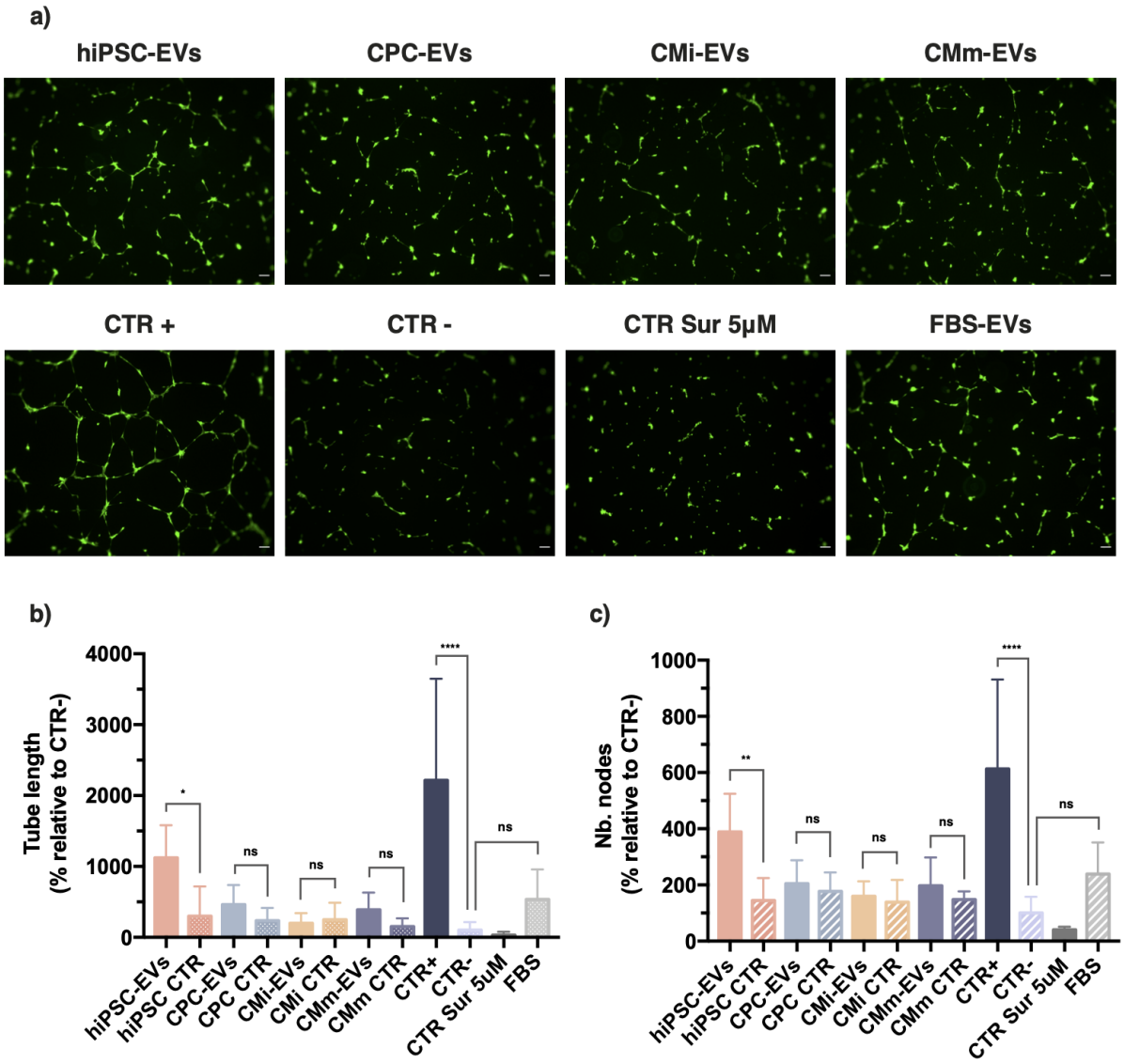


Figure 3.5: **Effect of EVs from hiPSCs and hiPSC-cardiac derivatives on HUVEC angiogenesis, quantified by the tube formation assay. a)** Representative images of tube formation at timepoint 8h post-seeding. Scale bar: 100 µm. Quantification of tube formation as percentage of **b)** tube length and **c)** number of nodes, relative to untreated control (CTR-) corresponding to 100%. All data shown as mean ± SD (n=3). *p $<$ 0.1, **p $<$ 0.01, ****p $<$ 0.0001, ns non-significant.

3.4 MicroRNA profile of EV cargo

EVs are established mediators of cell-cell communication, acting within the cardiovascular system to regulate biological processes through transfer of their cargo to recipient cells. EV content has been shown to be target-specific and variable according to the recipient cell and respective physiological stimulus [72, 107]. To better interpret the results obtained from functional assays, which show a stronger effect from hiPSC-EVs treatment, and gain insight into the specific signals directed to the cells, transcriptomic analysis of EV cargo was performed.

Next Generation Sequencing (NGS) of EV samples was performed by Norgen Biotek Corporation. Differential Expression (DE) data for each group comparison (Figure 3.6 a) 1 and 2), with significance for $|\log \text{fold change}|>2$, indicates distinct microRNA expression profiles for EVs from different cell groups. Interestingly, the further apart these cell sources stand in the differentiation and maturation timeline, the more their respective EV microRNA profiles diverge, seen by the increasing number of DE miRNAs from hiPSC-EVs when compared to CPC, CMi and CMm-EVs.

This suggests specificity of EV cargo and specialized stimuli for each destined target according to its cell source. Furthermore, DE data is distinctly observed through the Volcano plot displayed in Figure 3.6 b), which shows the relationship between fold change (X-axis) and statistical significance (Y-axis). To obtain this plot, miRNA expression data of cardiac derivative samples (CPC/CMi/CMm-EVs) was compared as a whole to that of hiPSC-EVs, given that results from functional assays advocate for a greater therapeutic value for the latter group. Such comparison method upholds for the remaining analyses performed with transcriptomic data.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a series of integrated databases on gene functions and regulatory pathways, widely used for modulation of biological networks from genomic information. Figure 3.7 a) shows the results obtained for KEGG pathway analysis of miRNA targets. For every KEGG class, each containing a set of related pathways, the number of targeted genes is shown for CPC/CMi/CMm-EVs (purple) and hiPSC-EVs (orange) miRNA data. Results show a number of relevant pathways in Signal Transduction enriched in both samples. Among these are MAPK, Wnt, TGF-β and PI3K/AKT signalling pathways, that regulate vital biological events such as cell differentiation, proliferation, migration, growth and apoptosis, and therefore are involved in cardioprotective activity mediated by pro-survival mechanisms.

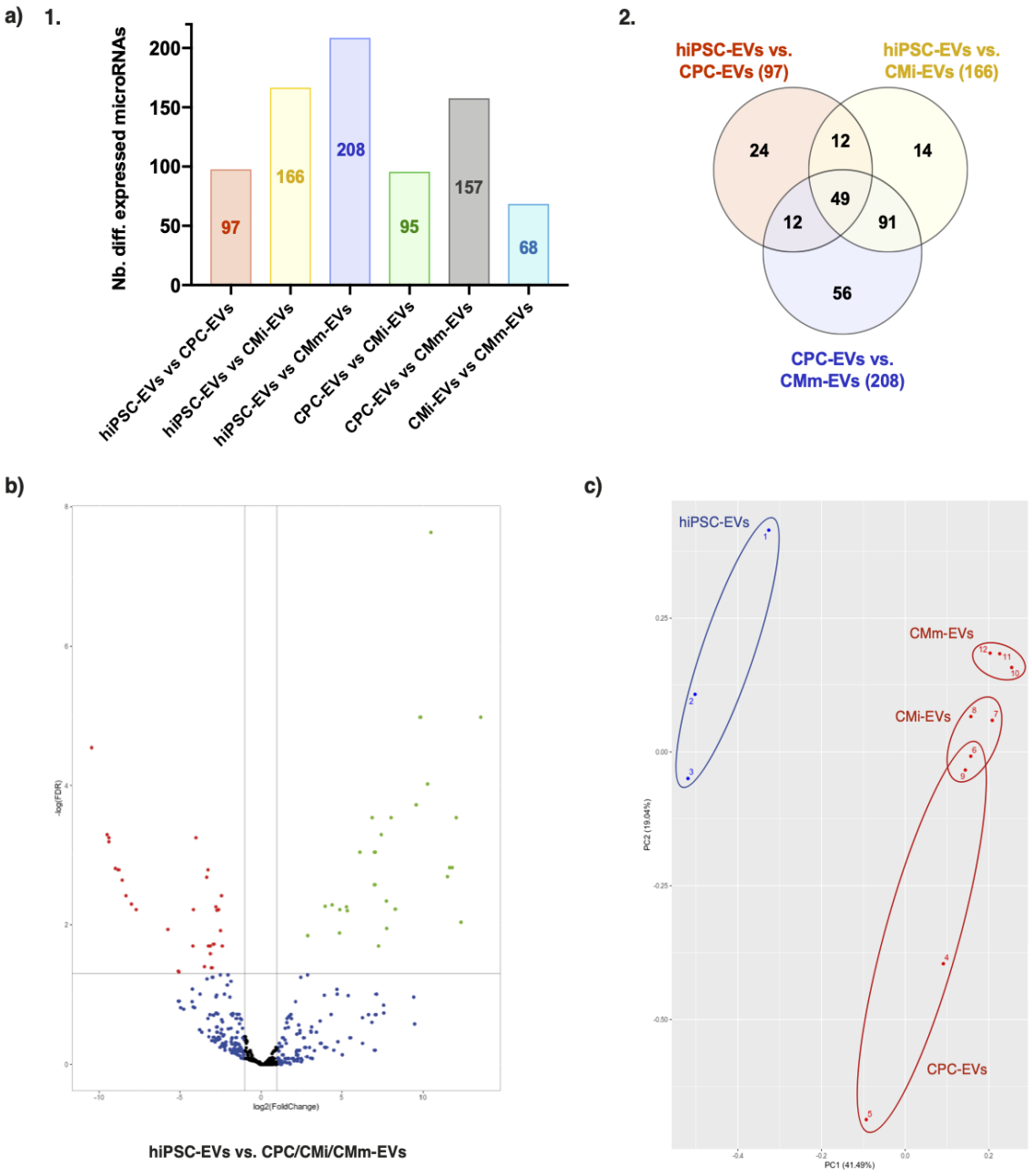


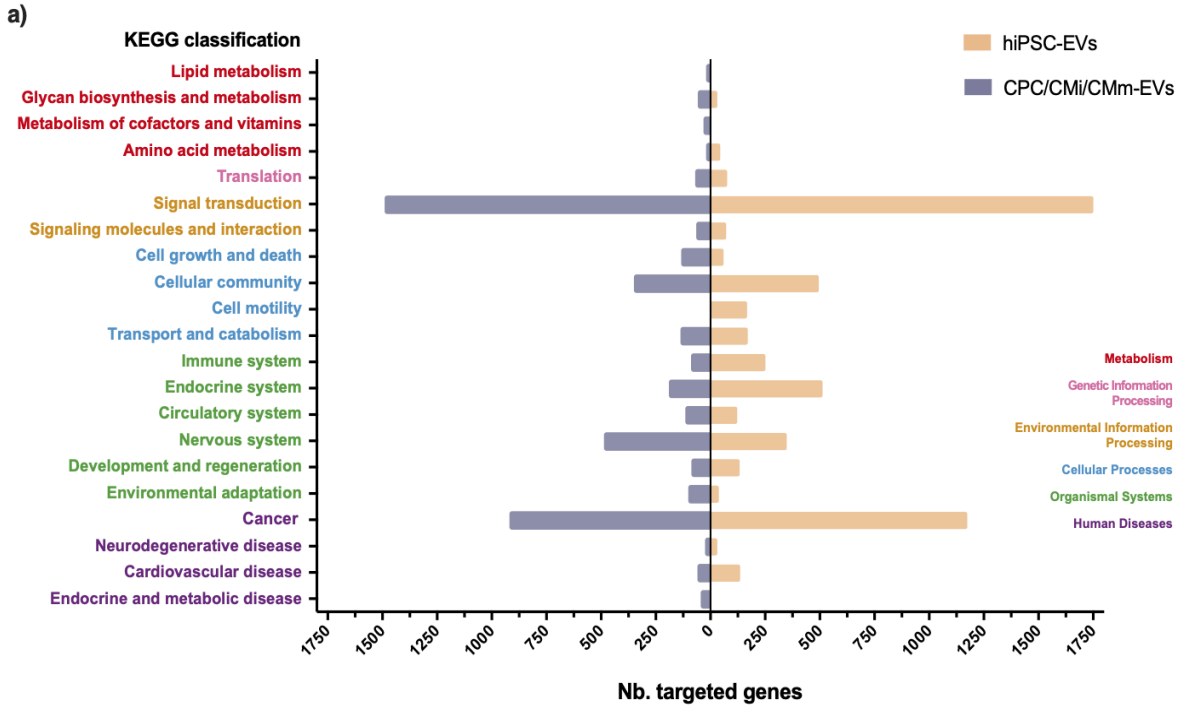
Figure 3.6: **Differentially expressed microRNAs in EV samples derived from hiPSCs and hiPSC-cardiac derivatives.** **a)** Number of differentially expressed microRNAs 1. within EV groups and 2. between hiPSC-EVs and the remaining samples. Differential expression considered for log fold change (FC) $\geq$ 2 or $\leq$ -2 and p-value $\leq$ 0.05. **b)** Volcano plot displaying statistical significance of microRNA differential expression (DE) between hiPSC-EVs and CPC/CMi/CMm-EVs. Y-axis shows $-\log_{10}$ of the false discovery rate (FDR) and X-axis the $\log_2$FC between compared groups. Statistical significance increases with Y; Up and down regulated microRNAs in CPC/CMi/CMm-EVs compared to hiPSC-EVs are shown in green and red, respectively. DE data obtained considering CPC, CMi and CMm-EVs samples as replicates of the same group. **c)** Principal Component Analysis (PCA) plot obtained with the 50 microRNAs with highest coefficient of variation (%CV). Cluster groups are plotted against the two highest components of variation (PC1 and PC2).

Importantly, Cellular Processes implicated in wound healing and angiogenesis are significantly enriched in hiPSC-EVs (Figure 3.7 a). These include cell motility and community processes, such as regulation of the actin cytoskeleton, junctional proteins and focal adhesion, all key activities for vascular angiogenic response [161]. Additionally, hiPSC-EVs show increased targeting of signalling pathways regulating pluripotency of stem cells, further advocating for EV specificity.

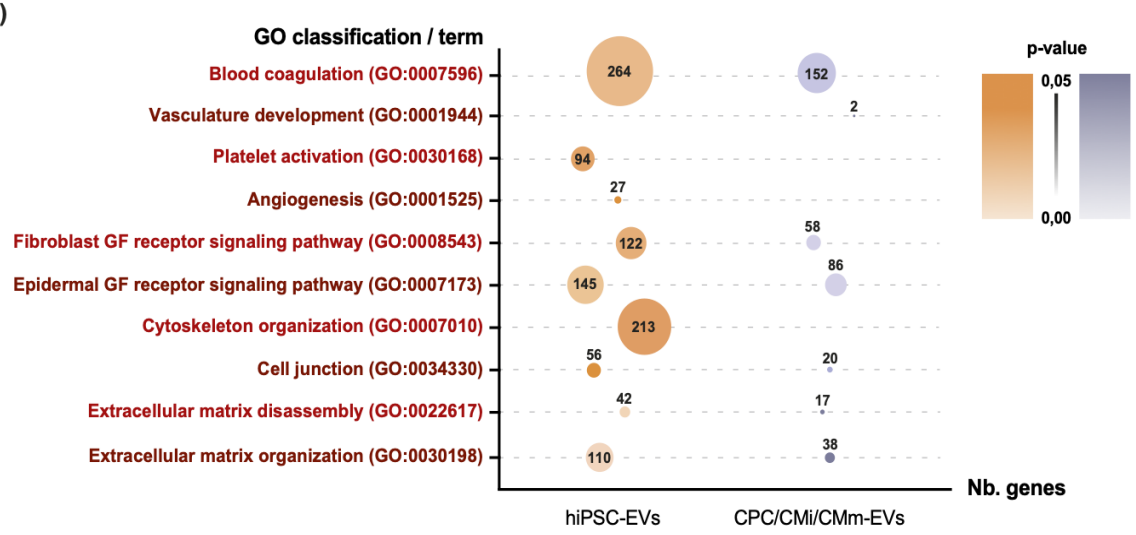
Gene Ontology (GO) enrichment analysis yielded an extensive array of biological processes associated with targeted genes. Figure 3.7 b) shows biological processes contained within the categories of circulatory system development, response to wounding and cellular component organization, identified for hiPSC-EVs and CPC/CMi/CMm-EVs groups, more specifically GO terms related to wound healing and angiogenesis. Results suggest enrichment of relevant biological functions, such as blood coagulation, platelet activation, GF signalling and cytoskeleton and ECM organization in hiPSC-EVs. Platelets are important modulators of angiogenesis and their activation is vital for subsequent release of effector molecules [162], including FGF and other bioactive factors. FGF signalling has been shown to play a central role for the maintenance of junctional proteins and overall vessel integrity [163]. Furthermore, overexpression of reported pro-angiogenic miRNAs was detected in hiPSC-EVs, including miR-18a, miR-20a and miR-17-5 of the miR-17-92 cluster. Taken together, such findings, although not conclusive, could attest to the more pronounced angiogenic effects observed for hiPSC-EVs. However, from this first set of results, no evident trace stands out to firmly substantiate the outcome of functional assays, and a more in-depth analysis, along with experimental confirmation through overexpression or inhibition of identified miRNAs, would be required to reach any conclusions.

In addition to the wound healing/angiogenesis GO-term analysis, specific differentially expressed miRNAs involved in cardiac muscle development and maturation were identified (Figure 3.7 c). Importantly, some of the most well-known and relevant muscle-specific miRNAs, also entitled myomiRs, were found to be highly overexpressed in CPC/CMi/CMm-EVs. These include miR-1, miR-133, miR-208 and miR-499a [164, 165], all showing fold changes of over 100 when compared to hiPSC-EVs. Additionally, several other DE miRNAs involved in cardiac commitment and development were detected, including miR-199a, miR-145 and miRs from the let-7 family.

a)


KEGG classification
hiPSC-EVs
CPC/CMi/CMm-EVs
Lipid metabolism
Glycan biosynthesis and metabolism
Metabolism of cofactors and vitamins
Amino acid metabolism
Translation
Signal transduction
Signaling molecules and interaction
Cell growth and death
Cellular community
Cell motility
Transport and catabolism
Immune system
Endocrine system
Circulatory system
Nervous system
Development and regeneration
Environmental adaptation
Cancer
Neurodegenerative disease
Cardiovascular disease
Endocrine and metabolic disease
Metabolism
Genetic Information Processing
Environmental Information Processing
Cellular Processes
Organismal Systems
Human Diseases
1750
1500
1250
1000
750
500
250
0
250
500
750
1000
1250
1500
1750
Nb. targeted genes

b)


GO classification / term
Blood coagulation (GO:0007596)
Vasculature development (GO:0001944)
Platelet activation (GO:0030168)
Angiogenesis (GO:0001525)
Fibroblast GF receptor signaling pathway (GO:0008543)
Epidermal GF receptor signaling pathway (GO:0007173)
Cytoskeleton organization (GO:0007010)
Cell junction (GO:0034330)
Extracellular matrix disassembly (GO:0022617)
Extracellular matrix organization (GO:0030198)
264
152
2
94
27
122
58
145
86
213
56
20
42
17
110
38
p-value
0,05
0,00
Nb. genes
hiPSC-EVs
CPC/CMi/CMm-EVs

c)

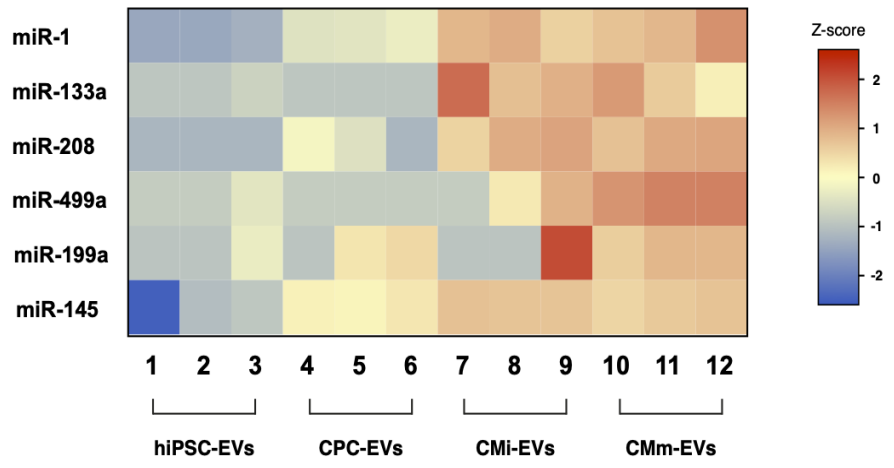

miR-1
miR-133a
miR-208
miR-499a
miR-199a
miR-145
Z-score
2
1
0
-1
-2
1
2
3
4
5
6
7
8
9
10
11
12
hiPSC-EVs
CPC-EVs
CMi-EVs
CMm-EVs

Figure 3.7: **KEGG pathways and GO biological processes analyses. a)** KEGG pathway classification associated with microRNAs upregulated in hiPSC-EVs and CPC/CMi/CMm-EVs. Data shown as the number of targeted genes in each KEGG class for each group. Associated pathways considered significant for p-value < 0.05. **b)** GO classification and term of relevant biological processes associated with microRNAs upregulated in hiPSC-EVs and CPC/CMi/CMm-EVs, presented in a bubble plot. Size of bubbles varies with the number of targeted genes for each biological process, indicated inside or above each bubble; color varies with p-value according to the presented color scale. KEGG and GO data obtained using the DIANA-miRPath v3 web-based analysis software. **c)** Heatmap of RNA-Seq expression Z-scores computed for relevant cardiac muscle-miRNAs differentially expressed between hiPSC-EVs and CPC/CMi/CMm-EVs groups.

These findings suggest specificity of CPC/CMi/CMm-EVs miRNA content towards cardiac muscle development and are further supported by results from the Principal Component Analysis (PCA) displayed in Figure 3.6 c), which show two major clusters for EVs from hiPSCs (blue) and cardiac-derivative cells (red) along Principal Component 1 (PC1). Taking this pronounced distribution in miRNA content of EVs from cardiac and non-cardiac committed cells, DE data between these groups could provide insight into novel cardiac-specific exosomal miRNAs.

It is important to highlight that all of the described *in silico* results regarding KEGG pathway and Gene Ontology analysis were obtained using a widely used web-based tool, miRPath v3, which infers biological pathways and processes from an implemented miRNA target prediction algorithm and predicted/validated miRNA-gene interaction datasets. Such tools, however well conceived, are known to yield questionably reliable results, given that many of the considered interactions lack experimental validation and also because of intrinsic bias in the constructed algorithm [166]. This bias has been known to lead to a systematic detection of a given group of biological functions, such as cancer biology and related pathways. Thus, there is limited dependability of the obtained results and respective interpretations.

Furthermore, experimental validation would be required to confirm these preliminary conclusions. As an example, overexpression or inhibition of the most promising identified miRNAs could elucidate their role in angiogenesis. Such approach has been followed to determine the anti-angiogenic action of exosomal miR-320 from CMs of diabetic mice, an upregulated miRNA in comparison to healthy CMs, by noticing reduced migration and tube formation capacity of ECs under miR-320 overexpression, as well as restored function upon miR-320 inhibition [167]. Similarly, the pro-angiogenic effects

of miR-143 and miR-222 from ischemic CM-derived EVs were inferred by EC stimulation with these miRNAs [154]. Furthermore, stress stimuli such as ischemia, hypoxia or glucose deprivation may trigger an acute reparative response in cells and thus lead to more specific EV cargo with greater angiogenic potential [168, 169]. Taken that unsupervised clustering of exosomal miRNAs from hypoxic and normoxic CPCs by PCA indicate distinct populations depending on treatment condition [122], the application of such stimuli to our cell sources could change the landscape of results and show therapeutic properties not only for hiPSC-EVs but also for cardiac derivatives.

Chapter 4

Conclusions

4.1 Thesis Achievements

In this thesis, the therapeutic potential of EVs from cardiac and non-cardiac cell sources in the cardiovascular context has been elucidated. Growing evidence suggests a relevant role for paracrine signalling in the cardioprotective effects observed in cell-based therapies [52, 158], which has recently led to a shift in clinical focus from cell-based to cell-free approaches. EVs are considered important messengers in the process of tissue rehabilitation, and numerous studies have tried to unravel their intrinsic mechanisms and mode of action in endogenous cardiac repair. EVs from various hiPSC-derived cell sources [139, 157, 159, 160], primary human cardiac cells [115, 153] and animal cell lines [154, 168] have been experimentally tested for their therapeutic potential in the cardiovascular context. The use of hiPSC-derivatives as cell sources for EV production presents advantages over alternative adult cells given their self-renewal and proliferation properties, however few comparative studies have been conducted to define the most promising cell candidates within hiPSC-derivatives.

EVs were isolated from CCMed of four well-defined cell populations: hiPSCs, hiPSC-CPCs, immature and mature hiPSC-CMs, as confirmed by flow cytometry and gene expression analysis. The isolation procedure was conducted according to ISEV-defined standards [82], through a combination of differential centrifugation and iodixanol-based density gradient ultracentrifugation, a technique that has shown great capacity to recover EVs with high purity levels [143]. Indeed, TEM and Western blot results indicated an adequate isolation of EV populations and absence of contaminants. Furthermore, particle descriptor analysis provided information regarding EV production capacity for

each cell source, revealing a higher EV yield for CM-derived EVs, contrary to literature reports which favor hiPSC-CPC production [139].

The therapeutic potential of EV populations was assessed by means of functionality assays, to test their wound healing and tube formation abilities. EVs from hiPSCs and derived CPCs and CMs have been found to sustain angiogenic properties *in vitro* and *in vivo* [139, 157]. Indeed, all four EV samples showed wound healing ability, with more significant effect observed for hiPSC-EVs. However, no tube formation capacity was detected for cardiac-derived groups, with only hiPSC-EVs showing significant, albeit modest, angiogenic properties.

EVs have been found to exert their cardioprotective action through the transfer of microRNAs involved in the pro-angiogenic response [154, 167]. Thus, transcriptomic analysis of EV cargo was conducted and analysed for the detection of gene targets implicated in angiogenesis. Differential expression data and unsupervised clustering analysis advocated for specificity of EV miRNA contents according to cell source, similarly to multiple literature reports [107]. Furthermore, KEGG pathway and Gene Ontology analysis hinted at the increased targeting of pro-angiogenic pathways in hiPSC-EVs.

These results suggest hiPSC-EVs as a suitable candidate for therapeutic use in cardiac repair, although with no pronounced beneficial effects in comparison with the remaining cardiac-derived EV groups. Multiple studies have reported the use of stress stimuli to potentiate EV bioactivity [168, 169]. An interesting approach that could lead to an increase in angiogenic potential of cardiac-derived EVs would be to expose the cells to hypoxic conditions prior to CCMed collection. Additionally, enrichment of EVs with specific pro-angiogenic miRNAs could further enhance cardioprotective effects, using miRNAs identified from a deeper analysis of RNA-seq data.

4.2 Limitations and future work

In spite of the proven beneficial effects of EVs in the context of cardiovascular repair seen in this study and in a number of literature reports, there are many limitations hindering the translation of this work into clinical applications. Firstly, the choice of functional assays adopted for this work may not represent the most suitable models for the study of the therapeutic potential of EVs, therefore limiting the observed effects of EV treatment and leading to moderate results.

Secondly, the isolation process adopted for this work is both time and labour consuming, and although effective at obtaining a pure EV population, it lacks the necessary efficiency for a clinical setting. This does not pose a problem for this particular project, given that its focus lies solely on uncovering the therapeutic value of EVs, but makes it impractical for large-scale production in later stages of medical application. In this regard, multiple studies are underway to defining GMP-compliant optimized cell culture platforms and high-yield isolation procedures for the scalable production and purification of EVs for cardiovascular therapeutics [170, 171].

Furthermore, strategies involving cell preconditioning for the modulation of EV signalling towards a more specific pro-angiogenic and cardioprotective response have been employed in numerous studies [154, 168, 169] and reveal promising results for boosting the effects of EV treatment. Such strategies will be essential to validate the therapeutic potential of EVs from hiPSC-cell derivatives and demonstrate their advantages over more well-established sources, such as adult MSCs and CPCs.

Lastly, combined bioengineering approaches must be explored to bridge the gap of clinical translation regarding EV delivery and retention. Several groups have made progress in this domain by developing efficient systems for the sustained delivery of EV treatments [128, 129], leading to enhanced cardiac repair. Many other intrinsic and extrinsic strategies are being explored [106], such as the promotion of specific cell targeting and improved biodistribution by means of EV modifications.

Bibliography

[1] Benjamin, E. J., Muntner, P., Alonso, A. et al. (2019). Heart disease and stroke statistics-2019 update: a report from the American Heart Association, Circulation, 139(10), 56–528. doi:10.1161/CIR.0000000000000659.

[2] Naghavi, M., Abajobir, A. A., Abbafati, C., Abbas, K. M., Abd-Allah, F., Abera, S. F.,... Agrawal, A. (2017). Global, regional, and national age-sex specific mortality for 264 causes of death, 1980–2016: a systematic analysis for the Global Burden of Disease Study 2016. The Lancet, 390(10100), 1151–1210. doi:10.1016/s0140-6736(17)32152-9

[3] Colvin, M., Smith, J. M., Hadley, N., Skeans, M. A., Uccellini, K., Goff, R.,... Kasiske, B. L. (2020). OPTN/SRTR 2018 Annual Data Report: Heart. American Journal of Transplantation, 20(s1), 340–426. doi:10.1111/ajt.15676

[4] Lund, L. H., Khush, K. K., Cherikh, W. S., Goldfarb, S., Kucheryavaya, A. Y., Levvey, B. J.,... Stehlik, J. (2017). The Registry of the International Society for Heart and Lung Transplantation: Thirty-fourth Adult Heart Transplantation Report—2017; Focus Theme: Allograft ischemic time. The Journal of Heart and Lung Transplantation, 36(10), 1037–1046. doi:10.1016/j.healun.2017.07.019

[5] Eschenhagen, T., Bolli, R., Braun, T., Field, L. J., Fleischmann, B. K., Frisén, J., Hill, J. A. (2017). Cardiomyocyte Regeneration. Circulation, 136(7), 680–686. doi:10.1161/circulationaha.117.029343

[6] Shimizu, I., Minamino, T. (2016). Physiological and pathological cardiac hypertrophy. Journal of Molecular and Cellular Cardiology, 97, 245–262. doi:10.1016/j.yjmcc.2016.06.001

[7] Bergmann, O., Zdunek, S., Felker, A., Salehpour, M., Alkass, K., Bernard, S.,

Frisén, J. (2015). Dynamics of Cell Generation and Turnover in the Human Heart. Cell, 161(7), 1566–1575.doi:10.1016/j.cell.2015.05.026

[8] Barreto, Hamel, Schiatti, Yang, George. (2019). Cardiac Progenitor Cells from Stem Cells: Learning from Genetics and Biomaterials. Cells, 8(12), 1536.doi:10.3390/cells8121536

[9] Soonpaa, M., Koh, G., Klug, M., Field, L. (1994). Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science, 264(5155), 98–101. doi:10.1126/science.8140423

[10] Malliaras, K., Marban, E. (2011). Cardiac cell therapy: where we've been, where we are, and where we should be headed. British Medical Bulletin, 98(1), 161–185. doi:10.1093/bmb/ldr018

[11] Chiu, R. C.-J., Zibaitis, A., Kao, R. L. (1995). Cellular cardiomyoplasty: Myocardial regeneration with satellite cell implantation. The Annals of Thoracic Surgery, 60(1), 12–18. doi:10.1016/s0003-4975(95)00374-6

[12] Taylor, D. A., Atkins, B. Z., Hungspreugs, P., Jones, T. R., Reedy, M. C., Hutcheson, K. A., Kraus, W. E. (1998). Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. Nature Medicine, 4(8), 929–933. doi:10.1038/nm0898-929

[13] Menasche, P., Alfieri, O., Janssens, S., McKenna, W., Reichenspurner, H., Trinquart, L., Hagege, A. A. (2008). The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) Trial: First Randomized Placebo-Controlled Study of Myoblast Transplantation. Circulation, 117(9), 1189–1200. doi:10.1161/circulationaha.107.734103

[14] Wollert, K. C., Meyer, G. P., Lotz, J., Ringes Lichtenberg, S., Lippolt, P., Breidenbach, C., Drexler, H. (2004). Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. The Lancet, 364(9429), 141–148. doi:10.1016/s0140-6736(04)16626-9

[15] Hare, J. M., Traverse, J. H., Henry, T. D., Dib, N., Strumpf, R. K., Schulman, S. P., Sherman, W. (2009). A Randomized, Double-Blind, Placebo-Controlled, Dose-Escalation Study of Intravenous Adult Human Mesenchymal Stem Cells (Prochymal)

After Acute Myocardial Infarction. Journal of the American College of Cardiology, 54(24), 2277–2286.doi:10.1016/j.jacc.2009.06.055

[16] Hare, J. M., Fishman, J. E., Gerstenblith, G., DiFede Velazquez, D. L., Zambrano, J. P., Suncion, V. Y., Heldman, A. W. (2012). Comparison of Allogeneic vs Autologous Bone Marrow–Derived Mesenchymal Stem Cells Delivered by Transendocardial Injection in Patients With Ischemic Cardiomyopathy. JAMA, 308(22), 2369. doi:10.1001/jama.2012.25321

[17] Houtgraaf, J. H., den Dekker, W. K., van Dalen, B. M., Springeling, T., de Jong, R., van Geuns, R. J., Duckers, H. J. (2012). First Experience in Humans Using Adipose Tissue–Derived Regenerative Cells in the Treatment of Patients With ST-Segment Elevation Myocardial Infarction. Journal of the American College of Cardiology, 59(5), 539–540. doi:10.1016/j.jacc.2011.09.065

[18] Hatakeyama, M., Kanazawa, M., Ninomiya, I., Omae, K., Kimura, Y., Takahashi, T., Onodera, O., Fukushima, M., Shimohata, T. (2019). A novel therapeutic approach using peripheral blood mononuclear cells preconditioned by oxygen-glucose deprivation. Scientific reports, 9(1), 16819. doi:10.1038/s41598-019-53418-5

[19] Keighron, C., Lyons, C. J., Creane, M., O'Brien, T., Liew, A. (2018). Recent Advances in Endothelial Progenitor Cells Toward Their Use in Clinical Translation. Frontiers in Medicine, 5. doi:10.3389/fmed.2018.00354

[20] Smith, R. R., Barile, L., Cho, H. C., Leppo, M. K., Hare, J. M., Messina, E., Marban, E. (2007). Regenerative Potential of Cardiosphere-Derived Cells Expanded From Percutaneous Endomyocardial Biopsy Specimens. Circulation, 115(7), 896–908. doi:10.1161/circulationaha.106.655209

[21] Li, Q., Guo, Y., Ou, Q., Chen, N., Wu, W.-J., Yuan, F., Bolli, R. (2011). Intracoronary administration of cardiac stem cells in mice: a new, improved technique for cell therapy in murine models. Basic Research in Cardiology, 106(5), 849–864. doi:10.1007/s00395-011-0180-1

[22] Cheng, K., Shen, D., Smith, J., Galang, G., Sun, B., Zhang, J., Marbán, E. (2012). Transplantation of platelet gel spiked with cardiosphere-derived cells boosts structural and functional benefits relative to gel transplan-

tation alone in rats with myocardial infarction. Biomaterials, 33(10), 2872–2879. doi:10.1016/j.biomaterials.2011.12.040

[23] Tseliou, E., Pollan, S., Malliaras, K., Terrovitis, J., Sun, B., Galang, G., Marbán, E. (2013). Allogeneic Cardiospheres Safely Boost Cardiac Function and Attenuate Adverse Remodeling After Myocardial Infarction in Immunologically Mismatched Rat Strains. Journal of the American College of Cardiology, 61(10), 1108–1119. doi:10.1016/j.jacc.2012.10.052

[24] Bolli, R., Chugh, A. R., D'Amario, D., Loughran, J. H., Stoddard, M. F., Ikram, S., Anversa, P. (2011). Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. The Lancet, 378(9806), 1847–1857. doi:10.1016/s0140-6736(11)61590-0

[25] Makkar, R. R., Smith, R. R., Cheng, K., Malliaras, K., Thomson, L. E., Berman, D., Marbán, E. (2012). Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. The Lancet, 379(9819), 895–904. doi:10.1016/s0140-6736(12)60195-0

[26] Kobold, S., Guhr, A., Kurtz, A., Löser, P. (2015). Human Embryonic and Induced Pluripotent Stem Cell Research Trends: Complementation and Diversification of the Field. Stem Cell Reports, 4(5), 914–925. doi:10.1016/j.stemcr.2015.03.002

[27] Reisman, M., Adams, K. T. (2014). Stem cell therapy: a look at current research, regulations, and remaining hurdles. P&T : a peer-reviewed journal for formulary management, 39(12), 846–857.

[28] De Los Angeles, A., Ferrari, F., Xi, R., Fujiwara, Y., Benvenisty, N., Deng, H., Daley, G. Q. (2015). Hallmarks of pluripotency. Nature, 525(7570), 469–478. doi:10.1038/nature15515

[29] Thomson, J. A. (1998). Embryonic Stem Cell Lines Derived from Human Blastocysts. Science, 282(5391), 1145–1147. doi:10.1126/science.282.5391.1145

[30] Bjorklund, L. M., Sánchez-Pernaute, R., Chung, S., Andersson, T., Chen, I. Y., McNaught, K. S., et al. (2002). Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc. Natl. Acad. Sci. U.S.A. 99, 2344–2349. doi: 10.1073/pnas.022438099

[31] Prokhorova, T.A., Harkness, L.M., Frandsen, U., Ditzel, N., Schrøder, H.D., Burns, J.S. (2009). Teratoma formation by human embryonic stem cells is site dependent and enhanced by the presence of Matrigel. Stem Cells Dev.18(1):47–54. doi: 10.1089/scd.2007.0266

[32] Nussbaum, J., Minami, E., Laflamme, M.A., Virag, J.A.I., Ware, C.B., Masino, A., Muskheli, V., Pabon, L., Reinecke, H. and Murry, C.E. (2007), Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. The FASEB Journal, 21: 1345-1357. doi:10.1096/fj.06-6769com

[33] Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S. (2007). Induction of Pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872. doi: 10.1016/j.cell.2007.11.019

[34] Raab, S., Klingenstein, M., Liebau, S., Linta, L. (2014). A Comparative View on Human Somatic Cell Sources for iPSC Generation. Stem cells international, 2014, 768391. doi:10.1155/2014/768391

[35] Zhang Y., Wang D., Chen M., Yang B., Zhang F., Cao K. (2011). Intramyocardial transplantation of undifferentiated rat induced pluripotent stem cells causes tumorigenesis in the heart. PloS One 6, e19012. doi: 10.1371/journal.pone.0019012

[36] Mummery, C. L., Zhang, J., Ng, E. S., Elliott, D. A., Elefanty, A. G., Kamp, T. J. (2012). Differentiation of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells to Cardiomyocytes: A Methods Overview. Circulation Research, 111(3), 344–358. doi:10.1161/circresaha.110.227512

[37] Correia, C., Koshkin, A., Duarte, P., Hu, D., Carido, M., Sebastião, M. J., Gomes-Alves, P., Elliott, D. A., Domian, I. J., Teixeira, A. P., Alves, P. M., Serra, M. (2018). 3D aggregate culture improves metabolic maturation of human pluripotent stem cell derived cardiomyocytes. Biotechnology and bioengineering, 115(3), 630–644. doi:10.1002/bit.26504

[38] Ménard, C., Hagège, A. A., Agbulut, O., Barro, M., Morichetti, M. C., Brasselet, C., Menasché, P. (2005). Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study. The Lancet, 366(9490), 1005–1012. doi:10.1016/s0140-6736(05)67380-1

[39] Ye, L., Chang, Y.-H., Xiong, Q., Zhang, P., Zhang, L., Somasundaram, P., Zhang, J. (2014). Cardiac Repair in a Porcine Model of Acute Myocardial Infarction with Human Induced Pluripotent Stem Cell-Derived Cardiovascular Cells. Cell Stem Cell, 15(6), 750–761. doi:10.1016/j.stem.2014.11.009

[40] Menasché, P., Vanneaux, V., Hagège, A., Bel, A., Cholley, B., Cacciapuoti, I., Larghero, J. (2015). Human embryonic stem cell-derived cardiac progenitors for severe heart failure treatment: first clinical case report: Figure 1. European Heart Journal, 36(30), 2011–2017. doi:10.1093/eurheartj/ehv189

[41] Menasché, P., Vanneaux, V., Hagège, A., Bel, A., Cholley, B., Parouchev, A., Larghero, J. (2018). Transplantation of Human Embryonic Stem Cell–Derived Cardiovascular Progenitors for Severe Ischemic Left Ventricular Dysfunction. Journal of the American College of Cardiology, 71(4), 429–438. doi:10.1016/j.jacc.2017.11.047

[42] Cyranoski, D. (2018). “Reprogrammed” stem cells approved to mend human hearts for the first time. Nature, 557(7707), 619–620. doi:10.1038/d41586-018-05278-8

[43] Aicher, A., Brenner, W., Zuhayra, M., Badorff, C., Massoudi, S., Assmus, B., Eckey, T., Henze, E., Zeiher, A. M., Dimmeler, S. (2003). Assessment of the tissue distribution of transplanted human endothelial progenitor cells by radioactive labeling. Circulation, 107(16), 2134–2139.

[44] Smits, A. M., van Laake, L. W., den Ouden, K., Schreurs, C., Szuhai, K., van Echteld, C. J., Mummery, C. L., Doevendans, P. A., Goumans, M. J. (2009). Human cardiomyocyte progenitor cell transplantation preserves long-term function of the infarcted mouse myocardium. Cardiovascular research, 83(3), 527–535. doi:10.1093/cvr/cvp146

[45] Askari, A., Unzek, S., Goldman, C. K., Ellis, S. G., Thomas, J. D., DiCorleto, P. E., Topol, E. J., Penn, M. S. (2004). Cellular, but not direct, adenoviral delivery of vascular endothelial growth factor results in improved left ventricular function and neovascularization in dilated ischemic cardiomyopathy. Journal of the American College of Cardiology, 43(10), 1908–1914. doi:10.1016/j.jacc.2003.12.045

[46] Hahn, J. Y., Cho, H. J., Kang, H. J., Kim, T. S., Kim, M. H., Chung, J. H., Bae, J. W., Oh, B. H., Park, Y. B., Kim, H. S. (2008). Pre-treatment of mesenchy-

mal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. Journal of the American College of Cardiology, 51(9), 933–943. doi:10.1016/j.jacc.2007.11.040

[47] Singelyn, J. M., DeQuach, J. A., Seif-Naraghi, S. B., Littlefield, R. B., Schup-Magoffin, P. J., Christman, K. L. (2009). Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering. Biomaterials, 30(29), 5409–5416. doi:10.1016/j.biomaterials.2009.06.045

[48] Formiga, F. R., Pelacho, B., Garbayo, E., Abizanda, G., Gavira, J. J., Simon-Yarza, T., Mazo, M., Tamayo, E., Jauquicoa, C., Ortiz-de-Solorzano, C., Prósper, F., Blanco-Prieto, M. J. (2010). Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model. Journal of controlled release : official journal of the Controlled Release Society, 147(1), 30–37. doi:10.1016/j.jconrel.2010.07.097

[49] Menasché, P., Vanneaux, V., Fabreguettes, J.-R., Bel, A., Tosca, L., Garcia, S., Larghero, J. (2014). Towards a clinical use of human embryonic stem cell-derived cardiac progenitors: a translational experience. European Heart Journal, 36(12), 743–750.doi:10.1093/eurheartj/ehu192

[50] Neofytou, E., O'Brien, C. G., Couture, L. A., Wu, J. C. (2015). Hurdles to clinical translation of human induced pluripotent stem cells. The Journal of clinical investigation, 125(7), 2551–2557. doi:10.1172/JCI80575

[51] Maxeiner, H., Krehbiehl, N., Müller, A., Woitasky, N., Akintürk, H., Müller, M., Weigand, M. A., Abdallah, Y., Kasseckert, S., Schreckenberg, R., Schlüter, K. D., Wenzel, S. (2010). New insights into paracrine mechanisms of human cardiac progenitor cells. European journal of heart failure, 12(7), 730–737. doi:10.1093/eurjhf/hfq063

[52] Chimenti, I., Smith, R. R., Li, T. S., Gerstenblith, G., Messina, E., Giacomello, A., Marbán, E. (2010). Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. Circulation research, 106(5), 971–980. doi:10.1161/CIRCRESAHA.109.210682

[53] Banai, S., Jaklitsch, M. T., Shou, M., Lazarous, D. F., Scheinowitz, M., Biro, S.,

Epstein, S. E., Unger, E. F. (1994). Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. Circulation, 89(5), 2183–2189. doi:10.1161/01.cir.89.5.2183

[54] Heeschen, C., Aicher, A., Lehmann, R., Fichtlscherer, S., Vasa, M., Urbich, C., Mildner-Rihm, C., Martin, H., Zeiher, A. M., Dimmeler, S. (2003). Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood, 102(4), 1340–1346. doi:10.1182/blood-2003-01-0223

[55] Deindl, E., Zaruba, M. M., Brunner, S., Huber, B., Mehl, U., Assmann, G., Hoefer, I. E., Mueller-Hoecker, J., Franz, W. M. (2006). G-CSF administration after myocardial infarction in mice attenuates late ischemic cardiomyopathy by enhanced arteriogenesis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 20(7), 956–958. doi:10.1096/fj.05-4763fje

[56] Simons, M., Annex, B. H., Laham, R. J., Kleiman, N., Henry, T., Dauerman, H., Udelson, J. E., Gervino, E. V., Pike, M., Whitehouse, M. J., Moon, T., Chronos, N. A. (2002). Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. Circulation, 105(7), 788–793. doi:10.1161/hc0802.104407

[57] Henry, T. D., Annex, B. H., McKendall, G. R., Azrin, M. A., Lopez, J. J., Giordano, F. J., Shah, P. K., Willerson, J. T., Benza, R. L., Berman, D. S., Gibson, C. M., Bajamonde, A., Rundle, A. C., Fine, J., McCluskey, E. R., VIVA Investigators (2003). The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation, 107(10), 1359–1365. doi:10.1161/01.cir.0000061911.47710.8a

[58] Lin, Y. D., Luo, C. Y., Hu, Y. N., Yeh, M. L., Hsueh, Y. C., Chang, M. Y., Tsai, D. C., Wang, J. N., Tang, M. J., Wei, E. I., Springer, M. L., Hsieh, P. C. (2012). Instructive nanofiber scaffolds with VEGF create a microenvironment for arteriogenesis and cardiac repair. Science translational medicine, 4(146), 146ra109. doi:10.1126/scitranslmed.3003841

[59] Jackson, R., Tilokee, E. L., Latham, N., Mount, S., Rafatian, G., Strydhorst, J., Ye, B., Boodhwani, M., Chan, V., Ruel, M., Ruddy, T. D., Suuronen, E. J., Stewart, D. J., Davis, D. R. (2015). Paracrine Engineering of Human Cardiac Stem Cells With Insulin-Like Growth Factor 1 Enhances Myocardial Repair. Journal of the American Heart Association, 4(9), e002104. doi:10.1161/JAHA.115.002104

[60] Viereck, J., Bang, C., Foinquinos, A., Thum, T. (2014). Regulatory RNAs and paracrine networks in the heart. Cardiovascular research, 102(2), 290–301. doi:10.1093/cvr/cvu039

[61] Hullinger, T. G., Montgomery, R. L., Seto, A. G., Dickinson, B. A., Semus, H. M., Lynch, J. M., Dalby, C. M., Robinson, K., Stack, C., Latimer, P. A., Hare, J. M., Olson, E. N., van Rooij, E. (2012). Inhibition of miR-15 protects against cardiac ischemic injury. Circulation research, 110(1), 71–81. doi:10.1161/CIRCRESAHA.111.244442

[62] Deatherage, B. L., & Cookson, B. T. (2012). Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. Infection and immunity, 80(6), 1948–1957. https://doi.org/10.1128/IAI.06014-11

[63] Chargaff, E., & West, R. (1946). The biological significance of the thromboplastic protein of blood. The Journal of biological chemistry, 166(1), 189–197.

[64] Pan, B.-T., Johnstone, R. M. (1983). Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: Selective externalization of the receptor. Cell, 33(3), 967–978. doi:10.1016/0092-8674(83)90040-5

[65] Harding, C., Heuser, J., Stahl, P. (1983). Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. The Journal of Cell Biology, 97(2), 329–339. doi:10.1083/jcb.97.2.329

[66] Johnstone, R., Adam, M., Hammond, J., Orr, L., Turbide, C. (1987). Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem, 262(19), 9412–20.

[67] Raposo, G., Nijman, H., Shoorvogel, W., Liejendekker, R., Harding, C., Melief, C., Geuze, H. (1996). B lymphocytes secrete antigen-presenting vesicles. Journal of Experimental Medicine, 183(3), 1161–1172. doi:10.1084/jem.183.3.1161

[68] Kalra, H., Adda, C. G., Liem, M., Ang, C.-S., Mechler, A., Simpson, R. J., Mathivanan, S. (2013). Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. PROTEOMICS, 13(22), 3354–3364. doi:10.1002/pmic.201300282

[69] Gould, S. J., & Raposo, G. (2013). As we wait: coping with an imperfect nomenclature for extracellular vesicles. Journal of Extracellular Vesicles, 2(1), 20389. doi:10.3402/jev.v2i0.20389

[70] Keerthikumar, S., Gangoda, L., Liem, M., Fonseka, P., Atukorala, I., Ozcitti, C., Mathivanan, S. (2015). Proteogenomic analysis reveals exosomes are more oncogenic than ectosomes. Oncotarget, 6(17). doi:10.18632/oncotarget.3801

[71] Sotelo, J. R. & Porter, K. R. (1959). An Electron Microscope Study of the Rat Ovum. The Journal of Cell Biology, 5(2), 327–342. doi:10.1083/jcb.5.2.327

[72] Colombo, M., Raposo, G., Théry, C. (2014). Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. Annual Review of Cell and Developmental Biology, 30(1), 255–289. doi:10.1146/annurev-cellbio-101512-122326

[73] Carayon, K., Chaoui, K., Ronzier, E., Lazar, I., Bertrand-Michel, J., Roques, V., Joly, E. (2011). Proteolipidic Composition of Exosomes Changes during Reticulocyte Maturation. Journal of Biological Chemistry, 286(39), 34426–34439. doi:10.1074/jbc.m111.257444

[74] Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., Raposo, G. (2013). Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. Journal of Cell Science, 126(24), 5553–5565. doi:10.1242/jcs.128868

[75] Stuffers, S., Sem Wegner, C., Stenmark, H., Brech, A. (2009). Multivesicular Endosome Biogenesis in the Absence of ESCRTs. Traffic, 10(7), 925–937. doi:10.1111/j.1600-0854.2009.00920.x

[76] Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Simons, M. (2008). Ceramide Triggers Budding of Exosome Vesicles into Multivesicular Endosomes. Science, 319(5867), 1244–1247. doi:10.1126/science.1153124

[77] Van Niel, G., D'Angelo, G., Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. Nature Reviews Molecular Cell Biology, 19(4), 213–228. doi:10.1038/nrm.2017.125

[78] Stein, J. M., & Luzio, J. P. (1991). Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles. Biochemical Journal, 274(2), 381–386. doi:10.1042/bj2740381

[79] Cocucci, E., Meldolesi, J. (2015). Ectosomes and exosomes: shedding the confusion between extracellular vesicles. Trends in Cell Biology, 25(6), 364–372. doi:10.1016/j.tcb.2015.01.004

[80] Kalra, H., Drummen, G. P., Mathivanan, S. (2016). Focus on Extracellular Vesicles: Introducing the Next Small Big Thing. International journal of molecular sciences, 17(2), 170. https://doi.org/10.3390/ijms17020170

[81] Tang, D., Kang, R., Berghe, T. V., Vandenabeele, P., & Kroemer, G. (2019). The molecular machinery of regulated cell death. Cell Research. doi:10.1038/s41422-019-0164-5

[82] Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., Atkin-Smith, G. K. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. Journal of Extracellular Vesicles, 8(1), 1535750. doi:10.1080/20013078.2018.1535750

[83] Meehan, B., Rak, J., Di Vizio, D. (2016). Oncosomes – large and small: what are they, where they came from? Journal of Extracellular Vesicles, 5(1), 33109. doi:10.3402/jev.v5.33109

[84] Akers, J. C., Gonda, D., Kim, R., Carter, B. S., Chen, C. C. (2013). Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. Journal of Neuro-Oncology, 113(1), 1–11. doi:10.1007/s11060-013-1084-8

[85] Zaborowski, M. P., Balaj, L., Breakefield, X. O., Lai, C. P. (2015). Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. BioScience, 65(8), 783–797. doi:10.1093/biosci/biv084

[86] Llorente, A., Skotland, T., Sylvänne, T., Kauhanen, D., Róg, T., Orłowski, A., Sandvig, K. (2013). Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1831(7), 1302–1309. doi:10.1016/j.bbalip.2013.04.011

[87] Laulagnier, K., Motta, C., Hamdi, S., Roy, S., Fauvelle, F., Pageaux, J.-F., Record, M. (2004). Mast cell- and dendritic cell-derived exosomes display a specific lipid

composition and an unusual membrane organization. Biochemical Journal, 380(1), 161–171. doi:10.1042/bj20031594

[88] Ikonen, E. (2001). Roles of lipid rafts in membrane transport. Current Opinion in Cell Biology, 13(4), 470–477. doi:10.1016/s0955-0674(00)00238-6

[89] Ratajczak, J., Miekus, K., Kucia, M., Zhang, J., Reca, R., Dvorak, P., Ratajczak, M. Z. (2006). Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia, 20(5), 847–856. doi:10.1038/sj.leu.2404132

[90] Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature Cell Biology, 9(6), 654–659. doi:10.1038/ncb1596

[91] Cheng, L., Sharples, R. A., Scicluna, B. J., Hill, A. F. (2014). Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. Journal of Extracellular Vesicles, 3(1), 23743. doi:10.3402/jev.v3.23743

[92] Skog, J., Würdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Sena-Esteves, M., Curry, W. T., Jr, Carter, B. S., Krichevsky, A. M., Breakefield, X. O. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nature cell biology, 10(12), 1470–1476. doi:10.1038/ncb1800

[93] Montecalvo, A., Larregina, A. T., Shufesky, W. J., Beer Stolz, D., Sullivan, M. L. G., Karlsson, J. M., Morelli, A. E. (2011). Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood, 119(3), 756–766. doi:10.1182/blood-2011-02-338004

[94] Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Milito, A., Fais, S. (2009). Microenvironmental pH Is a Key Factor for Exosome Traffic in Tumor Cells. Journal of Biological Chemistry, 284(49), 34211–34222. doi:10.1074/jbc.m109.041152

[95] Mulcahy, L. A., Pink, R. C., Carter, D. R. F. (2014). Routes and mecha-

nisms of extracellular vesicle uptake. Journal of Extracellular Vesicles, 3(1), 24641. doi:10.3402/jev.v3.24641

[96] McMahon, H. T., & Boucrot, E. (2011). Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nature Reviews Molecular Cell Biology, 12(8), 517–533. doi:10.1038/nrm3151

[97] Escrevente, C., Keller, S., Altevogt, P., & Costa, J. (2011). Interaction and uptake of exosomes by ovarian cancer cells. BMC Cancer, 11(1). doi:10.1186/1471-2407-11-108

[98] Newton, A. J., Kirchhausen, T., & Murthy, V. N. (2006). Inhibition of dynamin completely blocks compensatory synaptic vesicle endocytosis. Proceedings of the National Academy of Sciences, 103(47), 17955–17960. doi:10.1073/pnas.0606212103

[99] Nabi, I. R., Le, P. U. (2003). Caveolae/raft-dependent endocytosis. The Journal of Cell Biology, 161(4), 673–677. doi:10.1083/jcb.200302028

[100] Lipardi, C., Mora, R., Colomer, V., Paladino, S., Nitsch, L., Rodriguez-Boulan, E., Zurzolo, C. (1998). Caveolin Transfection Results in Caveolae Formation but Not Apical Sorting of Glycosylphosphatidysitol (GPI)-anchored Proteins in Epithelial Cells. The Journal of Cell Biology, 140(3), 617–626. doi:10.1083/jcb.140.3.617

[101] Nanbo, A., Kawanishi, E., Yoshida, R., Yoshiyama, H. (2013). Exosomes Derived from Epstein-Barr Virus-Infected Cells Are Internalized via Caveola-Dependent Endocytosis and Promote Phenotypic Modulation in Target Cells. Journal of Virology, 87(18), 10334–10347. doi:10.1128/jvi.01310-13

[102] Svensson, K. J., Christianson, H. C., Wittrup, A., Bourseau-Guilmain, E., Lindqvist, E., Svensson, L. M., Belting, M. (2013). Exosome Uptake Depends on ERK1/2-Heat Shock Protein 27 Signaling and Lipid Raft-mediated Endocytosis Negatively Regulated by Caveolin-1. Journal of Biological Chemistry, 288(24), 17713–17724. doi:10.1074/jbc.m112.445403

[103] Fitzner, D., Schnaars, M., van Rossum, D., Krishnamoorthy, G., Dibaj, P., Bakhti, M., Simons, M. (2011). Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. Journal of Cell Science, 124(3), 447–458. doi:10.1242/jcs.074088

[104] Feng, D., Zhao, W.-L., Ye, Y.-Y., Bai, X.-C., Liu, R.-Q., Chang, L.-F., Sui, S.-F. (2010). Cellular Internalization of Exosomes Occurs Through Phagocytosis. Traffic, 11(5), 675–687. doi:10.1111/j.1600-0854.2010.01041.x

[105] Christianson, H. C., Svensson, K. J., van Kuppevelt, T. H., Li, J.-P., Belting, M. (2013). Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. Proceedings of the National Academy of Sciences, 110(43), 17380–17385. doi:10.1073/pnas.1304266110

[106] de Abreu, R. C., Fernandes, H., da Costa Martins, P. A., Sahoo, S., Emanueli, C., & Ferreira, L. (2020). Native and bioengineered extracellular vesicles for cardiovascular therapeutics. Nature reviews. Cardiology, 17(11), 685–697. doi:10.1038/s41569-020-0389-5

[107] Del Fattore, A., Luciano, R., Saracino, R., Battafarano, G., Rizzo, C., Pascucci, L., Muraca, M. (2014). Differential effects of extracellular vesicles secreted by mesenchymal stem cells from different sources on glioblastoma cells. Expert Opinion on Biological Therapy, 15(4), 495–504. doi:10.1517/14712598.2015.997706

[108] Théry, C., Ostrowski, M., Segura, E. (2009). Membrane vesicles as conveyors of immune responses. Nature Reviews Immunology, 9(8), 581–593. doi:10.1038/nri2567

[109] Minciacchi V. R., You S., Spinelli C., Morley S., Zandian M., Aspuria P., Cavallini L., Ciardiello C., Sobreiro M., Morello M., Kharmate G., Jang S. Chul, Kim D. (2015). Large oncosomes contain distinct protein cargo and represent a separate functional class of tumor-derived extracellular vesicles. Oncotarget, 6(13), 11327-11341. doi:10.18632/oncotarget.3598

[110] Waldenström, A., Gennebäck, N., Hellman, U., Ronquist, G. (2012). Cardiomyocyte Microvesicles Contain DNA/RNA and Convey Biological Messages to Target Cells. PLoS ONE, 7(4), e34653. doi:10.1371/journal.pone.0034653

[111] Bang, C., Batkai, S., Dangwal, S., Gupta, S. K., Foinquinos, A., Holzmann, A., Thum, T. (2014). Cardiac fibroblast–derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. Journal of Clinical Investigation, 124(5), 2136–2146. doi:10.1172/jci70577

[112] Luo, H., Li, X., Li, T., Zhao, L., He, J., Zha, L., Qi, Q., Yu, Z. (2018). Exosomes/microvesicles microRNA-423-3p derived from cardiac fibroblasts mediates the cardioprotective effects of ischemic postconditioning. Cardiovascular research, 115(7), 1189-1204. doi:10.1093/cvr/cvy231.

[113] Datta, R., Bansal, T., Rana, S., Datta, K., Datta Chaudhuri, R., Chawla-Sarkar, M., Sarkar, S. (2016). Myocyte-Derived Hsp90 Modulates Collagen Upregulation via Biphasic Activation of STAT-3 in Fibroblasts during Cardiac Hypertrophy. Molecular and Cellular Biology, 37(6). doi:10.1128/mcb.00611-16

[114] Chung, A. S., Lee, J., & Ferrara, N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. Nature Reviews Cancer, 10(7), 505–514. doi:10.1038/nrc2868

[115] Barile, L., Lionetti, V., Cervio, E., Matteucci, M., Gherghiceanu, M., Popescu, L. M., Vassalli, G. (2014). Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction. Cardiovascular Research, 103(4), 530–541. doi:10.1093/cvr/cvu167

[116] Bian, S., Zhang, L., Duan, L., Wang, X., Min, Y., Yu, H. (2013). Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model. Journal of Molecular Medicine, 92(4), 387–397. doi:10.1007/s00109-013-1110-5

[117] Barile, L., Cervio, E., Lionetti, V., Milano, G., Ciullo, A., Biemmi, V., ... Vassalli, G. (2018). Cardioprotection by cardiac progenitor cell-secreted exosomes: role of pregnancy-associated plasma protein-A. Cardiovascular Research, 114(7), 992–1005. doi:10.1093/cvr/cvy055

[118] Zhang, Z., Yang, J., Yan, W., Li, Y., Shen, Z., & Asahara, T. (2016). Pretreatment of Cardiac Stem Cells With Exosomes Derived From Mesenchymal Stem Cells Enhances Myocardial Repair. Journal of the American Heart Association, 5(1), e002856. doi:10.1161/jaha.115.002856

[119] Chaturvedi, P., Kalani, A., Medina, I., Familtseva, A., Tyagi, S. C. (2015). Cardiosome mediated regulation of MMP9 in diabetic heart: role of mir29b and mir455 in exercise. Journal of cellular and molecular medicine, 19(9), 2153–2161. doi:10.1111/jcmm.12589

[120] Yang, Y., Li, Y., Chen, X., Cheng, X., Liao, Y., Yu, X. (2016). Exosomal transfer of miR-30a between cardiomyocytes regulates autophagy after hypoxia. Journal of Molecular Medicine, 94(6), 711–724. doi:10.1007/s00109-016-1387-2

[121] De Jong, O. G., Verhaar, M. C., Chen, Y., Vader, P., Gremmels, H., Posthuma, G., … van Balkom, B. W. M. (2012). Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. Journal of Extracellular Vesicles, 1(1), 18396. doi:10.3402/jev.v1i0.18396

[122] Gray, W. D., French, K. M., Ghosh-Choudhary, S., Maxwell, J. T., Brown, M. E., Platt, M. O., … Davis, M. E. (2014). Identification of Therapeutic Covariant MicroRNA Clusters in Hypoxia-Treated Cardiac Progenitor Cell Exosomes Using Systems Biology. Circulation Research, 116(2), 255–263. doi:10.1161/circresaha.116.304360

[123] Sokolova, V., Ludwig, A.-K., Hornung, S., Rotan, O., Horn, P. A., Epple, M., Giebel, B. (2011). Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. Colloids and Surfaces B: Biointerfaces, 87(1), 146–150. doi:10.1016/j.colsurfb.2011.05.013

[124] Harane, N. E., Correa, B. L., Gomez, I., Hocine, H. R., Vilar, J., Desgres, M., Silvestre, J.-S. (2020). Extracellular Vesicles from Human Cardiovascular Progenitors Trigger a Reparative Immune Response in Infarcted Hearts. Cardiovascular Research. doi:10.1093/cvr/cvaa028

[125] Colao, I. L., Corteling, R., Bracewell, D., & Wall, I. (2018). Manufacturing Exosomes: A Promising Therapeutic Platform. Trends in Molecular Medicine, 24(3), 242–256. doi:10.1016/j.molmed.2018.01.006

[126] Witwer, K. W., Soekmadji, C., Hill, A. F., Wauben, M. H., Buzás, E. I., Di Vizio, D., Théry, C. (2017). Updating the MISEV minimal requirements for extracellular vesicle studies: building bridges to reproducibility. Journal of Extracellular Vesicles, 6(1), 1396823. doi:10.1080/20013078.2017.1396823

[127] Yu, B., Ashraf, M., Xu, M. (2015). Therapeutic Potential of Stem Cell-Derived Extracellular Vesicles in Cardioprotection and Myocardium Repair. Mesenchymal Stem Cell Derived Exosomes, 119–138. doi:10.1016/b978-0-12-800164-6.00006-x

[128] Chen, C. W., Wang, L. L., Zaman, S., Gordon, J., Arisi, M. F., Venkataraman, C. M., ... Atluri, P. (2018). Sustained release of endothelial progenitor cell-derived extracellular vesicles from shear-thinning hydrogels improves angiogenesis and promotes function after myocardial infarction. Cardiovascular Research, 114(7), 1029–1040. doi:10.1093/cvr/cvy067

[129] Hamada, T., Dubois, J. L. N., Bellamy, V., Pidial, L., Hagège, A., Pereira, M. N., & Menasché, P. (2020). In vitro controlled release of extracellular vesicles for cardiac repair from poly(glycerol sebacate) acrylate-based polymers. Acta Biomaterialia. doi:10.1016/j.actbio.2020.08.015

[130] Lener, T., Gimona, M., Aigner, L., Börger, V., Buzas, E., Camussi, G., Chaput, N., Chatterjee, D., Court, F. A., Del Portillo, H. A., O'Driscoll, L., Fais, S., Falcon-Perez, J. M., Felderhoff-Mueser, U., Fraile, L., Gho, Y. S., Görgens, A., Gupta, R. C., Hendrix, A., Hermann, D. M., Giebel, B. (2015). Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper. Journal of extracellular vesicles, 4, 30087. doi: 10.3402/jev.v4.30087

[131] Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Amigorena, S. (1998). Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes. Nature Medicine, 4(5), 594–600. doi:10.1038/nm0598-594

[132] Escudier, B., Dorval, T., Chaput, N., André, F., Caby, M.-P., Novault, S., Zitvogel, L. (2005). Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of the first phase I clinical trial. Journal of Translational Medicine, 3(1), 10. doi:10.1186/1479-5876-3-10

[133] Morse, M. A., Garst, J., Osada, T., Khan, S., Hobeika, A., Clay, T. M., Lyerly, H. K. (2005). A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. Journal of Translational Medicine, 3(1), 9. doi:10.1186/1479-5876-3-9

[134] Besse, B., Charrier, M., Lapierre, V., Dansin, E., Lantz, O., Planchard, D., Le Chevalier, T., Livartoski, A., Barlesi, F., Laplanche, A., Ploix, S., Vimond, N., Peguillet, I., Théry, C., Lacroix, L., Zoernig, I., Dhodapkar, K., Dhodapkar, M., Viaud, S., Soria, J. C., Chaput, N. (2015). Dendritic cell-derived exosomes as mainte-

nance immunotherapy after first line chemotherapy in NSCLC. Oncoimmunology, 5(4), e1071008. doi: 10.1080/2162402X.2015.1071008

[135] Wolfers, J., Lozier, A., Raposo, G., Regnault, A., Théry, C., Masurier, C., Zitvogel, L. (2001). Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nature Medicine, 7(3), 297–303. doi:10.1038/85438

[136] Dai, S., Wei, D., Wu, Z., Zhou, X., Wei, X., Huang, H., Li, G. (2008). Phase I Clinical Trial of Autologous Ascites-derived Exosomes Combined With GM-CSF for Colorectal Cancer. Molecular Therapy, 16(4), 782–790. doi:10.1038/mt.2008.1

[137] Kordelas, L., Rebmann, V., Ludwig, A.-K., Radtke, S., Ruesing, J., Doeppner, T. R., Giebel, B. (2014). MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. Leukemia, 28(4), 970–973. doi:10.1038/leu.2014.41

[138] Nassar, W., El-Ansary, M., Sabry, D., Mostafa, M. A., Fayad, T., Kotb, E., Adel, H. (2016). Umbilical cord mesenchymal stem cells derived extracellular vesicles can safely ameliorate the progression of chronic kidney diseases. Biomaterials Research, 20(1). doi:10.1186/s40824-016-0068-0

[139] El Harane, N., Kervadec, A., Bellamy, V., Pidial, L., Neametalla, H. J., Perier, M.-C., Renault, N. K. E. (2018). Acellular therapeutic approach for heart failure: in vitro production of extracellular vesicles from human cardiovascular progenitors. European Heart Journal, 39(20), 1835–1847. doi:10.1093/eurheartj/ehy012

[140] Wang, Y., Zhang, L., Li, Y., Chen, L., Wang, X., Guo, W., Tang, Y. (2015). Exosomes/microvesicles from induced pluripotent stem cells deliver cardioprotective miRNAs and prevent cardiomyocyte apoptosis in the ischemic myocardium. International Journal of Cardiology, 192, 61–69. doi:10.1016/j.ijcard.2015.05.020

[141] Khan, M., Nickoloff, E., Abramova, T., Johnson, J., Verma, S. K., Krishnamurthy, P., Kishore, R. (2015). Embryonic Stem Cell-Derived Exosomes Promote Endogenous Repair Mechanisms and Enhance Cardiac Function Following Myocardial Infarction. Circulation Research, 117(1), 52–64. doi:10.1161/circresaha.117.305990

[142] Correia, C., Koshkin, A., Duarte, P., Hu, D., Teixeira, A., Domian, I., Serra, M., Alves, P. M. (2017). Distinct carbon sources affect structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. Scientific Reports, 7(1). doi:10.1038/s41598-017-08713-4

[143] Van Deun, J., Mestdagh, P., Sormunen, R., Cocquyt, V., Vermaelen, K., Vandesompele, J., Hendrix, A. (2014). The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling. Journal of Extracellular Vesicles, 3(1), 24858. doi:10.3402/jev.v3.24858

[144] Brennan, K., Martin, K., FitzGerald, S. P., O'Sullivan, J., Wu, Y., Blanco, A., ... Mc Gee, M. M. (2020). A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. Scientific Reports, 10(1). doi:10.1038/s41598-020-57497-7

[145] Turbendian, H. K., Gordillo, M., Tsai, S.-Y., Lu, J., Kang, G., Liu, T.-C., ... Evans, T. (2013). GATA factors efficiently direct cardiac fate from embryonic stem cells. Development, 140(8), 1639–1644. doi:10.1242/dev.093260

[146] Cai, C. L., Liang, X., Shi, Y., Chu, P. H., Pfaff, S. L., Chen, J., & Evans, S. (2003). Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Developmental cell, 5(6), 877–889. doi:10.1016/s1534-5807(03)00363-0

[147] Patel, G. K., Khan, M. A., Zubair, H., Srivastava, S. K., Khushman, M., Singh, S., & Singh, A. P. (2019). Comparative analysis of exosome isolation methods using culture supernatant for optimum yield, purity and downstream applications. Scientific Reports, 9(1). doi:10.1038/s41598-019-41800-2

[148] Lötvall, J., Hill, A. F., Hochberg, F., Buzás, E. I., Di Vizio, D., Gardiner, C., Gho, Y. S., Kurochkin, I. V., Mathivanan, S., Quesenberry, P., Sahoo, S., Tahara, H., Wauben, M. H., Witwer, K. W., & Théry, C. (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. Journal of extracellular vesicles, 3, 26913. doi:10.3402/jev.v3.26913

[149] Chuo, S. T.-Y., Chien, J. C.-Y., & Lai, C. P.-K. (2018). Imaging extracellular vesicles: current and emerging methods. Journal of Biomedical Science, 25(1). doi:10.1186/s12929-018-0494-5

[150] Dehghani, M., Gulvin, S. M., Flax, J., & Gaborski, T. R. (2020). Systematic Evaluation of PKH Labelling on Extracellular Vesicle Size by Nanoparticle Tracking Analysis. Scientific Reports, 10(1). doi:10.1038/s41598-020-66434-7

[151] Pužar Dominkuš, P., Stenovec, M., Sitar, S., Lasič, E., Zorec, R., Plemenitaš, A., ... Lenassi, M. (2018). PKH26 labeling of extracellular vesicles: Characterization and cellular internalization of contaminating PKH26 nanoparticles. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1860(6), 1350–1361. doi:10.1016/j.bbamem.2018.03.013

[152] Patan S. (2000). Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. Journal of neuro-oncology, 50(1-2), 1–15. doi:10.1023/a:1006493130855

[153] Vrijsen, K. R., Sluijter, J. P., Schuchardt, M. W., van Balkom, B. W., Noort, W. A., Chamuleau, S. A., & Doevendans, P. A. (2010). Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells. Journal of cellular and molecular medicine, 14(5), 1064–1070. doi:10.1111/j.1582-4934.2010.01081.x

[154] Ribeiro-Rodrigues, T. M., Laundos, T. L., Pereira-Carvalho, R., Batista-Almeida, D., Pereira, R., Coelho-Santos, V., Silva, A. P., Fernandes, R., Zuzarte, M., Enguita, F. J., Costa, M. C., Pinto-do-Ó, P., Pinto, M. T., Gouveia, P., Ferreira, L., Mason, J. C., Pereira, P., Kwak, B. R., Nascimento, D. S., & Girão, H. (2017). Exosomes secreted by cardiomyocytes subjected to ischaemia promote cardiac angiogenesis. Cardiovascular research, 113(11), 1338–1350. doi:10.1093/cvr/cvx118

[155] Grada, A., Otero-Vinas, M., Prieto-Castrillo, F., Obagi, Z., & Falanga, V. (2017). Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay. Journal of Investigative Dermatology, 137(2), e11–e16. doi:10.1016/j.jid.2016.11.020

[156] Staton, C. A., Stribbling, S. M., Tazzyman, S., Hughes, R., Brown, N. J., & Lewis, C. E. (2004). Current methods for assaying angiogenesis in vitro and in vivo. International Journal of Experimental Pathology, 85(5), 233–248. doi:10.1111/j.0959-9673.2004.00396.x

[157] Dougherty, J. A., Kumar, N., Noor, M., Angelos, M. G., Khan, M., Chen, C. A., & Khan, M. (2018). Extracellular Vesicles Released by Human Induced-Pluripotent Stem Cell-Derived Cardiomyocytes Promote Angiogenesis. Frontiers in physiology, 9, 1794. doi:10.3389/fphys.2018.01794

[158] Adamiak, M., Cheng, G., Bobis-Wozowicz, S., Zhao, L., Kedracka-

Krok, S., Samanta, A., ... Zuba-Surma, E. K. (2017). Induced Pluripotent Stem Cell (iPSC)–Derived Extracellular Vesicles Are Safer and More Effective for Cardiac Repair Than iPSCs. Circulation Research, 122(2), 296–309. doi:10.1161/circresaha.117.311769

[159] Ding, Q., Sun, R., Wang, P., Zhang, H., Xiang, M., Meng, D., ... Chen, S. (2018). Protective effects of human induced pluripotent stem cell-derived exosomes on high glucose-induced injury in human endothelial cells. Experimental and Therapeutic Medicine. doi:10.3892/etm.2018.6059

[160] Liu, B., Lee, B. W., Nakanishi, K., Villasante, A., Williamson, R., Metz, J., ... Vunjak-Novakovic, G. (2018). Cardiac recovery via extended cell-free delivery of extracellular vesicles secreted by cardiomyocytes derived from induced pluripotent stem cells. Nature Biomedical Engineering, 2(5), 293–303. doi:10.1038/s41551-018-0229-7

[161] Bayless, K. J., & Johnson, G. A. (2011). Role of the Cytoskeleton in Formation and Maintenance of Angiogenic Sprouts. Journal of Vascular Research, 48(5), 369–385. doi:10.1159/000324751

[162] Brill, A., Elinav, H., & Varon, D. (2004). Differential role of platelet granular mediators in angiogenesis. Cardiovascular Research, 63(2), 226–235. doi:10.1016/j.cardiores.2004.04.012

[163] Murakami, M., Nguyen, L. T., Zhuang, Z. W., Moodie, K. L., Carmeliet, P., Stan, R. V., & Simons, M. (2008). The FGF system has a key role in regulating vascular integrity. The Journal of clinical investigation, 118(10), 3355–3366. https://doi.org/10.1172/JCI35298

[164] Thum, T., Catalucci, D., & Bauersachs, J. (2008). MicroRNAs: novel regulators in cardiac development and disease. Cardiovascular Research, 79(4), 562–570. doi:10.1093/cvr/cvn137

[165] Sluijter, J. P. G., van Mil, A., van Vliet, P., Metz, C. H. G., Liu, J., Doevendans, P. A., & Goumans, M. J. (2010). MicroRNA-1 and -499 Regulate Differentiation and Proliferation in Human-Derived Cardiomyocyte Progenitor Cells. Arteriosclerosis, Thrombosis, and Vascular Biology, 30(4), 859–868. doi:10.1161/atvbaha.109.197434

[166] Godard, P., & van Eyll, J. (2015). Pathway analysis from lists of microRNAs: common pitfalls and alternative strategy. Nucleic Acids Research, 43(7), 3490–3497. doi:10.1093/nar/gkv249

[167] Wang, X., Huang, W., Liu, G., Cai, W., Millard, R. W., Wang, Y., ... Fan, G.-C. (2014). Cardiomyocytes mediate anti-angiogenesis in type 2 diabetic rats through the exosomal transfer of miR-320 into endothelial cells. Journal of Molecular and Cellular Cardiology, 74, 139–150. doi:10.1016/j.yjmcc.2014.05.001

[168] Borosch, S., Dahmen, E., Beckers, C., Stoppe, C., Buhl, E. M., Denecke, B., ... Kraemer, S. (2017). Characterization of extracellular vesicles derived from cardiac cells in an in vitro model of preconditioning. Journal of Extracellular Vesicles, 6(1), 1390391. doi:10.1080/20013078.2017.1390391

[169] Garcia, N. A., Ontoria-Oviedo, I., González-King, H., Diez-Juan, A., & Sepúlveda, P. (2015). Glucose Starvation in Cardiomyocytes Enhances Exosome Secretion and Promotes Angiogenesis in Endothelial Cells. PLOS ONE, 10(9), e0138849. doi:10.1371/journal.pone.0138849

[170] Andriolo, G., Provasi, E., Lo Cicero, V., Brambilla, A., Soncin, S., Torre, T., Milano, G., Biemmi, V., Vassalli, G., Turchetto, L., Barile, L., & Radrizzani, M. (2018). Exosomes From Human Cardiac Progenitor Cells for Therapeutic Applications: Development of a GMP-Grade Manufacturing Method. Frontiers in physiology, 9, 1169. doi:10.3389/fphys.2018.01169

[171] Patel, D. B., Luthers, C. R., Lerman, M. J., Fisher, J. P., & Jay, S. M. (2018). Enhanced extracellular vesicle production and ethanol-mediated vascularization bioactivity via a 3D-printed scaffold-perfusion bioreactor system. Acta Biomaterialia. doi:10.1016/j.actbio.2018.11.024

[172] Chen, C. W., Wang, L. L., Zaman, S., Gordon, J., Arisi, M. F., Venkataraman, C. M., ... Atluri, P. (2018). Sustained release of endothelial progenitor cell-derived extracellular vesicles from shear-thinning hydrogels improves angiogenesis and promotes function after myocardial infarction. Cardiovascular Research, 114(7), 1029–1040. doi:10.1093/cvr/cvy067

Supplementary information

Materials and Methods

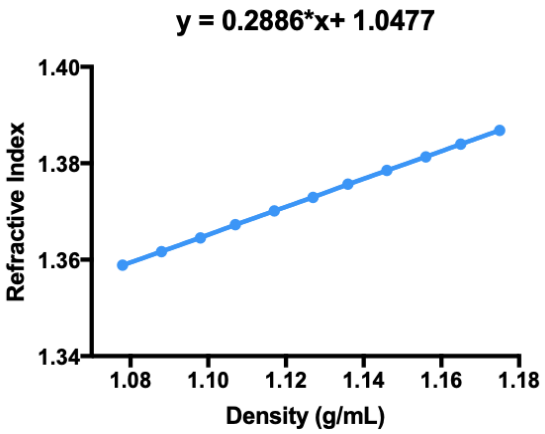


Figure 4.1: Linear regression curve relating refractive index values to density of iodixanol solutions.

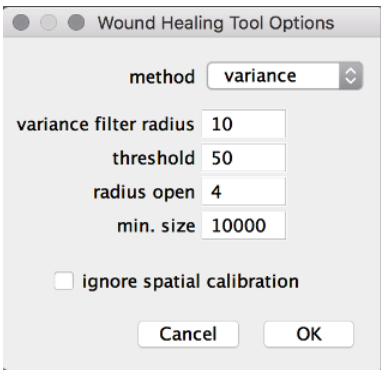


Figure 4.2: Settings used for MRI Wound Healing tool of Fiji software to determine percentage of wound closure.

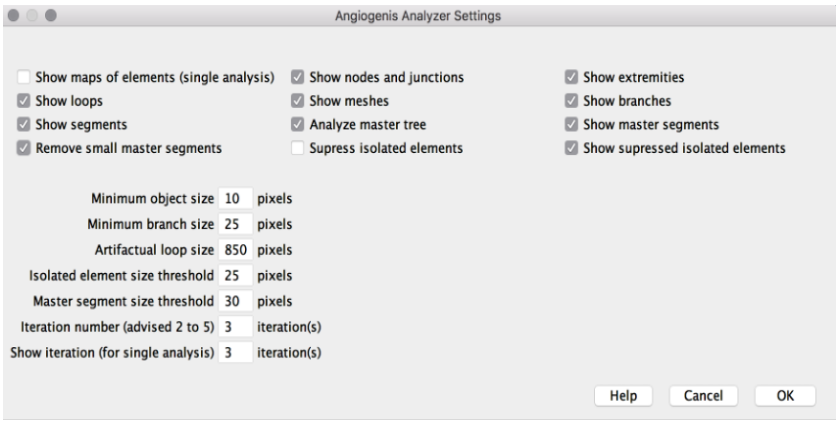


Figure 4.3: Settings used for Angiogenesis Analyzer plugin of Fiji software to determine tube formation parameters.

Results

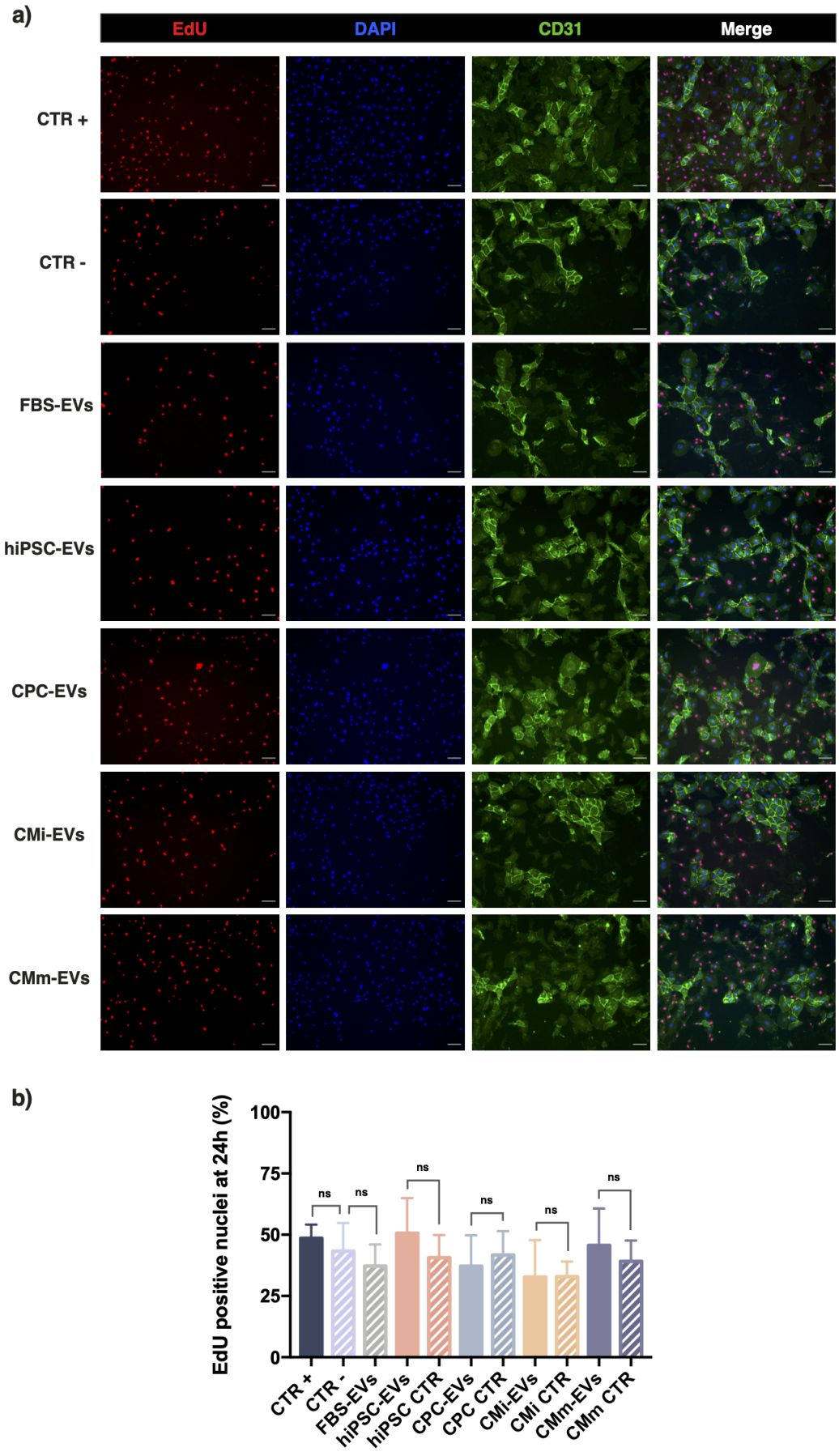

a)
EdU
DAPI
CD31
Merge
CTR +
CTR -
FBS-EVs
hiPSC-EVs
CPC-EVs
CMi-EVs
CMm-EVs
b)
EdU positive nuclei at 24h (%)
100
75
50
25
0
ns
CTR +
CTR -
FBS-EVs
hiPSC-EVs
hiPSC CTR
CPC-EVs
CPC CTR
CMi-EVs
CMi CTR
CMm-EVs
CMm CTR

Figure 4.4: **Effect of EVs from hiPSCs and hiPSC-cardiac derivatives on HUVEC proliferation, quantified by EdU staining. a)** Immunofluorescence images of EV and control samples from the 24h timepoint of the wound healing assay, after incubation with EdU reagent. HUVECs were stained for transmembrane protein CD31 (green) and nuclei were counterstained with DAPI (blue). Merged images show HUVEC proliferation in cells with pink nuclei. Scale bar: 100 µm. **b)** Percentage of EdU-positive nuclei, quantified from a minimum of 1.000 DAPI-stained nuclei per condition, with the respective comparison to negative or vehicle control. All data shown as mean ± SD (n=1). ns non-significant.

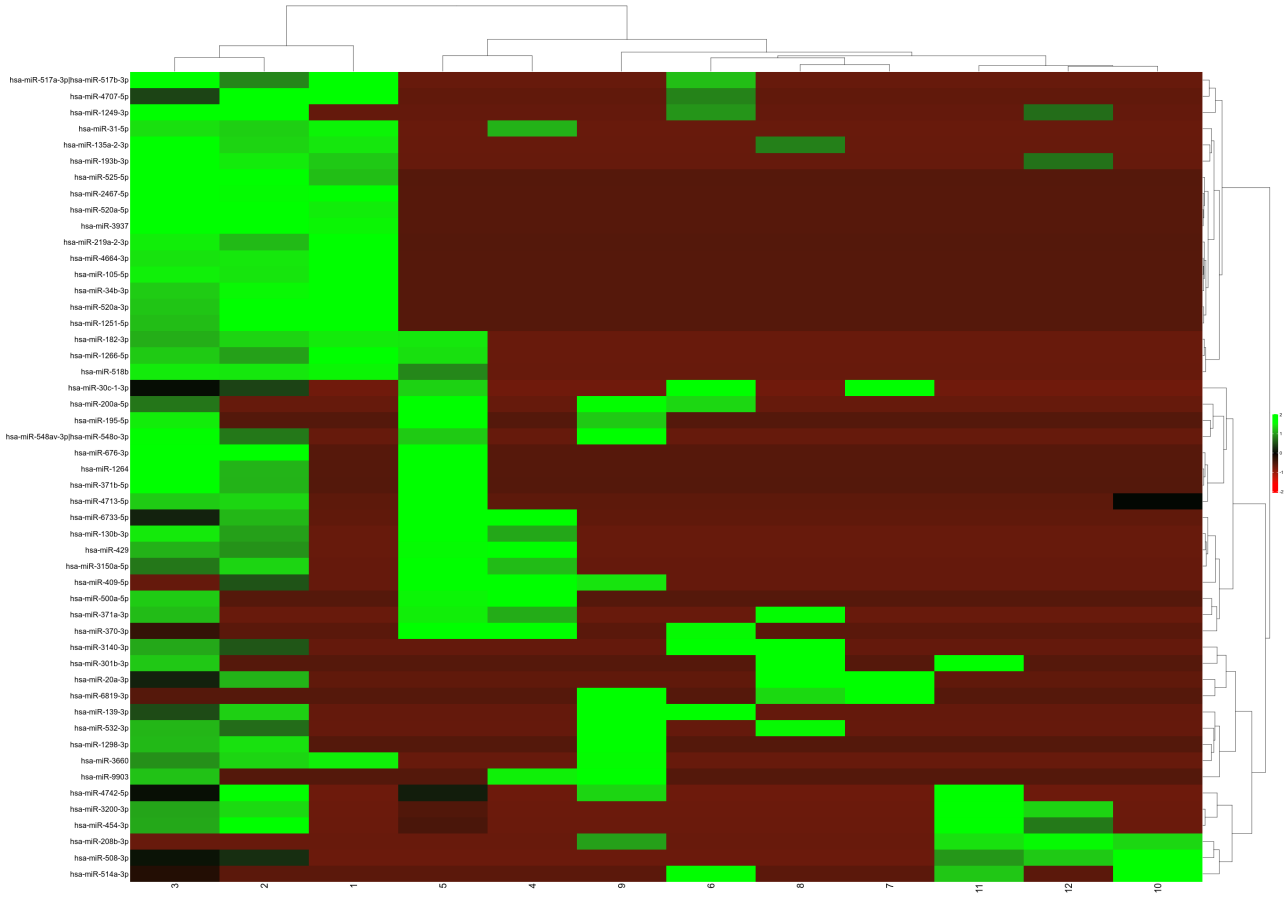


Figure 4.5: **Heat map of differential microRNA expression of EV samples.** Heat map was generated from the 50 microRNAs with highest %CV between hiPSC-EVs (samples 1-3) and CPC/CMi/CMm-EVs (4-12). Color scale indicates microRNA expression level relative to the mean, where green and red indicate higher or lower expression, respectively.