Strategies To Improve Cardiac Cell Therapy

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to my loved one and our little one

"We have to continually be jumping off cliffs
and developing our wings on the way down."

Ray Bradbury

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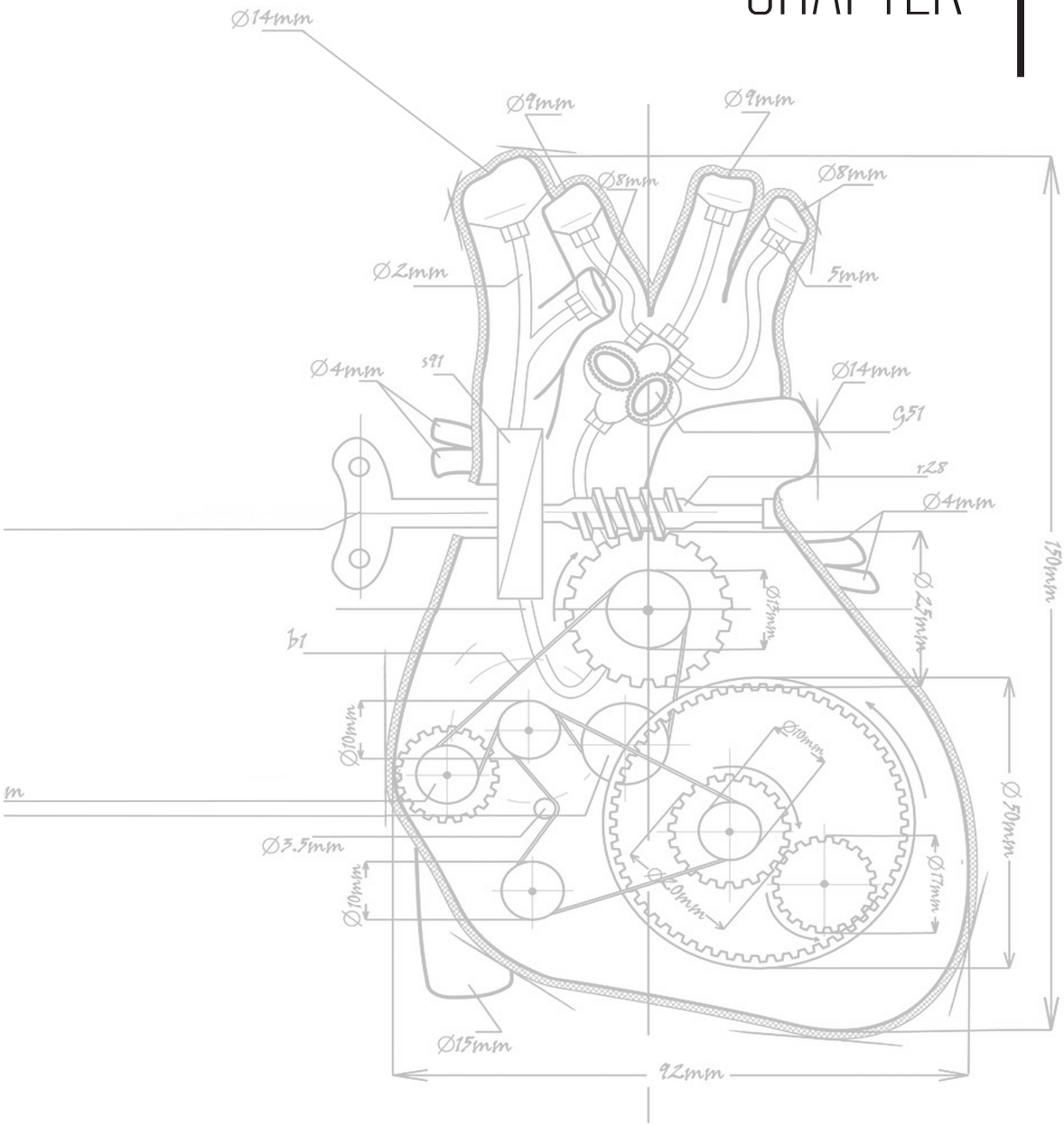
Stem cells: a complex therapeutic

CHAPTER 1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At present day, stem cell therapy is a thriving area of research in almost all clinical fields. Ever since their discovery and initial isolation, the scientific community has embraced stem cells for their potential to change the therapeutic approach for chronic diseases; moving from a disease management towards a regenerative framework. However, this concept is not new since it has been successfully implanted in the clinic in the form of organ transplantation [1]. As a proof of concept, this technique has shown the possibility to replace malfunctioning organs with unscathed donor organs, which are able to take over their functions and provide the patients with an extended and better quality of life. Nonetheless, this approach is severely hindered by the shortage of donor organs and therefore will never become a viable medical treatment option for the majority of patients. The progression and refinement in the isolation and culturing procedures of stem cells over the past 15 years has reopened the door for regenerative medical approaches, in the hope to heal chronically damaged organs of patients that are now on an organ donor waiting list.

In this regard, a cell is one of the most complex biopharmaceuticals. Protein and gene therapy approaches are based on relatively simple macromolecules. Amino acids, and their folded structures, provide the necessary components to induce an intended target response. Similarly, the delivery of nucleic acids to the nucleus of the target cells will allow for the production of the encoded gene. Although steps have to be taken to ensure their stability and targeting after administration, the molecular mechanisms behind protein and gene therapy are generally well understood. Nevertheless, these approaches are best fitted to target a single defect rather than eliciting a complex biological regenerative response, for which stem cells seem to be better suited. However, cells are far more complex to utilize as a therapeutic. A simple reason is the fact that they are composed, among other, of thousands of proteins and an entire genome worth of nucleic acids. Merely based on the proteins that they secrete, cells can already be targeting a multitude of receptors. However, another crucial difference is that cells are dynamic and are able to react to their environment. Upon transplantation, the transcriptome, proteome, and even secretome profile of cells can change, thereby altering their function compared to what is observed *in vitro*. All this makes cells a unique multi-dimensional therapeutic well-fitted for regenerative medical approaches, but also a much more complex and challenging entity to utilize and study. In this thesis, we will take a pharmacological perspective to discuss the current state of cardiac stem cell therapy.

Cardiac cell therapy

As the blood supply to myocardium wanes, the lack of oxygen and nutrients in the highly energetic heart muscle cells quickly leads to a path towards cellular death [2]. Although the exact number of cardiomyocytes that will die is dependent upon the location and duration of the heart infarction, it has been estimated that up to a billion cells can be lost during this pathological process [3]. The loss of these contractile cells and the subsequent formation of scar tissue leads to physiological compensatory mechanisms that ensure that the remaining cardiomyocytes take over the pumping burden of the lost cells. However, over time this process has detrimental effect on the heart, often leading to hypertrophic cellular responses, cardiac remodeling, which progressively leads to heart failure. In the latter case, the patient's heart is

unable to pump the blood effectively around the body and becomes dependent on a life-long use of drugs possibly requiring the implantation of mechanical assist devices. But even on this regiment of drugs, symptoms often worsen since the diseases cannot be cured. For this reason, coronary heart disease is a huge burden on society estimated to cost the EU economy 60 billion a year, of which 33% is due to direct health care costs, 29% to productivity losses and 38% to informal care [4]. Therefore, there is an emphasis to provide these patients with a therapeutic option to halt the progression of the disease and/or repopulate the lost cells in order to regenerate the heart.

The heart has an intrinsic regeneration capability by means of resident cardiac progenitor cells [5, 6], which during the physiological state ensures the turnover over of cardiomoycoytes, albeit at a very low rate [7]. Nevertheless, in the pathophysiological situation these endogenous stem cells are unable to contribute significantly to the restoration of the myocardium. Therefore, the basis of cardiac regenerative therapy is to use an exogenous cell source to provide the heart with building blocks for its reconstruction. This concept has taken many shapes, from using bone-marrow derived cells to embryonic stem cells (for more detail on cell types see chapter 2], and has moved swiftly from animal model to the clinic. In order to highlight the advancements in this area, we will break up this chapter into two parts. Firstly, we will discuss what the effect is of the body on the stem cell (pharmacokinetics), and thereafter, the effect of the stem cell on the body (pharmacodymanics).

Pharmacokinetics

The term 'pharmacokinetics' stems from the ancient Greek and is loosely defined as "drug motion". In the study of small molecule drugs it is often divided into four step; absorption (administration), distribution, metabolism and excretion, also known as ADME [8]. These steps follow the faith, or motion, of a drug from its administration until its clearance from the body. Although stem cells will be unable to fit all these categories perfectly, we use a similar approach to describe the faith of stem cells in the body.

Administration

Administration is of critical importance for subsequent bio-distribution of a drug. The oral administration route is preferred for small compound drugs, since the ease of intake is most desirable for patients. However, similarly to other biopharmaceuticals [9], cells are not incapable of surviving the harsh acidic environment of the stomach, and subsequently even less likely to be able to permeate through the intestine wall for absorption in the blood stream.

The least invasive manner to inject cells towards the heart is directly into the circulation by means of an intravenous injection [10]. Based on the homing potential of hematopoietic stem cells after intravenous bone marrow transplantation in leukemia patients [11], it was hypothesized that cardiac cell therapy could rely on similar chemo-attractant signals stemming from the damaged heart to attract the circulating cells to the site of injury. Although this administration route is most desirable and cost effective, the cells are faced with many obstacles prior to their arrival in the damaged heart. A more local delivery strategy, intracoronary injection allows for the administration of stem cells directly into the cardiac coronary circulation. A beneficial aspect

of this approach is that a large number of heart patients will undergo percutaneous coronary intervention (PCI) or angiography, during which time an intracoronary injection could be performed [12]. The injection can also be tailored by targeting a specific coronary region which was affected by the ischemic event. Nonetheless, in such an approach the injected cells are faced with high coronary flow and are required to transmigrate through the capillaries to reach the myocardium. Lastly, intra-myocardial injection is the administration of stem cells directly into the myocardial tissue. The preferred approach for this technique is with a minimally invasive thoracoscopic procedure or catheter-based needle injection systems [13]. This administration route is not restricted by boundaries for cellular uptake from the coronaries, and therefore seems to be the most direct and reliable manner to get the cells into the myocardium. Nonetheless, cell spillage can occur at the site of injection, and it can be difficult to bring the cells into a preferred location in order to avoid injecting them into a remote region too far from the injury or into an infarcted area deprived of oxygen and nutrients [14].

Distribution

Distribution monitors the movement of drugs to its effector sites. This process is critical to the effectiveness of the drug, since its localization to extravascular target sites will allow it to carry out its function. The distribution is dependent upon the administration route, therefore we will discuss the bio-distribution of stem cells within the body and the myocardium as it relates to the above mentioned injection techniques.

Although a few studies have shown cell engraftment in the myocardium after intravenous injection (IV) [15, 16], the overall scientific consensus is that it remains a very inefficient process, which has rarely been pursued in clinical practice. The inability to trap intravenously injected cells could come from the lack of secreted chemo-attractant from the injury, and/or the limited cardiac output dedicated to supplying the coronary arteries. In bio-distribution studies [16, 17], only trace amount of radioactively labeled cells are found back in the heart with the predominated signal stemming from the lungs, liver and spleen. Substantiating the benefits of a local administration route, a comparative bio-distribution study [18] of radioactively labeled bone marrow mononuclear cells (BM-MNC) showed a superior engraftment rate in the infarct region after intracoronary delivery (IC) compared to intravenous infusion. Interestingly, in this study they also observed an increased myocardial retention when the enriched CD34 fraction (25%) was injected compared to entire bone marrow mononuclear cell population (3%). Therefore, this study highlighted that not all cell types will behave similarly and enter the injured areas after administration. Differences in integrin expression or other cell surface protein could lead to altered adhesion properties, thereby changing their engraftment kinetics. Nonetheless, with both cell populations the majority seem to be extracted from the circulation by the liver and spleen during the first passage (IV 85 %, and IC 55% of total radioactivity).

Another major barrier to proper cell distribution in the body are the lungs. Especially in studies with larger cell types [16] (e.g. mesenchymal stem cells (MSCs)) there is a noticeable accumulation in this organ, likely stemming from these cells inability to move beyond a given capillary bed size. In a comparative study from van der Spoel et al [19], radioactively labeled MSCs were injected intra-coronary or intra-myocardially and traced for their bio-distribution

after 4 hours. The highest accumulation from both modalities was observed in the lungs (~ 25 percent of total cells), while also noting off-target accumulation in the liver, spleen and kidney. Total retention in the heart did not differ between the two techniques, with the myocardium amassing 12 percent of the entire radioactive tracer pool in the body. Interestingly, local inspection of cell distribution by *ex vivo* γ-scan and histology revealed a stark distinction between the two techniques. Whereas intramuscular injection tended to accumulate in a site-specific manner (high signal at the mid-papillary level) with clusters of cells in the infarcted region and border zone, intracoronary infusion led to a scattered and diffused distribution throughout the targeted myocardium. Overall, bio-distribution studies have agreed that administration of stem cells, independent of the route, remains an ineffective process. On average, intracoronary and intramuscular delivery systems achieve a 15 percent acute engraftment rate, however, consideration must still be taken since the individual cell types and myocardial distribution of cells can still be effected by the administration route.

Metabolism and excretion

Metabolism, the body's ability to transform a drug, and excretion, the removal of the drug from the body, are the final steps of the drug's movement in the body. In the case of most pharmacological compounds, metabolic changes to their structure (most notably through the actions of cytochrome p450) will deactivate them directly or by enabling their excretion. Although cells will not be catalyzed in such a manner, once delivered to the heart their integrity is affected both directly and indirectly by the body.

After suffering an ischemic event, the damaged myocardium is under oxidative stress as the native cells die and the immune cells infiltrate to start clearing the debris. The stem cells, therefore, arrive in a hostile pro-inflammatory milieu and thereby susceptible to pro-apoptotic signaling. A study by Zhang et al [20] showed the profound effect of this ischemia damage on transplanted cells. Although grafted cells remained viable within hours of injection [2% apoptotic cells), TUNNEL positive cells drastically increased to 30 percent over the first 24 hours. Overall, they calculated that up to 90 percent of engrafted cells succumbed to these external stimuli over the course of one week. Nonetheless, limited apoptotic cells are observed after one week, therefore cells which did survive seem to be stably integrated and can potentially contribute therapeutically. This observation has been further supported by numerous studies in which pro-survival strategies have been successfully implemented to make stem cells more resilient against external stimuli. Pharmacological pretreatment and genetic modification targeting and inhibiting pro-death pathways have shown the ability to increase the number of cells in the myocardium [21-23], highlighting the indirect environmental burden that stem cells are confronted with upon their transplantation.

Within the immune system's defenses is the ability to recognize and remove foreign entities in the body. As has been observed in organ transplantation therapy, HLA complexes on cells present patient specific glycoproteins to the host immune system which is able to distinguish "self" from "foreign", and has made it crucial to match donor and recipient organs before the procedure [24]. For stem cells, there exists a similar threat from this direct involvement of the body immune system, although it is slightly more complex [25]. Firstly, many of the approaches

are using the patient's own stem cells for treatment, thereby circumventing the risk. Although this is an option for adult stem cells, such as bone marrow derived or resident progenitor cells, it remains a complication when establishing a therapy with embryonically derived stem cells. Secondly, certain stem cells, most notably MSC, express low levels of HLA on their surface and have inherent ability to avoid detection by the body [26]. These "immune privileged" cells can even act upon and modulate the immune system [27], consequently reducing inflammation at the site of injury (see pharmacodynamics section). Nonetheless, the immune system is a crucial aspect to consider for the successful engraftment of the stem cells, since a mismatch between donor and recipient will results in complete clearance of the therapeutics from the body [28]. It must be noted that the studies mentioned above have focused their research on exploring the elimination of the cells from the body, mostly because it happens in such a large percentage of injected cells and the read-out is rather straight forward. However, little is known about how the surviving stem cells are affected by their new environment. There is evidence that the body can direct cardiac differentiation of stem cells (see pharmacodynamics section), this is especially apparent in studies using undifferentiated adult cardiac progenitor cells (CPCs). However, the mechanisms and triggers behind this process are poorly understood. Furthermore, recent investigations have started to explore other interactions such as the effect of heart contraction on the alignment of cells in the myocardium [29]. These types of studies could be important for the optimization of cardiac cell therapy, perhaps shedding more light on the ideal administration location for an improved therapeutic distribution.

Pharmacodynamics

Pharmacodynamics is focused on how the drug affects the body. The complexity of stem cells allows them to target a multitude of processes, thus both agonistic and antagonistic actions on several biochemical pathways can be expected. Furthermore, their potential participation in direct tissue regeneration further increases their therapeutic interaction with the myocardium. In this section, we will examine the current understanding of this dynamic process.

Effect on the myocardium

On the basis of their regenerative potential, preclinical studies were undertaken to evaluate the therapeutic effectiveness of restoring the myocardium with stem cells. The first therapeutically viable stem cells to be injected were derived from the bone marrow, which provided an easy accessible and patient specific cell source. Spearheaded by the findings with c-kit+ selected bone marrow cells from Orlic et al [30], which demonstrated remarkable increase in myocardial viability and hemodynamic performance of the injured heart after cell transplantation, an explosion of preclinical trials were commenced to study the role of bone marrow cells in cardiac repair.

In experiments with BM-MNCs, cell injections led to a reversal in ventricular remodeling, reduction in collagen density, and a decrease in scar size in both rat [31, 32] and porcine [33, 34] models of MI. In addition, a greater angiogenic response was also observed in animals that received these cells, demonstrated by a higher vessels number in the myocardium and an augmented myocardial perfusion. Most notably, from a recent meta-analysis [35] of BM-MNC

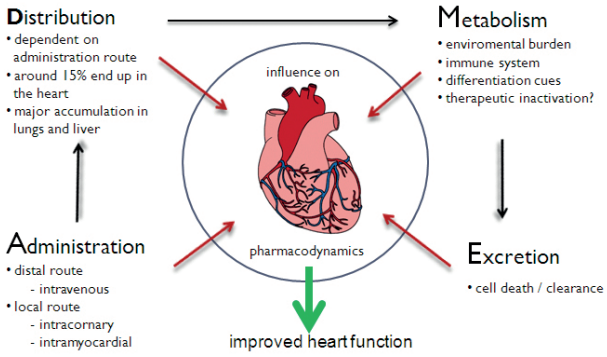

Figure 1. Schematic overview of pharmacokinetics for cardiac cell therapy. The administration of stem cell will affect the subsequent distribution of cells, which could influence the metabolism and excretion of the biotherapeutics. Each has an overall influence on the pharmacodynamics properties of the stem cells, which can influence the efficacy of the treatment.

injections in large animal models, it was shown that the global LV function was markedly increased after cell therapy. On average, a 6 percent increase in left ventricular injection fraction (LVEF) was observed. MSCs are a more homogenous cell population that can be isolated from the bone marrow, but also from other sources such as adipose tissue [26]. These multipotent stem cells, being able to differentiate towards different cell-lineages, are easily isolated and propagated from a donor and therefore provide a robust cell source for therapeutic application. Similarly to the physiological effects induced by the BM-MNC, MSC injection led to increased capillary formation and reduced infarct size, while improving regional and global cardiac contractile function [36, 37]. Evidence suggest a possible role of MSCs in targeting extracellular matrix remodeling [38], which improves the pathological process and leads to better cardiac function. Overall, MSCs seem to be a more potent cell type compared to BM-MNC, illustrated by an average LVEF increase of 8 percent in studies performed in large animals model.

In studies utilizing CPCs, improved cardiac performance was also noted after their injection into the myocardium [39-41]. This was associated with a decreased collagen content, increased myocardial viability and greater angiogenic response. Actions against pathological remodeling seem to be playing a role as well, since increased wall thickness was observed in cell treated animals. Pre-clinical studies with ES cells have also shown to be promising [42, 43]. However, in studies with longer follow-up times issues were raised about the transient nature of the effects [44]. During the first month better functional recovery was observed, but over the course of the next two month these effects reverted back to baseline. This highlights the problems of the poor pharmacokinetics, and how it could impede the therapeutic actions of the stem cells. The exciting results from the animal studies were followed up by many clinical trials [45, 46]. For bone marrow derived cells, these studies offered variable results. In some trials no beneficial effects were observed, while in others only transient effects were noted. Nevertheless, in a meta-analysis of 29 randomized control trials using bone marrow cells a 3 percent overall improvement in LVEF was established [47]. The STAR-heart study even reported a decrease in mortality in patients treated with stem cells compared to placebo [48]. Recent phase I clinical trials with CPCs (SCIPIO [49] and CADUCEUS [50]) demonstrated the safety and feasibility of injecting autologous heart derived stem cells into the coronary beds. Astoundingly, within their

small sample size the SCIPIO trial demonstrated an 11 percent increase in LVEF in patients treated with the stem cells persisting for over 1 year follow-up.

Mechanisms of action

Despite the speed at which stem cells have arrived in the clinic to treat patients with heart disease, the therapeutic mechanism by which they operate remains poorly understood. At the turn of the millennia, the conceptual design to use stem cells to repopulate the heart after injury generated much excitement. Nonetheless, almost 15 years later that concept remains elusive amid the growing evidence for their beneficial therapeutic effects.

The evidence for stem cell participation in direct tissue regeneration has existed since the initial transplantation experiments. The study by Orlic et al [51] showed that a subpopulation of bone marrow cells was able to substantial increase myocytes in the infarct zone by direct transdifferentiation into cardiomyocytes. However, follow-up studies [52, 53] refuted this observation, stating that bone-marrow cells transplanted into the myocardium adopted hematopoietic fates rather than transdifferentiating into myocytes. Interestingly, in those studies they still observed the therapeutic actions of the stem cells, thereby raising major questions about the underlying mechanism of action. In studies using stem cells with established cardiogenic potential, such as CPCs, differentiation into cardiomyocytes has been firmly established after transplantation. Nonetheless, up to 10 million stem cells are normally administered into the infarct myocardium that has just lost billions of myocytes due to ischemia. According to biodistribution studies only 15 percent or less of the total injected cells engraft, of which only a fraction differentiates into cardiomyocytes. Therefore, their differentiation can never account for the total improvement in cardiac function.

In this respect, the study by Chimenti et al [54] investigated the contribution of differentiation to the overall regeneration observed in the heart after CPC transplantation. They looked into angiogenesis, as well as myogenesis, within areas in which the stem cells were detected and tried to attribute to what extent the transplanted cells were directly participating in these processes. Surprisingly, although they did find differentiated cells, these only accounted for ~20-50 percent of the total increase in newly formed capillaries and myocardial viability. The other effects seem to be brought about by the cells ability to stimulate endogenous pathways, inducing angiogenesis, reducing apoptosis or maybe even activating the endogenous repair mechanisms, thereby attenuating the damage brought about by the myocardial infarction. This ability has now become known as the paracrine effects of stem cells, and covers among others an array of cytokines and growth factors released by the cells upon their transplantation. The most striking example of paracrine effects are found in studies in which the *in vitro* culturing medium of stem cells (also known as conditioned medium] is used as a therapeutic agent. The study by Timmers et al [55] demonstrated that MSC conditioned medium alone is sufficient to protect the myocardium and stimulate endogenous regenerative processes.

The paracrine factors secreted by stem cells stimulate various pathways. Most notably, activation of PIK3/Akt [56] and ERK1/2 [57] pathways has been observed after myocardial delivery of various cell types. These pathways are activated by growth factor receptors, such as EGFR and VEGFR, and elicit powerful pro-apoptotic and angiogenic signals to the heart. Some cytokines and

growth factors can target remodeling and hypertrophic processes, while others can even stimulate the proliferation and migration of resident progenitor cells and thereby initiate endogenous regeneration. Vesicles released by cell types, such as exosomes [58], have also been shown to play a potent role in cardiac healing, by delivering cytosolic stem cell cargo containing microRNAs and various other intracellular components [59]. Lastly, stem cells, such as MSCs and CPCs, have immune-modulatory properties. *In vitro* MSCs [27] and CPCs [60, 61] have been shown to suppress the proliferation of immune cells, such as T-cells and B-cells, while also being able to selectively expand immunosuppressive regulatory T-cells. Since inflammation after the injury can lead to adverse remodeling in the myocardium, the ability of stem cells to reduce or modulate the overall inflammatory reaction in the heart can lead to therapeutic benefits.

At the moment, the exact mechanism of action behind cardiac stem cell therapy remains unclear. It is clear, however, that the direct involvement of cells in tissue regeneration had been overestimated during the initial years of research. The lack of differentiation has led researchers to uncover a multitude of other targets and mechanisms that were not part of the original hypothesis. Nonetheless, the mechanism of action is also dependent upon the cell type administered. Bone marrow derived cells trans-differentiation seems unlikely to contribute in any significant way, and thus these cells rely heavily on paracrine factors for their therapeutic effects. On the other hand, CPCs appear to be able to exert their effect both directly and indirectly on the regeneration process of the heart.

Safety

Skeletal myoblast were one of the first cell types to be examined for potential use in cardiac transplantation due to their intrinsic contractile nature. Although preclinical studies demonstrated that skeletal myoblasts effectively prevented left ventricular remodeling and preserved global parameters, a higher incidence of arrhythmic events was observed in myoblast-treated patients in early phase clinical trials [62]. This side-effect seems to be caused from the inability of myotubes to couple to the native cardiomyocytes, and thus serves as stark reminder of the potential complications in introducing a non-cardiac cell type in the heart. Nonetheless, the clinical trials with bone-marrow or adipose derived stem cells have proven cellular transplantation to be a very safe procedure for patients [63].

Several safety issues had been raised about stem cell therapy, especially in concern with the delivery route. For example, there is a possibility of myocardial perforation after an intra-myocardial injection or a blockage of the coronary circulation after intracoronary infusion, which could lead to more myocardial damage. In regards to the latter, autologous MSCs from dog bone marrow injected into normal coronary arteries demonstrated micro-infarctions [64]. However, these issues seem to be limited in their effects, and masked by the effective therapeutic actions of the stem cells. Concerns about tumor formation, such as angioma or myoma, have also not been linked to adult stem cells thus far, although teratomas remain a concern for pluripotent cells [10]. A critical issue remains the *ex vivo* handling of stem cells prior to transplantation, since contaminated cells injected into the myocardium could potentially lead to devastating infections such as myocarditis. The use and take up of animal products

during the culturing process can also lead to unwanted side-effects. Therefore, all stem cell studies should abide by isolation and culturing protocols using good manufacturing practice facilities to minimize the risk.

Drug interaction

Patients who suffer from heart failure are prescribed a multitude of drugs to alleviate symptoms and during interventional procedures [2]. However, certain medication in their regiment can have direct effect on the function and therapeutic efficiency of transplanted stem cells. For examples, statins have received attention for positively influencing stem cell behavior. Studies have shown that statins can inhibit the senescence of cells [65], while also promoting cells survival by the activation of the PI3K/Akt pathway [66]. In regards to stem cell transplantation, the use of rosuvastatin in a rat model of MI increased MSC survival and differentiation after transplantation, which led to a better functional recovery compared to the rats only treated with MSCs [67]. Similarly, the use of β-Blockers, in particular carvedilol, during cell transplantation is also advantageous, since its antioxidant properties can increase the MSC engraftment rate [68].

There also exist negative interactions between stem cells and drugs. Heparin, for example, has proven to attenuate the migratory capacity of BM-MNC by inhibition of stromal cell-derived factor-1 /chemokine receptor type 4 signaling [69]. Such signals are imperative for the homing and migration of cells upon administration. Furthermore, the widespread use of COX inhibitors in the patient population can also be detrimental to their behavior. COX-2 inhibitor has been shown to considerably decrease PGE2 production in ES cells [70], which are dependent on PGE2 synthesis for pathways involved in differentiation and proliferation. In addition, aspirin has been proven to reduce signaling of the Wnt/β-catenin pathway in MSCs, thereby reducing the proliferation rate of the cells [71].

Scope of this thesis

The intent of this thesis was to undertake some of the critical underlying issues of conventional cardiac cell therapy approaches, especially in regard to the use of cardiac progenitor cells. As highlighted above, there is a need to bolster the poor pharmacokinetic properties of stem cells. In this respect, we explored various techniques that tackle some of the observed pitfalls; from protecting the cells from pro-apoptotic signaling in the ischemic myocardium to avoiding cell leakage after administration. These experiments were performed with a critical eye towards their pharmacodynamics properties by monitoring their faith and effects on the myocardium. Overall, we created a platform which future cellular therapies could utilize to create a more robust and efficient regenerative therapy for the heart.

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Cardiac stem cell transplantation: exogenous or endogenous repair?

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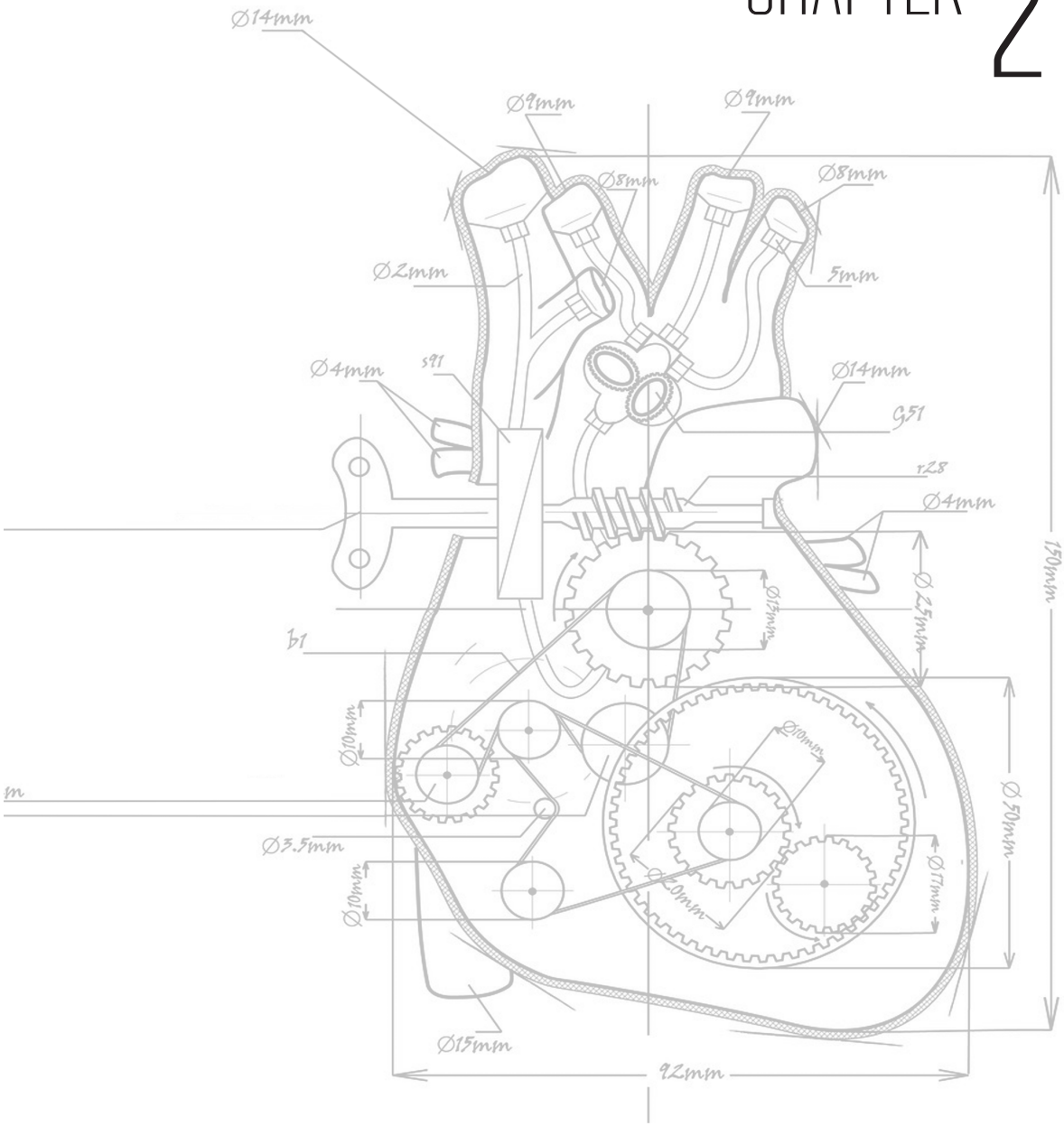
CHAPTER 2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Despite medical treatment and revascularization options, heart failure is associated with significant morbidity and mortality rates. As the demand for cardiac transplantation by far exceeds the availability of donor hearts, heart assist devices are used as a bridge to transplant. However, transplantation and assist devices are only available for a minority of individuals. Consequently, new therapeutic strategies are being explored, of which cell transplantation therapy has emerged as a promising option to restore cardiac performance. Several *in vivo* experiments have demonstrated improved cardiac function and reduced remodeling upon cell injection into injured hearts. This article summarizes the types of stem or progenitor cells that are currently used in cardiac cell therapy, and reviews evidence of their beneficial effects on cardiac function and remodeling after myocardial infarction (MI) from preclinical and clinical studies. In addition, the potential modes of action of cell differentiation of the engrafted cells and/ or activation of resident stem cells (SCs) by paracrine factors are discussed.

Cell sources

Important features of stem cells include self-renewal, clonogenicity, and the potential to differentiate into multiple cell lineages. Many different types of SCs have been described as potential sources for cardiac therapy. These cells can be classified in many ways; for instance, embryonic versus somatic SCs based on their origin, and omni-, pluri-, ormultipotent versus progenitor cells with a restricted differentiation potential. For the regeneration of cardiac muscle, differentiation into cardiomyocytes, endothelial cells, and vascular smooth muscle cells is essential.

Pluripotent SCs

Embryonic SCs (ESCs) or germline SCs are, in theory, ideal candidates for cell transplantation therapy as they are pluripotent and can differentiate into all cell lineages including endothelial cells and spontaneously beating cardiomyocytes [1–4]. Other pluripotent cell types that have been described are the so-called induced pluripotent SCs (iPSs), which originate from differentiated fibroblasts [5,6]. To generate iPSs, fibroblasts are transfected with various transcription factors that are associated with pluripotency (e.g. Oct-3, Oct-4, Nanog, Sox2, LIN28, Klf4, and c-Myc), thereby creating reprogrammed cells that are able to differentiate into cardiomyocytes [5,7,8]. An advantage of this approach is the use of autologous cells for cell therapy, thus obviating the need for immunosuppressive strategies. Another form of pluripotent stem cells are the very small embryonic-like cells (VSELs), which are isolated from the bone marrow (BM) and express genes that are associated with pluripotency [9]; these cells have been reported to differentiate into cardiomyocytes and capillaries *in vivo* [10]. A summary is provided in Table 1.

BM-derived SCs

BM-derived SCs (BMSCs) were originally thought to only contribute to the BM and give rise to hematopoietic cells, but it is now claimed that they are essential in repairing the myocardium after damage [11,12]. BMSCs can be mobilized from the BM and migrate to the myocardium upon release of several chemokines and growth factors, such as stromal cell-derived factor-1

(SDF-1) and vascular endothelial growth factor, when this tissue becomes injured [11–13]. The different types of SCs that can be isolated from the BM include the above-mentioned VSELs, mesenchymal SCs (MSCs), hematopoietic SCs (HSCs), multipotent adult progenitor cells (MAPCs), and endothelial progenitor cells (EPCs). MSCs have been shown to lack expression of hematopoietic (CD45) and endothelial (CD34) markers and to differentiate into cardiomyocytes in a porcine model, based on the expression of several other molecular markers such as α-actinin and troponin I [14]. Among the HSCs, only c-kit+ and lineage-negative cells are described to differentiate into cardiomyocytes and vascular cells in the murine myocardium [15,16]. MAPCs that have been isolated from the BM have also been demonstrated to have the ability to differentiate into a cardiomyogenic lineage [17]. Finally, EPCs (CD34+ mononuclear cells) are present in the BM and circulate in peripheral blood. Although it is unlikely that these cells can differentiate into cardiomyocytes, they are able to form vascular networks and improve perfusion [18–21]. Despite cardiomyocyte differentiation having been described for some of these cells *in vivo*, it is important to realize that these events are rare and difficult to prove. More evidence exists for the important role of circulating BMSCs in endothelial repair.

Table 1 Stem cell sources

Cell type	Markers of differentiation	Ability to differentiate into fuctional cardiomyocytes	
		In vitro	*In vivo*
Pluripotent stem cells			
Embryonic stem cells	Connexin-43, sarcomeric proteins, myosin, α-actin	Yes	Yes
Very small embryonic-like cells	Troponin T, α-actin	Yes	Yes
Induced pluripotent stem cells	Myosin light chain 2v, α-MHC, atrial natriuretic peptide, and α-actinin	Yes	Yes
Bone marrow stem cells			
Mesenchymal stem cells	Vimentin, troponin T, troponin I, α-actinin, connexin-43, α-MHC, desmin	Yes	Yes
Multipotent adult progenitor cells	Desmin, α-actinin, and skeletal muscle myosin	Yes	Yes/No
Endothelial progenitor cells	Desmin, troponin T, connexin-43	Yes	Yes/No
Cardiac stem cells			
c-kit	α-actin, cardiac myosin, desmin, connexin-43	Yes	Yes
Islet-1	Troponin T, α-cardiac actinin	ND	ND
Cardiospheres	Troponin, α-MHC	ND	ND
Sca-1	Troponin I, α-actin, cardiac myosin, α-MHC, desmin, connexin-43	Yes	Yes
Oct-4	α-MHC, α-actin, connexin-43	Yes	Yes

MHC: myosin heavy chain; ND: not determined

SCs derived from other tissues

MSCs can also be isolated from adipose tissue. These MSCs have comparable characteristics to BMSCs and can differentiate into several cell lineages, including cardiomyocytes and vascular endothelium [22]. In theory, a common feature in all tissues is their vascularization; therefore, several groups have sought to identify a progenitor cell stemming from the blood vessels. Recently, pericytes were shown to be a common progenitor cell in the vasculature of multiple human organs with CD146, NG2, and platelet derived growth factor β-receptor expression and absence of hematopoietic, endothelial, and myogenic cell markers [23]. Although no research has been published on their potential to be used for cardiac therapy, the vascular affiliation of these progenitor cells hints towards a possible therapeutic application.

Cardiac-specific SCs

The heart is not a terminally differentiated organ. There is ongoing DNA synthesis in the heart and limited cardiomyocyte renewal [24]. In addition, the adult human heart harbors progenitor cells that can be extracted and cultured. It has been suggested that these progenitors are perfect candidates to regenerate the heart owing to their localization [25,26]. Many different types of cardiac progenitor cells or cardiac SCs (CSCs) have been isolated from the heart including SC antigen-1 positive (Sca-1+), c-kit+, islet-1 positive (isl-1+), and cardiosphere cells. CSCs are lineage-negative and also negative for the hematopoietic markers CD34 and CD45. Sca-1+ CSCs were initially described in mice [27]; however, recently, Goumans *et al.* isolated human cardiomyocyte progenitor cells based on Sca-1+ selectivity, and these cells could differentiate were able to into beating cardiomyocytes upon stimulation with 5-azacytidine and transforming growth factor-β [28,29].

In addition to cardiomyocytes, Sca-1+ CSCs can form vascular-like structures *in vitro* [28,29]. Beltrami *et al.* identified a cell population in rat hearts that expressed myogenic markers, indicating their commitment to the cardiomyocyte lineage [30]. These cells were able to differentiate into three lineages (cardiomyocytes, smooth muscle cells, and endothelial cells). Ott *et al.* also identified Oct-4 (an SC marker) cells in the adult rat myocardium [31]. Messina *et al.* demonstrated that human cardiospheres, consisting of Sca-1+, c-kit+, flk-1+, and CD31+ cells, exhibited beating upon co-culture with rat cardiomyocytes [32]. Laugwitz *et al.* demonstrated that isl-1+ cells from humans, mice, and rats express cardiomyogenic genes, and display contraction upon co-culture with myocytes [33]. A thorough comparison of these CSCs at the level of gene expression or proteomics is lacking, but is crucial to prove that there is one or more type of CSC found in the human heart.

Cell therapy

With the discovery of SCs and progenitor cells, many *in vivo* experiments have been initiated in an attempt to improve cardiac function. These experiments have focused on *in vivo* differentiation towards cardiomyocytes and cardiac-supporting tissue such as vascular endothelial cells and smooth muscle cells. Typically, cardiac function and morphology improves slightly (or does not deteriorate) after SC injection. Incorporating this therapy into clinical

practice is of great importance; however, before SC therapy can be translated to standard patient care there are still many limitations to overcome. It is currently unclear whether any cell type is capable of differentiation *in vivo* in a reproducible way, producing significant numbers of cardiomyocytes to replenish the injured myocardium. Furthermore, it is unclear as to whether cardiomyocytes injected following *in vitro* differentiation would couple with the host myocardium. Most studies have failed to show such coupling. Moreover, the cells, having become activated *in vitro*, produce a substantial amount of extracellular matrix, resulting in electrical insulation [34].

Other issues that may hinder the use of SC therapy in clinical practice include the need for high quantities of autologous cells and the prevention of cell death after injection. Therefore, in most settings, SC research is focused on optimizing cell delivery and improving proliferation, survival, and differentiation of the delivered cells. Clinical studies carried out thus far have demonstrated limited improvements in cell therapy. A meta-analysis of randomized controlled trials on BMSC transplantation revealed an increase of 2–4% in ejection fraction compared with controls [35]. It is of great importance to understand and explain the observed results at the molecular level.

Evidence for differentiation after cell transplantation

In 2007, Tomescot *et al.* showed that human ESC-derived cardiomyocytes survived transplantation and were observed in the infracted rat hearts after 2 months [36]. Myogenic differentiation was induced in human ESCs by bone morphogenic protein-2 (BMP-2) and an inhibitor of the fibroblast growth factor (FGF) receptor (SU5402). These pretreated cells were injected into rat myocardium at 15 days post-infarction; at 2 months post-transplantation, newly formed cardiomyocytes were detected. Unfortunately, these engrafted and differentiated cells had shorter sarcomeric lengths compared with the host cardiomyocytes and no gap junctions or connexin-43 expression. Gap junctions propagate electrical signals and conduct contraction in the heart, and are essential for incorporation and coupling of the engrafted cells. Interestingly, van Laake *et al.* demonstrated that human ESC-derived cardiomyocytes injected into infarcted murine hearts (a severe combined immunodeficiency model) were functionally incorporated after 13 weeks and did express gap junctions [37]. However, the investigators failed to detect coupling between the human and murine cardiomyocytes.

Orlic *et al.* reported that injection of BMSCs into mice with MI resulted in a reduction in infarct size of 40% [38]. BMSCs were capable of forming new cardiomyocytes, smooth muscle cells, and endothelial cells, thereby expressing cardiacspecific transcription factors and proteins, including myosin light chain, troponin T, and connexin-43. Wang *et al.* isolated MSCs from rat BM and injected them into the left ventricular wall of rat hearts after MI [39]. The MSCs engrafted successfully at 9 days post-transplantation. At 4 weeks, expression of myosin heavy chain protein, organized contractile proteins, and connexin-43 was observed in these MSCs. Two other studies in pigs have also demonstrated MSC differentiation towards a myogenic phenotype, with expression of myogenic markers such as transcription factors, troponin T, myosin light chain 2A, and β-myosin heavy chain confirmed [40,41]. In addition to direct cardiac repair, transplanted MSCs can mediate paracrine and immunomodulatory effects [42].

MSC injections can blunt the tissue immune response after both autologous and allogeneic cell or tissue transplantation. However, the efficacy of MSC therapy depends on the pro-inflammatory state prior to cell injection [43].

Dawn *et al.* described the formation of enhanced green fluorescent protein-positive (eGFP+) cardiomyocytes after injection ofeGFP+ c-kit+ CSCs into infarcted rat hearts [44]. Furthermore, these latter cells were capable of differentiating into epithelial and smooth muscle cells. As a result, infarct size was reduced in CSC-treated mice after35 days [44, 45]. Bearzi *et al.* reported that injection of human c-kit+ CSC into infracted myocardium in immunodeficient mice and immunesuppressed rats led to the formation of a chimeric heart containing human myocardial tissue [46]. Differentiation of SCs into cardiac cells can be enhanced by pretreatment with specific GFs or hypoxia. Canine MSCs cultured in the presence of FGF, insulin-like GF-1 [IGF-1), and BMP-2 are able to differentiate into a cardiac lineage and show expression of early muscle and cardiac markers, such as desmin and transcription factors [47]. In a chronic canine MI model, GF-pretreated autologous BMSCs improved morphology and function of the infarcted hearts, and troponin I and cardiac myosin co-localized in the engrafted BMSCs 4 weeks after transplantation [48]. Uemura *et al.* injected hypoxia-stimulated BMSCs into the infarcted myocardium ofmice and found improved survival of BMSCs and residing cardiomyocytes as compared with normoxia-cultured BMSCs [49]. After 4 weeks, the mice that were treated with hypoxic BMSCs displayed improved cardiac morphology and function over those treated with normoxia-cultured BMSCs or placebo. These investigators also demonstrated that survival genes, such as the Akt and endothelial nitric oxide synthase (eNOS) genes, were up regulated in hypoxic BMSCs *in vitro* as compared with non-stimulated cells.

The observed beneficial effects on morphology described above cannot solely be explained by the number of surviving cells. MI generally leads to a loss of 2×10^9 myocytes in a medium-sized infarct. Typically, only 1×10^6 to 1×10^7 cells are used in cell transplantation. Owing to inefficient delivery techniques, cell death, and migration of cells to other tissues, only a small number of cells, approximately 3% of those transplanted, will be retained in the infarcted myocardium [50]. Of this fraction, only a small portion can actually differentiate into cardiomyocytes, but despite this, SC injection can lead to a considerable reduction in infarct size and an increase in ejection fraction [44,51]. This indicates that other factors, possibly paracrine effects, are of importance in both the survival and differentiation of engrafted SCs and their surroundings (Figure 1). The paracrine effect may even be the crucial component of SC treatment.

Evidence for paracrine effects

Activation of resident cells is a possible explanation for the improved cardiac morphology observed with SC transplantation. Moreover, it has been demonstrated that the number of resident CSCs in end-stage human heart failure is increased, as indicated by a 29-fold increase in their mitotic index and enhanced initiation of differentiation into myocyte, smooth muscle, and endothelial cell lineages [51]. Paracrine factors released by SCs in a hostile environment trigger or activate resident SCs, and stimulate anti-apoptotic or survival pathways in resident cells. In addition, these paracrine factors can mobilize and attract SCs from the BM to the site

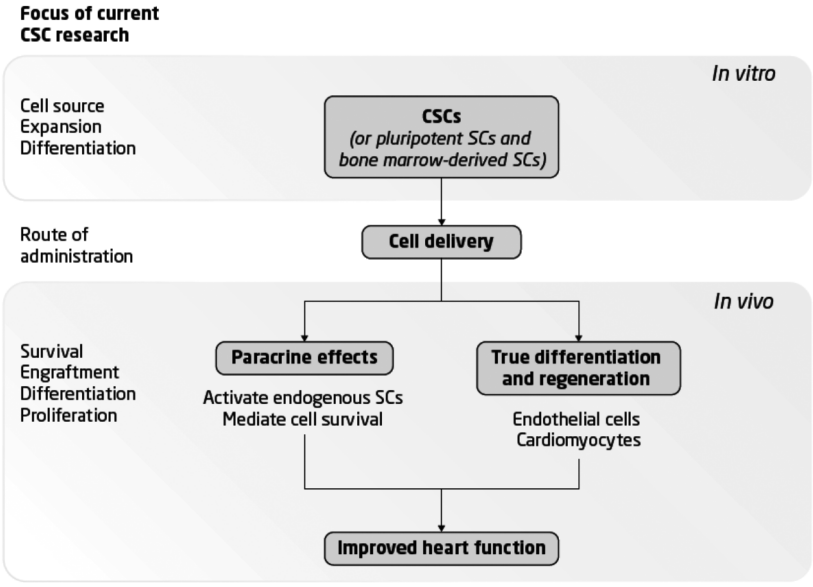

Figure 1 An overview of CSC therapy. This schematic highlights two modes of action *in vivo*: the differentiation and integration into the myocardium, and the secretion of beneficial paracrine factors. The left-hand column describes current areas of research undertaken to improve stem cell therapy.

of injury (i.e. the heart). Researchers have found that new vascular structures and cardiomyocytes, which do not originate from the engrafted cells, develop in the infracted region [38,52]. After MI, the myocardium releases chemokines such as SDF-1, which stimulate the mobilization of BMSCs [53,54]. Haider *et al.* conducted experiments in which IGF-1 was overexpressed in MSCs; these cells were administered to infracted rat cardiac tissue leading to increasedSDF-1 release and subsequent increased mobilization of circulating SCs (compared with the administration of MSCs deficient inIGF-1) [55]. Moreover, IGF-1 over-expression also led to increased survival of the injected MSCs and further reduction in infarct size compared with normal MSCs.

Upon MI, many paracrine factors (e.g. GFs) that can affect the differentiation and survival of SCs are released. Other than survival and differentiation, these factors are likely to stimulate angiogenesis and thus have a cyto-protective role [49,56]. When *Akt*-transduced MSCs were injected into infarcted animal myocardium, increased numbers of surviving MSCs and reduced rates of apoptosis in the myocardium were observed [49,57]. In addition, MSCs transduced with Akt-1 restored the myocardial tissue volume significantly better than *LacZ*-transduced MSCs (control cells) [57].

Currently, the application of paracrine factors that are released by SCs, without injection of the cells themselves, is being considered. In 2008, Timmers *et al.* administered intravenous and intracoronary injections of conditioned medium from cultured human ESC-derived MSCs into a porcine ischemic/reperfusion injury model [58]. They observed a 60% reduction in infarct size and a short-term improvement in cardiac performance with these injections. Identification of the key paracrine factors involved in the abovementioned studies could enable the

development of protein-based therapies involving injection of these factors as a primary treatment, or help to enhance SC therapy to improve cardiac function [59]. Such therapy could promote differentiation of SCs into cardiomyocytes and vascular structures, as well as the activation of autologous cells, thus initiating and enhancing anti-apoptosis and angiogenesis, and consequently regenerating the injured heart.

CONCLUSION

Paracrine factors could promote differentiation, angiogenesis, and anti-apoptosis of engrafted SCs. Under physiological conditions, such factors are responsible for mobilization of SCs from the BM. It is suggested that paracrine factors released from SCs can enhance this effect; therefore, the identification and subsequent application of these factors may lead to increased beneficial effects in cell therapy. Treatment with these factors alone to achieve the paracrine effects observed with cell transplantation is being considered to replace the transplantation therapy itself. However, true regeneration and replacement of injured tissue may still have additional values. Further research is needed to establish the true effects of SC transplantation therapy, in particular, whether improving cell delivery, survival, proliferation, and differentiation will also lead to enhanced clinical relevancy for the treatment of ischemic heart failure.

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Different types of cultured human adult cardiac progenitor cells have a high degree of transcriptome similarity

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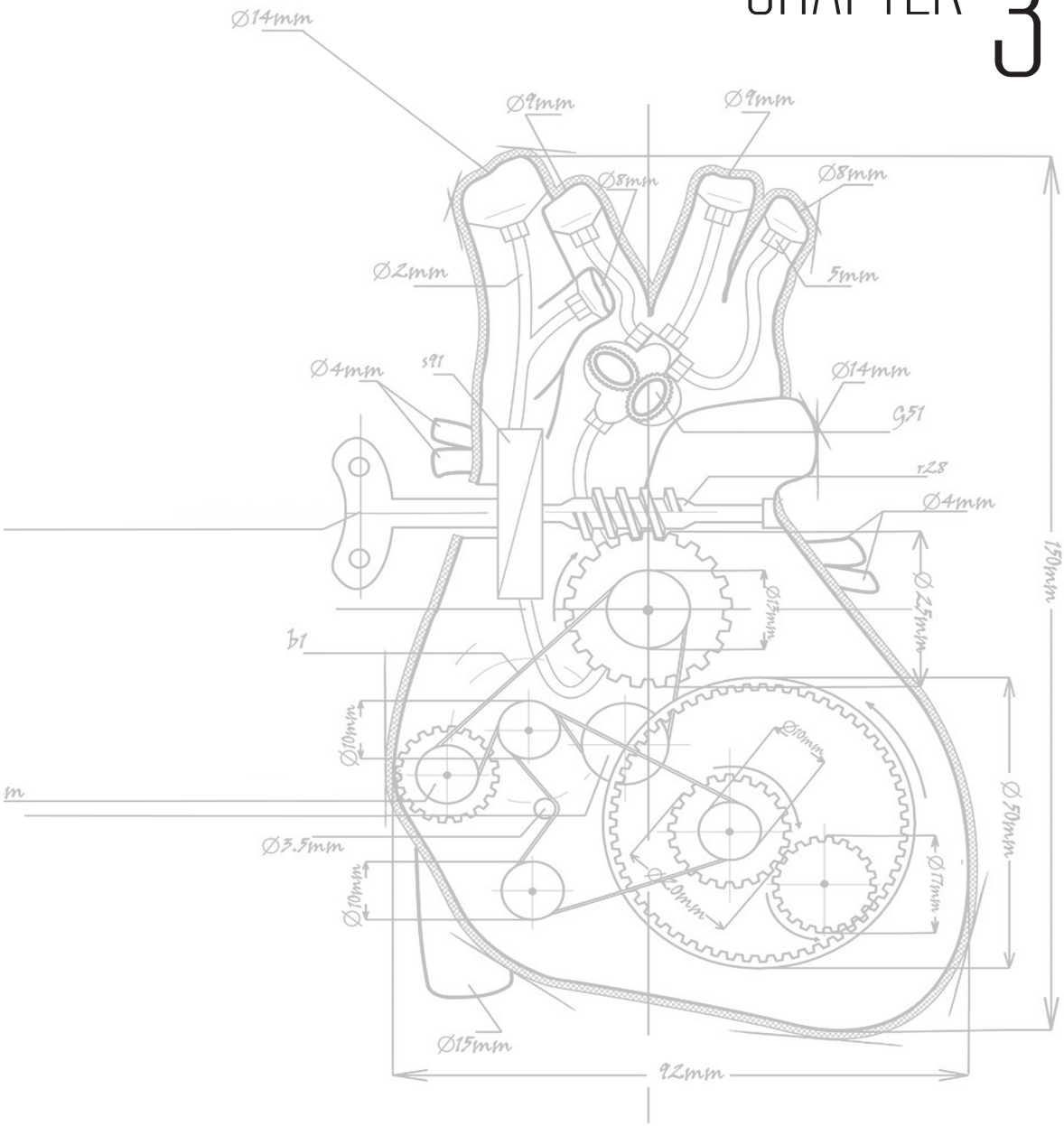
CHAPTER 3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ABSTRACT

The discovery of different resident cardiac progenitor cells (CPCs) a decade ago, described by several research groups, stimulated the quest, isolation, and use of these cells for cardiac regeneration. Human CPCs are moving towards the clinic as one of the most promising cell types for cardiac repair, but the extent to which their molecular profiles vary as a result of donor heterogeneity or different isolation method is unclear. Here, human CPCs were isolated based on c-kit or Sca-1 expression, or were derived from explant cultures, and were subsequently propagated in a panel of different media formulations. Comparative genomic profiling revealed that CPCs isolated with different methodologies shared a very high degree of correlation in gene expression patterns (≥0.96). Notably, only Cardiospheres, being cultured in a 3D culture environment, were molecularly more distinct. This suggests that different human-derived CPC populations, at least upon propagation in culture, have similar gene expression programs.

INTRODUCTION

Despite the large effort in the development of new strategies that is aiming at the prevention or treatment of cardiovascular diseases (CVD), they are still the leading causes of death among the western society. Among the different strategies, cell-based cardiac transplantation therapy has been put forward during the last ten years for the stimulation of repair of ischemic heart disease creating enthusiasm and hopes in the scientific as well as public community [1,2]. Several clinical trials, employing different stem cell types, have been performed showing encouraging results in term of safety but conflicting data in term of efficacy [2,3]. Among the different cell types, resident cardiac progenitors cells (CPCs) have been recognized as one of the most promising cellular source for cardiac cell therapy. Different CPCs have been isolated from human adult cardiac biopsy samples using different methodologies and culture conditions [4–9]. CPCs showed therapeutic efficacy in different animal models and three clinical trials for cardiac regeneration by resident autologous CPCs showed a solid proof of concept in terms of safety and therapeutic potential [10,11]. Despite CPCs are moving forward to clinical application, still many question are open regarding their molecular profile and intern-relationships or the influence of different culture conditions. Defining a common molecular profile that defines CPC's is therefore an important goal. Similarly, identifying robust and multi-laboratory isolation and culture protocols that generate reproducible cell populations from genetically diverse donors is critical for their translational success.

METHODS AND RESULTS

For our comparison, we collected human auricle biopsy samples anonymously from 20 different adult patients that underwent bypass surgery and generated a total number of 33 different cardiac derived progenitor cell (CPC) lines (Supplementary Table 1). The cardiac left auricles were enzymatically digested and immune-magnetically selected to isolate either Sca-1 positive (+) [6,7], c-kit+ [4,5] CPCs (Figure 1), or auricles were cut in $1mm^3$ parts and cultured as explants to obtain Cardiospheres (CSps) [8] and Cardiosphere Derived Cells (CDCs) [9]. After CDCs expansion, both Sca-1+ and c-kit+ cells were again obtained by immune-magnetic selection and expanded further (Figure 1 and Supplementary Methods). Different CPC lines were cultured in their originally described culture media [4,6,8,9] or switched to the other used media and culture coatings. Only CSps were cultured exclusively in their original culture condition due to their 3D structure and suspension growing conditions (Figure 1). Upon culturing, the different monolayer growing CPCs had a similar morphology and were all characterized by a large nucleus to cytoplasm ratio (Figure 1b-l). When cells were cultured in GEL-SP++, containing a high level of growth factors, their proliferation rate was higher compared to the other culture conditions but no clear differences were observed between different CPC subtypes cultured in the same conditions. RNA from all the isolated CPCs samples was examined by Illumina HumanHT-12v4 whole genome array analysis (Figure 1 and Supplementary Table 1). To explore gene expression profiles of different CPC lines and observe global differences, unsupervised

hierarchical clustering and Pearson's correlations were performed. When comparing individual CPC cell-lines, isolated with different methodologies, they shared a high degree of similarities and correlation in gene expression patterns (Figure 2a). By averaging expression profiles of individual CPC conditions, thereby reducing donor variability, similarities increased even more, ranging from 0.92 to 0.988 (Figure 2b and c). These results show that individual donor differences were larger than influences of isolation and medium conditions. Moreover, the

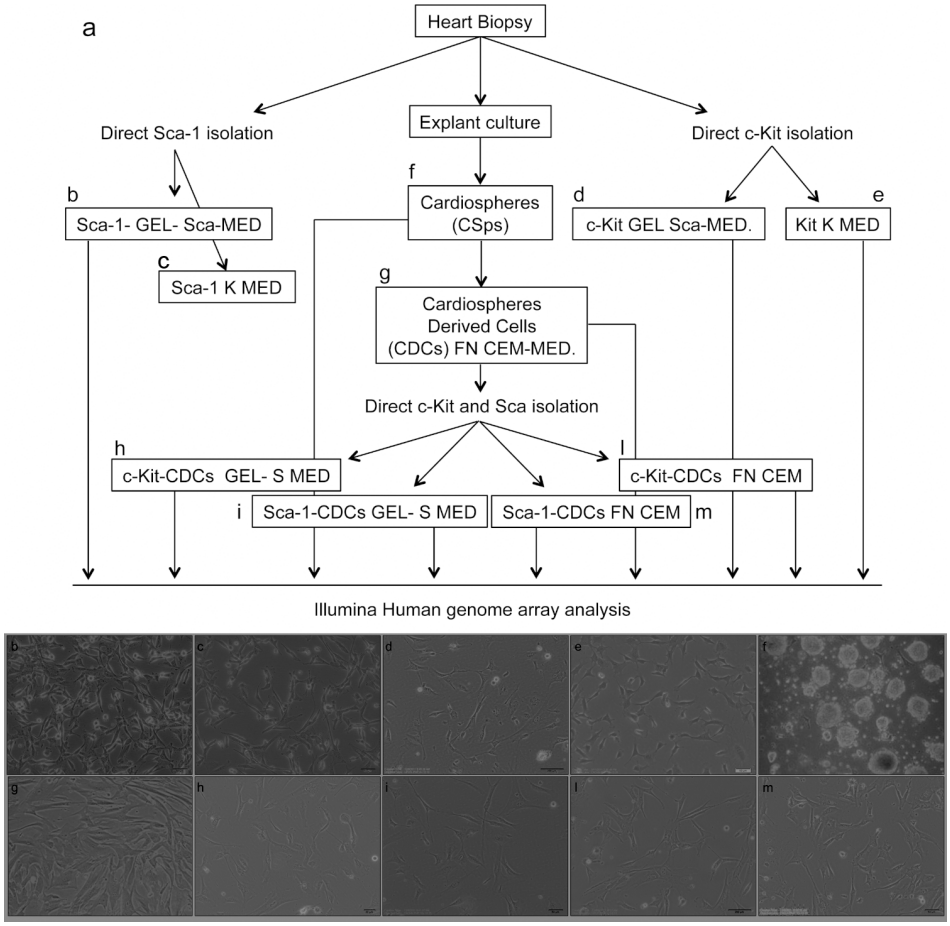

Figure 1 (a) Experimental design of the project. (b) Sca-1+ cells were isolated from human auricle biopsy and cultured in gelatin coated flask and Sca- medium (Sca GEL S MED [8]. (c) After expansion cells were also cultured in c-Kit culture condition (Sca K-Med)[6]. (d) C-Kit+ cells were isolated and cultured in Kit-CPCs Medium [60] (Kit K-Med) and after expansion (e) cultured in gelatin coated flask and Sca- medium (Kit- GEL S MED) [8]. Human auricle samples were cultured as explant to form Cardiospheres (CSps)[10] (f). CSPs were expanded as Cardiospheres derived cells (CDCs) in Fibronectin and Complete Explant Medium (FN CEM; g) [11] or in gelatin coated flasks and Sca medium (CDCs GEL S MED) [8]. After expansion c-kit+ (h/l) or Sca-1+ (i/m) were isolated from CDCs FN CEM and cultured or in FN CEM (Kit-CDCs FN/CEM (h) or Sca-CDCs FN/CEM (i)) [11] or in GEL S MED condition (Kit-CDCs GEL S MED (f) or Sca-CDCs GEL S MED (h) [8]. Representative pictures of individual cultures are presented for each used condition. Scale bar= 20µm.

strongest correlations between the different CPC lines were observed when cells were isolated and cultured in the same conditions (Figure 2b and c). Among the different CPCs, spheres-growing CSps showed the least correlation (0.91- 0.96), while monolayer-growing CPCs shared higher correlations among them (0.96- 0.98); (Figure 2c and Supplementary Figure 1). We performed a moderated t-test to evaluate significant differentially expressed genes between the individual samples (Supplementary Table 2). Out of the 13073 analyzed genes, we found only few genes differentially expressed in 5 out of 20 different monolayer-cultured CPC cell-lines comparisons (Supplementary Table 2a and 3). Only when CSps were compared with the other CPCs more differently expressed genes were identified (Supplementary Table 2b and 8). To further explore potential differences between CPCs, in spite of any statistical significant differences, individual scatter plots were generated for each monolayer-cultured CPC sub-condition and all the genes that displayed a 2-fold or more expression difference between different conditions were selected (Figure 3 and Supplementary Figure 2 and 3). These genes were used for a gene ontology (GO) analysis and classification by employing Panther Classification System (http://www.pantherdb.org; Applied Biosystems;) to further explore if clear gene clusters or biological processes could be identified. However, only for the CSps analysis, we selected statistical differentially regulated genes. GO analysis showed that all differentially expressed and selected genes are distributed over 17 different biological processes (Supplementary table 4 and 7), 11 different molecular functions (Supplementary Table 5 and 7), or 28 different protein classes (Supplementary Table 6 and 7). Among the biological processes, most genes were present in cellular and metabolic processes but with very little differences in total percentage of genes between the different CPC groups (Supplementary Table 4 and 7). Similar observations were evident for clustering on molecular functions, displaying the majority of the genes involved in binding processes and catalytic activities (Supplementary Table 5 and 7). Among the different protein classes, enzyme modulators, extracellular matrix proteins and adhesion molecules were the most prominent (Supplementary Table 6 and 7). GO analysis suggest that the different CPCs showed similar distribution of their most different genes, as percentage, in different biological processes, molecular functions and protein classes.

Since we are studying different progenitor populations from the heart, we further explored if we could identify differences in gene patterns between the different CPC populations, based on selected genes important for stem cell- maintenance, growth, and biology. In particular, we evaluated genes involved in the regulation of different stem cell pathways like TGF-β, Wnt, NFkB, p53, JAK/STAT, Notch and Hedgehog (Figure 4A), cell cycle (Figure 4B), stem cell transcription factors (Figure 4C), and growth factors, cytokines and chemokines (Figure 4D). Detailed heat map analysis showed again, however, a very similar profile among all samples, with small differences mainly related to individual donors and not to different cell types or conditions (Figure 4). Since CSps and monolayer growing CPCs have differently expressed patterns, we selected all the significantly differentially expressed genes that displayed a 2-fold or more difference and compared them with CDCs, and c-Kit and Sca-1+ CPCs monolayer-cultures (Supplementary Table 8). Ingenuity pathway analysis was used to generate Molecular Networks, representative of the identified genes (Supplementary Table 9-10-11 and Supplementary Figure 4-5-6). Ingenuity pathway analysis identified a gene network in CSps that is enriched in genes

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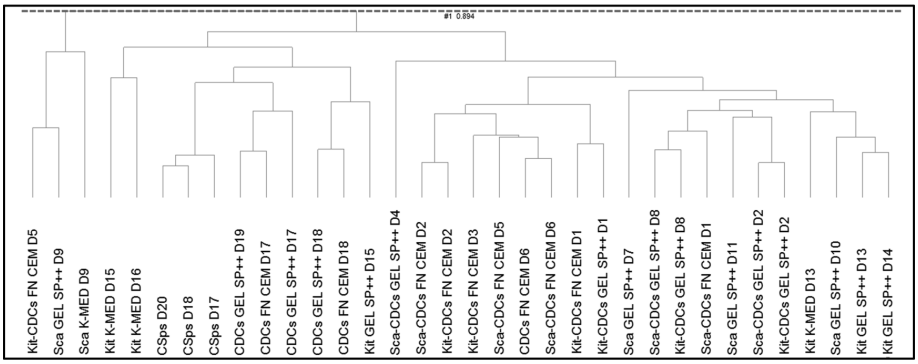

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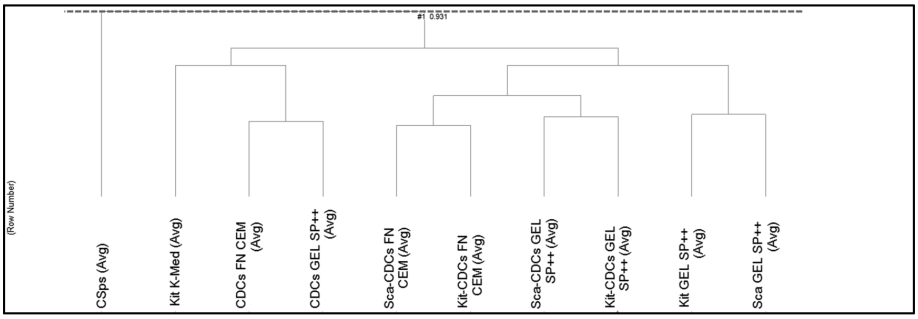

c

	Sca GEL SP++	Sca-CDCs GEL/SP++	Kit-CDCs GEL/SP++	Kit GEL/SP++	Kit K-Med	Sca-CDCs FN/CEM	Kit-CDCs FN/CEM	CDCs FN CEM	CDCs GEL SP++	CSps
Sca GEL SP++	-	0.976	0.974	0.986	0.951	0.966	0.98	0.956	0.953	0.912
Sca-CDCs GEL/SP++	0.976	-	0.987	0.968	0.959	0.98	0.98	0.962	0.96	0.923
Kit-CDCs GEL/SP++	0.974	0.987	-	0.967	0.954	0.981	0.984	0.966	0.958	0.92
Kit GEL/SP++	0.986	0.968	0.967	-	0.954	0.958	0.972	0.961	0.964	0.917
Kit K-Med	0.951	0.959	0.954	0.954	-	0.966	0.961	0.971	0.969	0.946
Sca-CDCs FN/CEM	0.966	0.98	0.981	0.958	0.966	-	0.988	0.978	0.962	0.925
Kit-CDCs FN/CEM	0.98	0.98	0.984	0.972	0.961	0.988	-	0.974	0.959	0.923
CDCs FN CEM	0.956	0.962	0.966	0.961	0.971	0.978	0.974	-	0.987	0.953
CDCs GEL SP++	0.953	0.96	0.958	0.964	0.969	0.962	0.959	0.987	-	0.961
CSps	0.912	0.923	0.92	0.917	0.946	0.925	0.923	0.953	0.961	-

Figure 2 Cluster dendogram and pair wise comparison of CPCs samples. (a) cluster dendogram of all CPCs samples; (b) cluster dendogram of averaged CPCs samples and (c) pair-wise comparison of averaged values. (a) n= 1 ; (b/c) n=4 for Kit/ Sca-CDCs FN CEM or Sca-GEL SP++; n=3 for Kit/ Sca -CDCs GEL SP++; n=2 for Kit GEL SP++; n=1 for Kit K-Med.

encoding for growth factor production and signaling molecules involved in the development of cardiac muscle, vasculogenesis and angiogenesis (Cardiovascular system development and function network, Supplementary Table 9a, 10a and 11c and Supplementary Figure 4a, 5a and 6c). Another identified gene network is Cellular Growth and Proliferation, particularly involved in embryonic development of the cardiac and vascular system (Supplementary Table 9b, 10c and 11a and Supplementary Figure 4b, 5c and 6a). These findings are in accordance with other previously published observations, in which was demonstrated that CSps have a more robust secretome and stem cell phenotype compared to other CPCs [12–14]. Additionally, our analysis also identified a gene network involved in the regulation of lipid metabolism and small molecule biochemistry and transport (Supplementary Table 9c, 10b and 11b and Supplementary Figure 4c, 5b and 6b).

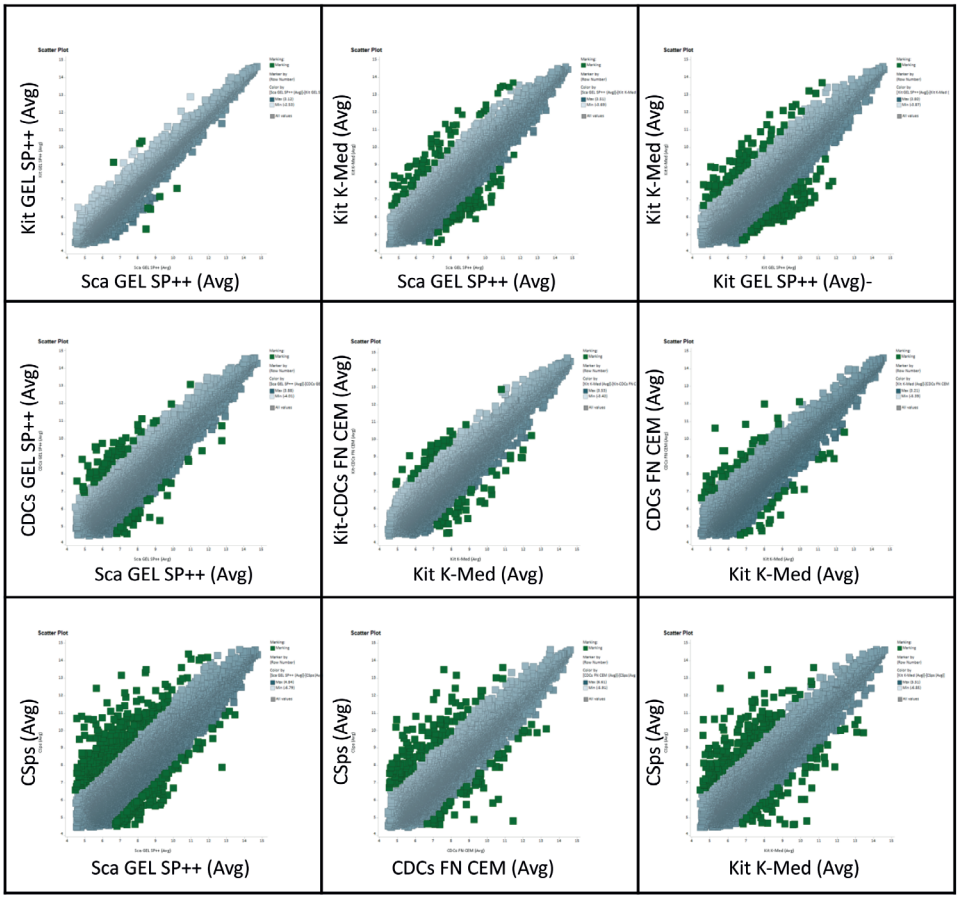

Figure 3 Scatter plot comparison of CPCs samples. (a) Scatter plot comparison of averaged CPCs cell lines; genes with ≥ 2 fold difference are in highlighted in green.

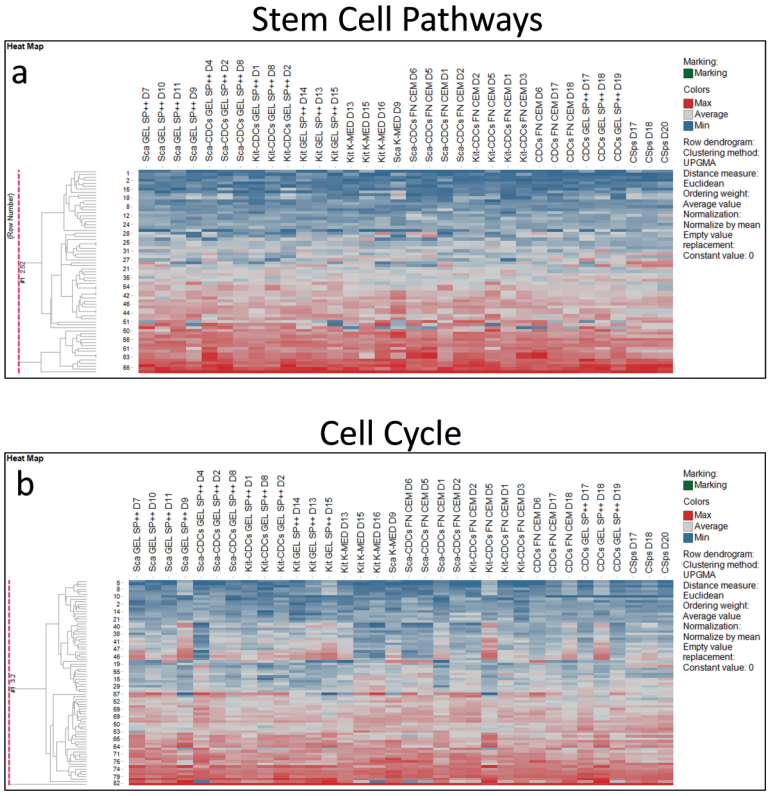

Figure 4 (a, b) Genome guided analysis of CPCs samples; Heat map of genes involved in (a) stem cell related pathways (TGF-ß, WNT, NFκB, p53, JAK/STAT, Notch, Hedgehog); (b) cell cycle and proliferation (G1 Phase and G1/S Transition; S Phase and DNA Replication; G2 Phase and G2/M Transition; M Phase; Cell Cycle Checkpoint and Arrest and Cell Cycle Regulators); (c) Stem cell transcription factors; (d) Growth Factors, Cytokines and Chemokines .

DISCUSSION

Taken together, our data suggest that human CPCs can be isolated from patient heart biopsies using different markers, such as c-kit or Sca-1- like, and alternative methodologies, via direct cell isolation or via explant culture, such as CSps and CDCs. For the first time, however, we showed that upon culture expansion, these cell populations are very similar at their gene expression level, even more pronounced when cultured in comparable culture conditions and even transcended by donor differences.

Although different methodologies can be applied to isolate these cell populations, many reports did report a co-expression of these markers *in vivo* or when isolated [15–17] .Co- expression of stem cell markers (c-Kit, MDR1 and Sca-1-like) within CPCs was demonstrated by several groups [15,17], suggesting that these cells co-exist in the adult myocardium [16]. Recently, Dey *et al.* isolated CPCs, based on different surface markers, from age and sex matched mouse hearts[12].

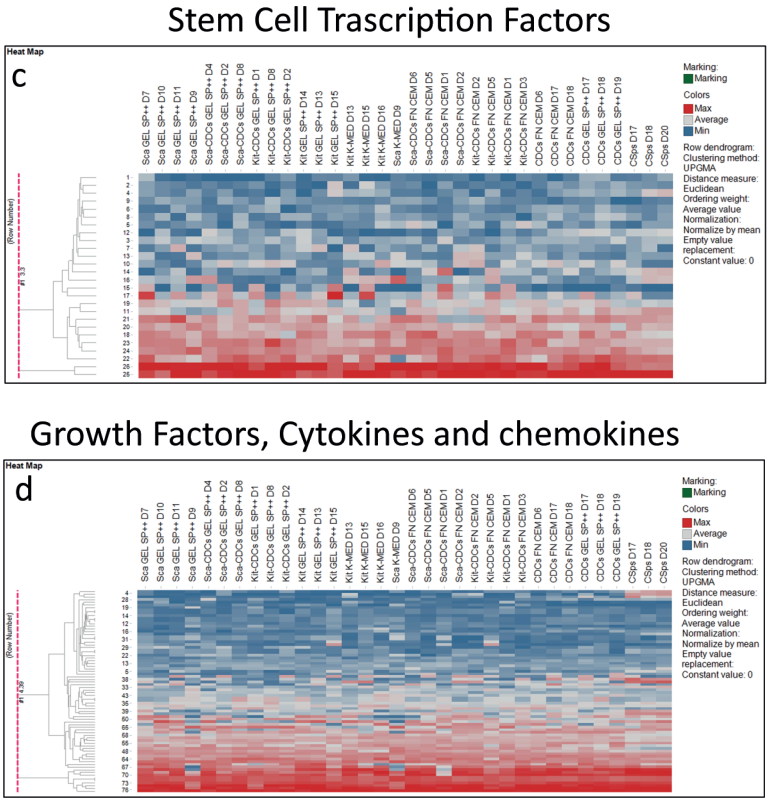

Figure 4 (c, d)

The authors showed that c-kit+, Sca-1+ and SP CPCs represent three progenitor cell populations at different stage of cardiac commitment and identified cKit+ cells as the most primitive population, and Sca-1+ as the most committed CPCs [12]. In our study, we did not observe differences between the different monolayer CPCs population upon culture propagation. A similar stage difference, however, might be present *in situ* in humans as well but lost upon culture expansion. We believe that the expression of these different stem cell markers and their co-expression probably represent different developmental and/or physiological stages of CPCs, rather than intrinsic different CPC populations. The needed proliferative culture conditions in which all the cells are exposed to environmental signals could minimize the possible *in vivo* physiological differences that might be present.

Among the different CPCs analyzed, CSps are the most different, probably due to the unselected cell populations that form CSps and their particular 3D culture structure and thereby different

interactions and growing conditions. The combination of mixed cell population and 3D culture conditions give CSps a stem cell niche-like structure, as it was demonstrated in a previous study [14,18], and might be responsible for the differences observed in our gene analysis. Surprisingly CDCs, which is a cell population derived from CSps, are more similar with other antigen selected CPCs rather than with CSps, confirming the idea that monolayer and high proliferative culture condition might play an important role in minimizing the differences among the different CPCs analyzed. For future translation for cardiac cell therapy with these cells, however, it remains crucial to obtain enough cell numbers. Moreover, from a clinical point of view, our results also suggest that we need to take into account the cell donor variability between patients and further study the correlation between CPC characteristics and e.g. the diseased status of a patient, such as chronic or acute heart disease, diabetes, or other co-morbidities [19].

Our findings are of fundamental importance to create more consensus among different scientists in the field of myocardial regenerative potential, and, together with other possible transplantation strategies such as tissue engineering, gene modification or pre-conditioning should further align approaches to improve the reported beneficial effects of cell therapy for heart disease by using cardiac derived progenitor cell populations.

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SUPPLEMENTARY

MATERIALS AND METHODS

Cell isolation and Culture

Human cardiac progenitor cells were isolated from anonymous adult human auricle samples from 20 different patients Standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht for surgical waist material were obtained. For direct c-Kit and Sca-1 isolation, the cardiac left auricles were cut in small pieces and enzymatically digested in 1 mg/ml collagenase A (Roche 103578) for 2 hours at 37°C. The digested tissue was placed in a 40μM cell strainer to obtain single cell suspension and CPCs were isolated by Magnetic cell sorting as described before [5,7].

Sca-1+ cells were plated at 0.1% gelatin coated wells in growth medium consisting of 25% EGM-2(3% EGM-2 single quotes (Cambrex, cat. no. CC-4176) in EBM-2 (Cambrex, cat. no. CC-3156)) and 75% M199 (BioWhittaker, cat. no. BE12- 119F), 10% FBS (Hyclone, cat. no. CH30160.30), 1x MEM non-essential amino acids (BioWhittaker, cat. no. BE13-114E) and 1x penicillin/streptomycin (Sigma, cat. no. P4458) [7].

c-Kit+ cells were cultured in HAM'S F12 (Fisher, SH3002601), 10% FBS (Fisher, cat. no. SH3040602), 0,2mM L-Glutatione (Sigma, cat. no. G6013), 5mU/ml human Erythropoietin (Sigma, cat. no. E5627), 10ng/ml basic FgF (peprotech, cat. no. 100-18B) and 1x penicillin/streptomycin (Sigma, cat. no. P4458) [10].

Alternatively, human auricle biopsy samples were cut in small pieces and cultured as explants in 4 mg/mL Fibronectin coated dishes in Complete Explant Medium (CEM) consisting of IMDM (Gibco, cat. no.21980), 20% FBS (Hyclone, cat. no. CH30160.30), and 1x penicillin/streptomycin (Sigma, cat. no. P4458). Cardiosphere were cultured in Cardiosphere-growth medium (CGM): 35% IMDM and 65% DMEM/F-12 Mix, 3.5% FBS, 1% penicillin–streptomycin, 1% l-glutamine, 0.1 mM 2-mercaptoethanol, 1 unit/mL Thrombin (Sigma cat. no. T4393), 2% B-27 supplement (Invitrogen cat no. 17504-044), 80 ng/mL bFGF (Peprotech cat. no. 100-18B), 25 ng/mL EGF (Peprotech cat. no. AF-100-15) and 4 ng/mL cardiotrophin-1 (Peprotech cat. no. 300-32). CDCs were cultured in CEM and Fibronectin coated flasks [11, 12].

c-Kit (Miltenyi cat no. 130-091-332) and Sca-1+ (Miltenyi cat no. 130-092-529) cells were subsequently isolated by MiniMACS Magnetic cell sorting (Miltenyi Biotech) and cultured as above.

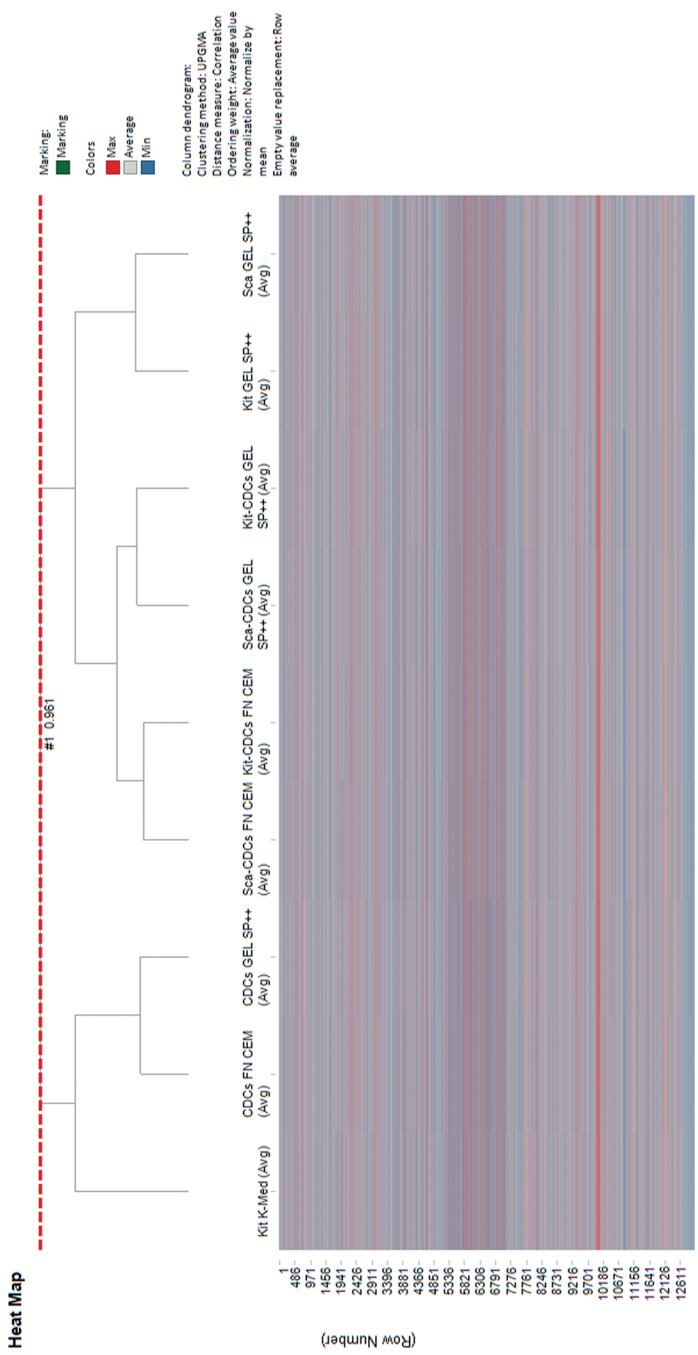
RNA isolation, quality control, and Microarray analysis:

Upon culture expansion, RNA was isolated by NucleoSpin RNAII column (Macherey-Nagel, cat no. 740955.250). RNA isolation was performed at passage 7 to 10 after isolation. The Quality control, RNA labeling, hybridization and data extraction were performed at ServiceXS B.V. (Leiden, The Netherlands). RNA concentration and purity was measured using the NanoDrop ND-1000 Spectrophotometer. Only samples with a 260/280 ratio between 1.8 and 2.1 were used for further analysis. RNA integrity was measured using the Agilent 2100 Bioanalyzer. After

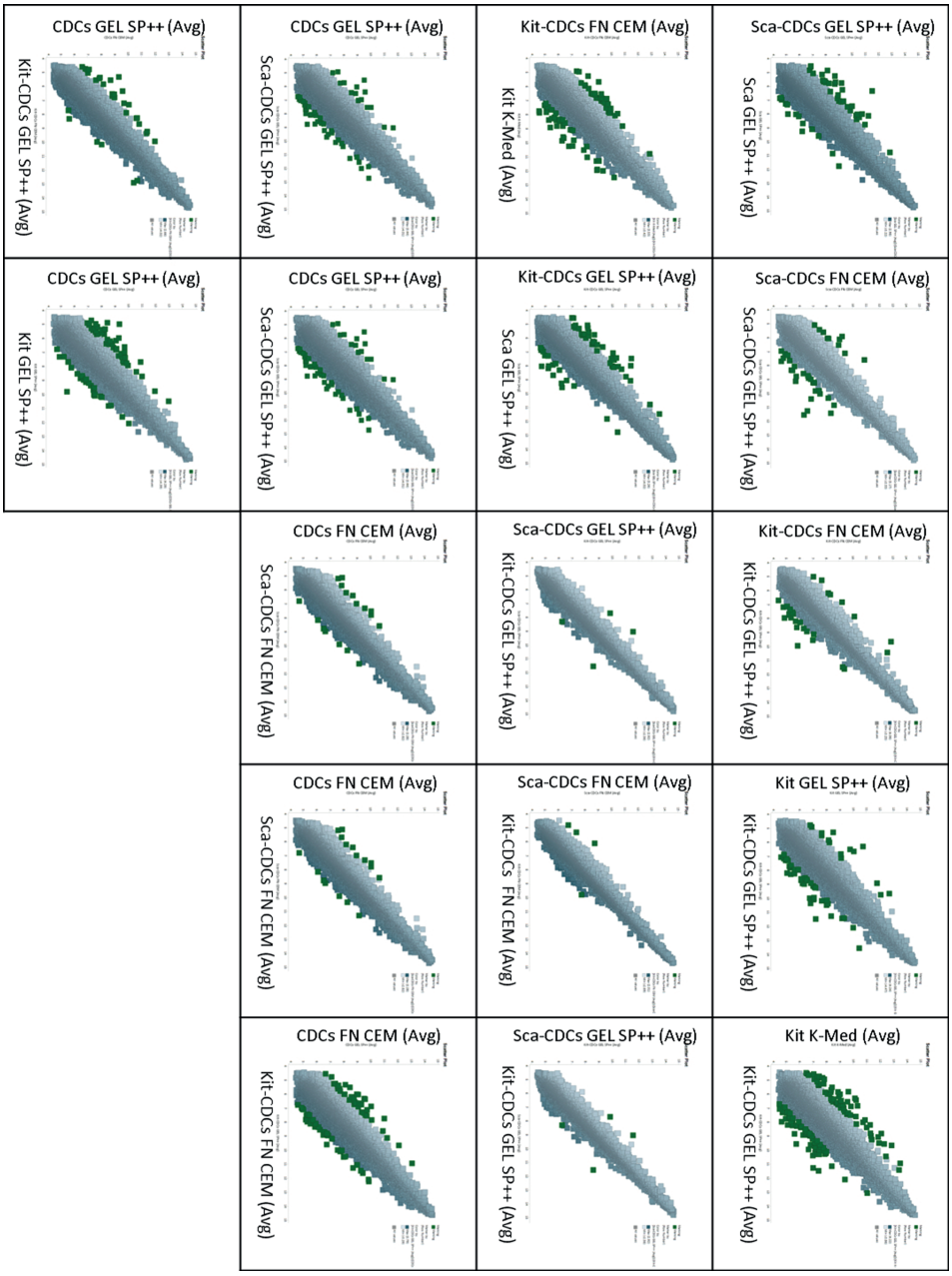
visual inspection of the Bioanalyzer electropherograms, only samples with a RNA Integrity Number (RIN) ≥ 9 were accepted for amplification and labeling process.
Amplification and labeling of the RNA samples was performed according to the manufacturer's specifications. For Illumina RNA profiling experiments, the Ambion® Illumina TotalPrep RNA Amplification Kit (Ambion, cat no. IL1791) was used. Labelled cRNA was generated according to the Illumina "whole-genome gene expression direct hybridization assay" protocol and hybridized on Illumina HumanHT12-v4 Beadchips. Fluorescence intensity data obtained from Illumina Beadstudio was processed using the "R" bioconductor with the "lumi" package using variance-stabilizing transformation within technical replicates and robust spline normalization to normalize between samples25. Quality control was performed on all microarrays to exclude technical errors and only genes that passed the detection call in >1 sample were included in the analysis.

Statistical analysis

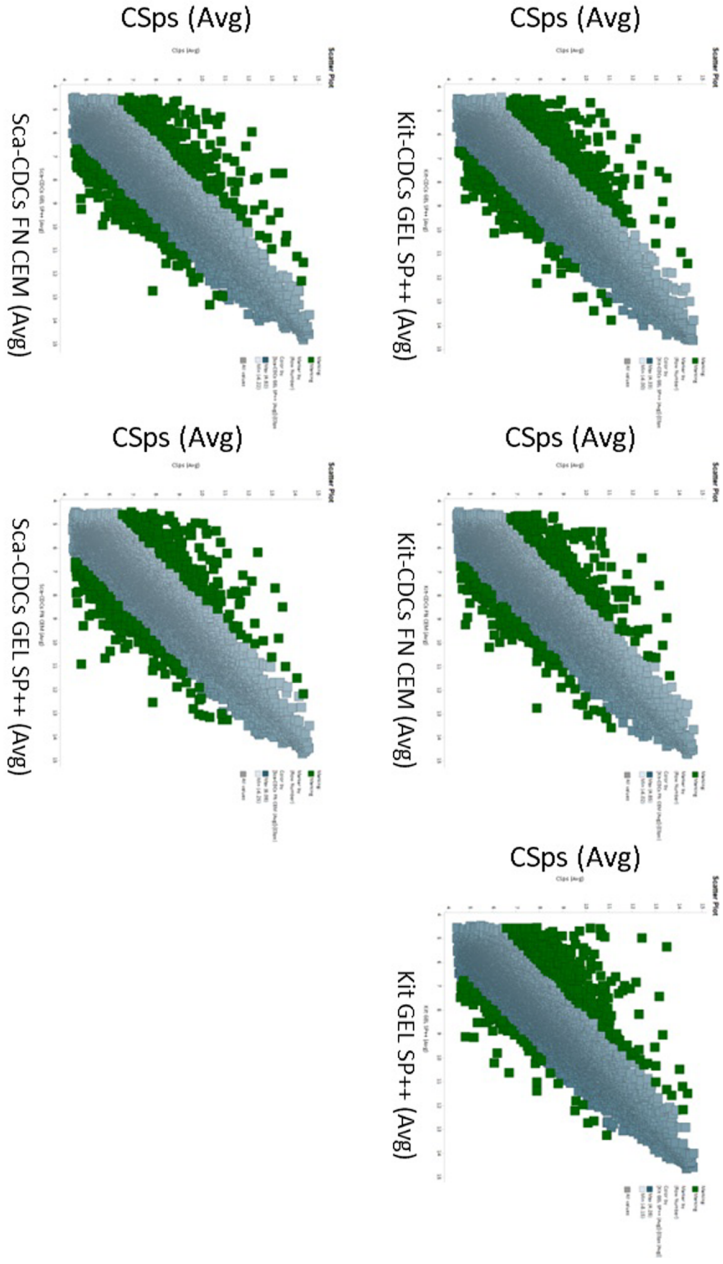
To identify differentially expressed genes, pairwise comparisons between progenitor cells obtained using different culturing methods were performed using the moderated T-test employed in the "limma" package [26]. The False Discovery Rate was controlled using the method by Benjamini and Hochberg [27]. Sample relationships were investigated by hierarchical agglomerative clustering using the unweighted pair group with arithmetic mean (UPGMA) algorithm, with sample correlation as distance measure. For cluster analysis within defined cellular processes, a similar method employing Euclidean distance was used.



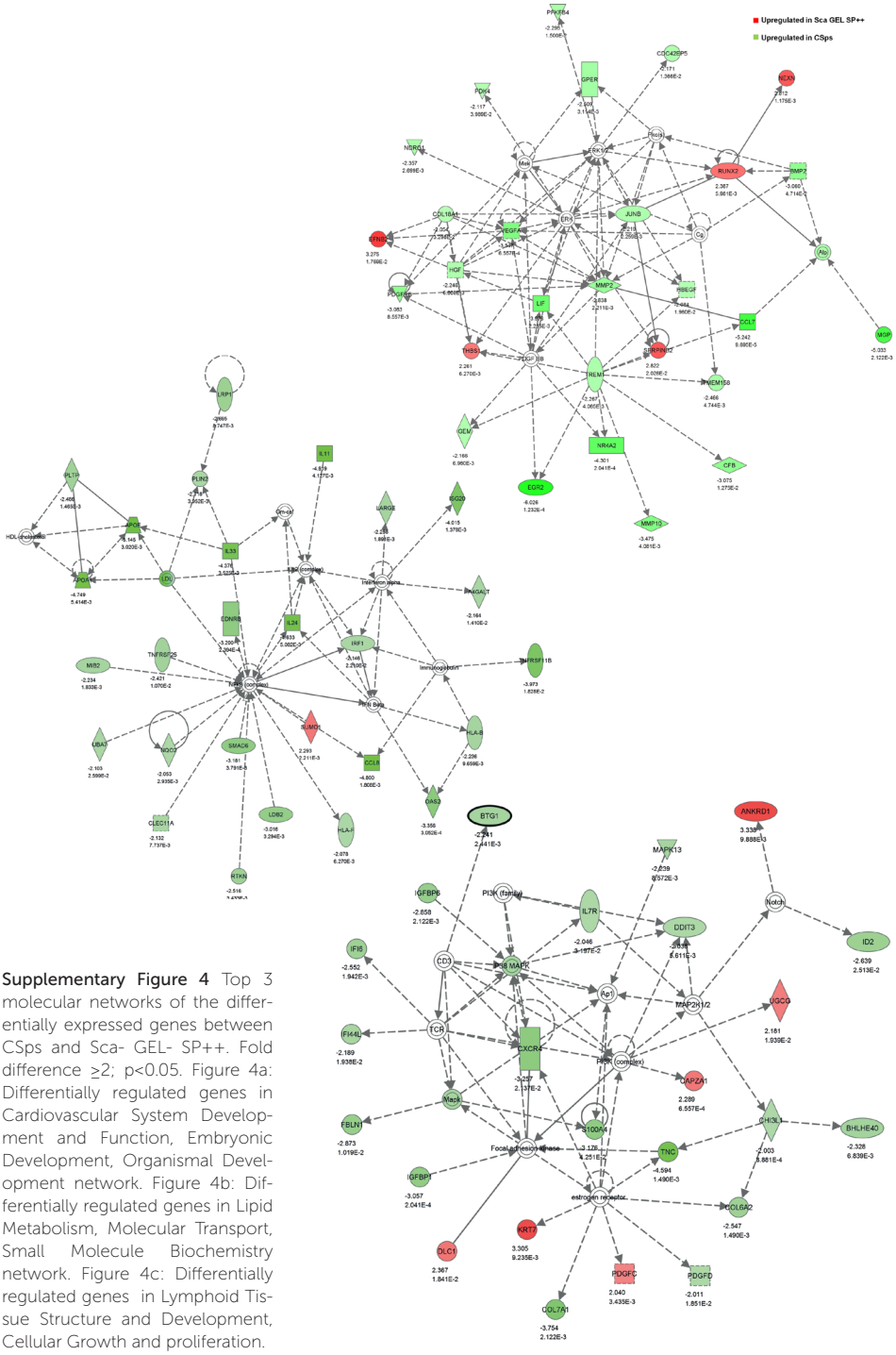
Supplementary Figure 1 Heat map analysis of monolayer growing CPCs.



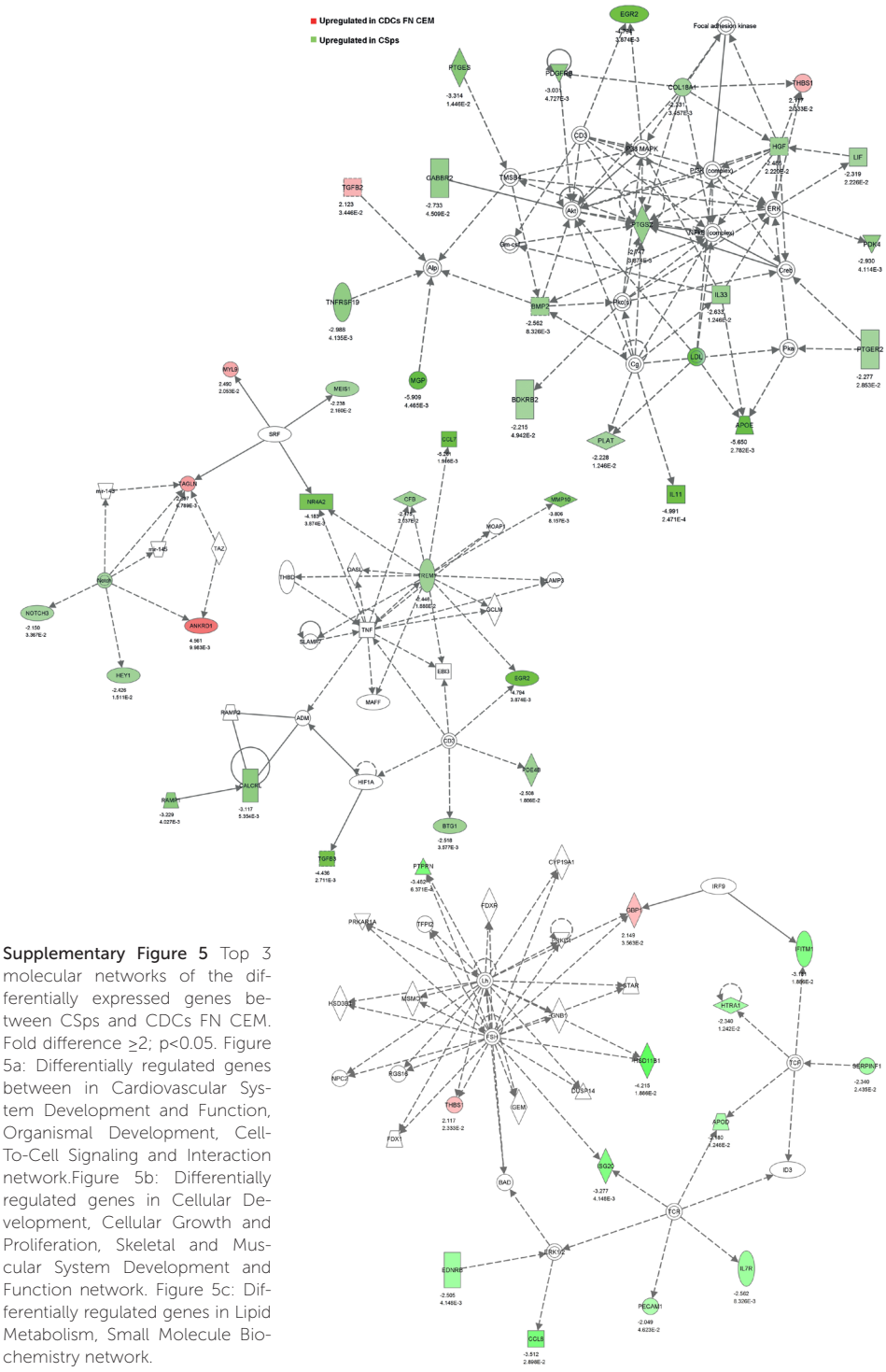
Supplementary Figure 2 Box plot comparison of averaged monolayer growing CPCs cell lines; genes with ≥ 2 fold difference are in highlighted in green.



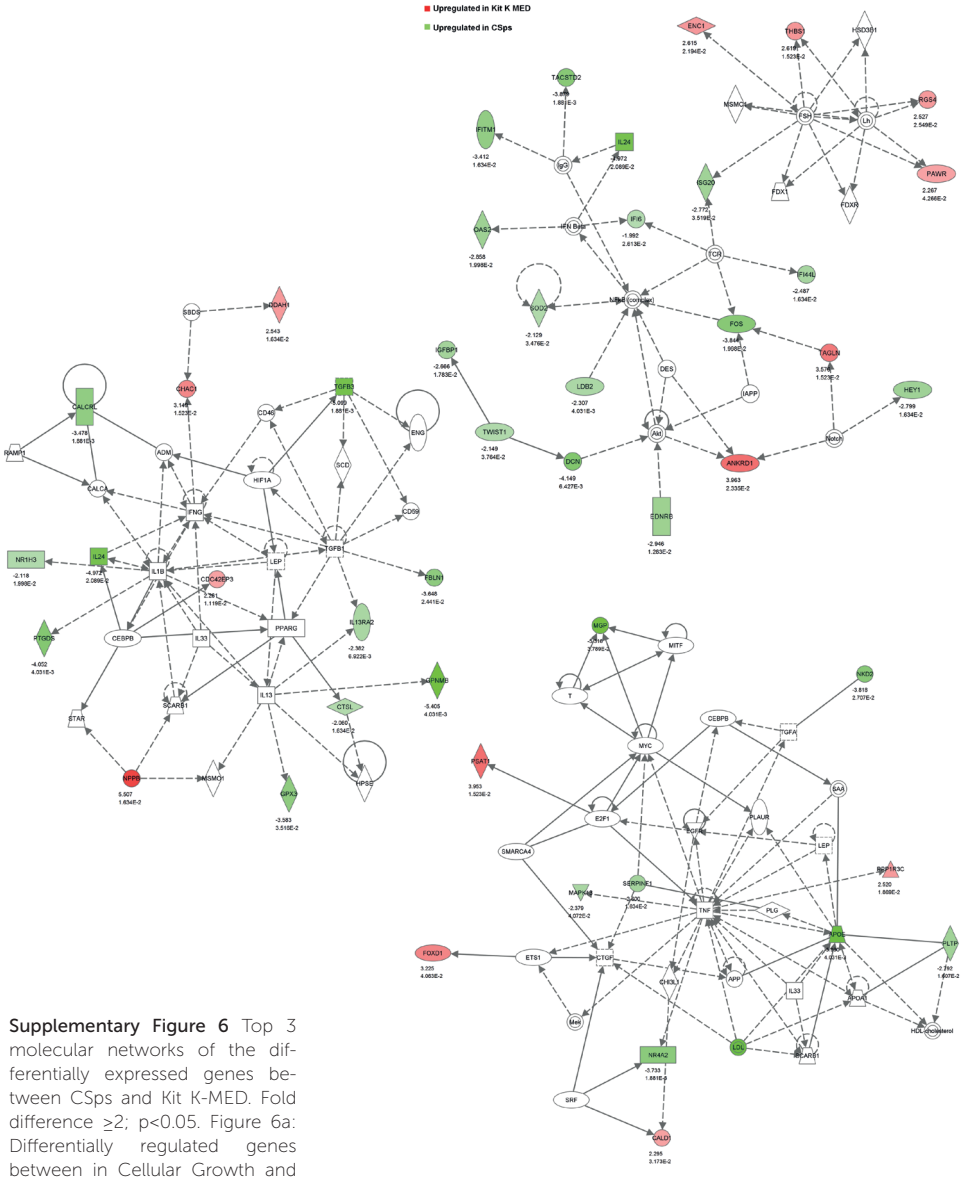
Supplementary Figure 3 Box plot comparison of CSps and monolayer growing CPCs cell lines; genes with ≥ 2 fold difference are in highlighted in green.



Supplementary Figure 4 Top 3 molecular networks of the differentially expressed genes between CSps and Sca- GEL- SP++. Fold difference ≥2; p<0.05. Figure 4a: Differentially regulated genes in Cardiovascular System Development and Function, Embryonic Development, Organismal Development network. Figure 4b: Differentially regulated genes in Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry network. Figure 4c: Differentially regulated genes in Lymphoid Tissue Structure and Development, Cellular Growth and proliferation.



Supplementary Figure 5 Top 3 molecular networks of the differentially expressed genes between CSps and CDCs FN CEM. Fold difference ≥2; p<0.05. Figure 5a: Differentially regulated genes between in Cardiovascular System Development and Function, Organismal Development, Cell-To-Cell Signaling and Interaction network.Figure 5b: Differentially regulated genes in Cellular Development, Cellular Growth and Proliferation, Skeletal and Muscular System Development and Function network. Figure 5c: Differentially regulated genes in Lipid Metabolism, Small Molecule Biochemistry network.



Supplementary Figure 6 Top 3 molecular networks of the differentially expressed genes between CSps and Kit K-MED. Fold difference ≥2; p<0.05. Figure 6a: Differentially regulated genes between in Cellular Growth and Proliferation, Carbohydrate Metabolism network. Figure 6b: Differentially regulated genes in Lipid Metabolism, Molecular Transport, and Small Molecule Biochemistry network. Figure 6c: Differentially regulated genes in Cardiovascular System Development and Function, Organismal Development, Tissue Morphology network.

Mesenchymal stromal cells to treat cardiovascular disease: Strategies to improve survival and therapeutic results

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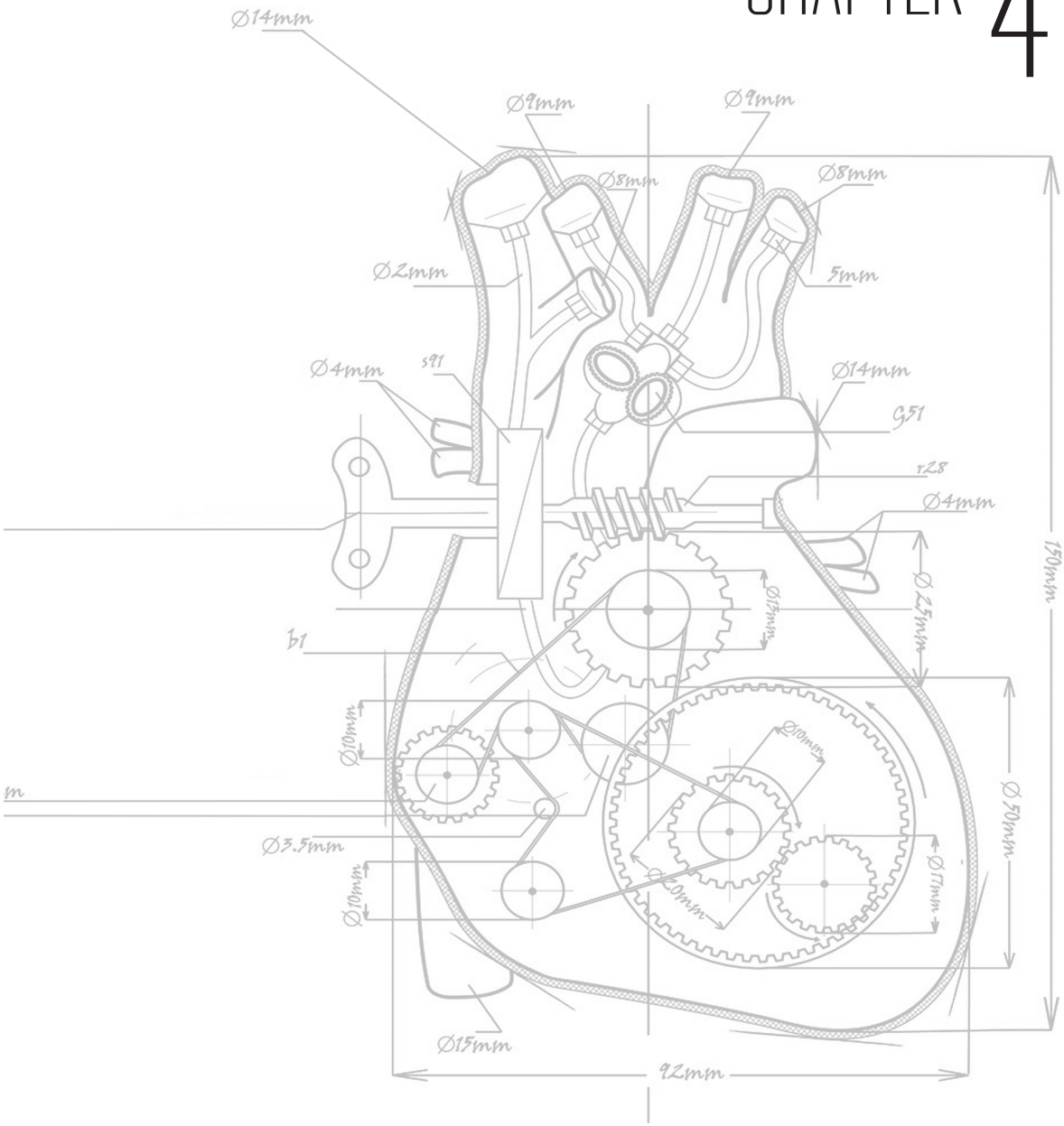
CHAPTER 4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ABSTRACT

Following myocardial infarction, damage due to ischemia potentially leads to heart failure. Stem cell transplantation has emerged as a potential treatment to repair the injured heart, due to the inherent characteristics of stem cells such as self-renewal, unlimited capacity for proliferation and ability to differentiate to various cell lineages. Most promising results have been reported thus far on mesenchymal stem cells (MSC). Following transplantation in the heart, stem cells are expected to (1) reduce the damage, (2) activate the endogenous regenerative potential of the heart, and (3) participate in the regeneration of the tissue. Until now, the results of intervention with stem cells in animals were promising, but clinical studies have failed to live up to those expectations. Current problems limiting the efficacy of cellular therapy are 1) limited knowledge on the time and mode of administration, 2) loss of homing receptors on culture-expanded cells as a consequence of the culture conditions, 3) massive cell death in the transplanted graft in the damaged heart, due to the hostile environment, 4) lack of knowledge on MSC behaviour in the heart. Since generally only 1–5% of delivered cells were found to actually engraft within the infarct zone, there is an urgent need for improvement. In animal models, strategies to precondition MSC before transplantation to survive in the damaged heart were applied successfully. These include exposure of cells to physical treatments (hypoxia and heat shock), pharmacological agents, "priming" of cells with growth factors, and genetic modification by over-expression of anti-apoptotic proteins, growth factors or pro-survival genes. To develop the strategy with maximal engraftment, survival and function of cells in the heart is the ultimate challenge for years to come.

INTRODUCTION

Ischemic heart disease is one of the leading causes of death worldwide [1]. During ischemia there is a shortage in supply of oxygen and nutrients to heart tissue, causing both necrosis and apoptosis of cells2. The dying cells in the heart are cardiomyocytes and non-myogenic cells, like vascular cells [2]. With the loss of contractile myocytes there is a reduction in cardiac function, increased production of fibrotic connective tissue, ultimately leading to the formation of rigid hypocellular scar tissue [3,4]. Consequently, this inevitably may lead in time to heart failure. Once heart failure is diagnosed, no curative therapy exists other than heart transplantation. The regular medication prescribed is a symptom suppressing treatment [5]. Therefore, a compelling clinical need exists for new cardiovascular therapies. For more than a decade, the ideal concept is to develop cellular therapy based on multipotent stem cells in order to replace dying cardiomyocytes and prevent further ischemic damage to the heart [6]. Although several stem/progenitor cell types have been studied in an effort to find the best source for cardiac regeneration, the debate is unresolved. Are embryonic stem cells needed (ESC), could 'adult stem cells' be used as an autologous source or is the induced pluripotent stem (iPS) cell the answer [7,8]?

Most stem cells reside in the bone marrow (BM), but after damage stem cells will be mobilized from the BM and migrate to the side of injury, including the heart [9]. Studies have shown mobilization of hematopoietic stem cells (HSC) and of endothelial progenitor cell (EPC). Mobilization of MSC is less clear since it might be that the numbers of mobilized cells fall below the detection limits [10,11]. Apart from migration to the heart, stem cells also reside in the heart. It has been suggested that the heart is not a terminally differentiated organ, but is capable of self-renewal, which makes cellular therapy more likely to be successful and therefore more appealing [12,13]. Thus the tissue specific stem cells in the heart are close to the injury site and will come into action for repair, however, these cells need to be adequately stimulated and afterwards replenished with cells from the BM9. Obviously, the endogenous repair potential of the heart is not sufficient in case of extensive damage. To what extent cellular therapy can initiate and stimulate the repair of impaired heart function is subject of study in several clinical trials [14,15]. It is still unclear whether adult stem cells, can differentiate into cardiomyocytes *in vivo*. Until now, the results of interventions with stem cells in mice experiments have been promising, while in human studies the results were usually below expectation [14].

CELLULAR THERAPY; CELLS, EFFECTS AND EXPECTATIONS

At first, skeletal muscle myoblasts were used for transplantation, demonstrating some improvements in cardiac function. However, a lack of electrochemical coupling and the possibility of uncontrolled arrhythmic events made myoblasts undesirable [16]. A lot of attention has been focused on embryonic stem cells (ESC), because of their pluripotent and unlimited self-renewal potential. However, complete differentiation of ESC is hard to induce *in vitro*, likely due to still unknown timing events in development. Undifferentiated ESC will develop teratomas.

In addition, the use of ESC is subject to ethical discussion. BM-derived stem cells were considered as candidates due to the fact that, after BM-transplantation, donor cells were found integrated in the host heart [17]. Although the nature of the regenerating cell was unclear, it stimulated several assays and clinical trials with BM-derived cell preparations [18]. Initially, the attention was focussed on the HSC from BM, which were found to differentiate into cardiomyocytes both *in vivo* and *in vitro*. As summarized in a review of Segers and Lee, the results until now of several clinical trials demonstrate marginal improvements, e.g. an increase of 8% in the ejection fraction in the most favourable case with BM-derived cells (TOPCARE-AMI) [14]. The longest follow-up in these trials was 18 months, indicating that little is known about long-term effects of these treatments [19]. In BM, other progenitor cells like MSC are present, but these frequencies are less than that of HSC (~ 3%). MSC are usually considered as a supporting cell for HSC in the BM. MSC can differentiate *in vitro* and *in vivo* into osteoblasts, chondrocytes, adipocytes and according to some studies to cardiomyocytes [20,21]. They produce many paracrine factors [22] which reduce inflammation, cell death and fibrosis [20]. Finally, BM also contains an endothelial progenitor cell (EPC). A variety of EPC were described in literature, which differ greatly in maturity, and this has raised a debate on their identity [23,24]. EPC are thought to induce angiogenesis via release of growth factors and cytokines. Thus, BM is a heterogeneous tissue, containing low percentages of stem/progenitor cells, and therefore it seems highly unlikely that the functional improvements in the heart following BM injections are due to stem cells.

Expansion of progenitor cells in culture was successful for MSC, and large numbers were cultured under good manufacturing practice (GMP) conditions for clinical applications. MSC therapy showed variable effects on cardiac function in patients after myocardial infarction [25,26]. Thus the question, "which cells are best to use?' is still unanswered, as well as the cellular mechanism responsible for the observed improvement in myocardial perfusion. A recent breakthrough in the reprogramming of cells, showed that somatic cells, such as fibroblasts, could be reprogrammed into induced pluripotent stem (iPS) cells, thereby creating cells indistinguishable from ESC at the epigenetic and functional levels [8,27]. In future, human iPS cells might be obtained and expanded and serve as 'embryonic stem'-like autologous adult cells for cell therapy.

Recently, stem cells were also discovered located in niches in the heart. These cardiac progenitor cells (CPC) were shown to differentiate into myocytes, smooth muscle and endothelial cells, both *in vitro* and *in vivo* [18, 28]. Due to their origin in the heart and differentiation potential, with a pre-directed cardiac fate, these cells are suggested to be the preferred cell type for cardiac regenerative therapy. However, several different isolation methods are described and no consensus on a surface marker for isolation and identification of this CPC population is defined, although the most well known are c-Kit and Sca-1. This further intensified the search for the most potent cell in the heart. Although the CPC show an impressive proliferation potential *in vitro*, relatively few endogenous CPC proliferate *in vivo* after ischemic damage [18]. This might be due to the cells themselves, which become senescent within the heart and do not receive the required signal [18].

The focus in this paper will be on MSC since these cells have already been applied clinically and the results make them valuable for cardiac regeneration especially when strategies are utilized to boost their survival in the post-infarct environment. To repair the ischemic heart, cellular therapy and in particular MSC-therapy is expected to: (1) reduce cardiac damage, (2) activate the endogenous regenerative potential of the heart, and (3) participate in the regeneration of the tissue. Cellular therapy with MSC in animal models have shown that these cells inhibited apoptosis, had immunomodulatory effects, stimulated angiogenesis, and reduced fibrosis and scar formation [29].

Reducing injury following ischemia

After an infarction, different phases can be recognized in the healing process [30,4]. First, due to deprivation of nutrients and oxygen, irreversible cell death and tissue necrosis is followed by an inflammatory response. Neutrophils are recruited to the infarct zone within 24h, followed by monocytes/macrophages, which will degrade extracellular matrix constituents, macromolecules released by injured cells, as well as necrotic cardiomyocytes. The wall of the infarcted ventricle is now highly susceptible for rupture. Then, after 5 days monocytes/macrophages and endothelial cells coordinately regulate angiogenesis and promote blood supply to the forming granulation tissue. Type I collagen, synthesized by myofibroblasts, strengthens the infarct to protect it against rupture. By 2–3 wk after MI, monocytes/macrophages disappear and granulation tissue matures into a scar [4]. In humans, scar maturation takes at least 8 weeks [30]. During this process, the healing heart undergoes profound changes in ventricular geometry and function. The combination of inflammation and adverse remodeling promotes heart failure.

MSC therapy has been performed in small and large animal models of MI [31-39]. The overall consensus is that paracrine factors produced by MSC are responsible for the improvements observed after MSC therapy. No convincing evidence of MSC differentiation into cardiomyocytes was shown [31], engrafted cell numbers are low, and the benefits of MSC therapy seemed to be a temporal effect [33]. Finally, the concept that paracrine factors produced by MSC were of significant importance resulted in a study in pigs in which MSC conditioned medium was used for the treatment of acute myocardial infarction [40]. In these experiments significant reductions in infarct size (60%) and marked improvement in systolic and diastolic cardiac performance were demonstrated upon reperfusion injury.

Anti-Apoptotic effect

Following a period of ischemia, many myocardial cells die by apoptosis or necrosis. It was suggested that the apoptotic cell is not inevitably committed to death, but in a meta-stable transition state, that might be reversible with appropriate therapy [41]. Cells go into apoptosis by "suicide;" that is, cells programmed to die would do so autonomously (intrinsic pathway). Apoptosis can also be mediated by an external trigger, such as Fas ligand (FasL) or tumor necrosis factor (TNF). Those factors induce apoptosis after binding to their receptors (extrinsic pathway) [42]. The Bcl-2 proteins form a family of proteins involved in the intrinsic pathway with anti- and pro-apoptotic members, i.e. Bcl-2 and Bax, respectively. MSC were found to

secret a great number of anti-apoptotic cytokines, such as VEGF, stem cell derived factor (SDF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF-1), and platelet-derived growth factor (PDGF) [31, 32, 43-45]. In several *in vivo* studies, it was shown that MSC transplantation resulted in a reduced number of apoptotic cardiomyocytes and/or smaller infarct size after MI as compared to PBS injected controls [31,-33, 35, 36]. In addition, after MSC transplantion the amount of cleaved-caspase-3 was decreased, and the ratio of Bcl-2/ Bax proteins increased in the infarcted heart as compared to the MI mice receiving PBS injection. This higher level of Bcl-2 expression represents an excess of anti-apoptotic proteins. Interestingly, Jiang *et al.* [33] demonstrated that the maximum reduction in number of apoptotic cardiomyocytes was not found when cells were transplanted 1hr after MI, but after 1 week. When MSC were transplanted later, i.e. at 2 wks after MI, this protective effect of MSC on cardiomyocytes was significantly decreased [33]. This data was confirmed by Hu and colleagues [46] and indicated that transplantation was most effective in the late phase after MI in which late inflammation, early angiogenesis and forming of granulation tissue occurred4.
More recently, MSC were found to be activated by apoptotic cells to secrete stanniocalcin-1 (STC-1). This protein reduced and inhibited apoptosis of UV-irradiated fibroblasts and lung cancer epithelial cells, respectively [47]. STC-1 is expressed in multiple organs suggesting that it might function in an autocrine and/or paracrine manner, whereas its localization in the heart and skeletal muscle suggested a role in myocyte function. It is thought that upregulation of STC-1 in the failing heart may be cardioprotective initially, because it reduces the ventricular workload. However, sustained upregulation might become maladaptive, because it could potentially reduce the ejection fraction [48].

Reducing inflammatory responses
During necrosis, cells disintegrate and the remaining debris will trigger an inflammatory response. The inflammatory response attracts many cells of the immune system, which damage more cells in the vicinity and generally results in the formation of scar tissue. MSC have been reported to regulate inflammatory responses, in particular their immunosuppressive effect on T cells have been clinically applied for the treatment of acute Graft-Versus-Host- Disease (GVHD) [49]. MSC are important immunoregulatory cells in the body, with additional effects on NK cells, B-cells, macrophages, monocytes, dendritic cells, neutrophils [50]. Their impact on inflammatory responses in the heart is not known yet. Recently, transplanted MSC were reported to block recruitment of lymphocytes and neutrophils into the injured lung [51]. It was shown that MSC protected its environment by blocking the production and/or activity of TNF-P and IL-1P. The interleukin 1 receptor antagonist (IL1RN) was described as the potential mediator, produced in high levels by a subpopulation of MSC51.

Decreased formation of fibrosis
The inflammatory phase of healing with resultant granulation tissue formation is followed by a fibrogenic phase that eventuates in scar tissue [52]. Scar tissue is a sturdy layer of cells and collagen, which does not contract. MSC transplanted in an animal model of MI resulted in decreased fibrosis in the infarcted heart as compared to PBS injected MI animals [32, 35, 53].

This was also noticed in other injured organs [54]. *In vitro* results from Ohnishi and colleagues [55] showed that MSC-conditioned medium upregulated anti-proliferation-related genes in cardiac fibroblast and downregulated their type I and III collagen expression. This leads to an inhibition in scar formation, improved compliance and elasticity of the infarct zone. According to Li and colleagues (2009) [53], MSC secrete anti-fibrotic factors such as adrenomedullin, responsible for the resulting decrease in myocardial fibrosis, and scar formation. Others studies reported that MSC stimulate cardiac fibroblast to produce MMPs [54,57] thereby indirectly reducing fibrosis, or that MSC themselves produce MMPs [43, 58].

Activation of the endogenous regenerative potential of the heart

It is essential to restore blood flow as soon as possible after a MI to supply injured tissue and surrounding cells of oxygen and nutrients, and stimulate blood vessel formation. Impaired perfusion will stimulate CPC and adult endothelial cells, that need to proliferate to contribute to the revascularisation of the heart [59]. Stem cells are nourished in niches, and elucidating the nature of the signals regulating the niche sheds new light on how to control, or more importantly, stimulate resident stem cells [60].

Stimulation of angiogenesis

Although it has been claimed that MSC can differentiate into endothelial cells the evidence has not been convincing [61,62]. The contribution of MSC towards angiogenesis has been described mostly as a result of trophic support, e.g. the secretion of growth factors like VEGF, HGF and bFGF, which contribute to angiogenesis and formation of collaterals [20, 63, 64]. Several groups showed in animal models that the significant reduction in vessel density after MI as compared to sham-operated animals, was significantly less after MSC treatment as compared to controls [31-33,37,38]. Benefits of MSC therapy, in relation to stimulating angiogenesis post-MI, could be obtained by injecting the cells in a restricted period, i.e. one week after MI [33].

CPC in niches in the heart

In recent years several subsets of cardiac stem/progenitor cells were described, each type carrying different markers. It is unknown yet which signals control the niches in which they reside in the heart, but stimulation of CPC in its niche might activate the endogenous repair potential of the heart. The stem and early lineage committed cells are found in niches in heart, similar to those found in the BM and the brain. They are structurally and functionally connected to cardiomyocytes and fibroblasts via connexins and cadherins [60]. Comparable with the supporting MSC in the bone marrow, do the supporting cells in the heart, i.e. myocytes and fibroblasts, regulate the CPC via cell-cell interaction. Therefore, by transplanting MSC in the injured heart, again a 'stem cell supporting' cell is available to maintain the 'stem cellpool' of the heart, offering a rationale for MSC therapy in the heart.

CPC will be activated after ischemia, which can be either acute or chronic, and then start to regenerate the heart. This regeneration usually takes in border zone, but was also found in remote areas [60]. However, the sheer number of lost cells in a myocardial infarction is high and the endogenous CPC cannot replenish them. In addition, it is likely that the tissue

destruction following ischemia not only affects the adult cardiomyocytes, but also the niches containing the CPC [65].

Participation in the regeneration of the tissue

Ideally, injected cells applied for therapeutic purposes should be able to replace lost cells and restore function of the tissue. The number of lost cardiomyocytes after an infarct has been calculated to be in the order of 1 billion cardiomyocytes [66]. The number of MSC reported to be injected in clinical trials varied between $3x10^6$ and $6x10^{10}$, of which only the later dose, i.e. the highest number of MSC, showed significant improvements in ejection fraction [25, 26]. Although the issue on cell numbers injected has not yet been addressed in larger clinical studies with BM cells, the results of a recent trial in acute infarction patients suggested a relation between dose of transplanted cells and functional outcome [67].

To determine whether MSC participate in the regeneration of heart tissue, studies paid attention to their potential to differentiate into cardiomyocytes, endothelial cells and smooth muscle cells. Their differentiation potential to adipocytes, chondrocytes and osteoblasts is inappropriate in the heart and could be detrimental [68]. This issue is not well-reported, but is important in an evaluation on safety and feasibility of MSC transplantation for cardiac therapies.

The *in vitro* cardiomyogenic potential of MSC as demonstrated by some investigators encouraged the expectations on their capacities to regenerate heart tissue *in vivo* [29, 69-71]. Cardiomyogenic differentiation of MSC ranged *in vitro* from no differentiation and proliferation of MSC72 to formation of spontaneously contracting cardiomyocytes [69, 70]. To determine myocardial differentiation of cells, their expression of cardiac markers, proteins and organisation were studied. Most convincing were the expression of the Z-band protein alpha-actenin, the beta-myosin heavy chain, cardiac troponin-T, and the presence of cytoplasmic myofilaments [69, 21, 73, 74]. Even though the presence of these and other proteins clearly indicates myocardial differentiation, true cardiomyocyte differentiation needs a sarcomeric organisation and contraction. Several others suggest that following injection *in vivo* in the heart, MSC would receive stimuli from the environment to mature which leads to electrical and mechanical activity. Several studies reported that the transplanted MSC differentiated to immature cardiomyocytes [75,76]. Others observed full differentiation [77,78] and formation of connections with native/host cardiomyocytes [79], or even a trilineage differentiation (smooth muscle cells, endothelial cells and cardiomyocytes) [80]. Also Nagaya and colleagures [32] showed in rats, that engrafted MSC were positive for cardiac markers: desmin, cardiac troponin T, and connexin43, but some MSC were positive for von Willebrand factor and formed vascular structures. Dai *et al.* [34] showed that allogeneic rat MSC survived in infarcted myocardium as long as 6 months and expressed markers that suggest muscle and endothelium phenotypes. In their study MSC improved global LV function already after 4 weeks; therefore they suggested a possible early paracrine effect of the transplanted MSC. Evidence for *in vivo* differentiation of human MSC came from studies in small animals of xenogeneic transplantation [81, 82]. Although a lot of effort has been put into the study of cardiomyogenic differentiation of MSC, it is clear that for true regeneration the number of incorporated MSC as demonstrated in these animal studies are too low.

CURRENT PROBLEMS DIMINISH RESULTS OF CELLULAR THERAPY

In addition to the choice of which cell type is the best for transplantation therapy, current problems with regard to an effective cell-based therapy in cardiovascular disease are fourfold:
1) The first problem concerns the time and mode of administration. Only few experimental and clinical studies addressed the optimal timing of cell injections after an acute MI [33,46,83,84]. Animal studies and clinical trials showed that late treatment (4-8 days) was more effective in left ventricular recovery compared to early treatment (up to 4 days). In models of chronic ischemia, cell injection was found encouraging, and perfusion was improved [85]. These time-related questions need further attention. Cells can be delivered via different routes: intravenous, intracoronary or intramyocardial. Even the most effective manner, intramyocardial injection, showed that up to 40% of cells were lost due to leaking out of the injection site entering into the circulation [86]. Terrovitis and colleagues showed that retention of epicardial myocardial injected stem cells could be significantly improved by applying fibrin glue on top of the injection site thereby providing a seal87. Also the use of an injectable scaffold, as a delivery vehicle for cells, improved cell retention [88,89].
2) A second problem in cellular therapy is massive cell death of the transplanted graft. The hostile environment, in which cells are injected, deprived of oxygen and nutrients, with inflammation, and no matrix survival signals and cell–cell interactions, ultimately leads to cell death. Injected cells are not able to adapt rapidly to the stress and at the same time integrate in the dying tissue [86,90]. Toma *et al.*81 estimated that more than 99% of human MSC had died at day 4 after transplantation. It is generally accepted that 1–5% of delivered cells actually engraft within the injected region [90,91].
3) A third problem is related to loss in homing receptors on culture-expanded cells. Blau and colleagues showed that a loss in homing receptors on satellite cells was prevented for a large extent when the cells were cultured on extracellular matrix like hydrogel instead of directly on plastic [92]. This might be important for the homing and engraftment into the heart of other cultured cells like MSC as well.
4) Lastly, due to a lack of knowledge on MSC behavior in the heart, it is unknown how the cells regulate their beneficial effects. Some researchers, like Torella *et al.*, argue that the mechanistic actions of MSC need to be elucidated before further progressing clinical trials [93].

PRECONDITIONING STRATEGIES

The problem of massive cell death, has resulted into the development of *in vitro* preconditioning strategies to enhance survival of injected cells in the damaged heart. These include exposure of cells to physical treatments, like hypoxia and heat shock, treatment with pharmacological agents, "priming" of cells with growth factors, and genetic modification by overexpression of anti-apoptotic proteins, growth factors or pro-survival genes [66, 94]. Since cell death is a multi-factorial phenomenon, addressing one single factor responsible for cell death may not be an effective strategy. Next to that, some of the pre-treatments seem to activate similar signalling

pathways in the cell, for example the growth factor IGF and hypoxia, were found to activate the pro-survival gene Akt95. Overall, most of these preconditioning strategies improved the survival rates of treated cells *in vitro*, and when administered to the post-MI heart, this resulted in an enhanced functional improvement as compared to controls [78]. The combination of several preconditioning strategies demonstrated impressive additional effects *in vivo* [96].

Physical Treatment

After being harvested, MSC are cultured under normoxic conditions (20% O2). It is generally assumed that normoxy is beneficial for the viability and proliferation of MSC. However, in their physiological niche MSC are exposed to relative hypoxia (2-7% O2), and after injection into ischemic tissue severe ischemia (0.4-2.3% O2) is present [97]. This sudden drop in oxygen causes MSC to rapidly go into apoptosis. However, preconditioning of MSC in a less severe form of hypoxia (1-3% O2) can prepare them for the hypoxia-related stress, and thereby stimulate their survival97. There have been reports of MSC withstanding severe hypoxia (pO2 < 1%) for 48 hours [98]. Interestingly, MSC viability and proliferation is not decreased upon long-term hypoxia (16h); instead, this process increased MSC migration towards ischemic tissue.

The effects of hypoxia are balanced by upregulation of various pro-survival systems. One of these is hypoxia inducible factor, HIF-1alpha. This transcription factor, in turn, stimulates an increase in p38 mitogen– activated protein kinase (p38MAPK) activity and phosphorylation of Akt [97,98]. At the same time, there is an upregulation in homing-signal receptors, such as c-Met, CX3CR1, and CXCR498. In fact, it is postulated that high level of CXCR4 expression commonly found in BM stem cells is partly due to the relative low oxygen tension in their niches [99, 100]. CXCR4 can specifically bind to SDF-1 and is expressed on lymphocytes, as well as various progenitor and stem cells. MSC expressing high levels of CXCR4, are thought to mobilize and home towards the ischemic myocardium, in response to high levels of cardiac SDF-1 secretion. This chemotactic activity of cardiac SDF-1 has been demonstrated for cardiac progenitor cells [101]. SDF-1 activities include induction of motility and homing, chemotactic responses, expression of adhesion molecules, secretion of MMPs and angiogenic factors, such as VEGF102. In addition, cell proliferation and survival are modulated as well by rapidly activating the PI3k-Akt-NFkB pathway. By targeting the HIF-1alpha gene this will result in an upregulation of gluconeogenesis in the cell, thereby managing survival under both hypoxia and serum-deprivation [103]. Additionally, HIF-1alpha induced a variety of growth factors, including VEGF, offering the benefits of increased angiogenesis [103]. Heat shock is known to protect various aspects of cell metabolism and function. Research with skeletal muscle cells showed that hyperthermia of 42°C over a period of 1h protected the cells against apoptosis [104]. Furthermore, skeletal myoblasts that received this heat shock treatment prior to transplantation, doubled cell survival in the heart at all time points examined after administration into the coronary artery. Similar results have been observed with embryoid body–derived human cardiomyocytes [105]. The heat shock treated cells showed a fourfold increase in graft size when directly injected into the uninjured rat hearts. Yet survival rates *in vivo* are not available for hyperthermically treated MSC. Recent studies [106,107] showed *in vitro* that when MSC were heat-shocked at 43°C for 45 minutes, only mild transient morphological changes of the

cytosol occurred, with no alteration in expression of CD44, CD90, and CD105.
Heat shock proteins (Hsp) are synthesized as a response to various damaging stimuli, including temperature fluctuations [108]. In mammalian cells, the stress response involves the upregulation of five major groups of Hsp, namely Hsp27, Hsp60, Hsp70, Hsp90, and Hsp104. They exert their effect in the anti-apoptotic pathways as chaperones, by preventing the aggregation and promoting the folding of crucial pro-survival proteins. For example, Hsp70 interacts with BAG-1, an anti-apoptotic protein which binds to Bcl-2 to promote cytoprotection. This interaction plays a central role in Bcl-2 activation in a stress induced environment108. Hsp20, on the other hand, associates with Akt1 and protects its kinase activity from heat stress and serum deprivation [109]. Akt1 is a serine threonine kinase, and provides a strong survival signal in many pathways related to apoptosis and cell proliferation. It inhibits several pro-apoptosis molecules, such as Bad, Caspase–9, Forkhead transcription factors, IKK-alpha, GSK-3 and is even thought to negatively influence p21 and p53. In addition, it stimulates NF-kB nuclear translocation, leading to the upregualtion of pro-survival genes such as Bcl-xL and caspase inhibitors [110].

Pharmacological agents

Various pharmacological entities can help protect MSC in the harmful environment of the post infarct heart. Statins, inhibitors of HMG-CoA reductase, are used in the clinical setting for their cholesterol-lowering function, but can also exert a variety of other biological functions such as anti-oxidant effects, protection of endothelial function, and anti- inflammatory action [111]. These drugs have provided beneficial effects when used after an acute myocardial infarction (AMI) and reperfusion, by improving survival and cardiac function, while decreasing cardiac hypertrophy and pulmonary edema [112]. Although statins have a limited effect on angiogenesis, the administration prior to AMI revealed a decrease in the area of risk. It suggests that the beneficial role of statins may come from an improvement of the local cardiac environment. Research on smooth muscle cells and cardiomyocytes has revealed that the observed improvement is linked to the anti-apoptotic mechanisms of statins. In MSC stimulated by hypoxia and serum depravation, the addition of lovastatin rescued the cells from apoptosis through inhibition of the mitochondrial apoptotic pathway [111]. They noticed increased phosphorylation of Akt and ERK1/2 in MSC treated with lovastatin. Other studies revealed similar findings on pro-survival signals upon statin treatment, such as the work done by the group of Yang *et al.*[113]. In combination therapies, using MSC transplantation and administration of simvastatin or atorvastatin in a miniswine infarct model, they enhanced the survival and myogenesis of implanted MSC. Other chemical entities, such as the anti-oxidant Berberine114 and anti-anginal drug Trimethoxybenzyl (TMZ) [113, 115], have also been used to aid the survival of MSC under stress conditions. Both treatments significantly attenuated hypoxia induced apoptosis *in vitro*, through a PI3K/Akt-dependent pathway. Transplantation of MSC pre-treated with TMZ showed a significant increase in the recovery of cardiac function and decrease in fibrosis in a rat infarct model. This process seemed to be mediated through the upregulation of pAkt and Bcl-2.

Growth factor priming

VEGF added to the medium in which murine MSC were cultured, resulted in an improvement in viability and reduced senescence in high passaged MSC (P10) [116]. This was measured by a decrease in MSC expression of p21 and p19ARF, but not by expression of p16INK. p16INK, p21 and p19ARF are cell cycle inhibitors and cellular aging markers, and an increase in expression relate to a decrease in both growth and function [117]. As found in the same study, MSC cultured with VEGF showed activation of the pro-survival factor, Akt. Akt signaling mechanisms are linked to the pro-survival factor, Bcl-xL, and the pro-apoptotic factor, Bax. Testing for these factors in MSC cultured with VEGF, showed increased and decreased expression of Bcl-xL and Bax, respectively [116]. *In vivo*, co-injection of MSCs from EGFP transgenic mice and VEGF peptide immediately after MI showed an increased MSC survival at 1 and 4 weeks in combination with an improved cardiac function. However, in these *in vivo* experiments an indirect effect of VEGF, i.e. reducing the hostile environment of the damaged heart, might improve the survival of cells as well. Consequently, it is not obvious whether the *in vivo* results are due to VEGF-preconditioning of MSC or direct VEGF effects [116]. TGF-P expression by macrophages during wound healing has shown proliferative induction and anti-apoptotic effects. Herrmann and colleagues [118] used this as a rationale to treat MSC with TGF-P. *In vitro*, TGF-P was found to significantly increase the production of VEGF by MSC and, to an even greater extent when a combination of TNF-P or hypoxia was used. TGF-P preconditioning improved MSC-mediated postischemic myocardial functional recovery as demonstrated in isolated rat hearts.

To improve efficacy of MSC transplantation has been performed to induce cardiomyogenic differentiation of MSC. Hahn *et al.* [118] showed in rats that a combination of fibroblast growth factor (FGF)-2, IGF-1, and bone morphogenetic protein (BMP)-2 enhanced *in vitro* the expression of cardiac transcription factors and improved survival of MSC under hypoxia. This induction of cardiac specific markers only occurred if GF-primed MSC were cocultured with cardiomyocytes, during which gap junctional communication developed between MSC and cardiomyocytes. According to Hahn and colleagues [119], this coupling through gap junctions might be particularly beneficial *in vivo* and explain their finding of improvements in LV systolic function after transplantation of primed MSC in a chronic rat model of myocardial ischemia. However, it is not clear from this study how many MSC survived transplantation, and how this is increased by "priming". The fact that cytoprotection by MSC is not only mediated by paracrine factors but also by direct cell-to-cell communication through gap junctions, is a new and important message [119].

Another effect described for IGF-1 stimulation of MSC, was an increase in the expression levels of chemokine receptor CXCR4 [120], thereby increasing the migration response of MSC towards SDF-1. Since SDF-1 will be produced at the site of injury, an increase in homing capacity of cells will improve their effectiveness *in vivo* to migrate to the site of injury. GF 'priming' will induce transient changes in MSC, and this will be reversed after *in vivo* transplantation in the body. A solution for retaining these changes induced in MSC by GF was found in their genetic modification for self-supply of certain growth factors.

Genetic manipulation

Heme oxygenase-1 (HO-1), an anti-apoptotic and anti-oxidant enzyme, possesses potent cytoprotective activity in an ischemic environment. To enhance MSC resistance to ischemia, MSC have been modified with hypoxia-regulated HO-1 gene expression [121]. OH-1-MSC survival was found to be increased *in vitro* under hypoxic conditions as compared to control MSC, as illustrated by a 2-fold reduction in the number of TUNEL positive cells. The incorporation of HO-1 also increased the survival *in vivo* of MSC 5-fold, 7 days after implantation in infarcted mouse hearts.

Some studies have also revealed that upregulation of heat shock proteins (Hsp), similarly to a heat shock treatment, can have beneficial effects on MSC survival in the infarcted heart. Wang and colleagues [122] used Hsp20, which they inserted transiently into rat MSC via an adenovirus. These cells, Hsp20-MSC, showed increased survival under oxidative stress conditions *in vitro*, and improved cardiac function *in vivo* compared to control cells. Furthermore, increased secretion of VEGF, FGF-2, and IGF-1 was noticed. Therefore, the conditioned medium of Hsp20-MSC offered protection in co-cultures with adult cardiomyocytes in a hypoxic environment. Chang *et al.* [122] demonstrated the beneficial effect of Hsp 70, by fusing the protein to a Protein Transduction Domain (PTD). PTD has the ability to very efficiently transport bound proteins into eukaryotic cells both *in vitro* and *in vivo*. By delivering a PTD-Hsp70 construct into MSC *in vitro*, the cells displayed higher viability in hypoxic conditions, as can be seen from increased Bcl2 expression and a decrease in pJNK and caspase-3 activity. *In vivo*, the PTDHsp70- MSC led to a decrease in TUNEL-positive myocardial cells and fibrosis, while increasing angiogenesis and cardiac function.

The anti-apoptotic pathways have also been directly targeted by genetic manipulation. Several groups [123,124] genetically modified MSC by overexpressing the pro-survival gene Akt1. When exposed to hypoxia and serum deprivation, the Akt-MSCs showed higher viability *in vitro*, which manifested in an 80% reduction of apoptosis at 24h. When transplanted into the hearts of rats 1h after coronary ligation, almost 3 times more Akt-MSC than control-MSC were found to be present 24h after injection, containing more viable cells. Furthermore, transplanted Akt-MSC reduced inflammation, collagen deposition and cardiomyocyte hypertrophy, while restricting the loss of myocardial volume up to 90% [123, 124]. Whereas Gnecchi *et al.*[124] found the effects of Akt-MSC to be due to paracrine signalling, Mangi and colleagues [123] noted that MSC either disappeared within two weeks, or turned into cardiac myocyte-like cells in the infarct zone, forming connections with native cardiomyocytes, thereby indicating a strong electromechanical coupling. One explanation for the effect of Akt-MSC is the production of secreted frizzled related protein 2 (Sfrp2) [125, 126]. Sfrps are secreted glycoprotein molecules that structurally resemble cell surface frizzled receptors but lack the transmembrane domain. The binding of Wnt3a to the frizzled receptors induced apoptosis via the canonical pathway through the activation of various caspases [126]. By competing with this receptor, Sfrp2 sequesters Wnt3a, thereby preventing the activation of the caspases. In fact, *in vitro* studies demonstrated that cells exposed to sfrp2 showed a decrease in apoptotic activity after exposure to hypoxia and subsequent reoxygenation [126].

Several groups have genetically modified MSC to stimulate homing to the heart [77, 127]. CXCR4, for example, was overexpressed in MSC via retroviral transduction. Although proliferation was only slightly increased [127], a significant increase in homing to the infarct zone was found77,127. This resulted in beneficial effects by reducing wall thinning, generating smaller infarcts, and preservation of left ventricular function and fractional shortening. In addition, transplanted CXCR4-MSC as well as control-MSC seemed to have an effect on the collagen composition of the scar, favouring collagen III over collagen I [127]. Overexpressing the CXCR4 ligand, SDF-1, in MSC and administrating these cells via tail vein led to 3.5 fold improvement of cardiac function as compared to control-MSC injection. This beneficial effect was mediated not through cardiac regeneration, but rather cardiac preservation. Between 24h and 48h after AMI, an 80% reduction was observed in cardiomyocyte apoptosis in the border zone. Furthermore, SDF-1 overexpression in MSC also resulted in increased neovascularisation of the infarct zone [128]. Since several other groups demonstrated that late MSC treatment (4-8 days) was more effective for functional improvements in the heart, than early treatment (up to 4 days), it is tempting to speculate on an even higher potential of SDF-1- MSC if transplanted late.

CONCLUSIONS

MSC are an attractive adult-derived cell population for cardiovascular repair. A significant amount of work is being conducted in animal models, and even clinical studies have been started. Differences in isolating, culturing, and administration of cells among studies makes it difficult to compare, and therefore questions relating to cell type, cell dose, mode of administration, timing and number of treatments continues to exists. However, to realize the true potential of MSC therapy for clinical application these issues will have to be solved. As became clear, paracrine activities of MSC *in vivo* explains many of the observed effects, i.e. anti-apoptotic, reduced inflammation, and decreased fibrosis. The stimulation of angiogenesis following MSC transplantation supports their role in the activation of the endogenous regenerative potential of the heart. The reported finding *in vivo* of direct cell-to-cell communication between MSC and CPC through gap junctions is an exiting message. Active participation of MSC in regeneration of tissue is highly controversial.

After transplantation, massive cell death in the graft will diminish the efficacy of cellular therapy. In several preconditioning strategies, a significant improvement of cell survival was obtained, *in vitro* as well as *in vivo*. Since cell death is a multi-factorial phenomenon, targeting one pro-apoptotic factor might not be sufficient. Instead, simultaneous targeting of multiple pro-survival factors, in a sort of "pro-survival cocktail", might be a more effective strategy. Furthermore, tackling, not only the aspects related to cells as described in this paper, but also related to their environment, will enhance engraftment of transplanted cells. To maximize engraftment, survival and function of stem cells in the heart is the challenge for coming years.

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Increasing short-term cardiomyocyte progenitor cell (CMPC) survival by Necrostatin-1 did not further preserve cardiac function

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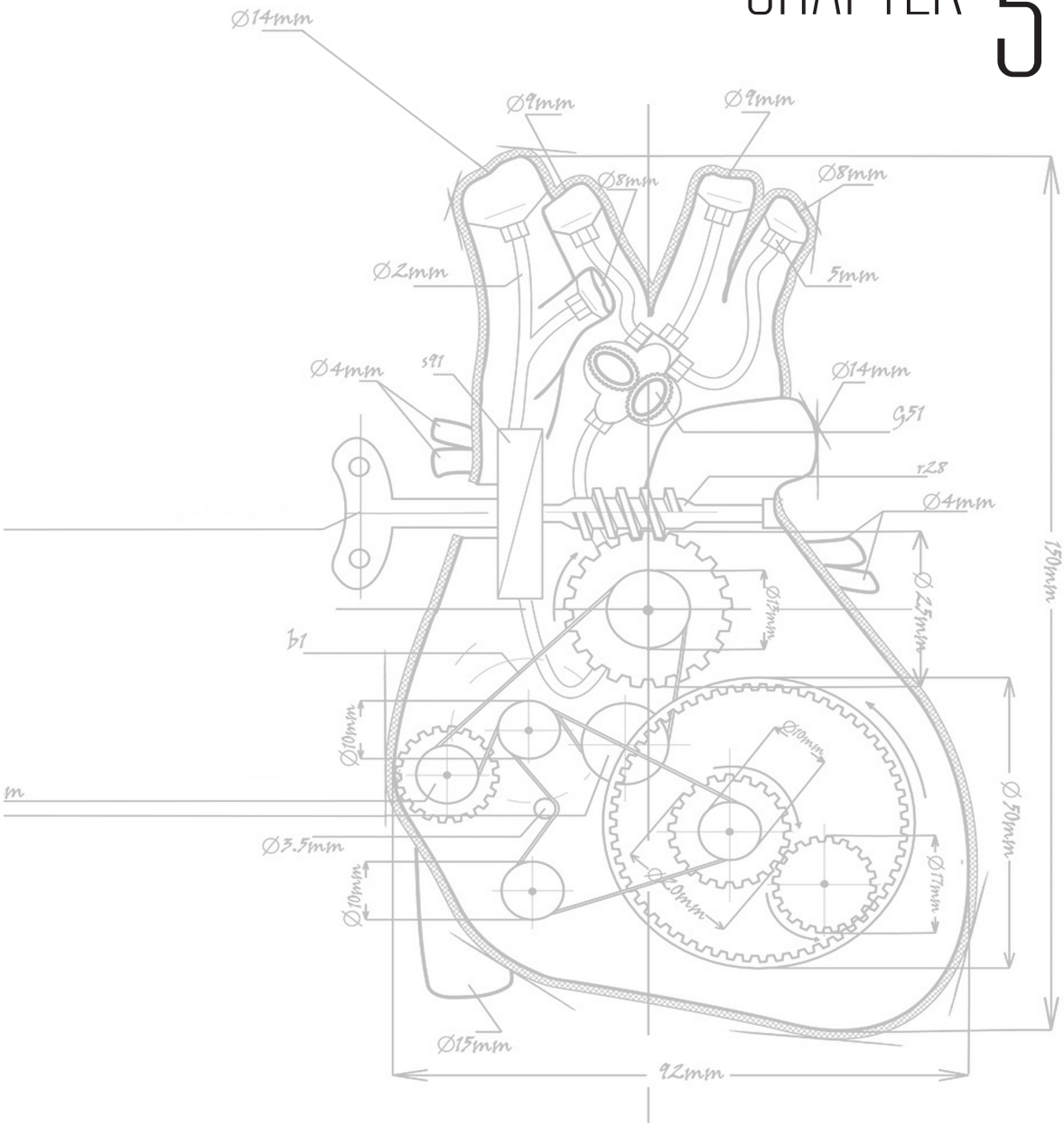
CHAPTER 5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ABSTRACT

Aim

One of the main limitations for an effective cell therapy for the heart is the poor cell engraftment after implantation, which is partly due to a large percentage of cell death in the hostile myocardium. In the present study, we investigated the utilization of Necrostatin-1 (Nec-1) as a possible attenuator of cell death in cardiomyocyte progenitor cells (CMPCs).

Methods and Results

In a mouse model of myocardial infarction, survival of CMPCs 3 days after intra-myocardial injection was 39±9% higher in cells pretreated with the Nec-1 compound. However, the increase in cell number was not sustained over 28 days, and did not translate into improved cardiac function (EF %, 20.6±2.1 vs 21.4±2.5 for vehicle and Nec-1 treated CMPC, respectively). Nonetheless, Nec-1 rescued CMPCs remained functionally competent.

Conclusion

A pharmacological pretreatment approach to solely enhance cell survival on the short-term does not seem to be effective strategy to improve cardiac cell therapy with CMPCs.

INTRODUCTION

Cardiac cell therapy is emerging as a treatment for ischemic heart disease. Various cell types have been successfully employed in the recovery of cardiac damage by reducing inflammation, activating endogenous regenerative responses, and, in some cases, directly participating in vasculogenesis and cardiomyogenesis (1, 2). Nevertheless, many studies observed only a small percentage of engrafted cells in the myocardium upon injection, thereby possibly limiting their therapeutical effects (3-5).

In the last decade, several landmark papers have disproven the notion of the human heart as a post-mitotic organ (6, 7). This paradigm shift has led to the discovery of cardiac progenitor cells (CPCs), which can, to a certain extent, repopulate the heart after injury. On the basis of cell surface markers (c-kit, Sca-1) (8, 9), but also transcription factors (Islet-1) (10), ability to efflux hoechst (side population) (11), and culture conditions (cardiospheres) (12), several different CPC populations have been isolated. Most of these cells are clonogenic and have the capability to differentiate into different cardiac cells, like myocytes, smooth muscle cells, and endothelial cells (13). Previously, we reported the isolation of human Sca-1+-like cardiomyocyte progenitor cells (CMPCs) (14) and their potential to differentiate into cardiomyocytes and vascular structures *in vitro* (15) and *in vivo* (3). Upon injection in a mouse model of myocardial infarction they preserved cardiac function. Although very promising, their long-term engraftment and subsequent direct participation in cardiac tissue regeneration was limited to around 3 percent of injected cells at 3 months post-injection (3). Probably due to the hostile environment of the ischemic myocardium, a large percentage of cells die within the first 48 hours post-injection (16). Therefore, it could be of great benefit to provide a pro-survival stimulus prior to implantation. We have shown that under oxidative stress conditions, necrosis is the main cause of cell death in CMPCs (17). Although previously thought to be non-regulated, inhibiting the receptor interacting protein 1 (RIP1), a death domain protein required for activation of necrosis, cell death could be attenuated (18). In the current study, we attempted to improve the survival of CMPCs by pretreatment with RIP-1 inhibitor, Necrostatin-1 (Nec-1) (19), prior to injection into the myocardium and monitored cardiac function to see the effect of improved survival on myocardial damage.

METHODS

CMPC isolation and transduction

CMPCs were isolated and propagated as previously described (20). For the use of human fetal tissue, individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht were obtained. CMPCs were transduced with a lenti-viral construct, containing pLV-CMV-luc-GFP (21), with the addition of 8ug/ml of polybrene (Millipore). After 24 hours, medium was refreshed with standard CMPC culture medium and transduction efficiency was determined and enriched by FACS sorting for GFP (BD Bioscience).

In vitro apoptosis and necrosis induction and assessment

CMPCs were pre-treated with vehicle or 30μM Nec-1 (Santa Cruz) for 30 min prior to the addition of 75μM tert-Butyl hydroperoxide (Sigma) for 16–20 hrs. Analysis was performed as described previously (17).

Animals

All experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*, with prior approval by the Animal Ethical Experimentation Committee, Utrecht University.

Myocardial infarction and cell transplantation

Male NOD-SCID mice, aged 10–12 weeks, were anesthetized (i.p. injection; fentanyl 0.05 mg/kg; dormicum 5 mg/kg; dormitor 0.5 mg/kg) and myocardial infarction (MI) was induced by ligation of the left coronary artery, as described previously (3, 22). 30 min after MI, 10 μL phosphate-buffered saline (PBS) containing 0.5×10^6 CMPCs, either pretreated with the vehicle (n=7) or 30μM Nec-1 for 30 min (n=8), was divided over two injection-sites in the infarct-borderzone. Painkiller (s.c. temgesic 0.15 mg/kg) was given to mice for 2 days (every 8hours) post-operatively.

Bioluminescent Imaging (BLI)

The detection of emitted photons by transduced CMPCs was performed by the sensitive photon imager from Biospace Laboratory. BLI images were obtained with the Biospace CCD camera and analyzed by Photovision software (Biospace Laboratory). The substrate D-luciferin sodium salt (Promega), dissolved in PBS, was injected intra-peritoneal at a dose of 125 mg/kg 15 min prior to measurement. Mice were anaesthetized with 1.5% isoflurane and positioned onto the stage inside the light-tight camera box. BLI images were subsequently acquired over a period of 15min. Exposure conditions (time, aperture, stage position, binning, and time after injection) were kept the same in all measurements. For quantification, standard regions of interest were defined for the heart. For BLI measurement of cultured cells, luciferin was added to the plate, incubated for 1 minute, and inserted into the BLI for a 5 min image acquisition. For quantification, standard regions of interest were defined for each well.

MRI measurements

In addition to BLI imaging, cardiac parameters were determined prior to MI, and at 7 and 28 days post-MI for each mouse. End-diastolic volume (EDV), end-systolic volume (ESV), and ejection fraction (EF) were measured by high-resolution magnetic resonance imaging (MRI; 9.4 T, Bruker Biospin) as described previously (22). Analysis was performed using Q-mass for mice digital imaging software (Medis) by a blinded investigator.

Immunofluorescence

Mice were sacrificed 1 month post-MI by cervical dislocation; hearts were flushed with PBS and fixed as described previously (3) and thereafter cut into 7 μm cryosections. All sections were

numbered to identify the position of human grafts. Sections were stained as previously described (3) using the following antibodies: human integrin-β1, troponin- I (Santa Cruz), and Alexa488-labelled (Invitrogen) secondary antibodies. For quantification, all human β1 integrin-positive cells throughout each heart were scored by a blinded investigator. To calculate the area occupied by human cells, microscope images were transformed into binary image file by Image-J, and thereafter quantified.

CMPC functionality

CMPCs were pre-treated with vehicle or Nec-1 for 30min prior to the addition of 75μM tert-Butyl hydroperoxide in serum-free M199 medium for 16–20 hrs. All cells were collected, counted, and the same number of living cells, as determined by trypan blue, was used in the subsequent functional assays. Proliferation of surviving cells was monitored by WST-1 reagent (Roche) (23), release of VEGF, IGF-1 and HGF was monitored by ELISA (R&D Systems), and tubule formation was performed in micro-slide for angiogenesis (Ibidi) coated with ECMatrixTM (Millipore)(24). To induce differentiation, cells were treated with 5 μM 5'-azacytidine (Sigma) for 72 h in differentiation medium, followed by TGF-β1 stimulation (1 ng/ml; Sigma)(15).

Statistics

Data is presented as mean ±SEM and were compared using the two-tailed paired Student's t test. A difference of $p<0.05$ was considered to be statistically significant.

RESULTS

Necrostatin-1 pretreatment of CMPC *in vitro*

Under oxidative stress conditions, CMPC mainly displayed a necrotic phenotype (figure 1A) and by pretreatment with Nec-1, we observed a 37 ± 8% reduction in necrotic cell death in CMPCs compared to vehicle (figure 1B). As was established previously (17), we did not find differences in apoptotic mediated cell death between the 2 groups. Therefore, Nec-1 increased the survival of CMPCs by inhibiting necrotic cell death (figure 1B).

Lenti-viral transduction and Nec-1 effects on CMPC functionality

Following lenti-viral transduction, GFP-positive CMPCs were enriched by FACS to attain a 92% GFP-positive cell population (suppl. figure 1A). Morphologically, CMPCs retained their original phenotypic appearance (suppl. figure 1B). Proliferation was monitored over a 16 day period, in which no changes were observed between non-transduced, transduced, and GFP-sorted CMPCs (suppl. figure1C). All cells had a doubling time of approximately 24 hours, which was sustained throughout the period of the experiment.

To exclude a direct effect of Nec-1 on luciferase expression, CMPC were pretreated either with vehicle or Nec-1 and subsequently measured by BLI. The BLI signal observed in the cells was equally strong in both groups (suppl. figure2A, 2.0 ± 0.4 x107 vs 2.1 ± 0.3 x10^7 ph/s/cm2/sr, respectively). Furthermore, pretreated CMPCs were also passed through multiple functional

assays, which tested their potency as well as potential therapeutical mode of actions, in order to exclude off-target effects of Nec-1. Proliferation remained robust between the groups (doubling time ~24h), while tubule formation, cardiogenic differentiation potential, and the secretion of VEGF, IGF-I, and HGF remained unaffected by the Nec-1 treatment (suppl. figure2B-E). Lastly, we quantified CMPC (Nec-1 and vehicle pretreated) numbers in the myocardium directly (15min) after injection, in order to exclude a possible influence of Nec-1 on initial cell retention. The histological analysis revealed that Nec-1 did not interfere with this process. We therefore concluded that the viral transduction, as well as the Nec-1 pretreatment, did not affect CMPC functionality *in vitro* and initial cell engraftment *in vivo*.

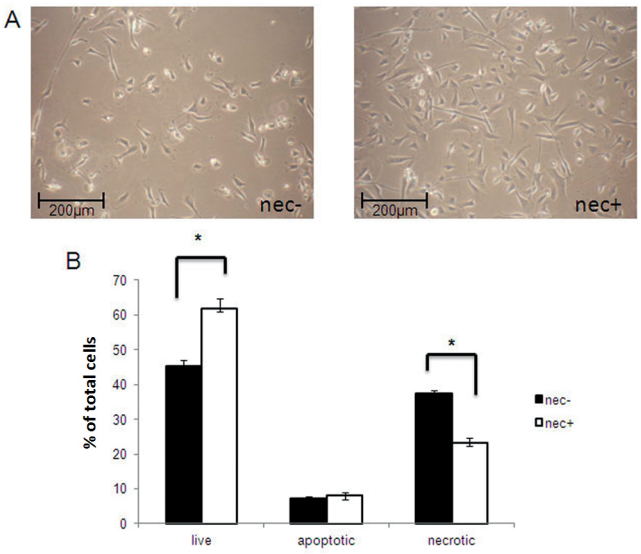

Figure 1 CMPCs were pretreated with 30µM Nec-1 (or vehicle), an inhibitor of RIP1, followed by H2O2 stimulation. **A)** After 16h, more adherent CMPCs were observed in culture dishes pretreated with Nec-1 (nec+). **B)** Live, apoptotic and necrotic cells were detected by AnnV/7-AAD staining using flow cytometric analysis, and demonstrated the ability of Nec-1 to attenuate necrotic cell death in CMPCs. *p<0.05

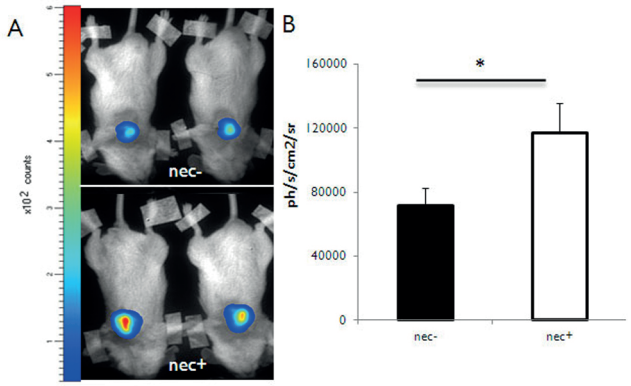

Figure 2 Monitoring cell survival by BLI at 3 days post-injection. **A)** Representative BLI image of mice which received vehicle (nec-) or Nec-1 pretreated (nec+) CMPCs after LAD ligation. **B)** Quantitative analysis of BLI signal demonstrated that Nec-1 pretreated cells had better engraftment 3 days post-injection. *p<0.05

Short-term CMPC survival *in vivo*

Initial experiments were carried out to test whether BLI technology could be used as a reliable measure for different CMPC numbers. *In vitro*, we plated CMPCs in increasingly higher numbers, which correlated strongly (R^2=0.971) with the subsequent BLI measurements (suppl. figure3A). Additionally, we tested this relationship *in vivo* by titrating different numbers of myocardial injected CMPCs, ranging from 2x15,000 to 2x 240,000. By increasing CMPCs numbers, we observed higher BLI signals, which led to a high correlation (R^2=0.967) between the two parameters (suppl. figure 3B). The results of these correlation experiments validated the use of BLI to monitor CMPC numbers *in vivo*.

To check the effect of Nec-1 on CMPC survival, mice underwent left anterior descending artery ligation and received an intra-myocardial injection with (1) vehicle pretreated CMPCs; (2) Nec-1 pretreated CMPCs. *In vivo* bioluminescence imaging (figure 2A), 3 days post injection, demonstrated a 39% higher signal (71,665 $\pm$ 11,165 versus 117,138 $\pm$ 18,567 ph/s/cm2/sr, respectively) in mice which received the Nec-1 pretreated cells (figure2B). The presence of cells was confirmed by histological analysis, which revealed an increased number of human specific cells in the myocardium of mice which received the Nec-1 pretreated CMPCs (suppl. figure 3C). This data clearly showed the ability of Nec-1 to increase the short-term engraftment of CMPCs.

LV function and wall thickness

Cardiac functional measurements were carried out by MRI prior to MI, and at day 7 and 28 post-MI. Baseline measurements revealed comparable ESV, EDV, and EF between the two groups (figure 3A-D). Also on day 7 no significant difference could be observed in all cardiac parameters between the groups. Interestingly, in our final follow-up time of 28 days, we do not see improved cardiac recovery in the Nec-1 treated group, in which more cells initially survived. Although a small decrease in EDV and ESV, and increase in EF in the Nec-1 treated group was observed, these changes were non-significant. Nevertheless, when comparing these values to previous injection control groups carried out in our lab (3, 25-27), it is clear that transplantation of cells in both groups had a positive effect on the preservation of cardiac dimensions (3).

Preservation of the cardiac structure also plays a crucial role in preventing further remodeling. However, the thickness of the LV wall remained unaffected by increased CMPC survival (figure 4B; 0.49 $\pm$ 0.05 versus 0.51 $\pm$ 0.08, vehicle versus Nec-1 pretreated, respectively). Lastly, to see if there was a correlation between the observed CMPC survival by BLI and the preservation of cardiac dimensions by MRI, we plotted the EDV, or EF values from day 28 against the BLI signal from day 3, but also here no significant correlation could be observed (suppl. figure 4). Therefore, we concluded that increased short-term CMPCs survival did not translate into better preservation of cardiac dimensions.

Long-term CMPC survival *in vivo*

Engrafted CMPCs were identified in histological sections with a human-specific antibody recognizing integrin-β1, which is expressed on the human cell membrane. The transplanted cells were found back in patches located in the borderzone, as well as in the infarcted region, as described before (3). We identified 2.1 $\pm$ 0.5 % of the initially injected vehicle pretreated CMPCs,

compared to a similar 2.2 ± 0.6 % of the Nec-1 treated cells (figure 5A). Additionally to cell number, we also analyzed the area occupied by the staining. However, also in this quantitative analysis we did not observe differences between the two groups at 28 days (figure 5B).
Furthermore, for the quantification of human DNA present in the murine myocardium we used a primer specific for human Alu sequences as previously described (28) . We detected human DNA in all mouse hearts, however, similar levels of expression were again observed between the groups (figure 5B). This data indicated that the observed higher short-term CMPCs engraftment in the Nec-1 group was not sustained over the 28 day follow-up period.

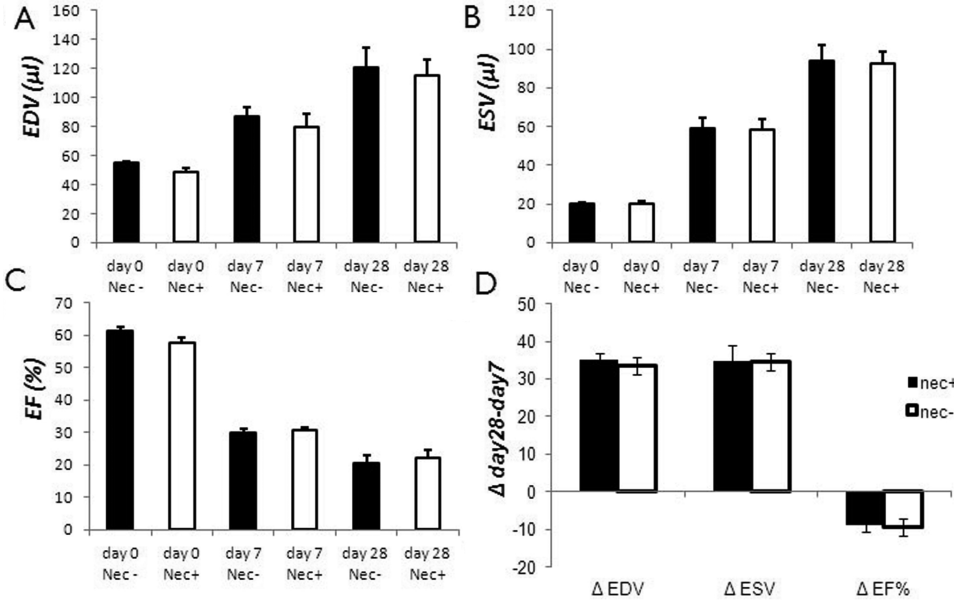

Figure 3 Assessment of cardiac function by MRI at baseline, and 7 and 28 days post-injection. End-diastolic volume (EDV) **(A)**, end-systolic volume (ESV) **(B)**, and percent ejection fraction (%EF) **(C)** remained unaltered between vehicle and Nec-1 pretreated CMPCs at all time points. **D)** Quantifications of the change in the 3 parameters between day 28 and day 7 showed no significant difference between the vehicle (nec-) and Nec-1 pretreated (nec+) CMPCs.

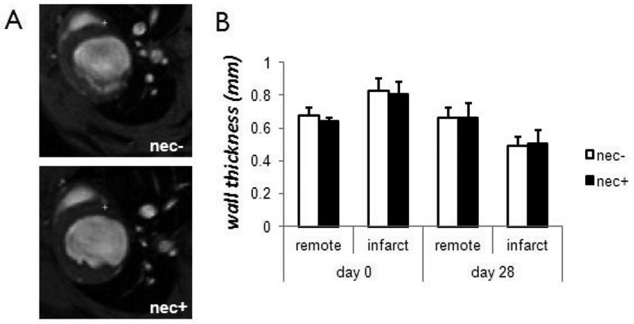

Figure 4 Evaluation of wall thickness by MRI. **A)** MRI images showing an axial view in the diastole of animals which received vehicle (nec-) or Nec-1 (nec+) pretreated CMPCs at 28 days post-injection. **B)** Wall thickness was measured at two time-points, and in two regions (remote and infarct). Wall thinning between the groups remained constant.

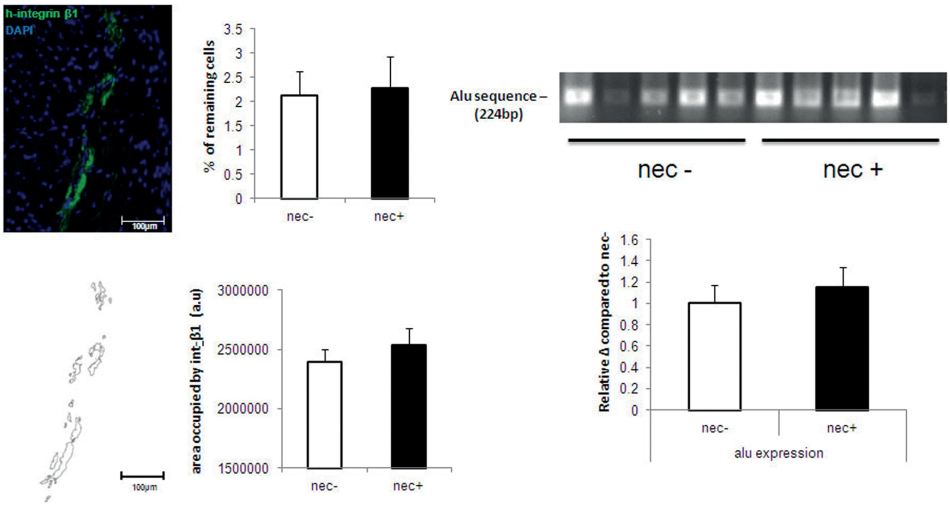

Figure 5 *In vitro* assessment of long-term CMPC engraftment. **A)** Representative fluorescent image (top left) of human specific integrin-β1 staining (green) in the murine myocardium. By histological analysis, the total number of cells in the heart was determined, and represented as the percentage of the total injected cells (top). Binary pictures (bottom left) were utilized to determine the area occupied by the staining (bottom). Both values were not significantly different between the 2 groups (n=5, both). **B)** A semi-quantitative PCR was preformed with human specific Alu sequence primers (top right). We observed comparable Alu expression in both groups (n=5) (bottom right). (nec-, vehicle pretreated cells; nec+, Nec-1 pretreated cells)

Functionality of Nec-1 "rescued" CMPCs

Since we did not observe additional therapeutical beneficial effects in mice with more surviving CMPCs, we explored whether the cells that are rescued by the Nec-1 compound are as functionally competent as before the oxidative stress stimulus. For this, we put the CMPCs under oxidative stress conditions, after which they were placed in several functional assays. We did not observe any decrease in function in rescued CMPCs. Their ability to proliferate (figure 6A) was comparable to non-rescued CMPCs (doubling time ~36h). Similarly, the secretion of several growth factors, which seem to be important for the therapeutical benefit exerted by the paracrine factors of CMPCs, remained similar compared to non-rescued cells (figure 6B). Furthermore, as can been seen from the tubule formation images (figure 6D), rescued CMPCs were able to form vessel-like structures with similar size and length to those of non-rescued cells, and their cardiogenic differentiation potential *in vitro* (figure 6C) remain unaffected.

DISCUSSION

In this study, we demonstrated the ability of Necrostatin-1 to attenuate necrotic cell death in CMPCs after intra-myocardial injection. At three days post-injection BLI-signal and histological analysis showed increased presence of CMPCs pretreated with the compound. However, in our histological/PCR analysis at 28 days, similar numbers of cells are recovered in both groups.

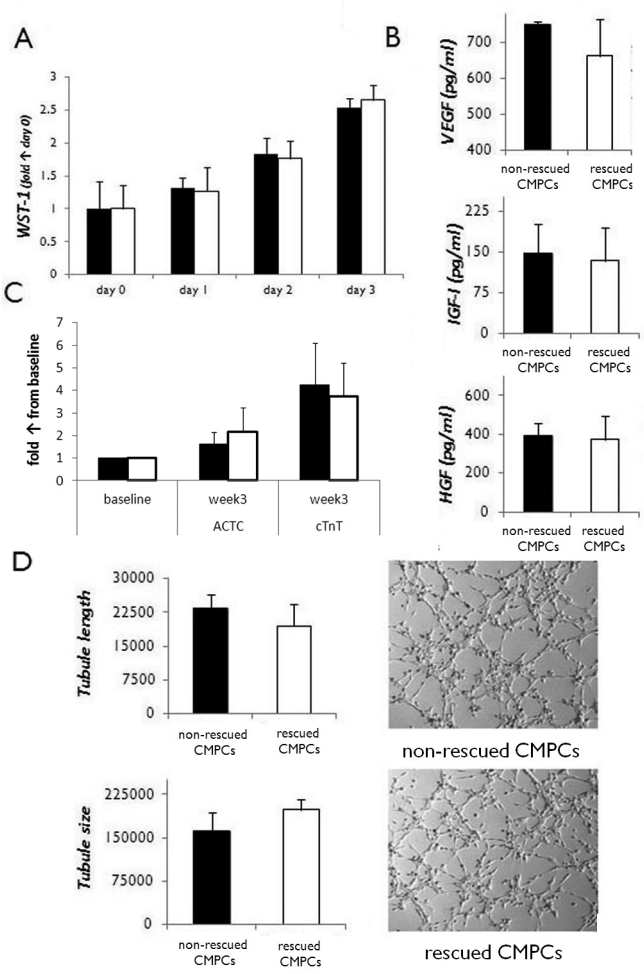

Figure 6 Characterization of Nec-1 rescued cells. **A)** Proliferation, as determined by WST-1, was monitored over a 3 day period. The ability of rescued CMPCs to proliferate during this time remained unchanged. **B)** Secretion of VEGF, HGF, and IGF-1, in medium collected from CMPCs after 16h incubation, was determined by ELISA. Levels of all three growth factors were similar between non- and rescued CMPCs. **C)** CMPC were differentiated by 5-aza and TGF-beta stimulation. mRNA expression was determined prior to differentiation (baseline) and after 3 weeks by RT-PCR for cardiac actinin (ACTC) and troponin T (cTnT). The expression of cardiac markers was not significantly different between groups. **D)** Tubule formation in matrigel is represented in terms of tubule length and size. Representative images of matrigel experiment (bottom left) illustrated the functional competence of Nec-1 rescued cells.

Moreover, the short-term increase in cell survival did not lead to an improved preservation of LV function. In our hands, we showed that CMPCs that are treated and rescued by Nec-1 seem to be still functionally competent. Therefore, we concluded that short-term increase in survival achieved with this pharmacological approach was not an effective strategy to further improve CMPC transplantation therapy.

The lack of improvement in cardiac function seen in the group with increased short-term survival could be due to several reasons. Firstly, it is possible that we already reached a threshold dose in the non-treated CMPC group, and that the effect of cell therapy plateaus with increased cell numbers. While CMPCs do participate to a certain degree in vasculogenesis and cardiomyogenesis in the injured myocardium, their paracrine factors are probably their main beneficial therapeutical feature. In this context, it is feasible that there is a saturation of the receptors involved in the paracrine mediated effects. Secondly, we observed at 28 days that

the increase in cell number is not sustained by the Nec-1 pretreated group. This observation could be due to the transient nature of our pro-survival stimulus and the prevalence of cell death 3 days post-injection. The leveling off of survival benefits over the 28 days follow-up period could lead to similar therapeutical effects in both groups. Lastly, Nec-1 pretreatment potentially leads to only a relatively small increase in dosage. In the present study, as well as in multiple other studies (29-31), it has been shown that about ~15% of intra-myocardial injected cells remain in the heart directly after injection. In our study, we injected $5x10^5$ CMPCs into the myocardium, which would lead to an acute cell engraftment of about ~$5x10^4$ cells. Therefore, with our pretreatment approach we could be observing only 1-3 $x10^4$ additional cells in the Nec-1 treated group and it might require a greater increase in cell numbers to see dose-dependent effects of CMPCs.

Interestingly, in literature there are also numerous contradictory findings about increased cell number and the benefit it exerts on preservation of cardiac function. This is especially apparent in the clinical arena were a consensus has yet to be reached on the dose-dependency of cardiac cell therapy (32-36). In animal models, there have been various studies investigating pretreatment or genetic manipulation options to improve cell survival after intra-myocardial injection. In studies by Niagara et al (37) and Afzal et al (38), they preconditioned skeletal myoblast and MSC, respectively, with a pharmalogical compound, diazoxide, and noticed improvements in cardiac function when using preconditioned cells. Although diazoxide improves cell survival, it also increases the agiogenic potential of the cells, which makes it difficult to attribute the improved LV function solely to the increase in cell number. In the study by Kutschka et al (39), they used a Bcl-2 viral vector to attenuate cell death in cardiomyoblasts. Although measurements by BLI showed significantly increased signals in cells overexpressing Bcl-2, no functional differences were observed between groups at one month follow-up. Interestingly, a similar approach was taken by Li et al (40), in which a 2-fold increase in survival in Bcl-2 overexpressing MSCs was associated with smaller infarct sizes and better functional recovery.

Prosurvival stimuli have been investigated for CPCs as well. Huang et al (41) made use of a novel microRNA survival cocktail to improve the viability of CPCs, which led to a sustained higher BLI signal for up to 28 days. Although the prosurvival signaling led to a significant improvement in LV function compared to the PBS control group, they did not observe significant differences in function between the CPC-control and CPC- microRNA treated cells. More recently, however, in a study by Liu et al (42) they stratified the CPC injected mice into high and low engraftment groups based on PET imaging data. By using this early cell engraftment data from day 1, they were able to predict the subsequent functional improvements in mice with higher cell engraftment. In regard to the 39% higher BLI signal we noted in our present study, the stratification carried out by Liu et al resulted in an ~30% higher day 1 PET signal for the high engraftment group, however they initially injected a higher dose (1×10^6 CPCs), thereby possibly increasing the initial engraftment.

The incongruency in the above mentioned studies can be partly attributed to the imperfection in intra-myocardial delivery of cells, especially in the small rodent studies, but also in the large variety of cells type being studied. In the case of prosurvival stimuli, there is also a chance of off-target effects on e.g. increased paracrine secretion or proliferation, which can influence cell behavior,

and are therefore not always a true representation of a dose-dependent action of cells. Lastly, the differences in the injected cell numbers (and in the case of dose-response studies the range between cell doses) also makes it difficult to compare studies. Therefore, to truly understand the influence of cell survival in the myocardium, and put our study in a boarder perspective, a comprehensive large scale dose-response studies will need to be carried out for each individual cell type, which at the moment is still lacking. Nonetheless, these discrepancies also highlight the lack of knowledge in the mechanism of action behind the cardio-protective effects of cell therapy. In conclusion, even though Nec-1 pretreatment of CMPCs increased their tolerance to oxidative stress *in vitro* and improved cell survival *in vivo*, no beneficial effects were observed on preservation of cardiac dimensions through this manipulation. Since the retention of cells after the initial wash out period is low (~15%), prosurvival strategies are therefore targeting an already diminished cell number, which might be impeding their therapeutical potential. New approaches using extracellular matrix or synthetic polymers for the entrapment of cells could potentially target this problem (43), and attempt to reclaim the large number of cells lost to the circulation. Survival strategies could conceivably be more useful in such settings, as they can exert their effects on larger number of cells.

Funding

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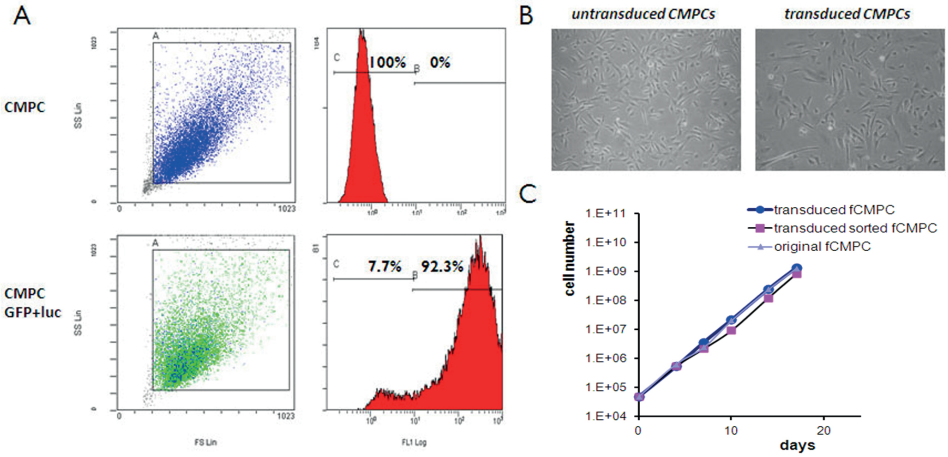
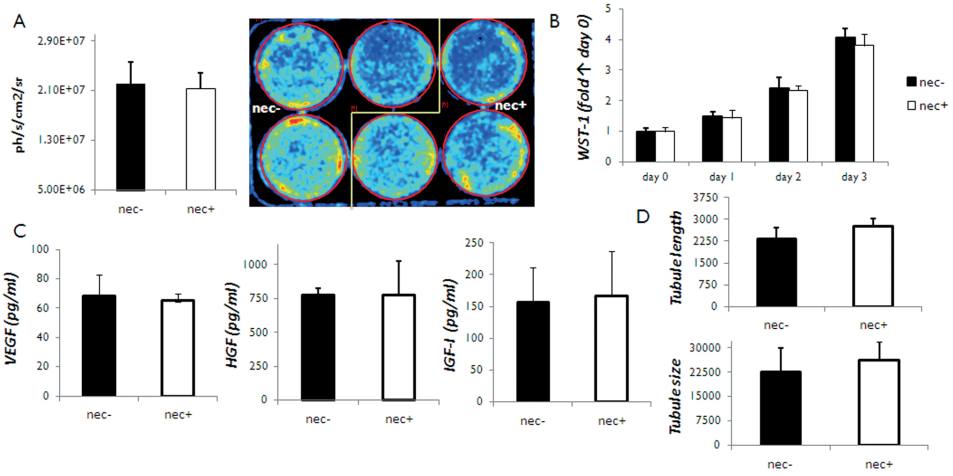
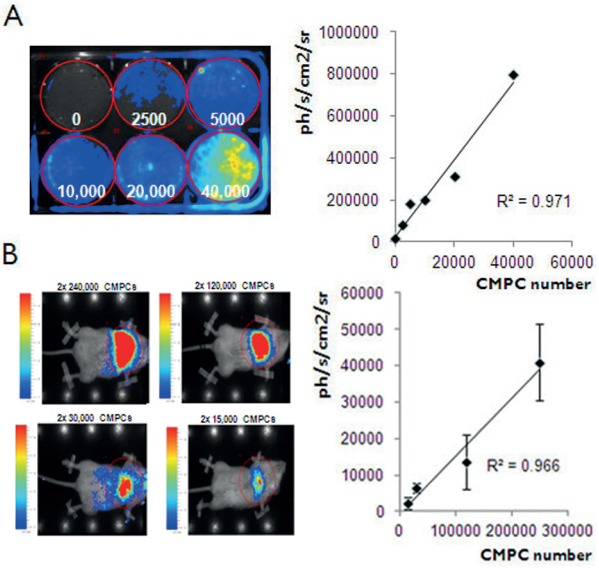
SUPPLEMENTARY



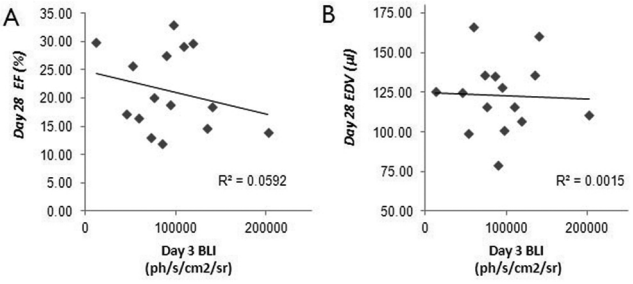
Supplementary Figure 1 Characterization of transduced CMPCs. **A)** FACS plots of non- (top), and tranduced (bottom), which were also sorted for GFP, CMPCs evaluated for GFP expression. **B)** Morphological assessment of tansduced CMPCs. C) Proliferation of CMPCs over a 16 day period remained similar between non-, transduced, and GFP enriched cells.



Supplementary Figure 2 Assessment of Nec-1 off-target effects in CMPCs. **A)** Luciferase expression was unaffected by the pretreatment of Nec-1. Illustrative image of BLI measurement, in which vehicle (nec-) and Nec-1 (nec+) pretreated CMPCs were compared. Functionally, the proliferation **(B)**, paracrine secretion **(C)**, tubule formation **(D)**, and cardiogenic differentiation potential **(E)** were unchanged after pretreatment with Nec-1 (ACTC; cardiac actin, cTnT; cardiac troponin T). Histological evaluation **(F)** of acute CMPC retention 15 min post-injection. Values were not significantly different between the nec- (n=3) and nec+ (n=3) groups.



Supplementary Figure 3
Relationship between BLI signal and CMPC number. **A)** CMPCs, from 0 to 4x10^4 cells, were plated in a 6-well dish and measured by BLI. Quantification of the BLI signal revealed a robust correlation (R^2=0.971) with CMPC number. **B)** Directly after myocardial injection of 2x 0.15x10^5(n=3), 0.3x105(n=3), 1.2x10^5(n=2), or 2.4x10^5 (n=2) CMPCs, animals were measured by BLI. We determined a strong correlation (R^2=0.966) between injected cell number and subsequent BLI measurement. **C)** At 3 days post-injection, quantitative analysis of BLI signal demonstrated that Nec-1 pretreated cells (nec+; n=5) had better engraftment compared to vehicle pretreated CMPCs (nec-; n=4). The histological analysis, represented as the percentage of the total injected cells remaining in the heart, showed a clear increase in cell numbers in the Nec-1 preatreated group. *p<0.05



Supplementary Figure 4
Evaluation of relationships between short-term CMPC survival (BLI) and long-term functional follow-up (EDV, EF). In both correlation plots **(A,B)** we observed no relationship of day 28 MRI measurements with day 3 BLI signal.

3D tissue printing with cardiac progenitor cells preserves cardiac performance in a murine model of myocardial infarction

Sumitted to Biomaterials

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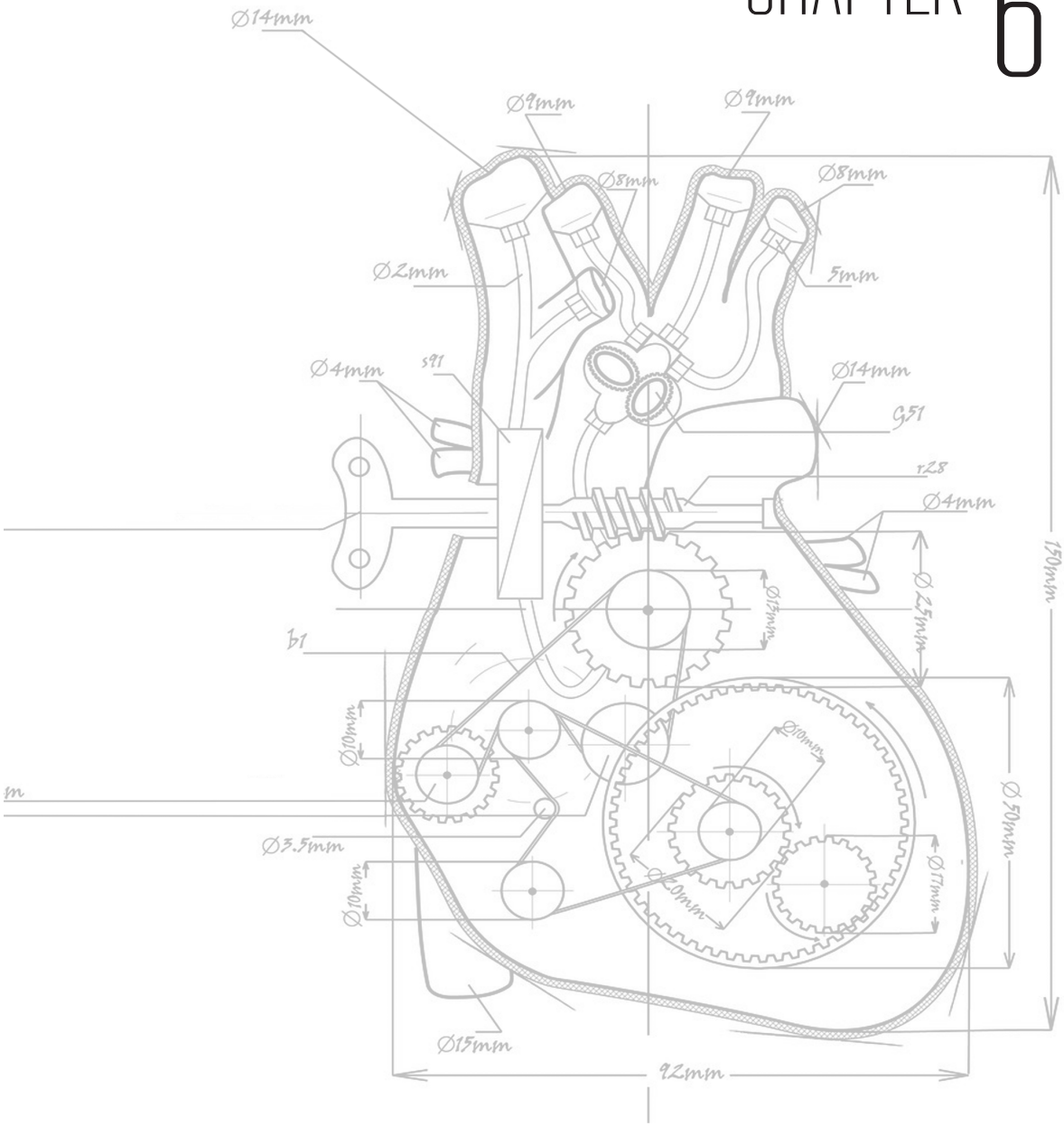
CHAPTER 6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ABSTRACT

Aim

Cardiac cell therapy suffers from limitations related to poor engraftment and significant cell death soon after transplantation. *Ex vivo* tissue engineering was demonstrated to increase cell retention and survival, as well as, to mechanically support the damaged heart. The aim of our study was to evaluate the 3D printing of hCMPCs in hyaluronic acid/ gelatin based matrix for *in vivo* application.

Materials and Methods

hCMPCs were printed in a gelatin/hyaluronic acid matrix ($30x10^6$ cells/ ml) to form a biocomplex made of 6 perpendicularly printed layers with a surface of 2cm x 2cm and a structure of 500 µm thick, and retained their viability, proliferation and differentiation capability. The printed biocomplex was transplanted in a mouse model of myocardial infarction (MI), and MRI measurement revealed reduction in adverse remolding and preservation of cardiac performance. On the histological level, we showed increased wall thickness and myocardial tissue viability in matrix treated animals. Furthermore, the matrix supported the long-term *in vivo* survival and engraftment of CMPCs, in which we detected cardiac troponin and CD31 expression after 4 weeks.

Conclusions

We showed that hCMPCs can be printed and cultured in Gel/HA. Transplantation in a mouse model of MI contributed to an improvement in cardiac function and a better remodeling upon injury. Overall, we developed an effective and translational approach to enhance CMPC delivery and action in the heart.

INTRODUCTION

An ischemic event in the myocardium has dire consequences for the heart, since the death of cardiomyocytes within the infracted area leaves the heart with less contractile elements. This shifts the pumping burden to the remaining viable myocardium and can have deleterious consequences for patients suffering from the disease, potentially leading to the development of heart failure. Current therapies are focused on limiting secondary damages to slow down the progression of the disease [1, 2]. Therefore, end-stage heart failure patients often require heart transplantation, for which donor hearts are in short supply and carry risks of rejection [3]. Although the existence of endogenous stem cell populations in the heart has been well documented [4, 5], these cells are unable to repair the injury and restore the function of the heart.

The need to replenish the heart with new myocytes is of critical importance to avoid the chronic manifestation of the disease. In this regard, stem cell transplantation therapy offers a new therapeutical avenue to create *de novo* cardiomyocytes either *in vitro*, *ex vivo*, or *in vivo*. A wide variety of stem cells have shown the ability to differentiate into cardiomyocytes. Of these, the multipotent Sca1+ cardiac-derived cardiomyocyte progenitor cells (CMPCs) offers a desirable combination of a patient specific cell source with cardiogenic potential, both *in vitro* as well as *in vivo* [6, 7]. Besides direct involvement in tissue repair, the plethora of factors secreted by CMPCs can also activate the endogenous stem cell pools, thereby making it a well suited cell type for the implementation of cardiac regenerative strategies [8, 9].

Although promising results have come out of pre-clinical and clinical studies [10-12], cardiac stem cell therapy still suffers from inefficient delivery, engraftment, and differentiation of cells in the myocardium [13-15]. Furthermore, in ischemic heart disease not only the cardiomyocytes are destroyed or modified but also the extracellular matrix. Therefore, combining procedures aiming at regenerating both myocardial cells and the extracellular matrix could improve the effectiveness of cellular therapy. For this reason, hybrid therapies that include *in vivo* and *in vitro* tissue engineering (TE) are being developed as potentially new therapeutic approaches for repairing myocardial tissue [15, 16]. We recently showed that tissue printing technology can be used with alginate in combination with CMPCs to create, *in vitro*, a cardiogenic patch with precise pore size and microstructure which allow a better cell viability over time [17].

Here, we improved this tissue engineering approach by creating a cardiogenic scaffold of CMPCs and a gelatin/hyaluronic acid based biomaterial. With the aid of 3D printing, we were able to build a customized patch that can harbor CMPCs without affecting their growth and differentiation potential. Furthermore, this scaffold was able to be transplanted on the murine heart, leading to excellent cell survival and engraftment. Lastly, mice which received the scaffold showed improved cardiac function after MI.

METHODS AND MATERIALS

CMPC isolation and culture

Human fetal Cardiomycytes Progenitor Cells (CMPCs) were isolated by magnetic cell sorting by based on Sca-1 positive selection and propagated as previously described [7]. Briefly, cells were plated at 0.1% gelatin coated wells in growth medium consisting of 25% EGM-2 (3% EGM-2 single quotes (Cambrex,) in EBM-2 (Cambrex)) and 75% M199 (BioWhittaker), 10% FBS (Hyclone), 1x MEM non-essential amino acids (BioWhittaker) and 1x penicillin/streptomycin. Differentiation medium consisted of 50% IMDM (GIBCO), 50% HamF12 GlutaMAX-1 (GIBCO), 1x MEM nonessential amino acids, 2% horse serum (GIBCO), 1x insulin-transferrin-selenium (GIBCO), 1x penicillin/streptomycin. 5-azacytidine (5μM; Sigma), l-ascorbic acid (10-4 M; Sigma) and TGF-β (1 ng/ml; Peprotech) were added according to our previously published protocol [6].

Standard informed consent procedures and prior approval of the ethical committee of the University Medical Center Utrecht were obtained.

Tissue preparation by 3D printing

A Bioscaffolder tissue printer (Sys + Eng, resolution 5 μm/step, working area 200 x 150 x 90 mm) was used to print a mixture of gelatin and hyaluronic acid matrix (HyStem matrix; Sigma) with or without CMPCs in a design created with the Rhinoceros software. HyStem matrix was prepared according to the manufacturer's protocol. CMPCs were combined with the matrix to a final concentration of 40x106 cells per mL. Directly after addition of the crosslinker, the biocomplex was loaded into a syringe for use in the 3D printer. The scaffold was composed of 6 perpendicular layers, and each layer is made of 7 strands printed at a distance of 2.5 mm from each other. The construct had a final size of 2x2cm. This patch was cultured up to 28 days for *in vitro* analysis and for 7 days in growth medium prior to *in vivo* transplantation. Differentiation assays were started as well 1 week after printing.

Live/dead assay

To determine cell viability of printed CMPCs, scaffolds were cut (0.5 cm × 0.5 cm), washed with PBS and subsequently stained with 2 μM calcein AM and 4 μM ethidium bromide (EthD-1) solution (live/dead assay; Invitrogen Corp.), as described by the manufacturer. After staining, the tissue was washed again and analyzed by confocal microscope analysis.

qPCR

Total RNA was isolated from the scaffolds and heart cryo-sections using Tripure isolation reagent (Roche), according to manufacturer's protocol. To homogenize the scaffolds/tissue, microbeads were added to tripure samples and mixed by a tissue homogeniser. cDNA was synthesized by using iScript cDNA synthesis Kit (Bio-rad) and quantitative RT-PCR amplification was detected in a MyIQ single-color real-time polymerase chain reaction system using iQ SYBR Green Supermix (170–8884, Bio-Rad) and specific primers (see suppl. table 1) as described before [18].

Animal experiments

All experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*, with prior approval by the Animal Ethical Experimentation Committee, Utrecht University.

Mouse model and matrix application

Female NOD-SCID mice, aged 10–12 weeks, were anesthetized (Fentanyl 0.05 mg/kg; dormicum 5 mg/kg; domitor 0.5 mg/kg) and myocardial infarction (MI) was induced by ligation of the left coronary artery as previously described [19]. In this study, the mice were divided into three groups; control (LAD ligation only), empty matrix (HyStem matrix without CMPCs), and CMPCs matrix (HyStem matrix with CMPCs). Directly after coronary ligation the matrix was applied epicardially with the aid of fibrin glue (1µl of each component; Sanquin).

MRI measurements

Cardiac parameters were determined prior to MI, and at 3 and 28 days post-MI for each mouse. End-Diastolic Volume (EDV), End-Systolic Volume (ESV), and percentage ejection fraction (%EF) were measured by high-resolution magnetic resonance imaging (MRI; 9.4 T, Bruker Biospin). Analysis was performed using Q-mass for mice digital imaging software (Medis) by a blinded investigator [19].

Bioluminescent Imaging (BLI)

CMPCs were transduced with a lenti-viral construct, containing pLV-CMV-luc-GFP as previously described[20] and used to monitor printed CMPCs survival upon *in vivo* transplantation. For detection of luciferase activity, mice were treated with 125 mg/kg of D-luciferin sodium salt in PBS via intra-peritoneal injection, 15 min prior to measurement. The detection of omitted photons by transduced CMPCs was performed by the sensitive photon imager from Biospace Laboratory. Exposure conditions (time, aperture, stage position, binning, and time after injection) were kept constant in all measurements. For quantification, standard regions of interest were defined for the heart.

Histological analysis

Mice were sacrificed 1 month post-MI by cervical dislocation; hearts were flushed with PBS, embedded in Tissue Tek (Sakura), and cut into 7 µm cryosection.

Haematoxylin & Eosin (H&E): Cryosections were fixed in 4% paraformaldehyde (PFA) followed by staining in haematoxylin solution (Boom) and subsequently in eosin solution (BDH Certistain). Sections were thereafter dehydrated and mounted with Entellan (Merck). Wall thickness was measured with Cell^P 2.8 software.

Masson's Trichrome: Cryosections were fixed in Bouin's fixative, and subsequently in 4% PFA. After fixation, slides were stained in Weigert's Haematoxylin, biebrich scarlet acid fuchsin solution (Sigma), phosphotungstic / phosphomolybdic acid (Sigma), aniline blue, and then incubated in acetic acid before being dehydrated and mounted with Entellan (Merck). Stainings were analysed with Cell^P 2.8 software and quantified with ImageJ 1.46 software.

Picrosirius-red: Sections were fixed in 4% PFA. Subsequently, sections were stained in picrosirius-red and washed 0.2 N HCl (Sigma-Aldrich). Lastly, sections were dehydrated and mounted with Entellan (Merck). Collagen depositions were visualized with polarized light and quantified as previously described [18].
Immunofluoresence: Sections were dried, fixed with acetone, and permeabilized with 0.1% Triton X-100 (Sigma), dissolved in 1% BSA in PBS for 10 minutes and blocked with 10% goat serum in PBS for 60 minutes. Then, the slides were incubated o/n with primary antibody diluted in 0.1% BSA in PBS at 4°C. Secondary antibody incubation was performed at room temperature for one hour, followed by 5 minutes Hoechst incubation and mounting with Fluoromount (Southern Biotech). Analysis was done by Olympus BX60 microscope, quantified using Cell^P imaging software (Olympus). Control slides were incubated only with the secondary antibody. Details of primary, secondary antibodies, and dilutions used can be found in suppl. table 2.

Data analysis
All data are expressed as mean – standard error of the mean. An unpaired Student's t-test was performed to compare two groups. One-way analysis of variance and Tukey's pot hoc test was used for multiple group comparisons. A p-value of less than 0.05 was considered significant.

RESULTS

3D Tissue Printing
Using a 3D Tissue printer Bioscaffolder, we were able to generate a biocomplex consisting of CMPCs in Hyaluronic acid/ gelatin matrix. The construct had a final size of 2cm by 2cm and 400 µm tick (figure 1A+B). CMPCs were homogenously present throughout the biocomplex. Live-dead assay was performed 2 hours after printing and showed that the biocomplex generation and printing process did not affect cell viability (figure 1C+D).

Biocomplex *in vitro* characterization
Expression of cardiac markers were analyzed by PCR analysis at 1 day, and 1 and 4 weeks to evaluate whether the printing process and 3D culture in gelatin and hyaluronic acid matrix influenced the cardiogenic properties of the printed CMPCs. We analyzed the expression of both early cardiac transcription factors Nkx2.5 and GATA-4, as well as late sarcomeric marker cardiac troponin and actin. We did not observe significant differences in the expression of Nkx2.5, TnT and cardiac actin after 1 month in culture compared to day 1 after printing, while a significant temporal increase in GATA-4 expression was observed after 1 week (suppl. figure 1A). Protein expression of Nkx2.5 was also confirmed by immunofluorescence analysis after 1 week in culture (figure 2A). Ki67 immunofluorescence analysis was performed to evaluate proliferation capability of printed CMPCs and showed that after 1 week in culture a large majority of cells (64% $\pm$ 2.5%) were still positive for Ki67 (figure 2B).
To analyze if CMPCs are able to remodel their 3D scaffold-environment by matrix deposition, collagen was detected by picrosirius red staining. After 7 days of culturing, a slight but not

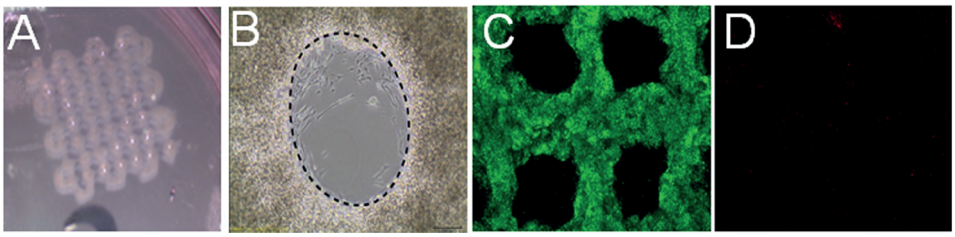

Figure 1 3D tissue printing. Seven rows of Hy-Stem/ CMPCs mixture were printed at a distance of 2.5mm from each other, both horizontally and vertically (A). CMPCs were homogenously present throughout the biocomplex (B). Live-dead assay performed 2 hours after printing showed the vast majority of CMPCs to be alive (green; C) and only a few were dead (red; D).

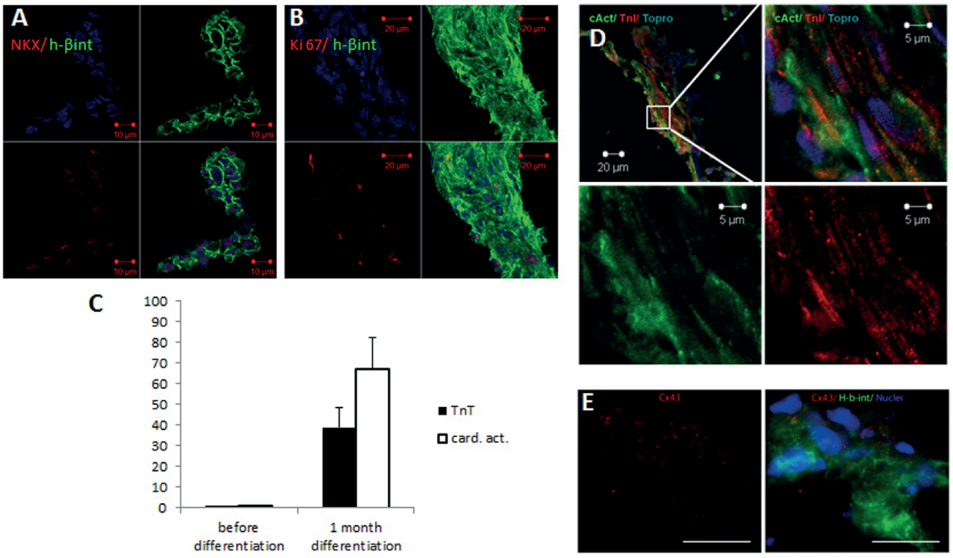

Figure 2 *In vitro* biocomplex characterization. Histological analysis after one week in culture revealed that CMPCs maintained Nkx2.5 expression (A) and a large majority of the cells continued to proliferate (Ki67+) (B). After standard cardiac differentiation procedure, we observed significant increase in cardiac troponin (TnI) and actin (card. act.) gene expression (C). Protein expression of TnI, cardiac actin (cAct) (D) and Cx43 (E) was also confirmed by immunofluorescence analysis.

significant increase in total collagen content was observed (Suppl. Fig. 1B). However, at day 28, the total amount of collagen deposition was significantly increased compared to day 1 (507.2 $\pm$ 168.6 and 27.9 $\pm$ 11.5, $P<0.05$, $n=4$); (suppl. figure 1C). Lastly, to understand the cardiogenic differentiation capability of printed CMPCs, standard differentiation assays were started 1 week after printing. After 1 month of differentiation, a significant increase in cTnT and cardiac actin was observed by PCR analysis, 38.96 $\pm$ 9.6 and 67.31$\pm$ 15.1, respectively (figure 2C). Protein expression of TnI, cardiac actin and Cx43 was also confirmed by immunofluorescence analysis (figure 2D+E).

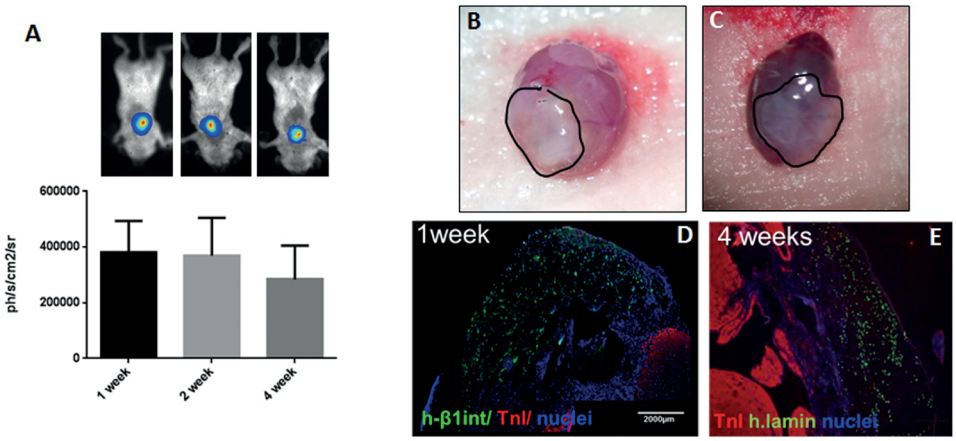

Figure 3 *In vivo* CMPC survival. Assessment of cell survival by bioluminescent imaging (BLI) (A) showed no significant drop in signal over the one month follow-up period. Transplanted scaffolds were visible in all treated mice as a patch on the infarcted area of the ventricular wall both at 1 (B) and 4 (C) weeks. The presence of human transplanted cells was confirmed by immunofluorescence analysis for human-specific β-integrin (D) and human Lamin A/C (E), which reveled robust presence of CMPCs inside the matrix at all time points.

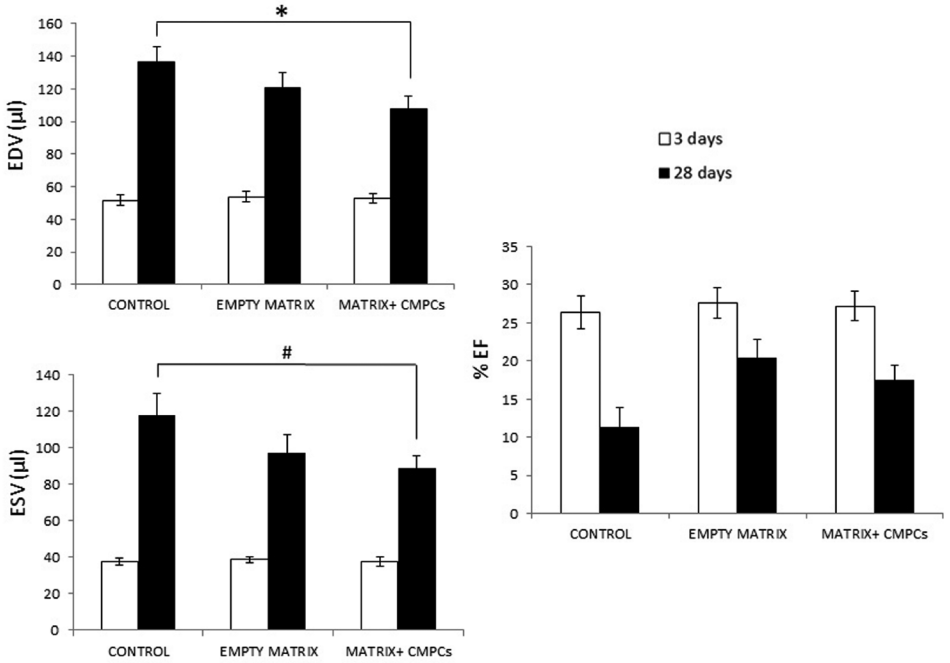

Figure 4 Assessment of cardiac parameters by MRI after patch transplantation. While the same at 3 days post-transplantation, both end diastolic and systolic volumes (EDV and ESV respectively) showed a pronounced decrease in matrix + CMPCs treated (n=9) treated animals compared to control group (n=8) after 28 days. A similar but lesser trend was observed for the empty matrix group (n=7). For the percentage ejection fraction (%EF), a similar strong tendency was observed for the patch treated animals (*p<0.05, # p<0.07).

***In vivo* CMPC survival**

To evaluate cell survival upon epicardial transplantation of the biocomplex, luciferase-expressing CMPCs were printed and transplanted on top of the infarcted ventricular wall. Detection at 1, 2 and 3 weeks showed a slight but non-significant decrease in BLI signal, indicating that transplanted cell were able to survive up to 1 month *in vivo* (figure 5). Transplanted scaffolds were always visible in all treated mice as a small patch on the infarcted area of the ventricular wall (figure 3B+C). The presence of human transplanted cells was confirmed by immunofluorescence analysis for human-specific β-integrin and human Lamin A/C (figure 3D+E). Transplanted CMPCs were mostly localized in the transplanted construct and only few cells migrated from the scaffold into the infarct region 4 weeks after transplantation.

Printed biocomplex ameliorates infarct remodeling

To evaluate cardiac parameters after patch application, MRI analysis was conducted at 3 and 28 days post-MI. Although no short-term effects were observed between the groups, a significant reduction in EDV in mice treated with the cellularized patch (107.87 ± 7.41 µl) compared to the control group (136.48 ± 9.08 µl) was detected at 28 days, along with a strong trend for smaller ESV (89.01 ± 6.63µl versus 123.53 ± 10.64 µl respectively). The empty matrix induced similar trends in EDV and ESV (120.79 ± 9.5 µl and 97.02 ± 10.2 µl), however not to the extent of CMPC containing patches (figure 4A+B). Furthermore, on average the mice treated with a patch (both empty, 19.96 ± 2.38 % and CMPCs, 17.44 ± 2.06 %) had an increased EF compared to control (11.41 ± 2.51 %), however these fell slightly out of the range of significance (figure 4C).

The improved effect on cardiac remodelling in the CMPCs- matrix group was also confirmed at a histological level by measuring infarct wall thickness (figure 4B). Mice treated with matrix and CMPCs showed a significantly thicker infarct wall as compared to control mice (587.5 ± 103.74 µm vs 253.4 ± 30.04 µm), and a trend towards thicker wall thickness compared to the empty matrix group 457.5± 12.17 µm (figure 5A). The empty matrix treatment also increased infarct wall thickness significantly compared to the control group (587.5µm vs 253.4µm, respectively). To evaluate whether the matrix and the CMPCs are able to preserve endogenous cardiomyocytes viability in the infarct region, tissue sections were stained with Masson's Trichrome staining (figure 5D). Treatment of the myocardial infarction with matrix and CMPCs significantly increased the amount of viable tissue in the infarct as compared to empty matrix and control group (33.9% vs 31.4% and 29.4%, respectively). Empty matrix did not significantly improve viability in the infarct compared to control (figure 5C). PCR analysis performed on cryosections by using human specific primers confirmed that CMPCs still expressed VEGF and IGF-I at a gene level 4 weeks after transplantation (figure 5E), suggesting the possibility for paracrine signalling towards the endogenous myocardium.

To evaluate the potential of the CMPCs to stimulate vascularization in the infarcted region and the border zone, tissue cryo-sections were stained for CD31 (PECAM) and smooth muscle actin but no differences were found in the number of vessels in the infarct- and border-zone as determined by CD31+ / SMA+ double positive staining (suppl. figure. 2).

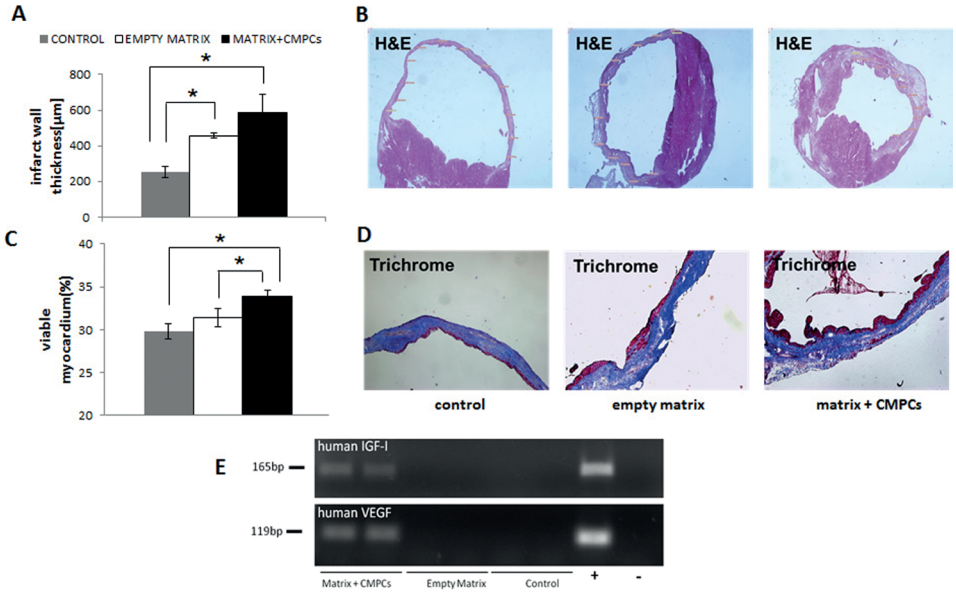

Figure 5 Effect of matrix application on the myocardium. Treatment of the myocardial infarction with matrix+ CMPCs significantly increased the wall thickness (A+B) and viable myocardial tissue (C+D) in the infarct compared to control. This was established by H&E and Masson's Trichrome staining respectively. After isolation of RNA from the hearts, PCR revealed the presence of human VEGF and IGF-I 4 weeks after transplantation only in mice which were treated with the cellularized matrix (* $p<0.05$).

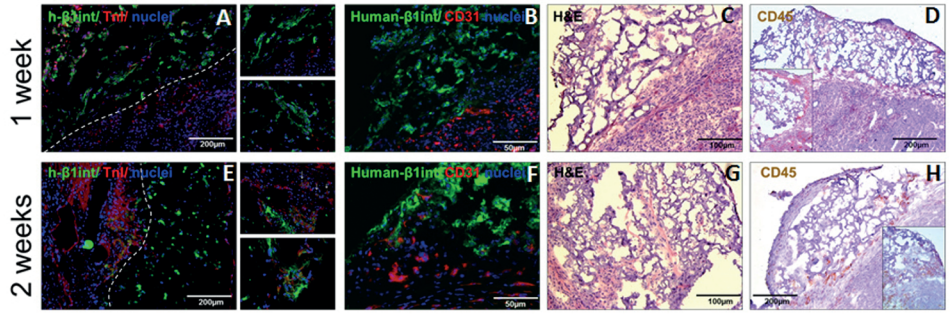

Figure 6 *in vivo* CMPCs characterization at one and two weeks. At 1 week after transplantation only few CMPCs (human integrin-β1; h-β1int) were positive for cardiac troponin (TnI) (A) and their number increased at 2 weeks (E), while CD31 expression in CMPCs remained low at both time points (B+F). H&E analysis showed an optimal integration of the transplanted patch along the ventricular wall (C+G). Furthermore, CD45+ cells were predominantly present in the infarct region and on the border of the biocomplex with only few CD45+ cells migrating inside the scaffold (D+H).

In vivo CMPCs characterization

In vivo histological analysis was carried out at week 1, 2 and 4 to evaluate the fate and the phenotype of transplanted cells. Detailed H&E analysis showed an optimal integration of the transplanted patch along the ventricular wall in all time-points analyzed (figure 6 and 7 C+G). At 1 week only few CMPCs were positive for TnI (figure 6A) and their number increased at 2 weeks (figure 6E). After 1 month, a large number of transplanted CMPCs differentiated even further, as demonstrated by co-expression of human specific markers (β-integrin and Lamin A/C) and TnI (figure 7A-E). Among the TnI+ CMPCs, some of them showed a sarcomeric structure indicating that cells were able to differentiate into cardiomyocytes (figure 7B+C), other transplanted CMPCs showed an intermediate phenotype between an immature cardiac progenitor cell and a fully mature cardiomyocytes (figure 7E+G) as demonstrated by Nkx2.5 expression (figure 7M). Expression of Cx43 was also detected 4 weeks after transplantation, indicating a maturation process towards cardiomyocyte phenotype and internal coupling of the transplanted CMPCs (figure 7L).

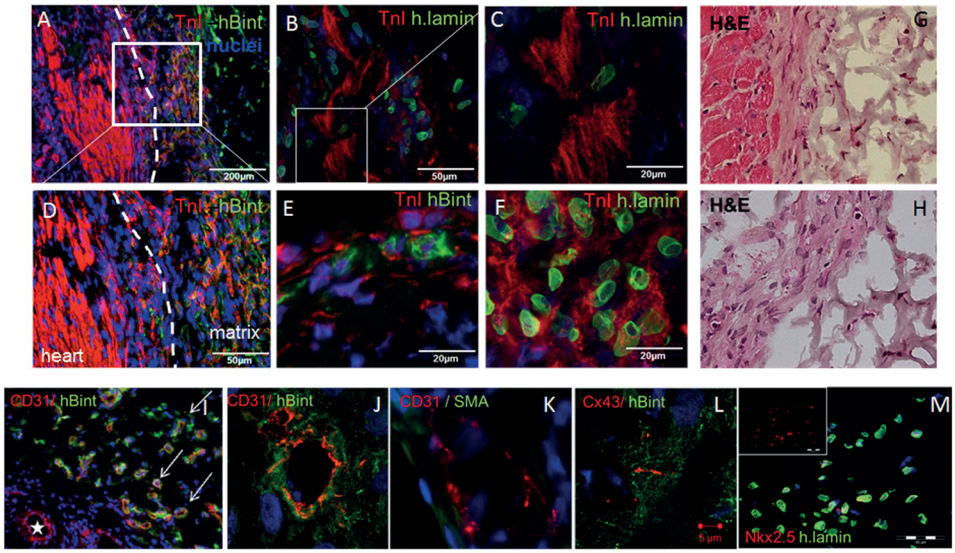

Figure 7 *in vivo* CMPCs characterization at four weeks. In the patch, a large number of transplanted CMPCs (human integrin-β1; hBint or human Lamin A/C; h.lamin) co-expressed cardiac troponin (TnI) (A-F). A few TnI+ CMPCs organized into sarcomeric structures (B+C). H&E analysis showed an optimal integration of the transplanted patch along the ventricular wall (G+H). CD31+ CMPCs were found in the matrix (I+J), and formed tubular structures. Some SMA+ cells were visible surrounding CD31+ endothelial cells (K) indicating vessels formation within the patch. Expression of Cx43 was also detected in CMPCs (L) indicating a maturation process towards cardiomyocyte phenotype, however other transplanted CMPCs showed an intermediate phenotype as indicated by Nkx2.5 expression (M).

We also evaluated whether transplanted CMPCs differentiated towards the vascular lineage. CD31 expression was very low at 1 and 2 weeks after transplantation (figure 6B+F). Interestingly, as observed for TnI expression, after 1 month of transplantation CD31+ CMPCs increased in the transplanted patch, with circular expressions patterns, suggesting vessel-like structures (figure 7I+J). Occasionally, SMA+ cells were visible surrounding CD31+ cells indicating vessels formation within the patch (figure 7K). Lastly, to evaluate the inflammatory response to the transplanted patch, we investigated the presence of CD45+ cells within the scaffold. We found that CD45+ cells were predominantly present in the infarct region and on the border of the biocomplex with only few cells migrating inside the scaffold. After 14 days, we did not observed a further increase of endogenous CD45 cells indicating that the transplanted patch did not induce any sustained inflammatory response in the host (figure 6D+H).

DISCUSSION

Recent evidence has accumulated showing that stem cells delivered to the heart have limited retention and poor survival in the myocardium [13, 14, 21]. The cells can fail to engraft and get washed out by vascular channels. For those that are retained, the new environmental surroundings are often detrimental to their viability and may not be inductive for the most optimal therapeutic cellular response. In this respect, tissue engineering has proven to be an effective method to improve the delivery, engraftment, and differentiation of stem cells in the injured heart [22]. For such applications, biomaterials have to be carefully selected and constructed to provide proper support and guidance to stem cells upon transplantation [23, 24]. Biological derived materials have been extensively investigated and used based on their intrinsic properties to support cell attachment and growth, but also for their biocompatibility limiting graft rejection and promoting integration and degradation after implantation.
In the present study, we designed a cardiac patch consisting of hyaluronic acid/gelatin matrix and CMPCs. The porous 3D printed design supported the attachment and growth of CMPCs, while not affecting their cardiac differentiation potential *in vitro*. In a mouse model of MI, the scaffold proved to be an excellent vehicle for cell survival, engraftment, and the induction of differentiation, which led to increased myocardial tissue viability and improved cardiac function.

Matrix application improved cardiac function

As previously reported in other studies [25-27], cardiac repair was observed in hearts which received a biomaterial or matrix only. A material can provide cardio-protective effects as a mechanical wall support by thickening the infarct wall and reducing wall stresses, thereby preventing negative LV remodeling [28]. In our study, it is evident from both MRI and histological data that our hyaluronic acid/gelatin matrix provided similar beneficial effects. However, cellularizing the biomaterial patch with CMPCs had an additional advantage for the injured myocardium. On a histological level, patches containing progenitor cells were able to better promote pro-survival signaling for cardiomyocytes, improving the overall viability of the tissue. Furthermore, the addition of cells had a positive effect on adverse remodeling, leading to smaller

EDV and ESV, and thicker LV walls. Similar beneficial therapeutic performance of cell-laden patches have been shown in several other studies. Chi *et al.* [29] showed that the incorporation of mesenchymal stem cells markedly improved LV remodeling and myocardial viability compared to treatment of silk fibrin/hyaluronic acid patch alone, while added systolic contractility was noted by Qiuan *et al.* [30] in epicardially grafted cell patches.

Since very few cells invaded and coupled with the native myocardium in our study, it is unlikely that the basis for the observed effects was the formation of new force-generating cardiomyocytes from the progenitors in the patch which contributed to the contractility of the injured heart. Therefore, it seems that indirect contribution from paracrine factors were behind the observed therapeutic effects. In this regard, we were able to detect expression of human-specific VEGF and IGF-1 in the hearts that received the cellularized patch, showing that CMPCs were actively expressing paracrine factors one month post-transplantation. IGF-I in particular is a well-established cardioprotective growth factor since it is able to promote the survival of cardiomyocytes [31]. Nevertheless, pro-angiogenic growth factor VEGF appeared to have less of an effect, since no increase in tissue vascularization was observed in the endogenous myocardium. One additional difference between cell and cell-free patches that cannot be ruled out is the remodeling of the applied matrix, which can lead to different physical properties. The CMPCs are constantly transforming the matrix, as can be observed from the collagen stainings *in vitro*, which will affect matrix stiffness. Also the *in vivo* grafts retained a better shape when cells were present in the patch. In this respect, recently published data showed that intramyocardial administration of a stiffer matrix led to better functional recovery after acute MI [32]. Even though the delivery route is different, it highlights the need for future investigation into the therapeutic effects of matrix stiffness for epicardial patches as it is well known that stiffness influence stem cell behavior and differentiation [33] [34].

Cardiac and vascular differentiation of CMPCs after transplantation

The histological characterization of CMPCs after transplantation revealed dynamic temporal changes in their phenotype. Starting with a few TnI+ positive human cells in the matrix in the initial weeks, we noticed progressive increase in the number of positive cells over time together with a more mature cardiomyocyte-like phenotype. For the vascular differentiation of CMPCs, a similar progressive differentiation was observed which resulted in humanized CD31+ tubular structures containing lumens after on month. Since this spontaneous differentiation was not observed in the matrix *in vitro*, these effects are likely arising from exposure to environmental cues. Mechanical forces, such as contractile stimulation, and electrical fields present in the heart can be translated into biological responses and have proven to be efficient at progressing the *in vitro* differentiation and maturation of stem cells [35-37]. Furthermore, CMCPs might also be triggered by biological factors such as TGF-beta, VEGF or hypoxia to start their differentiation into endothelial lineage.

Similar effects were noted in a recent study with adipose cardiac-derived progenitor cells [38]. Delivered epicardially in a fibrin patch, the cardiac and endothelial faith of these cells was monitored by bioluminescence imaging procedures, which showed differentiation into both lineages after transplantation. Nonetheless, not all epicardial delivery strategies observed this

effect. In a study by Hamdi *et al.* [39], a collagen-based patch harboring cardiomyogenic SSEA-1+ human embryonic cells was transplanted, and although cardiac markers were present in the patches before implantation, none of them could be subsequently identified in the grafted tissue *in vivo*. This is likely due to the different cell types used, or it could be the material itself that might be more conductive for the differentiation cues than others. Of interest to the present study, is to extend the follow-up time to check if the differentiation can be pushed even further under the cardiac contractile circumstances.

Clinical translation of designed tissue engineered approach

The developed cardiogenic matrix is highly adjustable to trials in larger animal models and translatable for clinical use. Since immune-deficient mice were used to prevent rejection of transplanted human cells, we were unable to evaluate the true immunogenicity of the material in the current study. Nonetheless, the thiol-modified hyaluronic acid and gelatin material used here is commercially available in GMP-grade quality under the name Renevia. The Spanish Agency of Medicines and Medical Devices (AEMPS) recently approved a clinical trial to test Renevia for subcutaneous delivery of autologous adipose derived cells in patients with lipoatrophies [40]. Furthermore, the 3D printed approach offers the possibility to fully customize the size and shape of the scaffold in order to accommodate future large animal studies, and even individualize the therapy to the specific pathological requirements of patients in the clinic. In addition, CMPC can be isolated directly from patients and be used as an autologous cell source for clinical application, as has been shown for clinical trials with other cardiac progenitor cells [10, 11]. However, this technique could easily be applied to other cell types or genetically modified cells. Due to the long term retention of cells on the myocardial surface, therapies could be designed with powerful paracrine secreting cells such as Akt modified MSC, which have been shown to produce potent cardio-protective factors [41]. Overall, the developed design can be uniquely tailored to various regenerative applications.

CONCLUSION

In this study, we developed an effective and translational approach to enhance CMPC delivery to the heart. The attachment of the cardiogenic 3D printed patch preserved heart function by reducing LV remodeling and improving myocardial viability. Future investigation follow-up studies will be needed to evaluate the progression of CMPC differentiation and overall therapeutic benefit over a longer follow-up period. Nonetheless, this study highlights the beneficial effects of incorporating biomaterials in order to advance regenerative medical applications with stem cells.

Aknowledgements

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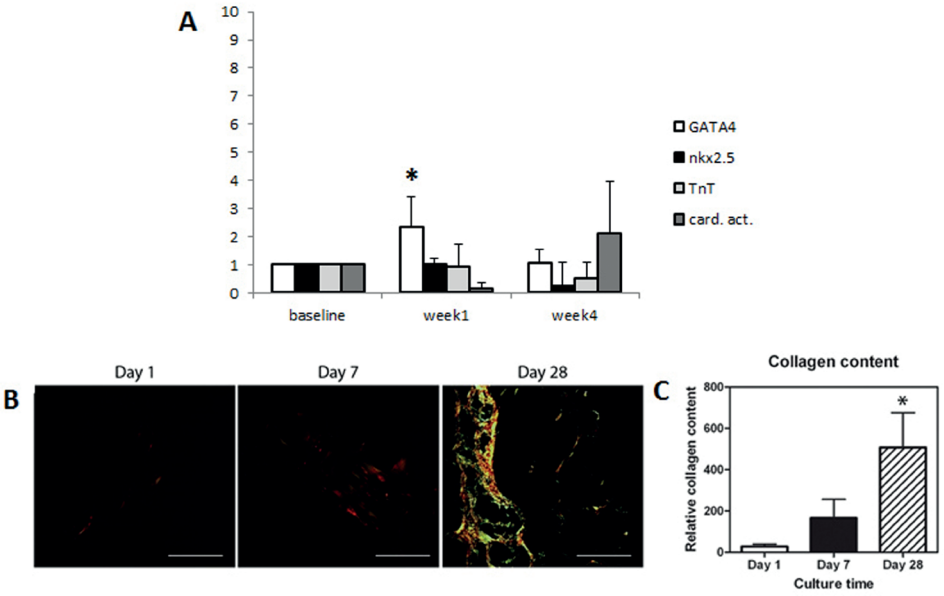
SUPPLEMENTARY

Supplementary Table 1 Primer sequences.

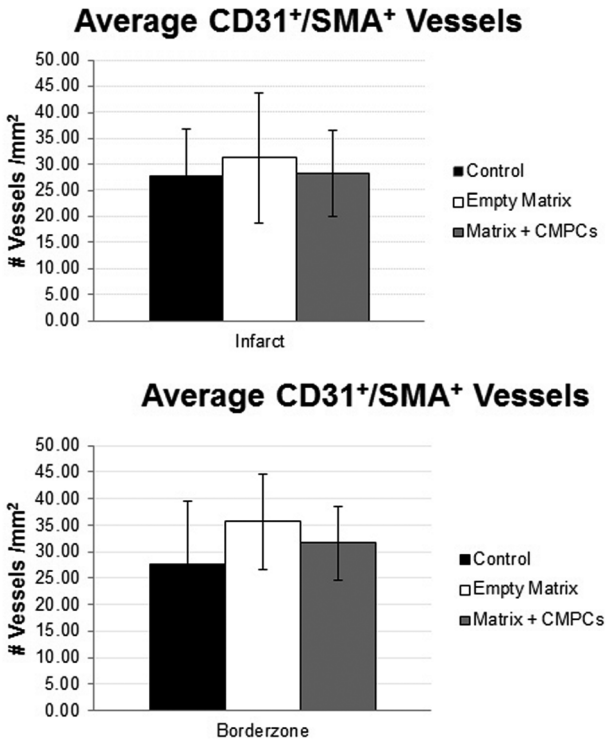
	Primer sequence	T
GATA-4.4 FW	GTTTTTTCCCCTTTGATTTTTGATC	59
GATA-4.5 RV	AACGACGGCAACAACGATAAT	59
hNkx2.5-4 FW	CCCCTGGATTTTGCATTCAC	59
hNkx2.5-5 RV	CGTGCGCAAGAACAAACG	59
ACTC1 F	GTCGGGACCTCACTGACTAC	60
ACTC1 R	CAATTTCACGTTCAGCAGTG	60
hTNNT2(1f)	GCGGGTCTTGGAGACTTTCT	60
hTNNT2(1r)	TTCGACCTGCAGGAGAAGTT	60
hVEGF Fw	CCTTGCTGCTCTACCTCCAC	62
hVEGF Rv	GCAGTAGCTGCGCTGATAGA	62
hIGF1 Fw	TCTGCACGAGTTACCTGTTA	62
hIGF1 Rv	CAATCTACCAACTCCAGGAC	62
hGAPDH Fw	CTCTGACTTCAACAGCGACA	59
hGAPDH Rv	TCTCTCTCTTCCTCTTGTGC	59

Supplementary Table 2 Antibody information.

Primary				
Target	**Host**	**Dilution**	**Company**	**Cat. Nr.**
Connexin 43	Rabbit	1:500	Zymed	71-0700
CD31	Rabbit	1:50	Santa Cruz	sc-1506
Integrin-β1	Mouse	1:50	Santa Cruz	sc-59827
Ki-67	Rabbit	1:100	Abcam	ab833
Lamin A/C	Mouse	1:100	Vector	VP-L550
Nkx2.5	Rabbit	1:200	Santa Cruz	sc-14033
Smooth muscle actin	Mouse	1:400	Sigma Aldrich	F3777
Troponin-I	Rabbit	1:50	Santa Cruz	sc-15368
Secondary				
Label	Host	Dilution	Company	Cat. Nr.
Alexa-488	Goat	1:400	Invitrogen	A11029
Alexa-555	Goat	1:400	Invitrogen	A21458
Biotin	Goat	1:1000	Vector	BA-1000



Supplementary Figure 1 Expression of cardiac markers (A) were analyzed by qPCR analysis at 1 day, and 1 and 4 weeks after printing and showed no difference in Nkx2.5, cardiac troponin (TnT) and cardiac actin (card. act.) in the embedded CMPCs. GATA4,however, had significantly higher expression after one week, but returned back to baseline after one month. Picosirius red staining of the cellularized matrix revealed collagen deposition by CMPCs, which increased significantly after 28 days (*p<0.05).



Supplementary Figure 2 Evaluation of vascularization in the infarcted region and border zone after patch application. Tissue sections were stained for CD31 and smooth muscle actin (SMA) but no differences were found in the number of vessels in the infarct and border zone as determined by CD31+ / SMA+ double positive staining.

Sustained retention of cardiomyocyte progenitor cells (CMPCs) on the myocardium by 3D printed patch

In preparation

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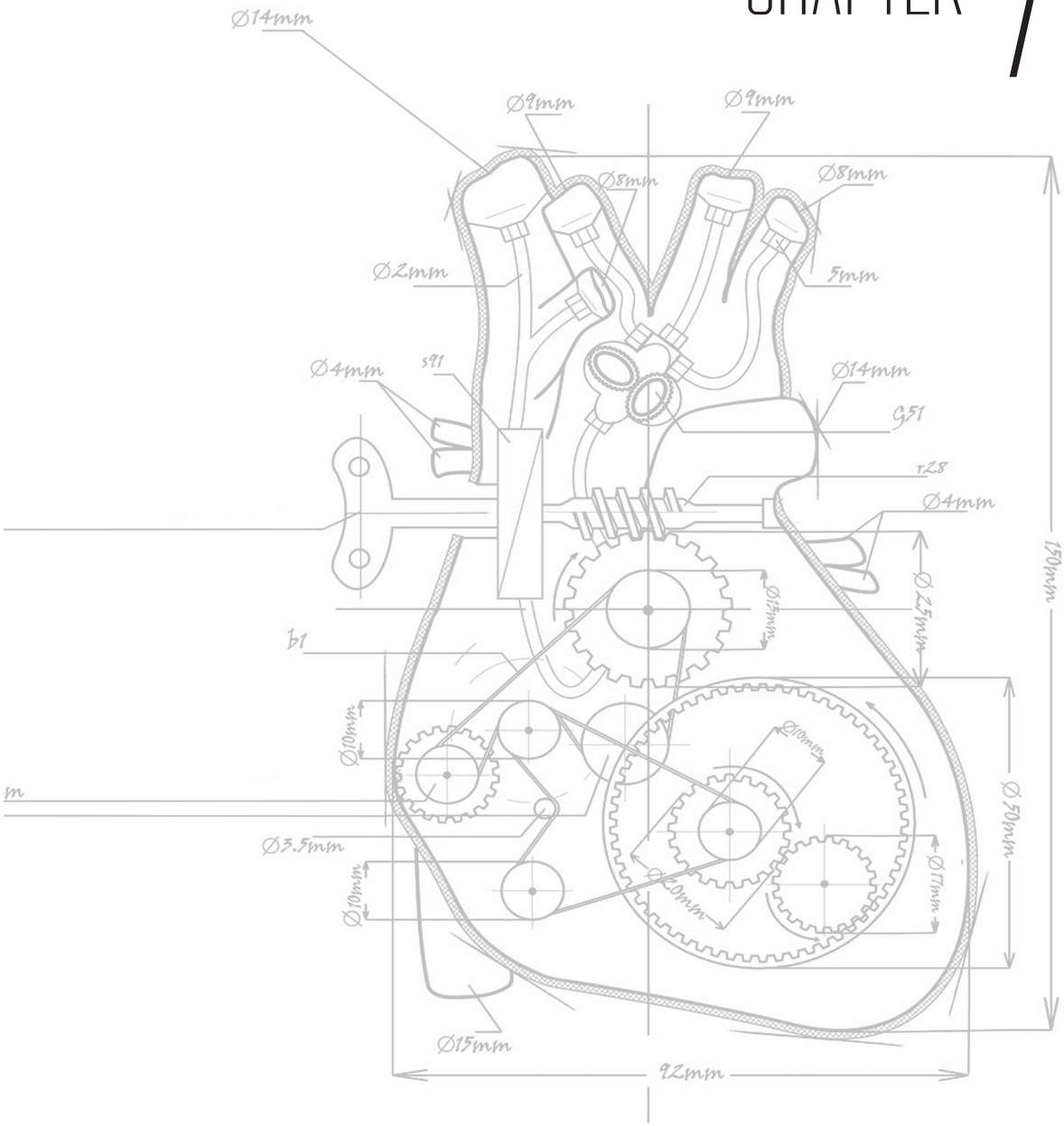
CHAPTER 7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ABSTRACT

Aim

Cardiac stem cell therapy aims to repair the heart after myocardial infarction (MI). Current stem cell therapy approaches lack efficacy, however, due to low stem cell engraftment, low stem cell survival and limited differentiation. Here, we evaluated the potential of a 3D printed patch, consisting of cardiomyocyte progenitor cells (CMPCs) and a hyaluronic acid/gelatin-based matrix, to improve long-term stem cell survival, differentiation, and cardiac repair after epicardial application in a mouse model of MI.

Methods and Results

Monitoring cell survival by bioluminescent imaging (BLI) demonstrated a stable engraftment of the CMPCs over the course of the experiment. After three months, histological analysis revealed the robust presence of CMPCs in the matrix, but no cells migrated from the epicardial patch into the myocardium. Furthermore, the cells did not fully differentiate into cardiomyocytes, but did participate in the formation of new vasculature in the matrix. The application of the patch did no significantly affect myocardial viability or vessel formation in the infarcted region but did improve wall thickness.

Conclusion

The delivery of CMPCs in a 3D printed cardiac patch is an efficient approach to increase the long-term cell survival and retention in the heart. Although it helped preserve wall thickness, poor matrix integration and cell differentiation/migration are areas of research requiring further refinements, in order to unlock the full therapeutic potential of cardiac tissue engineering approaches with CMPCs.

INTRODUCTION

Cardiovascular diseases are still a major cause of death in the western world [1]. Many of those deaths are caused by either the short- or long-term effects of coronary artery disease. After a myocardial infraction (MI), the cardiomyocytes downstream of the affected coronary vessel are soon following a path towards cellular death, leading to scar formation and decreased cardiac contractility. In response to the physical changes, the heart is forced to start a remodeling process in order to preserve its performance. However, paradoxically these changes can lead to adverse structural alteration, such as thinner walls and ventricle dilatation. Over time, adverse remodeling will cause a decrease in cardiac function, which can ultimately lead to heart failure [2]. Stem cell therapy is a regenerative approach which aims to replace the cardiomyocytes that were lost as a consequence of the MI. The restoration of the myocardium should hereby help prevent the adverse remodeling process and lead to better clinical outcome for patients [3]. Recent clinical stem cell studies have shown small, short term or transient improvements in cardiac function [4]. The disappointing results in cellular therapy so far have been attributed to low cell engraftment, survival, and differentiation in the infarct area.

Cardiac tissue engineering (CTE) is being explored in order to address the aforementioned shortcomings of stem cell therapy [5]. Biocompatible matrixes are the basis for CTE, and serve as an environmental support for the incorporated stem cells. The higher viscosity of the matrix, compared to a standardly used saline preparation, can reduce the early washout of cells that is observed after cardiomyoplasty [6]. As major cell death after transplantation has been noted to be caused by the inflamed and toxic environment within the infarct area, the matrix may also create a healthy environment in which the cells are protected from pro-apoptotic signaling. Furthermore, materials can also be designed with inherent cardiogenic properties [7], to promote the formation of new heart muscle by the injected stem cells. Besides the function of the matrix as a modulator of the stem cells, recent studies suggest that materials can provide biomechanical support to the infarcted wall [8, 9].

Several types of stem cells have been used in cardiac cell therapy applications, amongst them are a group of cardiac-present endogenous cardiac progenitor cells (CPCs) [10-13]. CPCs are self-renewing and multi-potent stem cells that are committed to the cardiac lineage, and thus have the ability to differentiate into cardiomycoytes, endothelial cells, and smooth muscle cells. Since these cells can be isolated directly from patients, they have attracted considerable clinical interest due to their superior differentiation potential compared to other adult stem cells. In this study, we used Sca1+ selected cardiomyocyte progenitor cell (CMPCs) [14], which have been shown to improve cardiac function after their transplantation. Nonetheless, these cells still suffer from a low engraftment rate, with only a few percent found back one month after transplantation [15].

A new approach for the construction of CTE constructs is 3D printing. This technology enables precise and layered production of CTE constructs, which can, for example, allow for superior nutritional diffusion and avoid necrosis at the center of thick matrices [16]. As previously described (chapter 6), we have created a 3D based construction system for the combination

of CMPCs with a biodegradable hyaluronic acid and gelatin matrix. Although the epicardial application of this cardiogenic construct led to reduced pathological remodeling and high cell retention one month after transplantation, the CMPCs did not undergo full differentiation and integration into the myocardium. In this study, we used the same setup to investigate the long-term, 3-month, therapeutic effectiveness of this cardiogenic patch. The aim was to determine if the 3D printed cardiac patch could retain CMPCs in the heart and stimulate their differentiation further over this longer follow-up time.

METHODS AND MATERIALS

CMPC isolation

CMPCs were isolated and propagated as previously described. For the use of human fetal tissue, individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht were obtained. CMPCs were transduced with a lenti-viral construct, containing pLV-CMV-luc-GFP, as described previously [15].

Tissue preparation by 3D printing

A Bioscaffolder tissue printer (Sys + Eng, resolution 5 µm/step, working area 200 x 150 x 90 mm) was used to print the HyStem matrix (with or without CMPCs) in a design created with the Rhinoceros software. Briefly, HyStem matrix (Sigma; HYSHP020) was prepared according to the manufacturer's protocol. CMPCs were combined with the matrix to a final concentration of $4x10^6$ cells per mL. Directly after addition of the crosslinker, the biocomplex was loaded into a syringe for use in the 3D printer. The scaffold was composed of 6 perpendicular layers, and each layer is made of 7 strands printed at a distance of 2.5 mm from each other. The construct had a final size of 2x2cm. This patch was cultured for a further 7 days in growth medium prior to in vivo transplantation. For additional detail see chapter 6.

Animals

All experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*, with prior approval by the Animal Ethical Experimentation Committee, Utrecht University.

Mouse model and matrix application

Female NOD-SCID mice, aged 10–12 weeks, were anesthetized (Fentanyl 0.05 mg/kg; dormicum 5 mg/kg; domitor 0.5 mg/kg) and myocardial infarction (MI) was induced by ligation of the left coronary artery, as described previously [17]. In this study, the mice were divided into three groups; control (LAD ligation only), empty matrix (HyStem matrix without CMPCs), and CMPCs matrix (HyStem matrix with CMPCs). Directly after ligation the matrix was applied epicardialy with the aid of fibrin glue (Cryolijm, Sanquin).

Bioluminescent imaging (BLI)

The detection of emitted photons by transduced CMPCs was performed by the sensitive photon imager from Biospace Laboratory. BLI images were obtained with the Biospace CCD camera and analyzed by Photovision software (Biospace Laboratory), as described previously [15].

Histological analysis

Mice were sacrificed 3 month post-MI by cervical dislocation; hearts were flushed with PBS, embedded in Tissue Tek (Sakura), and cut into 7 µm cryosection.

Haematoxylin & Eosin (H&E)

Cryosections were fixed in 4% paraformaldehyde (PFA) followed by staining in haematoxylin solution (Boom) and subsequently in eosin solution (BDH). Sections were thereafter dehydrated and mounted with Entellan (Merck). Wall thickness was measured with Cell^P 2.8 software.

Masson's Trichrome

Cryosections were fixed in Bouin's fixative, and then in 4% PFA. After fixation, slides were stained in Weigert's Haematoxylin (Sigma), biebrich scarlet acid fuchsin solution (Sigma), phosphotungstic / phosphomolybdic acid (Sigma), aniline blue (BDH), and then incubated in acetic acid before being dehydrated and mounted with Entellan (Merck). Stainings were analyzed with Cell^P 2.8 software and quantified with ImageJ 1.46 software.

Immunofluoresence

All sections were numbered to identify the position of human grafts. Sections were stained as previously described [15] using the following antibodies: human integrin-β1 (Santa Cruz), human lamin A/C (Vector), troponin- I (Santa Cruz), CD31 (Santa Cruz) and Alexa488- and 555-labelled secondary antibodies (Invitrogen).

Statistical analysis

All values are presented as mean ± SEM. Analysis was performed with GraphPad Prism 5.0 and groups were compared with one-way ANOVA with Bonferroni post-hoc test. A difference with a p-value < 0.05 was considered significant.

RESULTS

Assessment of CMPC survival

Directly after permanent LAD ligation, the printing matrix was applied epicardially over the injured murine myocardium. In order to monitor CMPC survival over the course of three month, we used BLI technology to acquire light emission from the luciferase transduced CPCs (figure 1A). Measurements were performed at 14, 28, 56 and 84 days post-transplantation (figure 1B). Signal was detected throughout the follow-up time, although we noticed a steep decline between two and four weeks. However, after one month the signal stabilized and sustained similar levels until the end of the experiment.

On histological level, the CMPCs containing matrix was traced by its characteristic sponge-like architecture on the epicardial surface of the heart (figure 1C). Although variation in the matrix application between the mice was observed, in all cases the patch covered at least a part of the infarcted myocardium. Engrafted CMPCs were identified in histological sections with a human-specific antibody recognizing lamin A/C. Individual human cells were found populated throughout the matrix (figure 1D). However, no migration of CMPCs into the myocardium was observed.

Faith of transplanted CMPCs

Subsequently, we evaluated the status of the CMPCs after transplantation by co-staining cardiac and vascular differentiation markers with human specific integrin-β1. For their cardiogenic differentiation, we used an antibody against troponin-I to visualize the presence of sarcomeric elements in the cells. Although some troponin-I expression was observed in the CMPCs, it was not as abundant or organized as that seen in the native myocardium (figure 2A). In certain locations in the matrix, there were human grafts exhibiting higher troponin expression (figure 2B). Nonetheless these cells were not fully matured and lacked the phenotypic sarcomeric organization characteristic of cardiomyocytes.

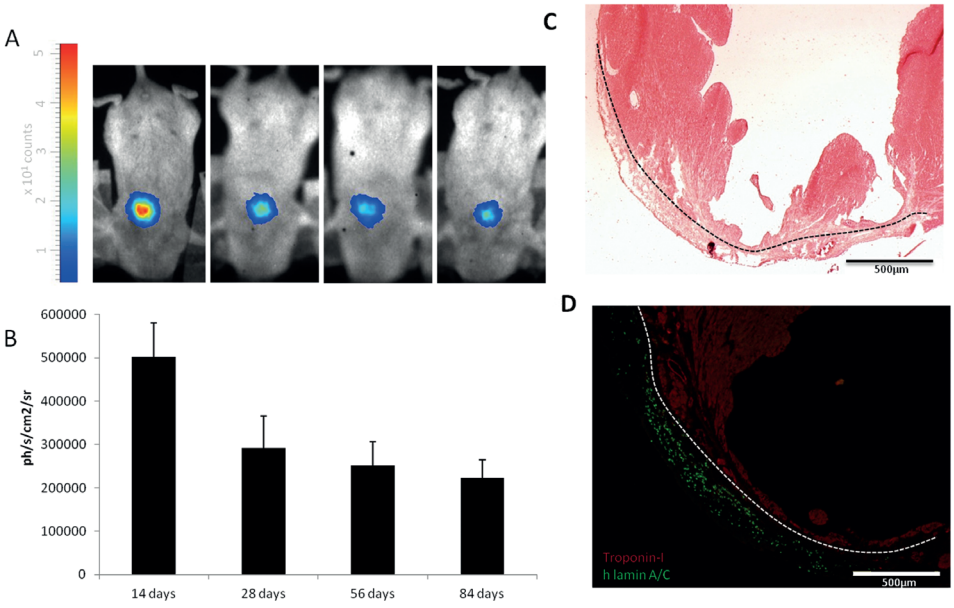

Figure 1 Assessment of *in vivo* CMPC survival. Representative BLI images (A) of mice (n=4) which received matrix CMPCs after LAD ligation. Quantitative analysis (B) of BLI signal demonstrated survival of CMPCs up to 3 month post-transplantation. Histological analysis (C) of application showed presence of matrix on the epicardial surface of the heart after three months. Representative fluorescent image of human specific lamin A/C (green) (D) in the murine myocardium, showed a robust presence of human cells after 3 months (dashed line outlines border between matrix and myocardium).

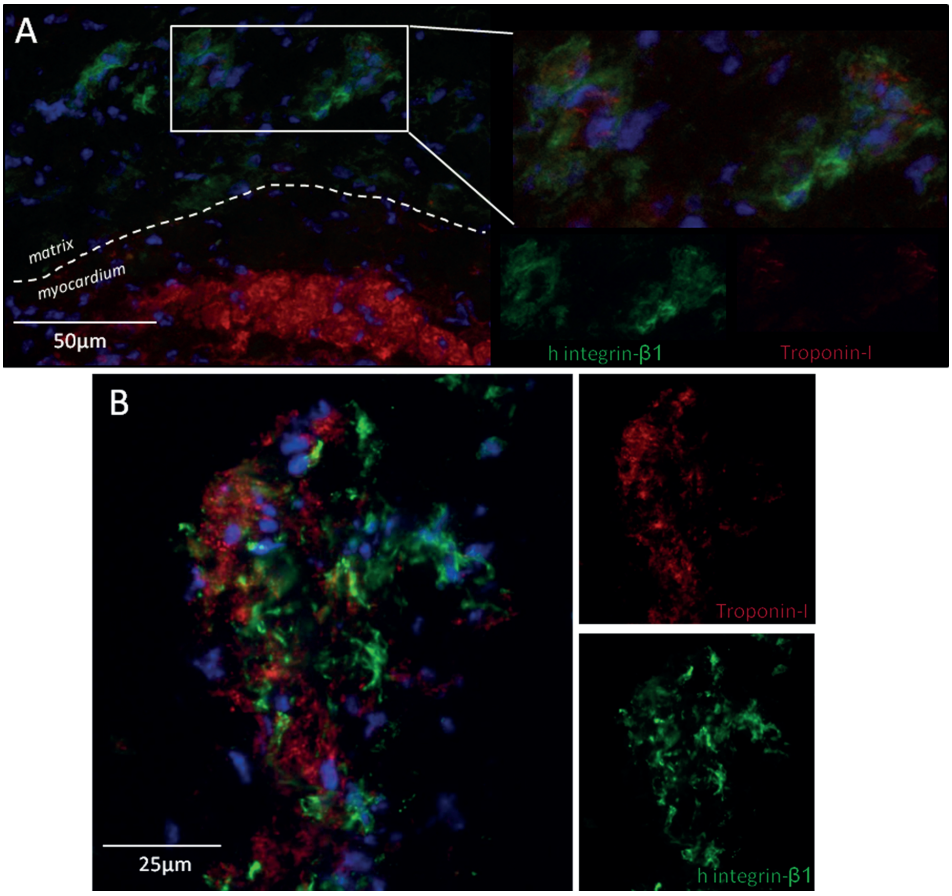

Figure 2 Histological analysis (troponin-I, red; human integrin-beta1, green) of cardiogenic differentiation in human grafts 3-month post-transplantation. On the border (dashed line) of matrix and myocardium clear differences are observed between the endogenous expression of troponin-I in the cardiomyocytes and the partially differentiated CMPCs (A). In certain locations in the matrix, there were human grafts exhibiting higher, but not organized, troponin-I expression (B).

Furthermore, we assessed the vascular differentiation by co-staining the treated hearts with CD31, with which we could observe the formation of fully humanized-vessels in the matrix. The vascular differentiation did not occur homogenously throughout the patch and was limited to local areas in the matrix which possessed an abundance of CMPC derived vessels. While not as organized in comparison to their endogenous mouse counterparts (figure 3A), these vascular structures displayed high levels of CD31 expression and containing a tubular organization surrounding a lumen (figure 3B). Since we did not observe the presence of erythrocytes within the new vasculature, it remains unclear whether these *de novo* generated vessels functionally contributed in the supply of oxygen and nutrients to the matrix.

Effect of matrix application on the myocardium

Lastly, we studied the local effects of the matrix application on the myocardium. We used three groups to determine the efficacy of the treatment on the infarcted myocardium; no treatment (control), treated with matrix only (empty matrix), and treated with matrix containing CMPCs (matrix + CMPCs). The application of the cellularized matrix helped preserve cardiac structure, since wall thickness was significantly increased (402 ± 90 µm) compared to both control mice (253 ± 24 µm, p=0.046) and mice treated with the empty matrix (254 ± 30 µm, p=0.047) (figure 3B). Interestingly, the morphology of the transplanted acellular patch was thinner and contained more pores compared to the more compact and dense structure observed in the cellularized matrix group, perhaps indicating a higher susceptibility of the a-cellular matrix towards the actions of proteolytic enzymes.

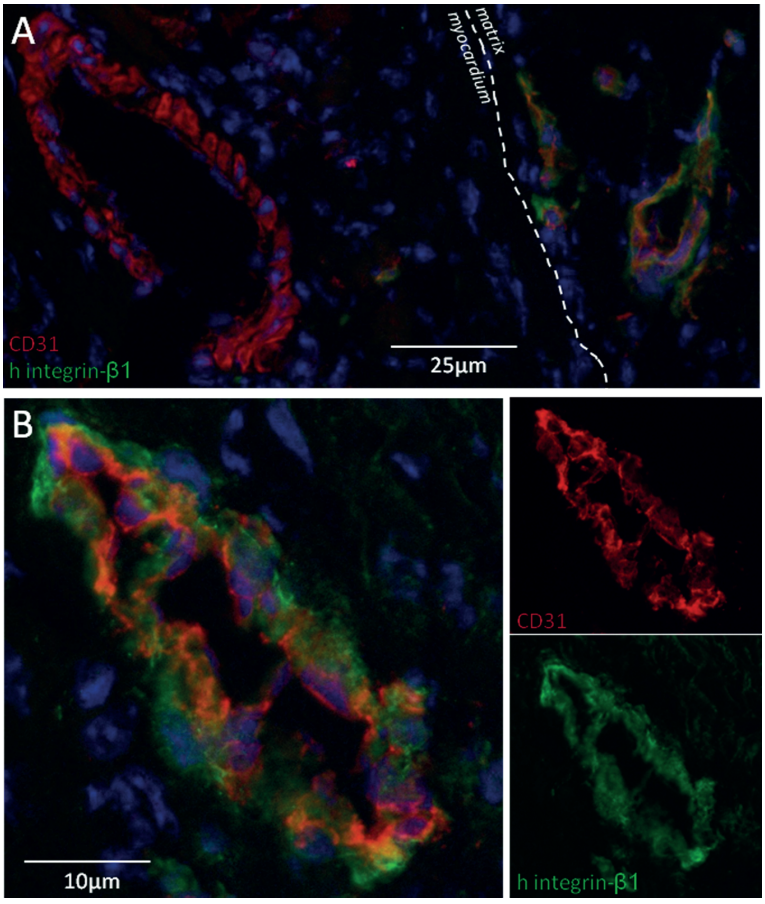

Figure 3 Histological analysis (CD31, red; human integrin-beta1, green) of vascular differentiation in human grafts 3-month post-transplantation. On the border (dashed line) of matrix and myocardium, humanized vessel structures were observed with similar CD31 expression compared to their murine counterpart (A). A close up view of the CMPCs differentiated vessel with clear tubular structure and lumen (B).

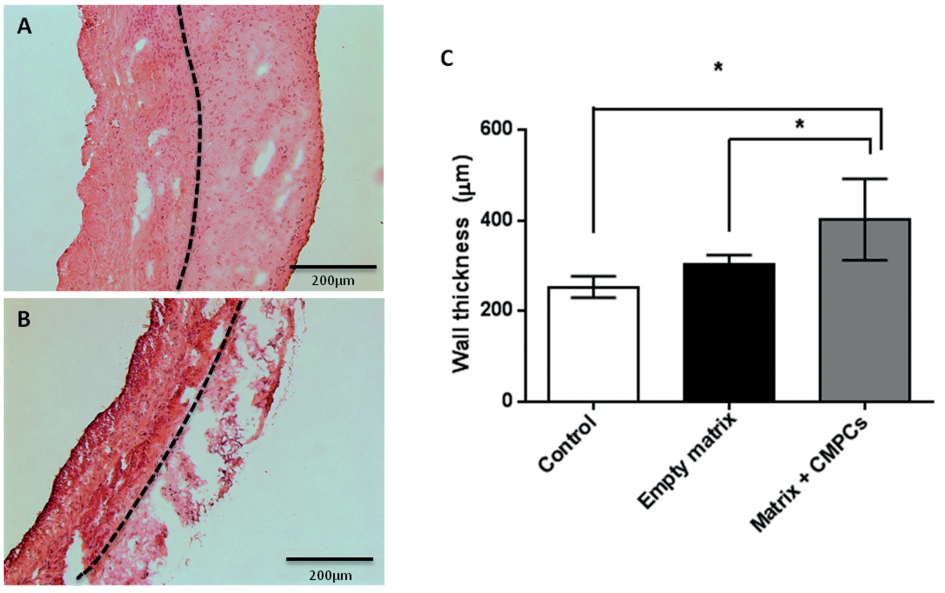

Figure 4 Histological analysis of matrix application and influence on wall thickness. Representative H&E images of mice treated with either matrix containing CMPCs (A) or empty matrix (B). Quantitative analysis of wall thickness (C) showed a pronounced increase in mice treated with matrix CMPCs (n=5), while the no difference were observed between empty matrix (n=3) and control (n=3).

A staining for CD31 and smooth muscle actin was performed in order to determine if the matrix and CMPCs could increase vascularisation in the infarct area (figure 4A). The mice treated with matrix and CMPCs showed a trend towards increased vascularisation (26 ± 5 vessels/mm^2) compared to control mice (17 ± 2 vessels/mm^2) in the infarct area although this difference was not significant (figure 4B). Interestingly, the material alone was sufficient to promote a similar therapeutic effect (27 ± 5 vessels/mm2) in the injured myocardium. Furthermore, Masson's trichrome staining was performed (figure 5A) to determine if the application of the matrix and the CMPCs could protect the viability of the myocardium. The staining revealed a slight trend towards a decrease in viable myocardium in mice treated with matrix and CMPCs (28.1 ± 6.7%) and empty matrix (27.3 ± 3.2%) compared to non-treated animals (30.9 ± 4.4%) (figure 5B). However this difference was found to be non-significant.

DISCUSSION

Cardiac tissue engineering is an emerging field of research that aims to utilize biocompatible materials in order to advance stem cell based regenerative medical approaches. In the present study we evaluated the potential of a 3D printed patch, consisting of cardiomyocyte progenitor cells (CMPCs) embedded in a hyaluronic acid/gelatin-based matrix, to improve long-term stem cell survival, differentiation, and cardiac repair after epicardial application in a mouse model of MI.

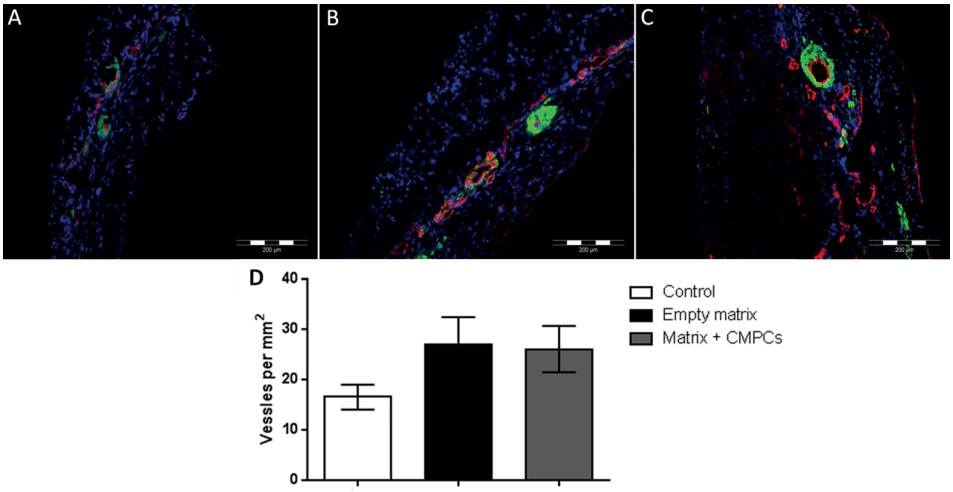

Figure 5 Assessment of vascularization in the injured myocardium. Representative CD31 (red) and SMA (red) fluorescence images of control mice (n=3) (A) and those treated with either empty matrix (n=3) (B) or matrix CMPCs (n=5)(C). Quantitative analysis of vascularization (D) showed an increased response in mice which received both empty and CMPC matrix.

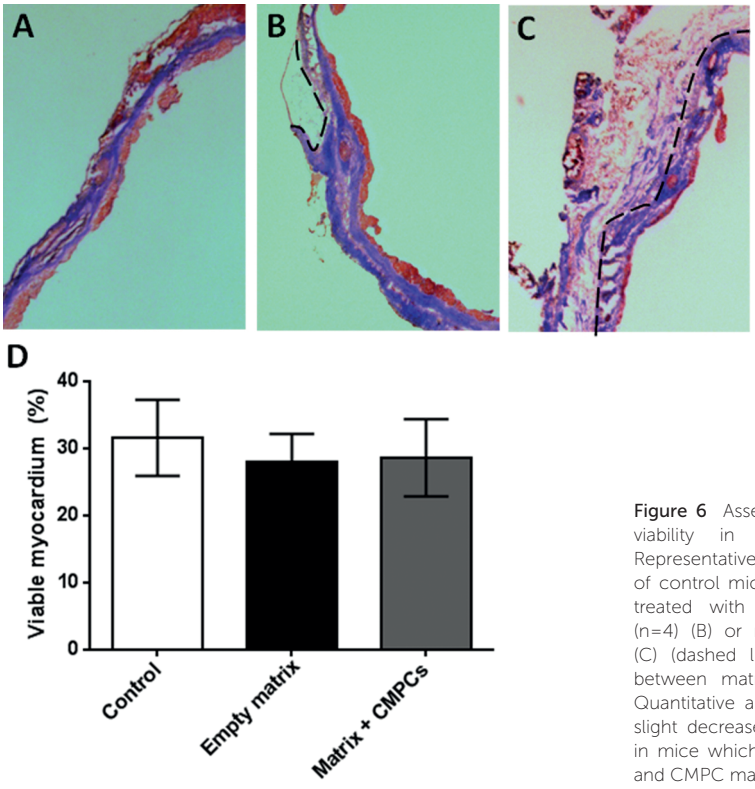

Figure 6 Assessment of myocardial viability in the infarct region. Representative trichrome images of control mice (n=4) (A) and those treated with either empty matrix (n=4) (B) or matrix CMPCs (n=6) (C) (dashed line represents border between matrix and myocardium). Quantitative analysis (D) showed an slight decrease of viable myocardium in mice which received both empty and CMPC matrix.

Although the designed 3D printed matrix proved to be efficient at improving CMPC delivery and survival to the heart, no clear long-term therapeutic effect was observed on myocardium. As indicated by the stable BLI signal, the epicardial patch application sustained the long-term survival of cells by providing them with an environmental support after transplantation. Since the detection of photons emitted by the luciferase CMPCs is restricted by tissue thickness, the drop in signal over the course of the first month is likely attributed to the dramatic weight gain in the mice during their recovery from surgery (although cell death cannot be excluded). Nonetheless, the robust stem cell presence after three months indicated that the 3D designed matrix allowed sufficient nutritional diffusion and structural support to promote the survival of the majority of the transplanted cells. These observations are in agreement with others like Cheng et al [18] and Tokunaga et al [19], strengthening the suggestion to use CTE to effectively improve the targeting of cardiac progenitor cells to the heart.

Nonetheless, the long-term therapeutic efficiency of the approach was not evident. Even though we observed an increase in wall thickness and established a trend towards increased vessel formation in the infarcted myocardium in patch treated animals, there was no overall benefit for viability of the tissue. Strangely, even a slight decrease in myocardial viability in the mice treated with the patch (both with and without cells) was observed. The exact nature of this observation is unknown, especially after data from our one-month study showing improved cardiac performance and viability. This might be linked to the asymmetrical matrix application resulting in uneven structural support for the injured myocardium, leading to increased wall stress on the infarcted areas devoid of matrix, and thereby promoting adverse remodeling over the longer follow-up period, and thus neglecting the beneficial effects noted in the previous study. In this case, it could be an important observation for the potential side-effects of cardiac patch applications, which will need to be carefully evaluated and confirmed in future follow-up studies. On the other hand, the lack of clear differences might be due to the transient nature of the therapy as observed in earlier stem cell therapy studies, using cardiac-differentiated embryonic stem cells [20]. Alternatively, although the CMPCs remained alive, they might lose their potency to exert therapeutic effects on the myocardium, such as the active secretion of paracrine factors.

Furthermore, the matrix did not fully integrate with the myocardium, since it sponge-like architecture was still detected on the epicardial surface. Although the cellularized scaffold had a denser "tissue-like" physical property compared to the empty matrix, CMPCs maintained their residence in the matrix and did not establish an interaction with the native myocardium. A potential obstacle for CMPC migration could have been the barrier created by the fibrin glue, which was used to fix the patch to the myocardium. However, in literature fibrin has been shown to support cellular invasion [21-23] and thus was unlikely to be the culprit for this effect. Therefore, it seemed that the hyaluronic acid based matrix did not promote cellular migration out of the patch, thereby possibly losing the inherent ability of cardiac derived stem cells to response to damage signals from the heart [24]. Additionally, physical and environmental cues from the myocardium were not sufficient to stimulate cardiac differentiation inside the patch, as was previously shown upon intramyocardial injection of CMPCs [14]. Since CMPCs inside the matrix were able to respond to *in vitro* cardiac-differentiation stimulation (chapter 6), it is

likely that their epicardial location was not inductive for the desired cardiogenic response. Therefore, to generate *de novo* cardiomyoctes with our currently designed approach, an *in vitro* differentiation and maturation step will be required prior to transplantation.

The observed vascular differentiation of CMPCs falls in line with a recent study in which showed the predisposition of c-kit selected cardiac progenitor cells to contribute more to cardiac endothelial cell turnover rather than cardiomyocytes [25]. Even though the humanized endothelial cells had the phenotypic vessel appearance (tubular organization), it was unclear if they were functionally coupled to the myocardium. Nonetheless, an interesting observation was that stem cell generated vessels were present in confined areas of the matrix. Therefore, these cells must have been exposed to unique set of cues that were absent for the other transplanted cells. The signals could be related to the material properties (e.g. high mechanical stress), environmental factors (e.g. hypoxia), or unique CMPC interaction in that particular area. Further investigation into these underlying triggers could be utilized to fortify future tissue engineering applications that often lack proper diffusion of nutrient and oxygen [26-28].

In conclusion, the delivery of CMPCs in 3D printed cardiac patch is an efficient approach to increase cell survival and retention in the heart. Although it helped preserve wall thickness, poor matrix integration and cell differentiation/migration are areas of research requiring further refinements in order to unlock the full therapeutic potential of this approach. Closer investigations into materials that can better accommodate the desired cellular response, while providing similar environmental support as the presently used hyaluronic acid/gelatin, would be subject for future research.

Funding

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Gelatin microspheres as carriers for cardiac progenitor cell (CPC) and growth factor to the ischemic myocardium

In preparation

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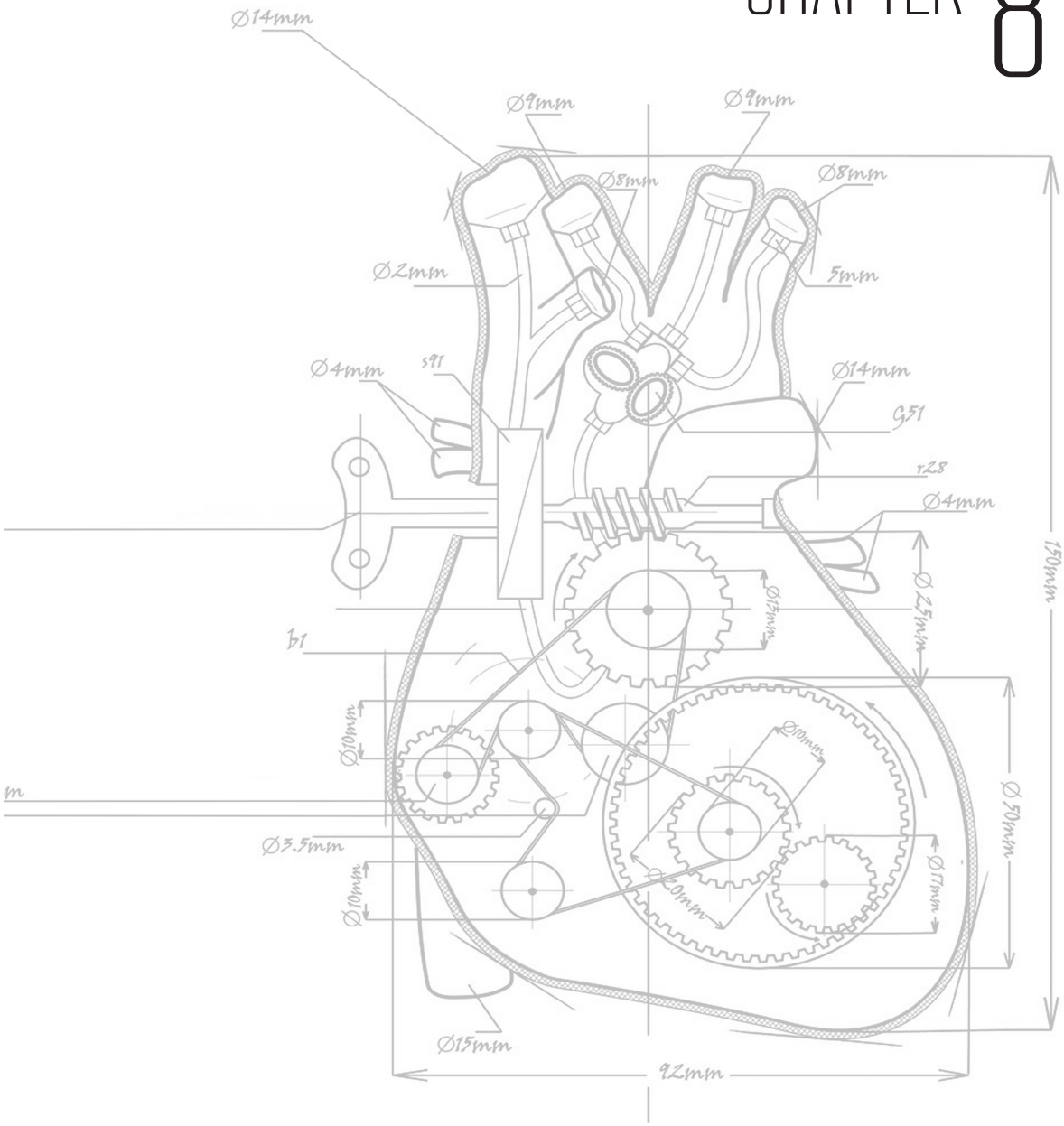
CHAPTER 8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ABSTRACT

Aim

The poor cell retention and survival in classical cardiac stem cell therapy approaches seem to be reducing the therapeutic efficacy of the injected stem cells. In order to ameliorate their regenerative effects, various biomaterials are being investigated for their potential directive and supportive properties. Here, we aimed to utilize gelatin microspheres (MS) as micro-carriers for the co-delivery of cardiac progenitor cells (CPCs) and growth factors to the myocardium

Methods & Results

The gelatin MS, generated from a water-in-oil emulsion, were able to accommodate the attachment of CPCs, and support their cardiogenic potential. In a mouse model of myocardial infarction, we demonstrated the ability of these micro-carriers to substantially enhance cell engraftment in the myocardium as indicated by bioluminescent imaging (BLI) and histological analysis. Furthermore, we also showed the ability of micro-carriers to incorporate IGF-I and HGF, which promoted pro-survival signaling and proliferation of the attached CPCs.

Conclusion

Overall, we developed a simple and effective translational approach to increase the retention of CPCs in the myocardium. Furthermore, the ability to incorporate IGF-I and HGF into the microspheres should further promote the endogenous and exogenous regenerative properties of the therapy.

INTRODUCTION

Although classical cell therapy approaches for the injured heart show therapeutic promise by improving cardiac performance, poor pharmacokinetics seem to be impeding the full regenerative effect of injected stem cells [1]. Independent of the delivery route, cell injection results in only 10 to 15 percent acute engraftment in the myocardium [2]. The highly vascularized organ seems to be the culprit of this effect, washing away the cells before they have time to engraft. Furthermore, the small percentage of myocardially retained cells is directly confronted with a hostile ischemic environment, resulting in a further decrease of surviving stem cell numbers. Within a few days, the presence of cells is reduced to just a few percent of the originally injected dose [3, 4]. Therefore, the development of new approaches is necessary to make cells less vulnerable to these processes and improve cardiac cell therapy.

In this respect, biologically compatible materials have been at the forefront of regenerative medical advancements [5]. Materials can contain beneficial cues to modulate stem cell behavior and optimize the desired cellular response, but can also provide cells with a protective environment directly after *in vivo* administration. In the cardiac arena, many initial approaches with materials have been shaped in the form of patches for epicardial application [6]. While exciting histological as well as functional data were presented, these approaches are limited in their clinical scope, since these large constructs require open heart surgery for their application. A more suitable delivery method is based on a minimally invasive catheter approach, often used already in early clinical phase studies for the intracoronary [7] or endocardial [8] administration of stem cells. Nonetheless, the increased viscosity of most cell-material constructs makes them very difficult to inject through the long and narrow lumen of the catheter. Researchers are currently designing tunable cross-linking systems that can circumvent this issue [9, 10]. These tunable systems can make use of the local physiological state of the myocardium (e.g. temperature, pH) for triggering a switch from a liquid injectable to a gel state, in order to ensure the encapsulation of the stem cells in the heart. Nonetheless, these approaches remain difficult to design, due to incompatibility between the cells and the usually harsh chemical nature of quick cross-linking materials.

Another approach for designing catheter compatible cell-material complexes are micro-carriers [11]. These small entities, such as microspheres (MS), are well-suited for catheter delivery systems, since their size allows them to travel unhindered through the catheter lumen. Furthermore, simple water-in-oil emulsion techniques allows for MS fabrication from natural materials [12]. Due to its low immunogenicity and biodegradability, gelatin has a well-regarded biocompatible nature. It has been widely used in the clinic for various applications as in for example the formulation of drugs [13] and sealant in vascular prostheses [14]. Additionally, over the past decades its unique biochemical structure has been investigated for the ability to sustain the release of bioactive substances [12]. Depending on the extraction method from collagen the process can yield either negatively or positively charged gelatin, enabling polyion complexation with oppositely charged therapeutics, such as growth factors. In this respect, successful slow-release strategies were developed using gelatin MS and various growth factors (bFGF [15], VEGF [16]).

In this study, we aimed to develop a micro-carrier based strategy for the delivery of cardiac progenitor cells (CPCs) and cardioprotective growth factors (IGF-I/HGF) to the heart [17-20]. Previously, we have shown that endogenous cardiac-derived Sca-1+ isolated CPCs have the capacity to both participate in and stimulate cardiac regeneration, leading to a robust increase in cardiac performance [4]. Nonetheless, these cells succumb to poor delivery and survival, resulting in ~1-3 percent survival after one month [3]. Gelatin MS, with an approximate size of 50-75μm, are perfectly fitted to function as catheter based micro-carriers, while their biocompatibility serves as a match for the loading of CPCs. The incorporation and release of IGF-I and HGF through MS should ultimately aid the survival of CPCs *in vivo*, but also act as therapeutic molecules for endogenous cardiac regeneration [21]. Overall, we think that this approach will eventually lead to better functional recovery and tissue healing after CPC transplantation in the ischemic myocardium.

METHODS AND MATERIALS

CPC isolation, culture, and transduction

CPCs were isolated and propagated as previously described [22]. For the use of human fetal tissue, individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht were obtained and conforms to the principles outlined in the Declaration of Helsinki for the use of human tissue or subjects. CPCs were transduced with a lenti-viral construct, containing pLV-CMV-luc-GFP as described previously [3].

Gelatin microsphere (MS) preparation

MS were prepared as previously described [23, 24]. Briefly, an aqueous 10 wt% solution of gelatin type B (Sigma; G9382) was made. This solution was added to heated olive oil, stirred at 400 rpm and chilled on ice while stirring. Speed was reduced to 300 rpm, chilled acetone was added and the solution was filtered. The gelatin microspheres were collected, washed in chilled acetone and sieved to separate 50-75μm gelatin MS. MS were cross-linked with 25% glutaraldehyde (Sigma; G5882) solution overnight and the reaction was stopped with glycine (100mM). Next, the gelatin microspheres were washed in chilled acetone again and freeze dried.

MS loading with CPC and growth factors

Prior to loading, gelatin MS were sterilized under UV for at least 30 minutes. To load the gelatin microspheres with IGF-I and HGF (Preprotech; 100-11 and 100-39), 5 mg 50-75μm gelatin microspheres were weighted and 5 μl of mixed growth factor suspension was added (500ng each) and incubated over night at 4°C. To load the gelatin MS with CPCs, either 3 mg gelatin MS were mixed with 5 million CPCs in one well of a 6-well ultra-low attachment plate, or 5 mg gelatin MS were mixed with 8 million CMPCs in a 10cm ultra-low attachment plate. The gelatin microspheres and CPCs were cultured together for 18-24 hours to let the

cells attach to the MS. CPC-laden gelatin MS were size selected and collected by running the suspension through a 100 µm cell strainer.

Monitoring CPC behavior on MS *in vitro*

For immunostaining, cultured CPC-MS microcarriers were prepared as stated above, embedded in Tissue-Tec, and cut into 7 µm cryosections. Sections were stained as previously described using the following antibodies: human integrin-β1 (Santa Cruz; sc-59827), Nkx2.5 (Santa Cruz; sc-14033), and Alexa488 + 555 labelled (Invitrogen; A11029 and A21429) secondary antibodies. CPCs-MS were stained with a live/ dead staining (Invitrogen, L-3224) directly upon culturing, according to manufacturer's protocol.

Differentiation of CPCs was performed by 5-aza and TGF-β stimulation as described previously [3]. Total RNA was isolated with TriPure (Roche; 11667157001) based on manufacturer's instructions. One µg of DNA-free RNA was taken to generate cDNA (iScript cDNA synthesis kit, BioRad 170-8891). Gene expression was established by quantitative PCR with the obtained cDNA (Sybr®Green Supermix Biorad 172-5006). ß-actin and GAPDH were used as housekeeping mRNA and Nkx2.5, GATA4, TnT, and ACTC1 were used as described previously [25].

MMP activity in medium collected from single CPCs culture or CPC co-culture with MS was established with zymography with small modification as described before [26]. In short, samples were mixed with Tris-Glycine SDS Sample Buffer and loaded on a 10% zymogram gel (Novex; EC61755). The gel was run for 120 minutes at 124V in Tris-Glycine SDS Running Buffer. The gel was recovered in Renaturing Buffer for 1 hour and completed in Developing buffer O/N at 37°C. All solutions were prepared according to manufacturer's protocol. The gel was stained in 0.1% Coomassie blue.

Animals

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, with prior approval by the Animal Ethical Experimentation Committee, Utrecht University.

Myocardial infarction and cell/microcarrier transplantation

Male NOD-SCID mice (Harlan), aged 10–12 weeks, were anesthetized (i.p.; fentanyl 0.05 mg/kg; midazolam 5 mg/kg; medetomidine 0.5 mg/kg) and myocardial infarction (MI) was induced by ligation of the left coronary artery, as described previously [27]. Before any incision was made, the adequacy of anesthesia was monitored by testing rear foot reflexes. Continual observation of respiratory pattern, rectal temperature, and responsiveness to manipulations was carried out throughout the procedure. Directly after MI, 20 µL phosphate-buffered saline (PBS) containing 1 × 106 CPCs or CPCs-MS, was divided over two injection-sites in the infarct-borderzone. Painkiller (s.c.; temgesic 0.15 mg/kg) was given to mice for 2 days (every 8 hours) post-operatively.

Bioluminescent imaging (BLI)

The detection of emitted photons by transduced CPCs was performed by the sensitive photon imager from Biospace Laboratory. BLI images were obtained with the Biospace CCD camera and analyzed by Photovision software (Biospace Laboratory) as described previously [3].

Histological analysis

Mice were sacrificed by cervical dislocation; hearts were flushed with PBS and fixed as described previously [4] and thereafter cut into 7 µm cryo-sections. All sections were numbered to identify the position of human grafts. Sections were stained as previously described [22] using the following antibodies: human integrin-β1 (Santa Cruz; sc-59827), human lamin A/C (Vector; VP-L550), troponin- I (Santa Cruz;sc-15368), and Alexa488- and 555-labelled secondary antibodies. For quantification, the presences of human grafts throughout the heart were scored as previously described [3, 4].

Statistics

Data is presented as mean ±SEM and were compared using the two-tailed paired Student's t test. Pearson correlation coefficient was used to assess correlation. A difference of $p<0.05$ was considered to be statistically significant.

RESULTS

Loading CPCs on micro-carriers

Gelatin MS were sieved out of a water-in-oil emulsion, resulting in the formation of a homogenous spherical population with a diameter of 50 to 75 µm (figure 1A). A minor presence of smaller MS was present in the preparation due to the imperfections in the sieving process. However, no MS larger than 75 microns were observed.

The loading of CPCs onto MS was performed on low-attachment surfaces. In long static culture settings, the binding of CPCs to MS led to the aggregation of the micro-carriers (suppl. figure 1), most likely due to the increased adhesiveness of the MS upon CPC attachment. In order to avoid this phenomenon, the preparation time was reduced to < 24hours and complexes were filtered (100 µm pore size) prior to therapeutic utilization. Cryo-sections of the prepared micro-carriers revealed the attachment of the CPCs to the outside of the MS (figure 1B).

The ratio between the amount of MS and number of CPCs was crucial in the binding dynamics. In a 10 cm culture dish, the plating 0.4 million CPCs per mg of MS led to only a small fraction of the micro-carrier being covered with the cells. The vast majority of MS only had a few cells attached. Alternatively, 4 million cells per mg, led to the formation of cell spheroids devoid of a micro-carrier support, since numerous cells did not find gelatin beads to attach to. In our hands, the optimal ratio for covering MS and avoiding cell spheroid formation was 1.6 million cells per mg (figure 1C).

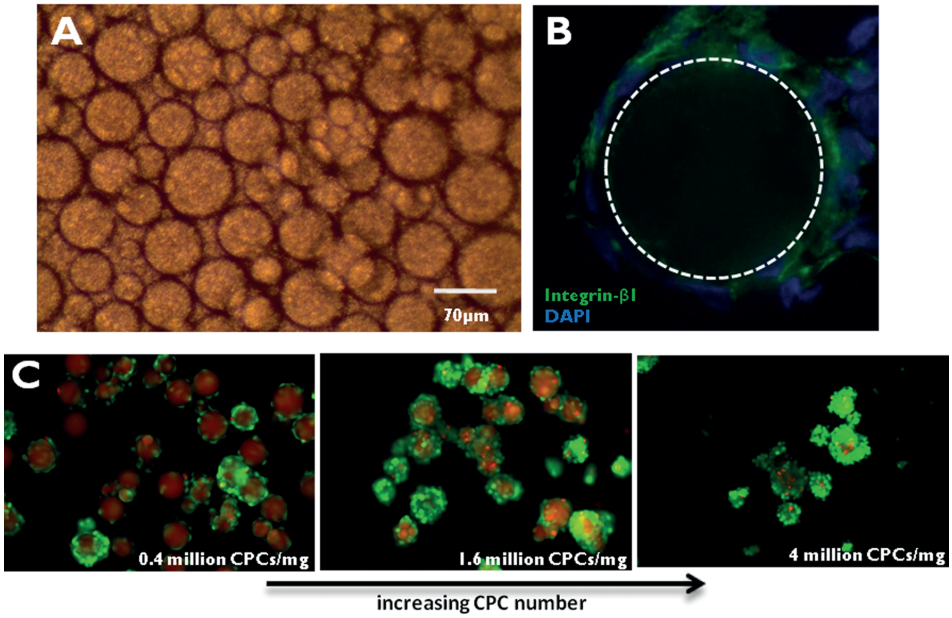

Figure 1 Loading CPCs on microcarries. Gelatin MS are spherical population with an approximate diameter of 50-75µm (A). Crysosections of CPC loaded microcarriers reveal the presence of cells on the outside of the MS (B). Binding dynamics of CPCs to MS visualized by live (green) dead (red) staining (note: MS are auto-fluoresent) (C). Changing the ratio of cells to MS (millions of CPCs per mg of MS) altered the MS covering.

CPC behavior on microcarriers

Next, to ensure the biocompatibility of gelatin MS, we tested several aspects of CPC behavior after the formation of the complexes. In order to rule out an influence of MS on the cardiogenic potential of the progenitor cells, we analyzed cardiac gene expression over the course of a two week culturing period, and three weeks after the induction of cardiomyocyte differentiation. Standard culturing procedure with MS did not influence the expression of Nkx2.5 and GATA4, two early cardiac transcription factors at the heart of CPC's lineage commitment ((figure 2A+B). As indicated before [3, 28], the stimulation of differentiation led to a robust increase in troponin gene expression, demonstrating that MS did not seem to interfere with the cardiogenic potential of the stem cells (figure 2C). Furthermore, transplanting the CPC laden micro-carriers onto Matrigel led to the migration of cells away from the MS, forming tubular-like structures, demonstrating the maintenance of their previously reported angiogenic potential (suppl. figure 2) [3, 29]. Moreover, this also established the ability of CPCs to detach from their micro-carrier, which for *in vivo* application will be crucial for the invasion into the injured myocardium.

Since an interplay is reported between gelatin and cell-integrin binding and subsequent MMP expression [30], we also wanted to investigate whether the attachment of CPCs to the gelatin moieties would elicit an altered enzymatic profile. Interestingly, such changes did occur in CPCs co-cultured with MS. The detection of secreted proteolytic enzymes from the CPCs was established by zymography (figure 2D), which revealed a major increase in MMP-2 release in

medium containing the CPC-MS complexes (figure 2E). The increased production of MMP-2 was maintained over the course of the three day follow-up period.

Functionalizing microcarriers with IGF-I and HGF

Next, we examined the possibility of loading cardiac protective growth factors (IGF-I and HGF) into the MS. The isoelectric point of acidic gelatin (pI~5) should permit for the complexation with oppositely charged IGF-I (pI~9) and HGF (pI~8). To test this hypothesis, we allowed for the absorption of the two growth factors into the MS overnight at 4°C. Subsequently, we monitored their release into PBS over the course of 6 days by ELISA. As described in literature [12], an initial burst release of both growth factors was observed predominately during the first day, which was followed by a plateau in the release rate (figure 3A). Overall, around 80 percent of the loaded HGF was retained in the MS, while 30 percent of IGF-I remained. To ensure the biological stability of the growth factors after interaction with gelatin MS, we stimulated CPCs with the

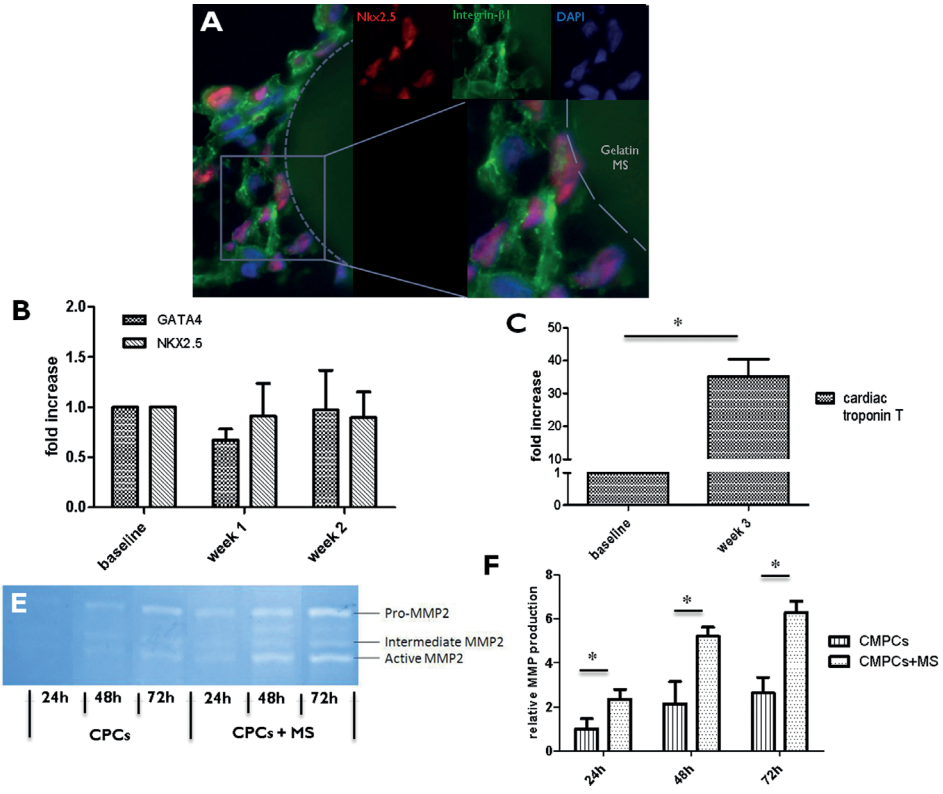

Figure 2 Behavior of CPCs on microcarriers. CPCs retain expression of early cardiac markers (A,B) for up to 2 weeks when cultured on gelatin microcarriers. Upon differentiation, we noticed increased cardiac troponin expression (C), showing that the CPCs do not lose their cardiogenic potential upon interaction with gelatin microcarriers. The detection of secreted hydrolytic enzymes from the CPCs was established by zymography (E), which revealed a major increase in MMP-2 production in medium containing the CPC-MS complexes (F).

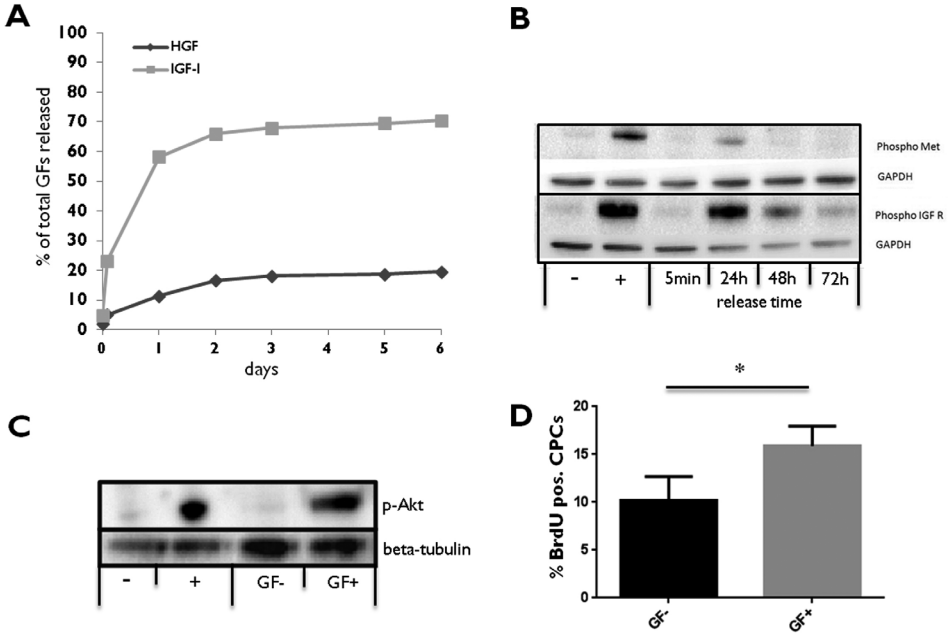

Figure 3 Loading growth factors in gelatin MS. ELISA measurement (A) of HGF or IGF growth factor release over the course of 6 days. Western blot (B) demonstrating activation of IGF-R and c-met receptor in CPC after stimulation with release medium. Culturing of CPCs on growth factor loaded MS (GF+) resulted in the activation of phospho-Akt (similarity to CPCs stimulated directly with the growth factors (+)), whereas CPCs on MS without growth factor (GF-) did not (C). Furthermore, increased BrdU incorporation was observed in CPCs cultured on MS with growth factors (D).

PBS from the release experiment. Phosphorylation of IGF-1 and HGF-1 receptors IGF-IR and c-met, subsequently, was established with release solution from the first 72 hours (figure 3B).
In order to test the response of CPCs to IGF-I and HGF stimulation, we setup various functional assays based on their known cellular effects. By monitoring proliferation, we noticed an increased division rate by CPCs after stimulation with both IGF-I and HGF, although no synergistic effects were observed indicating probably maximal stimulation (suppl. figure 3A). Pro-survival signaling was also observed after IGF-I and HGF stimulation, illustrated by increased phopho-Akt levels. However, only IGF-I was able to significantly reduce necrotic cell death of CPCs in an oxidative stress environment (suppl. figure 3C). Lastly, chemotactic potential of the growth factor was tested in a Boyden-chamber assay, in which HGF led to increased CPC migration (suppl. figure 3B). Therefore, the combinational stimulation with IGF-I and HGF leads to increased proliferation, resistance to oxidative stress, and migration of the CPCs.
Next, we tested if incorporated growth factors could modulate attached CPCs. As was observed in the stimulation experiments, CPCs loaded on the growth factor MS showed an induction in phosphor-Akt levels compared to CPCs loaded on MS without (figure 3C). Furthermore, increased proliferation was attained with the growth factors, highlighted by increased BrdU incorporation in the CPCs attached to MS-GF. Therefore, incorporation of IGF-I and HGF into MS could functionally influence the cells attached to its surface.

Microcarriers improved CPC delivery to the injured myocardium

To investigate the feasibility and efficacy of delivering CPCs to the heart with micro-carriers, we used a mouse model of MI. Despite the sheer stress endured during the injection procedure, histological analysis directly after intra-myocardial injection of the CPC-MS complexes revealed the presence of CPCs in close proximity to their micro-carriers (figure 4A and B). Although a few cells were detected further away (data not shown), the vast majority of cells were clustered around the MS. Two weeks post-injection, we observed MS and CPCs distributed in the border zone and infarct region. At this time point, the CPCs had detached from the MS and invaded into the surrounding myocardium (figure 4C and D). Furthermore, no increased mortality was observed in mice which received the microspheres (data not shown) proving the safety and feasibility of the method.

Subsequently, we compared the engraftment rate between CPCs in a single cell suspension and CPCs loaded on micro-carriers by BLI (figure 5A and B). At 3 days, the photon signals coming from the mice which received the CPC-MS were 10-fold greater than those of mice which received CPCs alone (figure 5A). At one week, although the signal dropped drastically compared to 3 days, the same significant difference was observed between the two groups. One month after CPC transplantation, the mice were sacrificed and cells histologically traced. The quantification of cells numbers revealed a 30-fold increase in engrafted CPCs when they were loaded onto the MS (figure 5C).

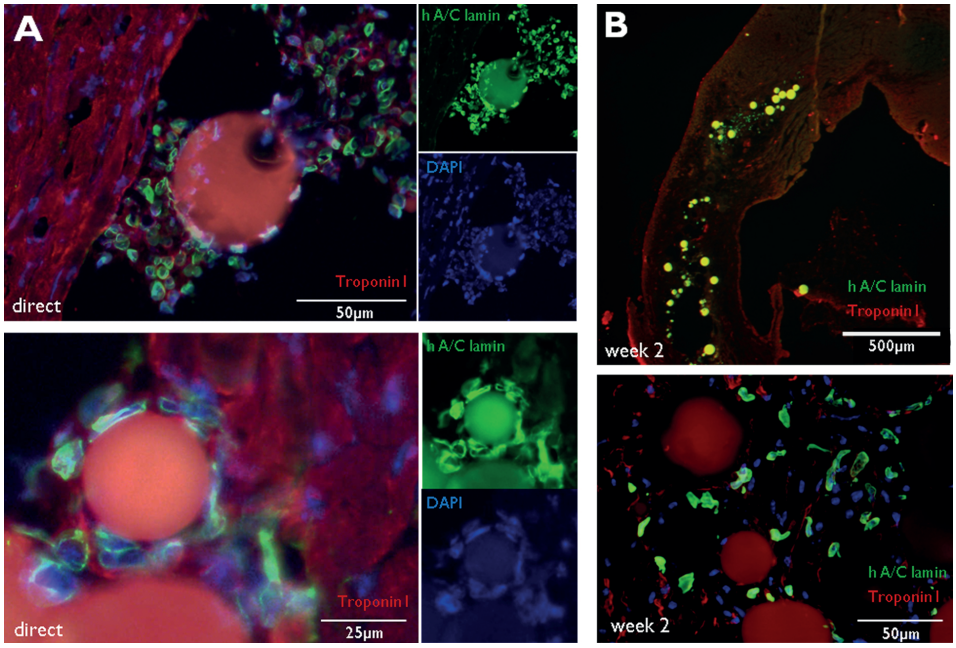

Figure 4 Injection of CPC laden gelatin MS into murine myocardium. Histological analysis of CPC-MS complexes directly after injection into the heart (A), revealed CPCs in close proximity to MS. Two weeks post-injection (B), MS are distributed throughout the infarct and border zone and CPCs have detached and invaded into the surrounding myocardium.

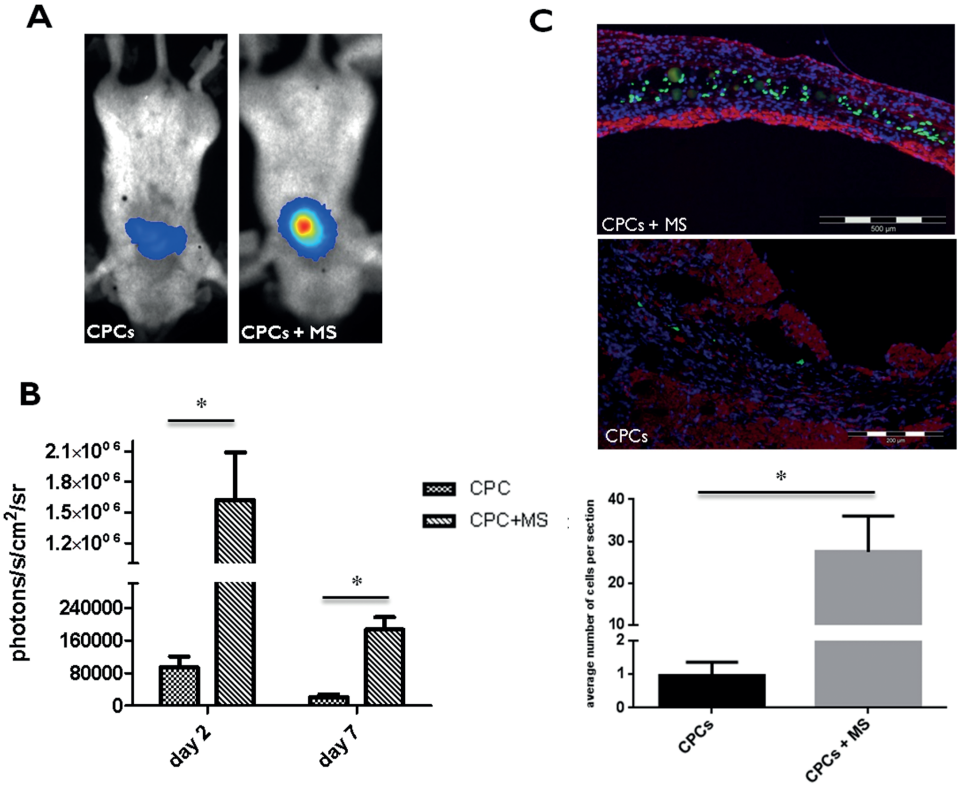

Figure 5 Comparison of engraftment after CPC intra-myocardial injection with/without gelatin MS. Representative images from BLI measurements at day 2 post-injection (A). Quantification of BLI signal (B) revealed dramatic increases in cell retention for the CPC-MS group at day 2 and day 7 post-injection. Histological analysis for human cells (green) in the mouse myocardium (red) at one month post-injection (C) showed superior long-term cell engraftment in mice which received micro-carrier transplanted CPCs.

DISCUSSION

The use of biomaterials in cell therapy approaches has gained attention to improve the overall therapeutic benefits from the treatment. Various techniques have been employed to combine cells and materials, with the aim of improving upon the limitations of standard administration procedures, such as poor engraftment and survival of cells in the myocardium. Here, we investigated gelatin microspheres as a possible vehicle for the delivery of CPCs and growth factors to the heart. Firstly, we realized an *in vitro* procedure that promoted the attachment of CPCs to the surface of the MS and showed that this interaction did not interfere with the cardiogenic profile of the stem cells. Thereafter, in a mouse model of MI, we showed the feasibility of injecting CPC loaded micro-carriers into the injured heart, which significantly increased cell retention in the tissue compared to the standard injection procedure.

Directly after administration, histology revealed CPCs surrounding the MS in the myocardial tissue, displaying the ability of cells to maintain the anchorage to the material over the course of the injection procedure. The preservation of this interaction is crucial for the efficacy of the approach, since the detachment of cells might eventually lead to their clearance, similarly to that observed with standard cell preparations. Monitoring this process by BLI, we observed a 10-fold MS mediated increase in cell engraftment at 2 days post-injection. Although the overall signal intensity decreased dramatically on day 7, it remained higher compared with CPC injected alone, even relative to early day 2 measurements. Furthermore, up to 30 times more CPCs were histologically traced back in the myocardium after one month. In literature, injectable scaffolds have been repeatedly found to increase cell retention [9, 10, 31-34]. These approaches were based on formulations that help to encapsulate the cells in a matrix network after transplantation, which on average documented a 2 to 5 fold increase in myocardial cell presence. Although different techniques and time points were used to assess cell retention, our novel micro-carrier method showed to be a more efficient approach to improve the cell delivery. A likely contributor to this effect is the requirement of injected scaffolds to polymerize into gels after transplantation, during which time an early wash out of cells is likely to occur. In comparison, MS-cell complexes are formed *in vitro* and will be retained without further modifications, abolishing the initial cell clearance from the myocardium.

Furthermore, we noticed a clear increase in MMP2 activity in the media collected from CPCs co-cultured with MS. The binding of integrin αVβ3 of CPCs on RGD moieties of gelatin is likely stimulating an invasion response, which triggered the expression of matrix-degrading enzymes [30]. On a global level MMPs are known to promote adverse remodeling in heart failure [35], however in a cellular context these proteases have been shown to be key regulators of homing and invasion processes. In the study by Zheng *et al*. [36], pretreatment of CD34+ umbilical cord blood stem cells with a specific MMP inhibitor completely blocked cell engraftment to the bone marrow, while Iwakura *et al*. [37] demonstrated the requirement of MMP9 for endothelial progenitor cell mobilization and incorporation into the injured myocardium. Furthermore, the activation of MMP2 was shown to be crucial in mesenchymal stem cell invasion into myocardial tissue after intracoronary injection [38]. Therefore, increased MMP expression in CPCs loaded on MS might be an additional mechanism by which MS promoted the retention of the stem cells in the heart. In this regard, the CPCs were "primed" for invasion *in vitro* through the interaction with gelatin which might have improved their subsequent myocardial engraftment. The molecular properties of the gelatin MS could also support the slow-release of cardioprotective growth factors; IGF-I and HGF. As observed in the release experiment, up to 80 percent of the total HGF and 20 percent IGF-I are retained inside the micro-carriers, which can be released upon enzymatic degradation of the gelatin network. Thus, as the body starts to degrade the MS after transplantation the retained proteins will be slowly released into the tissue, wherein the peptides will be able to promote anti-apoptotic effects on the cardiomycoytes [17-20]. Furthermore, IGF-I and HGF have recently been shown to also stimulate cardiac repair by the activation of endogenous CPCs [21, 39]. In these studies, a single bolus injection of the growth factors led to increased cardiomyogenesis and significant improvement in cardiac function. Additionally, recent reports [40, 41] showed the added value of combining these

growth factor with biomaterials for the creation of a slow-release platform, thereby improving the functional outcome of the therapy. Similarly, sustained IGF-I/HGF release from MS should offer the injured heart comparable long-term protection and regenerative stimulation.
Moreover, the functionalization of the CPCs-laden micro-carriers with IGF-I and HGF induced pro-survival Akt phosphorylation and increased the proliferation of the attached stem cells. As shown in the study by Iruegas *et al*. [42], the co-injecting of CPCs with IGF-I/HGF led to better functional recovery than cells alone, partly due to the improved therapeutic action of the stem cell. According to our *in vitro* data we can expect similar benefits, since the proteins will help to protect and promote the proliferation of the transplanted cells, thereby possibly reducing the vast amount of cells lost between day 2 and 7 post-transplantation. Therefore, the MS mediated release of IGF-I and HGF should be an effective therapeutic complement to CPC therapy by directly improving the state of transplanted cells and indirectly assisting at healing the heart.
Overall, we developed a simple and effective translational bio-degradable approach to increase the retention of CPCs in the myocardium. By anchoring CPCs on gelatin MS, we were able to substantially increase the presence of cells in the injured myocardium. Furthermore, the ability to incorporate IGF-I and HGF into the microspheres should further promote the endogenous and exogenous regenerative properties of the therapy. Future follow-up studies will evaluate the therapeutic benefit of designed approach, in order to validate it as a way to improve CPC based cell therapy.

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SUPPLEMENTARY

Growth factor stimulation experiment

Proliferation assay

Proliferation of CPCs was monitored by WST-1 reagent (Roche, 11644807001). Briefly, 2,000 CPCs were seeded per well in a 96 well plate with or without 100ng/ml IGF-1, HGF or both, and grown in M199 medium containing 2% serum. WST-1 was used according to the manufacturer's protocol.

Migration assay

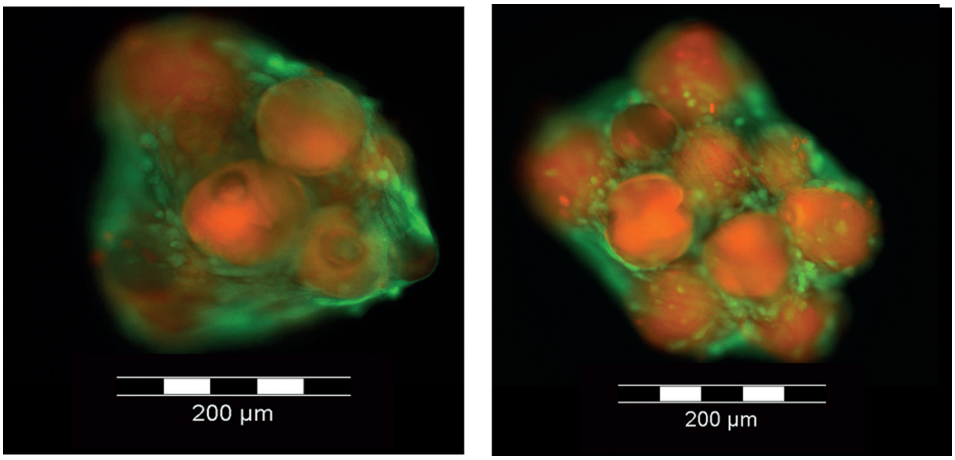
CPCs (10,000 cells/ml) were placed in upper well of boyden chamber, while serum-free medium with or without 100ng/ml IGF-I, HGF, or both was placed in the lower chamber as chemotactic attractant. After 6 hour incubation, the mesh was removed, stained with dapi and mounted on microscope slide. Cells were counted to determine the number of migrate CPCs.

Survival assay

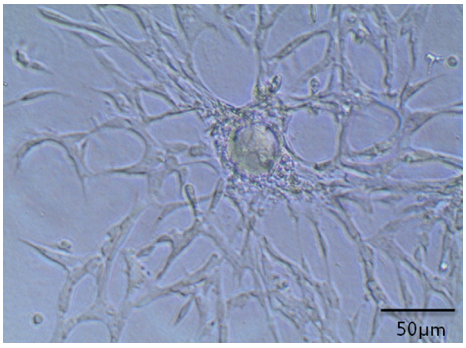
CPCs were seeded in a 12-well plate and grown to 80% confluence. CPCs were treated with 75μM tert-Butyl hydroperoxide (H202) (Sigma; 458139) in serum-free M199 medium for 16–20 hrs with 100ng/ml IGF-I, HGF, or both. All cells were collected and subsequently washed with phosphate-buffered solution (PBS). For apoptosis and necrosis detection, cells were incubated with AnnexinV-PE and 7-Amino-actinomycin-D in 1x binding buffer (BD Pharmingen, apoptosis and necrosis detection kit I; 559763) for 25 min in a dark container. After incubation, apoptotic, necrotic and live cell populations were detected by flow cytometric analysis.

Western blot

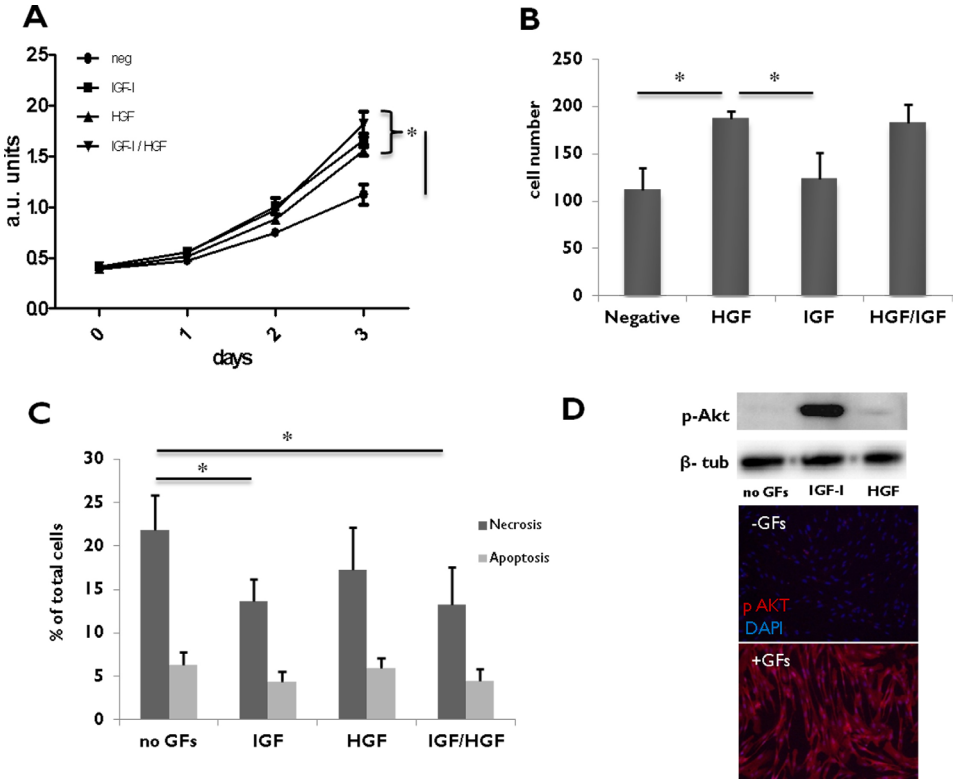
CPCs were serum starved overnight prior to 30min stimulation with growth factors (100ng/ml; IGF-1 and HGF). Cells were lysed in SDS sample buffer (Cell Signaling, 7722), sonicated and heated to 95°C for 5 minutes. Samples were mixed with NuPage LDS Sample Buffer (Novex, NP0008) to make the samples heavier and loaded on a 4-12% Bis-Tris gel (Novex, NP0335). The gel was run in MOPS Buffer and proteins were electrotransferred to a PVDF membrane. The membrane was blocked in blocking buffer for 1 hour and washed. Next, the membrane was incubated with rabbit monoclonal anti Phospho-Met (1:1000, Cell signaling 3077) O/N at 4°C, rabbit polyclonal anti Phospho-IGF-IR (1:1000, Cell signaling 3021) O/N at 4°C or rabbit monoclonal anti GAPDH (1:2000, Cell signaling 2118) 30 minutes at RT. Incubation with secondary antibody solution Polyclonal goat anti rabbit immunoglobins/ HRP (1:1500, Dako P0448) against Phospho-Met and Phospho-IGF-IR for 1 hour at RT and against GAPDH for 45 minutes at RT. Blot was developed in ECL substrate for 7 minutes for the Phospho-Met and Phospho-IGF-IR blot and approximately 30 seconds for the GAPDH blot.



Supplementary Figure 4 Live (green)/ dead (red) staining of CPCs cultured in static condition on low-attachment plate with microspheres (auto-fluorescent) for one week.



Supplementary Figure 2 Invasion of CPCs into matrigel after attachment to gelatin MS.



Supplementary Figure 3 The effects of IGF-I and HGF stimulation on CPCs. We noticed an increased division rate by CPCs after stimulation with both IGF-I and HGF (A). Chemotactic potential of the growth factor was tested in a Boyden chamber assay, in which HGF led to increased CPC migration (B) Pro-survival signaling was also observed after IGF-I and HGF stimulation, illustrated by increased phopho-Akt levels.(D) However, only IGF-I was able to significantly reduce necrotic cell death of CPCs in an oxidative stress environment (C).

Can cardiac reprogramming factors be used to improve cardiac cell therapy? A pilot experiment

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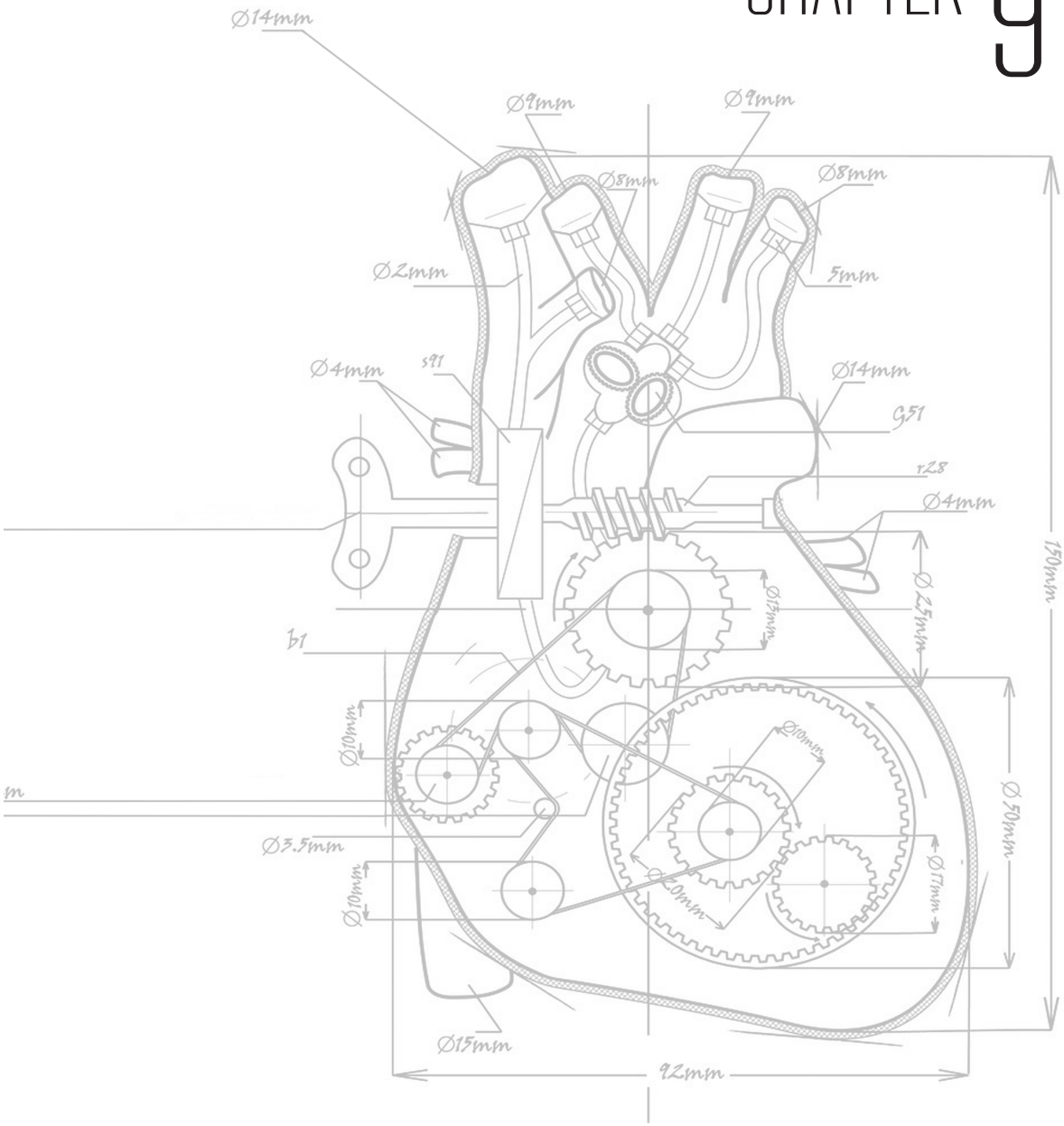
CHAPTER 9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The lack of engraftment in classical cellular therapy approaches has long been seen as an obstacle for realizing the full therapeutic potential of stem cells. Therefore, the optimization of cardiac progenitor cell (CPC) delivery strategies discussed in this thesis has provided a platform to further enhance the effects of cellular therapies to the heart. By increasing CPC presence in the injured myocardium, these stem cells are be able to secrete more paracrine factors to further stimulate endogenous repair pathways and also provide additional *de novo* myocardium and vessels by direct differentiation. Nonetheless, the latter process seems to be hampered by the inefficacy of CPCs to participate in it. In our experiments (chapter 6 and 7), we observed sarcomeric protein expression in the stem cells after administration, however, the phenotypic analysis showed that these proteins were not properly organized in a striated pattern as observed in mature cardiomyocytes. Therefore, future research should focus on improving the maturation and electrical coupling of stem cell derived cardiomyocytes once delivered to the myocardium.

In this regard, recently discovered cardiac reprogramming factors could be utilized to address this problem [1]. The initial discovery of transcription factors that were able to transform fibroblast into pluripotent stem cells [2], technology now referred to as induced pluripotent stem cells (iPS), shattered the preconceived notion that cells were irreversibly committed to their lineage fate. This powerful tool allowed for the creation of patient specific regenerative constructs (through the intermediary pluripotent state), but also highlighted a remarkable plasticity of cells to undergo changes toward other somatic identities. In recent years, other sets of transcription factors were discovered that were able to modulate lineage fates [3-5], but interestingly they showed that the creation of a pluripotency was not always a prerequisite for this process. Direct reprogramming technology utilizes a set of defined transcription factors for the direct conversion of fibroblast to specific somatic cell types. For the reprogramming of fibroblast to cardiomyocytes, knowledge from developmental biology led to the discovery of GATA4, Mef2C and Tbx5 (also known as GMT) as key regulator for cardiogenic induction in these cells [1]. These three factors were sufficient for the initiation of cardiac gene expression in fibroblast, which acquired functional properties characteristic of cardiomyocytes *in vitro*. If the factors were introduced into the fibroblast prior to transplantation into the myocardium, the cells adopted a cardiomyoyte-like phenotype with a mature sarcomeric protein expression profile. Hence, GMT factors are a potent cardiogenic tool which might be useful in other therapeutic applications, such as improving stem cell therapy with CPCs.

CPCs are committed to the cardiac lineage and thus maintain expression of GATA4, Mef2C and Tbx5 in their undifferentiated state [6]. Therefore, if introduced, GMT factors enter a cell with a different epigenetic landscape compared to that of a fibroblast. Nonetheless, modulating levels of these transcription factors with GMT factors might, similarly to fibroblast, initiate biological pathways towards cardiogenic differentiation. To investigate this hypothesis, we setup a pilot experiment in which we transduced CPCs with GMT factors and compared cardiac gene expression to the CPCs that received a transfecting control containing dsRED. Astoundingly, after only three days of culture in regular growth medium, GMT factors increased cardiac troponin expression 500 fold (figure 1A). Cardiac actin levels were also increased over this short time period (figure 1B), showing the ability of these factors to initiate sarcomeric gene expression

in the CPCs. After three weeks, no further increases were observed in GMT treated CPCs. However, similar levels of expression were sustained and did not decrease over the follow-up time period. Immunohistological analysis at 3 weeks revealed a some alpha actinin positive cells (around 1 in 1000) in the GMT treated cells, which had striated organization reminiscent of cardiomyocytes derived from embryonic stem cells (figure 1C).

In order to better understand the mechanism underlying the observed effects, we setup an experiment in which we treated CPCs with the individual factors. Furthermore, we also cultured these cells in a differentiation medium (figure 2A) in an attempt to augment their cardiac differentiation. Similarly to the initial study, a strong induction in cardiac gene expression was observed three days (day 0 of differentiation,) after the addition of the GMT factors. However, in respect to the individual factors, only Mef2c had the ability to increase cardiac actin levels during the same time period. GATA4 and Tbx5 were unable to change the saracomeric profile of the CPCs, maintaining expression levels similar to those of dsRED treated cells (figure 2B). Additionally, two samples were taken at 3 and 5 weeks after the start of the differentiation protocol. The differentiation medium did not affect the expression of cardiac actin in GMT cells, since the difference between GMT and dsRED did not change as compared to the initial time point. There was also little alteration in the action of Mef2c over time. However, unlike their day 0 measurements, there was an effect from GATA4 and Tbx5, which seemed to be able to engage cardiac gene expression at these later time point, albeit at much lower levels (GATA4

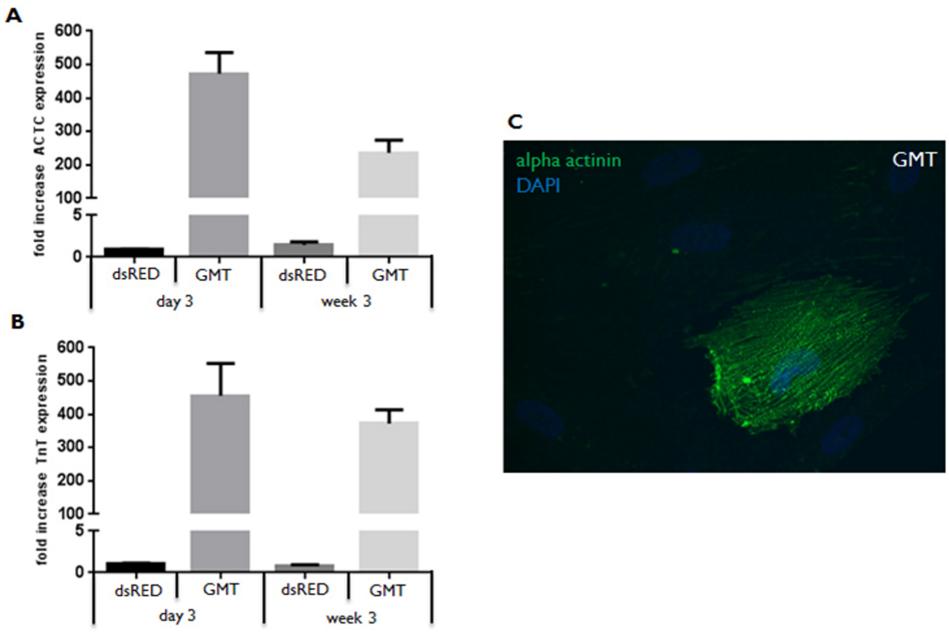

Figure 1 Effect of GMT factors in CPCs. qPCR analysis of ACTCI (A) and TNNT2 (B) gene expression in CPCs after transduction with GMT or dsRED after 3 and 21 days. Alpha actinin staining (C) of GMT-CPCs after three weeks of culture.

and Tbx5, 11 fold increase; Mef2c, 100 fold increase, respectively). At the moment it remains unclear if GATA4 and Tbx5 act synergistically with the differentiation medium, or if their effects on cardiac actin gene expression requires a longer incubation time.

Overall, cardiac reprogramming factors seem to be a tool well worth while investigating for improving cardiac cell therapy. The strong influence of GMT factors, in particular Mef2c, on sarcomeric gene expression offers a new opportunity to enhance the differentiation of CPCs *in vivo*, thereby possibility increase their therapeutic effects. Nonetheless, further careful investigation is still required in order to validate these results and optimize the conditions for introducing the factors into CPCs. It might be most fruitful to introduce individual factors at different time points, and/or to utilize the differentiation protocol at the most optimal moment. Furthermore, cardiogenic miRNAs [7] and additional cardiac reprogramming factors for human fibroblasts [8, 9] have recently been published, which could be useful in designing the optimal factor cocktail for the cardiac differentiation of CPCs.

Acknowledgements

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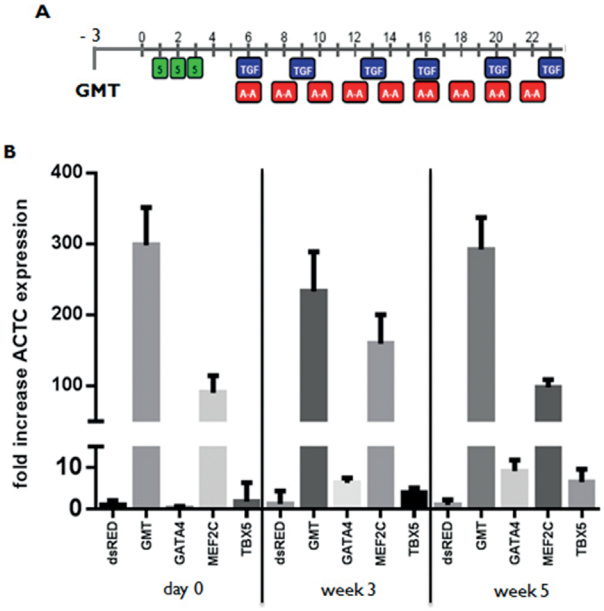

Figure 2 Effect of individual reprogramming factors and differentiation medium on ACTC expression. Schematic representation of the differentiation protocol (A) over the course of 4 weeks. qPCR analysis of ACTCI (B) gene expression at 0, 3, and 5 weeks after the start of differentiation.

METHODS AND MATERIALS

CPC isolation and culture

CPCs were isolated as previously described. CPCs were propagated in 0.1% gelatin coated flasks in SP++ medium (a mixture (1:3) of EGM-2 (Lonza) and M199 (Lonza), supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin and streptomycin (P/S; Gibco), and non-essential amino acids (Lonza)).

To induce differentiation, cells were treated with 5 μM 5'-azacytidine (Sigma) for 72 hours in differentiation medium (Iscove's Modified Dulbecco's Medium /HamsF12 (1:1) (Gibco)) supplemented with L-Glutamine (Gibco), 2% horse serum, non-essential amino acids, Insulin-Transferrin-Selenium supplement , and 10-4 M Ascorbic Acid (Sigma)), followed by TGF-β stimulation (1 ng/ml; Sigma) (8). The medium was changed every 3 days.

Retrovirus production and transduction

pMXs- constructs for overexpression 7 transcription factors GATA4, MEF2C, TBX5, ESRRG, MESP1, MYOCD, and ZFPM2 have been described previously [1]. The pMXs retroviral vectors and retroviral packaging vectors pAmpho (Clontech) were transfected into HEK293 cells using lipofactamine 2000 (Life technologies). Virus containing supernants were collected and incubate with human CMPC with 1x transDux (SBI, System Biosciences) at 30% confluency using DsRed as a control for efficiency. 24 hours after infection, viral supernants were replaced with fresh SP++ for 1 days before switching to differentiation media IFDIF.

RNA isolation and quantitative PCR

RNA was isolated using Trizol (Invitrogen) and reverse transcribed using iScript cDNA Synthesis kit (BioRad). RT-PCR amplification was detected in a MyIQ single-color real-time polymerase chain reaction system using iQ SYBR Green Supermix (BioRad). The primer sequences have been described previously.

Immunohistochemistry

CPC were homogenized with collagenase A, and thereafter plated on cover slips. Once attached, cells were fixed with 4% PFA and stained as previously described using α-sarcomeric actin (Santa Cruz) and Alexa488-labelled (1:500, Invitrogen) secondary antibody.

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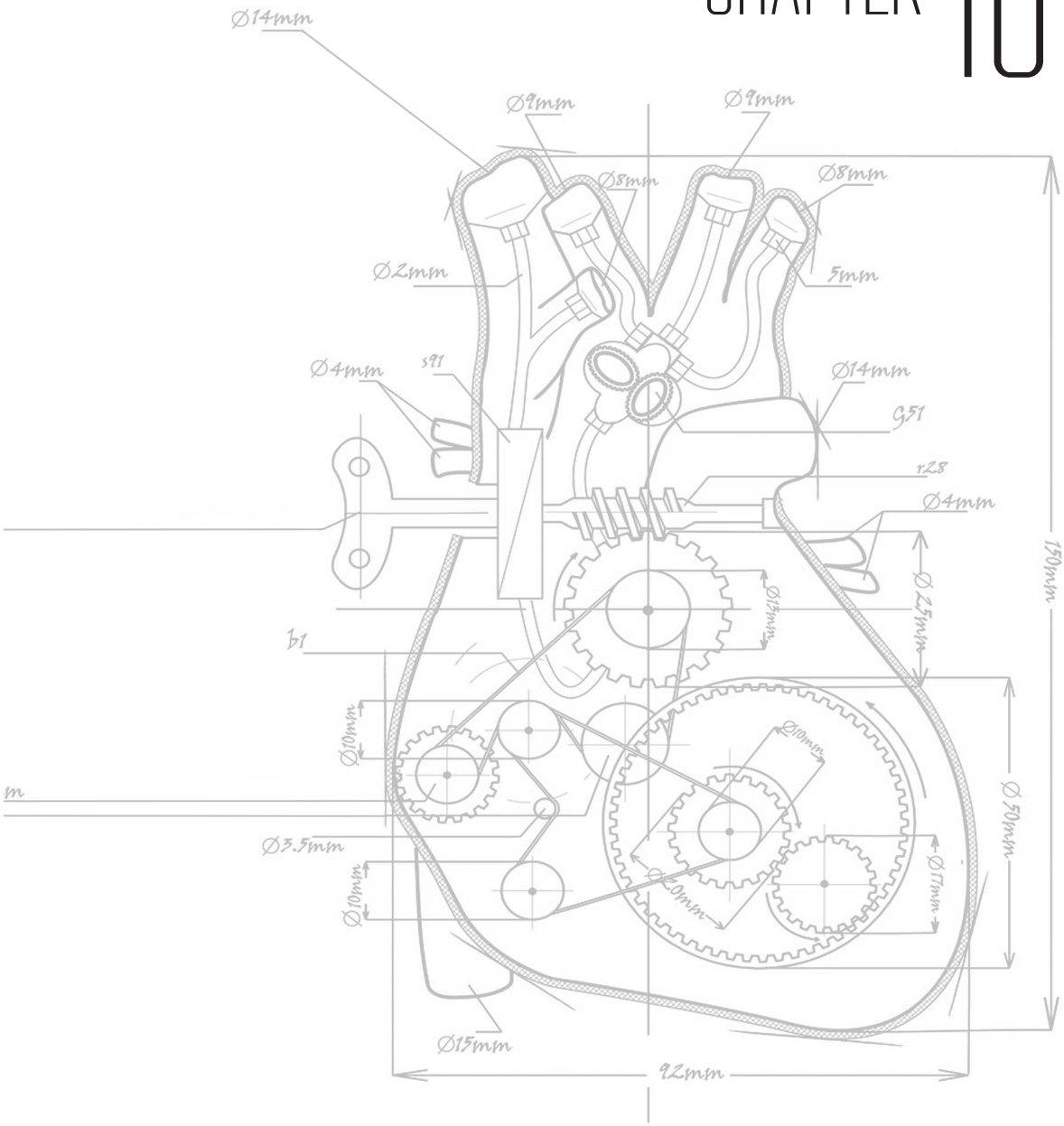
General Discussion

"The universe is a big place, perhaps the biggest."

Kurt Vonnegut

CHAPTER 10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Despite the occurrence of *de novo* cardiomyocyte generation over the course of a human lifespan [1], the heart remains one of the least regenerative organs in the body, with a yearly turnover rate of less than 1 percent. However, since the loss of cardiomyocytes underlies the pathology of heart failure [2], approaches aimed at increasing its regenerative ability could provide a cure to the millions of patients affected by this epidemic. In this respect, advancements in stem cell biology have opened the door to a potential exogenous source for new cardiac muscle. In the last decade, researchers have engaged and explored the concept of cardiac stem cell therapy, which has led to the development of a highly dynamic interdisciplinary field wherein experts from medical, biological, chemical and physical sciences attempt to create new therapeutic approaches to repair the damaged heart. This discussion, will highlight the field from where it started (1.0), to how it is progressing (2.0) and its possible final destination (3.0).

Cardiac cell therapy 1.0

The initial investigations were started with the concept that stem cells could be administered into the heart in order to repopulate the damaged organ. Cardiac myocytes were theoretically the best candidate to repair the heart, however their limited proliferative capability and survival after transplantation made them ill-fitted for cardiac cell therapy approaches [3, 4]. Nonetheless, driven by the magnitude of the clinical issue, investigators turned their attention to non-cardiac progenitor cells, such as bone marrow cells and skeletal myoblasts. A rather simple and practical approach was taken to deliver these cells to the myocardium. The biological therapeutics were homogenized, creating a single cell suspension that was diluted in phosphate buffered saline (PBS), the isotonic nature of which provided a stable environment for the cells and was compatible for injection into the body. The emphasis of early studies was on the biodistribution of stem cells [5-7], in order to establish the optimal administration route. Intravenous injection was soon deemed as suboptimal [5, 8] due to the low-homing potential of stem cells, while more promising results were obtained by injecting cells through the coronary circulation or directly into the heart muscle. However, even in the more local delivery techniques the engraftment of stem cells still suffered from undesirable pharmacokinetics. On average 10 percent of the stem cells were found back in the heart only hours post-injection.

Surprisingly, early studies showed that bone marrow cells [9] and skeletal myoblast [10] seemed to have the plasticity necessary to differentiate into cardiac cells. These findings were further supported by their therapeutic efficacy in pre-clinical studies. Histological analysis showed increased capillary formation and reduced infarct size in animals treated with cell injection, and led to improvements in regional and global cardiac contractile function [9, 11-13]. However, follow-up investigation soon refuted the mechanism of action behind these effects. It was observed that the cells were actually incapable of cardiac differentiation [14, 15], therefore exogenous regeneration did not seem to be the basis of the observed benefits. Nonetheless, these results did not slow down the translation of the original findings to the clinic.

Although the injection of bone marrow cells was subsequently reported to be safe, clinical trials with skeletal myoblast were soon arrested due to arrhythmic events in treated patients [16]. This was most likely due to the fact that these stem cells become skeletal myoblast in the heart, probably being unable to properly couple with the native myocardium. Furthermore, no

significant functional improvement was reported in the patient population which received the myoblasts [16]. In the trials with bone marrow cells the associated benefits with cell injection were variable. While some studies [17, 18] reported improvements in cardiac function, others only showed short-lived effects [19] or no effects at all [20, 21]. Overall, meta-analysis reported that long-term outcome from randomized control trials studying the treatment of acute myocardial infarction with injection of bone marrow cells resulted only in a small (2-4%) improvement in left ventricular ejection fraction [22-24].

The first decade of research into cardiac regenerative therapies ended with mixed results. It did, however, show therapeutic promise and eased some safety concerns associated with stem cell application. The encouraging results yielded by the original transplantation experiments probably led to a "hastened" initiation of clinical trials without fully understanding the complex interaction between stem cells and their myocardial targets. Furthermore, it also highlighted some of the major drawbacks in the pragmatic setup of conventional stem cell therapy such as poor engraftment, inadequate differentiation and limited cell survival.

Cardiac cell therapy 2.0

In order to address the aforementioned shortcomings, a reevaluation of stem cell therapy was needed. Especially in regards to their mode of action, the paradigm shift from the direct participation in tissue regeneration to stimulating endogenous processes (also known as the paracrine hypothesis), was important to better understand the physiological response to the therapy. It was observed that the production and secretion of growth factors and cytokines from the stem cells were able to elicit responses from various cell types *in vivo*. They were able to provide pro-survival stimulus to cardiomyocyte in the border zone, by activating the AKT signaling pathway [25]. Secretion of VEGF and other angiogenic factors promoted the formation of new vessels [26] and provided the ischemic heart with oxygen and nutrient so desperately needed. Furthermore, stem cells also seemed able to interact with the immune system [27], which seems to be involved in the pathological remodeling process after injury. Research today is focusing on key players from this vast array of factors released. In a study [28] with bone marrow derived mesenchymal stem cells (MSC), fractionation of their *in vitro* culture medium and subsequent injection into the heart showed that much of the cardioprotective effect is actually not mediated by proteins, but nano-sized vesicles called exosomes. Future research in the paracrine factor will be paramount in figuring out the exact mode of action by which the stem cells operate.

The emergence of endogenous cardiac progenitor cells (CPCs) as a viable therapeutic stem cell (scalable, safe and patient specific cell type) has finally provided researchers with a cardiac cell source for repairing the heart [29-31]. Unlike their non-cardiac predecessor, differentiation into cardiomyocytes, (but also endothelial cells and smooth muscle cells) after administration was unequivocally demonstrated. Nonetheless, their participation in tissue regeneration does not account for their full therapeutic effect, since only small percentages became cardiomyocyte [26, 32]. Thus, similarly to bone marrow stem cells, CPCs secrete paracrine factors that are likely contributing to their function *in vivo*. Furthermore, over the years several different isolation methods have been used to establish CPCs line. Initially, it seemed that there was little overlap

among CPCs identified by the different methods, and some scientists suggested that several populations of CPC exist. More recent studies indicate shared markers among once-distinct populations or different stages of maturation in the same line of cells [33, 34] . In chapter 3, we investigated these discrepancies by characterizing the known CPC populations in a clinically relevant fashion. Since these cells have to be expanded to attain sufficient numbers for transplantation into the patient, we compared their gene expression profile after tissue culture propagation. Nonetheless, we found limited variance between the different identified populations on the transcriptome level, and strikingly found a major influence of the culture conditions on the differently isolated progenitor cells. Therefore, they likely represent different developmental and/or physiological stages of CPCs, rather than intrinsic different CPC populations. Furthermore, more emphasis should be placed on the ex vivo culture settings of CPCs in order to reduce the variability and build a consensus in the scientific field.

A major drawback to cell therapy was the limited engraftment and survival of stem cells in the heart after administration (only 1-3 percent of all cells are found one month post-injection) [32, 35], which appears to be impeding their full regenerative potential. In this respect, many strategies have been developed to provide the cellular therapeutics with the necessary components to ensure their long-term retention. Pro-survival factors were the first to be studied. The pretreatment with pharmacological compounds [36] and/or growth factors [37, 38] to avoid apoptotic and necrotic cell death showed to be capable of increasing cell numbers in the heart, and in some cases even improve the therapeutic effects of the injected stem cells. Virus based vector for the overexpression of pro-survival factors were also utilized to improve cell retention [39, 40]. In chapter 5, we utilized necrostatin-1, a potent inhibitor of necroptosis in CPC, in an attempt to improve CPC cell therapy by attenuating cell death. Although we noticed increased cell survival on the short-term, no beneficial effects were observed on cardiac performance. We hypothesis that this could be due to the fact that pro-survival strategies are targeting a diminished cell population, since around 90 percent of all injected cells are removed by the circulation directly after injection.

Cardiac tissue engineering applications have proved to be extremely useful in addressing the limited cell retention after transplantation [41]. The utilization of biomaterials provides the cells with an environment with which they can interact and ensures their encapsulation or entrapment after injection. Different approaches have been taken to combine cells and materials, including cardiac patches [42-44], which are *in vitro* engineered constructs, and injectable scaffolds [45-47], which can act as vehicles to support the delivery of cells. In chapters 6 and 7, we developed a 3D printed approach to create a cardiogenic patch composed of hyaluronic acid matrix and CPCs. After the epicardial application of this construct, we were able to maintain robust CPC survival for up to three-month, a period of time during which graft of normal cell injection dwindles to few surviving cells. Although promising functional results were obtained with this intervention, incomplete incorporation of the matrix into the myocardium and limited cardiac differentiation of the CPCs are aspects that will need to be addressed in future follow-up studies. Furthermore, cardiac regenerative patches are limited in their clinical translation due to the invasive surgical requirements needed for their application. Therefore, in chapter 8 we shifted our attention to a catheter based tissue engineering approach with gelatin microspheres. These

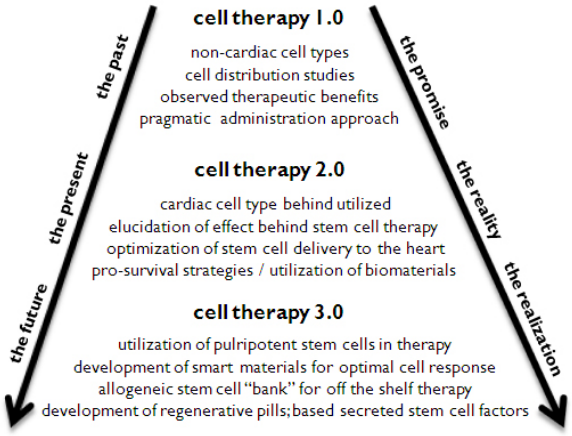

Figure 1 Schematic overview of the discussion of this thesis

spherical micro-carriers can accommodate the binding of CPCs on their surface and their size allows them to flow through the lumen of a catheter. The injection of CPC-laden micro-carriers into the myocardium dramatically reduced the wash-out of cells and increased the retention compared to CPCs injected alone. After implantation into the tissue we observed cells detaching from the material and invading into the surrounding areas, thus the microspheres served as a vehicle for the delivery of the progenitor cells to the damaged heart. An advantage of this micro-carrier approach is the proximity of delivery in respect to the injury, especially compared to an epicardial patch in which the cells are required to migrate out of the matrix. However, the CPCs are not sheltered by a protective environment, such as the hyaluronic acid in the patch, and thus susceptible to the harsh ischemic environment. This was apparent in the temporal decrease of bioluminescent signal in microsphere delivered CPCs, compared to the stable signal stemming from patch encapsulated CPCs. Nevertheless, bioactive molecules, such as growth factors, can be incorporated and released by the microspheres. In this regard, the functionalization of the beads with cardio protective growth factors, IGF-I and HGF [48], stimulated pro-survival signaling and proliferation in the attached CPCs *in vitro*, which can serve as beneficial signals for the cells after transplantation to overcome the environmental burden. Overall, of the approaches investigated in this thesis, micro-carriers are best suited to contribute to improving cardiac cell therapy in the clinic. The biocompatibility of gelatin and the ease of administration can make this approach a simple and effective tool to improve the pharmacokinetics properties of stem cells. Furthermore, combining this approach with pro-survival factors, such as necrostatin-1, can further advance the therapeutic output. For *in vitro* engineered patches, although it is an efficient technique to deliver cells to the heart, additional research will be required to optimize the matrix degradability to ensure its integration with the myocardium. Nonetheless, if these issues can be resolved it can become a powerful tool to rejuvenate the heart of chronic heart failure patients, for whom these constructs can be applied when required to undergo open chest surgery.

Cardiac cell therapy 3.0

The future of cellular therapies will rely heavily on improving the knowledge of stem cell biology, in order in to be able to form new cardiac muscle. Although the regenerative field has developed techniques to efficiently differentiate stem cells *in vitro*, to date limited data exists on approaches that provide a robust source of fully electromechanically integrated cardiomyocytes *in vivo*. The ability to increase the presence of CPCs, as shown in chapters 6-8, should provide the basis for further refinements in this area. In chapter 9, we showed the potential of reprogramming factors to modulate CPC behavior *in vitro*. This bio-engineering tool could also further be utilized to boost differentiation *in vivo*, possibly making the stem cells more susceptible to environmental cues after administration. Investigation into cardiogenic biomaterials, such decellularized heart matrix [49], could also serve to guide differentiation of cells, while still providing them with environmental support to minimize cells lost after injection.

The focus on adult stem cells has stemmed from their ease to isolate and propagate. These cells, however, are restricted in their differentiation potential, which can be helpful to lessen safety concerns, but might also be detrimental for their prospective therapeutic effects. Pluripotent stem cells will be part of the next movement of more "powerful" regenerative therapies, especially with the ability to now create patient specific (induced) pluripotent stem cells (iPS) [50]. Harnessing their potential has been central to the ability to use them efficiently and safely. As can be seen by the complex setup of *in vitro* differentiation protocols, mimicking developmental stages is a very complex undertaking. It is now the challenge to translate this knowledge to the *in vivo* situation; to be able to bring the cells into the heart and integrate them with the native myocardium, which will provide the heart with new contractile machinery. Such endeavors will require close cooperation between scientific fields. Biologically active scaffolds will need to be developed that can accommodate pluripotent derived cardiomyocytes, and help guide their maturation and integration into the heart. Expending our knowledge on the physiological process of cardiomyocytes coupling could lead the uncovering of new players involved in the process, which can thereafter be overexpressed by the cells or assimilated into the materials. The preferred delivery location will have to be carefully selected based on cellular response to the environment, and delivery devices developed to administer the biological therapeutic in the proper manner. The quest for true cardiac regeneration will take time, but if the past decades of cell therapy research have taught us anything it would be not too hasten the translation, rather carefully examine and optimize cell behavior in order to truly reap the benefits of these powerful therapeutic entities.

Even with non-cardiac stem cells therapeutic progress can still be made. While it is clear now that these stem cells are unable to create new heart muscle, steps can be taken to optimize therapies around their paracrine effects. In this regard, one of the remaining hurdles is the heterogeneous profile of stem cells derived from patients. The function of stem cells can be impaired in patients with comorbidities, such as diabetes, or even with advanced age, which is likely contributing to the different outcomes of clinical trials [51, 52]. However, for immunological and safety reasons, the use of patient's own stem cells (autologous cells) is seen as most optimal. Nonetheless, there needs to be future consideration for the use of allogeneic stem cells. Similarly to organ transplantation procedures, allogeneic stem cells could carefully be

matched with recipients to avoid graft rejection after administration. This would allow for the construction of an allogeneic stem cell "bank", which could be derived from young healthy donors (possibly from fetal origin) with robust stem cell profiles. It would also offer patients an "off-the-shelf" option for direct application after arrival in the hospital with a myocardial infarction, thereby avoiding delays associated with bone marrow aspiration and tissue culture procedures. This timeframe would also make best use of the anti-apoptotic properties exhibited by non-cardiac stem cells, thereby possibly increasing their therapeutic output.
Furthermore, a better understanding of the biological targets behind paracrine mediate effects could lead to the development of new class of "regenerative" pharmaceutics. The pharmaceutical industry, which has recently been struggling to develop new blockbuster drugs, could benefit greatly from the findings of stem cell research. The effects exerted onto the myocardium seem to activate a myriad of responses. Future research will need to focus on the exact factors that are responsible, and the effector sites which are targeted. Comprehensive examination into the pathways involved in the regeneration response should open the doors for chemists to design and synthesize new chemical entities that will offer similar pharmacodynamic properties as stem cells. As of today, the cost of implementing cardiac cell therapy in the clinic is large, due to the need for GMP culturing facilities and expensive delivery aperture. Therefore, the development of regenerative "pills" would vastly reduce those costs and make them available to a wider patient population.

CONCLUSION

Overall, regenerative medicine has emerged as a potential life altering therapeutic approach for patients suffering from congestive heart failure. Although no consensus has yet been reached on how to best utilize this new found knowledge, the recent advancements in the field offer great hope for the development of a new genre of medicines. The increasing ability to modulate stem cells with various factors and materials has led to the refinement of cellular therapies. However, the multiplicity of their actions in the myocardium remain a therapeutic "black box", by which researchers still have difficulties attributing the precise cellular process to the observed physiological effects. Moreover, predicting (or controlling) their behavior *in vivo* remains a great challenge. Therefore, future insightful gains into their functionality outside and inside the body will be key to the establishment of a viable cardiac regenerative therapy.

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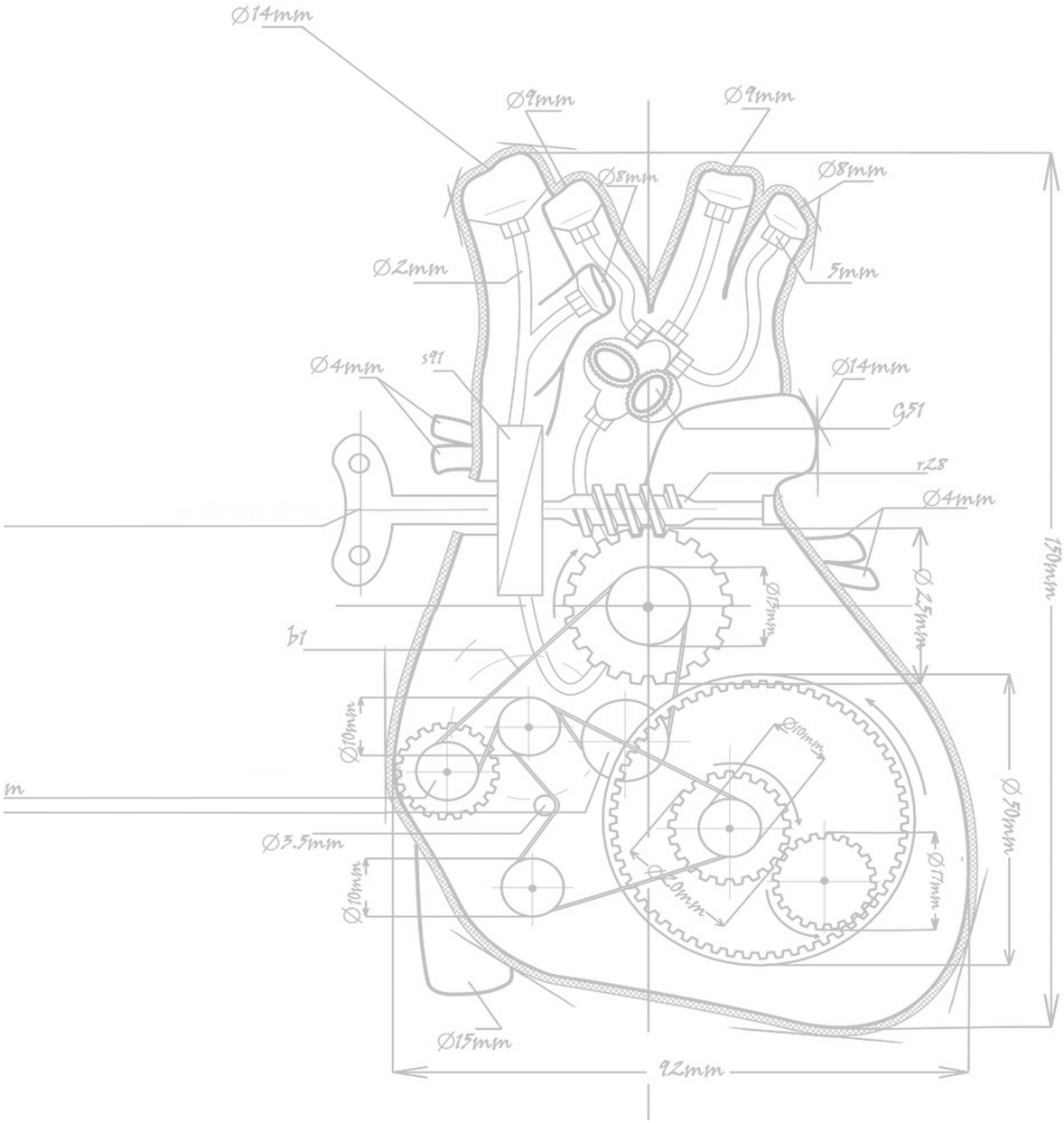
APPENDIX



NEDERLANDSE SAMENVATTING

Een hart infarct (MI) ontstaat doordat een gedeelte van het hart niet genoeg bloed krijgt, vaak door vaatvernauwing ten gevolge van aderverkalking (atherosclerose). Dit deel van het hart sterft daardoor af en er wordt een litteken gevormd. Aangezien hartcellen niet meer kunnen delen, kan dit litteken zich niet zelf herstellen en gaat de pompkracht van het hart omlaag.
Op dit moment bestaat er nog geen therapeutische mogelijkheid om dit probleem te herstellen, en veel patiënten krijgen in de loop van tijd na hun hart infarct last van hartfalen. Het is daarom nodig een nieuwe therapie te ontwikkelen die de hartspier en het andere hartweefsel kan herstellen. Stamcellen lijken hier een goede kandidaat voor. Stamcellen kunnen beschadigde organen herstellen, doordat ze kunnen differentiëren naar nieuwe celtypes, zoals hartspiercellen en bloedvatcellen, die afgestorven zijn door het hart infarct. Er zijn tot op heden al klinische studies gedaan, die zowel positieve als negatieve resultaten laten zien. In mijn thesis heb ik gekeken naar de tekortkomingen van de huidige methodes van stamcel therapie en hoe we deze gebreken aan kunnen pakken om het therapeutische voordeel te vergroten.
In deel I van mijn thesis heb ik de diverse soorten stamcellen samengevat die gebruikt worden om het beschadigde hart te behandelen. Deze stamcellen kunnen uit verschillende delen van het lichaam geïsoleerd worden. Mesenchymale stamcellen (MSC) kunnen bijvoorbeeld verkregen worden uit het beenmerg of vetweefsel, terwijl myoblasten uit skeletspieren gehaald worden. In deze thesis is het grootste deel van mijn werk gefocust op cardiomyocyte progenitor cells (CMPCs), stamcellen die direct uit het hart geïsoleerd worden. In hoofdstuk 2 geef ik een overzicht van hoe al deze cellen het hart kunnen repareren. Stamcellen uit het beenmerg participeren niet direct in de weefsel regeneratie, maar stimuleren de in het hart aanwezige cellen om het herstelproces te starten, door groeifactoren en andere signaal moleculen te produceren. Ook CMPCs kunnen deze stoffen produceren, maar daarnaast kunnen zij ook nieuwe vaten en hartspier vormen en dus actief participeren in het herstel van het hart. In hoofdstuk 3 hebben we ons gefocust op verschillende soorten CMPCs. Deze cellen zijn geïsoleerd met verschillende technieken door verschillende onderzoeksgroepen en er is weinig bekend over hoe gelijk of verschillend deze cellen onderling zijn. Wij hebben daarom de genexpressie profielen van al deze CMPCs bestudeerd en zijn tot de conclusie gekomen dat deze cellen onderling vrij gelijk zijn. De grootste verschillen werden niet gevonden door de verschillende isolatietechnieken, maar door variatie tussen de donoren waar de cellen van kwamen en de manier waarop ze gekweekt werden. Voor de implementatie van CMPC therapie is het daarom belangrijk dat er meer focus komt op het omgaan met de cellen dan de isolatie zelf.
In deel II van deze thesis heb ik gekeken naar 'pro-overleving' strategieën voor stamcellen. Een groot deel van de stamcellen overlijdt na de injectie in het hart, omdat ze in een vijandige omgeving terechtkomen. De schade die ontstaat door een MI veroorzaakt een toxische omgeving waarin de cellen hun werk moeten gaan doen. De pro-overleving strategieën pogen de cellen te beschermen tegen deze 'aanval' en verhogen de overleving en de aanwezigheid van cellen in het hart. In hoofdstuk 4 heb ik een overzicht gemaakt van een aantal methodes en technieken die in het verleden gebruikt zijn om MSC te beschermen, en heb ik wat mogelijke

nieuwe methodes voorgesteld. In hoofdstuk 5 hebben we een studie uitgevoerd waarin de een farmacologische stof, Necrostatin-1 (Nec-1) hebben gebruikt om de overleving van CMPCs in muizen te stimuleren. We zagen dat muizen die stamcellen kregen die behandeld waren met Nec-1, 50% meer CMPC overleving in het hart hadden dan de muizen die onbehandelde stamcellen kregen. Helaas bleek bij met meten van het functioneren van het hart met MRI, dat dat niet verschilde tussen de groepen. Ondanks een betere overleving van de cellen vonden we dus geen verbeterde effectiviteit van stamcel therapie voor het hart.

In deel III van mijn thesis hebben we een andere aanpak gekozen om stamcel therapie te verbeteren. Na injectie verdwijnt bijna 90% van de cellen in de vaten van het hart, omdat ze de tijd niet hebben om zich te hechten en deze cellen eindigen in de bloedstroom. In plaats van ons te focussen om celsterfte, wilden we nu het wegspoelen van de stamcellen naar de bloedstroom limiteren. Dit hebben we gedaan met behulp van biomaterialen, een onderdeel van het wetenschappelijke veld genaamd 'tissue engineering' (weefselkweek). In hoofdstuk 6 en 7 hebben we een 3D print methode opgezet waarin we een gel-achtig materiaal en CMPCs combineren in een georganiseerde structuur, die we daarna als een soort pleister op het beschadigde hart kunnen plakken. We kregen goede resultaten met deze aanpak, en de retentie van CMPCs in de muizen met een stamcel-pleister was stukken hoger dan bij injectie zonder biomateriaal. Daarnaast konden we met MRI-metingen en histologie ook zien dat de algemene gezondheid en conditie van de harten die de stamcel-pleister gekregen hadden, beter was dan die van onbehandelde muizen. Echter, de CMPCs die in de gel zaten, waren niet volledig gedifferentiëerd tot nieuwe hartspiercellen; een aandachtspunt voor toekomstige benaderingen.

In hoofdstuk 8 hebben we naar een nieuw materiaal gekeken: gelatine bolletjes. Dit zijn kleine bolletjes die geïnjecteerd kunnen worden in het hart, maar in tegenstelling tot stamcellen zijn ze groot genoeg om vast te lopen in de hartspier en worden ze dus niet weggespoeld. Groeifactoren en CMPCs kunnen zich hechten aan deze bolletjes, waardoor ze als een hulpmiddel gebruikt kunnen worden om de toediening van stamcellen in het hart te optimaliseren. Met de injectie van deze CMPC-gelatine bolletjes in het hart zien we duidelijke verbeteringen in stamcel overleving. Daarnaast kunnen deze gelatine bolletjes toegediend worden via katheters die in de dagelijkse praktijk ook gebruikt worden bij hartpatiënten, wat het extra aantrekkelijk maakt voor klinische toepassing.

In conclusie, hoewel er tot op heden al vele veelbelovende resultaten zijn met stamcel injecties in het hart, bleven de slechte overleving en retentie aandachtsgebieden om stamcel therapie efficiënter te maken. De strategieën die ik in mijn thesis heb onderzocht kunnen helpen om een uitvoerbare regeneratieve therapeutische optie te ontwikkelen voor patiënten met hartfalen. Verder onderzoek zal zich moeten zichten op het vormen van nieuwe hartspier door stamcellen. In hoofdstuk 9 heb ik 'reprogrammeer factoren' geïntroduceerd; eiwitten die een cel direct kunnen sturen om een hartspiercel te worden. Een integratie van deze technologie kan het therapeutische effect van stemcellen verder versterken. Goed gezien hebben we momenteel niet meer dan een oppervlakkig idee van de regeneratieve potentie van stamcellen. Hopelijk kunnen de komende tientallen jaren betere stamcel therapie benaderingen ontworpen worden om een scala aan ziekten te behandelen, zodra onderzoekers meer inzicht hebben van de onderliggende stamcel biologie.

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CURRICULUM VITAE

Dries Feyen was born May 8th, 1985 in Leiden, the Netherlands. He then went on to live in Bettlach, France and New Hope, USA where he completed his high school education. Upon graduation he returned to the Netherlands to attend the international honors university, University College Utrecht in 2003. He received his bachelors in 2006 with the double majors of Biology and Chemistry. Immediately after receiving his degree, Dries traveled to Windhoek, Namibia where he spent four months completing volunteer work with PAY, providing educational support for intercity youth. In 2007, he returned to the Netherlands and enrolled in the Master's program Drug Innovation at Utrecht University. During his Masters work he completed his first internship at the lab of Membrane Enzymology where he studied lipid trafficking in epithelial cells. His second internship was performed at the department of Pharmaceutics, where he focused on non-viral gene therapy approaches. During these two internships his interest in stem cell therapy grew which led him to continue his education as a PhD researcher at the lab of Cardiology under the supervision of Prof. Pieter Doevendans and Joost Sluijter. Here he focused on stem cells therapy for the heart. The results of his research are described in this thesis.