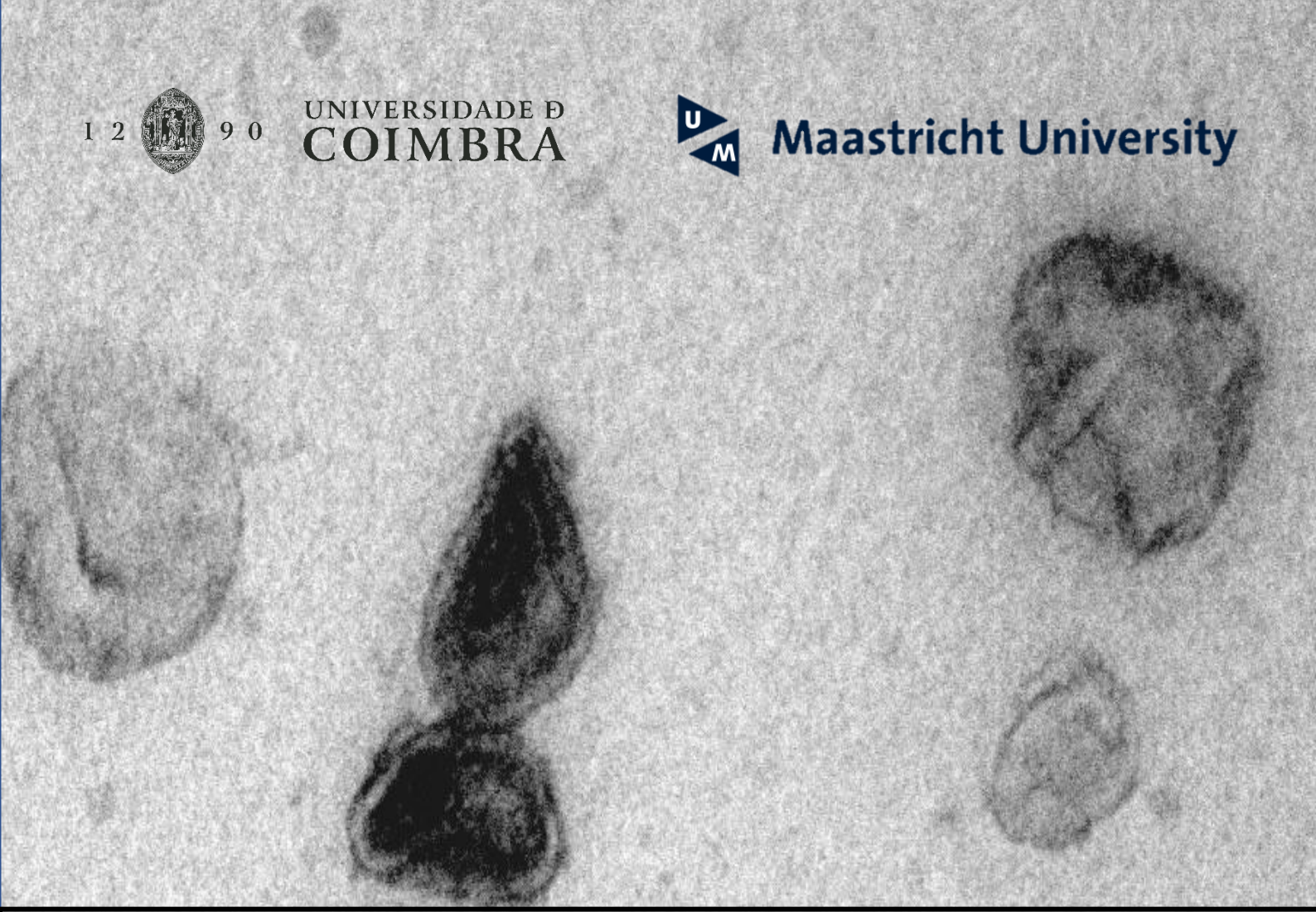
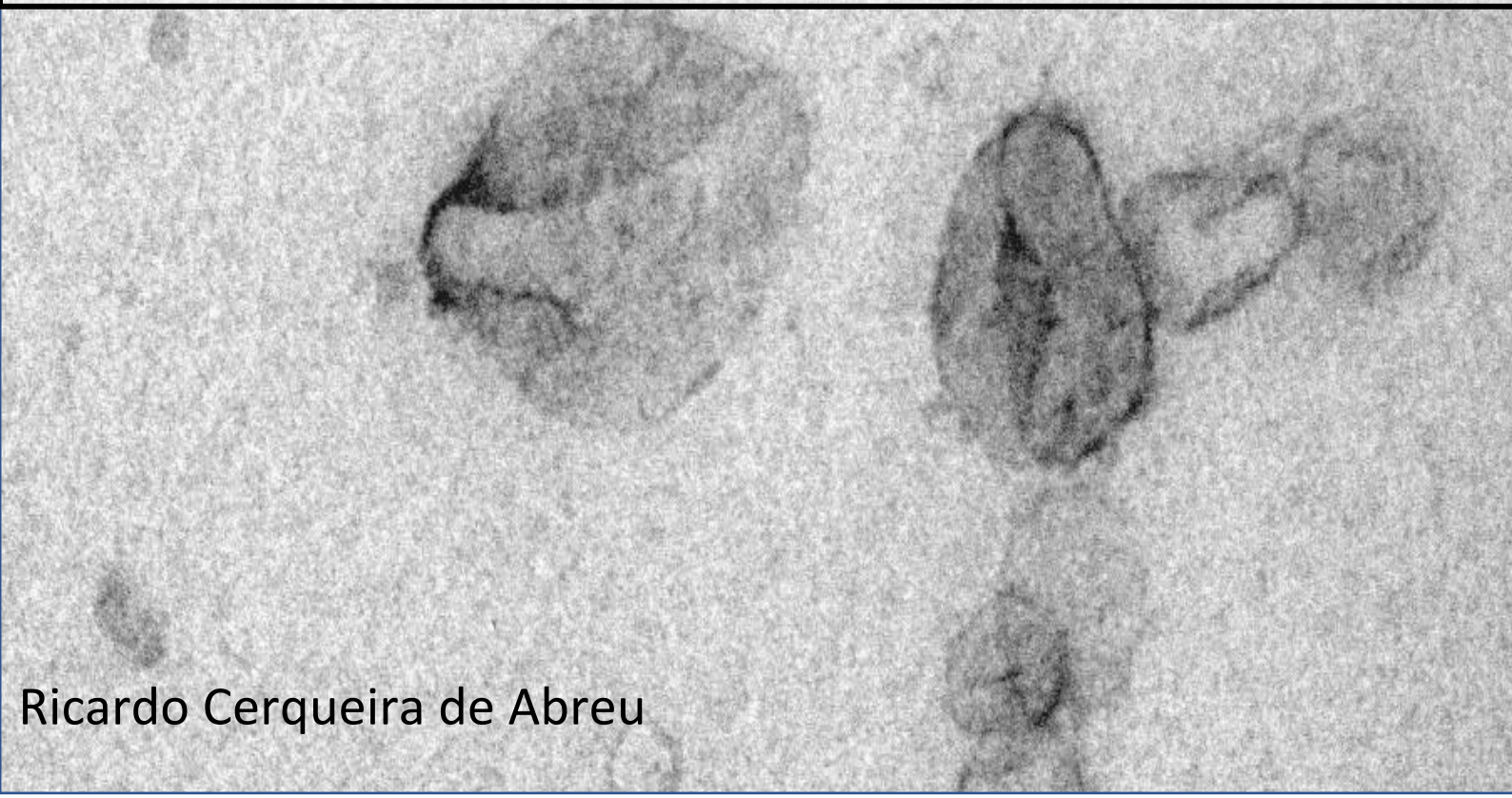


# Extracellular Vesicles as Platforms for Therapeutic microRNA delivery



Ricardo Cerqueira de Abreu

# Extracellular Vesicles as Platforms for Therapeutic microRNA delivery

Ricardo Cerqueira de Abreu

PhD thesis co-supervision between the University of Maastricht and the University of Coimbra

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**Extracellular Vesicles as Platforms for Therapeutic microRNA delivery**

DISSERTATION

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and Doutor at the University of Coimbra

on the authority of the Rector Magnifici,

Prof. dr. Pamela Habibović and Prof. dr. Amílcar Falcão,

in accordance with the decision of the Board of Deans,

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# CHAPTER I - General Introduction

Adapted from

**cROSsing the cardiac MIRe: fibroblast-cardiomyocyte ex(o)press**

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**Preamble**

Life does not exist in a vacuum. In other words, for there to be life, there had to be life. Historically, that notion may be challenged by life's very origins. In the modern age, life may now be synthetized. Nevertheless, enshrined in the tenets of cell theory, this idea offers some solace in the face of the cosmic inconsequence that underlies existence. Life does not exist in a vacuum; therefore, life is never alone. From unicellular beings to complex organisms, life exists and thrives in tandem with itself. We could lose ourselves in the complexities of Archaea processes or the nuance of Bacteria interaction. A bit closer to home perhaps, we might ponder on the necessity of communities and hierarchies in plants and animals. The idea of family. All of that is brought together in a single aspect. Communication. All of life needs something to "speak" to. In fact, it depends on it. Exchange of molecular signals, establishment of functional populations, food chains, interpersonal relationships all depend on effective communication. And life has devised a number of unique forms of communication, including the subject of this thesis: extracellular vesicles. If life had a purpose, extrinsic to its self-perpetuating nature, would it then not be to be understood? We may never know, but in research we make use of the tools at our disposal to decode life's speech and understand it. Whether life craves communication and understanding, or that is our observer's bias, once a question is posed, what choice do we have but to answer it?

That is what this thesis is, questions that were asked, and answers, perhaps always incomplete. Perhaps not as romanticized as my view of research and biology, but certainly an attempt at understanding a topic and communicating it.

**Extracellular vesicles origins and function**

Extracellular vesicles (EVs) are an heterogeneous group of small, droplet-like, cell membrane-derived particles which cannot replicate[1]. Although early findings may have pointed to the existence of EVs already in the 17th and 18th centuries, through studies regarding blood clotting, a modern conception of EVs was first established in 1967, where they were described as "platelet-dust" by Peter Wolf[2]. Twenty years later, Johnstone and his group used the term "exosome" to describe membrane-derived vesicles arising from differentiating reticulocytes[3]. Since then, several subtypes of EVs have been considered under the broader classification of "extracellular vesicles". The two most prevalent are exosomes and microvesicles (ectosomes), although other subtypes exist (i.e. apoptotic bodies[4], large oncosomes[5], exophers[6] and exomeres[7]). Exosomes and microvesicles may be distinguished via biophysical properties (e.g. size, shape, surface charge, density), biochemical signature (e.g. protein markers, RNA cargo, lipid profile) or by a combination of these properties. However, most EV subtypes exhibit overlapping features, which confounds both the nomenclature and their classification[8]. For example, typical EV markers include mainly tetraspanins (e.g. CD9, CD63, CD81), integrins (e.g. LAMP1/2) and lipid-binding proteins (e.g. endosomal sorting complexes required for transport (ESCRT)-associated proteins). Nonetheless, both exosomes and microvesicles, as well as other vesicle subtypes, may present some of these markers. Consequently, aside from detecting them during their biogenesis, which is laborious and challenging, EVs can be categorized according to their physical properties in a broader sense[9]. EVs with a diameter under 200 nm may be classified as small EVs (sEVs), whereas bigger EVs are classified as large EVs. This type of empirical classification is also associated with common EV-enriching techniques, such as differential or gradient ultracentrifugation or size-exclusion chromatography[10], which may yield EVs samples enriched in a specific size and/or density).

Both microvesicles and exosomes are lipidic structures derived from the cytoplasmatic membrane. Microvesicles are formed upon outward budding and fission of the cell membrane, whereas exosomes require the membrane to bud inward to form endosomal complexes. Those intracellular vesicles then invaginate again to produce intraluminal vesicles (ILVs) within, becoming multivesicular bodies (MVBs). When the MVBs fuse again with the cytoplasmatic membrane, they release ILVs, which become exosomes[11]. During these processes, both types of EVs are loaded with cytoplasmatic cargo and membrane

components, either stochastically (i.e. the EV composition will reflect, proportionately, that of the donor cell) or selectively, whereby the EV will be enriched or depleted in a specific factor[12]. Canonically, protein and RNA cargo rely on the endosomal sorting complexes required for transport (ESCRT) machinery in order to localize to the EVs. The ESCRT complexes are capable of binding numerous cytoplasmatic compounds, which may then be specifically included in EV composition[13].

EVs can only exert their biological function on recipient cells upon interacting with them. While EVs are known to deliver their contents to recipient cells via membranar fusion, the most prevalent process for EV uptake is thought to be endocytosis (including clathrin-mediated endocytosis, phagocytosis, macropinocytosis and caveolin-dependent endocytosis)[14] (**Fig. 1**). Consequently, a large proportion of internalized EVs is processed in the endolysosomal pathway[15,16] where a fraction of the EVs may fuse with the endosomal membrane, leading to "endosomal escape" and the release of their lumen contents into the cytoplasm. However, the majority of EVs is thought to be processed into the lysosomal compartments, where the EV cargo is degraded and/or recycled[16]. To deliver functional molecules (proteins, nucleic acids, metabolites) and modulate the recipient's cell fate, EVs must inevitably avoid this intracellular outcome.

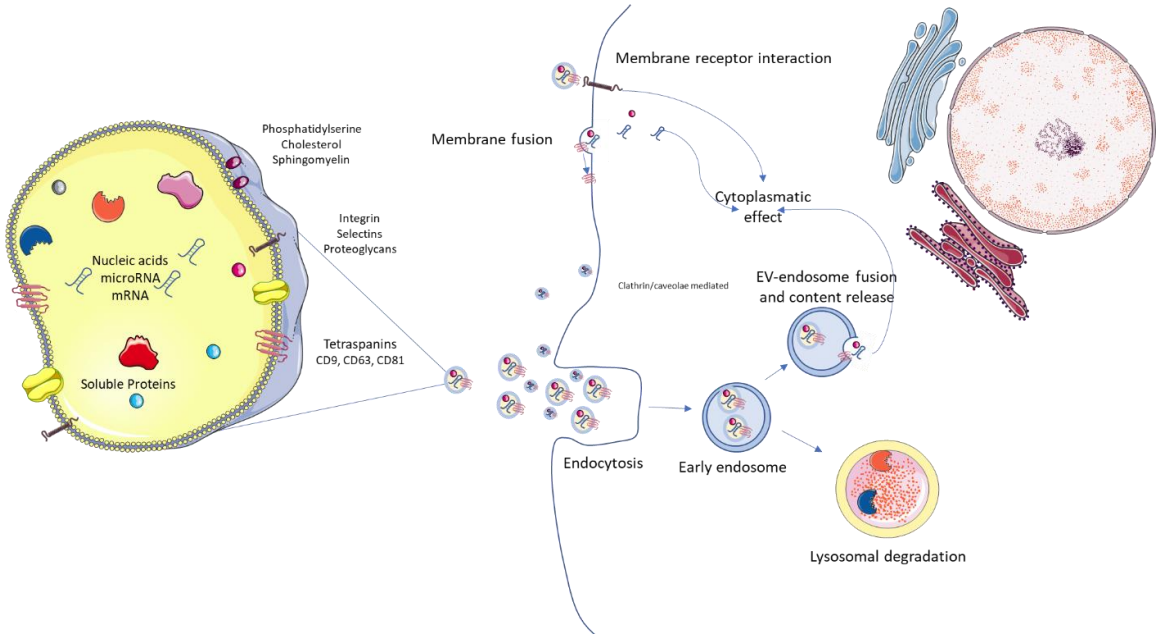


**Figure 1. EV internalization and intracellular processing.** EVs are most commonly internalized via one of the endocytic pathways of the recipient cell, although direct membrane fusion or interaction with surface membrane receptors may be sufficient to elicit a cellular response. After endocytosis, EVs find themselves in the endosomal pathway, in which they can either fuse with the endosome to release

their contents into the cytoplasm, or be degraded upon endosomal fusion with the lysosomal compartments. Drawings adapted from the Servier Medical Art image library.

Regardless of how EVs deliver their contents to recipient cells, their origin and composition determines their function. Initially, EVs were thought of as a cellular waste secretion mechanism[3]. However, many studies have since then ascribed new functions and importance to these vesicles, both in pathology and homeostasis. Fundamentally, EVs are key mediators of intercellular communication, delivering cellular molecules in an autocrine, paracrine or endocrine-like fashion through an organism[17]. EVs can act as a positive feedback mechanism such as, for example, in delivering pro-oncogenic molecules and thus contributing to the progression of tumorigenesis[18] or by delivering immunomodulatory cargo and modulating, immune responses in diabetes[19]. On other hand, EVs may also act as compensation mechanisms, countering disease progression. Notably, this process has been documented in cardiovascular disease, where, following an ischemic event, EVs carrying microRNAs were shown to exert a cardioprotective effect[20-22].

**MicroRNA primer**

MicroRNAs (miRNA) are small (~22 nucleotides in length) non-coding RNA. In their mature form, miRNA are typically single-stranded and capable of binding complementarily to target sequences in messenger RNA (mRNA) to stop translation[23]. miRNA are transcribed from genomic DNA by RNA-polymerase II/III, typically yielding stem-loop structures flanked by single-stranded overhangs called primary microRNA (pri-miRNA)[24] (**Fig. 2**). The overhangs flanking the stem-loops are cleaved at their ends by a protein complex composed of Drosha (a nuclear RNase III-type endonuclease) and its cofactor DiGeorge syndrome chromosome region 8 (DGCR8)[25,26]. This processing creates the precursor microRNA (pre-miRNA), which can then by exported to the cytoplasm via exportin 5[27]. Once in the cytoplasm, the pre-miRNA is further processed by the transactivation response element RNA-binding protein (TRBP) and Dicer (a RNase III endonuclease), which cleaves the stem-loop, exposing the 22 nucleotide-long, double stranded, mature miRNA sequences[28]. Those sequences then complex with Argonaute 2 (AGO2) to form the RNA interference silencing complex (RISC), where one of them will be selected as the active strand. The RISC then can target specific mRNAs and affect

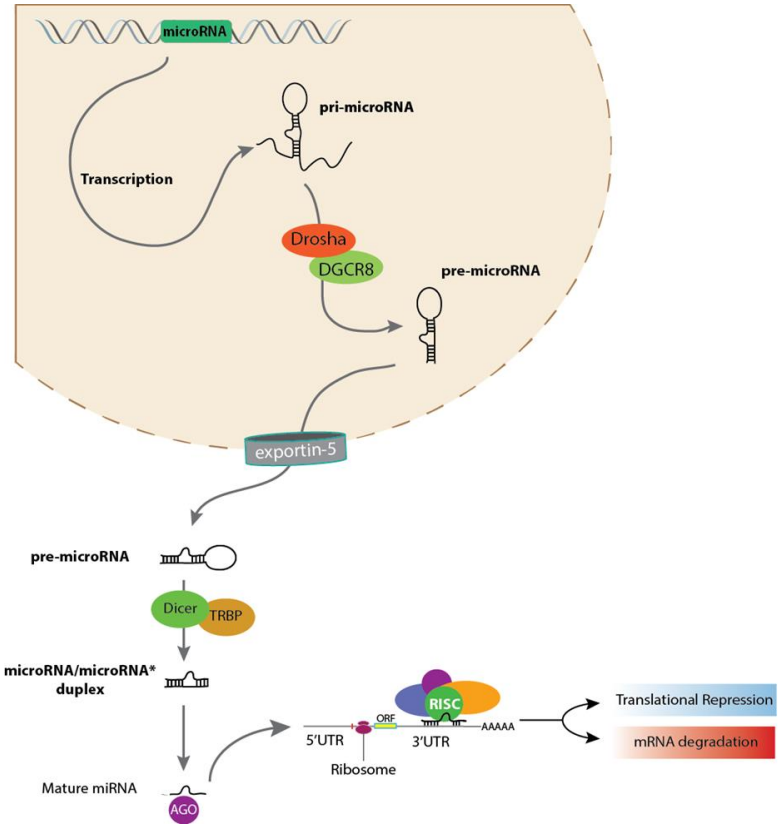


**Figure 2. Canonical microRNA biogenesis and mode of action.** miRNAs are transcribed into pri-microRNA, which are processed in the nucleus by Drosha and DGCR8 generating the pre-microRNA. This precursor is then exported to the cytoplasm, where Dicer, TRBP and AGO2 further process it into its active form. Finally, the mature miRNA is loaded into the RISC, to exert its function on the target mRNA. (From Abreu, R., M.Sc. thesis, University of Coimbra, Coimbra, 2016).

their translation[29]. Despite reports of atypical binding[30], normally, the miRNA 5' end sequence of 6 to 8 nucleotides (known as the seed sequence) binds to matching regions in the 3' untranslated region of their target mRNA[31]. This binding signals for translation arrest or degradation of the mRNA, effectively downregulating or abrogating the expression of a specific protein (**Fig. 2**).

In recent years, the gene expression regulatory potential of miRNA has been harnessed and applied therapeutically in different contexts[32-34]. From therapeutic and translational perspectives, compared to other gene expression inhibitors, such as genome editing technologies, miRNA offer several advantages. Therapeutically, for example, miRNA activity can be transient and controlled in time and space, depending on their administration[35,36]. MiRNA also offer another layer of gene regulation potential, as miRNA themselves can be inhibited by using silencing RNAs such as antagomiRs (oligonucleotides designed to block miRNA action)[37]. Additionally, miRNA, aside from being able to target a majority of relevant transcripts[38], are also capable of interfering with the expression of several mRNA species in the same pathway[39], granting them redundancy effectiveness. On the other hand, from a technology development and translational point of view, owing to miRNA's predictable and reproducible binding and form of action, a host of bioinformatic tools is now available to facilitate miRNA studies[40,41]. This is possible because many miRNA are widely conserved[42,43], which means that their natural inhibition cascades are likely to be preserved in animal models[44]. Finally, miRNA

are small nucleic acids which are inexpensive to manufacture, easy to transfect[45,46] and may be loaded on delivery systems to further boost their efficacy[47-49], *in vitro* and *in vivo*. All these attributes render miRNA a potent cell fate regulator and a promising tool for fundamental, translational and clinical research.

**Thesis aims and overview**

As previously mentioned, both EVs and miRNA are naturally produced by cells and can be employed in several pathophysiological contexts[50]. While their overlap in terms of attributes and functions at the cellular level is clear (e.g. ability to modulate cell fate), the question of how synergistic they may be remains open. On the one hand EVs themselves may carry miRNA species that, depending on the context, may be used as therapeutic agents[51]. On the other hand, cell-extrinsic stimuli may stimulate EV and/or miRNA production, which may yield beneficial downstream outcomes[52,53]. Conversely, the opposite may be true[54]. For example, EVs secreted in a pathological context may carry a mix of miRNAs or other molecules that have counteracting effects on a particular pathway or disease[55]. Additionally, microenvironment stimuli (i.e. extracellular cues that influence cell fate, such as hypoxia) responsible for the secretion of EVs enriched with a particular miRNA may themselves interfere with the biology

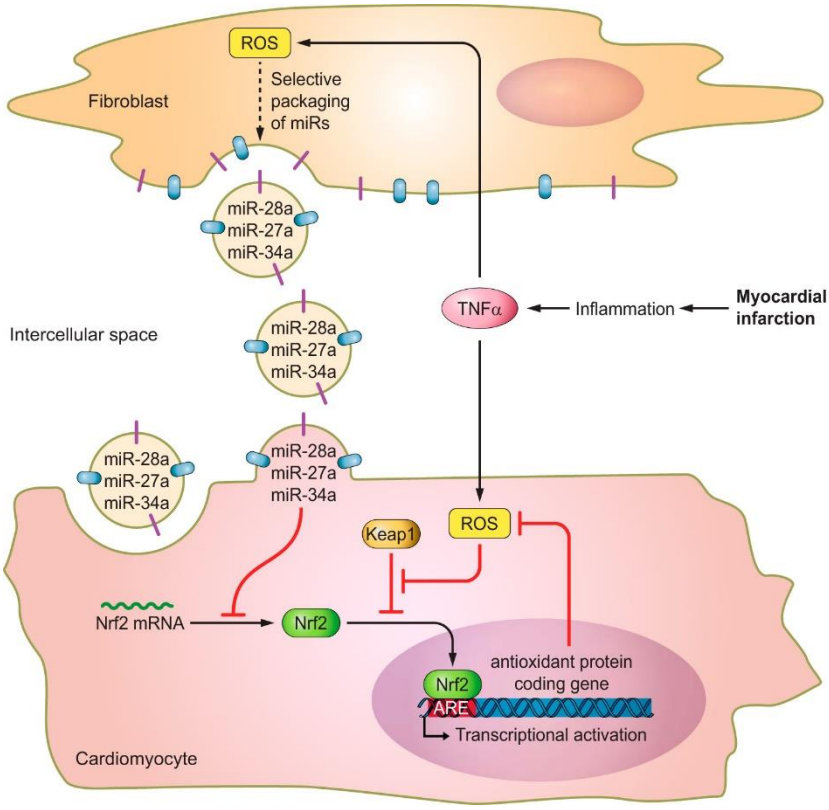


**Figure 3. Example of potential interplay between EVs and miRNA in myocardial infarction.** Upon injury to the heart, proinflammatory cues such as tumour necrosis factor (TNF)-α induce oxidative stress on cardiac myocytes and fibroblasts via reactive oxygen species (ROS). In cardiomyocytes, ROS is able to stop Kelch-like ECH-associated protein 1 (Keap1) from arresting nuclear factor E2-related factor-2 (Nrf2), which is then able to translocate to the nucleus, where it activates an antioxidant transcriptional program, creating a negative feedback loop. However, in fibroblasts, ROS seem to induce the selective loading of miRNA (miR)-27a, miRNA-28a, and miRNA-34a into exosomes. These extracellular vesicles are then released and absorbed by cardiomyocytes. Here, microRNAs are able to inhibit Nrf2 mRNA translation into protein, stopping its signalling cascade, allowing ROS to accumulate and damage the cell, which can eventually lead to chronic heart failure. ARE, antioxidant-responsive element. From Abreu, R.; da Costa Martins, P. A.; 2017. American Journal of Physiology: Heart and Circulatory Physiology.

of the recipient cell in a distinct manner[56]. Consequently, the interplay between EVs and miRNA in disease is far from trivial (**Fig. 3**).

Notwithstanding, using bioengineering tools, EVs can be modulated in numerous ways in order to alter their cargo and surface. This modulation procedure is especially useful to produce a complex therapeutic entity which can offer the advantages of miRNA functional effect at the cellular level. This approach also protects miRNA and enhances their biodistribution via the EV encapsulation and delivery system. Pathological contexts that may benefit the most from the development of EV-miRNA-based therapies include situations where there are hard-to-reach and poorly regenerative injured organs or tissues, such as cardiovascular or neurological diseases. Consequently, the core idea and aim of this thesis is to assess and develop methods for EVs to be efficiently and reproducibly enhanced and enriched to deliver specific and bioactive miRNA of interest in regenerative situations, and ascertain whether such modifications alter EV biology.

In **Chapter II**, the literature on EV modulation is extensively reviewed, particularly in the context of cardiovascular diseases (CVDs). This review lays the groundwork for studies using EVs as delivery agents, especially for miRNA delivery. The use of EVs in their native (non-modulated) state is discussed, as well as current strategies used to modulate EV cargo and/or surface. The discussion provides some insight into possible venues for future research and solutions on this topic.

**Chapter III** dives deeper into the role and applications of non-coding RNAs, including miRNA, in the context of CVDs. This study reviews the endothelial-cardiomyocyte-miRNA-EV axis in the context of CVD. The role of non-coding RNAs and EVs in boosting angiogenic function for the treatment of heart failure is explored.

**Chapter IV** is an original research study describing a comparison between the different approaches to modulate EVs with pre-selected miRNA, and their applications. This chapter discusses the various methodologies available for enriching EVs and the impact of that process on EV biology.

**Chapter V** describes an *in vivo* study where the EV-miRNA formulation defined in the previous chapter was used on a diabetic wound healing mouse model. Then, this study describes the

development of a high-throughput approach specifically designed to screen for novel molecules capable of enriching EVs with pre-selected miRNA.

**Chapter VI** summarizes and discusses the main findings and outcomes of this thesis. This work is put into the context of the broader field of EV and miRNA research, and how novel therapies can be developed by combining both fields particularly for the treatment of CVD.

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# CHAPTER II - Native and bioengineered extracellular vesicles for cardiovascular therapeutics

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**ABSTRACT**

Extracellular vesicles (EVs) are a heterogeneous group of natural particles with relevance for the treatment of cardiovascular diseases. The endogenous properties of these vesicles allow them to survive in the extracellular space, bypass biological barriers and deliver their biologically active molecular cargo to recipient cells. Moreover, EVs can be engineered to enhance their stability, bioactivity, presentation and capacity for on-target binding at both cell type and tissue levels. The therapeutic potential of native (i.e., EVs that were not modified via donor cell or direct modulation) and engineered EVs (i.e. EVs that were modified either pre or post-isolation or whose pharmacokinetics/presentation was altered using engineering methodologies) is still limitedly explored in the context of cardiovascular diseases. Efforts to tap into the therapeutic potential of EVs will require innovative approaches and a comprehensive integration of knowledge gathered from decades of molecular compound delivery. In this review, we outline the endogenous properties of EVs that make them natural delivery agents as well as those features that can be improved using bioengineering approaches. We also discuss the therapeutic applications of native and engineered EVs for cardiovascular applications and examine the opportunities and challenges that need to be addressed to advance this research area with an emphasis on clinical translation.

**Key points**

EVs secreted from stem/progenitor cells as well as differentiated somatic cells have regenerative properties in the context of myocardial infarction, ischemic limb, chronic wounds and stroke.

Despite the advantage of native EVs as delivery agents, their applicability in the cardiovascular context is hindered by intrinsic limitations, such as their undefined and heterogenous nature and limited tropism.

Targeting, bioactivity, kinetics and biodistribution of EVs may be improved by engineering approaches using both pre- and post-isolation methodologies to functionalize and/or otherwise enrich EVs.

Enhancing EVs is key to unlock their clinical potential for cardiovascular applications.

## 1. Introduction

Cardiovascular diseases have been the most prevalent cause of death and morbidity in the world for decades despite numerous breakthroughs and the discovery of novel therapies[1]. In the last 20 years, several therapeutic interventions have been initiated, including cell-based therapies; however, poor survival and/or engraftment of transplanted cells in the ischemic milieu of the cardiac tissue limited their clinical efficacy[2]. Mechanistically, the functional improvements observed with cell therapies are poorly understood; however, several pieces of experimental data indicate that they may act by paracrine action, mediated by the release of extracellular vesicles (EVs) and/or other factors[3,4]. Therefore, more recently, interest has been placed in cell-free therapies, in particular, those based in EVs, obviating the need of transplanting large number of cells whilst having a better-defined and less expensive product.

EVs are lipid bilayer-enclosed extracellular structures[5] secreted by virtually all cell types known, and include two major classes, namely exosomes and microvesicles[6]. Exosomes (30-150 nm), intraluminal vesicles formed via invagination of the membrane of multivesicular endosomes (MVEs), are released into the extracellular space upon fusion of MVEs with the cell membrane. Microvesicles (MVs) (50-1000 nm), a very heterogeneous class of EVs, are characterized by their origin and secretion via outward budding of the plasma membrane. Given the complexity involved in identifying their biogenesis, the size of the vesicles is the most widely used parameter to distinguish both types and, on that basis, we either have small EVs (sEVs) or medium/large EVs (m/lEVs)[7,8]. In this review, EVs represent sEV-enriched samples (many studies are not conclusive relatively to the biogenic origin of EVs), being the exception MV examples which are clearly defined in the text.

EVs carry proteins, RNAs and/or microRNAs (miRNAs), among other molecules, and they act as vehicles in cell-to-cell communication[9]. A large body of evidence suggests that EVs are involved in many physiological and pathological cardiovascular processes, including the regulation of angiogenesis[10,11], blood pressure[12,13], cardiomyocyte hypertrophy[14] and apoptosis/survival[15-17] and cardiac fibrosis[18]. Given their ubiquitous presence in body fluids, EVs have been used as potential biomarkers of cardiovascular diseases[19]. Moreover, because EVs are an important component of the paracrine effect of stem cell-based therapies[20], they are candidates as a standalone therapy in the context of cardiovascular diseases. Pioneering

work from the group of Lim suggested the therapeutic potential of EVs in protecting the heart from ischemic injury[20]. Shortly after, the group of Sahoo unravelled the pro-angiogenic potential of EVs in the setting of limb ischemia[10]. Ever since, a number of pre-clinical studies have reported the advantages of EVs for cardiovascular regeneration and protection[21-24]. Yet, several challenges need to be addressed before clinical translation of these therapies including (i) the development of platforms to monitor EVs (both the membrane and the cargo) *in vivo* to determine and optimize the EV dosage regimen, the route of administration, the biodistribution, potential toxicity, immunogenicity as well tumorigenesis, (ii) the characterization of EV cargo in order to use well-defined EV formulations and (iii) the development of strategies to modify the membrane of EVs in order to improve their accumulation in specific organs and tissues. To overcome these limitations, researchers developed pre- and/or post-isolation techniques capable of modulating the intrinsic properties of native EVs and modified their surface to enhance their targeting efficiency and track them *in vivo*. Based on these studies, the field is converging on the hypothesis that the modulation of EVs by engineering approaches may unlock their clinical potential for cardiovascular applications.

In this review, we cover initially the EV biophysical properties (e.g., size, charge, membrane composition and cargo content). This will be followed by the presentation of the therapeutic potential of native EVs for cardiovascular applications. Then, we describe engineering strategies to modulate the content of EVs in order to favourably alter their bioactivity, biodistribution, delivery, targeting and intracellular trafficking. Finally, we present the studies performed using engineered EVs as cardiac therapies and discuss how this area can move forward.

## 2. EV properties

The biophysical properties of EVs are briefly described below. These properties may be modified using engineering tools, a topic that is covered in section 4. EV biogenesis is not covered in this review and readers are referred to recent reviews related to this topic[25,26].

### 2.1. EV size

EV size is one of the parameters adopted to categorize EVs[7,8]. Typically, exosomes are smaller than microvesicles and their size distribution more uniform. EV size is critical for their study and application due to most of the protocols used for isolation and characterization relying on EV density and/or diameter[27]. Moreover, EV size, precluded by their biogenic pathway, correlates with EV composition, as does the cell line of origin[28]. Finally, the efficiency in tissue biodistribution, cell internalization and intracellular trafficking of EVs are size-dependent[29-32]. Indeed, it has been shown that upon systemic administration, large and/or aggregated vesicles (>200 nm) may be trapped in the lung, liver and spleen, taken up by macrophages or unable to extravasate and interact with non-vascular cells and tissues[29,30]. Additionally, aggregation renders EVs less spherical, which may decrease their margination and extravasation from circulation[31]. For example, it has been shown that the vasculature has enhanced permeability after ischemic injury; however, only small-size particles (<200 nm) cross the endothelial barrier[33].

At a cellular level, particles with different sizes may elicit different uptake mechanisms. For example, particles with a diameter smaller than 100 nm may be taken up via clathrin- or caveolae-mediated endocytosis, while larger complexes may require macropinocytosis[34,35]. Therefore, larger aggregates are more likely to be directed towards lysosomal degradation or membrane recycling[32], while smaller vesicles may exhibit higher rates of effective intracellular delivery. In the cardiac setting, particularly for EVs administered systemically, this is of utmost importance as EVs are required to extravasate successfully to the cardiac tissue and then be efficiently taken up by the relevant cell types.

### 2.2. EV charge

Another important property of EVs is their surface charge. As part of EV membrane composition comes from the plasma membrane[25], which is rich in phosphate groups, a global negative charge is the norm for EVs, just as in cells. However, the charge is also highly dependent on the sugar composition of the plasma membrane which is highly dependent in the expression level of sialyltransferase in the endoplasmic reticulum and Golgi apparatus[36,37]. Changes in surface charge can be used to infer stability of EVs in suspension, as low absolute values are thought to be typical of EVs more prone to aggregation due to lessened repulsion.

This, however, must be balanced with the fact that closer to neutral nanoparticles are more stable in circulation, compared with highly charged ones[38]. Both EV size and surface charge are crucial in specifying the mechanism of interaction between the EV and a host of potential ligands, as well as their uptake by target cells[39]. Finally, the presence of contaminants (e.g. protein or lipid aggregates) in an EV sample may affect multiple functions and parameters. Given the heterogeneity in surface charge of these contaminants, aggregates may be formed between them and EVs. Therefore, proper purification protocols must be ensured. This, however, is aside from the scope of this review, and we direct the reader to other excellent papers on the matter[40,41].

### 2.3. EV membrane composition

Exosome membranes have higher content of cholesterol than donor cell membranes[42], making them less susceptible to the permeation of small solutes. In addition, exosome membranes contain higher levels of phosphatidylserine, glycosphingolipids and sphingomyelin and lower levels of phosphatidylcholine than the corresponding donor cells[42]. Moreover, exosomes have low protein to lipid ratios compared with, for example, microvesicles[43]. Indeed, high cholesterol and sphingolipid content makes exosomes more resistant to detergents and high temperatures than microvesicles[43-45]. Aside from their lipidic composition, exosomes are decorated with proteins and sugars which, on one hand, contribute to exosome charge and maintenance of membrane structure and, on the other hand, mediate the interactions of exosomes with the target cells[25]. For example, tetraspanins are a class of membrane proteins abundantly present, in clusters, in exosomes and some of them, namely CD63, CD9 and CD81, are considered general exosome markers. Functionally, tetraspanins are involved in membrane fusion and cellular adhesion, and, as such, play a key role in exosome internalization. Other classes of proteins, such as chemokine receptors (e.g. CXC chemokine receptor type 4)[46], adhesion molecules[47-49] and proteoglycans (e.g. heparan sulfate)[50], have been shown to play a role in mediating EV interactions with the cell surface. When these protein and sugar-based components were deleted or masked, EV internalization[51] as well as EV biodistribution was affected[52,53]. EVs have their transmembrane proteins in an identical topology to that of the secreting cell, conferring them a degree of cellular identity and possible tropism[10,54].

### 2.4. EV lumen

From late 1990s, it has been proposed that EVs act as important players in intercellular communication, particularly in the context of immune responses and cancer[55,56]. This concept was further confirmed in 2007, after the discovery that exosomes contain microRNAs (miRNAs), as well as other types of RNAs, and can transfer their content to target cells ultimately affecting their activity[9]. Recent studies using high-resolution density gradient fractionation and direct immunoaffinity analyses further dissected EV composition[57]. EVs contain different proteins and RNAs in the lumen, including long and small non-coding RNAs, transfer RNA and ribosomal RNA[28,57-59]. Exosomes do not contain DNA, although it may be present in larger EVs or present in exosome-enriched samples due to the co-precipitation with histones[57]. Proteins, on the other hand, may be sorted into EVs via post-translational modifications, such as ubiquitination[60] and glycosylation[61] and these pathways can be hijacked in order to specifically target proteins onto EVs. Upon studying the interaction networks that can be functionally established between EV proteins, the intravesicular protein content has been proposed as an organized "nanocosmos" and not cellular scraps[62].

## 3. Native EVs for cardiovascular applications

The first study describing the use of EVs as a potential therapeutic intervention for cardiovascular diseases was published in 2010[20]. In early 2000s, several groups have shown that upon MI, transplantation of different cell types, including mesenchymal stem cells[63] and hematopoietic progenitor/stem cells ($CD34^+$ cells)[64], improved heart repair. A few years later, it was shown that the positive effects of those stem/progenitor cells were mediated not by direct contribution of the engrafted cells but by paracrine factors[65,66], in particular EVs[10,20] secreted by the surviving cells (**Box 1**). Since these pioneer studies, several groups have demonstrated the regenerative properties of EVs secreted from stem/progenitor cells[67-70] as well as differentiated somatic cells[71,72] in the context of MI[69,70,72,73], ischemic limb[67], chronic wounds[71], among others. While there are several studies on cardiovascular diseases such as atherosclerosis[74] and stroke[75,76], most research with EVs has pertained to ischemic heart disease and MI.

EVs with cardiovascular efficacy have been isolated from different cell sources such as mesenchymal stem cells[77-83], cardiac progenitor cells[15,21,68-70,84-88], cells differentiated from pluripotent stem cells[20,72,89-93] and differentiated somatic cells[71,94-96]. The impact of allogenic EVs has been evaluated in mice[69,79,81,87], rats[70,78,85,86,88] and pigs[21,84], through intravenous[78,82,97] and intramyocardial[21,68-70,77,80,81,83,84,86,88] administration. The EV dosage regimen was highly variable as between 30 to 1300 μg[78,85] and 4 to 4000 μg[89,92] of EVs per kg of animal have been tested for rats (300 g was assumed as the weight of a rat) and mice (25 g was assumed for the weight of a mouse), respectively. Given the fact that EVs are less immunogenic than their cellular counterparts (with the exception of exosomes released from dendritic cells[98]), it is not surprising to see several studies where EVs isolated from human cells have been tested into non-immunosuppressed animals such as mice[89,99], rats[80,85] and pigs[21].

Box 1. **Using extracellular vesicles in cardiovascular therapeutics**

**Advantages**

The use of extracellular vesicles (EVs) in cardiac regeneration was preceded by extensive research into cell- based therapies. As non- living entities, EVs overcame a major limitation of cell- based therapies, namely the poor survival after implantation. Of note, methods that were used for cell engineering can now be effectively applied to modulate EVs. In addition, given the hostile environment (that is, inflammatory, hypoxic and pro-apoptotic conditions) of the injured myocardium, poor engraftment hinders clinical success. Although EVs must contend with the low infiltration and endocytic rates of cardiomyocytes, EVs can overcome the poor engraftment by being internalized directly by the recipient cells. Furthermore, cell- based therapies are often associated with adverse immune responses, in particular when using allogeneic cells. This effect can be ameliorated, absent or even beneficially modulated by the use of EVs, depending on their source and/or modulation strategy. The faster clearance of EVs than their cellular counterparts can be seen as a safeguarding mechanism, while effective targeting of EVs to the tissue or cell type of interest facilitates their therapeutic success.

**Limitations**

The limitations of EVs in cardiovascular therapeutics relate to the lack of tools to target the injured myocardium efficiently, which limits their clinical use. Production and standardization of analytical parameters (that is, storage, isolation and purification procedures) are some of the challenges that the field needs to overcome to translate EV therapies from the bench to the bedside. Given that the physicochemical properties of EVs are determined by their cell, tissue or fluid of origin, the heterogeneity present in the source is reflected in the composition of the EVs. Therefore, methods to reduce this EV heterogeneity before and after isolation are needed.

EV tracking studies indicate that the intramyocardial delivery of EVs yields higher EV retention in the heart than intracoronary or intravenous administration routes[21]. The pre-clinical data collected so far indicate that EVs, regardless of their origin, may improve the ejection fraction (EF) (up to 1.3-fold increase[15,77,86,88] relatively to non-treated group) and reduce infarct size (up to 3-fold[79,81,84,85] decrease relatively to non-treated group). The therapeutic effect of EVs and donor cells has been evaluated/discussed and the results collected until now indicate that EVs are as effective as the donor cells in the context of MI[21,80].

The therapeutic role of EVs in acceptor cells has been mostly ascribed to the delivery of proteins and/or non-coding RNAs, in particular miRNAs. For example, the cardiovascular

protective role of exosomes has been attributed to miRNA-19a-3p[100], miRNA-21[79,90], miRNA-24[16], miRNA-22[81], miRNA-29a[18], miRNA-143[96], miRNA-146[69], miRNA-181b[84], miRNA-210[90], miRNA-222[96], miRNA-294-3p[93], mir-126[67], among others. Some of the miRNAs have been identified in previous studies of cell-based therapies (e.g. miRNA-19, miRNA-21, miRNA-24, miRNA-210)[101] to be relevant for cardiovascular repair while others are novel. Another important component of EVs, also associated with their bioactivity, are proteins such as platelet-derived growth factor D[78] and pregnancy-associated plasma protein-A[15]. Some of these proteins are at the EV surface and therefore, do not need to be delivered to the cytoplasm of the acceptor cell. For example, pregnancy-associated plasma protein A (also known as pappalysin-1) is a protein highly expressed in exosomes of cardiac-resident progenitor cells[15]. It has been shown that this protein mediates the cardioprotection and angiogenesis of cardiac-resident progenitor cell-derived EVs by cleaving the insulin-like growth factor binding protein-4 in insulin-like growth factor-1 which, in turn, activates the insulin growth factor receptor, ultimately leading to the phosphorylation of Akt and ERK1/2 and subsequently to decreased caspase activation and reduced cardiomyocyte apoptosis. In other cases, the proteins are in the lumen of EVs and need to reach the acceptor´s cell cytoplasm to elicit a biological affect. For example, EVs secreted by

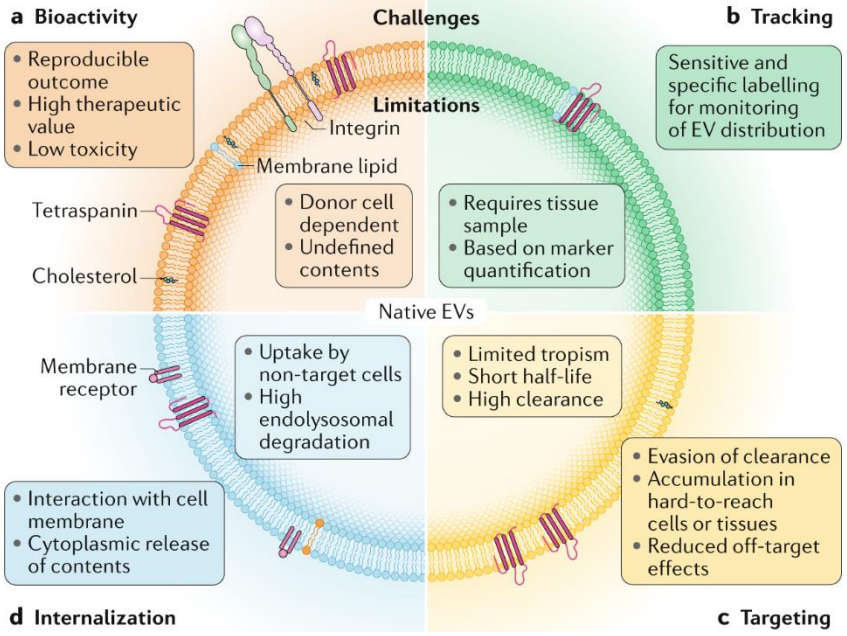


**Figure 1. Limitations and challenges in the use of native EVs as cardiovascular therapies**. While EVs have been shown to elicit functional responses on target cells, their potential is limited in several regards, including bioactivity, tracking, internalization and targeting. Therefore, several challenges should be addressed to harness the full potential of EVs. (a) **Bioactivity**. EVs must elicit a bioactive response in their target tissue/organ, which requires that EV cocktail of factors is potent enough to have therapeutic value. Yet, native EVs are limited by the nature of their content being intrinsically dependent on their donor cell features. (b) **Tracking**. The tracking of EVs is necessary to monitor their in vivo biodistribution and targeting. Yet, without modifications, EVs are only detectable after a biopsy is removed, limiting their monitorization. (c) **Targeting**. EVs should interact with specific cells and deliver their content. Although native EVs have some tropism, depending on the epitopes expressed by their donor cell, the process is limited. (d) **Internalization**. EVs should be taken up by target cells and deliver their cargo in the cell cytoplasm. Yet, native EVs have reduced endolysosomal escape and thus the ability to release their content in the cell cytoplasm.

stem/progenitor cells in mice under systemic inflammation conditions contain integrin-linked kinase that activate the NF-kB pathway[102]. The knockdown of integrin-linked kinase in inflamed exosomes attenuated their inflammatory response and enhanced the endothelial progenitor cell-derived exosome therapeutic activity in ischemic heart.

The mechanisms triggered by EVs depend on the source of EVs (as well as their content) and include: (i) an improvement in cardiomyocyte and endothelial cell survival[21,89,90,95,103] by the regulation of autophagy[104], activation of pro-survival signalling pathways (e.g. Akt, ERK, toll-like receptors)[95,105] and a decrease in oxidative stress[90], (ii) modulation of the inflammatory response[85,106] by influencing immune cell polarization (i.e. inducing a more reparative state rather than an inflammatory state)[84] and cytokine secretion[85] as well as increasing the activation of CD4-postive T cells[94] (iii) a decrease in scar content[21] and (iv) enhancement of angiogenesis[107,108]. For example, cardiosphere-derived EVs improved heart function in a mouse model of MI via miR-146, decreasing apoptosis and inflammatory response and increasing cardiomyocyte proliferation and angiogenesis[69]. It has also been shown that extracellular matrix-derived EVs carry miR-199a-3p, which, by regulating GATA-binding 4 acetylation, were able to rescue electric function in engineered and *in vivo* atria[109].

The above-mentioned studies underscore the potential of native EVs for cardiovascular therapy. However, their clinical potential has not been met yet and important limitations must be overcome before their establishment as an effective therapeutic tool (**Fig. 1**). These limitations may be surpassed by enhancing native EV using bioengineering approaches, as detailed in the following sections.

## 4. Engineering the EVs

Despite the therapeutic efficacy of native EVs to treat cardiovascular diseases, in recent years, technologies have been developed to modulate EV and thus to enhance their bioactivity, stability, targeting and presentation (by the development of EV-delivery systems) to the cardiovascular system (**Fig. 2**). In the subsequent sections, we explore how EVs may be enhanced or modified and used for the treatment of cardiovascular diseases.

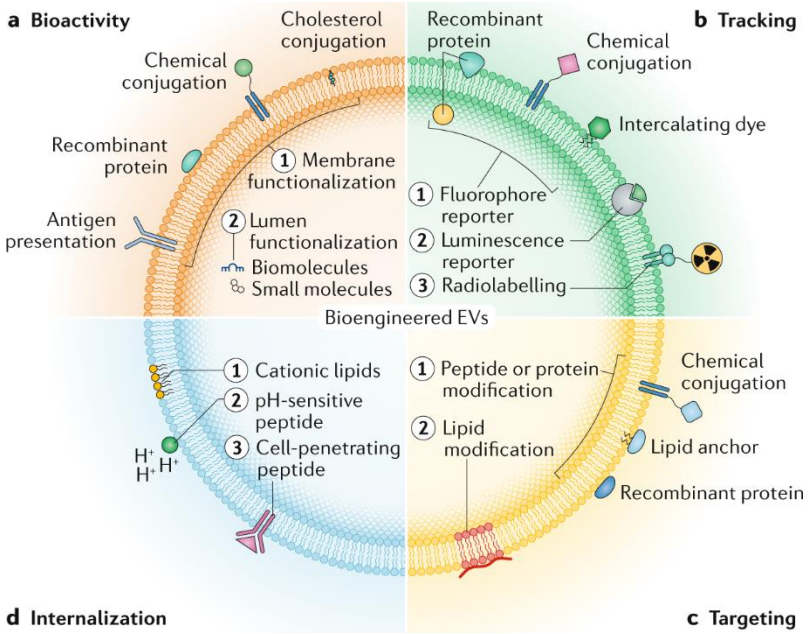


**Figure 2. Modulation of EVs for cardiovascular therapies.** Several strategies have been used to overcome limitations inherent to native EVs. These modifications can be categorized, from a technical standpoint, depending on whether they were performed on the donor cell prior to EV secretion, or after the purification of EVs from the medium or fluid of origin. From a biological standpoint, these modifications may pertain to the membrane or lumen of the EVs. In the cardiovascular context, several approaches have been used to modulate EV bioactivity, cell internalization and targeting as well as to allow EV tracking. (a) **Bioactivity.** To enhance the therapeutic potential of EVs, functionalization of the membrane and the lumen have been used. (b) **Tracking**. To track in vivo EVs, these formulations have been labelled with fluorophores, luminescence reporters or radiotracers. (c) **Targeting**. To enhance the targeting efficacy of EVs, they have been modified with exogenous peptides, proteins or lipids. (d) **Internalization**. To enhance EV internalization and endolysosomal escape, the vesicles have been modified with cationic lipids, pH-sensitive peptides and cell-penetrating peptides.

### 4.1. Tracking the EVs

Methods to track EVs *in vivo* and follow their biodistribution are very important to fully evaluate their cardiovascular therapeutic potential. Fluorescence[47,54,71,110,111], luminescence[112], positron-emission tomography (PET)/magnetic resonance imaging (MRI)[113] and single-photon emission computed tomography (SPECT)[114,115] imaging techniques have been used to monitor *in vivo* EVs. In most of the cases, the EVs were isolated and modified with chemical ligands[113,114]. In few cases, the EV-secreting cells were genetically modified to express a reporter. For example, human embryonic kidney 293T cells were engineered to express in their membrane a Gaussian luciferase fused to a biotin receptor domain[112]. The EVs could be monitored either *in vitro* or *in vivo* by luminescence or fluorescence (by the interaction with fluorescent streptavidin). Although fluorescence and luminescence imaging techniques are easy to operate and available in most laboratories, they do not offer high sensitivity and absolute quantification. In contrast, the methods that rely in PET/MRI or

SPCET/computed tomography offer higher sensitivity and absolute quantification while allowing the acquisition of images with anatomical details. In general, the intravenous administration of labelled EVs (without any further modification besides the labelling) isolated from different cell sources indicate that less than 10% of the injected EVs accumulate into the non-injured heart[54,112-115]. Yet, the accumulation of EVs in the heart is influenced by the delivery route, concentration of EVs and the identity of the EV-secreting cell[54,112-114].

### 4.2. Modulation of EVs bioactivity

#### 4.2.1. EV-secreting cell modulation

EV-secreting cells may be modulated by two different procedures: (i) culturing them in stress-induced conditions (e.g. hypoxia[80,88,96], serum starvation[71,96], inflammation[116]) and (ii) modulating them with exogenous compounds, such as nucleic acids, especially miRNAs[23,117,118], miRNA antagonists[119], Y-RNA[85], plasmid DNA[77,78,120,121] and small molecules[121] to enhance their bioactivity (**Table 1**). For example, EVs collected from cardiac progenitor cells cultured under hypoxia conditions increased the capacity of cardiac endothelial cells to form tube-like structures *in vitro*, reduced the expression of pro-fibrotic genes in cardiac fibroblasts cultured *in vitro* and improved function of the infarcted heart (in the acute phase, increased the fractional shortening from 30.6% to 36.4% and in the chronic phase increased from 27.6% to 34.2%) as compared to EVs collected from cells cultured under normoxia conditions[88]. The effect was mediated by several miRNAs including miRNA-292, miRNA-210, miRNA-103, miRNA-17, miRNA-199a, miRNA-20a and miRNA-15b[88]. The modulation of EV-secreting cells might also be achieved by changing their culture medium. For example, EVs collected from adipose-derived stem cells (ADSC) cultured in endothelial differentiation medium showed an increase in miRNA-31 and the resulting EVs enhanced endothelial cell migration, tube formation and aortic ring outgrowth compared to EVs collected from ADSC grown in normal medium and thus not-enriched for miRNA-31[122]. Finally, as an example of EVs collected from cells that were modulated by external agents, EVs collected from mesenchymal stem cells transfected with miRNA-181a increased the pro-reparative state of peripheral blood mononuclear cells and, upon administration in infarcted mice hearts, the miR-181a enriched EVs increased EF (12% relatively to the baseline)[23].

Table 1. **Modulation of EV bioactivity**

| EV Source | EV type | Method | Animal model | *In vivo* outcome | Ref. |
|---|---|---|---|---|---|
| ***Modulation of EVs bioactivity: EV-secreting cell modulation*** | | | | | |
| Human amniotic fluid stem cells | sEVs | Hypoxic preconditioning | Mouse MI | Improved cardiac repair | [127] |
| Human bone marrow mesenchymal stem cells | sEVs and MVs | Hypoxic preconditioning | Rat MI | Improved angiogenesis and cardiac function and reduced infarct size | [80] |
| Rat cardiac progenitor cells | sEVs | Hypoxic preconditioning | Rat MI | Improved cardiac function and reduced infarct size | [88] |
| Rat cardiomyocytes | sEVs | Hypoxic preconditioning | Mouse MI | Improved angiogenesis | [96] |
| Human mononuclear cells | sEVs | Hypoxic preconditioning | Mouse diabetic wounds | Improved angiogenesis and healing | [71] |
| Human adipose-derived stem cells | MVs | Endothelial medium conditioning | Mouse subcutaneous Matrigel plug | Improved angiogenesis | [122] |
| Rat mesenchymal stem cells | sEVs | GATA4 overexpression | Rat MI | Restored cardiac contractile function and reduced infarct size | [77] |
| Human umbilical cord mesenchymal stem cells | sEVs | Akt overexpression | Chick allantoic membrane | Improved angiogenesis | [78] |
| Human mesenchymal stromal cells | sEVs | Fluorescent miR-181a overexpression | Mouse MI | Reduced infarct size, improved function, reduced inflammation | [23] |
| HEK 293T cells | EVs | miRNA-146a overexpression | NA | NA | [117] |
| Human cardiosphere-derived cells | sEVs | Y RNA overexpression | Rat MI | Reduced infarct size and CM apoptosis, reduced inflammation | [85] |
| ***Modulation of EVs bioactivity: post-isolation methods*** | | | | | |
| Human blood | sEVs | EV loading with microRNA: ExoFect | Mouse MI | Reduced fibrosis | [128] |
| Mouse cardiac progenitor cells | sEVs | EV loading with microRNA: Electroporation | Mouse MI | Improved angiogenesis | [129] |
| Human umbilical mesenchymal stem cells | sEVs | EV loading with microRNA: Electroporation | Rat MI | Reduced fibrosis and improved cardiac function | [118] |

EV, extracellular vesicle; HEK, human embryonic kidney; MI, myocardial infarction; MV, microvesicle; NA, not applicable; sEV, small extracellular vesicle.

Several platforms have been developed for customizable enrichment of EVs with specific proteins[123] and RNAs[124,125] of interest; however, most of these platforms were not yet evaluated for cardiovascular applications. One exception, however, showed that using a genetic approach, it is possible to take advantage of the cellular machinery to engineer EVs with the ability to be customized for the presentation of specific epitopes, which may be specifically targeted to the heart[126].

### 4.2.2. Post-isolation methods

Hijacking the cellular machinery to produce modulated EVs has the advantage of preserving the biophysical properties of EVs relatively intact, but also has drawbacks, namely overexpressing a given molecule in a cell may have unforeseen consequences for its biology, ultimately interfering with EV biogenesis. Modulation strategies based on post-isolation modification of EVs may be an alternative for effective control of EV loading, targeting, and

delivery, regardless of their cell of origin. However, post-isolation methods may mask or impair endogenous EV properties and ultimately compromise EV bioactivity.

Membrane-permeabilizing strategies, such as electroporation (for both nucleic acids[130-132] and drugs[133]), heat-shock or freeze-thaw procedures[134,135], detergent treatment[133] and sonication[136], developed over the past decades to load cells with exogenous material, have been readily applied to the EV field with a varied degree of success. Additional strategies exploited the hydrophobicity of EV membranes in order to passively load compounds of interest into them[137,138] and the modification of the molecule of interest with cholesterol[139,140]. Recent work focused on the use of well-defined chemical formulations, some of which commercially available, designed with the specific purpose of transfecting EVs directly[141-143]. Thus far, these studies reported loading efficiencies up to 70% but the impact on the biophysical properties of EVs and the exact mechanism of action remains to be elucidated.

Several EV formulations have been enriched using transfection agents or membrane-permeabilizing strategies and evaluated in the context of cardiovascular applications to decrease cardiac fibrosis, modulate the inflammatory response and to increase angiogenesis (**Table 1**). For example, miRNA-21-5p has a critical role in the development of fibrosis after MI, regulating several gene targets including SMAD family member 7 (Smad7), sprout RTK signalling antagonist 1 and phosphatase and tensin homolog (PTEN)[128]. Human peripheral blood-derived EVs enriched for miR-21 inhibitors reduced fibrosis in a mouse model of MI as compared to non-modified EVs. In a separate example, EVs collected from cardiosphere-derived cells and enriched for miRNA-322 using electroporation, reduced the infarcted area and fibrosis, and increased angiogenesis in a mouse model of MI as compared to non-modified EVs[129]. Together, these studies demonstrate the possibility to enrich EVs after their isolation and thus increase their bioactivity as compared to the non-modified EVs.

### 4.3. Modulation of EV biodistribution and targeting

Upon systemic administration of EVs in animal models, they are quickly cleared or trapped in the liver, spleen and lungs[137], with the EV half-life (minutes range), inherently dependent on the identity of the EV-secreting cell[144-146]. EV biodistribution is influenced by multiple factors,

including the delivery route and dosage[54]. It is possible that EVs retained in several organs induce a systemic anti-inflammatory effect improving the regeneration of the cardiovascular system, as already observed with cell-based therapies[4]. However, studies clearly indicate that increased EV efficacy is related with EV retention at the lesion area[21]. Therefore, in the last years, several strategies have been developed to control EV biodistribution and targeting to specific organs and tissues. One strategy to maximize the uptake is to increase EV stability in circulation and therefore, improve the likelihood of an interaction between the EVs and the cells/tissues to be targeted. Modifying EVs with polyethylene glycol (PEG), a strategy previously used for liposomes[147], enhanced their circulation time[45] and reduced their uptake by nonspecific cells. Another strategy relies on the modification of EV membrane with specific proteins[126] or peptides[22,110,111,130,148-152] that are able to interact with specific cellular receptors or extracellular matrix components expressed in the cardiovascular system (**Table 2**). Although it is known that tetraspanins present in EV membranes have preferential binding to specific cell lineages (e.g. Tspn8 binds to α4 and β4 integrin chains that are expressed by endothelial cells[153]) these might not be enough for selective and effective organ or tissue targeting.

Table 2. **Modulation of EV biodistribution and targeting**

| EV Source | Targeting peptide or construct | Model | Outcome | Ref. |
|---|---|---|---|---|
| ***Chemical modification of the EV membrane with peptides or proteins*** | | | | |
| Mouse mesenchymal stem cells | RGD | Mouse middle cerebral artery occlusion | Suppression of the inflammatory response and cellular apoptosis in the lesion region | 110 |
| Mouse mesenchymal stem cells | CSTSMLKAC | Mouse MI | Reduction of fibrosis and CM apoptosis and improvement of cardiac function | 22 |
| Human cardiosphere-derived cells | CSTSMLKAC | Rat MI | Reduction of fibrosis and CM apoptosis and improvement of cardiac function | 148 |
| Human cardiosphere-derived cells | CSTSMLKAC | Rat MI | Improved targeting to the heart | 126 |
| ***Genetic or chemical modification of the EV-secreting cell to express a peptide or protein*** | | | | |
| Mouse mesenchymal stem cells | LAMP2B–CSTSMLKAC | Mouse MI | Suppression of the inflammatory response, improved angiogenesis, reduction of myocardial apoptosis, improvement of cardiac function | 149 |
| Human cardiosphere-derived cells | LAMP2B–WLSEAGPVVTVRALRGTGSW | Mouse MI | Improved targeting to the heart | 151 |
| HEK 293T cells | LAMP2B–APWHLSSQYSRT | Mouse MI | Improved targeting to the heart | 150 |
| Human K562 cells | RGD | Vascular targeting in zebrafish | Improved targeting to the vasculature | 152 |

EV, extracellular vesicle; HEK, human embryonic kidney; LAMP2B, lysosome-associated membrane glycoprotein 2B; MI, myocardial infarction.

In the case of heart diseases, the intramyocardial administration of EVs has been reported in pre-clinical studies; however, this route of administration is not always clinically desirable because it involves a catheterization procedure, unless delivery is planned during cardiac surgeries[154]. The intravenous administration of EVs is a much simpler procedure and allows repeated applications; however, it is more prone to off-targeted binding, increasing the potential for unwanted effects. Moreover, the poor accumulation of EVs into the cardiac tissue (due to poor extravasation and lack of efficient and specific epitopes for recognition) remains an important barrier. Engineered EVs can eventually overcome these obstacles and deliver their therapeutic cargo into the injured heart. Two approaches have been used to modify the surface of EVs for targeting the heart. In one, the EV-secreting cell is genetically modified to express a peptide which is then incorporated in the membrane of the secreted EVs[149-151]. For example, EV-secreting cells were genetically modified with a lentivirus construct expressing a membrane protein (Lamp2b) fused with ischemic myocardium-targeting peptide CSTSMLKAC[149]. Although no absolute quantification was provided for the accumulation of EVs in the heart, the fluorescence imaging results indicate a higher accumulation of the peptide-modified EVs relatively to EVs without surface modification. As an alternative, many laboratories adopted the surface modification of EVs by chemical approaches[22,110], mainly by following two strategies: (i) physical incorporation of lipids modified with proteins (e.g. streptavidin)[126] or peptides[148] into the membrane of the EVs and (ii) chemical incorporation of linkers to functional groups (carboxylic[111] or amine[22,110] groups) present at the surface of EVs to which peptides are subsequently reacted by a copper-free click chemistry. These reactions can be performed in aqueous solutions, are rapid, selective and very efficient as compared to conventional bioconjugation schemes. In both strategies, the epitopes selected for targeting ischemic regions or a given cell type of interest were: (i) a cyclic RGD peptide with high affinity to integrin αvβ3 highly expressed in brain endothelial cells after an ischemic event[110], (ii) an ischemia-targeting peptide[22,126,148,149] and (iii) a cardiomyocyte-specific peptide[150]. According to one study, introduction of approximately 263 copies of the targeting peptide per exosome has been achieved[110]. Although in many reports, no absolute quantification of EV accumulation was performed, the results showed that the peptide-modifications can increase the EVs tropism to ischemic regions, including the brain[110] or the heart[22,148-151], and induce a higher therapeutic effect[22,148]. For example, the EF of mice

hearts after MI, treated with EVs modified with a myocardium-targeting peptide was approximately 46% while in mice treated with scramble peptide-modified EVs was approximately 38%[22]. Similar improvements were reported by others[148,149]. Both genetic modification of the EV-secreting cell and surface modification of EVs by chemical approaches have pros and cons. The genetic approach may allow for a more standardized product which is desirable to address regulatory expectations. However, this strategy has several limitations including (i) changes in the biological activity of the EV as a consequence of the genetic manipulation and (ii) difficulty to control the density of the targeting epitope in the surface of EVs as well as to control their glycosylation state. The chemical approach may offer an effective control of EV surface modification both in content (e.g. to include non-natural amino acids to prevent peptide degradation) and density (number of epitopes per surface area of EV) of the targeting epitope, regardless of their cell of origin. The chemical approach may be performed during the purification steps of EVs and thus amenable for clinical translation.

### 4.4. Modulation of EV uptake and intracellular trafficking

The internalization and intracellular trafficking of EVs can be studied using fluorescence imaging techniques and labelled EVs[155,156]. The cellular internalization of EVs seems to be influenced by both the interaction between EVs and the cell membrane[26,156] and the endocytic capacity of the acceptor cell[71,157]. Internalization of EVs may be mediated either by non-specific interactions, particularly endocytic processes such as macropinocytosis and micropinocytosis[51], or by specific interactions, such as receptor-dependent pathways (in the case of peptide-modified EVs, see section above). Little is known about the differences in the endocytic capacity of the cells, the impact of those EV surface modifications in the intracellular trafficking and which EV surface modifications may improve endolysosomal escape. Yet, these issues are critical because a large proportion of internalized EVs are processed in the endolysosomal pathway and ultimately degraded in the lysosome[51,158]. Indeed, approximately 60% of internalized EVs colocalize with lysosomes after 48 h of contact[156].

Strategies have been proposed to enhance the endolysosomal escape of EVs. In one case, EVs have been coated with a combination of cationic lipids and pH-sensitive fusogenic peptides

which enhanced the disruption of the endolysosomal membrane leading to the efficient cytosolic release of the EV cargo[159]. In another case, EVs have been coated with arginine-rich cell-penetrating peptides to induce active micropinocytosis and a more efficient release of EVs to the cell cytoplasm[160,161].

### 4.5. Modulation of EV delivery

Local administration of EVs at the injured site has been shown to increase the chance of cell targeting and uptake by the cells of interest. In some cases, EVs are washed out or taken up by non-relevant neighbouring cells. Interestingly, differential uptake of EVs by different cell types in an ischemic hind limb has been reported[67]. Local accumulation of EVs after few hours following intramyocardial, intramuscular or topical administration in an infarcted pig heart[21], an ischemic limb tissue[67] or in a mouse would healing model[71], respectively, has also been demonstrated. Several biomaterial-based strategies have been developed to engineer EV presentation by sustained the release of EVs in the injured site including hydrogels based in hyaluronic acid[71,162,163], alginate[164,165], chitosan[166,167], collagen[72] and amphiphilic peptides[168] (**Table 3**). Selection of hydrogel composition took in consideration its biomedical history, degradation, in situ jellification profile, mechanical and release properties. The EVs were incorporated in the hydrogels by several means. In the first case, EVs were mixed with a polymer solution without involving the reaction of both entities[72,162,163,165,167,168]. The solution was then injected in the tissue of interest and physically or chemically crosslinked in a few minutes retaining the EVs within the polymeric structure. In the second case, the EVs were mixed with a polymer solution for formation of a polymer-EV conjugate and initiate the chemical crosslinking process[71]. Then, the solution was administered in the tissue of interest for further crosslinking and formation of a hydrogel. In the third case, the EVs were physically incorporated in the hydrogel after its polymerization[169]. In this case the hydrogel was already formed and the pores were large enough to allow the diffusion of EVs within the polymeric structure. It has been demonstrated that a 7 mm hydrogel patch could retain up to $3\times10^{10}$ EVs and released the EVs for more than 7 days after implantation in the heart[72]. The hydrogel-releasing EVs significantly improved the activity of injured tissues relatively to EVs administered without a sustained release system. For example, in two similar studies, the EF of infarcted rat hearts treated with a hydrogel patch containing EVs was 40%[72] and 25% higher

than control[162]. Importantly, the kinetics of EV release from the hydrogel seems to play an important role in their therapeutic effect[71]. For example, the slow release of EVs from hydrogels implanted in skin mouse wounds was not as effective as the coordinated release of EVs during skin regenerative process using remotely triggerable hydrogels[71].

Table 3. **Modulation of EV delivery**

| EV Source | Method | Model | Outcome | Ref. |
|---|---|---|---|---|
| Rat endothelial progenitor cells | Injectable hyaluronic acid-based hydrogel | Rat MI | Improvement in angiogenesis and cardiac function, decrease in infarct size | [110] |
| Rat endothelial progenitor cells | Injectable hyaluronic acid-based hydrogel | Rat MI | Improvement in haemodynamics, cardiac function and decrease in infarct size | [163] |
| Human induced pluripotent stem cell-derived cardiomyocytes | Encapsulation of EVs in collagen gel followed by its transplantation | Rat MI | Improvement in cardiac function, decrease in CM apoptosis, reduction in infarct size and cell hypertrophy | [72] |
| Human mesenchymal stem cells | Injectable peptide amphiphile hydrogel | Rat MI | Improvement in cardiac function, reducing inflammation, fibrosis and apoptosis, enhancement of angiogenesis | [168] |
| Rat mesenchymal stem cells | Injectable alginate gel | Rat MI | Decrease in cardiac cell apoptosis, improvement in angiogenesis, improved cardiac function | [165] |
| Human platelet-rich plasma | Encapsulation of EVs in alginate gel followed by its transplantation | Skin wound healing diabetic rat model | Improved angiogenesis and re-epithelization | [164] |
| Human mesenchymal stem cells | Encapsulation of EVs in chitosan gel followed by its transplantation | Skin wound healing diabetic rat model | Improved angiogenesis and re-epithelization | [166] |
| Human mononuclear cells | Injectable hyaluronic acid-based hydrogel | Skin wound healing diabetic mouse model | Improved angiogenesis and re-epithelization | [71] |
| Human mesenchymal stem cells | Injectable chitosan-based gel | Mouse hindlimb ischemia model | Increased angiogenesis in ischemic hindlimbs and high limb salvage | [167] |

EV, extracellular vesicle; MI, myocardial infarction

## 5. Conclusions and perspectives

In the last decade, significant progress was made in understanding the biology of EVs as well as their application in the cardiovascular arena. We now have a better understanding of the composition of EVs[28,57], the role of EVs in the communication between cells of the same or different tissues in the body[14,17,47,96] and how the content of EVs secreted by stem/progenitor cells or other cardiac populations is affected by disease[17,102]. Regarding EV applicability, substantial advances have been made in the: (i) therapeutic effect of EVs in pre-clinical models of several cardiovascular diseases such as MI[21,84], skin wound healing[71,166], hindlimb ischemia[67] and stroke[110], (ii) how the therapeutic effect of EVs compares to the one obtained from the transplantation of stem/progenitor cells[80,162] and (iii) how to enhance the therapeutic effect of EVs by increasing the stability and targeting to a specific location, by

enriching their therapeutic content, by improving their internalization and intracellular trafficking and controlling their spatial and temporal release from within biomaterials (**Fig. 3**).

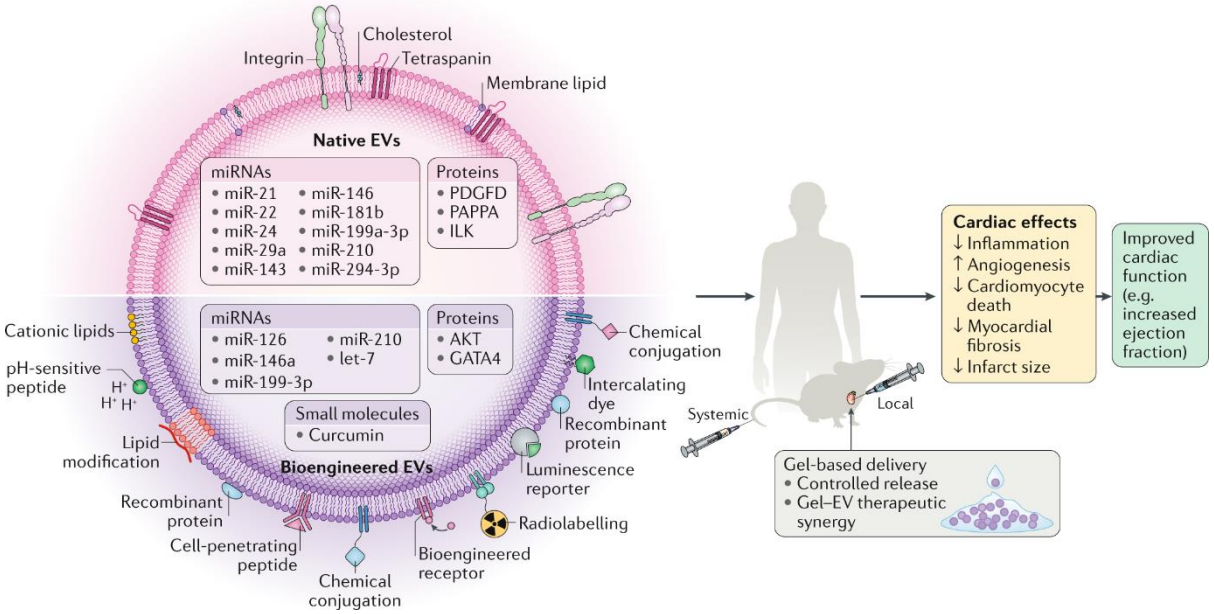


**Figure 3. Both native and modulated extracellular vesicles (EVs) have been used as cardiac therapies**. The modulated EVs have been enriched with therapeutically relevant compounds, such as microRNAs (miRs), proteins and small molecules, and have also been decorated with surface epitopes to improve their cardiac targeting and pharmacokinetics. EVs have been administered in animal models either locally or systemically. With local administration, biomaterial-based delivery systems have been used to control the release of EVs and to provide a supporting scaffold for tissue regeneration. Therapies involving native or modulated EVs have resulted in improved cardiac function (measured, for example, as an increase in left ventricular ejection fraction), mediated by a decrease in inflammation, cardiomyocyte death, fibrosis and infarct size, and an increase in angiogenesis. AKT, RACα serine/threonine-protein kinase; GATA4, transcription factor GATA4; ILK, integrin-linked protein kinase; PAPPA, pappalysin 1; PDGFD, platelet-derived growth factor D.

As consequence of these progresses, there are 2 observational and 2 interventional clinical trials actively running. In the observational clinical studies, changes in epicardial fat-contained EVs in patients with atrial fibrillation[170] and the miRNA expression profile in peripheral blood exosomes of patients with MI[171] will be investigated. In the interventional clinical studies, the therapeutic effect of exosomes on cutaneous wound healing[172] and in patients with acute ischemic stroke[173] will be evaluated.

Further translation of EVs as potential therapies will require further advances in the implementation of good practices related to EV separation and characterization, which implies the use of guidelines to track and organize data on EV separation and characterization, reference materials for normalization or calibration and inter-laboratory validation and reproducibility studies[174]. Some of these guidelines have been introduced in the last years by the scientific community[7,175]. In addition, further technological progresses will be needed to overcome the challenges related to the purification and characterization of EVs[28,57]. For

further clinical translation of EVs in the cardiovascular area, standard EV sources are needed to obtain EVs for therapeutic efficacy. EVs may be harvested from autologous (i.e. donor biologic fluids or harvested cells) or exogenous sources (i.e. allogeneic biological fluids or cell lines). Autologous EVs have the advantage of immuno-compatibility; however, these EVs cannot be harvested on demand, may have reduced/unpredictable bioactivity based on, for example, existing comorbidities and/or age of the donor and are much harder to standardize as a clinical product. EVs produced from an exogenous source have the advantage of being easier to standardize and store in larger quantities. Additionally, they may be standardized for any given application and modified either by pre- or post-isolation modifications. However, for clinical translation, further progresses are needed to optimize the administration route and the dosage regimen. A clinical study in a human has shown that $10^{10}$-$10^{11}$ EVs was an effective therapeutic dosage to treat one patient with graft-versus-host disease, with multiple administrations of increasing amounts[176]. This dose was estimated based on therapeutic dosages of transplanted MSCs for similar purposes (~$10^{7}$-$10^{8}$ MSCs); however, other studies indicate that clinical application of EVs may require > $10^{14}$ particles per dose[177]. High concentrations of EVs will require the use of bioreactors for cell culture. For example, the culture of $10^{8}$ MSCs for 2 weeks can generate approximately $10^{12}$ EV particles[178]. In this context, the use of engineering approaches may, however, decrease the amount of EVs required for the development of a therapeutic dose.

Overall, targeting technologies that increase EV accumulation in the cardiovascular system and thus decreasing the required dosage may be the key to unlocking their use in the clinical setting. This may be ensured by coupling tracking technologies, in order to mechanistically understand the biodistribution of EVs. Moreover, loading of EVs with exogenous molecules and controlling their *in vivo* delivery kinetics opens a number of opportunities to enhance EV bioactivity. Ultimately, engineered EVs represent a promising translational cell-free, robust and customizable platform to improve the outcomes in cardiovascular diseases.

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## 7. Author contributions

All authors contributed equally to all aspects of the article (researching data for article, substantial contribution to discussion of content, writing, review/editing of manuscript before submission).

## 8. Conflicts of Interest

The authors declare no competing interests.

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# CHAPTER III - Regulation of microvascularization in heart failure - an endothelial cell, non-coding RNAs and exosome liaison

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## ABSTRACT

Heart failure is a complex syndrome involving various pathophysiological processes. An increasing body of evidence shows that the myocardial microvasculature is essential for the homeostasis state and that a decompensated heart is associated with microvascular dysfunction as a result of impaired endothelial angiogenic capacity. The intercellular communication between endothelial cells and cardiomyocytes through various signaling molecules, such as vascular endothelial growth factor, nitric oxide, and non-coding RNAs is an important determinant of cardiac microvascular function. Non-coding RNAs are transported from endothelial cells to cardiomyocytes, and *vice versa*, regulating microvascular properties and angiogenic processes. Small-exocytosed vesicles, called exosomes, which are secreted by both cell types, can mediate this intercellular communication. The purpose of this review is to highlight the contribution of the microvasculature to proper heart function maintenance by focusing on the interaction between cardiac endothelial cells and myocytes with a specific emphasis on non-coding RNAs (ncRNAs) in this form of cell-to-cell communication. Finally, the potential of ncRNAs as targets for angiogenesis therapy will also be discussed.

## 1. Introduction

Heart failure (HF) is a final state of various cardiovascular conditions where the heart is no longer capable of supplying sufficient blood to support the physiological demand of the body[1,2]. Despite currently available therapies, HF remains a chronic condition with high morbidity and mortality rates. Since the pathogenesis of the disease is complex, there is a dire need for a comprehensive and thorough understanding of the pathophysiological processes that lead to the onset and progression of HF in order to improve or develop novel therapeutic options.

Pathological cardiac remodelling is marked by hypertrophic growth of the heart, formation of fibrotic tissue, infiltration of inflammatory cells, and reduced myocardial capillary number. HF is associated with vascular structural remodelling, which leads to disturbed blood flow and heart muscle tissue perfusion[3,4]. Reduction in capillary density, or capillary rarefaction, occurs in the heart of patients with HF[5,6], and is determined by the rate of angiogenesis in the heart where endothelial cells (ECs) play a significant role[7,8]. Despite the conflicting results from past and ongoing clinical trials, pre-clinical *in vivo* studies show clear improved cardiac function after neovascularization by enhancing capillary density with pro-angiogenic compounds[9-11]. One of the possible reasons for this discrepancy is the common use of single pro-angiogenic agents, mostly considered insufficient to boost angiogenesis in the heart[12,13].

MicroRNAs (miRNAs) are short non-coding RNAs, 21-23 nucleotide-long, that function by repressing the expression of their target genes[13,14]. miRNAs are able to bind complementarily to the 3' untranslated region (UTR) of more than one hundred target messenger RNAs (mRNAs)[15,16] involved in regulating common or diverse networks or pathways, allowing the occurrence of a synergistic effect. In contrast, long ncRNAs (lncRNAs) are more than 200 nucleotides in size and exert multiple functions, including functioning as scaffold for transcription factors, or acting as molecular sponges[17]. ncRNAs are established regulators of cardiac capillary formation[18-20] and their endothelial expression is influenced by various factors released by the adjacent cell types, forming an intercellular communication network. Exosomes have emerged as an essential intercellular communication tool among different cell types in the heart as they are able to carry various biological information, including proteins, lipids, and ncRNAs, from donor cells and affect the behaviour of the recipient

cells[21,22]. In this review, we discuss the contribution of microvascular remodelling in the development of HF. We outline the role of ECs and the significance of ncRNAs in the regulation of heart microvasculature. Moreover, we discuss the role of exosomes as an intercellular communication tool affecting endothelial angiogenic capacity. Finally, we discuss angiogenic ncRNAs as potential targets for neovascularization therapy to ameliorate HF.

## 2. Development of heart vascularization

ECs, among other cell types, are a major determinant of the homeostasis of the heart. They line the interior of myocardial capillaries, forming an endothelial contour, which serves as an anatomical and functional boundary between blood and the surrounding tissues. The formation of blood vessels starts with the formation of a linear heart tube consisting of several layers of cardiomyocytes (CMs) adhering to the EC layer, which develops at embryonic day (E.) 8 in the mouse and E.17-.19 in human. At this point, the heart is avascular and receives nutrients and oxygen supply from its surroundings through diffusion[8]. As the CM layer grows, the diffusion process can no longer maintain nutrient demand, and soon after the heart starts to contract, the primitive vascular plexus forms[23]. The plexus originates from angiogenic precursor cells coming from the pro-epicardial organ and sinus venosus, which will differentiate into ECs and form a primeval capillary network[8,23]. This early phase of blood vessel formation where blood vessels develop from non-existing vessels is termed vasculogenesis. The vascular plexus expands through EC sprouting from the pre-existing capillaries in a process known as angiogenesis, ultimately developing into an organized network of smaller and larger vessels. At E.13 in the mouse (E.42 in human) this network connects with the aorta, followed by colonization of smooth muscle cells and fibroblasts to form the media and adventitial layer in a process called arteriogenesis[8,24]. Myocardial thickness increases approximately fourfold during postnatal life due to CM proliferation up to day 7[25], and hypertrophic growth. The increased metabolic demand of proliferating and hypertrophying CMs is met by the expansion of myocardial capillary density three- to fourfold during the first 3 weeks of postnatal life[8]. Cardiac capillaries and CM growth are proportional to an increase in cardiac mass[26], suggesting that any alterations in these processes may result in myocardial hypoxia and/or ischemia leading to pathological remodelling and eventually HF.

## 3. Capillary rarefaction in the failing heart

Myocardial growth and angiogenesis are adaptive responses of the heart to an increase in hemodynamic demand. An upregulation of myocardial capillary density has been long observed in response to the presence of physiological stimuli, such as pregnancy and exercise, while an opposite effect occurs in HF[27-29]. Vascular endothelial growth factor (VEGF) is an angiogenic molecule with a pivotal role in vessel formation of several organs, including the heart[28,30]. CM growth induced by physiological stimuli through the Akt1 pathway[31-33] can promote CM growth with simultaneous upregulation of VEGF and promotion of angiogenesis, leading to an increase in capillary number[31]. Activation of Akt in heart muscle-restricted inducible Akt1 transgenic mice increases production of VEGF, with subsequent preservation of cardiac capillary density, indicating the paracrine effect of VEGF on the surrounding myocardial ECs[34]. Hypoxia inducible factor-1α (HIF-1α) and GATA4 are two major positive regulators of VEGF, that are involved in the regulation of VEGF in the heart by acting on distinct regulatory elements of VEGF[30,35]. An increase in capillary mass, in turn, can maintain cardiac muscle growth by enhancing the secretion of growth factors from the endothelium, such as nitric oxide (NO), which is transferred to CMs and results in the activation of PI3Kγ/Akt pathway[36-38].

Deregulation of PI3Kγ/Akt/VEGF pathway leads to cardiac dysfunction. VEGF deficiency has been observed during pressure overload, leading to a capillary rarefaction and transition to HF[30,39]. The myocardium is highly dependent on oxygen and nutrients, and therefore, displays a high capillary number to guarantee ample amount of supplies[37,40]. Vascularization of the myocardium is essential for cardiac homeostasis, and any abnormalities in this process will impair cardiac function. Capillary rarefaction has been observed in HF induced by various aetiologies, including myocardial infarction, hypertension, or cardiomyopathy[6,41,42]. Recent studies have also demonstrated capillary rarefaction in HF patients with preserved ejection fraction (HFpEF) and indicate that cardiac endothelial cell remodelling has a causal role in the onset and progression of the disease[30,43,44]. An increasing body of evidence shows that stimulation of blood vessel formation in the heart could exert a therapeutic benefit for a failing heart. Neovascularization therapies to induce canonical angiogenesis pathways through the PI3K/Akt1/VEGF axis has been advocated to improve capillary density and subsequent heart function[45,46]. However, and although animal studies showed promising results, clinical trials have shown none or only very modest effects[46-48]. This is due to several

factors, including the choice of one single pro-angiogenic growth factor, which is considered insufficient to boost angiogenesis in the heart[12,49]. From this perspective, ncRNAs, given their pleiotropic effects, serve as promising targets for neovascularization therapies in HF.

## 4. Non-coding RNAs in cardiac angiogenesis

ncRNA species are artificially divided into two groups depending on the length of their nucleotide sequence: small ncRNAs that are less than 200 nucleotide-long, and lncRNAs, which are more than 200 nucleotides in length[50,51]. Small ncRNAs are functionally subdivided into various categories, including small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), piwi interacting RNAs (piRNAs), and miRNAs. lncRNAs comprise the most heterogeneous and most poorly characterized group of non-coding transcripts to date. In this review, we focus on the contribution of miRNAs and lncRNAs in the regulation of cardiac microvasculature.

### 4.1. MicroRNAs

miRNAs are most likely the best studied functional, small ncRNAs. They are evolutionarily conserved ~22-nucleotide single-stranded RNA molecules that function by inhibiting the expression of mRNA targets through Watson–Crick base pairing with their binding site on the 3'UTR of the target transcript. Depending on the binding specificity, the target mRNA may be degraded (mRNA cleavage) or, more commonly, its translation is inhibited (mRNA decay). The varying degree of specificity in the complementarity between the miRNA and the target mRNA allows the same miRNA species to regulate several different mRNAs, simultaneously[52]. The human genome has been estimated to encode more than 1000 miRNA genes[53], which regulate over 60% of protein coding genes[49,54]. miRNAs are transcribed in the nucleus by RNA polymerase II as primary miRNA (pri-miRNA) transcripts[55]. The pri-miRNAs are then cleaved by the RNase III enzyme Drosha to generate precursor miRNAs (pre-miRNAs), which are subsequently exported to the cytoplasm by exportin-5[55,56]. Once in the cytoplasm, pre-miRNAs are further processed by RNase III enzyme Dicer into mature ~22 nucleotide single stranded miRNAs which are then incorporated into the RNA-induced silencing complex (RISC)[57]. Eventually, the mature miRNAs will guide this complex, through its complementary base pairing to degrade or to inhibit the translation of target genes[58].

miRNAs are involved in the regulation of various biological processes, such as cellular proliferation, differentiation, and migration[20,59]. Aberrant expression of miRNAs has been observed in different cardiac pathologies, including HF[60-62] and post-myocardial infarction remodelling[63,64]. In addition, miRNAs have been reported to regulate various aspects of the cardiac angiogenic response through their direct effect on ECs[18,65,66]. The first clue of the involvement of miRNAs in the regulation of angiogenesis was observed in Dicer knockout mice which displayed early mortality during embryonic development caused by impaired angiogenesis[67].

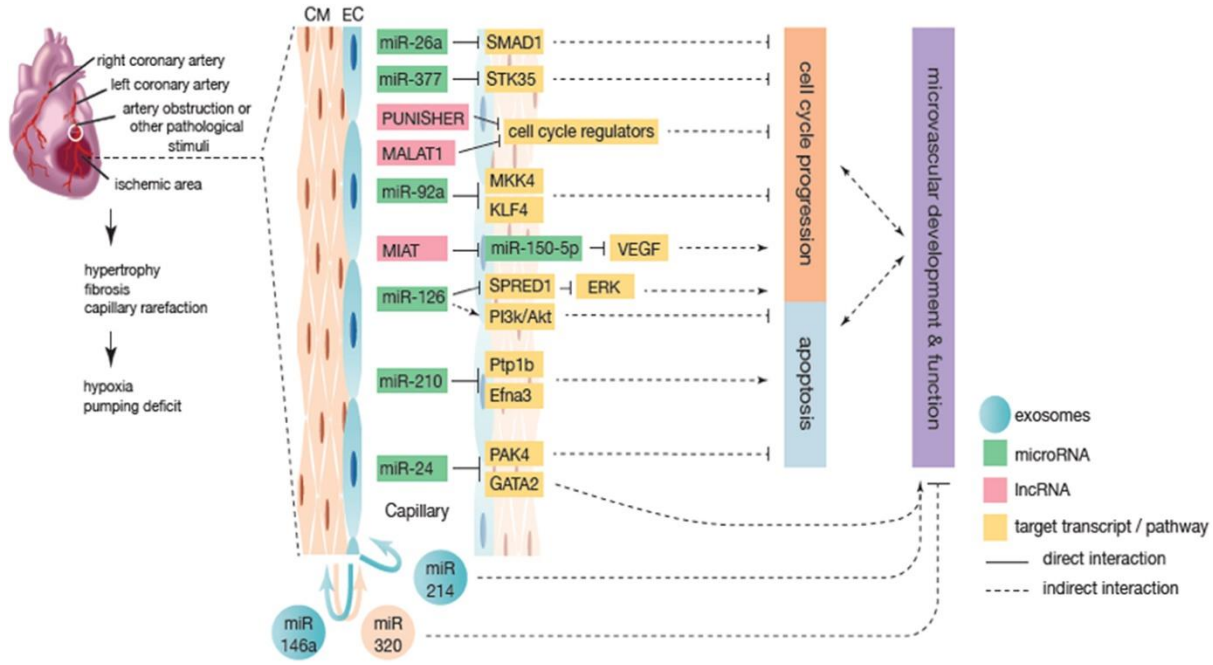


**Figure 1. The role of ncRNAs in cardiac EC function in HF**. In the injured heart, cardiac remodelling, including capillary rarefaction, takes place, leading to relative hypoxia in the heart. In response to these environmental changes, ECs are able to alter their gene expression profiles in a number of ways, including through ncRNA-mediated gene silencing. Interplay with other cell types, such as CMs also occur, which can be mediated by exosomal transfer. Several EC miRNAs and lncRNAs, and their target genes are shown to regulate EC cell cycle progression or apoptosis process, which determine the function and development of cardiac microvascularization.

Functional endothelial miRNAs can be categorized into those that impair (anti-angiogenic) and those that induce (pro-angiogenic) endothelial angiogenic properties. miR-92a and miR-24 are two abundant endothelial miRNAs with anti-angiogenic properties. Inhibition of miR-92a improved vascular function and proliferation, with subsequent amelioration of heart function, both in a pig model of ischemia/reperfusion injury[68] and a mouse model of myocardial infarction[18]. miR-92a interacts with MAP kinase kinase 4 (MKK4) and Kruppel-like factors-4 (KLF4), thus interfering with cell cycle progression in ECs[69]. miR-24 is enriched in cardiac ECs and upregulated after an ischemic insult. Inhibition of endothelial miR-24 leads to

reduced myocardial infarct size and improved heart function in mice, an effect that is mediated through prevention of EC apoptosis and enhanced vascularity[19]. miR-24 is also able to directly inhibit p21 protein (Cdc42/Rac)-activated kinase 4 (PAK4) and GATA binding protein 2 (GATA2) to improve EC survival and fitness[19] (**Fig. 1**).

Similarly, miR-26a[70] and miR-377[71] are two recently described miRNAs whose inhibition leads to recovery of microvascularization of the heart tissue. miR-26a expression is upregulated in a mouse model of acute myocardial infarction and in human subjects suffering from acute coronary syndromes[70]. SMAD family member 1 (SMAD1) was demonstrated to be the target gene of miR-26a responsible for these effects. Administration of a miR-26a inhibitor to mice subjected to myocardial infarction, led to an increase in SMAD1 expression with subsequent enhancement of angiogenesis, reduced infarct size and improved cardiac function[70]. Heart tissue from patients with HF revealed a significant upregulation of miR-377 expression, a miRNA that inhibits migration and the capacity of ECs and endothelial progenitor cells to form tubes *in vitro*. Implantation of miR-377-null endothelial progenitor cells into ischemic myocardium resulted in an attenuation of pathological cardiac remodelling[71], an effect that is mediated by the direct pro-angiogenic target of miR-377, serine/threonine kinase 35 (STK35),(Fig. 1). A similar role is exerted by miR-34 whose expression is upregulated in response to stress. Inhibition of all its family members (miR-34a, 34b, and 34c) results in increased capillary density in a mouse model of either myocardial infarction or pressure overload, accompanied by reduced fibrosis and improved heart function[72]. Downregulation of this miRNA leads to upregulation of several target genes, including VEGF, vinculin, protein O-fucosyltranferase 1, Notch1, and semaphorin 4B[72]. Even though the direct role of this miRNA and its target genes in the endothelium remains to be further investigated, it is clear that cardiac endothelial function is affected by the modulation of this miRNA[73].

Another anti-angiogenic miRNA is miR-503 whose expression is upregulated in myocardial microvascular ECs from type 2 diabetic Goto–Kakizaki rats, leading to impaired angiogenesis[74] as observed by reduced proliferation, migration, and network formation capacity of these cells *in vitro*. These effects were mediated by the suppression of target genes Cyclin E1 (CCNE1) and cell division cycle 25A (cdc25A). Inhibition of miR-503 in the ischemic muscle tissue of diabetic mice leads to improved post-ischemic angiogenesis and blood flow

recovery[75]. Given the fact that miR-503 is involved in the regulation of EC angiogenic capacity and that there is dysregulation of the expression of this miRNA in the hearts of diabetic mice, it is plausible that miR-503 also plays a role in regulating myocardial angiogenesis in the development towards HF.

miR-210, a strongly expressed miRNAs under cardiac hypoxic conditions, is considered to be a valid biomarker for chronic HF[76,77]. The function of miR-210 is, however, debatable. It has been advocated that this miRNA is pro-angiogenic and anti-apoptotic, through direct interaction with protein tyrosine phosphatase 1b (Ptp1b) and ephrin A3 (Efna3) (Fig. 1). Intra-myocardial injections of a minicircle vector carrying miR-210 precursor into a mouse model of myocardial infarction resulted in improved cardiac function in comparison to the untreated, control group. Histological analysis showed a decrease in cellular apoptosis and increased neovascularization[78]. In contrast, another study shows that miR-210 targets HIF-1α and induce a detrimental angiogenic response under hypoxic conditions while its inhibition improves heart function and survival of mice after myocardial infarction[79]. These diverging results could be a product of different transfection methods and efficiencies and/or technical bias, however this still requires clarification.

Another well-known endothelial-enriched pro-angiogenic miR is miR-126, an established fundamental player in the maintenance of vascular integrity and function[66]. This miRNA is encoded within the host gene epidermal growth factor like domain 7 (EGFL-7), known to regulate tubulogenesis[80]. miR-126 is able to potentiate endothelial pro-angiogenic pathways in multiple axis. It directly inhibits sprout-related, EVH1 domain, containing 1 (SPRED1), an inhibitor of the extracellular regulated MAP kinase (ERK), to prevent its anti-angiogenic action. miR-126 is also a potent anti-apoptotic factor through regulation of the PI3K/Akt[81-83]. While recent findings report a decrease in circulating levels of miR-126 after myocardial infarction, miR-126 expression profiles can also be correlated to cardiac function indexes, strongly supporting the contribution of miR-126 to regulation of proper cardiac function (**Fig. 1**)[81,84].

Several other miRNAs were reported to display differential expression and have biological relevance when exposing ECs to different types of stress, such as alterations in the blood flow, inflammatory response, hyperglycemia, and hypoxia (**Table 1**). While the significance of such

findings to the regulation of cardiac microvasculature has not yet been established, we speculate that many of these miRNAs may also play a role in the remodelling of the injured heart by impacting EC function.

**Table 1. Functional miRNAs in ECs exposed to pathological stimuli prevalent in cardiovascular diseases.**

| miRNAs | Targets | Pathological Stimuli | Model | Effect | References |
|---|---|---|---|---|---|
| miR-15a | FGF2<br>VEGF | Ischemia | Murine Hindlimb | Anti-angiogenic | [85] |
| miR-19a | CCND1 | Dysregulated Flow | HUVEC | Anti-proliferation | [86] |
| miR-19/221/222 | PGC-1α | Inflammation | HAEC | Pro-apoptotic | [87] |
| miR-21 | PTEN | Dysregulated Flow | HUVEC | Anti-inflammation<br>Anti-apoptotic | [88] |
| miR-92 | KLF2 | Dysregulated Flow | HUVEC | Anti-angiogenic<br>Pro-inflammation | [89] |
| miR-100 | mTOR | Ischemia | Murine Hindlimb | Anti-angiogenic | [90] |
| miR-101 | Cul3 | Ischemia | HUVEC | Pro-angiogenic | [91] |
| miR-101 | mTOR | Dysregulated Flow | HUVEC | Anti-proliferation | [92] |
| miR-106b~25 | PTEN | Ischemia | Murine Hindlimb<br>HUVEC | Pro-angiogenic | [93] |
| miR-107 | Dicer1 | Ischemia | MCAO mice<br>HUVEC | Pro-angiogenic | [94] |
| miR-132/212 | Rasa1<br>Spred1<br>Spry1 | Ischemia | Murine Hindlimb | Pro-angiogenic | [95] |
| miR-155 | AT1R<br>VEGFR2 | Ischemia | MCAO mice | Anti-angiogenic | [96,97] |
| miR-155 | AT1R<br>Ets1 | Inflammation | HUVEC | Anti-inflammation<br>Anti-angiogenic | [98] |
| miR-200c | ZEB1 | Ischemia | Murine Hindlimb<br>HUVEC | Anti-angiogenic | [99] |
| miR-221/222 | Ets1 | Inflammation | HUVEC | Anti-inflammation | [98] |
| miR-223 | RPS6KB1 | Ischemia | CMEC | Anti-angiogenic | [100] |
| miR-365 | Bcl2 | Inflammation | HUVEC | Pro-angiogenic<br>Anti-inflammation | [101] |
| miR-424 | Cul2 | Ischemia | HUVEC | Pro-angiogenic | [102] |
| miR-663 | KLF4<br>CEBPB<br>ATf3 | Dysregulated Flow | HUVEC | Pro-inflammation | [103] |

### 4.2. LncRNAs

lncRNAs differentially regulate gene expression. Prior to transcription, lncRNAs can act as a scaffold to recruit and coordinate the assembly of epigenetic complexes. They are able to interfere with transcription by serving as a decoy or competing with transcription factors, while potentially also being able to inhibit RNA polymerase II activity. Post-transcriptionally, lncRNAs can affect gene expression by interacting with mRNAs and causing their destabilization[104-106]. Moreover, lncRNAs can serve as sponges for miRNAs[107].

lncRNAs identified to be involved in cardiac microvascular dysfunction are myocardial infarction-associated transcript (MIAT) and PUNISHER (Fig. 1). Expression of MIAT was first reported as a predictor of myocardial infarction[108] and later related to pathological angiogenesis[109]. MIAT is able to sponge miR-150-5p, which is responsible for abnormal upregulation of VEGF, and thus promoting pathological reduced angiogenesis and microvascular dysfunction[109]. PUNISHER is a novel endothelial-specific lncRNA conserved in zebrafish, mice and human, named retrospectively according to the phenotype it induces in zebrafish. It appears to be an essential regulator of vessel formation as its inhibition results in severe vascular defects, which negatively correlates with cell cycle- and endothelial fitness-related gene expression, and positively correlates with cell adhesion-related gene expression[110].

While comprehensive studies on the role of lncRNAs in the regulation of cardiac microvasculature are scarce, these few reports provide some insights into the topic. Several lncRNAs are known to regulate EC function and behaviour, and despite not having been proven to exert their role in the heart, it is plausible that they play a role in fine tuning of cardiac function[17,111]. The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a pro-angiogenic lncRNA that is upregulated under hypoxic conditions and fundamentally controls the switch between endothelial proliferative and migratory phenotypes[112,113]. Another noncoding transcript that may impact on cardiac microvasculature is an antisense RNA transcript named ANRIL (antisense noncoding RNA in the INK4 locus)[114]. ANRIL is expressed in both vascular endothelial and coronary smooth muscle cells. Single nucleotide polymorphism variants of ANRIL have been linked to angiogenesis and atherosclerosis[115,116]. In addition to ANRIL, lncRNA maternally expressed gene 3 (MEG3) has been described to play a role in diabetes-related microvascular dysfunction. MEG3 expression levels are significantly low in the retina of streptozotocin-induced diabetic mice and MEG3 knockdown exaggerates retinal microvascular dysfunction, shown by increased microvascular leakage, inflammation, and capillary degeneration[117].

Other angiogenesis-related lncRNAs are LINC00323 and MIR503HG. Silencing of LINC00323 in human umbilical vein endothelial cells (HUVECs) inhibited cell proliferation, migration, and tube formation capacity. A direct interaction between LINC00323 and eIF4A3/GATA2 suggests

that LINC00323 acts as a scaffold controlling the expression of these two endothelial transcription factors. In its turn, MIR503HG induces miR-503 expression in an hypoxia-dependent fashion and its inhibition leads to reduced miR-503 expression levels and impaired EC proliferation and migration. MIR503HG and miR-503 seem to regulate endothelial angiogenic capacity by altering the expression of cell-cycle inhibitor p21 and GATA2[118].

SENCR is a human vascular-enriched lncRNA whose expression is abundant in ECs and positively correlates with Friend Leukemia Integration virus 1 (FLI1) expression, a regulator of endothelial development. The levels of this lncRNA are altered in vascular tissue and cells derived from patients with limb ischemia and with premature coronary artery disease. Overexpression of SENCR in HUVECs induces cell proliferation, migration, and tube formation capacity[119]. Two other lncRNAs which promote EC function are platelet-activating factor acetyl hydrolase 1B1 (PAFAH1B1, also known as Lis1) and NONHSAT073641. Downregulation of these lncRNAs leads to impaired endothelial tube formation and decreased sprouting. PAFAH1B1 promotes the expression of Matrix Gla Protein (MGP), a positive regulator of endothelial angiogenic capacity, as it is required for active histone marks and binding of RNA Polymerase II to the transcriptional start site of MGP. Although NONHSAT073641 positively regulates angiogenesis to a similar extent as PAFAH1B1, its molecular mechanism is still unknown[120].

## 5. Exosomes as an intercellular communication tool between cardiomyocytes and endothelial cells

ncRNAs can be transferred and influence the behaviour of other cells adjacent to their cell of origin. The mechanism of this transport can be either through direct cell-to-cell contact or through a paracrine-like action. Even though ncRNAs can travel through gap junctions from one cell to another[121-123], extracellular transportation has been the focus of most recent studies. The majority of secreted RNAs are chaperoned by another biological entity, including exosomes, which protects them from RNAse-mediated degradation[124,125]. Exosomes are small (ranging from 40 to 100 nm) cup-shaped, double-membraned extracellular vesicles secreted by the vast majority of human cell types[126]. They begin as intracellular vesicles, known as endosomes, which result from the inward budding of the cell membrane. Once the endosome

is formed, there is an invagination of its membrane, leading to accumulation of intraluminal vesicles within the larger multivesicular bodies (MVBs). The outer membrane of MVBs retains much of the plasma membrane composition, while its internal vesicles can incorporate cytosolic components[127]. MVBs can fuse with lysosomes for degradation of their cargos, or with the plasma membrane in order to release their internal vesicles, referred to as exosomes[128]. The process of exosome biogenesis differentiate these vesicles from other extracellular vesicles that arise from the outward budding of the cell membrane, apoptotic bodies, or necrotic blebs of the plasma membrane[129]. Exosomes can enter target cells through a variety of mechanisms such as ligand-receptor binding, membrane fusion, or endocytosis[130]. Exosomes mostly maintain the membrane characteristics of their parent cell which is enriched in cholesterol, sphingomyelin, glycolipids and ceramide[131]. Specific proteins, residing on the surface of the exosomes including tetraspanins (CD9, CD63, and CD81), can be used as exosomal markers. Exosome secretion depends on Rab27a and Rab27b which mediate the anchoring to the plasma membrane[132,133]. Several mechanisms are involved in the specific sorting of exosomal cargos, including endosomal-sorting complexes required for transport (ESCRT), tetraspanins and lipid-dependent mechanism. The ESCRT complex, which is composed of several sub-complexes, produce vesicles through inward budding of MVBs and sort mono-ubiquitinated proteins into them. Tetraspanins function to sort different cargos and interact with other transmembrane proteins, cytosolic proteins and lipids, and to organize the exosome membrane into tetraspanin-enriched domains[131,134]. Lipid-dependent mechanisms involve the synthesis of ceramide by a rate limiting enzyme neutral sphingomyelinase 2 (nSMase2), which can be inhibited by nSMase2 inhibitor compounds such as GW4869. Ceramide triggers the invagination of exosomes into MVBs and this pathway is considered responsible for the uptake of miRNAs into exosomes[135,136].

miRNAs have been reported to transfer within exosomes[137]. Following RNA-induced silencing complex (RISC) disassembly, some miRNAs are integrated into the intraluminal vesicles within MVBs[124]. Previous studies showed that miRNAs are selectively incorporated into exosomal vesicles[138] and can be enriched differently than the parent-cell type[139]. The different theories as to how the selection for exosomal transport occurs are still debatable[140]. Exosomes were initially described as a mechanism used by reticulocytes to discard redundant receptors and proteins complexes as they develop into erythrocytes[141]. In cancer, exosomes have been

shown to pass malignancy from cancer cells to the surrounding areas which are less or non-malignant[142,143]. In addition, exosomes are now emerging as important tools of intercellular communication among different cell types in the heart[21,131,133].

*In vitro* experiments established that the communication between CMs and cardiac ECs through exosomal miRNA transfer is an effective way of modulating gene expression and affecting the biology of these cells. Exosomes from diabetic rats-derived CMs are enriched in anti-angiogenic miR-320 but deficient of pro-angiogenic miR-126. Mouse cardiac ECs are able to incorporate these exosomes, which eventually lead to impairment of their proliferative, migration, and tube formation capacities (**Fig. 1**). This is associated with downregulation of the exosomal miR-320 target genes insulin-like growth factor 1 (IGF1) and E26 avian leukemia oncogene 2 (Ets2), in the recipient ECs[144].

Independent studies have postulated that vesicular miR-126-deficiency under pathological conditions can result in a decrease of endothelial neovascularization potential[145], and that this phenotypical change can be rescued by extracellular transfer of functional miR-126[146]. In line with these results, miR-126 along with miR-210, were found to be upregulated in the exosomes derived from cardiac ECs under hypoxic conditions. Overexpression of HIF on ECs also increases the expression of these miRNAs in the exosomes, which can be incorporated into cardiac progenitor cells to drive a pro-survival phenotype, proving their functionality across multiple cell types. Accordingly, delivery of these progenitor cells to mouse hearts after myocardial infarction leads to improved ejection fraction[147]. In a similar process, miR-146a is able to induce detrimental phenotypes in ECs, which results in the inhibition of angiogenesis in a mouse model for peripartum cardiomyopathy. Transfer of exosomal miR-146a to CMs changes their metabolic activity and contractility, leading to impairment of heart function[148]. A recent study determined the pro-angiogenic nature of miR-214[149], adding to the controversy in literature. While miR-214 seems to play a role in exosome-mediated signalling to promote EC angiogenic capacity[149], it can also function as an anti-angiogenic agent, once its direct transfection on ECs leads to reduced sprouting and tube formation[150]. Nevertheless, this miRNA was found to be secreted within EC-derived exosomes and be able to modulate endothelial angiogenic properties (**Fig. 1**).

lncRNAs have not yet been as much the focus of research as their smaller counterparts in the context of extracellular transport and intercellular communication, particularly in the field of cardiovascular diseases. However, interesting observations have been made, indicating that lncRNAs are selectively loaded and enriched in exosomes from cancer cell lines, including the previously described MALAT1[151]. Another lncRNA, LINC00152, is present in plasma-derived exosomes of gastric cancer patients. The majority of this lncRNA in plasma is derived from exosomes, establishing it as a diagnostic marker for gastric cancer[152]. Moreover, the lncRNA HOTAIR was found in exosomes isolated from serum of laryngeal squamous cell carcinoma patients and its expression is elevated in patients with lymph node metastasis in comparison to those without metastasis[153]. Furthermore, it has been demonstrated that hepatocellular cancer cells secrete exosomes containing various lncRNAs, including HOTAIR, HULC, linc-ROR and H19 which, *in vitro*, can be uptaken by ECs and induce angiogenesis by promoting EC re-organization into tubular-like structures and an increase in the expression of VEGF and its receptor[154].

Taken together, these studies emphasize the importance of crosstalk and subsequent exchange of gene expression regulators between multiple cell types in the heart. We expect that more comprehensive studies of exosomal ncRNA function in cardiovascular regulation in health and disease will keep yielding interesting and therapy-oriented results.

## 6. Angiogenic ncRNAs as potential therapeutic targets for heart failure

An increasing number of evidence supports the relevance of capillary rarefaction in the development of HF. Patients with end-stage HF due to idiopathic dilated cardiomyopathy[5,6], ischemic cardiomyopathy, and inflammatory cardiomyopathy[5] have demonstrated a reduction in cardiac capillary density. As capillary rarefaction leads to a decrease in coronary flow reserve[155], which is correlated with poor tissue perfusion and an abnormal oxygen consumption pattern[156], promotion of angiogenesis serves as a potential therapeutic tool for HF. In fact, studies have shown the efficacy of several pro-angiogenic factors in improving heart function in animal models of HF. Administration of VEGF and Ang-1 to a porcine model of myocardial infarction results in an increase in vascular density, myocardial perfusion and function[45]. Treatment of a rat model of chronic HF induced by coronary artery ligation with

combination of fibroblast and hepatocyte growth factor stimulates cardiac angiogenesis and arteriogenesis, leading to improved myocardial function and perfusion, as measured by magnetic resonance imaging[46]. In addition, several approved drugs, including pitavastatin and benidipine, categorized as statins and calcium-channel blockers, respectively, have been reported to induce myocardial angiogenesis and improve contractility in a mouse model of pressure overload[157] and in Dahl-salt sensitive rats[158]. Despite the efficacy of angiogenesis therapy observed in animal studies, conflicting results have emerged from clinical trials. Several randomized clinical trials, including AGENT[48], VIVA[46], and KAT[47], have shown only modest cardiac function improvement after fibroblast growth factor (FGF) or VEGF gene therapy. This may be due to several factors, including patients selection, delivery strategy, and the chosen growth factors[49]. A single pro-angiogenic gene that is commonly used to promote angiogenesis[12] might be considered insufficient to boost angiogenesis in the heart. From this perspective, modulation of ncRNAs offers a promising new potential therapeutic strategy. miRNAs are able to regulate multiple genes often coordinating one single signalling pathway, or several pathways, leading to a stronger synergistic effect than the effect of a single therapeutic target. miRNAs are also an interesting therapeutic option since they are comprised of only ≈22 nucleotides that can easily be inhibited or overexpressed. In addition, these nucleotide sequences are highly conserved across multiple species, favouring the translation from preclinical animal studies to clinical trials in humans[14].

The delivery of miRNAs to ECs as a mean of angiogenesis therapy has been reported. miR-126 delivery to ECs through circulating microparticles[146] or apoptotic bodies[82] induces vascular repair *in vivo*. Therapeutic delivery of miRNAs to ECs was also performed by intravenous injection of liposome-encapsulated miRNAs but this method did not only specifically target ECs, as miRNAs were also detected in leukocytes and other organs, including liver, spleen, and kidney[159]. Another delivery method so-called ultrasound-targeted microbubble destruction (UTMD) has been described to solve this cell specificity issue. Delivery of miR-126 to the ECs of vessels in ischemic rat hind limb with this method resulted in downregulation of known miR-126 target genes only in ECs and not in liver and spleen as common off-target organs, and increased vessel length, vascular density, and tissue perfusion[160]. UTMD is shown to be an efficient cell-specific method of miRNA delivery and less invasive in comparison to other delivery methods[13]. Although the study was performed in the model of hind limb ischemia, it

is likely that it can also be applied to study the effect of miRNA delivery to cardiac ECs in the context of pathological remodelling. An increasing number of evidence supports the notion of pro- and anti-angiogenic miRNAs exerting their effect during the development of cardiac pathologies. Even though there are less known regarding their therapeutic applications, lncRNAs are associated with EC function, and their pleotropic function, including being a sponge for miRNAs, renders their potential to modulate a specific process, including angiogenesis. In addition, therapeutically targeting lncRNAs could result in less off-target effects, due to their tissue specificity and their ability to regulate miRNA/mRNA networks[161]. Modulation of angiogenesis-related-ncRNAs is associated with improvement or worsening of heart function. Therefore, both miRNAs and lncRNAs have the potential to serve as therapeutic targets to restore cardiac microvascularization in the progression of HF.

## 7. Conclusion and future perspectives

Myocardial growth and angiogenesis response are reciprocal adaptive reactions of the heart. Physiological CM growth can induce an increase in capillary density in the heart, which in turn, enhances growth factors secretion from endothelium, maintaining heart muscle growth. This process indicates interplay between CM and cardiac ECs to maintain cardiac homeostasis. The canonical molecular mechanism involves the activation of PI3K/Akt1 pathway in CMs, leading to the secretion of VEGF and subsequent activation of ECs and stimulation of angiogenesis. Capillary rarefaction is a hallmark of a failing heart, which occur in the heart of patients with HF of different etiologies. Neovascularization therapies targeting PI3K/Akt1/VEGF axis are efficacious in animal models but unsuccessful in clinical trials, which could be due to the inefficiency of the single pro-angiogenic factors used in the studies.

ncRNAs emerge as new players involved in cardiac cellular crosstalk, affecting cardiac microvasculature homeostasis. The role of miRNAs is prominent in the myocardial vascularization with several miRNAs, including miR-92, -24, -26, -377, and 34, shown to be anti-angiogenic[18,68,70,71], and several others, such as miR-126 and -210, to be pro-angiogenic[82,83]. lncRNAs, being a larger counterpart of miRNAs, have emerged as new players involved in the regulation of heart tissue vascularization. MIAT and PUNISHER are two well-known lncRNAs involved in vessel formation in the heart[110]. Extracellular transportation

through exosomes has been shown to mediate miRNAs transfer between ECs and CMs and to affect critical functions on both cell types. lncRNAs, similar to miRNAs, can also be transported within exosomes and although their relevance in cardiac disease remains to be further elucidated, available data endorse their use as a biomarker or therapeutic target to treat pathologies of the heart.

The power of ncRNAs lies on their pleiotropicity, as they are capable to simultaneously regulate the expression of more than one gene, often regulating the same network or pathway, thereby synergistically enhancing the outcome of the regulation. Since angiogenesis therapy has been shown promising to treat HF, at least at the pre-clinical level, the potential of angiogenesis-related ncRNAs as strong modulators of angiogenesis in the heart should be further explored. The cell specificity and the applicability of the current delivery methods are also essential to be elucidated in order to extract the most beneficial effect of these angiogenesis-related ncRNAs not only in animal models, but also in clinical trials. Exosomes can be an alternative delivery method for ncRNAs. Both natural and modified exosomes, have been used as a tool to carry biological entities to target cells. It has been demonstrated that modified exosomes carrying a neuron-specific protein on their surface can transfer their siRNA cargo to mouse brains[162]. Exosomes carrying recombinant proteins and tumour antigens expressed by cancer vaccines were reported to have a therapeutic effect in phase I clinical trials on patients with melanoma[163]. Furthermore, several stem cell-derived exosomes were shown to induce tissue regeneration after ischemia[147,164]. Despite these interesting possibilities for the use of exosomal delivery to treat diseases, a better understanding of their complex structure and cargoes, and their potential off-target effects is necessary before translation into the clinic.

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# CHAPTER IV - Exogenous loading of miRNAs into small extracellular vesicles

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## ABSTRACT

Small extracellular vesicles (sEVs), through their natural ability to interact with biological membranes and exploit endogenous processing pathways to convey biological information, are quintessential for the delivery of therapeutically relevant compounds, such as microRNAs (miRNAs) and proteins. Here, we used a fluorescently-labelled miRNA to quantify the efficiency of different methods to modulate the cargo of sEVs. Our results showed that, compared with electroporation, heat shock, permeation by a detergent-based compound (saponin) or cholesterol-modification of the miRNA, Exo-Fect™ was the most efficient method with >50% transfection efficiency. Furthermore, qRT-PCR data showed that, compared with native sEVs, Exo-Fect™ modulation led to a >1000-fold upregulation of the miRNA of interest. Importantly, this upregulation was observed for sEVs isolated from multiple sources. The modulated sEVs were able to delivery miR-155-5p into a reporter cell line, confirming the successful delivery of the miRNA to the target cell and, more importantly, its functionality. Finally, we showed that the membrane of Exo-Fect™-loaded sEVs was altered compared with native sEVs and that enhanced the internalization of Exo-Fect™-loaded sEVs within the target cells and decreased the interaction of those modulated sEVs with lysosomes.

## Introduction

Extracellular vesicles (EVs) are biological particles secreted by most organisms and cell types[1]. In recent years, particular attention has been given to small EVs (sEVs), vesicles with a diameter between 30-200 nm capable of permeating biological barriers and deliver their cargo onto target cells[2]. There is an increasing interest to use these vesicles as vehicles for the delivery of biomolecules such as miRNAs, short (~22 nucleotides) non-coding nucleic acids that regulate gene expression at the post-transcriptional level, for the treatment of cardiovascular, neurodegenerative diseases, among others.

Early attempts to modulate the content of sEVs focused on modifications to the secreting cell such as, for example, transfection with the gene of interest or addition of small molecules to the culture medium[3-5]. This approach remains the most widely used strategy to enrich or deplete sEVs of any molecule of interest. However, this methodology is not applicable to sEVs isolated from biological fluids. Moreover, the establishment of *in vitro* cell cultures dedicated to sEV production is time consuming and costly. Therefore, the post-isolation modification of sEVs with exogenous biomolecules of interest has been investigated in recent years. Strategies used for the transfection of cells, such as electroporation[6-8], heat shock[9] and detergent-based[10] permeabilization of the membrane, were used for the modulation of sEVs. The results obtained indicated that small RNAs could be successfully introduced into sEVs and the modulated sEVs were capable of delivering their cargo to the target cell ultimately regulating their function. These results laid the groundwork for the modification of EVs after their purification. Yet, a direct comparison between the different methods of miRNA loading into sEVs has not been performed and, more importantly, several important questions remain unanswered such as, for example, whether the loaded molecule is in the lumen and/or at the membrane of sEVs and whether modulation of sEVs affects their biophysical properties and ultimately their intracellular trafficking properties and capacity to deliver the cargo.

In this work we compared, side-by-side, five different methodologies to load miRNAs into sEVs isolated from three different sources: (i) conditioned medium from human umbilical cord blood derived mononuclear cells (hUCBMNCs), (ii) human urine and (iii) commercially available foetal bovine serum. The methodologies tested were based in sEV electroporation[6,7], heat shock in the presence of calcium chloride[9], saponin

permeabilization[10], conjugation of the miRNA with cholesterol[11] and transfection with the commercial kit Exo-Fect™[12,13]. Firstly, the methodologies were ranked based on their effectiveness in loading a fluorescently labelled miRNA into sEVs, Exo-Fect™ being the most effective. Then, the selected method was compared with the transfection of the donor cell – used for the enrichment of miRNA in sEVs. Finally, the biophysical properties of Exo-Fect™-modulated sEVs, namely their size, zeta potential, membrane permeation, cytotoxicity, internalization and intracellular trafficking were characterized and the activity of the loaded miRNA was validated in a reporter cell line. Our results indicated that the loading of miRNAs with Exo-Fect™ was the most promising approach to modulate the content of sEVs and that upon modulation, sEVs retained their capacity to efficiently deliver their cargo into recipient cells. Additionally, compared to their native counterparts, Exo-Fect™-modulated sEVs showed decreased colocalization with lysosomal and early endosomal compartments.

**Materials and methods**

**sEV collection via differential ultracentrifugation**. All human umbilical cord blood (hUCB) samples were obtained upon signed informed consent, in compliance with Portuguese legislation. The collection was approved by the ethical committee of Centro Hospitalar e Universitário de Coimbra, Portugal (HUC-01-11). The samples were stored and transported to the laboratory in sterile bags with anticoagulant solution (citrate-phosphate-dextrose) and processed within 48 h after collection as previously described by us[14,15]. Briefly, mononuclear cells (MNCs) were isolated by density gradient separation (Lymphoprep™ - StemCell Technologies SARL, Grenoble, France). To obtain MNC-derived sEVs (mEVs), hUCB MNCs were cultured in X-VIVO 15 serum-free cell culture medium (Lonza) supplemented with Flt-3 (100 ng/mL, PeproTech) and stem-cell factor (100 ng/mL, PeproTech) under hypoxia (0.5% $O_2$) conditions for 18 h. Conditioned medium was collected and centrifuged at 300 g, for 10 min, at 4 ºC to remove cells followed by a centrifugation at 2.000 g, for 20 min, at 4 ºC to deplete cellular debris.

To obtain human urine-derived sEVs (uEVs), the first morning midstream urine was collected from healthy donors upon signed informed consent and upon approval from the Ethics Committee of the Faculty of Medicine, University of Coimbra (CE-070-2019). Samples were

centrifuged at 2.000 g, for 20 min, at 4 ºC to pellet cells and cell debris. After centrifugation, the supernatant was collected, diluted 1:3 with Tris-EDTA (20 mM, pH 9.0) and vortexed 90 s at 2.500 rpm to disrupt aggregates.

To obtain FBS-derived sEVs (fEVs), commercial FBS (#10270106, Gibco™) was thawed slowly at room temperature (RT) and diluted 1:4 in phosphate buffered saline (PBS).

Wharton-Jelly derived mesenchymal stromal cells (WJ-MSCs) were kindly donated by Crioestaminal. Cells were cultured at 5000 cells/cm$^2$ in MEM Alpha modification, with L-glutamine, ribo- and deoxyribonucleosides (SH30265, GE Healthcare) supplemented with 10% (v/v) sEV-depleted FBS (FBS was depleted of sEVs by ultracentrifugation at 100.000 g, for 18 h, at 4 ºC) and 0.5% (v/v) penicillin/streptomycin (P/S) for 24 h. Subsequently, WJ-MSCs were transfected with 25 nM of miR-155-5p (for some experiments miR-155-5p was labelled with Cy3 at the 3' of the passenger strand) using Lipofectamine RNAimax according manufacturer´s instructions. Non-transfected WJ-MSCs were used as a control. After 24 h of transfection, the transfection medium was discarded and WJ-MSCs were cultured on α-MEM supplemented with 10% (v/v) sEV-depleted FBS for further 48 h. Conditioned medium was collected and centrifuged at 300 g, for 10 min, at 4 ºC to remove cells followed by a centrifugation at 2.000g, for 20 min, at 4 ºC to deplete cellular debris.

Regardless of the source, sEVs were purified by differential centrifugation as described previously[16]. Briefly, samples were ultracentrifuged twice at 10.000 g, for 30 min, at 4 ºC, the pellet was discarded and the supernatant was submitted to an ultracentrifugation at 100.000 g, for 2 h, at 4 ºC, to pellet sEVs. Finally, the pellet from the last step was washed with cold PBS, ultracentrifuged again at 100.000 g, for 2 h, at 4 ºC, resuspended in 150 µL of cold PBS and stored at -80 ºC. Ultracentrifugation steps were performed using a swinging bucket rotor SW 32 Ti in an Optima™ XPN 100K ultracentrifuge (Beckman Coulter, California, U.S.A.) and 28.7 mL polyallomer conical tubes (Beckman Coulter).

**sEV purification via OptiPrep™ Density Gradient (ODG).** Native and modulated sEVs were purified using ODG according to standard protocols, described previously[17]. Briefly, discontinuous gradient solutions with 5%, 10%, 20% and 40% iodixanol were prepared by

mixing a working buffer [0.25 M sucrose, 6 mM EDTA, 60 mM Tris-HCl, (pH 7.4)], a homogenization buffer [0.25M sucrose, 1mM EDTA, 10 mM Tris-HCL, (pH 7.4)] and a stock solution of OptiPrep™ ([60% (w/v) aqueous iodixanol solution], in appropriate proportions. Specifically, to prepare the gradient, Optiprep was diluted 5:1 with working buffer to obtain a 50% Optiprep solution, hereafter denoted working solution. Then, 40%, 20%, 10% and 5% gradients were prepared by mixing 4, 2, 1 and 1 parts of working solution with, respectively, 1, 3, 4 and 9 parts of homogenization buffer. In a UC polyallomer tube, 6 mL of 10%, 20% and 40% solutions and 5 mL of the 5% solution were layered on top of each other in decreasing concentrations of iodixanol and subsequently 1 mL of sEV sample was carefully layered on top of the gradient. Preparations were ultracentrifuged at 100.000 g, for 18 h, at 4 ºC upon which 15 fractions of around 1.5 mL were collected and further analyzed. Ultracentrifugation steps were performed using a swinging bucket rotor SW 32 Ti in an Optima™ XPN 100K ultracentrifuge (Beckman Coulter, California, U.S.A.) and 28.7 mL polyallomer conical tubes (Beckman Coulter).

**sEV characterization by nanoparticle tracking analysis (NTA)**. Size and concentration of sEVs was performed through NTA using the NanoSight NS300 (Malvern Instruments, Malvern, U.K.). The system used an O-Ring Top Plate and the sample was injected manually at an approximate flow of 1 mL every 20 s. sEVs were diluted in PBS until a concentration between 15 and 45 particles/frame was reached. For each sample, 5 videos of 30 s were recorded with the camera level set at 16. All the videos were processed with NTA 3.2 analytical software, using the software threshold between 2 and 4 depending on the quality of the videos.

**sEV characterization by protein quantification**. sEV protein quantification was performed using the microBCA protein assay kit (Thermo Fisher Scientific, Massachusetts, U.S.A.), as per the manufacturer's instructions. Briefly, bovine serum albumin (BSA) was used to obtain a 10 points standard curve. Then, sEV samples were diluted 22 times in 2% (v/v) sodium dodecyl sulphate (SDS) to disrupt the sEV membrane and subsequently, 50 µL of the previous mix was pipetted, in duplicate, into a 96-well Corning® Costar® cell culture plates (Corning Inc., New York, U.S.A.). Reaction solution provided in the kit was added and incubated for 2 h at 37 ºC.

Next, the plates were equilibrated at room temperature for 15 min and finally, the absorbance at 562 nm was read in the microplate reader Synergy™ H1 (Biotek, Vermont, U.S.A.).

**Western blot analysis.** Western blot analysis for the detection of EV markers and contaminants was performed. Briefly, up to 15 µL of concentrated EV preparations in PBS (0.5 to 4µg) were mixed with 5 µL 4x Laemmli buffer (0.25M Tris base, 8% SDS, 40% glycerol, 200 mg bromophenol blue, 10% 2-mercaptoethanol) and boiled at 96ºC for 10 min. For the analysis of tetraspanins, Laemmli buffer was prepared without reducing agents. Samples were loaded in 30 µL wells, Any kD™ Mini-PROTEAN® TGX Stain-Free™ Protein Gel (Bio-Rad # 4568123) and gel electrophoresis was performed in 1× Tris/Glycine/SDS buffer prepared from a commercial 10× concentrated stock (10× Tris/Glycine/SDS Electrophoresis Buffer; Bio-Rad #1610772), at the constant voltage of 120V, for 75 min. Afterwards, gels were placed in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol in water) for 10 min to equilibrate. Then the gel was stacked on top of a nitrocellulose membrane (GE Healthcare #10600016) and both were assembled within a transfer system. Transfer was performed in wet conditions at 200 mA for 90 min. Afterwards, the membrane was removed and blocked in a 1:1 PBS-Tween 20 (0.2% (v/v)) with Intercept Blocking Buffer (Li-cor #927-70001) solution for 1h at room temperature. Membranes were then washed with PBS-Tween 20 and left to incubate overnight at 4ºC with the appropriate primary antibodies and according to the manufacturer recommendation (antibody details below). Then, membranes were washed 3 times with PBS-Tween and incubated for 1 h at room temperature with secondary antibodies. Membranes were then washed 3 times and viewed in the Odyssey CLx system (Li-cor) at the 700 nm and 800 nm wavelengths. Antibodies used in this study were: CD63 (BD Pharmingen #556019), ApoA-1 (Santa Cruz #sc-376818), GAPDH (Millipore, MAB374), Calnexin (Santa Cruz #sc-23954), Alix (Cell Signaling, #2171S), CD9 (BD Pharmingen #555370), THP (Santa Cruz #sc-271022) and IRDye® 800CW Goat anti-Mouse IgG Secondary Antibody (Li-cor #926-32210).

**sEV characterization by transmission electron microscopy (TEM)**. TEM analyses of sEVs were performed as previously described[16]. Briefly, samples were diluted 1:1 in 4% (v/v)

paraformaldehyde (PFA) and placed on Formvar-carbon coated grids (TAAB Technologies) for 20 min at RT. After washing 4 times with PBS, grids were placed on a drop of 1% (v/v) glutaraldehyde for 5 min, followed by 5 washes with distilled water, one minute each. In a dark environment, grids were incubated with uranyl-oxalate solution pH=7 for 5 min, and then placed on ice in contact with a solution of methyl cellulose (9:1) for 10 min. sEVs imaging was obtained using a Tecnai G2 Spirit BioTWIN electron microscope (FEI) at 80 kV.

**sEV characterization by Dynamic Light Scattering (DLS)**. DLS measurements were done on a Zetasizer Nano ZS (Malvern). The sample was pre-equilibrated at 37° C for at least 60 s and each measurement was the average of 11 runs. Three consecutive measurements were performed for each sample to evaluate its stability. The results were analyzed by the equipment software considering the viscosity and refractive index of water at the measurement temperature, and a refractive index of 1.59 for the scattering particles. The average size was taken from the analysis in volume distribution of particles.

**sEV characterization by pulse analysis light scattering (PALS).** NanoBrook ZetaPALS Potential Analyzer (Brookhaven Instruments Corporation, Long Island, U.S.A.) was used for sEV surface charge measurement. Briefly, 5 µL of purified sEVs were diluted in 1500 µL of biological grade ultrapure water (Fisher Scientific, New Hampshire, U.S.A.) and filtered twice through a 0.2 µM filter. sEVs were then placed in a disposable polystyrene cuvette and the electrode was immersed within the cuvette. Each sample was measured five times (using Smoluchowski module) at room temperature.

**sEV loading with fluorescently-labelled miRNA**. For the loading of sEVs with a miRNA using the different methods, $10^{10}$ sEVs were mixed with 10 pmol of miR-155-5p-Cy3 (custom product based on miRIDIAN from Dharmacon modified with 3'end guide strand Cy3) in PBS. To control for miRNA precipitation upon treatment, the miRNA was incubated in the same conditions as described below in the absence of sEVs. Electroporation was carried out in Gene Pulser Xcell™ Electroporation System (Biorad). $10^{10}$ sEV were resuspended in trehalose pulse

medium (50 nM trehalose in PBS), placed in 4 mm cuvettes and pulsed a single time (5 milliseconds) at 400 V. Heat shock was performed in the presence of 0.1 M calcium chloride[9]. $10^{10}$ sEV were placed on ice for 30 min, incubated at 42ºC for 1 min and immediately placed on ice for further 5 min. Detergent-induced membrane permeabilization was performed for 10 min at room temperature in a saponin solution (0.1 mg/mL of saponin in PBS) using $10^{10}$ sEVs[10]. Exo-Fect™ loading was carried out by incubating $10^{10}$ sEV for 10 min at 37 ºC with Exo-Fect™ (10 µL, in a final volume of 150 µL). Cholesterol was also used to complex miRNA with sEVs. In this case, samples were incubated with cholesterol-modified miRNA (custom product based on miRIDIAN from Dharmacon modified with 5'end passenger strand cholesterol TEG in addition to 3'end guide strand Cy3) for 1 h at 37ºC, in a final volume of 100 µL[11]. Regardless of the method used, all samples were purified using ExoQuick, as per the manufacturer's instructions. Briefly, samples were incubated with Exoquick reagent in 1:5 (v/v) (i.e. 1 ExoQuick volume to 5 sEV sample volumes) for 30 min on ice, centrifuged for 3 min at 13.000 g, the supernatant and the pellet were separated and fluorescence was measured on each fraction.

The emission spectra of all samples, excited at $\lambda_{ex=}$535 nm, was measured from $\lambda_{em}$=563 nm until $\lambda_{em}$=700 nm (incremental steps of 3 nm) in a microplate reader Synergy™ H1 (Biotek) and the highest point for each sample was considered to calculate the loading efficiency of each method. The loading efficiency on each condition, including the control without sEVs, was calculated using the formula: fluorescence intensity of the pellet/(fluorescence intensity of the pellet + fluorescence intensity of the supernatant). For each condition and each type of sEV, the fluorescence value of the respective control was subtracted to the measured value and this was expressed, in percentage, as the loading efficiency.

For experiments where detection of the miRNA was incompatible with fluorescence, i.e. RT-qPCR, labelling of miRNA-124-Cy5 was used to obtain a fluorescence profile of miRNA-labelled sEVs. In this case, samples were excited at $\lambda_{ex=}$633 nm, and emission was measured from $\lambda_{em}$=660 nm until $\lambda_{em}$=700 nm (incremental steps of 1 nm).

**sEV loading and RNase treatment.** mEVs ($2x10^{10}$ total particles) were incubated overnight at 4ºC with miRNA-124-Cy5 (10 pmol) for passive loading, or underwent Exo-Fect™ loading as

described above. As a control, the same amount of fluorescent miRNA in the absence of sEVs was used. Samples where then purified via ExoQuick as described above in the previous point, and their fluorescence was measured. Subsequently, purified mEV pellets or control pellets were subjected to 2 μg/mL RNAse (# R5125, Sigma- Aldrich), in a final volume of 150 μL, treatment for 30 min at room temperature and re-purified via ExoQuick. Finally, their fluorescence was measured and compared with the results prior to RNAse treatment.

**qRT-PCR analyses of miRNA content.** To evaluate miRNA expression in sEVs, total RNA was extracted using the RNeasy Micro Kit (#74004 Qiagen) as per the manufacturer´s instructions. cDNA was synthesized for each sample from the amount of RNA extracted from $2^{10}$ sEVs using the Mir-X™ miRNA First-Strand Synthesis Kit (#638313, Takara). Finally, qPCR was performed on the CFX Connect Real-Time System (Bio-Rad) using the NZYSpeedy qPCR Green Master Mix (2x) (#MB224, Nzytech). Reverse primer was the universal 3' mRQ primer (Takara). Forward primer sequences were: 5'-TTAATGCTAATCGTGATAGGGGT-3' (hsa-miR-155-5p) and 5'-GATCTCGTCTGATCTCGGAAG-3' (5s rRNA). For RNU6 (RNA, U6 small nuclear) amplification, the forward primer 5'-TCGGCAGCACATATACTAA-3' and the reverse primer 5'-GAATTTGCGTGTCATCCT-3' were used.

**sEV dye labelling**. Labelling of sEVs with the fluorescent probes 1-[4-(trimethylamino)pheny1]-6-phenylhexa-1,3,5-triene (TMA-DPH) and N-hexadecyl-7-nitro-2,1,3-benzoxadiazol-4-amine (NBD-$C_{16}$) was achieved through the addition of 1% (v/v) from a stock solution of the probe in DMSO, into a solution of sEVs in PBS while gently stirring in the vortex, followed by incubation overnight at 37 $^{o}$C. For a concentration of sEVs of $8.75 \times 10^{11}$ particles/mL a final concentration of 1 μM TMA-DPH and 0.1 μM NBD-$C_{16}$ was used. Loading of sEVs with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, #34554 Invitrogen) was performed as per the manufacturer's instructions. Briefly, CFDA-SE was dissolved in DMSO and sEVs were incubated in a solution of 20 μM of CFDA-SE in PBS with 2% (v/v) DMSO, for 90 min, at 37 ºC. The reaction was stopped by diluting the sample in 0.1% (v/v) BSA in PBS. The sEVs were then attached to CD9 immuno-labelled magnetic beads (#10620D Invitrogen) as per the manufacturer's protocol. Briefly, beads were washed in PBS and incubated with

sEVs overnight at 4 ºC. Then, samples were washed twice with PBS and the fluorescence of the sEVs was measure on a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with a thermostatted multicell holder. Before the measurements, the sEV solution was transferred to a 5 mm fluorescence cuvette and placed on top of a magnet for 5 min to sediment the sEVs. The cuvette was then transferred to the fluorimeter. The horizontal excitation beam was positioned above the sedimented sEVs thus measuring only fluorescence from CF-SE in the aqueous supernatant. Fluorescence intensity was followed over time at $\lambda_{exc}$=485 nm $\lambda_{em}$= 516 nm for incubation at 37 $^{o}$C. For *in vitro* cellular assays, sEVs were labelled with PKH67 (Sigma-Aldrich) as per the manufacturer´s instructions. Briefly, $2\times10^{10}$ sEVs were diluted in the kit buffer (diluent C) 1:1 and then PKH67 in diluent C (1:75) was mixed with the diluted sample. Subsequently, samples were incubated for 3 min at RT, followed by purification by ultracentrifugation as described above. As a control for PKH67 complexation with sEVs, the same protocol, in the applicable assays, was used in the absence of sEVs. In assays where Exo-Fect™-miRNA was used to modulate sEVs, that step was performed after PKH67 labelling. As a control for that setup, the Exo-Fect™-miRNA mix was incubated with PKH67 directly and processed was described above.

**Exo-Fect™ toxicity assays**. To assess the cytotoxicity of Exo-Fect™, human umbilical vein endothelial cells (HUVECs) were seeded on 1% (w/v) gelatin-coated porcine skin (Sigma-Aldrich) 96-well plates (Corning), at a density of $10^4$ cells per well in endothelial growth medium 2 (EGM2, Lonza) with EV-depleted FBS and left to adhere overnight. Cells were either modulated with Exo-Fect™-miR-loaded mEVs or native mEVs. Final concentration of miRNA was 25 nM per well. After 24 h, cells were washed with PBS, fixed with 4% (v/v) PFA and washed at RT with PBS. Then, cells were stained with 10 ng/mL Hoechst 33342 for 10 min at RT and imaged using the GE Healthcare™ InCell 2200 Analyzer imaging system, using a 20× objective, excitation wavelength of 405 nm. Per well, 8 different regions of interest were used to count the total number of nuclei and this was used as a proxy for the total number of cells within the different conditions. For the toxicity titration, cells were seeded and handled as detailed above with the exception that in the day following seeding, increasing concentrations

of Exo-Fect™, DMSO and ExoQuick were added to the cells and incubated for 24 h. Cells were then fixed and imaged as detailed above.

**sEV uptake assay.** HUVEC were plated in a 24 well plate at a density of $6\times10^4$ cells/well and left to adhere for 24 h. Cells were pre-incubated with different endocytosis inhibitors (details below) for 30 min followed by 4 h co-incubation with PKH67-labelled mEVs or Exo-Fect™-miR-155-modulated mEVs ($1.5\times10^9$ particles/mL). The following inhibitors were tested: nocodazole (5 µM), cytochalasin D (25 µM), filipin III (25 µM), chlorpromazine (25 µM) and dynasore (100 µM). The concentrations of the inhibitors were based in values previously reported in the literature[18,19] and validated to have no cytotoxic effect during the period of the assay. The toxicity elicited by each inhibitor upon 4.5 h exposure to the cells was evaluated using a CellTiter Glo kit (Promega). After incubation, cells were washed with PBS, trypsinized and centrifuged, followed by 5 min incubation with Trypan blue (0.004% (w/v)) to quench the fluorescence of non-internalized EVs[20]. Finally, cells were centrifuged, resuspended in PBS and cell fluorescence was quantified by flow cytometry (BD Accuri C6 Plus). As a control, cells were exposed to sEVs in the absence of inhibitors and to inhibit all forms of endocytosis, cells were incubated with sEVs at 4ºC.

**Intracellular trafficking of sEVs.** HUVEC were seeded in a 15 well IBIDI plate at a density of $10^4$ cells/well and left to adhere for 24 h. Cells were incubated with PKH67-labelled mEVs or Exo-Fect™-miR-155-modulated mEVs ($2.5\times10^9$ particles/mL) for 1, 2 and 4 h in EV-depleted EGM-2 medium (Lonza #CC-3162). After incubation, cells were washed and incubated with LysoTracker red DND-99 (Invitrogen, 100 nM) for 30 min followed by fixation with 4% (v/v) paraformaldehyde (PFA). To investigate the colocalization with early endosomes, after incubation with sEVs, cells were fixed with 4% (v/v) PFA. Next, cell membrane was stained with a mouse anti-human CD31 (DAKO, 1:50) primary antibody, followed by incubation with Alexa-fluor$^{633}$ rabbit anti-mouse (Invitrogen 1:1000) secondary antibody. In a different subset of experiments, early endosomes were labeled with rabbit anti-human EEA1 (Cell Signaling Technologies, 1:100) primary antibody followed by incubation with Alexa-fluor$^{633}$ goat anti-rabbit secondary antibody (Invitrogen, 1:1000). Cell nuclei were counterstained with DAPI and

imaged using the INCell analyzer (GE Healthcare) followed by image analysis using INCell Developer Tollbox. In addition, cells were imaged in a confocal microscope Zeiss LSM 710 to evaluate the colocalization between PKH67-labeled mEVs and lysotracker. Image acquisition was performed with Plan-Apochromat 40×/1.4 oil immersion objective and the images were analyzed with ImageJ software.

**miR functional transfer assay**. HEK-293T transfected with a reporter vector were kindly offered by Dr. Irvin Chen (David Geffen School of Medicine, University of California at Los Angeles). The reporter vector encodes EGFP conjugated to the binding sites of miR-302a and miR-302d, and mCherry conjugated to the binding sites of miR-142-3p, miR-155-5p and miR-223[21]. HEK-293T cells were cultured in T-75 culture flasks (2 million cells/flask) at 37 ºC in a humidified atmosphere of 5% $CO_2$ in DMEM cell culture media containing 10% (v/v) FBS and 0.5% (v/v) penicillin-streptomycin. For the mCherry knockdown experiments, HEK-293T cells were seeded in sEV-depleted medium in collagen-coated 96-well plate wells. Cells were left to adhere overnight and the following day, native sEVs (mEVs, uEVs or fEVs) ($1.5\times10^9$ particles/mL), freshly prepared or stored (>2 days at -80°C) Exo-Fect™-modulated sEVs ($1.5\times10^9$ particles/mL), cholesterol-miR-modulated mEVs ($1.5\times10^9$ particles/mL) or Lipofectamine RNAiMAx were used to transfect the cells with miR-155-5p or scramble miRNA at a final concentration of 25 nM. As a control for Exo-Fect™-modulated sEVs, the product of the sEV loading reaction (i.e. Exo-Fect™ protocol) performed in the absence of sEVs (fresh or stored) was used in the same proportions. After 24 h, transfection medium was discarded and medium containing 10 ng/mL Hoechst 33342 was added to the cells and after further 48 h medium, without Hoechst 33342, was refreshed. Cells were imaged alive every 24 h after transfection using the GE Healthcare™ InCell 2200 Analyzer imaging system. The analysis of the images was done using an InCell Investigator package based on the segmentation of the nuclei and quantification of mCherry within the nuclear periphery.

**Statistical analyses**. All the results showed in this work are presented as an average of the number of samples for each condition and standard deviation (SD). Statistical testing was performed using GraphPad Prism® 6.0 software. The statistical tests used in this work

consisted in student's t test and One-way ANOVA with Dunnet's multiple comparisons test correction. A P value <0.05 was considered statistically significant.

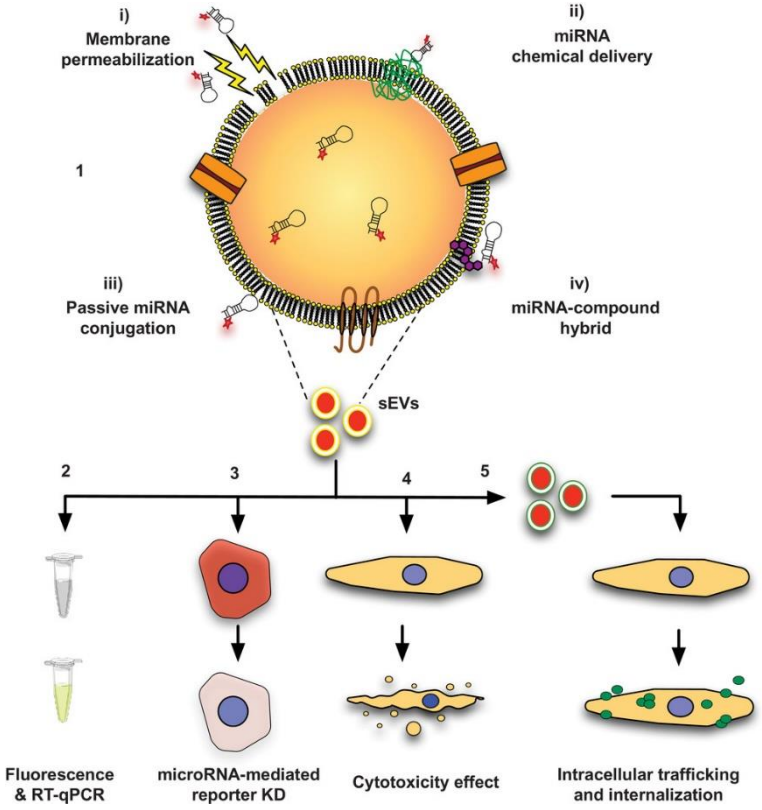


**Figure 1 – Schematic representation of the different methods used to modulate sEVs with a Cy3-labelled miRNA mimic and the follow-up assays performed to validate the modulation and assess the bioactivity of the modulated sEVs.** Five different methodologies have been used to load miRNAs into sEVs: transfection by Exo-Fect™ or cholesterol-modified miRNA and membrane permeabilization by a detergent (saponin), electroporation or heat shock. The modified sEVs were then characterized regarding their loading efficiency by fluorescence and qRT-PCR analyses (2), bioactivity in the HEK-293T reporter cell line (3), cell toxicity using a cell viability assay (4) and capacity to transfect cells (5).

## Results

### Exo-Fect™™ is effective in the loading sEVs with short non-coding RNAs

To identify the most efficient method for loading sEVs with a fluorescently-labelled miRNA, we decided to test, side-by-side, five methods previously reported in the literature. Follow-up experiments were performed to confirm the loading of the miRNA onto the sEVs and their bioactivity **(Fig. 1)**. Given the known variability in sEV composition depending on the cell/biofluid source, the most efficient loading strategy was further tested in sEVs isolated from (i) conditioned medium of hUCBMNCs, (ii) human urine and (iii) foetal bovine serum. sEVs secreted from hUCBMNCs (from now on named as mEVs) have been used because these

cells are easily obtained from multiple stem cell banks and their regenerative potential in the context of skin wound healing has been recently demonstrated by us[14]. sEVs obtained from human urine (uEVs) and bovine serum (fEVs) were used because these fluids are relatively easy to obtain and therefore one can obtain large numbers of sEVs for drug delivery applications. All sEVs were isolated using a standard differential ultracentrifugation protocol[16] and characterized by NTA (**Supp. Fig. 1a**), pulse analysis light scattering (PALS) (**Supp. Fig. 1b**) and TEM analyses (**Supp. Fig. 1c**). Regardless of the sEV source, TEM analyses showed the presence of cup-shaped structures, typical of sEVs. NTA analyses showed that the majority of sEVs had a size in the range of 100-200 nm, which is in accordance with sEVs reported in previous studies[22]. In addition, PALS analyses showed that mEVs had a zeta potential of -40.2+/-1.1 mV, while uEVs and fEVs had a zeta potential of -18.1+/-1.5 mV and -24.5+/-1.4 mV, respectively. These differences are likely due to differences in their membrane composition, which ultimately reflect their different origin. As for the purity of our samples, mEVs, uEVs and fEVs showed averages of $2.30\times10^9$ part/µg, $3.30\times10^9$ part/µg and $2.60\times10^9$ part/µg of protein, respectively. Based on previous studies[23], our samples fall within the same range of relative low purity, likely owed to the presence of some contaminants, as observed in TEM. To ensure that our preparations were enriched in sEVs, we performed western blot analyses to detect common EV markers and potential contaminants in two different batches of uEVs and mEVs (**Supp. Fig. 1d**). Our results showed that sEVs derived from both sources expressed the markers CD63, CD9 and GAPDH, although their expression level appeared donor-dependent. Alix was only detectable in uEVs and calnexin, an endoplasmic reticulum marker, was not detected in uEVs. ApoA-1, a contaminant found in high-density lipoproteins, was not found in mEVs. Urine sEV samples showed the presence of Tamm-Horsfall protein (THP), a protein highly present in urine samples[24]. Overall, our results showed that our samples were enriched in sEVs.

Next, we evaluated the efficiency of the different methods to load hsa-miR-155-5p-Cy3 into mEVs. The transfection procedures were based in protocols already published (e.g. electroporation, heat shock, saponin and cholesterol-modification)[6,7,9-11] or as per the manufacturer´s instructions (e.g. Exo-Fect™). Importantly, to render the results comparable across the different techniques, the same post-loading purification method, ExoQuick kit, was used thus yielding two fractions (pellet and supernatant) (**Fig. 2a**). To calculate the loading efficiency, after purification, we quantified the fluorescence of the pellet-containing sEVs and compared it to the total fluorescence (pellet + supernatant) (**Fig. 2b**). Overall, our results showed that the loading efficiency was higher for sEVs transfected with Exo-Fect™ than with the other selected methods (**Supp. Fig. 2a**). In the case of electroporation and heat shock in the presence of calcium chloride, our results suggested that the fluorescently-labelled miRNA precipitated in the absence of sEVs therefore leading to a sEV-non-specific fluorescent signal in the pellet fraction (10% of the total fluorescence for electroporation and 87% of the fluorescence for the heat shock in the

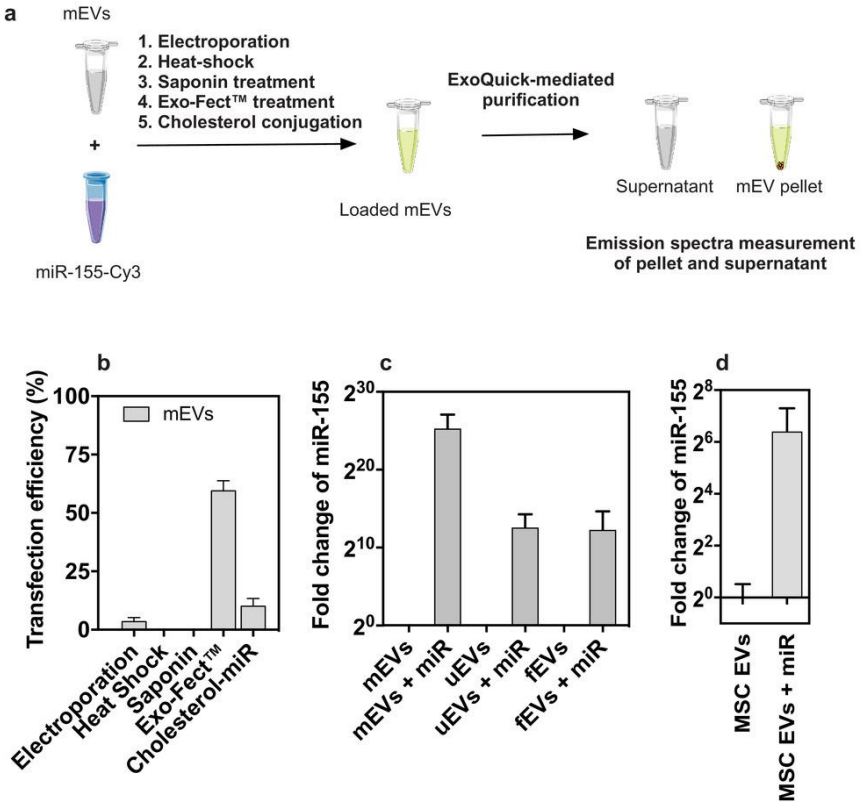


**Figure 2 – Modulation of sEVs.** (a) mEVs were loaded with miRNA-155-Cy3 using electroporation, heat shock, saponin permeabilization, Exo-Fect™ treatment and cholesterol conjugation. sEVs were then purified with ExoQuick and the fluorescence spectrum of the resulting pellet (sEVs) and supernatant (leftover probe) were quantified. The point of highest fluorescence of each condition was considered for calculating relative transfection efficiencies. (b) Transfection efficiencies were calculated for each condition as described in the Methods section (n=3 for all conditions tested). (c) qRT-qPCR analyses of miR-155-5p expression in Exo-Fect™-modulated and native mEVs. Results represent the fold change compared to non-modulated sEVs. The delta delta Cq method was used for the calculations and 5s RNA was used as a housekeeping control (n=2-3 with 2-3 technical replicates). (d) qRT-qPCR analyses of miR-155-5p expression in sEVs derived from MSCs or from MSCs transfected with miR-155-5p using lipofectamine RNAiMax (n=3 with 2 technical replicates).

presence of calcium chloride) (**Supp. Fig. 2a**). However, in the case of electroporation, after subtracting the fluorescence values of the control, we showed a 3% increase in fluorescence in the pellet fraction. Conversely, in the case of saponin, the vast majority of the fluorescent signal was present in the supernatant fraction, suggesting that it was not possible to load the miRNA into sEVs using this methodology. In the case of Exo-Fect™, our results showed that 50%, 21% and 30% of the fluorescence was found in the pellet fraction of mEVs, uEVs and fEVs, respectively (**Supp. Fig. 2b**), after normalizing to the control. Intriguingly, in the case of Exo-Fect™, we observed an overall decrease in total fluorescence (pellet combined with supernatant) suggesting an Exo-Fect™-mediated quenching effect, more pronounced in the presence of sEVs, that led to an underestimation of the overall effect of Exo-Fect™ (**Supp. Fig. 2c**). In addition, to assess whether the loaded miRNA was exposed or accessible to nucleases after Exo-Fect™ transfection, we treated sEVs loaded miR-124-Cy5 (through passive loading and Exo-Fect™) with RNAse (**Supp. Fig. 2d**). Our results showed that, in the absence of Exo-Fect™, there was a 73% reduction in the fluorescence of miR-124-Cy5, compared to a 11% reduction in fluorescence in the presence of Exo-Fect™. To confirm the loading of sEVs with the exogenous hsa-miR-155-5p, we have quantified by qRT-PCR the expression of hsa-miR-155-5p on Exo-Fect™-modulated sEVs from the three different sources (**Fig. 2c**). Our results showed >$2^{10}$-fold increase in miR-155-5p expression compared to native sEVs. Overall, our results showed that Exo-Fect™ was capable of efficiently transfecting sEVs with a miRNA of interest in all the three sEV sources herein tested. The larger differences observed between sEV loading are likely due to differences in the endogenous amounts of the miRNA and housekeeping tested and intrinsic biological properties of sEVs, which, as noted previously, differ, and may render some EV types more easily loadable. However, the fluorescence and miRNA expression patterns were globally similar, with mEVs being the most easily loaded source, followed by fEVs and uEVs.

To confirm that ExoQuick-mediated purification did not cause co-precipitation of the labelled miRNA, we performed loading of sEVs with a fluorescent miRNA and Exo-Fect™ followed by ODG purification. In total, we obtained 15 fractions (1.5 mL/fraction) of increasing density (**Supp. Fig. 3a**) and per fraction, we quantified the total number of particles and total fluorescence (**Supp. Fig. 3b**). Our results showed that the majority of particles (82%) and fluorescence (73%) localized to fractions 10 to 13 (**Supp. Fig. 3c**), corresponding to the 1.08

g/mL to 1.15 g/mL density range (sEV fraction). In addition, we used qRT-PCR to quantify the expression of the non-fluorescent miR-155-5p in native and modulated sEVs purified by ODG. Our results showed a $>2^{10}$-fold increase in miR-155-5p expression in modulated sEVs, a value comparable to the results obtained with the ExoQuick purification (**Supp. Fig. 3d**).

Transfection of EV-secreting cell with the precursor or mature miRNA has been investigated as a platform to enrich sEVs with a miRNA of interest[25]. In order to compare post-isolation modulation with modification of the secreting cell and subsequent harvesting of sEVs, we transfected mesenchymal stromal cells (MSCs) with lipofectamine complexed with a fluorescently labelled miRNA (hsa-miR-155-5p-Cy3) and isolated the sEVs from the conditioned medium (**Fig. 2d**). Although the fluorescence of sEVs was below the detection limit, we were able to quantify the level of miR-155-5p by qRT-PCR and our results showed a 22-fold increase in sEVs isolated from transfected MSCs compared to the control (non-transfected cells) (**Fig. 2d**). However, the concentration of miR-155-5p was several orders of magnitude lower than the concentration of miR-155-5p observed in sEVs modulated with Exo-Fect™. Based on these results, we decided to investigate in more detail the complex miRNA-Exo-Fect™-sEV regarding its biophysical structure and bioactivity.

**Exo-Fect™ interferes with sEV membrane structure**

Currently, it is unknown if Exo-Fect™ modulation results in the internalization of the miRNA of interest into the lumen of sEVs or fosters its interaction with the sEV surface. To address this question, we started by characterizing the Exo-Fect™-modulated mEVs by NTA, TEM and PALS analyses. In the absence of mEVs, Exo-Fect™ did not form observable nor quantifiable particles as measured by NTA (**Supp. Fig. 4a.1**) or seen by TEM analysis (**Supp. Fig. 4b.1**). Likewise, in the presence of miRNA, but in the absence of sEVs, no quantifiable particles were detected by NTA (i.e. <15 particles/frame). However, the Exo-Fect™ protocol appeared to induce mEV aggregation as observed by TEM (**Supp. Fig. 4b.2**) and NTA analyses (**Supp. Fig. 4a.1 and 4c**). Data from DLS analysis also supports this hypothesis, showing an increase in the average particle size which correlated with the percentage of Exo-Fect™ used with mEVs (**Supp. Fig. 4d**). In addition, the polydispersity of Exo-Fect™-modulated mEVs increased when higher amounts of Exo-Fect™ were used (**Supp. Fig. 4e**). Lastly, ExoQuick-based purification

of mEVs led to a small shift in zeta potential (**Supp. Fig. 4f**), which was further amplified by Exo-Fect™-mediated transfection of miRNA onto mEVs, from -40 mV to -20 mV (**Supp. Fig. 4g**). Collectively, these results suggest that Exo-Fect™ may interfere with the membrane of sEVs and ultimately promote their aggregation.

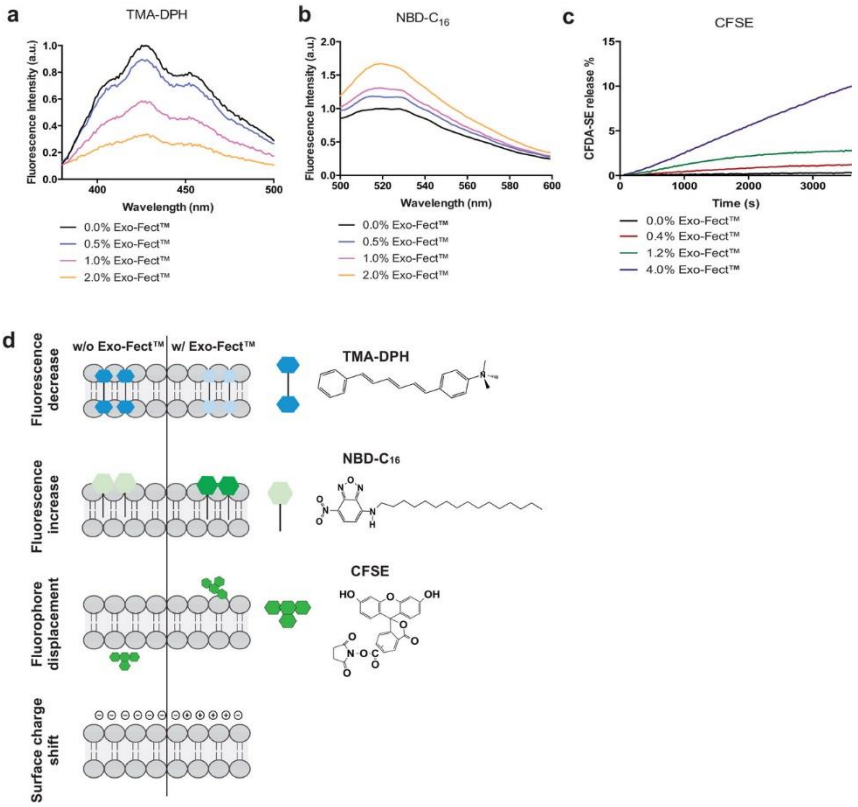


**Figure 3 – Exo-Fect™ interaction with sEV membrane**. Effect of increasing amounts of Exo-Fect™ on the fluorescence intensity of TMA-DPH (a) and NBD-C16 (b), and on the release of encapsulated CF-SE (b) where the inset shows the fluorescence spectra after 60 min incubation and the main plot shows the release % calculated from the fluorescence intensity increase. The final concentration of Exo-Fect™ in (a) and (b) is 0% (—), 0.5% (—), 1% (—) and 2% (—), and in (c) is 0% (—), 0.4% (—), 1.2% (—) and 4% (—). (d) Schematic representation of the proposed mechanism of interaction between Exo-Fect™ and sEV membrane regarding how it affects different fluorophores and the surface charge of the sEVs.

To confirm that Exo-Fect™ interacts with the membrane of sEVs, we performed biophysical analyses in which modulated mEVs were labelled with the fluorescent probes TMA-DPH or NBD-$C_{16}$. The fluorescence group of TMA-DPH is located at the hydrophobic core of the lipid membrane[26], while the one of NBD-$C_{16}$ is located at the membrane surface[27,28]. The fluorescent probes were equilibrated overnight with the mEVs, leading to a symmetric labelling of both membrane leaflets[29]. The next day, we quantified the changes in fluorescence intensity for both fluorophores in the presence and absence of Exo-Fect™ and our results showed that, upon addition of Exo-Fect™ to the mEVs labelled with TMA-DPH, the fluorescence dropped to a third of its initial value (**Fig. 3a**). Conversely, the fluorescence intensity of NBD-$C_{16}$ increased 3-fold upon addition of Exo-Fect™ (**Fig. 3b**). The observation that both probes were affected by Exo-Fect™ suggests that Exo-Fect™ alters the properties at the surface as well as in the core of the sEV membrane.

To further confirm that Exo-Fect™ interferes with the membrane of sEVs, we encapsulated the fluorescent molecule CFDA-SE inside mEVs, where it reacts with amino groups from proteins and other biomolecules[30]. We conjugated mEVs with anti-CD9 conjugated magnetic beads in order to isolate mEVs from the solution when required. In the absence of Exo-Fect™, we did not observe a significant increase in the fluorescence of the supernatant after incubation of mEV in PBS during 4 h at 37°C, indicating that there was no significant leakage of encapsulated CFDA-SE. However, the addition of Exo-Fect™ led to an increase in the fluorescence of the supernatant, suggesting that CFSE was leaking from the modulated mEV (**Fig. 3c**). We monitored the increase in fluorescence of the supernatant of these mEVs for several weeks to evaluate the fluorescence signal corresponding to the total leakage of CFDA-SE (results not shown). Based on this analysis, we were able to calculate the leakage efficiency at the different concentrations of Exo-Fect™ tested and we showed that, after only 1 h incubation of mEVs with 2% (v/v) Exo-Fect™ at 37°C, 10% of the CFDA-SE was released from the mEVs. Altogether, these results indicated that Exo-Fect™ interacted with the surface of mEVs (**Fig. 3d**) leading to the aggregation and perturbation of its barrier properties. The observation that CFDA-SE leakage was not instantaneous suggested that for concentrations up to 2% (v/v), Exo-Fect™ perturbation of sEV membrane did not lead to a disruption of sEV membrane integrity.

**Exo-Fect™ allows for functional transfer of miRNA to recipient cells**

Having established that Exo-Fect™ was an efficient method to modulate sEVs with a miRNA of interest, we decided to evaluate whether Exo-Fect™-modulated sEVs behaved similarly to their native counterparts in cellular assays. To that end, mEVs were loaded with the miRNA of interest or scramble miRNA (both at 25 nM) using Exo-Fect™ as a transfection agent, and administered to human umbilical vein endothelial cells (HUVECs) for 24 h. Our results showed that native mEVs or mEVs modulated with Exo-Fect™ at concentrations below □□□□□□v/v) had low (≤10%) impact in cell viability. mEVs modulated with Exo-Fect™ at concentrations above □□□□□□v/v) significantly decreased cell viability (**Supp. Fig. 5a**) likely due to the presence of Exo-Fect™. Indeed, Exo-Fect™ was toxic for cells in concentrations above 0.5% (v/v) (**Supp. Fig. 5b**). Altogether, our results suggest that sEVs modulated with Exo-Fect™ can

be used for miRNA delivery with residual cell toxicity for concentrations of Exo-Fect™ below 0.5% (v/v), at least in endothelial cells, and this concentration was used for subsequent studies.

To evaluate the bioactivity of Exo-Fect™-modulated mEVs we used a HEK-293T reporter cell line coding for the mCherry protein, with the target sequence for miR-155-5p expressed in its 3'-UTR[21]. Upon successful transfection of this cell line with miR-155-5p, the expression of mCherry was downregulated leading to a decrease in the fluorescent signal (**Fig. 4a**). Cells were transfected with Exo-Fect™-miRNA-155-modulated sEVs (mEVs, uEVs or fEVs) or with

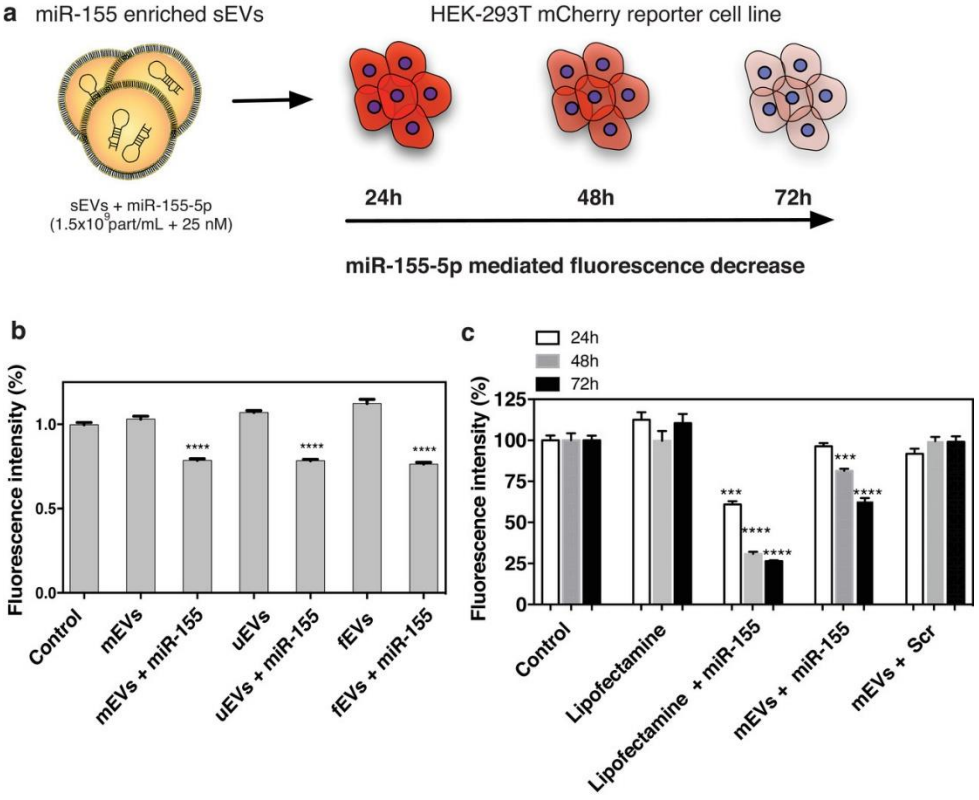


**Figure 4 – Exo-Fect™-modulated sEVs are functionally active in vitro**. (a) Schematic overview of the protocol used to determine the capacity of miRNA-modulated sEVs to deliver their cargo onto a HEK-293T reporter cell line. This reporter cell line constitutively expresses mCherry and contains a binding site for hsa-miR-155-5p on its sequence and thus, upon successfully transfection with miR-155-5p, the mCherry signal is reduced proportionally to the transfection efficiency. sEVs ($1.5\times10^9$ part/mL) loaded with miR-155-5p or Lipofectamine complexed with miR-155-5p was incubated with the reporter cell line (final miR concentration was 25nM) for 48 h, upon which the medium was changed. After 24 h, the nucleus was stained with Hoechst 33342, the cells were imaged and the fluorescence quantified every 24 h for 3 days. (b) Quantification of the average mCherry fluorescence intensity per cell at 72 h post incubation with mEVs, uEVs, fEVs or their Exo-Fect™-miR-155 modulated counterparts. Each condition was normalized to control (HEK-293T cells with no treatment). Statistical analysis reports to comparisons between each Exo-Fect™-miR-155 modulated condition and respective native sEV source. (n=2-3) (c) Quantification of the average mCherry fluorescence intensity per cell of native and modulated mEVs and control conditions. Per time point, all conditions were normalized to the control (HEK-293T cells without treatment). Results were obtained from one experiment with 3 technical replicates. Statistical significance test used was one-way ANOVA using Dunnet's correction, $P<0.05$.

their native counterparts, for 72 h (**Fig. 4b**) and regardless of the sEV source, the modulation with Exo-Fect™-miRNA-155 led to up to 24% decrease in the activity of the HEK-293T reporter cell line. We next investigated, only with mEVs, whether this effect was time dependent and how it compared with direct transfection of the reporter cell line with lipofectamine, a commonly used transfection agent. In this case, cells were transfected with Exo-Fect™-miRNA-155-modulated mEVs or lipofectamine complexed with the same miRNA and monitored every 24 h for up to 3 days. In cells that were non-transfected or transfected with lipofectamine alone, the fluorescence did not change. In contrast, cells transfected with miRNA-155, either with lipofectamine or Exo-Fect™-miRNA-155-modulated mEVs, showed a decrease of 74% and 28%, respectively, in cell fluorescence after 72 h (**Fig. 4c**). Although the efficiency of Exo-Fect™-miRNA-155-modulated mEVs was lower than lipofectamine, the results indicated that mEVs modulated with Exo-Fect™ retained their bioactivity.

Next, using the above-mentioned reporter cell line, we compared the loading efficiency of other methods to Exo-Fect™. To this end, sEVs loaded with cholesterol-miR-155, a strategy previously used to load sEVs with miRNAs[11], were incubated with the HEK-293T reporter cell line and our results showed that, compared to the control, no significant change in reporter activity was observed. These results suggest that, under the same testing conditions, this delivery strategy was less efficient (**Supp. Fig. 6a**). The differences observed between our results and previous results may be ascribed to differences in EVs: cholesterol-miR molecules ratio.

For many applications, the storage of sEVs is required before its use. Therefore, we evaluated whether the biological activity of Exo-Fect™-modulated sEVs could be compromised by the storage conditions[31]. To that end, freshly prepared mEVs were compared with the same batch of modulated mEVs preserved at -80℃ for over two days. The results showed that the biological activity, assessed using the above-mentioned reporter cell line, was largely preserved upon storage, with no statistical differences between time points across storage conditions (**Supp. Fig. 6b**). Moreover, in the absence of sEVs, Exo-Fect™-miR by itself, either used immediately or upon storage at -80℃ for over two days, was unable to elicit the knockdown of the reporter gene as described above for the formulations containing sEVs

(**Supp. Fig. 6b**) supporting the idea that sEVs are crucial for the functional transfer of the miRNA.

Next, we asked whether Exo-Fect™ could interfere with the intracellular trafficking of sEVs. To address this question, mEVs were labelled with PKH67, a fluorescent membrane amphiphilic dye commonly used to label sEVs[21,32]. We confirmed that PKH67 did not fluoresce in the absence of sEVs and that the presence of Exo-Fect™ in the sample did not alter sample fluorescence, prior to cell administration (**Supp. Fig 7a**). Furthermore, Exo-Fect™ did not form particles with either PKH67 and/or miRNA that could be localized in the sEV fractions upon purification by ODG (**Supp. Fig. 7b**). After establishing the adequacy of PKH67 to our purposes, HUVECs were incubated with native or Exo-Fect™-modulated mEVs, for up to 4 h, after which cells were fixated. These cells were subsequently labelled with DAPI (nuclei), CD31 (endothelial cell membrane) and with Lysotracker red (lysosomes – **Fig. 5a**) or EEA1 (early endosomes – **Fig. 5b**). sEV internalization was expressed taking into account the number of cells that had mEVs (green fluorescence) relative to the total number of cells labelled with CD31 (**Fig. 5c**). Approximately 70% of HUVECs internalized Exo-Fect™-modulated mEVs after 1 h while only 14% of cells internalized native sEVs (**Fig. 5c**). In addition, cells transfected with Exo-Fect™-modulated mEVs had higher fluorescence than cells transfected with native sEVs indicating that the number of sEVs per cell was higher in Exo-Fect™-modulated sEVs (**Supp. Fig. 8a**). In order to evaluate whether Exo-Fect™ modulation altered sEV intracellular trafficking, we compared the colocalization of mEVs either with lysosomal (Lysotracker$^{+}$) or early endosomal (Early Endosome Antigen (EEA1) 1$^{+}$) compartments. Exo-Fect™-modulated mEVs had lower co-localization with the endolysosomal compartment as compared to native sEVs, with a 37% difference at 1 h and a difference of 10% at 4 h (**Fig. 5d**). In addition, Exo-Fect™-modulated mEVs had also lower co-localization with early endosomes as compared to native sEVs between 2 and 4 h (2 h: 8% *vs* 3.4%; 8 h: 9.2% *vs* 4.75%) (**Fig. 5e**). To confirm that the results were not due to differences in the number of lysosomes between the two experimental groups or due to artifacts in the lysotracker staining, we quantified the fluorescence (**Supp. Fig. 8b**) and area of lysosomes per cell (**Supp. Fig. 8c**) with no statistical difference found.

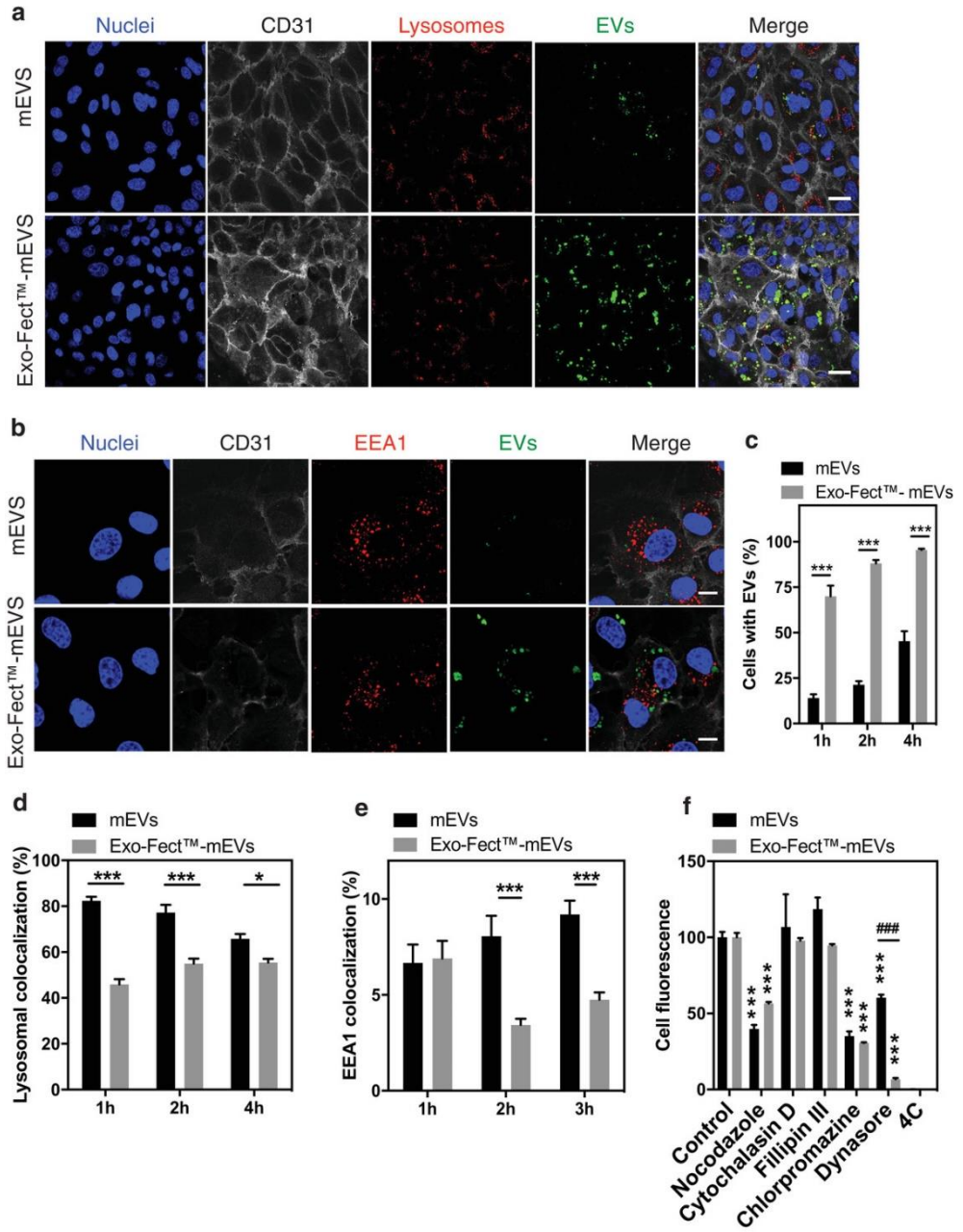


**Figure 5 – Internalization and intracellular trafficking of Exo-Fect™-modulated sEVs in endothelial cells.** Representative confocal images of HUVECs incubated for 2 h with mEVS (control) and Exo-Fect™-modulated mEVs, in a colocalization study with lysosomes (a; Lysotracker) and early endosomes (b; EEA1). Scale bar corresponds to 30 µm for lysosomal colocalization images and 10 µm for early endosome colocalization images. (c) Percentage of cells with internalized mEVs and Exo-Fect™-modulated mEVs as quantified by high content microscopy. (d) Quantification of colocalization with lysosomes and (e) early endosomes. (f) Assessment of internalization routes affected by endocytic pathway inhibitors. HUVEC were pre-incubated with endocytosis inhibitors for 30 min followed by 4 h co-incubation of PKH67-labelled mEVs or Exo-Fect™-modulated mEVs ($1.5\times10^9$ particles/mL) with each endocytosis inhibitor. After incubation, cells were washed with PBS, trypsinized and centrifuged, followed by 5 min incubation with Trypan blue (0.004% W/V) to quench the fluorescence of non-internalized sEVs. Cell fluorescence was quantified by flow cytometry. As control, cells were exposed to sEVs without any chemical inhibitor. To inhibit all forms of endocytosis, cells were incubated with sEVs at 4ºC. Results are expressed as mean±SEM (in c, d and f n=3, with 2 technical replicates per experiment; in e, n=1 with 3 technical replicates). Two-way ANOVA followed by Bonferroni´s post-test was used to compare mEVs and Exo-Fect™-modulated mEVs * and *** indicate $P<0.05$ and $P<0.001$, respectively. In f, comparison between mEVs and Exo-Fect™-modulated sEVs, ### indicates $P<0.001$. Comparison between control and inhibitors, *** indicates $P<0.001$.

To investigate whether Exo-Fect™ played a role in the internalization route of mEVs, HUVECs were pre-incubated with compounds known to inhibit specific endocytosis pathways (**Supp. Fig. 8d**), namely, nocodazole (microtubule-dependent endocytosis), cytochalasin D (actin-dependent endocytosis), filipin III (lipid raft-dependent endocytosis), chlorpromazine (clathrin-mediated endocytosis) and dynasore (dynamin-dependent endocytosis). The concentration of inhibitors used was based in previous studies[18,19]. Cells were then exposed to PKH67-labelled mEVs or Exo-Fect™-modulated mEVs for 4 h, after which their fluorescence was assessed via flow cytometry. Our results showed that cellular uptake of sEVs was mediated by endocytosis, as the cell incubation at 4ºC prevented sEV internalization. Moreover, endocytosis inhibition by nocodazole, chlorpromazine or dynasore was effective in reducing sEV uptake (**Fig. 5f**). Interestingly, dynasore was able to inhibit 93% the uptake of Exo-Fect™-modulated mEVs but only 40% of native mEVs.

**Discussion**

Here, we compared side-by-side five methodologies to load, post-isolation, exogenous miRNAs in sEVs obtained from three different sources. The methodology based in the transfection of vesicles with Exo-Fect™ yielded the most promising results based in the following parameters: (i) enrichment of miRNAs, (ii) capacity of the modified sEVs to transfer the exogenous miRNA to recipient cells and elicit a biological function (inhibition of the activity of a reporter cell line) and (iii) possibility to store the modified sEVs, for at least 2 days at -80℃. Yet, the methodology requires a critical selection of Exo-Fect™ concentration for sEV loading to avoid cytotoxicity given the fact that Exo-Fect™ remains adsorbed to the membrane of sEVs after purification with Exo-Quick (the method recommended by the manufacturer). In addition, we showed that Exo-Fect™ interferes with the membrane of sEVs.

Previous studies have highlighted the therapeutic potential of sEVs in different pathological contexts. In recent years, a lot of effort has been focused in enhancing the intrinsic potential of sEVs using a plethora of pre- and post-isolation methodologies[6-8,33]. Most of the work has been done in loading exogenous biomolecules in sEVs, in particular non-coding RNAs such as miRNAs[34]. Electroporation has been the most used methodology to load isolated sEVs[7,8,31]; however, the strategy presents important limitations. For example, electroporation may

induce miRNA and/or sEV aggregation and, overall, the loading efficiency within the sEVs is very modest[35-37]. In agreement with previous studies, our results indicated that electroporation promoted miRNA precipitation. Other loading strategies based on heat shock in the presence of calcium chloride[9] or the permeabilization of sEV membrane with saponin[10] have been used to load miRNAs into sEVs. According to our results, in the conditions herein tested, around 87% of the miRNA precipitated after heat shock, including in the absence of sEVs. Consequently, we cannot assess how much of that signal might be actual sEV modulation. Conversely, when we used saponin, we could not observe fluorescence in the sEV fraction. When comparing the size and concentration profiles of sEVs before and after treatment with saponin, no difference was found (data not shown), which indicates that sEV stability was not comprised by the detergent. Thus, whether the poor results with both these methodologies were caused by compound interference with ExoQuick remains to be determined and further purification procedures should be tested in future work.

Exo-Fect™ was the methodology that resulted in the highest loading of sEVs with an exogenous fluorescently-labelled miRNA. The loading was monitored using two different methods: (i) fluorescence of the exogenous miRNA loaded in sEVs and (ii) miRNA copies quantified by qRT-PCR. Different amounts of native miR-155-5p within each vesicle source likely contributed to variations in the enrichment of the miR-155-5p within each sample. Importantly, the enrichment of sEVs within the miRNA of interest was much higher using this post-isolation method than the classical transfection of the donor cell with the miRNA of interest followed by the isolation of sEVs from the culture medium. Interestingly, Exo-Fect™ methodology decreased the fluorescence of the initial miRNA likely due to a quenching resulting from the high concentration of miRNA loaded in sEVs[38]. Our results also showed that, depending on the sEV source, the loading efficiency varied which may be explained by the presence of contaminants in some samples. Urine-derived sEVs contained significant amounts of dark filaments observed by TEM. This is likely THP, a typical protein found in urine which may co-precipitate with sEVs isolated during ultracentrifugation and found by western blot in our samples[24]. Urine contaminants may interfere with different vesicle-dependent processes[39], and that may explain why miRNA-loading efficiency is reduced for this source of sEVs.

One possible explanation for the results reported herein was related with the possibility that ExoQuick purification could lead to the formation of Exo-Fect™ and miRNA complexes that could confound our results. To rule out this, we performed a series of controls where sEVs were absent from the process and showed that while such precipitation may occur (approx. 20%; **Supp. Fig. 2a)**, the effect that they may have in functional cellular assays is not measurable using our reporter cell model (**Supp. Fig. 6b**). Nevertheless, the ExoQuick-based protocol for purification warrants further scrutiny, especially in the context of translational applications. Overall, from a translational standpoint, the methodology presented has some pros and cons. First and foremost, the fact that sEVs may be used from any source post-isolation, without resorting to donor cell mass production and their respective modification with therapeutic compounds, is an important advantage. Additionally, the fact that the loading protocol is rapid and efficient, potentially capable of complexing different types of nucleic acids with sEVs, renders it a versatile solution. However, the fact that ExoQuick is not the best purification method in terms of sEV yield or purity[17], leaves space for further improvements to the protocol. Recent discussion has focused on scalable methods to yield high quality sEV preparations in the industrial and clinical scope[40]. These methods, such as tangential flow filtration and anion exchange chromatography, may be next step towards unlocking the translational potential of sEV formulations.

Our biophysical analyses of sEV modulated with Exo-Fect™ lead to a significant decrease in TMA-DPH fluorescence, which was indicative of a more polar environment around TMA-DPH[26]. In contrast, the fluorescence of NBD-$C_{16}$ increased indicating that the polarity around NBD was increased[28]. Taken together, these results indicate that Exo-Fect™ interacted and changed sEV membrane properties. In addition, Exo-Fect™ remained conjugated with sEVs after purification with ExoQuick and this can elicit cytotoxicity above a given concentration (in the case of endothelial cells above 0.5% (v/v)). Moreover, Exo-Fect™ presence in sEVs seems to protect the loaded miRNA from RNAse degradation. Further tests are necessary to understand whether the protection is due to the fact that the miRNA is located in the sEV lumen or due to a partial binding of the miRNA to the outer surface of the sEV while Exo-Fect™ acts as a protective layer against RNAses.

Functionally, miR-155 Exo-Fect™-modulated sEVs were able to inhibit the expression of mCherry in the HEK-293T reporter line, which, in our construct, had a binding site for this miRNA. While the extent of fluorescence decrease was lower than the one observed by cell transfection mediated by lipofectamine, it remains to be determined whether the limited knockdown effect of modulated sEVs was due to a limited endolysomal escape or a kinetic issue. Moreover, it would be interesting to pursue a similar functional study for all the different methods of sEV modulation, since methods with lower efficiency than Exo-Fect™ may still prove to be valuable in a given cellular model and/or therapeutic application. Nonetheless, preliminary tests with cholesterol-conjugated miR-155 on sEVs suggest that, under the conditions tested, Exo-Fect™ was the most efficient method of miRNA delivery.

Exo-Fect™-modulated sEVs displayed differences in cell internalization and intracellular trafficking. A previous study has shown that sEVs (without Exo-Fect™ modulation) are taken up by cells as single vesicles and a significant portion of sEVs (40-60%) seemed to accumulate in lysosomes after several hours and thus their content was likely degraded[41]. Our results showed that 1 h post transfection, sEVs without Exo-Fect™ modulation were slowly internalized by endothelial cells (approximately 14% of the cells were labelled with sEVs) but they showed high co-localization (82%) with the endolysosomal compartment and early endosomal compartments (6.7%). In contrast, within the same time frame, sEVs modulated with Exo-Fect™ were rapidly internalized by endothelial cells (approximately 70% of the cells were labelled with sEVs) and showed lower co-localization (45%) with the lysosomal compartment and similar profiles in endosomal inclusion (6.9% inclusion). At 4 h post transfection, the co-localization of native sEVs with the lysosomal compartment was still significantly higher than the one of Exo-Fect™-modulated sEVs (65% *vs* 55%, respectively). Likewise, the colocalization with early endosome marker nearly doubled for native sEVs when compared to modulated sEVs (9.2% *vs* 4.7% respectively). The lower co-localization of Exo-Fect™-modulated sEVs for early time points suggests that modulated sEVs may bypass the endolysosomal compartment more efficiently. Further studies are necessary to elucidate the endolysosomal escape mechanism. In addition, our results seem to indicate an impact of Exo-Fect™ on cellular uptake of sEVs. Upon inhibiting endocytosis pathways with different chemical compounds, we have found that both native sEVs and Exo-Fect™-modulated sEVs were internalized via dynamin and clathrin-mediated endocytosis given the impact of

dynasore and chlorpromazine, as well as nocodazole, a disruptor of microtubules that is also implicated in clathrin-mediated endocytosis[42]. Specifically, dynasore inhibited the uptake of Exo-Fect™-modulated mEVs at a higher level than for native mEVs. Dynasore is an inhibitor of dynamin-mediated membrane fission processes, such as clathrin and caveolae-dependent endocytosis[43] and our results suggest that these routes of cellular uptake play a larger role for Exo-Fect™-modulated sEVs than for their native counterparts.

Currently, approximately 30 independent studies have used Exo-Fect™ to load sEVs. The majority of these studies focused on loading small RNA duplexes (miRNAs, miRNA inhibitors and siRNAs)[44-48] in sEVs whereas others have attempted to load mitochondrial DNA[49], plasmid DNA[50], Y RNA[51] or small peptides[52]. These reports have established that Exo-Fect™ was a viable solution for the complexation of nucleic acids with sEVs. The studies of Pi *et al.* and Li *et al.*, using a quantification strategy similar to the one herein reported, showed that upon transfection of sEVs with Exo-Fect™, around 80% of the fluorescent signal remained in the sEV fraction of the reaction[13,53]. Nevertheless, we added a note of caution when interpreting fluorescent-based data for calculating the transfection efficiency since Exo-Fect™ consistently altered the emission spectra of fluorophores and may also induce a quenching-like effect. Ultimately, our data supports the idea that Exo-Fect™ is an efficient strategy to conjugate small nucleic acids within sEVs and can even enhance the intracellular trafficking and delivery of molecules of interest.

**Acknowledgments**

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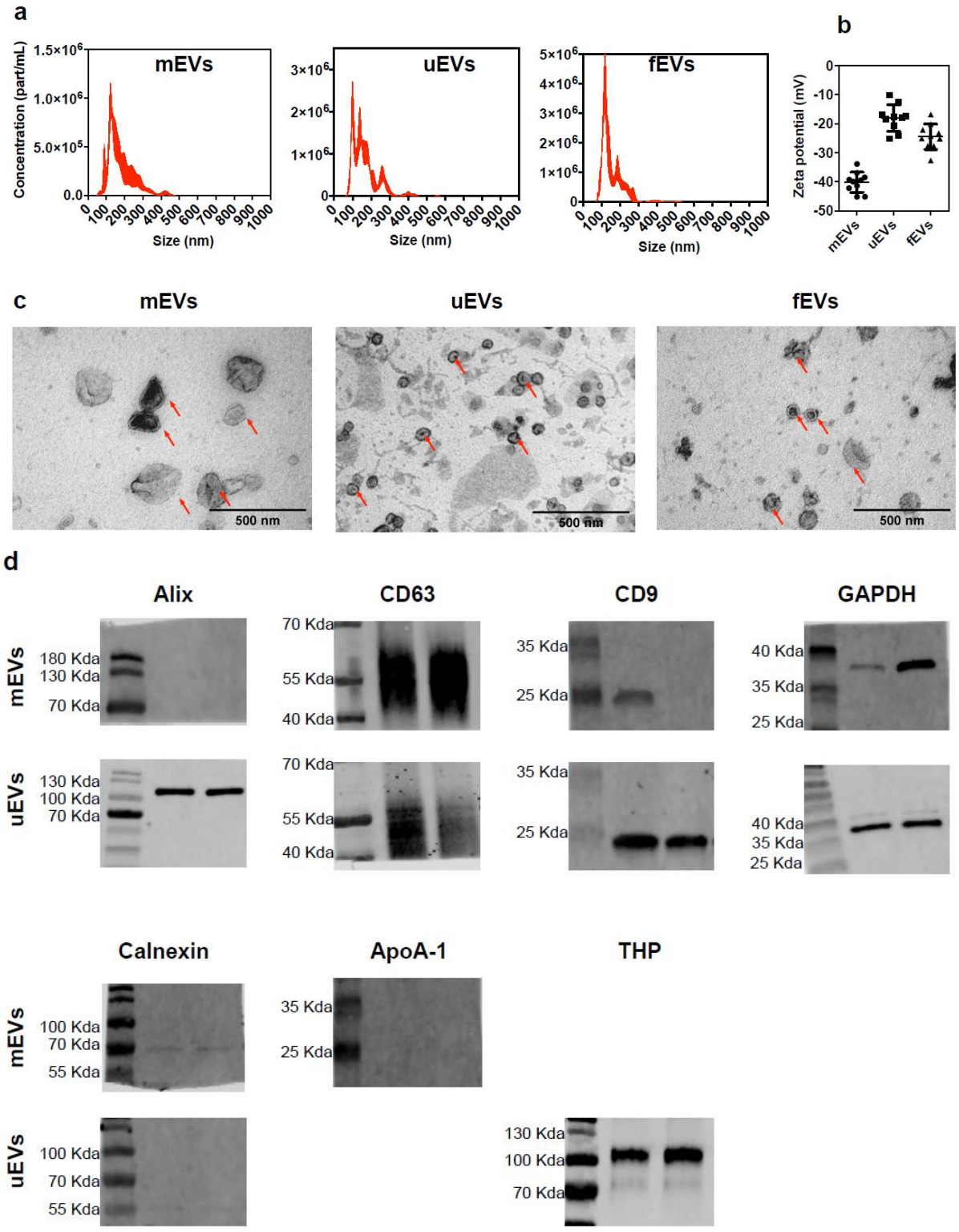
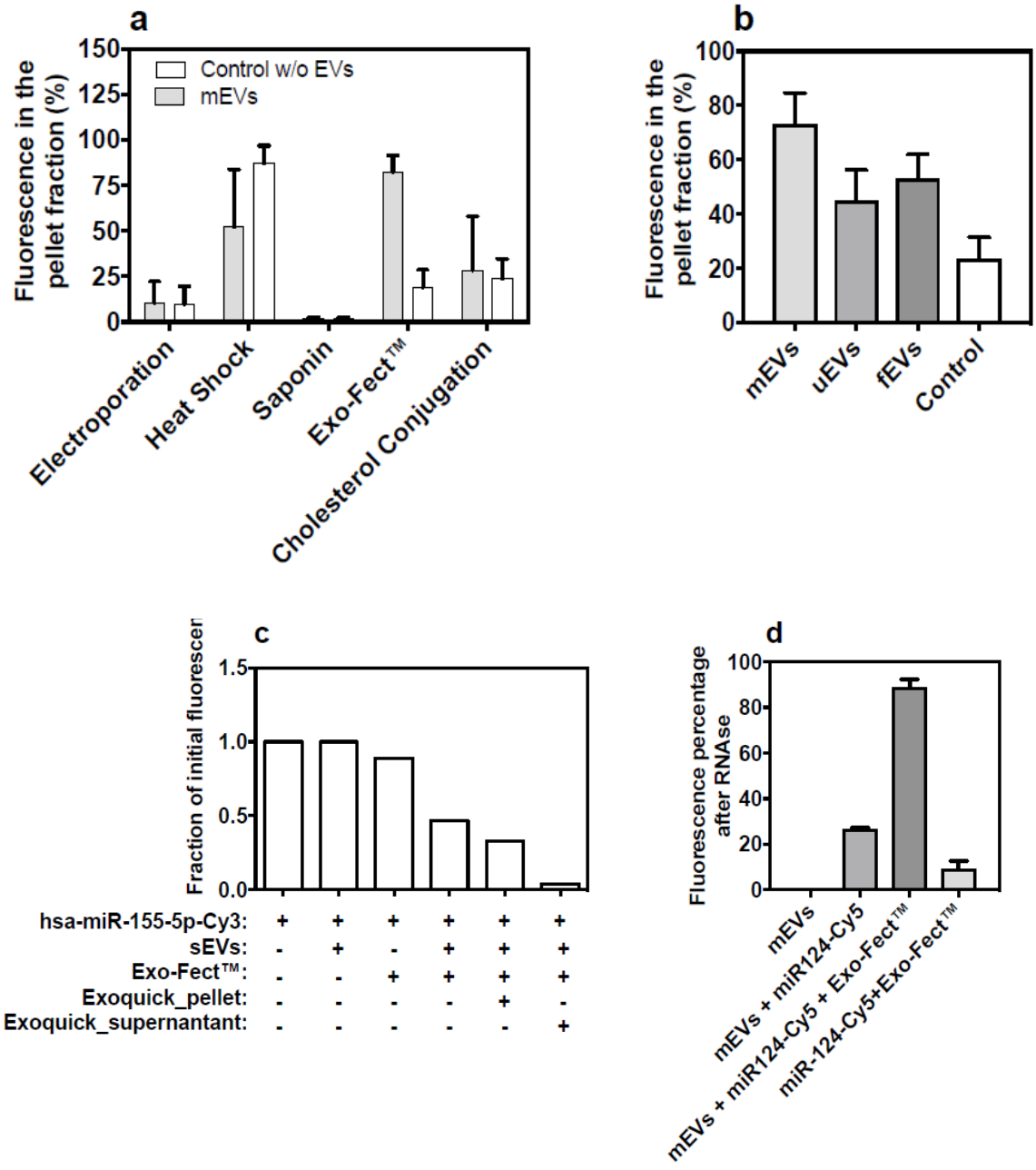
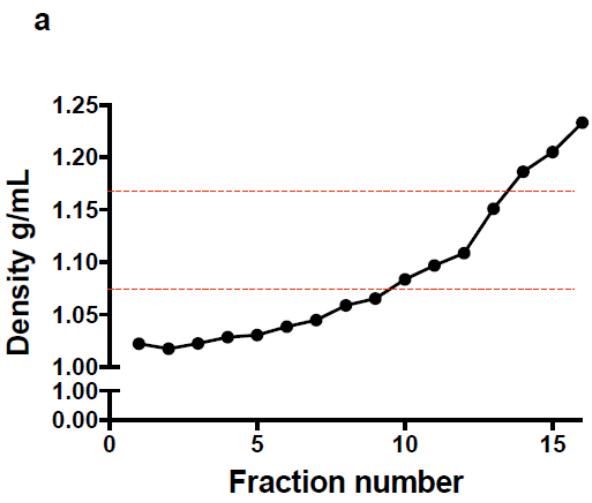
## Supplementary Material




**Supplementary Figure 1. Characterization of sEV isolated from different sources (mEVs, uEVs and fEVs)**. Samples of mEVs, uEVs and fEVs were analyzed via NTA (a), zeta potential (b), and TEM (c). mEVs and uEVs were further analyzed by Western Blot (d), where each lane represents a different donor. In all cases n=2.

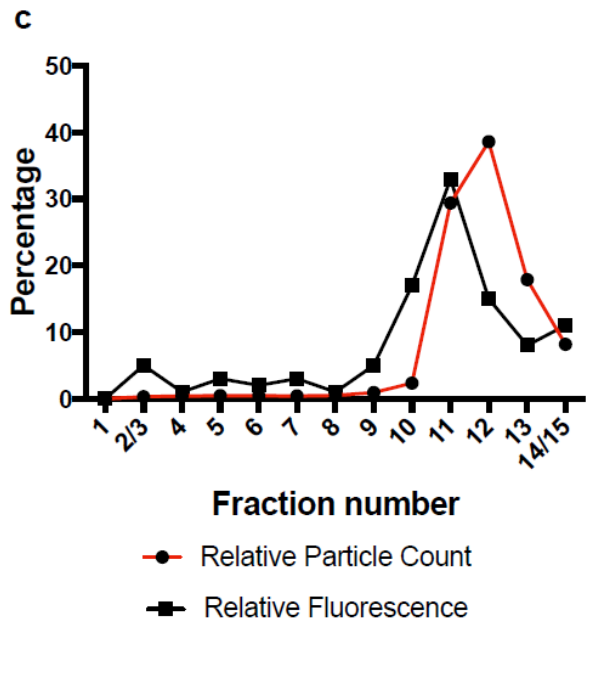


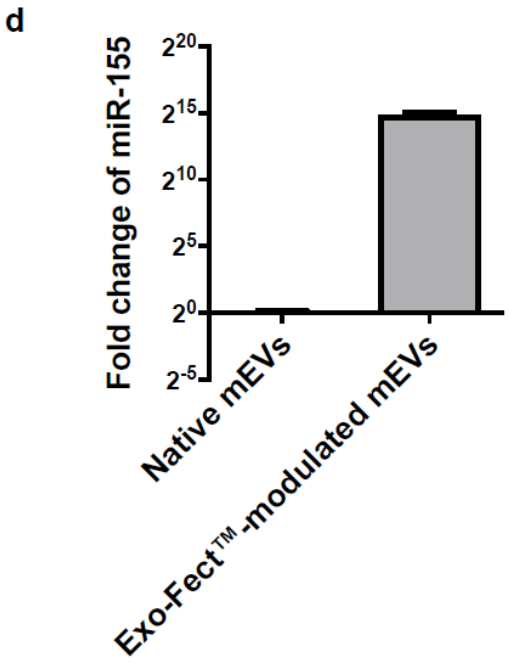

**Supplementary Figure 2. Characterization of sEVs from variable sources modulated by different methodologies.** (a) Fluorescence percentage in the pellet fractions of sEVs loaded with miR-155-5p-Cy3. Control indicates that the loading experiment was performed in the absence of sEVs. Results were obtained from 3 independent experiments. (b) Comparison of the transfection efficiency of Exo-Fect™ on vesicles isolated from different sources (mEVs, uEVs and fEVs). As a control the same procedure was performed but in the absence of sEVs (shown in white). Results were obtained from 3 independent experiments. (c) Fluorescence measurement of the different stages of sEV modulation with miR-155-5p-Cy3 via Exo-Fect™. Our results showed that immediately after addition of Exo-Fect™ to the mixture containing the fluorescently labelled miRNA and sEVs there was a decrease in the overall fluorescence. The majority of that fluorescence was preserved in the pellet (sEV) fraction after purification with ExoQuick. (d) mEVs loaded passively or with Exo-Fect™ and miR-124-Cy5 were treated with RNase and re-purified. The loss of fluorescence represents degradation or the miRNA on sEVs or Exo-Fect™, which is markedly lessened by the presence of Exo-Fect™ in the reaction.



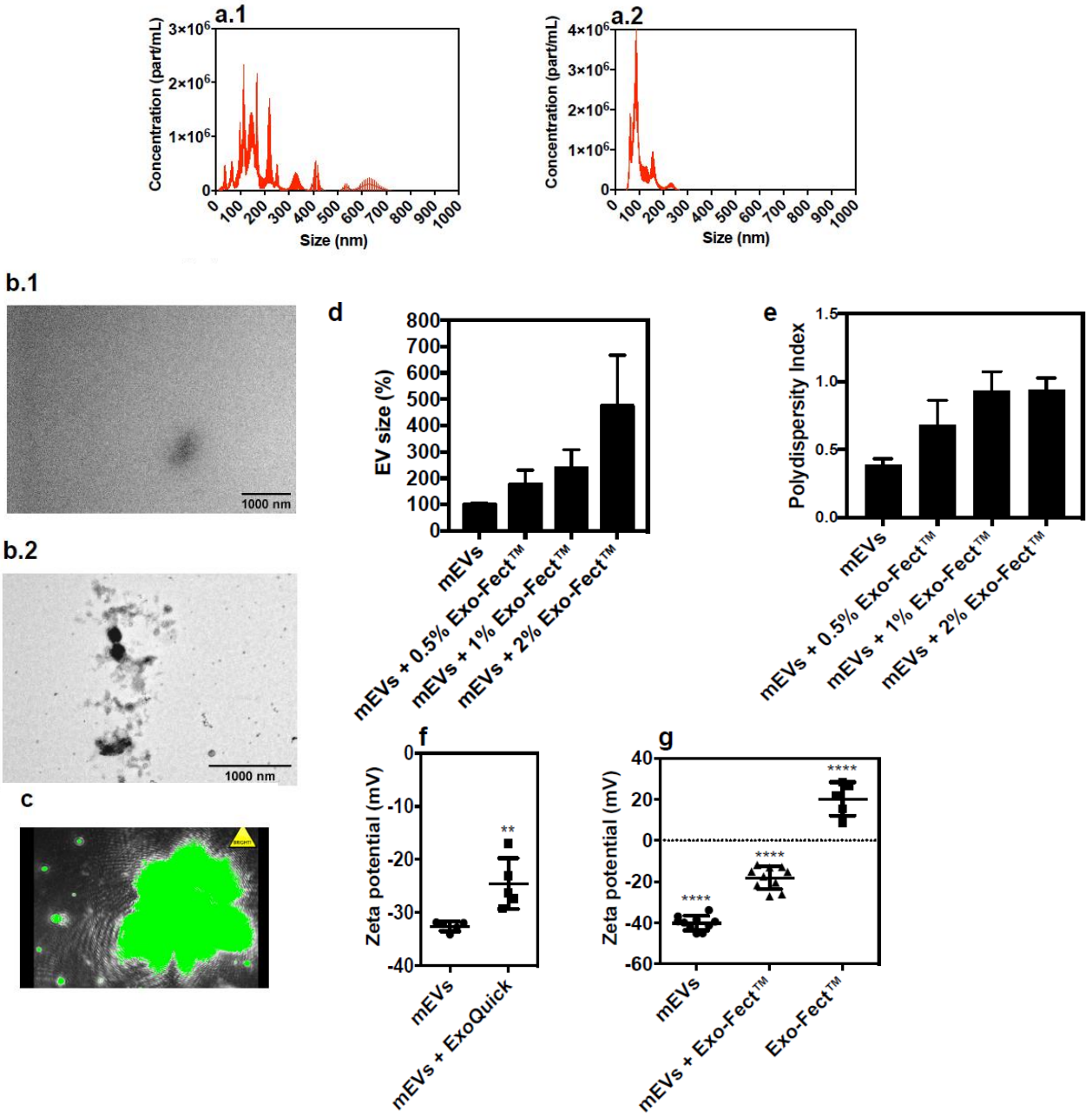

b

| Fraction | Relative Particle number | Relative Fluorescence |
|---|---|---|
| 1 | N/A | N/A |
| 2/3 | 0.32% | 5% |
| 4 | 0.43% | 1% |
| 5 | 0.47% | 3% |
| 6 | 0.47% | 2% |
| 7 | 0.42% | 3% |
| 8 | 0.53% | 1% |
| 9 | 0.94% | 5% |
| 10 | 2.35% | 17% |
| 11 | 29.39% | 33% |
| 12 | 38.59% | 15% |
| 13 | 17.91% | 8% |
| 14/15 | 8.18% | 11% |

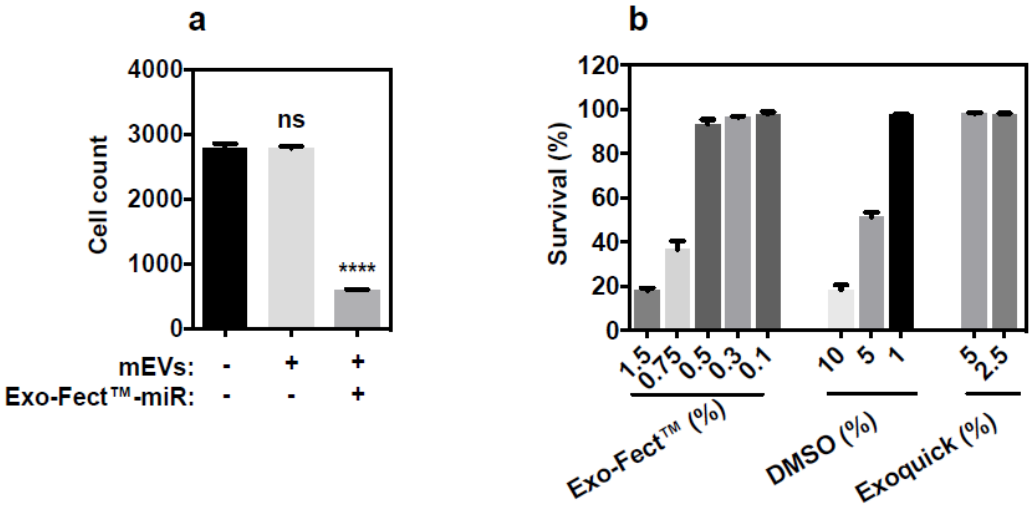


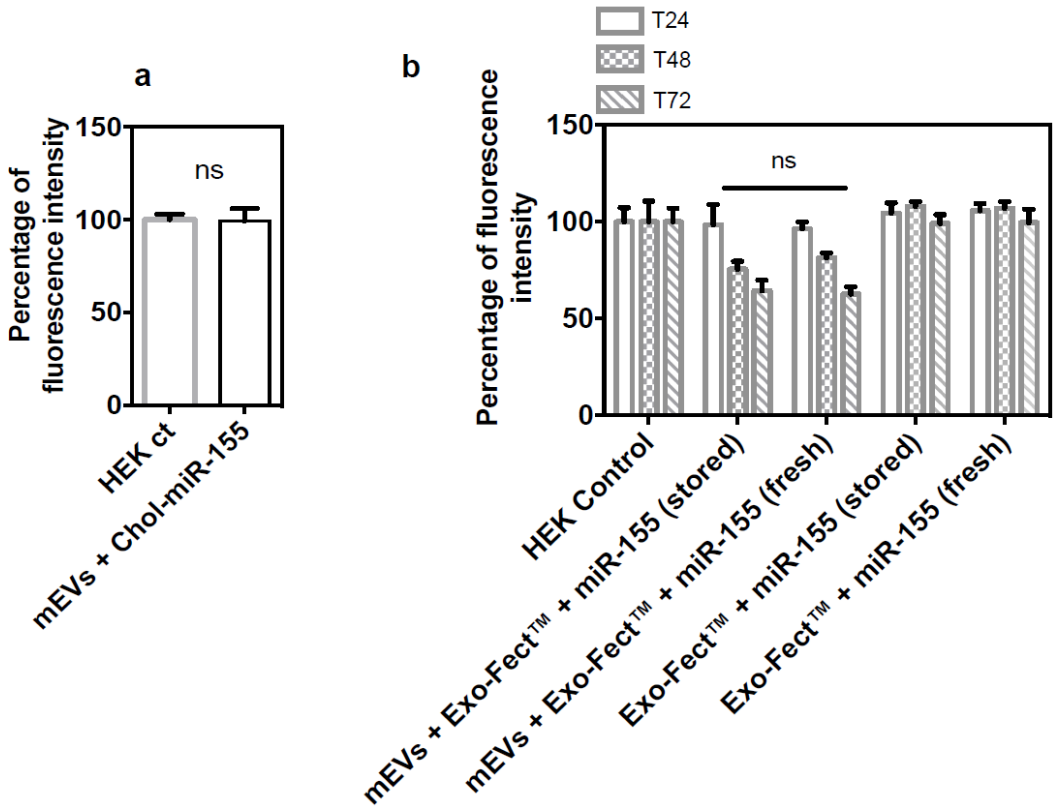

**Supplementary Figure 3. Purification and characterization of modulated sEVs by ODG.** For the simultaneous detection of miRNA by fluorescence and qRT-PCR in the same batch of sEVs, sEVs were loaded with both miR-124-Cy5 for detection by fluorescence and with miR-155 for detection by qRT-PCR analyses. (a) Density of each of the fractions obtained in mEV purification via ODG (n=3). Relative particle count, as measured by NTA, and relative fluorescence of miR-124-Cy5-labelled mEVs, as measured by fluorometer, of each ODG fraction is shown in (b) and (c) (n=3). Our results showed that most of the particles localized to fractions 10-13 and that the fluorescence from the labelled miRNA correlated with particle count, indicating that there was a conjugation between sEVs and miR, after Exo-Fect™-mediated loading. (d) Expression of miR-155 on fractions 10-13 measured by qRT-PCR (n=2). U6 was used as housekeeping gene.



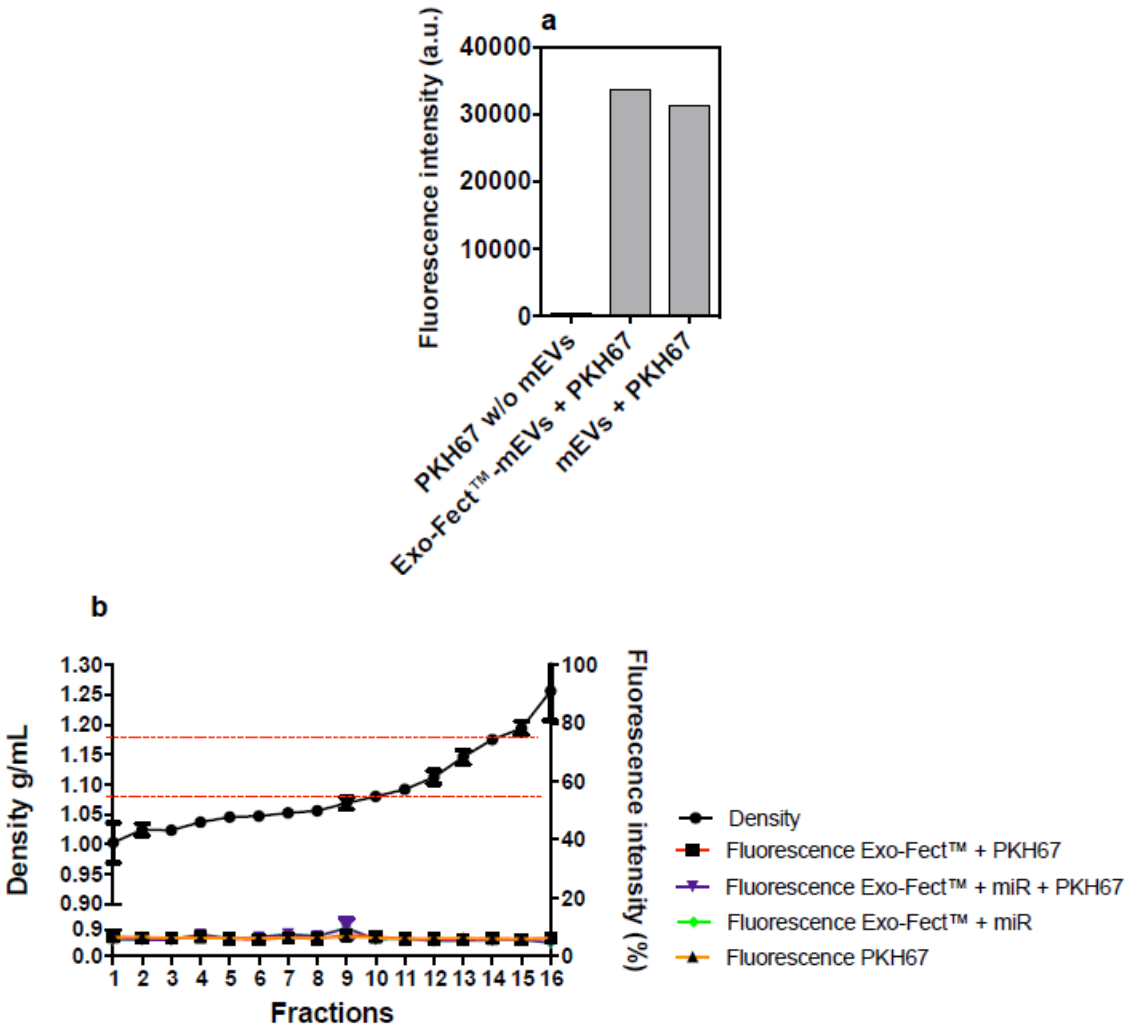

**Supplementary Figure 4. Characterization of Exo-Fect™-modulated sEVs.** (a) NTA particle size distribution profiles of Exo-Fect™ (1) and Exo-Fect™-modulated sEV (2). While Exo-Fect™ is within background levels, modulated mEVs can only be quantified in sizes generally smaller than 100 nm. (b) TEM images of Exo-Fect™ (1) and Exo-Fect™-modulated mEVs (2). Exo-Fect™ alone was not detected by TEM, but induced visible particle aggregation when complexed with mEVs. (c) NTA profile of Exo-Fect™-modulated mEVs. Large artefacts obstruct the field of view and mask sample distribution, explaining the results obtained via the quantification. (d) Exo-Fect™-modulated mEVs show an increase in average particle size, dependent on the Exo-Fect™ concentration used (0%, 1%, 2% and 4%). Results are normalized to control (0% Exo-Fect™) and expressed in percentage. This was done because sEVs with high concentrations of Exo-Fect™ show high level of aggregation and polydispersity. Results are the average of 3 technical replicates. (e) Polydispersity index of mEVs as measured by DLS, showing an increased heterogeneity dependent on Exo-Fect™ concentration. (f) Zeta potential profile of mEVs, mEVs after ExoQuick purification, and (g) mEVs, Exo-Fect™ and mEVs complexed with Exo-Fect™, 5, 5, 10, 10 and 5 technical replicates, respectively. Unpaired, two-tailed t-test or one-way ANOVA with Tukey's correction was used to compare all conditions with each other, ** indicates P<0.01 and **** indicates P<0.0001.



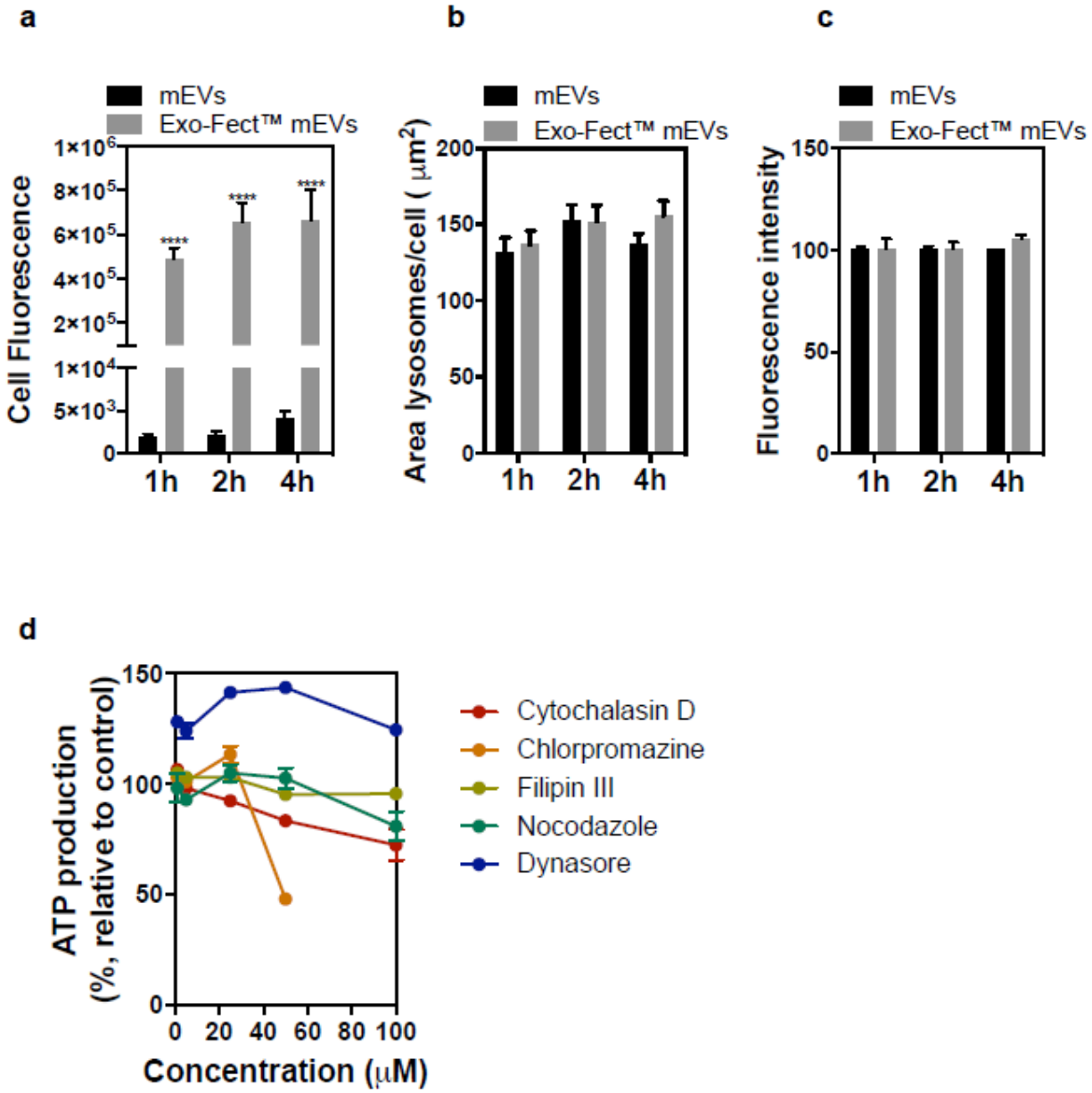

**Supplementary Figure 5. Cytotoxicity of Exo-Fect™-modulated sEVs against human endothelial cells.** (a) Effect of Exo-Fect™-modulated mEVs on endothelial viability. Endothelial cells were treated with native mEVs or Exo-Fect™-modulated mEVs. Cell viability was measured by cell counting after 24 h of incubation (n=1 with 3 technical replicates). Statistical analysis was performed comparing all experimental conditions to untreated control by one-way ANOVA using Dunnet's correction. **** indicates P<0.0001. (b) Effect of direct administration of Exo-Fect™ and ExoQuick on cells (n=1 with 3 technical replicates). DMSO was used as a positive control for the toxicity assessment based on cell survival.




**Supplementary Figure 6. Cholesterol-miR-modulated sEV efficiency and storage stability of Exo-Fect™-modulated sEVs.** (a) Assessment of the function of cholesterol-miR-155-modulated mEVs on the activity of the HEK-293T reporter cell line. Quantification of the average mCherry fluorescence intensity per cell at 72 h post incubation with cholesterol-miR-155 modulated. The cholesterol-miR-155 condition was normalized to control (HEK-293T cells with no treatment) (n=1 with 3 technical replicates). Unpaired, two-tailed t-test was used to assess statistical significance. (b) Comparison between fresh and frozen (-80 ºC for two days) Exo-Fect™-modulated sEVs or Exo-Fect™ with miR-155 on the activity of HEK-293T reporter. The quantification presented is the average mCherry fluorescence intensity per cell. Stored samples showed no statistical significance when compared to their fresh counterparts for each respective time point. Results are the average of 3 independent runs. Statistical analyses were performed between experimental groups at the same time using a one-way ANOVA test followed by Dunnet's correction.




**Supplementary Figure 7. PKH67 interactions with Exo-Fect™.** (a) Fluorescence quantification of the same initial amount of native and Exo-Fect™-modulated mEV samples prior to incubation with HUVECs for internalization experiments. Native sEVs were incubated with PKH67 as described in the methods section. After PKH67 labelling, sEVs were, in relevant conditions, modulated with Exo-Fect™, as described in the methods section. As a control, the same amount of PKH67 were used in solution, in the absence of sEVs. All conditions were purified via ultracentrifugation and their fluorescence was measured by fluorometry. Both samples showed similar levels of fluorescence, while in the absence of sEVs, PKH67 is non-fluorescent, indicating that its removal from samples was efficient. (b) Quantification of the fluorescence and density of each fraction of an ODG gradient where samples of PKH67 were loaded onto, with and without Exo-Fect™ (n=2), with and without miR-155-Cy3. The percentage of PKH67/Cy3 fluorescence relative to total fluorescence after ODG purification was calculated.




**Supplementary Figure 8. Internalization of Exo-Fect™-modulated mEVs in HUVECs.** (a) Cell fluorescence intensity was quantified after acquisition of images in a high content microscope (INCell analyzer, GE Healthcare) which were then analysed using INCell developer toolbox. (b) Quantification of the area occupied by lysosomes per cell and (c) normalized average intensity of lysosomal probe per cell. (d) Toxicity of each inhibitor used in the internalization studies was assessed after 4.5 h incubation with each inhibitor using CellTiter Glo kit (Promega). Results are expressed as mean±SEM (n=3, 2 technical replicates for a, b and c, and n=1 with 2 technical replicates for d).

# CHAPTER V –miR-loaded sEVs: *In vivo* application & discovery of novel loading agents

Adapted from

**Extracellular vesicles enriched with an endothelial cell pro-survival miRNA impact skin tissue regeneration**

Hugo Fernandes[1,2], Alessandra Zonnari[1], **Ricardo Abreu**[1,3,4], Sezin Aday[1], Marta Barão[1], Ana Branco[1], Miguel Lino[1], Cátia Seabra[1], Tania Barata[1], Inês Albino[1], Ermelindo Leal[1], Guilherme Tralhão[2], Lino Gonçalves[2], Margreet de Vries[5,6], Paul Quax[5,6] and Lino Ferreira[1,2]

*Submitted*

&

**High-throughput screening for loading sEVs with microRNA**

**Ricardo Abreu**[1,3,4], Patrícia A. T. Martins[1], Marta Barão[1], Clarissa Becher[1], Paula da Costa Martins[3,4], Hugo Fernandes[1,2], Lino Ferreira[1,2]

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**ABSTRACT**

Extracellular vesicles (EVs) are small cell-derived particles responsible for intercellular communication. In recent years, their capacity to transfer molecular cargo between cells has been harnessed for the delivery of specific therapeutic molecules, particularly microRNA. In this study, we used a chemical transfection agent to load the endothelial cell (EC) pro-survival miR-425-5p onto EVs and showed that compared to miR-scrambled modulated EVs, topical administration of miR-425-5p-modulated EVs during 10 days was able to accelerate wound regeneration in a streptozotocin (STZ)-induced diabetic wound healing mouse model. Because the chemical agent used for the modulation of EVs with miRNAs is not approved for clinical use neither displays bioactivity, we sought to discover novel molecules capable of efficiently loading pre-selected miRNAs onto EVs. To this end, we screened a library of ≈1300 compounds for their potential to load a fluorescent miR onto EVs. From the list of putative hits, we selected two for further validation and confirmed their capacity of significantly enrich EVs with a pre-selected miRNA of interest.

**Introduction**

Extracellular vesicles (EVs) are an heterogeneous group of small (up to 200 nm in diameter), cell-derived particles[1]. EVs carry a multitude of bioactive cargo[2] and are able to bypass biological barriers[3,4] making them promising therapeutic agents[5,6] and/or naturally-derived drug delivery systems[7-9].

The delivery of microRNA (miRNA) is particularly important in the field of regenerative therapy, with several miRNAs already in advanced stage clinical trials, poised for approval in clinical application[10]. miRNA comprises a functionally conserved, short (~22 nucleotide long), non-coding sequences[11] which are involved in posttranscriptional gene expression regulation[12]. EVs endogenously carry miRNA[13] and the successful internalization and intracellular processing of EVs will ultimately impact recipient´s cell fate and status[14,15]. Recent studies have explored EV-mediated miR delivery and its therapeutic properties[16-18]. Enrichment of EVs with specific miRNAs can be done indirectly by modulation of the secreting cell[19,20] or post-isolation[21]. However, there is no consensus on which methodology is the most efficient, scalable, reproducible to accomplish the post-isolation modification of EVs with miRNAs of interest. A recent study by our group has determined that the commercial kit Exo-Fect™ is a fast, reliable and efficient method to load miRNAs onto small EVs (sEVs)[7]. While this technology has been used in several previous studies[22-24], the effect of Exo-Fect-modulated sEVs has not yet been tested in wound healing.

In this study, we used the technology developed in our previous work[7] to establish whether Exo-Fect-miR-modulated EVs were capable of efficiently delivering a therapeutic miRNA (hsa-miR-425-5p) *in vivo* and stimulate tissue regeneration. To this purpose, an STZ-induced diabetic wound healing mouse model[6] was employed, and topical administrations of miR-425-5p-modulated EVs were evaluated for their ability to enhance wound healing.

Despite being an efficient transfection agent, Exo-Fect™ has several shortcomings, as it may cause significant changes in sEV biology, uptake and processing by recipient cells[7]. Therefore, the identification of novel formulations with similar miR-loading capabilities but without the detrimental effects of Exo-Fect™ is much needed. Here, we developed a fluorescence-based

high-throughput screening approach to identify molecules capable of loading a fluorescently-labelled miRNA onto EVs.

## Materials and Methods

**Isolation of human umbilical cord blood mononuclear cells (hUCBMNCs).** Umbilical cord blood was collected, stored and transported to the laboratory in sterile collection bags containing anticoagulant solution (citrate-phosphate-dextrose). All samples were processed within 48 h as previously described[25]. In brief, MNCs were isolated by density gradient separation (Lymphoprep™ – StemCell Technologies SARL, Grenoble, France) and cryopreserved in a solution consisting of FBS:IMDM:DMSO (55:40:5; % v/v) until further use.

**Isolation of sEVs from hUCMNCs.** Previously, hUCMNCs were stored at -80ºC, 1 million cells per cryovial. Cells were thawed in warm X-VIVO and immediately treated with DNAse I to degrade DNA arising from, mainly, co-isolated neutrophiles. Cells were then centrifuged at 300g for 5 min and recounted. Then, $2x10^6$ cells/mL were cultured in X-VIVO 15 serum-free cell culture medium (Lonza) supplemented with 100 ng/mL Flt-3 and 100 ng/mL stem cell factor (both from PeproTech) under hypoxia conditions (0.5% $O_2$) for 18 h. sEVs were purified from the conditioned media (CM) by differential centrifugation as previously described[26]. In brief, CM was collected and centrifuged at 300 g, for 10 min, at 4 ºC to remove cells followed by a centrifugation at 2.000 g, for 20 min, at 4 ºC to deplete cellular debris. Subsequently, samples were ultracentrifuged twice at 10.000 g, for 30 min, at 4 ºC, the pellet was discarded and the supernatant was submitted to an ultracentrifugation at 100.000 g, for 2 h, at 4 ºC, to pellet sEVs. Finally, the pellet from the last step was washed with cold PBS, ultracentrifuged again at 100.000 g, for 2 h, at 4 ºC, resuspended in 150 µL of cold PBS and stored at -80 ºC. Ultracentrifugation steps were performed using a swinging bucket rotor SW 32 Ti in an Optima™ XPN 100K ultracentrifuge (Beckman Coulter, California, USA.) and 28.7 mL polyallomer conical tubes (Beckman Coulter).

**Isolation of sEVs from human urine.** sEVs were purified from human urine by differential centrifugation as described previously[27]. All work with human samples was approved by the

Ethics Commission of the Faculty of Medicine of the University of Coimbra (CE-070-2019). Briefly, for the screening assay, the first urine in the morning (midstream) was harvested and collected from one healthy male donor. Elimination of cell debris and large contaminants was immediately performed by centrifugation at 2000 x g for 20 min and the supernatant was stored at -80°C until use. Thawed urine samples were diluted 1:3 in cold Tris-EDTA buffer (20 mM, pH 9.0, 4°C) and vortexed vigorously for 90 s. Then, samples were processed via differential ultracentrifugation as described above.

**sEV characterization by nanoparticle tracking analysis (NTA).** Size and concentration of sEVs was performed through NTA using the NanoSight NS300 (Malvern Instruments, Malvern, U.K.). The system used an O-Ring Top Plate and the sample was injected manually at an approximate flow of 1 mL every 20 s. sEVs were diluted in PBS until a concentration between 15 and 45 particles/frame was reached. For each sample, 5 videos of 30 s were recorded with the camera level set at 16. All the videos were processed with NTA 3.2 analytical software, using the software threshold between 2 and 4 depending on the quality of the videos.

**sEV protein quantification.** sEV protein quantification was performed using the microBCA protein assay kit (Thermo Fisher Scientific, Massachusetts, USA), as per the manufacturer's instructions. Briefly, bovine serum albumin (BSA) was used to obtain a 10 points standard curve. Then, sEV samples were diluted 22 times in 2% (v/v) sodium dodecyl sulphate (SDS) to disrupt the sEV membrane and subsequently, 50 µL of the previous mix was pipetted, in duplicate, into a 96-well Corning® Costar® cell culture plates (Corning Inc., New York, USA). Reaction solution provided in the kit was added and incubated for 2 h at 37 ºC. Next, the plates were equilibrated at room temperature for 15 min and finally, the absorbance at 562 nm was read in the microplate reader Synergy™ H1 (Biotek, Vermont, U.S.A.).

**sEV characterization by transmission electron microscopy (TEM)**. TEM analysis of sEVs was performed as previously described[26]. Briefly, samples were diluted 1:1 in 4% (v/v) paraformaldehyde (PFA) and placed on Formvar-carbon coated grids (TAAB Technologies) for 20 min at RT. After washing 4 times with PBS, grids were placed on a drop of 1% (v/v) glutaraldehyde for 5 min, followed by 5 washes with distilled water, one minute each. In a

dark environment, grids were incubated with uranyl-oxalate solution pH=7 for 5 min, and then placed on ice in contact with a solution of methyl cellulose (9:1) for 10 min. sEVs imaging was obtained using a Tecnai G2 Spirit BioTWIN electron microscope (FEI) at 80 kV.

**sEV characterization by Phase analysis light scattering (PALS).** Zeta potential measurements were done on a Zetasizer Nano ZS (Malvern). The sample was pre-equilibrated at 37°C for at least 60 s and each measurement was the average of 11 runs. Three consecutive measurements were performed for each sample to evaluate its stability. The results were analyzed by the equipment software considering the viscosity and refractive index of water at the measurement temperature, and a refractive index of 1.59 for the scattering particles. The average size was taken from the analysis in volume distribution of particles.

**Enrichment of sEVs with miR-425-5p and miR-scramble.** Transfection of sEVs was accomplished using Exo-Fect according to the manufacturer´s instructions. In brief, sEVs were incubated with Exo-Fect and miR-425-5p or miR-scramble for 10 min at 37ºC. sEVs were transfected with miR-425-5p and miR-scramble resulting in a total of 20 pmol of miRNA per 1 μg sEVs. Transfected sEVs were aliquoted in individual tubes for subsequent administration, on a daily basis, into the wound of STZ-induced diabetic wound healing mouse model.

**STZ-induced diabetic wound healing mouse model.** All the experiments were performed in accordance with the European Community law for Experimental Animal studies (86/609/CEE and 2007/526/CE) and approved by the Institutional and Governmental Research Ethical Board. C57BL/6J mice (male, 8 weeks) purchased from Charles River (Barcelona, Spain) were injected with 50 mg/kg of streptozotocin (STZ, Sigma-Aldrich, Sintra, Portugal) in citrate buffer (0.1 M), intraperitoneally (i.p.), for 5 consecutive days to induce diabetes. Seven days after the STZ injection, blood glucose was measured, using an Accu-Check Advantage glucometer (Roche, Amadora, Portugal), to confirm the diabetic state. Mice with blood glucose level above 250 mg/dL were considered diabetic. The animals were treated with 0.1–0.2 units of NPH insulin, subcutaneously (s.c.), as needed, to avoid weight loss. The animals were kept diabetic for 6 weeks prior to the wound healing experiments. On the day of the experiment,

mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), i.p. After removing the hair from the back of the mice, two 6-mm full-thickness excisional wounds were biopsied and the wound area was traced every day onto acetate paper to follow the rate of wound closure up to 10 days post wounding. The wound size was determined with the ImageJ software (NIH) and data was presented as percentage of original wound (day 0). sEVs were transfected with miR-425-5p or miR-scramble and twice a day, 0.1 μg sEVs transfected with 2 pmol miR-425-5p or miR-Scramble were topically administered onto the wound (daily: 0.2 μg sEVs and 4 pmol miR-425-5p or miR-Scramble), for 10 days. Animals were sacrificed at day 2, day 5 and day 10.

**High-throughput screening assay.** A high-throughput screening assay was designed and implemented in order in order to identify compounds capable of transfecting EVs with a fluorescently-labelled miRNA. The library used for the HTS was the Prestwick chemical library (Prestwick Chemical, Illkirch-Graffenstaden, France) enriched with additional, proprietary, research drugs available in the lab. For each compound, a 2 mM working solution was prepared in dimethyl sulfoxide (DMSO, Sigma Aldrich, USA). CD9-coated magnetic beads (5 μL beads/compound) (Invitrogen, Massachusetts, USA) were washed in PBS and complexed overnight at 4°C with $10^{10}$ EVs per compound in batch reaction, in 1.5 mL Eppendorf tubes. Following incubation, a magnetic field (DynaMag$^{TM}$-2 Magnet; Thermo Fisher, Massachusetts, USA) was applied to maintain the bead-EV complexes at the bottom of the tube whilst unbound EVs were removed using PBS. Next, the complexes bead-EVs were resuspended in 150 μL PBS, transferred into a 96-well PCR reaction plate (Life technologies, California, USA), the library compounds were added to the bead-EV complexes at a final concentration of 10 μM and incubated for 2 hours at room temperature (RT) on a microplate shaker (VWR, Pennsylvania, USA). After treatment, magnetic separation was used to separate the bead-EV complexes from the supernatant, the complexes were washed with PBS, resuspended in 150 μL PBS and incubated overnight at 4ºC with a fluorescently labelled microRNA (has-miR-155-5p-Cy3) at a final concentration of 20 pmol/well. Following overnight incubation, the supernatant was magnetically separated from the pellet and transferred to a Corning® 96-well black plate (Costar® Corning, New York, USA). Fluorescence intensity was measured using

a microplate reader (Synergy H1 Microplate Reader, BioTek, USA), with an excitation at 535 nm and an emission spectrum from 563 nm to 700 nm and emission steps of 2 were recorded. Finally, the pellet containing bead-EV complexes was resuspended in 150 µL PBS, transferred to a 96-well black plate, and the fluorescence spectrum was determined as described before. The highest fluorescence point was chosen for each sample to calculate the transfection efficiency using the following formula:

$$\frac{Fluorescence\ intensity\ of\ the\ pellet}{Overall\ fluorescence\ intensity\ (pellet + supernatant)}$$

As a positive control we used Exo-Fect™ and bead-EV complexes incubated without the addition of compounds were used as negative control.

**miRNA-derived fluorescence detection by flow cytometry for bead-bound sEVs.** Flow cytometry was performed on uEV samples using the Exosome-Human CD9 Flow Detection Reagent (Invitrogen, Massachusetts, USA) as recommended by the manufacturer. Briefly, native EVs or EVs loaded with fluorescently labelled miRNA were incubated overnight with anti-CD9-coated magnetic beads ($2^{10}$ EVs/10 µL of beads) at 4°C with end-over-end mixing. The following day, samples were short-spin centrifuged, diluted in assay buffer and magnetically separated to pellet bead-EV complexes. This process was repeated to wash the samples and remove unbound EVs. Finally, samples were loaded into the flow cytometer BD Accuri 6 (BD Bioscience, New Jersey, U.S.A.) and analysed. Samples were gated in the region corresponding to clean (untreated) magnetic beads and $10^5$ events were counted for each sample. The data was analysed using Flowjo™ (v10, FlowJo, LLC).

## Results

### sEV characterization

EVs were isolated from hUCMNCs and human urine using differential ultracentrifugation and protocols already established by us[6]. EVs isolated from hUCMNCs exhibited the canonical cup-shape observable in TEM (**Fig. 1A**), with size range between 90-200 nm (**Fig. 1B**) and an average surface charge of -43 ± 8.6 mV (**Fig. 1C**). Similarly to EVs isolated from hUCBMNC, EVs isolated from human urine also exhibited a cup-shape structure (**Fig. 1D**). However, TEM

analysis revealed the presence of filamentous contaminants in uEVs, likely uromodulin, a protein commonly found in urine which can cause network-like aggregates in samples. The size distribution of these EVs was also within the expected range of 100-200 nm in diameter (**Fig. 1E**) and their zeta potential was size distribution of these sEVs was also within the expected range of 100-200 nm in diameter (**Fig. 1E**) and their zeta potential averaged at -26 ± 4.5 mV (**Fig. 1F**).

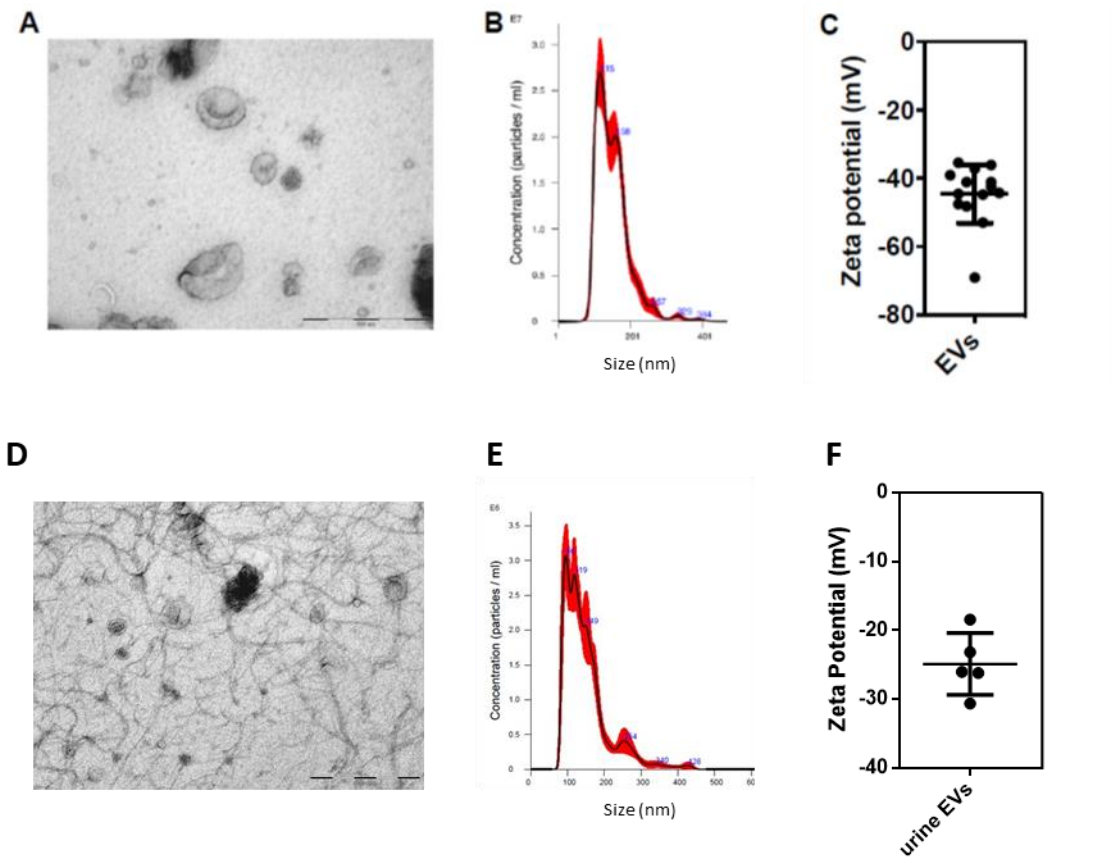



**Figure 1 - Characterization of EVs isolated from human umbilical cord blood-derived mononuclear cells (hUCBMNCs).** (A) TEM, (B) NTA and (C) zeta potential analyses of EVs isolated from hUCBMNCs. (D) TEM, (E) NTA and (F) zeta potential analyses of EVs isolated from human urine.

### *In vivo* testing of Exo-Fect-mediated miR-425-5p modulated EVs in a STZ-induced diabetic wound healing mouse model

Having previously established the ability of Exo-Fect™ to load miRNAs of interest onto sEVs we decided to evaluate the bioactivity miR-modulated EVs *in vivo* using a STZ-induced diabetic wound healing mouse model. For this purpose, the miRNA of choice was miR-425-5p which, in our hands, in a parallel study, stimulated a pro-survival phenotype in endothelial cells. Our results showed that bi-daily topical administration of 4 pmol of miR-425-5p- or miR-scrambled-modulated sEVs onto the wounds significantly accelerated wound closure during

a period of 10 days, with a statistically significant difference at day 3, 5-7 and 9 (**Fig. 2A-B**). Importantly, we showed that at day 2 and day 5, there was a statistically significant increase in the expression of miR-425-5p into the wound area compared to the control (miR-scrambled-treated wounds; **Fig. 2C**). These results demonstrate that Exo-Fect™ can be efficiently used to modulate the expression of selected miRNAs on EVs and, more importantly, transfer the miRNAs to target cells *in vivo*.

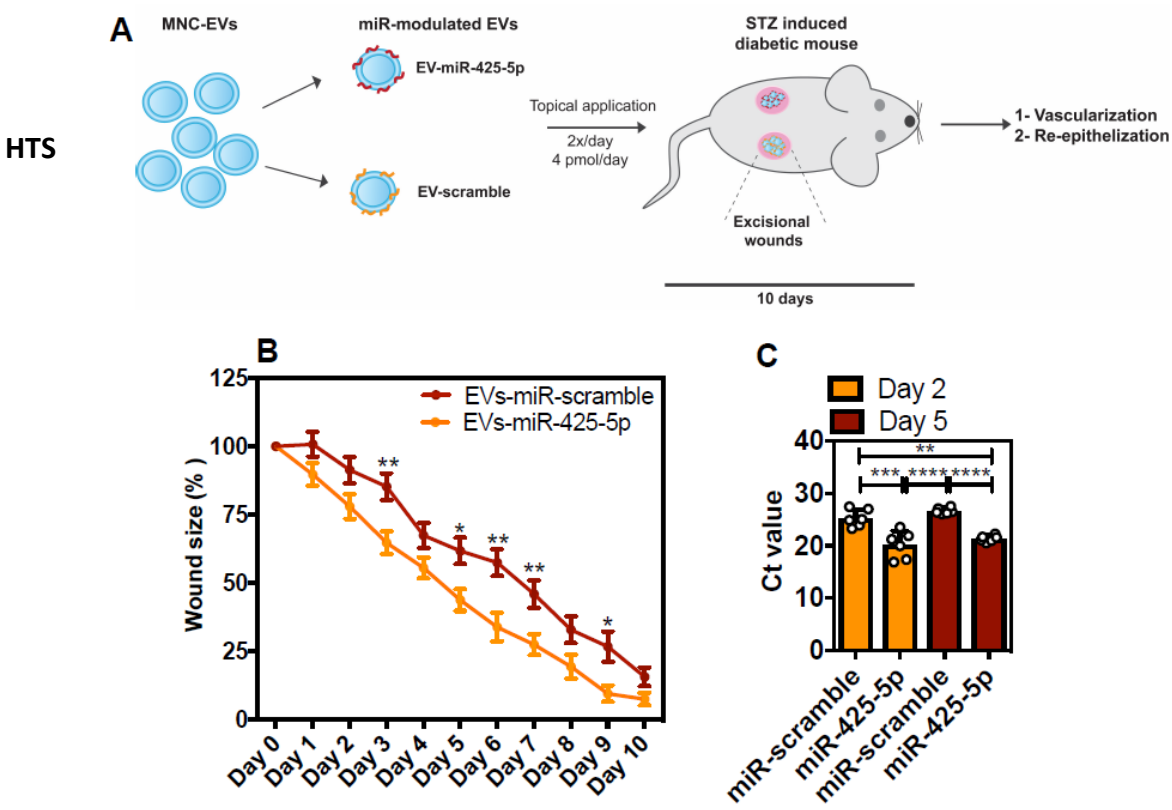
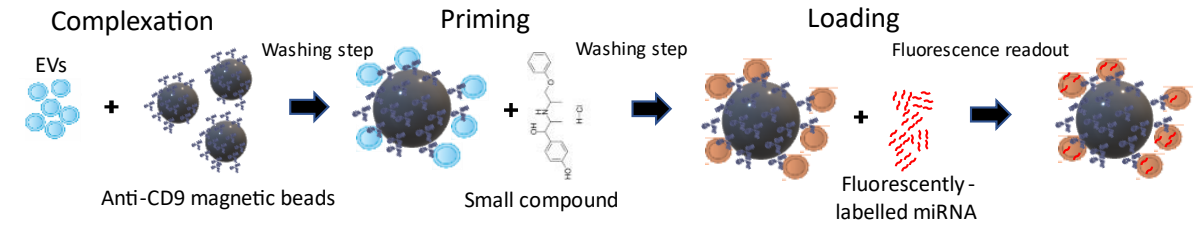



**Figure 2 - miR-425-5p-modulated EVs enhanced in vivo wound healing in STZ-induced diabetic mice.** (**A**), schematic representation of the experimental setup **(B)**, wound size percentage relative to the initial puncture at day 0. * P<0.05 using Unpaired t-test. Results are presented as mean±SD. **(C)** ct value of miR-425-5p in wound tissue at days 2 and 5. * denotes statistical significance: One-way ANOVA and Tukey´s multiple comparison test whereas * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001. Results are presented as mean±SD.

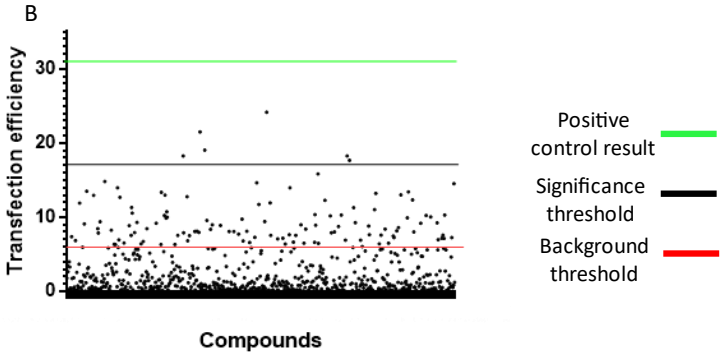
### Identification of compounds capable of modulating EVs with selected miRNAs

Our previous result, indicate that Exo-Fect is an efficient modulator of sEV cargo, being capable of functionally enriching sEVs with pre-selected miRNAs. However, our results also showed that, after modulation, Exo-Fect remains complexed with EVs (see Chapter IV), which can interfere with EV uptake, internalization and intracellular trafficking/processing. Consequently, in order to find novel compounds capable of modulating EVs with pre-selected miRNAs without negatively impacting EV biophysical properties, we implemented a HTS assay

A




B




C

| Compound name | Molecular weight | Formal Charge | Transfection efficiency |
|---|---|---|---|
| ***Chemical library compounds*** | | | |
| Isoxsuprine hydrochloride | 337.8 | 1 | 18% |
| Doxorubicin hydrochloride | 543.52 | 1 | 19% |
| Efavirenz | 315.675 | 0 | 21% |
| Digitoxigenin | 374.51 | 0 | 24% |
| ***Undisclosed compounds*** | | | |
| P3 | 1939.30 | 6 | 18% |
| P4 | 1583.02 | 5 | 18% |

D

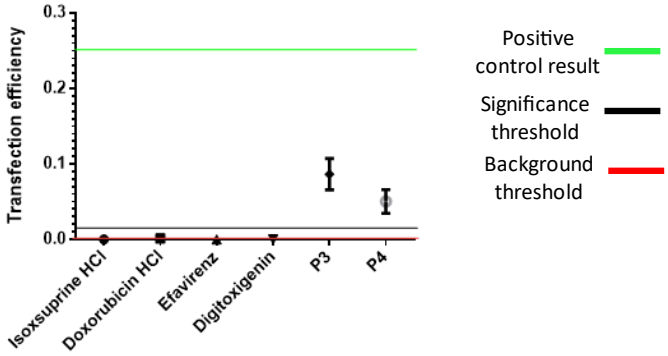



**Figure 3 – Screening for novel compounds capable of loading EVs with miRNA.** (A) Schematic representation of the screening protocol. Complexation was performed at 4 ℃ overnight, Priming of bead-bound EVs with small compounds was performed at room temperature for 2h, loading of the bead-bound EVs with fluorescently-labelled miRNA was performed at 4 ℃ overnight. Washing steps resorted to magnetic pull down of bead-bound EVs, aspiration of supernatant and resuspension of samples in PBS. The readout provided a ratio between the total fluorescence (last supernatant + pellet) and the fluorescence on EVs (pellet). (B) High-throughput screening results. Results are expressed as the pellet fluorescence divided by total fluorescence, in each compound, as a percentage. (C) Table summarizing the main features of the hit compounds which resulted from the screening. (D) Validation assay of the six hit compounds. Results are expressed as before. Positive control result lines represent the transfection efficiency of Exo-Fect and background threshold lines represent the upper limit of the confidence interval determining the background fluorescence intensity.

and tested a library of FDA-approved molecules enriched with some in-house available

proprietary research drugs (P1-5). The assay consisted in a series of complexations and washing steps, yielding a final pellet (containing the fluorescently-labelled miR complexed with EVs) and the supernatant (containing unbound fluorescently-labelled miR) (**Fig. 3A**). Our results showed that only 6 compounds (4 small molecules from the Prestwick Chemical library and 2 proprietary research drugs), were capable of complexing the fluorescently-labelled miRNAs with EVs (95% confidence interval threshold; **Fig. 3B**-**C**). However, after re-evaluating these 6 compounds using a more stringent protocol (including extra washing steps to reduce background signal) only two compounds were able to complex the fluorescently-labelled miRNA with EVs although not to the same extent as the positive control (**Fig. 3D**).

To further validate these results, we used flow cytometry to separate individual magnetic beads containing miR-enriched EVs and measured the fluorescence intensity after incubation of the fluorescently-labelled miRNA with three different concentrations of the compounds (10, 50 and 100 µM). For both compounds, our results showed an increase in the number of labelled events (**Fig. 4A-B**) positively correlating with compound concentration. Previously, it has been shown that modulation of EVs with miRNAs using electroporation led to precipitation of the miRNA without complexation with the EV[28]. To rule out this, we decided to analyse if the increase in fluorescence was specifically related to the interaction miRNA-EVs or could be due to precipitation. To that end, we tested the same three concentrations using a flow cytometry-based assay but now in the absence of EVs. Flow cytometry separates individual events (beads complexed with miR-labelled EVs), which ensures that no background, in the form of unbound fluorescent miRNA, is present. Our results showed that despite unspecific signal from the fluorescently-labelled miRNA and compound, the compounds were nonetheless capable of loading miR onto sEVs (**Fig. 4C**). Furthermore, the optimal concentration for miR loading onto sEVs differs, with P3 showing higher efficiencies at 100 µM while P4 was most efficient at 50 µM.

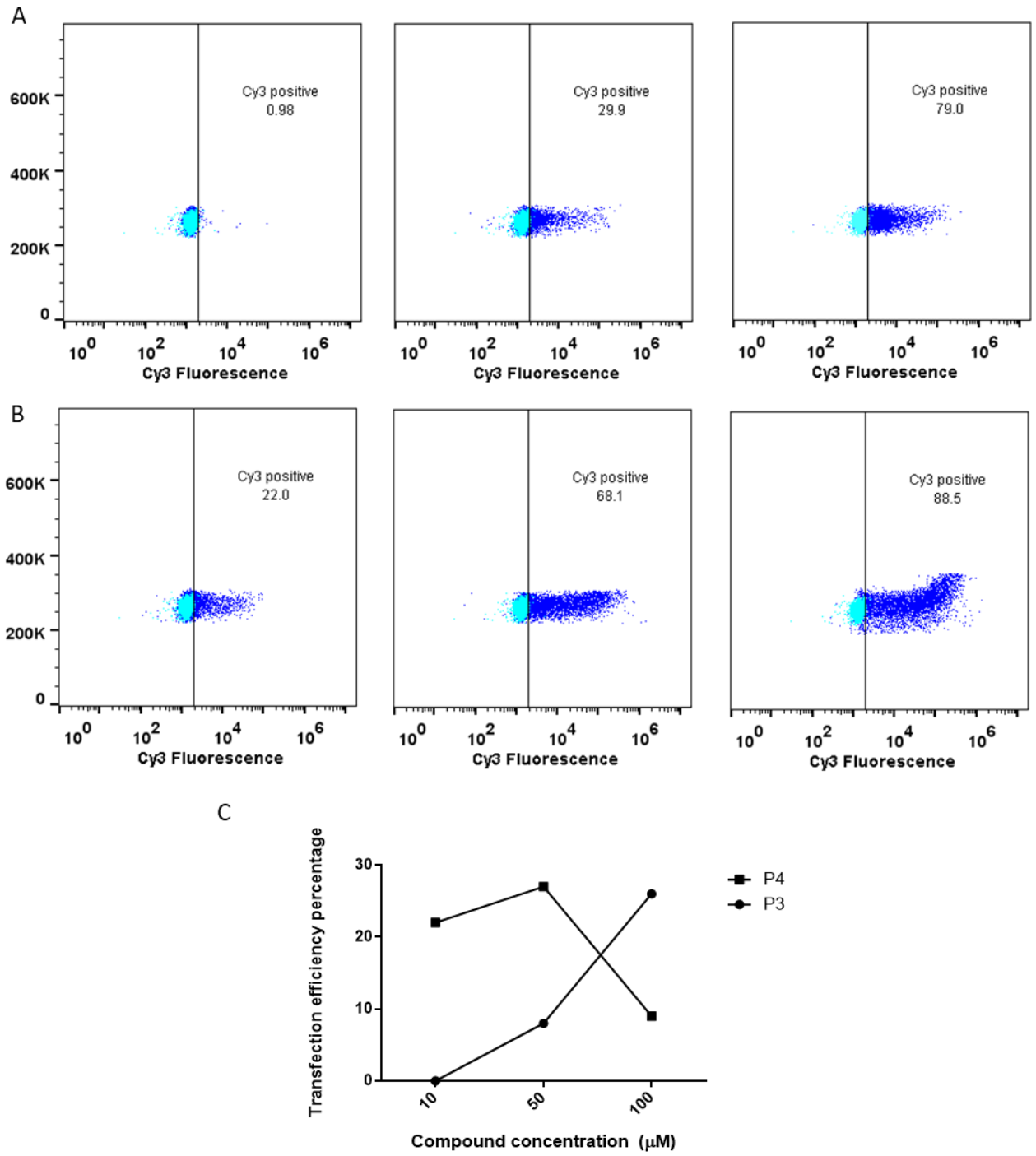



**Figure 4 – Flow cytometry validation of compounds capable of loading EVs with miRNA.** (A) P3 and (B) P4 sEV miR enrichment results for flow cytometry readout in 10, 50 and 100 μM concentrations of peptide. (C) Peptide concentration effect on transfection efficiency of sEVs with fluorescent miR, normalized to null control (absence of sEVs in reaction).

**Discussion**

Here we showed that miR-modulated sEVs were capable of eliciting a therapeutic response *in vivo*. Previously, we have used the STZ-induced diabetic wound healing mouse model herein described to assess the impact of native (unmodulated) sEV on wound healing[6].

In both the previous and current studies, a comparable regiment of sEV administration was used, where samples were topically administered bidaily on wounds (**Fig. 2A**). Both in the previous study and in this work, a comparable regimen of sEV administration was used: bi-daily topical administration of EVs on the wounds (**Fig. 2A**). This dosage was chosen because a single administration might be readily cleared from the system and bi-daily administration can elicit different biological responses based on the wound healing stage.

In the work of Henriques-Antunes et al.[6], it was found that native sEVs were sufficient to induce a significant increase in wound healing speed, with wounds healing by over 10% more area in treated versus control conditions at day 10. In comparison, when wounds were treated with EVs enriched with miR-425-5p, an endothelial pro-survival miRNA, not only were the wounds nearly completely closed at day 10, but also the overall wound healing kinetics seemed to have been accelerated by roughly a 3-day difference (**Fig. 2B**). Interestingly, our results showed that at day 2 and day 5, compared to the control, the expression of miR-425-5p was higher in the wounds of animals treated with miR-425-5p-modulated EVs suggesting a successful transfer of the miRNA into recipient cells (**Fig. 2C**). The fact that miR-425 was detected in control wound tissue indicates that either sEVs have some endogenous amounts of this miR, or that mouse miRNA was also being detected at the injury site. The latter option is more likely, as previous studies into the miRNA composition of these EVs found no evidence of miR-425-5p being present[6]. In conclusion, our results demonstrated that sEVs modulated with Exo-Fect™ can effectively deliver specific miRNAs to target tissues and ultimately elicited therapeutically relevant effects.

Following the establishment of Exo-Fect™ as a viable sEV transfecting agent, we then sought to discover novel compounds with similar or better miR-loading properties. This goal was motivated by the fact that, in a previous study conducted by us[7], we have found that Exo-Fect remains complexed with EV preparations and can elicit changes in EV-cell interaction and processing. Moreover, we also showed that above a certain concentration, Exo-Fect™ displays

cytotoxicity. To accomplish this goal, a quantitative high-throughput screening methodology was developed, where sEVs were complexed to magnetic beads, allowing for large scale processing of samples with low amounts of EVs and facilitating the washing steps throughput the different steps of the protocol (**Fig. 3A**). One of the limitations of this protocol is related to the fact that only CD9-expressing EVs were complexed with the beads and thus, CD9 negative EVs were excluded from the study limiting the total number of EVs one can use and eventually losing biologically relevant subpopulations. Previous work has shown that sEVs obtained from a given source may be heterogeneous and express different markers in different EV sub-populations[29] and therefore, the functional impact of selecting only the CD9-positive population of EVs is unknown. Nevertheless, we used the above-mentioned approach to screen the Prestwick library due to the fact that these compounds had already been approved by regulatory authorities, which would facilitate the translation of the results. Additionally, the diverse structure and function of the compounds present in the library could shed mechanistic insights into the interaction between miRNAs-EVs. Finally, the fact that these compounds may be active in a number of pathological contexts offered the possibility that the compound may act in a synergistic way with the EV and miR, allowing for a functional trinity of therapeutical agents complexed in a single entity (**Fig. 3B**). To broaden the scope of our strategy, 5 proprietary research drugs were added to the screening approach. Our results showed that only two compounds were capable of effectively load a fluorescently-labelled miRNA onto EVs (**Fig. 3D**). When further tested, those molecules exhibited concentration dependent effects on miR-loading of EVs (**Fig. 4A-C**), although the optimal settings for the use of these molecules in EV priming and loading have yet to be determined.

In conclusion, these novel compounds offer promising alternatives to Exo-Fect™, in the context of EV enrichment with miRNA. Furthermore, the establishment of the screening methodology as a valid approach to discover EV-reactive compounds may also prove useful for future studies in different contexts.

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# CHAPTER VI – Summary and General Discussion

The therapeutic potential of extracellular vesicles (EVs) resides both in their innate regenerative potential or as a drug delivery system. Firstly, as a therapeutic agent, EVs have shown to be useful in several pathological contexts. Initially, attention was fuelled by the fact that EV cargo mimicked the content of the donor cell[1] and therefore, the use of already established regeneration-inducing cell types, such as stem cells, sparked a paradigm shift towards EV use[2-4]. Stem cell transplantation for regenerative purposes has several shortcomings which the use of EVs eliminated or lessened[5,6], such as the poor scalability of the systems, low engraftment/survival rates, oncogenic potential, elicitation of adverse immune responses, undefined mechanisms of action and even ethical concerns[7]. The discovery that EVs were key mediators of the therapeutic effects observed with stem cell therapies[8-10] increased the interest in the field. The fact that EVs can bypass biological barriers[11,12] render them particularly useful in the context of hard-to-reach tissues and organs, such as the heart or the brain. Furthermore, the low regenerative potential of these organs makes them a prime target for therapeutic EV usage. Finally, the possibility to engineer EVs can be harnessed to improve their bioactivity and/or targeting potential making them highly attractive therapeutic agents and/or drug delivery systems for already established therapeutic molecules[13]. In this thesis, two approaches were explored, in which we've made contributions to the field and wider scientific community. Firstly, in **Chapter IV**, we compared previously reported EV-enriching methodologies, with this study being the first to compare these approaches side-by-side for EV enrichment with miRNA. The functional effect of modifying EVs, both from a phenotypical outcome on recipient cells/tissues and an EV biology point of view was then explored. For the first time, we showed that while Exo-Fect is an efficient and reproducible method to load EVs with miRNA, it causes changes to EV biology which likely result in different cellular uptake and processing. This approach then inspired us to seek novel molecules capable of loading EVs with therapeutic molecules. Consequently, in **Chapter V**, a high-throughput quantitative screening methodology was developed and implemented for this purpose. Our results ultimately yielded two compounds which may be explored from a biotechnological perspective and constitute innovative contributions to the field.

**Enhancement of EVs for therapeutic application**

In **Chapter II**, we performed an extensive literature review into the application of EVs in the cardiovascular setting. Given the epidemiological status of cardiovascular diseases (CVD)[14] and the lack of therapies capable of efficiently restoring cardiac function, EV-based therapies emerged as particularly promising alternatives. Despite the ongoing effort for the application of EVs to treat CVDs, their therapeutic efficacy remains limited[15] mostly because in the damaged myocardium, EVs must contend with a harsh inflammatory environment[16,17] and, regardless of the administration route, low accumulation rates[18]. Other limitations of EV-based technology pertain to the fact they are not easily trackable *in vivo* and although they may have some homing capacity for specific cell types, the majority of systemically administered EVs typically ends up in the liver and spleen[18]. Finally, the fact that the bulk of EVs that enter a recipient cell may end up being degraded by the endolysosomal system[19], means that the effective cargo that may be released in the cytoplasm is variable and may be an order of magnitude lower than required to elicit a strong therapeutic effect. Nonetheless, recent studies have attempted to modify and enhance EV function(s) by engineering EVs in an attempt to overcome these obstacles[20,21]. In this Chapter, we identified four key areas where EVs may be enhanced to yield superior therapeutical outcomes: tracking, targeting, internalization and bioactivity.

To improve EV tracking, the EV membrane has been modified with fluorophores which allow for their tracking and monitorization *in vivo*, ultimately informing about the best dosage and administration regimens. However, a common issue with this type of approach is the fact that the tracking moieties may have longer half-lives than the EV itself and therefore caution is required when interpreting data pertaining to labelled EVs, as it may be prone to artefacts[18]. Other opportunities may arise in this field, however, as the development of triggerable systems, inspired, by synthetic nanoparticle approaches[22], may allow for more specific visualization of EVs. Additionally, the development of fluorescence resonance energy transfer (FRET) in association with EVs may allow for specific tracking of the EVs in target tissues[23].

A number of strategies has been employed to improve **targeting and internalization** of EVs into a given cell/tissue/organ. Several of these strategies explore the functionalization of the EV surface with peptides that aim to either bind specifically to certain cell types[24,25], increasing

EV availability in the tissue of interest, or facilitating endosomal escape[26,27], increasing the cytoplasmatic availability of EV cargo in the recipient cell. Finally, enhancing EV bioactivity has been the area where most of the research has been conducted so far and several methods and approaches have been explored to that end[28-32]. Generally, the idea behind this type of EV enhancement entails loading EVs with therapeutically relevant molecules and harness their intrinsic capacity to cross biological membranes to effectively deliver the cargo in recipient cells. The dual use of EVs as drug delivery agents as well as therapeutic entities in their own right has become a new paradigm in EV research[21,33]. Most studies have focused on one of two possible approaches for the enrichment of EVs: modification of the donor cell followed by the isolation of EVs from the medium or post-isolation modification of EVs. While the former method tends to capitalize on the endogenous therapeutic potential of the donor cell[34,35], the gamut of possible modifications is less controllable and/or limited given the technologies available for genetic manipulation of cells. Post-isolation methods are much more versatile given the fact that they often utilize EVs isolated from highly available sources such as urine or plasma or even milk and/or plants and the number of molecules used for the modification can be broader since no modification of the secreting cell is required However, the methods employed for modification may cause structural changes to EVs[36,37], which may have unexpected effects in EV uptake and intracellular processing[38]. and ultimately their bioactivity. Which EV strategy is the most suitable to enhance cardiac function upon injury is still a matter of debate, and it was a question further explored in **Chapters IV** and **V**.

As previously established, EV action is contingent on effectively reaching the target cell. To this end, the interplay between EVs and endothelial cells is crucial, as these cells constitute the major interface between systemically delivered EVs and target tissues and organs. Consequently, microvascular health and development is key for effective EV action. On the other hand, as previously mentioned, non-coding RNAs are one of the bioactive cargoes present in EVs that can regulate the fate of the target cell. There is, therefore, an interest in understanding how all these entities interplay in health and disease. To this end, in **Chapter III**, we have explored the role of non-coding RNAs and EVs in microvasculature, particularly in the context of CVD. From this study, the role of non-coding RNA, especially microRNA, as highly relevant targets or treatments for cardiovascular repair and regeneration as been established. As discussed in **Chapter II**, several miRNAs have been used as therapeutic tools

to regulate gene expression in various contexts. Presently, despite over 500 clinical trials being underway/concluded, involving the use of microRNA in humans, only a handful are being pursued as promising clinical tools[39,40]. So far, only a few clinical trials making use of miRNAs have entered phase 3/4, and those that have intend to control endogenous levels of miRNA in order to produce a therapeutic effect. This is the case, for example, of a study using levosimendan to regulate the expression of miR-660-3p, miR-665 and miR-1285-3p, to ameliorate heart failure[41]. It remains to be seen whether the conjugation of the gene regulation power of microRNA can be translated into effective therapies using EVs as a delivery agent in clinic.

A caveat inherent to the compartmentalized view and study of EVs as presented in **Chapters II** and **III** is the fact that the EV is not construed as a whole entity. This is, EVs are mostly considered only in terms of their components (e.g. membrane, lumen, cargo) which may assessed or modulated. A more integrated conceptualization of EV study was alluded to in **Chapter II**, when the idea that multiple enhancements may be performed on the same vesicle was proposed. Nevertheless, even this multipronged approach lacks the context of the donor cell or the specific EV properties in question, for example. Perhaps a more holistic approach to EV study and application is necessary in order to achieve a true bionanoparticle with therapeutical value.

**Challenges in clinical application of EVs**

Having established the core concepts that demonstrate that EVs can be modulated to enhance their therapeutic activity, we explore in **Chapter IV** and **Chapter V** different methods and applications for EV enhancement. In **Chapter IV**, we showed that the commercial kit Exo-Fect is an efficient method to load EVs with a microRNA of interest and further showed that the modulated EVs were capable of downregulating the expression of target transcripts *in vitro*[38]. In **Chapter V** we also showed that this technology is a valid tool for *in vivo* treatment of diabetic wounds, as it compares favourably with previous treatments regarding wound closure kinetics[42]. However, we also noted that the Exo-Fect reagent/protocol, despite its unknown composition, sticks to the EVs and elicits particle aggregation and cytotoxicity above the threshold of 0.5% (v/v). These observations may explain why the uptake and processing

of Exo-Fect-modulated EVs differs from their native ultimately contributing to changes in EV biology. Consequently, in **Chapter V**, we developed a novel screening assay to identify compounds capable of loading miRNA onto/into EVs. This high-throughput screening protocol yielded two compounds of undisclosed nature that appear to be highly efficient in loading EVs with pre-selected miRNAs. More work will need to be done to elucidate whether these molecules have the potential to reach Exo-Fect levels regarding their capacity to load the EVs with miRNA and whether they influence EV biology in the same way or not.

The desirable traits of an ideal EV transfecting agent are not yet well understood. From our results, it appears the cationic nature of the molecules may play a role in bringing the miRNA and EV membrane, both negatively charged. Importantly, while, theoretically, it would be more efficient to have a loading agent bring therapeutic molecules into the lumen of the EV, thereby conferring them protection, little is known of how to achieve that goal. Additionally, the technical difficulty of confirming a molecule was internalized into an EV is still a considerable obstacle in addressing this question. Moreover, effective concentrations of miRNA, or other therapeutic compounds of interest, are mostly based on studies that do not rely on EVs for delivery, hence adding another layer of complexity. In this regard, it has been found that EVs, from several sources, may carry endogenously less than 1 copy of a single miRNA per EV[43] and that these EVs can yield beneficial phenotypical outcomes[44] in regenerative situations. However, when loading EVs with exogenous material, most studies, including our own, can only ascertain an average of molecules per EV, which may not allow for accurate interpretation of data. For instance, it is possible that a few of the EVs in a loaded sample hold the majority of the therapeutic agent (low occupancy/high concentration model[43]). In this case, it is expected that the downstream functional studies resulting from using these EVs are more variable, depending on whether those specific enriched EVs were more internalized than their counterparts or not. Single-EV technologies and platforms currently in development will be key to answering these questions[45]. Also, as alluded to before, modifying EVs post-isolation may incur in unexpected biophysical alterations on affected EVs. For instance, our current work hypothesis regarding the study of **Chapter IV** indeed admits that Exo-Fect may stick to EV membranes, after transfection. Whether this can occur with other loading molecules, such as the compounds discovered in **Chapter V**, is still not known. Interestingly, whether having loading agents stick to the EVs may appear strictly

detrimental at first glance, by virtue of altering normal EV processing and biology, there may be serendipitous upsides to this process. In the case of Exo-Fect, a higher cellular uptake percentage and lower lysosomal colocalization point to the idea that while Exo-Fect may cover some portion of EV membrane, it via that mechanism that those EVs can interact with recipient cells in a way that leads to higher cytoplasmatic release of their contents. Finally, most of the studies herein discussed are centred around miRNA and similar nucleic acids. However, other therapeutically relevant drugs may be more effective, depending on the specific pathophysiological context of application. In those cases, despite some preliminary work[46,47], it is still very much unknown whether the same principles as those current under development for small nucleic acids apply, or whether molecules of different natures, such as lipids or proteins, may require entirely distinct frameworks for EV loading, still remains to be explored.

From a technical standpoint, our work in **Chapter IV** sheds light on the difficulties of manipulating EVs and interpreting data. Several methodologies used for EV collection and modulation may introduce artefacts or otherwise alter normal EV biology. It is known that ultracentrifugation, the most used technique for EV purification, can collapse EVs[48] and co-purify unwanted contaminants[49]. Electroporation of EVs and microRNA causes precipitation of aggregates[36]. As we have also shown, Exo-Fect induces aggregation and quenching of fluorescent probes (both used for membrane and/or miRNA labelling) on EVs. The disparities caused by these and others techniques may confound EV research, particularly across different groups. These complications emphasize the need to improve and homogenize EV-related practices, an effort that has been conducted by the International Society for Extracellular Vesicles (ISEV)[50,51].

However, these are not the only obstacles hindering the translation of EV-based therapies into effective clinical alternatives. Another major bottleneck is still the scalability of EV production/collection systems. Many EV applications require the biosignature of their donor cell (i.e. the molecular, cytoplasmatic EV-sorted contents) to be therapeutic[52,53], and as such the mass production of standardized EV batches needs to be established[54]. On the other hand, relying on the mass production of donor cells for EV harvesting is a costly and laborious process. Another alternative may be the utilization of primary human EVs. In **Chapter V** we

also used human urine EVs, which may be one potential solution[55]. The use of these vesicles also allows for the use of EVs as autologous therapies reducing the risk of immune-rejection/reaction by the recipient organism[56]. Other options have focused on the use of EV-like particles from plants[57,58] or EVs from bovine milk[59,60]. These alternative sources of EVs may prove interesting and cost-efficient methods of obtaining high quantities of vesicles with a known bioactivity or prone to modification using bioengineering strategies.

Nevertheless, there is currently a race to the clinic focusing on EV-based platforms for drug delivery. In this regard, EVs compete with synthetic nanoparticles and viruses to some extent. Synthetic nanoparticles are generally more controllable systems, synthesized from known and reproducible reactions. Additionally, a large, easily modulable surface area makes them ideal candidates for compound conjugation[61]. However, typically the clearance of synthetic nanoparticles can either be too efficient, leading to poor biodistribution[62], or too inefficient, leading to toxic accumulation in target tissues[63]. On the other hand, the efficiency of viral-based strategies for gene regulation is typically more efficient than EVs or nanoparticles. Nevertheless, potentially low loading capacity, off-target effects and immunogenicity concerns[64] still render some of these viral approaches unsuitable for clinic. As we have established previously, EVs have properties that distinguish them from other drug delivery platforms. Whether they can be harnessed effectively enough to bring EVs to the clinic in lieu of alternatives remains to be seen.

**Concluding remarks**

In this thesis we have discussed the application of EVs in therapy, and how modulating them may increase their efficacy, for *in vitro* and *in vivo* applications, with a translational and clinical output in mind. We've focused primarily on the cardiovascular setting, which, despite challenging, is lacking in effective treatments. As such, it stands to benefit the most from advances in EV-based therapies, with multiple clinical trials are underway[65-69].

Disease contexts may favour the use of EV-based technologies. For example, some authors have described that during myocardial injury, rapid calcium influx into cardiomyocytes and fibroblasts triggers mass endocytosis[70]. This event could present an opportunity for potentiating EV uptake in the injured tissue and thereby boost treatment efficacy. However,

to make the most of these opportunities, there is a greater need for standardization and optimization of EV administration regimens and routes. Recent studies have used vastly different amounts of EVs across different administration routes[29,71-73], while aiming for similar functional outcomes. Further advances in the field of EV-based therapies are thus contingent on achieving gold standard practices for EV delivery.

Finally, it is important to underscore that while we have focused on the application of EVs in therapy, the clinical potential of these molecules is not restricted to treatment options. In fact, a majority of the work with EVs has focused on their promise as valid biomarkers for a number of diseases[74-77]. In line with these studies, the conjugation of these approaches has also shown the potential of EVs as theranostics[78]. Moreover, as mentioned previously, EVs play a crucial role in the normal homeostatic function of the human organism[79]. Consequently, any advances in basic EV research stand to benefit translational and clinical research, and vice-versa.

In conclusion, there is tremendous promise surrounding EV application. This thesis sought to contribute to the field by studying how to enhance the endogenous potential of EVs for the delivery of therapeutically relevant molecules. I am confident that in the coming years EVs will be translated into effective clinical treatments. Indeed, following the adage “life finds a way”, in this case I think it already has.

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Impact

## Socio-economic impact and scientific innovation

Extracellular vesicles are at the forefront of scientific interest and on the cusp of translational and clinical application. Consequently, a number of stakeholders can be identified that are affected by the work presented in this thesis. Firstly, the general public stands to benefit from advances in the development of novel therapies for high mortality/morbidity pathologies, such as cardiovascular disease[1] or cancer[2]. In line with this point, the lack of effective first line treatments in cardiac disease or some types of cancer makes these pathologies particularly difficult to deal with from a healthcare perspective, and consequently, from a governmental standpoint as well. Thereby, the standardization and optimization of EV-related technologies for therapeutic efficacy, as we have explored in **Chapters I-III**, will also contribute to the general easing of burden of disease globally. From an economic stance, pharmaceutical and R&D companies currently working with EVs and EV-related technologies will also benefit from the work here presented. Presently, over 45 companies[3] fill those criteria and as such they may incorporate findings in this thesis in their own pipelines and projects. Another major stakeholder associated to this work is the scientific community at large. The concepts and evidence herein presented clearly show EVs as promising tools for a multitude of purposes. More than that, however, the elucidation of some aspects of Exo-Fect role in EVs, as shown in **Chapter IV**, serve as a note of caution on the use of this reagent which may inform future research more accurately than previous studies would. Lastly, the establishment of a novel screening protocol for EV-related compounds, as outlined in **Chapter V**, will contribute directly to the development of novel EV-based products and the understanding of EV biology.

In order to reach the maximum impact and as many stakeholders as possible, the work presented in this thesis has been the subject of scientific discussion and dissemination, having been presented at multiple conferences (e.g., 2021 ISEV meeting, 2018 EMBO course on EVs) and also in the form of peer-reviewed scientific publications.

## References


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## Biography and Publications

Ricardo Abreu was born in Porto, Portugal, in 1993. After finishing his B.Sc. studies in Genetics and Biotechnology at the University of Trás-os-Montes e Alto Douro (Vila Real, Portugal) in 2014, he pursued his M.Sc. at the University of Coimbra (Coimbra, Portugal). Through the Erasmus mobility programme, he performed his master's degree dissertation at the laboratory of Prof. Paula da Costa Martins, at the University of Maastricht, where he worked in non-coding RNA technology in the context of angiogenesis. In 2016, he enrolled in a joint PhD programme between the University of Coimbra, through the group of Prof. Lino Ferreira, and the University of Maastricht, through the group of Prof. Paula da Costa Martins. His studies were focused on the modulation of extracellular vesicles for use in the therapeutic context of regenerative diseases.


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