Extracellular vesicle therapeutics for cardiac repair: A translational perspective

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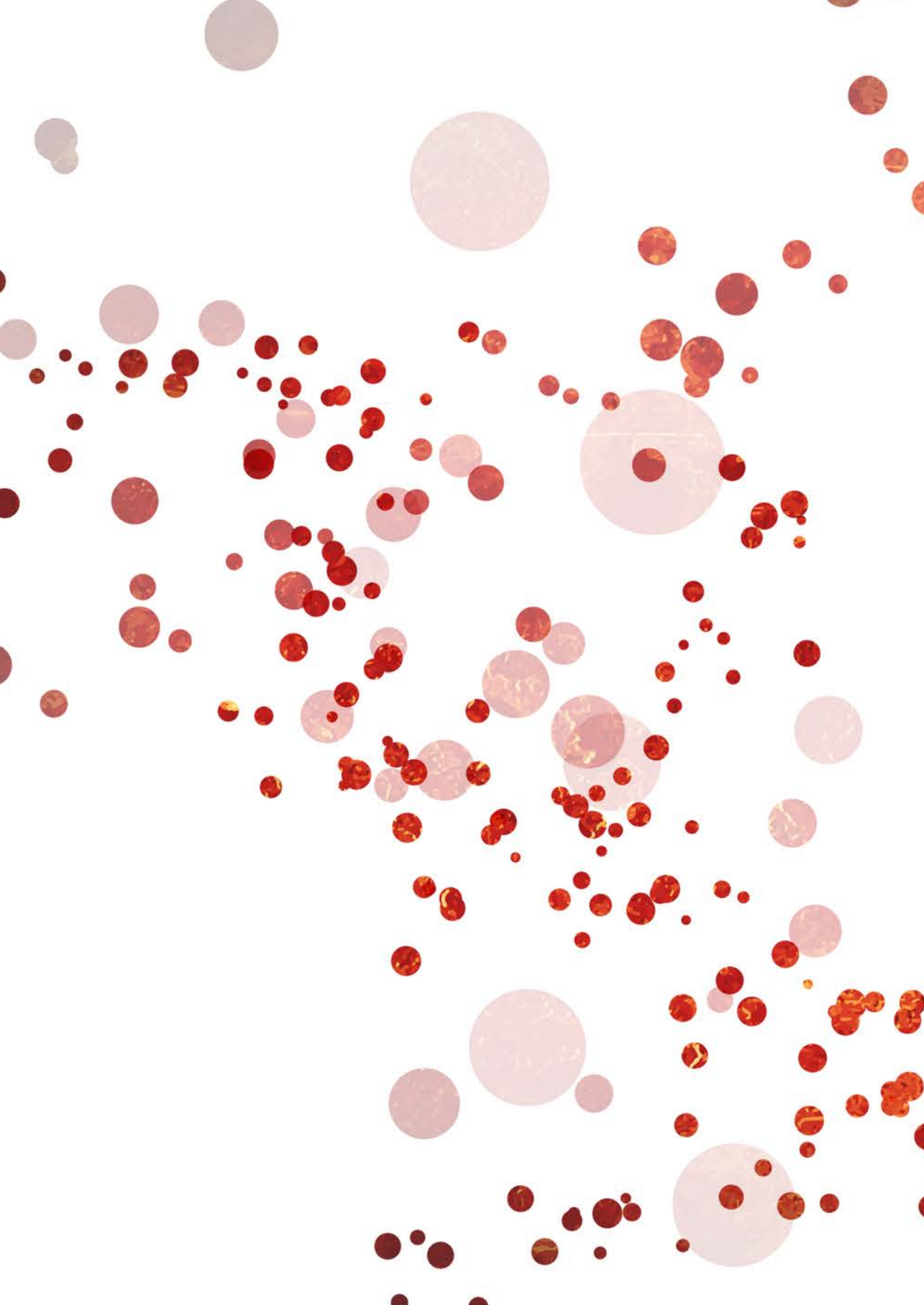


CHAPTER 6

Summary and general discussion

EXTRACELLULAR VESICLES FOR CARDIAC REPAIR

Cardiovascular disease (CVD) is the number one cause of death worldwide, accounting for 32% of death worldwide[1]. Ischemic heart disease has an estimated death rate of >9 million people a year. Over the last decade, acute mortality after MI has decreased due to better strategies including timely reperfusion. However, at the same time, approximately 25% of people that survive this initial ischemic event will develop heart failure (HF), which is characterized by the inability of the heart to provide a sufficient amount of blood to the body[2,3]. For this chronically ill patient population, no curative treatments are available besides heart transplantation. Therefore, other treatment options are needed and being explored that either focus on preventing the development towards HF, or, focus on treatment of this chronic HF-patient population. The use of progenitor cells as a regenerative therapy to restore the initial cell loss after MI has been a huge focus area for the last decade. Different types of progenitor cells have been considered as potential mediators of cardiac repair. Cardiac-derived progenitor cells (CPC) could be an attractive cell type to induce cardiac repair, since they originate from the heart itself and might be predisposed to activate internal cardiac reparative pathways. Furthermore, CPC are able to differentiate in all needed cardiac cell types[4,5]. Interestingly, direct injection of CPC into the damaged myocardium resulted in improved cardiac function after MI, despite only 3-4% engraftment of these transplanted cells[6]. An immediate wash-out of cells through the venous drainage system was observed, indicating involvement of paracrine factors[7]. When injecting conditioned medium from mesenchymal stromal cells (MSC), Timmers et al. found the same benefit in infarct reduction as compared to cell injection[8]. After separating the conditioned medium into fractions smaller and larger than 1000 kDa, it was observed that only fractions larger than 1000 kDa were responsible for infarct reduction upon MI[9]. In depth research on the secretome of progenitor cells identified extracellular vesicles (EVs) as important components of conditioned medium carrying reparative properties[10]. EVs are nanosized, lipid bilayer-enclosed particles that play important roles in intercellular communication in both health and disease, which makes them an interesting source for therapeutic applications. Progenitor-cell derived EVs have been shown to provide endogenous protection after MI by transferring their cargo, *e.g.* miRNAs, lipds, and proteins, to cardiac cell types, thereby stimulating repair processes in the ischemic myocardium[11–15]. An overview describing the potential of cardiac progenitor cell-derived extracellular vesicles (CPC-EVs) as therapeutics post MI was provided in **chapter 1**.

This thesis described the potential use of these cardiac progenitor-derived extracellular vesicles for cardiac repair and focused on translational aspects that could accelerate clinical implementation of EV-based therapeutics.

OPTIMIZATION OF EV PRODUCTION PROCESSES

EV isolation method

While EVs are increasingly being considered as potential therapeutics, some important aspects have to be addressed before their use as a product in clinical studies. First, one of the prerequisites for clinical application of EVs is a standardized, reproducible, and scalable isolation method[16,17]. The most widely used EV isolation method and the current golden standard is differential ultracentrifugation (UC). However, this method is time consuming, has a limited scalability, and EV yield is operator-dependent[18,19]. Furthermore, recent literature suggested that high speed centrifugation may induce aggregation and/or disruption of EVs due to high shearing forces, which might affect EV functionality[20,21]. For this reason, alternative EV isolation methods are currently being explored, one of which is ultrafiltration combined with size-exclusion chromatography (SEC)[22]. This size-based separation of EVs from other media components is more standardized and highly scalable for clinical production. In order to assess if EV isolation method leads to differences in EV functionality, we compared EVs isolated with ultracentrifugation (UC-EV) to EVs isolated with chromatography (SEC-EV) *in vitro* in **chapter 2**. Here, we used CPC-EV-induced extracellular signal regulated kinase (ERK) phosphorylation in endothelial cells as read-out to investigate possible differences in EV functionality[13]. We found that SEC-EV resulted in higher functionality compared to UC-EV, indicated by more pronounced ERK activation in endothelial cells. This may be a consequence of the high shear forces that UC-EV have to withstand during high speed centrifugation. Consequently, signaling molecules on the UC-EV surface may be destroyed, thereby preventing their ability to activate, bind, or be taken up by recipient cells.

In order to validate our *in vitro* results in an *in vivo* model, we compared functionality of EVs obtained using different isolation methods, in a permanent ligation model, as well as an I/R injury model (**chapter 3**). To our surprise, we observed no difference in infarct size between PBS, UC-EV, and SEC-EV treated groups after permanent ligation nor after I/R injury, indicating the absence of a therapeutic effect upon EV injection. As a result, we were not able to assess if EV isolation influenced EV functionality *in vivo*. Our data is in contrast with previously reported studies that did observe reduced infarct size upon treatment with CPC-derived EVs[23–25]. A recent meta-analysis of controlled animal studies showed that EVs derived from several types of progenitor cells reduced infarct size and improved cardiac function[26]. Therefore, finding the primary driving factor for this difference is of great importance for future employment of EV-based therapeutics. There are several possible explanations for the observed discrepancy, which will be discussed in separate subheadings below.

Cell source

As mentioned before, EVs derived from different progenitor cells have been used for cardiac repair[26]. MSC-derived EVs have been shown to exert beneficial effects after MI in multiple studies[8–10,27]. MSCs can be obtained from bone-marrow or blood, allowing for clinical translation. However, MSC-EV have their own technical limitations, as, in our hands, MSCs cultured in serum-free medium fail to produce sufficient numbers of EVs for further analysis.

Furthermore, a cell population originating from the heart itself, such as CPCs, might be more inclined to excrete beneficial mediators for the heart. Thus, although MSCs could be an interesting cell source, in this thesis we have focused on the use of CPC-EVs for cardiac repair. CPCs can be isolated from human biopsies using different methodologies that are either based on surface markers (Sca1$^+$-CPC or c-kit$^+$-CPC) directly, or via explant culture (cardiosphere-derived cells (CDC))[4,28,29]. When comparing the gene expression profiles of multiple CPC types, isolated using these different methodologies, no major differences were found between these CPC types[30]. Interestingly, small differences were mainly related to individual CPC clones rather than CPC isolation methodology. One main difference between our study and a previously reported study by Maring et al. was the use of a different Sca1$^+$ CPC clone for the *in vivo* studies[23]. Within the heart, multiple Sca1$^+$ CPC subpopulations have been discovered, which can be detected using different antibodies recognizing a glycosylation variant of their respective epitopes[31]. As different subpopulations may have different therapeutic efficacy, we compared the CPC clone used in our study to the CPC clone from the study by Maring et al. in a mouse study with long-term follow-up[23]. However, treatment with neither of the CPC clones was able to improve cardiac function 1 and 4 weeks after MI, suggesting this possibility is unlikely to explain the different outcomes (data not shown).

Culture methods

Another potential explanation could be differences in culture methods. In order to purify our EV population and optimize our culture procedure for future human application, we cultured our cells in serum-free culture media before EV collection, whereas cells were previously cultured in 'EV-depleted' FBS-containing medium[23]. EV-depleted serum is obtained via overnight ultracentrifugation of serum in order to deplete it from serum-derived EVs. It has been suggested that ultracentrifugation of serum does not deplete all serum-derived EVs, RNAs, and proteins[32–35]. Therefore, a possible explanation could be that serum-derived EVs, proteins and/or RNA are co-isolated when EV-depleted medium is used. This may, on its turn, be responsible for the observed functional difference of both EV types.

In order to investigate if differences in the presence of serum components could be the key explanation for the observed differences, we performed a pilot study. Here, we isolated CPC-EVs from either EV-depleted serum-containing medium (EV + serum) or serum-free medium (EV - serum) using ultracentrifugation and assessed EV purity and their biological effects using two *in vitro* angiogenesis assays (see Appendix). Our data suggest at least similar purity of EV + serum and EV - serum, as assessed by a similar ratio in particle number per 1 µg protein[36]. In terms of function, biological activity of EV - serum was slightly increased when compared to EV + serum, as assessed by two different *in vitro* angiogenesis assays. Therefore, these data are in contrast to our hypothesis that co-isolation of EV-depleted serum components could explain the observed differences in functional outcome *in vivo*. Yet, we may question the predictive value of these *in vitro* assays for therapeutic efficacy *in vivo*, since we observed no therapeutic benefits when injecting EVs derived from serum-free cultured cells after MI.

One important aspect that remains to be addressed, however, is the possibility that serum-free culturing might alter the composition and content of EVs, leading to reduced therapeutic

efficacy *in vivo*. It has been described that EV content can be altered after stimulation of cells with different environmental cues, for example hypoxia, TNF-α stimulation, high glucose concentrations, or serum-free culturing[37–39]. Thus, serum-free culturing of CPC may alter the content of CPC-EVs, reducing levels of key components needed to exert functional effects in post-MI models *in vivo*. Proteomic analysis of EV - serum and EV + serum could reveal possible differences in protein composition between EVs obtained from cells with different culture methods and could provide insights on how intracellular signaling pathways are potentially affected by serum-free cell culture, which eventually might lead to altered EV protein content.

There is much more diversity in culture and isolation methods when we examined studies in literature. One of these differences is the time after which conditioned medium is being collected. While we collected conditioned medium after culturing cells for 24 hours, other studies cultured cells in serum-free medium for 48 hours, or for even longer periods of 7 or 15 days[11,14,24,25,40]. Culturing cells in serum-free medium for such a long period of time should raise concerns about whether these cells are alive and healthy, and whether the EV isolates do not comprise apoptotic bodies. Furthermore, a variety of EV isolation methods are currently used to isolate EVs. Most studies either use ultracentrifugation, which is the current golden standard, or ultrafiltration to isolate EVs for assessing their therapeutic efficacy in *in vivo* studies[9,14,23,40]. However, some reported studies have used ExoQuick or similar poly-ethylene glycol (PEG) solutions to precipitate EVs[11,24]. While this method is simple and quick, there are concerns that this isolation method does not yield a pure population of EVs[41]. Moreover, differences can even be found within one isolation method. Although 1 hour of ultracentrifugation is sufficient to pellet EVs, some studies collect EVs after 4 hours of centrifugation[14], which should also raise concerns on the purity of the isolated EV population. Altogether, as there are many different methods to obtain EVs, examining their effect on functionality *in vivo* is key to accelerate clinical adoption of EV therapeutics.

In conclusion, a variety of culture methods are used to culture cells before EV collection. Although our data do not support the hypothesis that co-isolation of EV-depleted serum components would explain our differences in therapeutic efficacy *in vivo*, we should examine whether serum-free culturing alters EV composition and content. Furthermore, future studies should focus on the effect of different culture methods on EV functionality *in vivo* before clinical application of EVs.

MI model

An alternative explanation could be provided by differences in the experimentally induced MI model. Currently, we lack a standardized MI model to evaluate efficacy of new therapeutic strategies. As a result, reported studies that assess CPC-EVs' therapeutic efficacy have used a variety of MI models. For example, experiments have been performed in different animal species, have used a ligation or cryoinjury to induce cardiac damage, have used a permanent ligation model or I/R injury model, have been using different times of ischemia before reperfusion, or have been using a different time window or dosing regime. In addition, a plethora of different read-outs have been used to assess therapeutic efficacy, for example based on cardiac function or infarct size, which have in addition been assessed after different

periods of time. Comparing all those different studies with one another and assessing the most potent therapeutic strategy is therefore a huge challenge.
In our study, we observed high variability in infarct sizes within the PBS treated group. We could speculate that picking up small differences is difficult with such high variations in initial infarct sizes. However, similar variabilities in infarct size have been observed in other studies[25,42–44]. Therefore, technical differences in the experimentally induced MI model might not be the most likely explanation for the observed lack of therapeutic efficacy.

Dosing and timing of treatment

One of the challenges in EV research is to accurately quantify the number of EVs after isolation, which has resulted in the development of a large variety of technologies for EV quantification. Consequently, this has led to differences between studies with regards to the quantification method of EV dosing. Thus, while some studies applied an EV dose based on only the number of producing cells and not by a quantitative analysis, others based EV dosing on the number of particles, or amount of protein. The EV dose we employed differs from previously reported mouse studies using CPC-EV treatment after MI. Some examples are an EV dose of 2.8 x 10^9 particles[11], 6.5 x 10^8 particles[45], or an EV dose based on protein levels[23]. Generally, when comparing our EV dosing (10 x 10^{10} particles per injection) to previous studies, we used higher particle numbers. Thus, in our study we may have administered too high doses, thereby failing to achieve therapeutic benefits post-MI. Reduced therapeutic effects have previously been observed *in vitro* when using a high EV dose of CPC-EVs when compared to a lower EV dose[14]. Although dose-dependency was only tested *in vitro*, this could indicate that overdosing may be important issue *in vivo* as well. However, a critical note is that particle numbers and protein concentration of EV preparations are dependent on their purity upon isolation, making a direct comparison very challenging. Another explanation could be differences in the time of EV administration after MI. In our study, we performed intramyocardial injection of EVs 15 min after permanent ligation, while others perform intramyocardial injections after 60 minutes[14,25]. In our I/R model, we applied EV treatment at the moment of reperfusion, which is similar to one study[14], but differs from another study that administered EVs 30 minutes after reperfusion[24]. The optimal EV dose and timing of treatment needed for therapeutic efficacy post-MI has not been adequately covered by previous studies, and is therefore an important topic for future research. These variables can only be eliminated when performing dose-response experiments and investigating differences in timing of treatment in *in vivo* models of MI.
Altogether, several variables could be explaining the observed differences in therapeutic efficacy among studies. Future studies should focus on whether cell culture method, dosing, or timing of treatment could explain these apparent discrepancies. Once we have identified these key factor(s), we can proceed to validate if EV isolation methods lead to differences in EV functionality *in vivo*. For further investigation of EVs' therapeutic potential we propose to isolate EVs using SEC, although we were not able to assess if SEC-EVs have a beneficial therapeutic potential *in vivo* when compared to UC-EVs yet. Nevertheless, the biological activity of SEC-EVs has shown to be beneficial when compared to UC-EVs in multiple *in vitro* assays. Furthermore, EV isolation using SEC is a highly standardized, reproducible, and scalable method, which is essential to pursue EV therapeutics for clinical use.

EV storage

For therapeutic application, EVs have to be stored after isolation in order to use them immediately when required. The ability to store EVs upon isolation while maintaining their functionality is indispensable for future clinical application of EVs. Therefore, another important aspect to address within the EV production process is the effect of storage conditions on EV functionality. Currently, most studies describing the effect of storage on EVs have focused on physiochemical properties, while few studies are available that have assessed the effect of storage on EV functionality[46,47]. Therefore, in **chapter 5** we investigated the effect of storage conditions on EV physiochemical properties such as size and concentration, and EV functionality *in vitro* and *in vivo*. Physiochemical characteristics of 4°C or -80°C stored EVs were similar 1 day after storage when compared to freshly isolated EVs. Furthermore, we found no apparent differences between 4°C stored EVs and -80°C stored EVs, as assessed by *in vitro* angiogenesis assays. In contrast, other studies showed altered physiochemical properties and impaired function of EVs *in vitro* after storage at 4°C or -80°C for periods varying between 1 to 25 days[46–48]. These differences may have been the result of differences in storage buffers, freeze-thawing procedures, or cellular mechanisms of action when using EVs derived from different cell types. Moving towards therapeutic applications of EVs, the effect of storage temperature on EV functionality should be studied *in vivo*, especially as the field increasingly recognizes that *in vitro* potency assays do not predict *in vivo* efficacy yet. We studied the effect of different storage conditions on EV functionality *in vivo* using a Matrigel plug assay. We found no statistically significant difference in the number of CD31+ cells between PBS-loaded and EV-loaded Matrigel plugs although some EV-loaded plugs did show higher numbers of CD31+ cells. Furthermore, we observed elevated non-homogenous cell infiltration in Matrigel plugs loaded with 4°C or -80°C stored EVs when compared to PBS, indicating no apparent differences between 4°C and -80°C stored EVs. We have previously shown that CPC-EVs have strong pro-angiogenic effects, both *in vitro* and *in vivo*[13,49]. However, the increased total cell numbers in our study did not correlate with increased numbers of CD31+ cells. Possible explanations for this disparity could be that the infiltration cells are vascular cells, but we do not detect them as such due to technical limitations, or that the infiltrating cells express other vascular markers. Alternatively, these infiltrating cells could be of a different, non-vascular, origin. We should first investigate these two potential explanations and validate our *in vivo* findings in a larger number of animals to be able to draw definite conclusions. However, altogether, our data suggest that short-term storage of EVs at -80°C does not affect functionality of CPC-EVs when compared to storage at 4°C, which is useful information that could eventually contribute to faster clinical adoption of EV therapeutics.

EV retention

The injection of cellular therapeutics has often demonstrated only modest beneficial effects in different patients groups, as a result of retention problems[50–53]. Similarly, strategies to enhance EV delivery in chronically diseased patients and prolong exposure of EV therapeutics have yet to be optimized to achieve their full potential for therapeutic efficacy. Given the immediate flush-out of cells after intramyocardial injection[7], which may also be expected

for EVs, strategies are being developed aiming to increase retention of therapeutics. A potential method for sustained EV release and to prolong therapeutic exposure is provided in **chapter 4**. Here, we evaluated the use of a hydrogel based on ureido-pyrimidinone (UPy) units coupled to poly(ethylene glycol) chains (UPy-hydrogel) as potential gradual release system for EVs. We found that UPy-hydrogels provide gradual release of EVs *in vitro* measured over a period of 4 days and that EVs retained their biological activity after release from UPy-hydrogel. In addition, we showed that UPy-hydrogel enhanced local EV retention *in vivo* after subcutaneous application. Another potential delivery platform for EVs are porcine-derived decellularized extracellular matrix (ECM) hydrogels[54]. Here, the majority of released EVs were detected 1 day after encapsulation[55]. Ultimately, we aim to investigate if EV-loaded hydrogels can enhance retention of EVs upon intramyocardial delivery and whether sustained EV release could increase therapeutic efficacy after MI compared to a single EV dose. However, examining EV retention in the mouse heart is still limited by the fact that accurate injection of such a small volume is challenging. Thus, when exploring the use of UPy-hydrogel to enhance EV retention upon intramyocardial delivery in future studies, we might be dependent on controllable infusion pumps that can accurately administer small volumes into the myocardium or on the use of larger animal models. Ultimately, this could contribute to improved efficacy upon local delivery of EV therapeutics.

CHALLENGES IN THE TRANSLATION OF EV THERAPEUTICS

Since the discovery that EVs have potential reparative and/or regenerative capacity in multiple fields, the interest in EV therapeutics has expanded rapidly. However, as we move towards standardization and optimization of EV production processes and have successfully increased purity of our EV preparations, we are facing several challenges in translating these optimized preparations into therapeutically effective products on at least two different levels (Figure 1). Successful clinical translation starts with the discovery and assessment of EVs' biological effect using *in vitro* assays, followed by validation of these findings in preclinical animal models. In **chapter 3**, we showed that CPC-EVs were not able to reduce infarct size in two mouse models of MI. However, when assessing the functionality of the used CPC-EVs *in vitro*, we found that EVs were still able to activate ERK in endothelial cells, indicating some level of functionality *in vitro*. Therefore, one could question the predictive value of this *in vitro* assay. This lack of correlation between *in vitro* and *in vivo* functionality is one of the major challenges in the field of EV therapeutics, as we are currently not aware of an *in vitro* assay that is able to predict EV functionality *in vivo* for myocardial repair. Therefore, more effort in developing *in vitro* assays that are able to predict whether EVs induce cardiac repair *in vivo* is essential. Given that the therapeutic mode of action will likely be different and specific for each disease condition, we may need distinct *in vitro* assays for different therapeutic applications. Improving our understanding of CPC-EVs' mechanism of action (MoA) is essential for this. Functional assays are fundamental in order to proceed towards clinical use of EVs, as we must establish

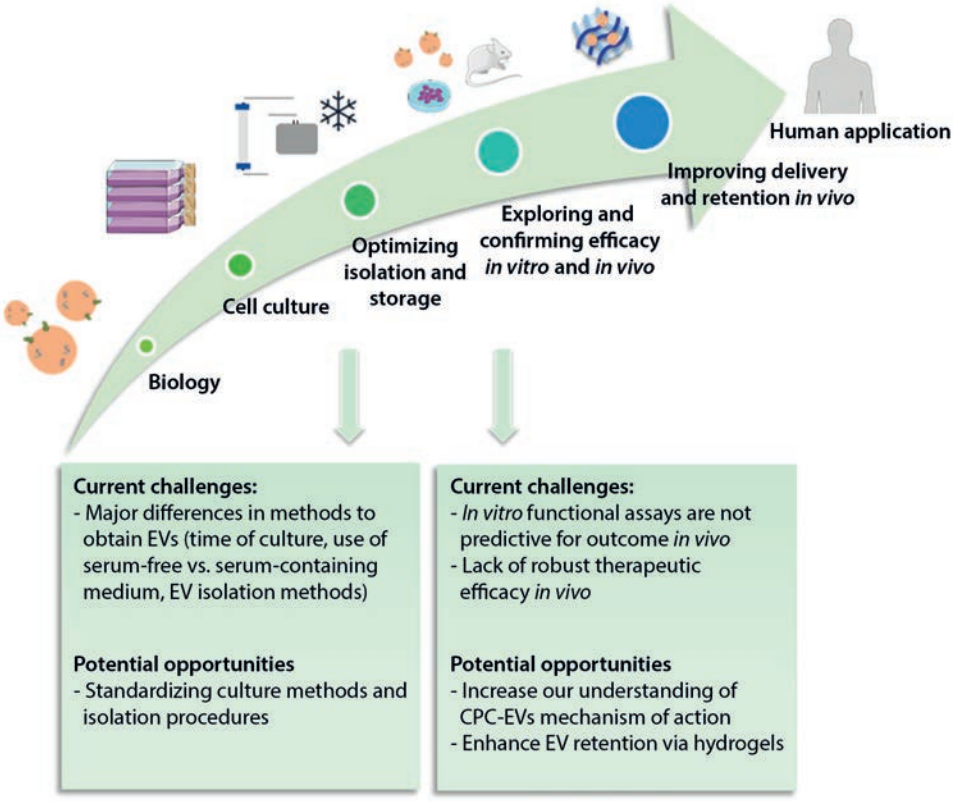


Figure 1 A translational perspective on EV therapeutics.

standardized validation methods of EVs' biological activity to be able to assess EV quality and batch-to-batch differences. To address these issues, members of four societies (SOCRATES, ISEV, ISCT, ISBT) proposed a potential quantifiable metrics to harmonize the definition of MSC-EVs and provide a guide to enable the comparison of EV manufacturing and define key physical and biological characteristics of MSC-EVs[56]. These criteria included that the MSC-EV preparation must be defined according to their cellular origin, the presence of membrane lipid vesicles, physical and biochemical integrity of the vesicles, and biological activity. The development of such standardized quality assurance assays is fundamental to characterize and compare EV preparations and to ensure further translation of EV therapeutics.

An additional challenge in the translation of EV therapeutics is that EVs' therapeutic effect may not be as robust as was initially anticipated on[26], as it may be dependent on factors as culture methods, MI models, or dosages. This scenario might remind us of the stem cell therapy 'hype'. The major initial promise of stem cells to favorably alter the clinical course of major cardiovascular disease has quickly led to progression into huge and costly clinical trials, without proper understanding of their biological mechanism. Although stem cell

therapy was found to be safe, these studies mostly found contradictory results in terms of efficacy[57–60]. Thus, as EV-based therapeutics are of somewhat similar complexity as stem cell therapeutics, we may take this as an example and improve understanding of CPC-EVs' mechanism of action before moving forward into clinical trials.
Moreover, exploring EVs' mechanism of action is essential for further clinical translation with respect to regulatory aspects[61]. With regards to the pharmaceutical classification of EV therapeutics derived from unmodified cells, EV therapeutics can be defined as biological medicines. Regulatory classification of drugs and most biological products is dependent on their pharmaceutically active substance. In contrast to pharmaceutical drugs, the active substance of cellular therapeutics does not necessarily have to be a defined molecule, but it can be defined as the cells themselves[62]. In that respect, the same definition could be applied to EV-based therapeutics. Although the MoA of EV-based therapeutics does not have to be sorted out completely before the start of the first clinical trials, an overview of a plausible hypothesized MoA must be provided when EV-based therapeutics are applied in the clinic[63,64]. Therefore, although EV therapeutics still hold enormous potential, we must improve upon our understanding of their biology and mechanism of action in order to further pursue use of EV-therapeutics in clinical trials.

FUTURE PERSPECTIVES

Unraveling CPC-EVs' mechanism of action
As touched upon in the previous paragraph, understanding the mechanism by which CPC-EVs exert therapeutic benefits after MI is essential for moving towards translational use of EVs. In **chapter 1,** we have described four processes that can be targeted by new therapeutic strategies to stimulate cardiac repair after MI, which are: preventing cardiomyocyte apoptosis, regulating the immune response, stimulating vessel formation/angiogenesis, and reducing fibrosis. Furthermore, we mentioned the key mechanisms by which CPC-EVs are able to stimulate cardiac repair, of which the most well studied mechanisms of action are inhibition of cardiomyocyte apoptosis and stimulation of angiogenesis.
More recently, a few new studies were reported that showed the potential cardioprotective capacity of CPC-EVs. One of those studies showed that CPC-EVs were able to suppress proliferation of activated T cells, indicating their potential to balance the immune response after MI[65]. Furthermore, the ability of CPC-EVs to lower cardiac fibroblast activation was shown in a 3D human fibrotic model[66]. Lastly, a surface protein with anti-apoptotic properties was recently discovered in CPC-EVs called pregnancy associated plasma protein A (PAPP-A). PAPP-A is a protease and has been demonstrated to release bioactive insulin growth factor-1 (IGF-1) via proteolytic cleavage of IGF-binding protein-4 (IGFBP-4)[14]. The release of IGF-1 subsequently activates the IGF-1 receptor, which triggers intracellular ERK1/2 and Akt activation in HL-1 cardiomyocytes, leading to decreased caspase activity and reduced cardiomyocyte apoptosis. siRNA-knockdown of PAPP-A in CPC-EVs reduced the functional benefit after permanent MI in rats when compared to control CPC-EVs, indicating a cardioprotective role for PAPP-A[14].

It is generally assumed that EVs exert their therapeutic effect via a combination of bioactive molecules, including *e.g.* miRNAs and proteins. However, an interesting study by Toh et al. showed that it is not likely that the MoA of EVs is regulated via miRNAs, as the number of functional miRNA copies per EV is probably too low to exert a therapeutic effect[67]. In contrast, proteins are usually present in a biologically relevant concentration, indicating that it is more likely that EVs exert their effect via a protein-based MoA. Therefore, future studies should focus more on this aspect. To date, most information on EVs' mechanism has been gathered by using proteomics or miRNA enrichment analysis, however, these analyses are usually limited by a lack of causal relation between the presence of the protein or miRNA and functional efficacy *in vitro* and *in vivo*. Two exceptions are studies on PAPP-A and on EMMPRIN that used knock-down experiments to show a causal relation between these proteins and a therapeutic effect[13,14]. Ultimately, we are aiming to unravel the MoA by which CPC-EVs provide cardioprotection after MI. For that reason, we developed a protocol to perform single cell sequencing of the mouse heart which may potentially be used to identify the specific cardiac cell types and affected mechanisms upon EV uptake. This could provide insights into the MoA of CPC-EVs' upon MI.

EV-mimetic therapeutics

Interestingly, we have the ability to engineer EVs' surface and cargo through chemical and biological techniques, which is already extensively explored in the drug delivery field[68,69]. If we are able to identify EVs' effector molecules in the coming years, we could use this knowledge to engineer EVs with more favorable characteristics. Furthermore, engineering cardiac homing peptides on EVs could lead to improved targeting to cardiac tissue. Ultimately, this may allow us to create unique EV-mimetic populations *e.g.* by engineering liposomes to carry cardioprotective molecules and targeting moieties. By using EV-mimetics, we could eliminate effects induced by culture conditions as a factor that can influence EV cargo. Furthermore, EV-mimetic therapeutics would contribute to enhanced standardization, scalability, and reproducibility for clinical application.

IMPORTANCE OF STANDARDIZATION IN SCIENCE

Chapter 3 in this thesis described results with an unexpected negative outcome. A potential contributor to this outcome may have been lack of standardization of experimental processes. This thesis described many discrepancies between reported studies on cell culture method, culturing time, EV isolation method, and MI models. As there are so many different variables introduced by using different experimental methods that could alter therapeutic outcome, this stresses the need to improve upon standardization of these processes.

Thus, when exploring innovative EV-based therapeutics for cardiac repair, we should standardize our EV production methods and establish a standardized and widely accepted MI model. Although this concept is widely acknowledged by others[61,70], major complications include the lack of understanding of the optimal culture conditions for EV collection, or the

most optimal MI model. A worldwide collaboration of key opinion leaders would be required to develop such standardized recommendations. For EVs, first steps have already been made by the International Society for Extracellular Vesicles to improve standardization by introducing guidelines on *e.g.* EV characterization, EV separation, and concentration[17,71]. More effort must be put in development of - and adherence to those guidelines for cell culture methods, EV isolation method, purity, quantity, and MI models. Eventually, this could contribute to improved translation of innovative EV-based therapeutic strategies towards future clinical application.

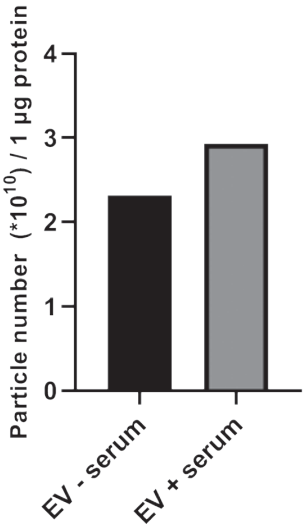
CONCLUSIONS

In this thesis, the first steps have been made in optimizing EV production processes such as EV isolation and storage, in order to accelerate clinical adoption of EV therapeutics. However, we did not observe therapeutic efficacy of our CPC-EV treatment after MI, which is in contrast to previously reported studies. Thus, future studies should focus on exploring the cause of this discrepancy. While CPC-EVs hold great potential to serve as cell-free therapeutic for cardiac repair, more effort must be put in understanding EVs' mechanism of action and increasing standardization of EV production processes in order to allow for further employment of EV-based therapeutics.

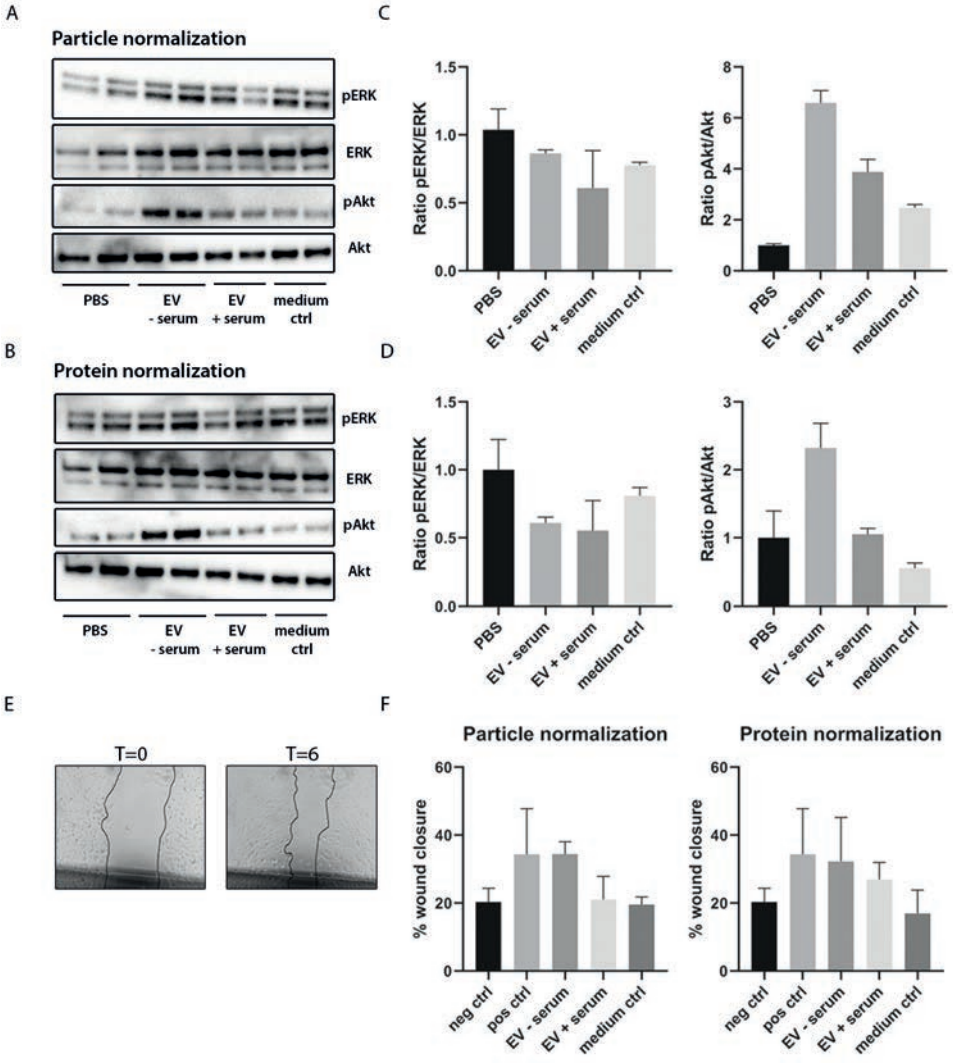
APPENDIX

In this pilot study, we aimed to investigate if co-isolation of serum-derived components when using EV-depleted serum-containing culture medium could explain the differences in therapeutic efficacy we found in comparison to the previous reported study by Maring et al[23]. Therefore, we isolated CPC-EVs either from cells cultured in EV-depleted serum-containing medium (EV + serum) or serum-free medium (EV - serum) using ultracentrifugation. To assess if serum components are isolated from EV-depleted culture medium, we used non-conditioned EV-depleted serum-containing medium (medium ctrl) that underwent ultracentrifugation steps similar to conditioned medium as additional control. First, we assessed the purity of EV - serum and EV + serum, expressed by the ratio of the number of EV particles in 1 µg EV protein, as described before[36]. EV + serum and EV - serum displayed no major difference in ratio between particle number per 1 µg protein, indicating comparable EV purity.

To compare EV functionality of both preparations of EVs, we used two *in vitro* angiogenesis assays. We assessed the ability of EV + serum and EV - serum to activate both ERK and Akt and performed a scratch migration assay in endothelial cells. As shown in Figure 2, EV - serum activated ERK and Akt to a higher extent than EV + serum and medium ctrl (Figure 2A-D). Similarly, EV - serum treatment resulted in higher percentage of wound closure when compared to EV + serum and medium ctrl (Figure 2E+F). Therefore, biological activity of EV - serum was higher when compared to EV + serum and medium ctrl. These results indicate that, despite our expectation, co-isolation of serum components after culturing with EV-depleted medium would not explain the observed differences in functional outcome *in vivo*.



Supplemental Figure 1 Assessment of EV purity.
EV purity of serum-free medium derived EVs (EV - serum) and EV-depleted serum-containing derived EVs (EV + serum), expressed as particle number per 1 µg EV protein.



Supplemental Figure 2 Comparison of serum-free medium derived EVs (EV - serum) and EV-depleted serum-containing medium derived EVs (EV + serum) using *in vitro* angiogenesis assays.
Non-conditioned serum-containing medium (medium ctrl) was used as additional control. A-B) HMEC-1 were stimulated with EVs, after which phosphorylation of ERK and Akt as well as total ERK and Akt were assessed using Western blotting. EVs were either normalized for similar number of particles (A) or similar protein content (B). C-D) Quantification of ratio pERK/ERK and pAkt/Akt for both particle and protein EV normalization. E) A scratch migration assay was performed in HMEC-1 and percentage of wound closure was assessed at baseline (T=0) and after 6 hours (T=6). F) Quantification of percentage of wound closure.

SUPPLEMENTAL METHODS

Cell culture

Human fetal heart tissue was obtained by individual permission using standard procedures for written informed consent and prior approval of the ethics committee of the University Medical Center Utrecht, the Netherlands. This is in accordance with the principles outlined in the Declaration of Helsinki for the use of human tissue. Cells were cultured on 0.1% gelatin-coated culture flasks. Human cardiac progenitor cells (CPCs) were cultured using EGM-2 (Lonza, CC-3162), with M199 (Gibco, 31150-030)) supplemented with 10% fetal bovine serum (Biowest, S1810-500), 1% Penicillin-Streptomycin (Gibco, 15140-122), and 1x MEM Non-Essential Amino Acids Solution (Gibco, 11140-035), as described before[5]. Human microvascular endothelial cells (HMEC-1) were cultured in MCDB-131 (Gibco, 10372-019) with 10 ng/mL human Epidermal Growth Factor (EGF) (Peprotech/ Invitrogen 016100-15-A), 1 µg/mL Hydrocortisone (Sigma H6909-10), supplemented with 10% fetal bovine serum (Biowest, S1810-500), and 10 mM L-Glutamine (Gibco, 25030-024). HL-1 mouse cardiomyocytes were cultured using Claycomb medium (Sigma, 51800C), supplemented with 10% FBS, 1% Glutamax, 1% Penicillin-Streptomycin (Gibco, 15140-122), 0.3 mM Vitamin C (Sigma, A4034), and 0.1 mM Phenylenephrine. Cells were incubated at 37˚C with 5% CO_2 and 20% O_2 and passaged at 80-90% confluency after digestion with 0.25% trypsin.

Collection of conditioned medium

Conditioned medium (CM) was collected either from CPCs cultured in serum-free medium for 24 hours or from CPCs cultured in EV-depleted FBS-containing medium for 3 days. EV-depleted FBS-containing medium was prepared as follows. First, 33% FBS was mixed with 67% Medium 199, followed by centrifugation at 120.000 x *g* using a type 50.2 Ti fixed-angle rotor for 16 hours. Next, additional M199, EGM2, 1% Penicillin-Streptomycin and 1% MEM Non-Essential Amino Acids Solution were added. As an additional control, non-conditioned EV-depleted FBS-containing medium was kept for 3 days at 37°C, followed by similar isolation procedures, to assess if additional media components were isolated.

EV isolation

EVs were isolation using ultracentrifugation. First, conditioned medium was centrifuged for 15 min at 2000 x g and 0.45 µm filtered (0.45 Nalgene filter bottles) to remove cell debris. Next, EVs were pelleted by a 1 hour centrifugation at 100.000 x *g* using a type 50.2 Ti fixed-angle rotor. EVs were filtered (0.45 µm) and washed by a second 100.000 x *g* centrifugation step. EV pellets were dissolved in PBS, after which particle count was determined using Nanoparticle Tracking Analysis (Nanosight NS500, Malvern), using a camera level of 15 and a detection threshold of 5. EV protein levels were determined using a microBCA assay kit (Thermo Scientific, 23235).

Angiogenesis assays

Methods of the ERK/Akt activation assay and scratch migration assay are described in chapter 5.

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