Extracellular vesicle therapeutics for cardiac repair: A translational perspective

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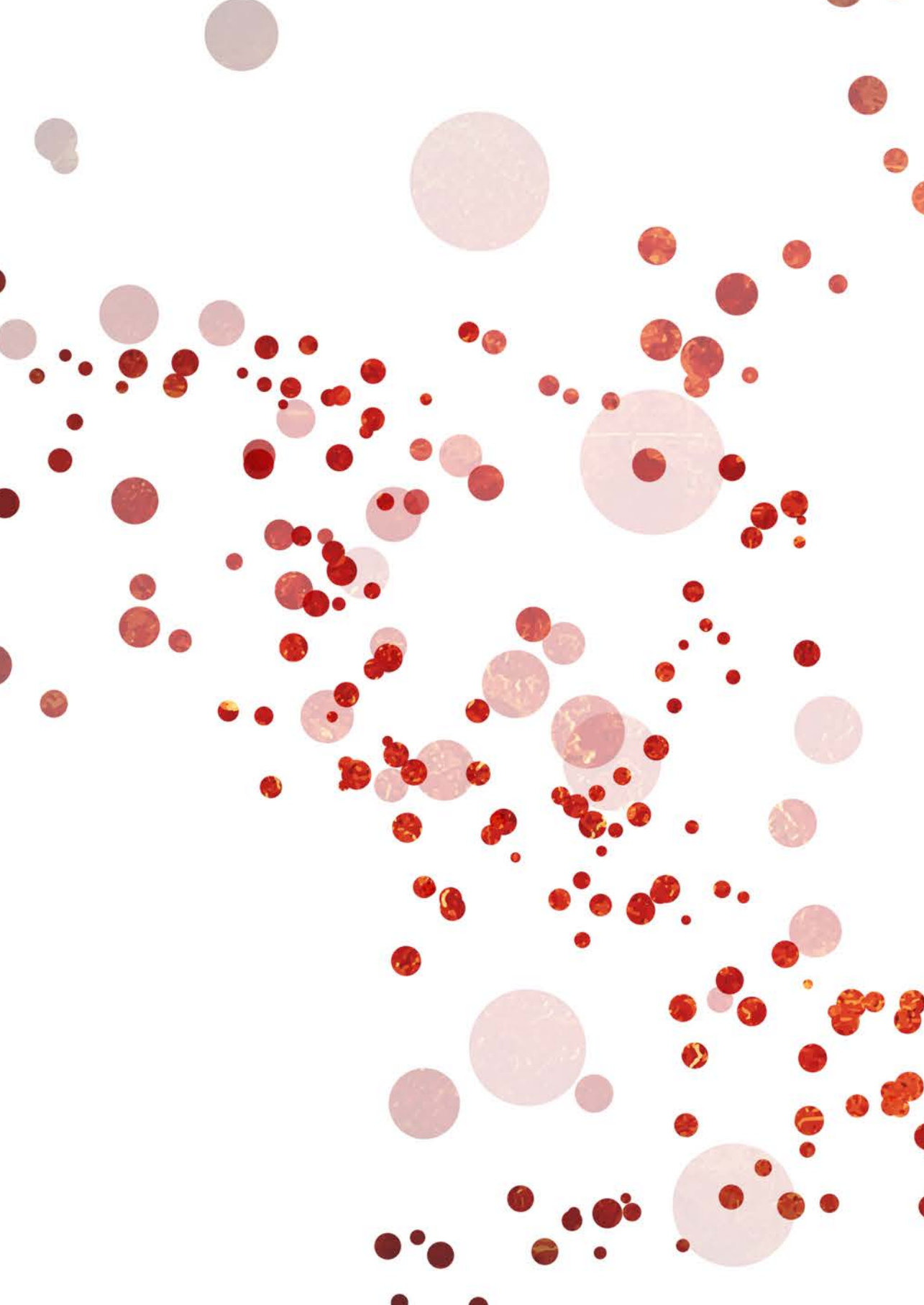

CHAPTER 1

Cardiac progenitor-cell derived exosomes as cell-free therapeutic for cardiac repair

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INTRODUCTION

Myocardial infarction (MI) is one of the leading causes of death in the western world[1]. MI is induced by occlusion of one or more coronary arteries that supply oxygen to the heart, resulting in necrosis and apoptosis of cardiomyocytes that are highly dependent of oxygen. As a result, different molecular and cellular mechanisms are activated roughly in two phases. First, as necrotic cardiomyocytes release danger signals into the myocardium, the immune system is activated via toll-like receptors and complement activation[2]. This inflammatory response causes the attraction of neutrophils and monocytes to the infarcted area and is necessary to remove cellular debris. An overactive immune system can promote further tissue damage and infarct expansion[3]. The second step is a reparative phase characterized by activated fibroblasts (myofibroblasts) that produce excessive amounts of extracellular matrix, resulting in the formation of scar tissue[4]. Initially this scar tissue replaces the lost cardiomyocytes and provides strength to the heart to maintain its integrity, however, later progressive matrix deposition by activated myofibroblasts might lead to myocardial stiffening and impaired contraction. Since the initial myocardial damage is caused by a perfusion defect, stimulating neovessel formation or promoting arteriogenesis could contribute to cardiac regeneration[5,6]. Cardiac repair mechanisms may be improved by interfering in these reparative mechanisms that play a role after MI by down-tuning the dentrimental processes, such as cardiomyocyte apoptosis, the inflammatory response, and fibrosis, and promoting further reparative signals like angiogenesis (Figure 1).

Of all patients that suffer from MI, approximately 25% will develop heart failure within one year[7]. Currently, the only long-term treatment option for heart failure patients is heart transplantation, but donor availability is limited. Although patients waiting for heart transplantation can benefit from a left ventricular assist device (LVAD) taking over the pump function of the heart, this is usually a temporary solution[8]. Therefore, new treatment options are explored to replace the lost cardiomyocytes and improve contractility in cardiac diseases, especially for heart failure patients.

Cardiac progenitor cells as potent potential cell type for myocardial repair

One of the first authors describing the existence of cardiomyocyte regeneration was Oberpriller et al[9]. Amputation of the ventricular apex of the newt heart resulted in the renewal of cardiomyocytes by re-entry into the cell cycle and proper engraftment in the myocardium. Also resection of the ventricular apex in zebrafish resulted in complete apical regeneration, mainly due to proliferation of progenitor cells in the heart and possibly also by dedifferentiation of residing cardiomyocytes[10,11].

For decades it was believed that the mammalian heart had no regenerative capacity. Recent studies provided evidence for a limited but true regenerative potential of the heart[12–14]. Bergmann et al. demonstrated the ability of the heart to regenerate by quantifying carbon-14 incorporation into the DNA of human cardiomyocytes[13]. Approximately 1% of the cardiomyocytes is renewed at an age of 25; this capacity is fast reduced upon aging and in sharp contrast to cardiac resident non-cardiomyocytes with a renewel rate of

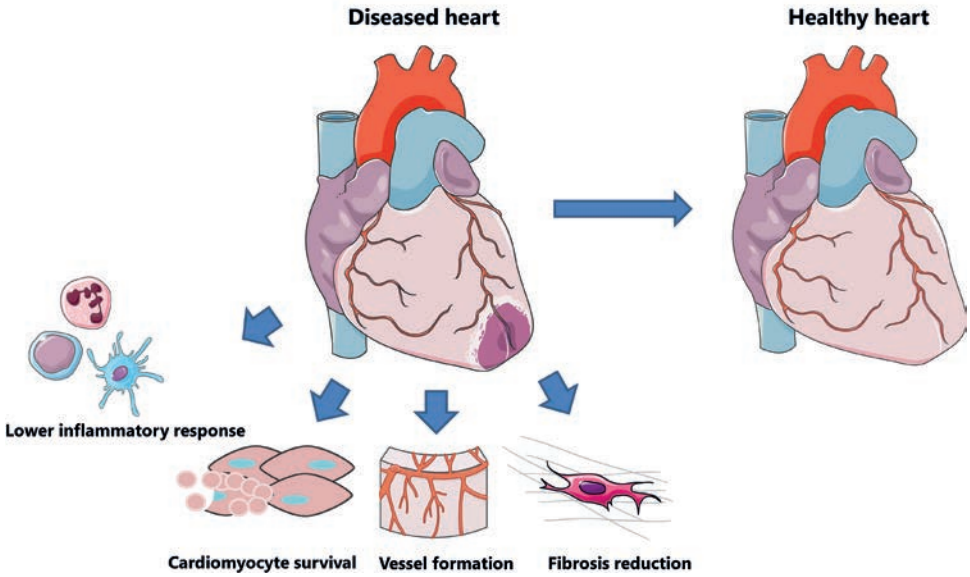

Figure 1 Processes that need additional adaptations to further induce cardiac repair after myocardial infarction.
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approximately 15%[15]. Recently, a human case study of a newborn reported functional recovery of the human heart suffering from MI at this early age[16]. As a result of these observations, several new strategies have been explored to stimulate the regenerative capacity of the mammalian heart.

One of the strategies is the use of progenitor cell treatment as potential therapy to improve cardiac repair and prevent further damage in cardiac diseases. Several cell sources have been studied over the years and used to stimulate myocardial repair; these so-called first generation patient-derived cells include bone-marrow mononuclear cells (BM-MNCs)[17–19] and mesenchymal progenitor cells (MSCs)[20,21]. The use of BM-MNCs and MSCs for cardiac repair are explored extensively due to their quick and relative easy clinical application. Furthermore, large numbers of cells could be achieved by culturing MSCs under good manufacturing practice conditions for clinical use[21,22]. Meta-analysis of pre-clinical and clinical studies showed that injection of MSCs, in contrast to BM-MNCs, resulted in beneficial effects on cardiac function[23,24]. MSC therapy was, however, limited to its potential to activate endogenous repair systems in the heart[25]. More recently, second-generation cells, including cardiac-derived progenitor cells (CPCs)[26–30] and induced pluripotent stem cell (iPSC)-derived cardiomyocytes[31,32], have gained interest as a cell source for myocardial repair, mainly because of their promising regeneration capacity and their intrinsic ability to form contractile cells. iPSC-derived cardiomyocytes are cardiomyocytes generated by reprogramming fibroblasts to pluripotent stem cells using several transcription factors[31,32]. Despite their true

potential to form cardiomyocytes, the main effect of second-generation cells, observed upon cardiac transplantation, has been of paracrine origin. Excellent recent reviews describing the most relevant results and current limitations of cell-based therapies have been recently reported[33,34].

The existence of progenitor cells in the heart was first described by Beltrami et al.[28], but since then several cardiac progenitor cell populations have been identified[26–30]. CPCs are potentially the most promising adult cells for cardiac therapy as they can generate all cardiovascular lineages *in vitro* and *in vivo*[27,35,36]. Since they originate from the heart itself, CPCs may be destined to activate endogenous repair mechanisms. Therefore, CPCs hold greater cardiac regeneration potential compared to BM-MNCs or MSCs.

In different animal models for myocardial infarction, injection of CPCs increased cardiac performance[37–40]. However, although cardiac function was improved, cell engraftment of the injected cells in the myocardium was low, as indicated before for BM-MNC and MSCs. To stimulate cell survival upon myocardial injection, pre-treatment of CPCs with e.g. pim-1 or necrostatin-1 before CPC injection has been investigated[41,42]. To further improve cell retention and prevent immediate flush-out[43], different approaches have been investigated, e.g. the use of cell clusters or a combination of cells with microcarriers[44,45]. These approaches resulted in increased cell retention and survival, however, the additional beneficial effects on cardiac function was minimal.

Comparison of CPC types

Although the heart has poor regenerative potential, many cardiac progenitor cell types have been identified based on marker expression/morphology, including Sca1+, c-kit+, cardiosphere-derived cells (CDCs) and cardiospheres (CSPs), and all these types can be isolated from the heart successfully[26–30]. As the existence of so many CPC populations is counterintuitive, Gaetani et al. have compared the different CPC types[46]. Using their individual isolation methods, several of these progenitor cell types have been cultured and the gene expression profiles were compared to define differences between culture propagated CPCs. The gene expression profile of CSPs was most distinct from the Sca1+, c-kit+, and CDCs, most likely due to the monolayer and 3D culture conditions. Additionally, the difference between individual patients was larger than differences between different cell types from a single individual and expression partners are highly overlapping. Interestingly, when these cells were freshly isolated directly from the rodent heart some differences could be observed, indicating that c-kit positive cells were the most primitive progenitor cell[47]. However, this difference is abolished upon culture propagation. Furthermore, Zwetsloot et al. recently compared effect sizes of different types of CPCs[48], and observed that small differences in effect size can be found based on cell type; CSP treatment resulted in the largest increase in ejection fraction after injection in different animal models compared to e.g. Sca1+ and c-kit+ CPCs, that showed a lower increase in cardiac function. Therefore, the mode of action of different CPC types on the myocardium is largely similar, although slight variations in effect size and transcriptome are described. Interestingly, a strong drop in functional benefit was observed upon their use in rodent and preclinical large animal models.

To date, two clinical trials have used CPCs as cell type for cardiac repair after MI. The SCIPIO (c-kit+ CPC)s and CADUCEUS trial (CDCs) showed that intracoronary infusion of CPCs is safe in patients and led to enhanced cardiac function[49,50]. Therefore, CPCs are a promising cell type for stem cell therapeutics.

Paracrine secretion

Originally, the concept for myocardial repair by progenitor cells was that they would engraft in the infarcted area and differentiate into functional cardiomyocytes upon injection. Recently, it has become more and more clear from both animal studies and clinical trials that injected progenitor cells do not engraft properly in the cardiac tissue, despite beneficial effects on cardiac function[37,44,49,50]. Moreover, cardiomyocyte, endothelial, and blood vessel numbers were increased, which led to the hypothesis that the injected progenitor cells exert their effect via release of factors into their environment, called paracrine factors[37,42,51]. To study the effect of paracrine secretions, Timmers et al. injected MSC conditioned medium intravenously at the moment of reperfusion in pigs after MI and showed that MSC secretions could mimic the increased cardiac function[52]. This paracrine effect was observed for bone-marrow derived-, and mesenchymal progenitors, but also CPC secretions have these effects. CPC conditioned medium lowered cardiomyocyte apoptosis, stimulated endothelial cell migration, and increased tube formation of endothelial cells *in vitro*[40,44,53,54].

In addition to paracrine molecules, the release of extracellular membrane vesicles such as exosomes are of increasing interest. Besides their use as biomarkers to detect early diseases[55], these nano-sized vesicles have also shown to be important mediators in repair after cardiac injury. Upon receiving stress signals, cells can influence their communication to other cells by adjusting membrane markers and vesicle content. Interestingly, Lai et al. identified the active cardioprotective component in the conditioned medium of MSCs to be exosomes[56]. They showed that upon separation of MSC conditioned medium in fractions of different sizes, the beneficial effects on ischemia/reperfusion injury observed after injection with fractionated MSC conditioned medium could only be reproduced by injecting the fraction containing complexes larger than 1000 kDa. Since progenitor-derived exosomes were found to be the paracrine factors mainly responsible for the observed beneficial effects after progenitor cell injection[56–59], the idea that CPC exosomes could be used for this purposes have emerged as potential off-the-shelf therapeutics.

CPC exosomes carry a variety of different proteins, growth factors, mRNAs, and microRNAs (miRNAs). MiRNAs are small non-coding RNAs that can inhibit or degrade mRNA, thereby preventing protein translation. Studies that investigate the effect of CPC exosomes on cardiac repair *in vitro* and *in vivo* are described below.

Functional benefits of CPC exosome treatment

To study the functional benefits of CPC exosomes, CPC exosomes were intramyocardially injected in mice undergoing ischemia-reperfusion of the left coronary artery[39]. Injection of CPC exosomes reduced cardiomyocyte apoptosis by 53%. In addition, Barile et al. showed that intramyocardial injection of CPC exosomes in mice improved cardiac function after MI[40]. Morphological analysis after CPC exosome treatment in the myocardium revealed

reduced scar tissue, lowered cardiomyocyte apoptosis, and increased blood vessel density. Injection of exosomes from autologous CPCs requires cell expansion *in vitro*, therefore, injection in the chronic phase is more clinically relevant. Therefore, while most studies investigate the effect of CPC exosomes in the acute setting after MI (within a few hours), Tang et al. studied the effect of CPC exosome treatment of an old infarct[38]. Intracoronary infusion of autologous CPC exosomes in rats one month after MI resulted in less fibrotic tissue and improved cardiac function. The fact that CPC exosomes still seem to have regenerative effects after a longer time period is promising for patients with chronic cardiac diseases.
To investigate if the release of exosomes from CPCs is critical for cardiac repair *in vivo*, Ibrahim et al. treated CPCs with GW4869, a reversible inhibitor of neutral sphingomyelinase that blocks, among others, exosome production[54]. The CPC-mediated benefits in mice after MI were completely abolished after treatment with GW4869, indicating that exosome release from CPCs is necessary to accomplish the beneficial effects on cardiac function. Altogether, these *in vivo* studies suggest that CPC exosomes induce cardiac repair, by interfering in processes such as cardiomyocyte apoptosis, fibrosis, and vessel formation. The following *in vitro* studies aim to identify the key cardioprotective processes stimulated by CPC exosomes.

Key mechanisms targeted by CPC exosomes

Targeting the different processes that either prevent or reduce cardiac injury or contribute to cardiac regeneration after MI might lead to new treatment options. As described before, MI induces a cascade of molecular and cellular mechanisms in mainly two phases. The first phase is characterized by cardiomyocyte apoptosis and subsequent activation of the immune system. Cardiomyocyte apoptosis is a large contributor to impaired cardiac function after MI, as the major loss of contracting cells is responsible for the reduced contraction capacity of the heart. Preventing cardiomyocyte apoptosis could therefore be one of the mechanisms to improve cardiac injury. Interestingly, CPC exosomes have shown to have anti-apoptotic effects. Chen et al., for example, showed that CPC exosomes prevent apoptosis of H_2O_2-treated cardiomyocytes *in vitro*[39]. Caspase 3/7 activity in cardiomyocytes was lowered after treatment with CPC exosomes, which is an important mediator of H_2O_2-induced apoptosis. To further identify how CPC exosomes affect oxidative-stress related apoptosis of cardiomyocytes, Xiao et al. focused on exosomal-derived miRNAs[60]. They found that miRNA-21 is upregulated in CPC exosomes exposed to oxidative stress compared to non-exposed CPC exosomes. Interestingly, miRNA-21 targets programmed cell death 4 (PDCD4) in cardiomyocytes, thereby reducing oxidative-stress related apoptosis. Furthermore, miRNA analysis revealed that miRNA-210, miRNA-132, and miRNA-146a are highly enriched in CPC exosomes compared to fibroblast exosomes[40]. By inhibiting downstream targets such as RasGAP-p120, ephrin A3, and PTP1b, these miRNAs inhibit cardiomyocyte apoptosis and enhance endothelial migration after MI. Likewise, CDC and CSP-derived exosomes promote cardiac regeneration, as was shown after injection of these exosomes in the ischemic myocardium[54]. MiRNA analysis comparing CDC exosomes to fibroblast-derived exosomes revealed that miRNA-146a was the most highly enriched in CDC exosomes. Reduced cardiac function after MI was observed for miRNA-146a knockout

mice compared to wild-type mice, indicating a role for miRNA-146a in cardiac repair. Pathway analysis revealed that miRNA-146a is involved in cell survival, cell cycle, and cellular organization, which are important processes involved in cardiac injury.

Upon MI, necrotic/apoptotic cardiomyocytes release danger signals into the environment, thereby activating the immune system via complement activation and toll-like receptors[2]. Although the immune response is required to clear tissue debris after MI, an overactive immune system might aggravate cardiac damage and infarct size[3]. Therefore, modulating the immune response might prevent/reduce cardiac injury. Progenitor exosomes might be able to modulate this balance in immune responses after MI by delivery of miRNAs, anti-inflammatory cytokines, or other molecules involved in inflammation. This anti-inflammatory response was described for MSC exosomes, as MSC exosomes were capable of switching the macrophage phenotype from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype and suppress T-cell activation[61]. Until now, the immune-modulating properties of CPC exosomes have not been described in literature yet.

The second phase after MI involves myofibroblasts that are responsible for reorganizing the structure of the heart, a process called remodeling. Reducing the fibrotic tissue may be a promising way to improve cardiac repair, however, as fibrosis is initially a reparative response, a fine balance between pro- and anti-fibrotic factors is needed. Interestingly, the physiological state of CPCs can influence the secretion and cargo of CPC exosomes. Culturing CPC exosomes under hypoxic conditions resulted in higher tube formation and lowered pro-fibrotic gene expression compared to exosomes cultured under normoxic conditions[62]. Indeed, administration of hypoxic CPC exosomes in mice reduced fibrosis and increased cardiac function compared to normoxic CPC exosomes in an ischemia-reperfusion model. Microarray analysis revealed that eleven miRNAs with anti-fibrotic and pro-angiogenic properties were upregulated compared to normoxic exosomes. Whether the observed beneficial effects of hypoxic CPC exosomes on cardiac function are established through these miRNAs only or if other molecules are also involved needs to be investigated[63]. Although several *in vivo* studies indeed observed anti-fibrotic effects of CPC exosome treatment after MI[38,62], to our knowledge there are no further studies addressing the possible anti-fibrotic mechanism of CPC exosomes so far.

Other cardioprotective mechanisms that could be important for cardiac regeneration are stimulating angiogenesis or arteriogenesis, since the initial myocardial injury is due to a perfusion defect[5,6]. Progenitor exosomes derived from several cell sources have been described to have pro-angiogenic effects. Sahoo et al., for example, showed that exosomes from human CD34+ progenitor cells mediate their pro-angiogenic activity[57]. After adding exosomes, derived from CD34+ progenitor cells to endothelial cells *in vitro*, they observed increased viability, proliferation, and tube formation of endothelial cells. Furthermore, subcutaneous injection of a matrigel plug containing CD34+ exosomes in mice showed higher vessel formation compared to injection of a matrigel plug alone. They found that the presence of a pro-angiogenic protein in CD34+ exosomes, sonic hedgehoc, was largely responsible for the preserved cardiac function after MI[58].

This pro-angiogenic property of exosomes was also observed for CPC-derived exosomes. Vrijsen et al. reported that CPC exosomes stimulated migration of endothelial cells in a

wound scratch assay[53]. Analyzing the presence of pro-angiogenic factors in CPC exosomes revealed high expression levels of extracellular matrix metalloproteinase inducer (EMMPRIN), which is present on the exosomal membrane. The migration of endothelial cells upon stimulation with CPC exosomes was not observed upon stimulation with exosomes depleted for EMMPRIN (KD EMMPRIN exosomes). Furthermore, KD EMMPRIN exosomes also inhibited angiogenesis *in vivo*, demonstrated by a reduced influx of cells into a matrigel plug compared to control exosomes after application in mice[64]. Therefore, EMMPRIN is an important mediator of the pro-angiogenic effect of CPC exosomes.

Future perspectives

Altogether, these studies provide insights into the ability of CPC exosomes to enhance cardiac repair after injury and the involved mechanisms. The key mechanisms that are influenced by CPC exosomes described so far are neovessel formation and cardiomyocyte apoptosis (Figure 2). Despite considerable efforts have been made to study the effect of CPC exosomes on cardiac repair, many challenges have to be overcome before deployment of exosomes in clinical trials. Firstly, most of the described studies investigated the effect of CPC exosomes on the acute setting after MI[39,40]. Due to better revascularization therapy and medication planning, the survival of patients after acute MI is increased last decades. These surviving patients, however, have a higher chance to develop a more chronic disease like heart failure. From a clinical perspective it would therefore be useful to study regeneration by CPC exosomes in these more chronic phases after cardiac injury. Another important challenge is retention of exosomes after injection. van den Akker et al. performed intramyocardial injection of stem cells and observed immediate flush-out of the cells upon injection[43]. It is thus likely that the same flush-out can be expected upon exosome injection into the myocardium, since the exosomes sizes are even smaller (30-100 nm) compared to cells (8-12 μm). Furthermore, accurate mapping of the *in vivo* biodistribution of exosomes after systemic injection is also an important objective before using exosomes in clinical trials. Lai et al. developed an excellent technique to allow multimodal imaging of exosomes *in vivo*. Membrane-bound Gaussia luciferase was combined with metabolic biotinylation to visualize exosomes after systemic injection in athymic nude mice via bioluminescent signals[65]. The highest uptake of exosomes was observed in the liver and spleen, therefore, systemic administration of exosomes might require targeted therapy towards the injured heart. Aiming to target exosomes to the brain, Alvarez-Erviti et al., engineered cells to express an exosomal membrane protein (lysosome-associated membrane glycoprotein 2b) fused to a brain-specific peptide that targets the acetylcholine receptor[66]. They showed increased delivery of functional exosomes to the brain. Thus, although some achievements have been made to engineer exosomes in a way that they target tissues aimed for, by using specific ligands, non-specific accumulation of exosomes in other tissues remains an issue to be solved[65–67]. Lastly, to cover the high demand of exosomes needed for clinical application, a reproducible and standardized exosome isolation technique is required that allows for upscaling[68]. In addition, the characteristics of exosome-based therapeutics have to be defined properly, which requires more in-depth research into the mechanism of how exosomes exert their therapeutic effects. Nonetheless, CPC exosomes can be considered

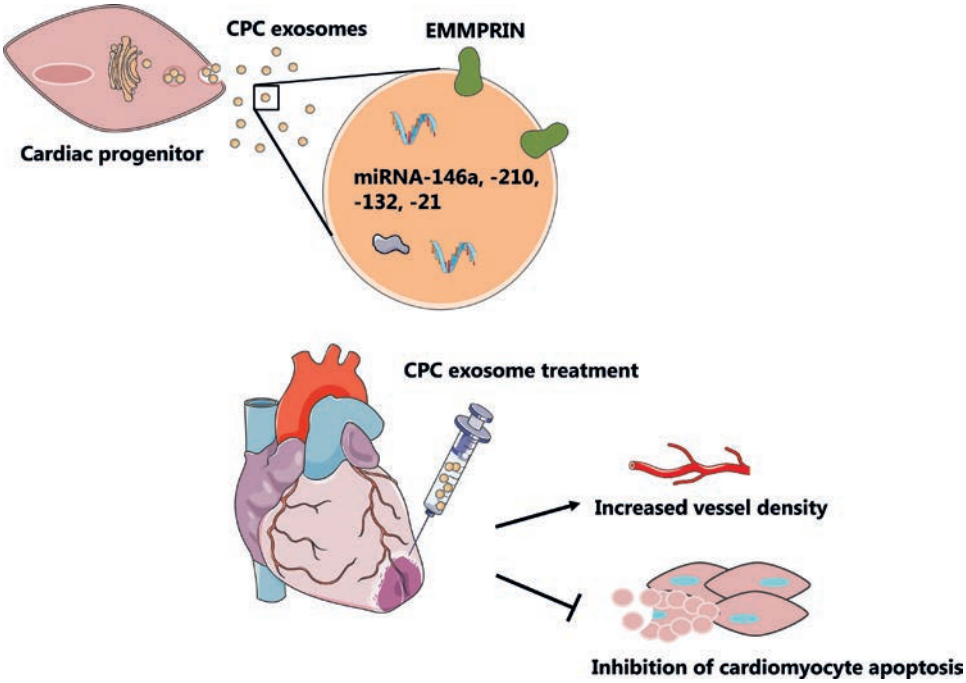

Figure 2 Key mechanisms targeted by CPC exosomes.
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as potential off-the-shelf therapeutics, as they are able to stimulate the regenerative capacity of the heart mainly by increasing vessel density and lowering apoptosis of cardiomyocytes.

THESIS OUTLINE

Extracellular vesicles (EVs) are nano-sized lipid bilayer-enclosed particles that are released by every cell type of the human body studied to date. Mammalian EVs can be classified in three major subclasses according to their intracellular origin[69]. Larger vesicles are generally more heterogeneous in size (50-1000 nm) and are referred to as microvesicles or ectosomes. These microvesicles are formed through direct budding from the plasma membrane. The smaller vesicle population (40-100 nm) is referred to as exosomes, which originate from intraluminal budding of multivesiclar endosomes (MVE) and are released upon fusion with the plasma membrane. The last subclass is also more heterogeneous in size (50 nm – 5000 nm) and consists of vesicles that are released when cells are compelled to undergo apoptosis, which are named apoptotic bodies. Despite differences in origin, these subclasses show overlapping characteristics in terms of size, and they lack subtype-specific markers as of

yet. As a result, it remains difficult to purify these subpopulations and therefore no uniform nomenclature is currently used[70]. In this thesis, we will use the term 'extracellular vesicles' from now on, to refer to all vesicle subtypes.

The aim of this thesis was to investigate if CPC-derived extracellular vesicles (CPC-EVs) can be used for cardiac repair, and to optimize EV production and delivery processes that could allow for faster clinical application of EV therapeutics.

An overview describing the potential of CPC-EVs as therapeutics post MI was provided in **chapter 1**. Moving towards the use of EVs for therapeutic applications, several aspects need to be addressed that will accelerate their clinical adoption. First, we need a standardized and scalable isolation method that yields sufficient amounts of EVs with maintained functionality. We hypothesized that EV isolation method could affect their functionality. Therefore, in **chapter 2** we compared physiochemical characteristics, as well as *in vitro* functionality of ultracentrifugation-isolated EVs (UC-EV) and ultrafiltration combined with size-exclusion chromatography-isolated EVs (SEC-EV). To validate our *in vitro* findings, we compared UC-EV and SEC-EV in a permanent ligation mouse model, as well as an I/R injury mouse model, in **chapter 3**. We assessed short term infarct size, myocardial deformation parameters, and plasma levels of troponin I after treatment with UC-EV or SEC-EV when compared to PBS treatment.

Strategies are being developed to prolong EV exposure to target organs in order to achieve optimal therapeutic effects. One promising approach to achieve this is using EV-loaded injectable hydrogels. In **chapter 4** we investigated if EV release can be prolonged using a pH switchable ureidopyrimidinone (UPy) hydrogel. First, we investigated the kinetics of EV release from UPy-hydrogel *in vitro*. Next, we explored if this UPy-hydrogel can be used to increase EV retention *in vivo*.

For clinical application of EV therapeutics, the ability to store EVs at different conditions is an important aspect. Currently, there is little information on the effect of storage on EV functionality. Therefore, in **chapter 5** we directly compared the functionality of CPC-EV that were stored at 4˚C and -80˚C. We first compared physiochemical characteristics of freshly isolated EVs to EVs stored at 4˚C or -80˚C. Additionally, we assessed EV functionality after different storage temperatures using *in vitro* and *in vivo* angiogenesis assays. Finally, **chapter 6** provides a summary and general discussion of the work presented in this thesis.

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