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TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BM	bone marrow
CAM	chorioallantoic membrane
CASC	cardiac atrial appendage stem cell
CDC	cardiosphere-derived cell
CSC	cardiac stem cell
CVD	cardiovascular disease
Cx43	connexin 43
EC	endothelial cell
ELISA	enzyme-linked immunosorbent assay
EPC	endothelial progenitor cell
ET-1	endothelin 1
ESC	embryonic stem cell
FBS	fetal bovine serum
HMEC-1	human microvascular endothelial cell line 1
HF	heart failure
IGFBP-3	insulin-like growth factor binding protein 3
IHD	ischemic heart disease
IFN-γ	interferon gamma
iPSC	induced pluripotent stem cell
LG-DMEM	low glucose Dulbecco's modified eagle medium
LV	left ventricle
LVEDV	left ventricular end diastolic volume
LVESV	left ventricular end systolic volume

LVEF	left ventricular ejection fraction
MAPC	multipotent adult progenitor cell
MHC	major histocompatibility complex
MI	myocardial infarction
MLC-2A	atrial myosin light chain 2
MLC-2V	ventricular myosin light chain 2
MSC	mesenchymal stem cell
PBMC	peripheral blood mononuclear cell
P/S	penicillin-streptomycin
TNF-α	tumor necrosis factor alpha
UM-SCC	University of Michigan Squamous Cell Carcinoma
VEGF	vascular endothelial growth factor

ABSTRACT

Heart failure as a consequence of myocardial infarction (MI) is one of the major causes of global morbidity and mortality. Stem cells promise to replace the damaged or lost cardiac muscle with functional healthy tissue. However, only moderate therapeutic effects were observed in clinical trials with various stem cell types, likely due to their limited cardiomyogenic differentiation. Recently a new cardiac stem cell population was discovered, called the cardiac atrial appendage stem cell (CASC). These CASCs have typical stem cell properties and possess a superior *in vitro* myocardial differentiation potential compared to other stem cell types. This project further explored the contribution of CASCs to myocardial regeneration.

The safety and therapeutic benefit of CASC transplantation in a minipig MI model was established. Improvement of both regional and global left ventricular function together with a reduction in scar mass were associated with extensive cell engraftment and the formation of fully differentiated cardiomyocytes with a mature contractile profile. Moreover, CASCs showed electromechanical integration into the host myocardium without observations of cardiac arrhythmias.

In the second part of this study, it was shown that CASCs do not contribute to neovascularization by differentiation into vascular cells, but by the secretion of numerous angiogenic growth factors. Combined myogenesis and angiogenesis enhances the therapeutic potential of CASCs, making them highly suited for the treatment of ischemic heart disease. Finally, the effect of the inflammatory

infarct environment on CASCs was investigated. CASC viability was not affected by inflammatory conditions and they showed a low immunogenic profile and immunomodulatory properties. These results might pave the way for allogeneic stem cell transplantation, which would allow development of an 'off-the-shelf' cell-based therapy.

In conclusion, this study yielded essential proof of the safety and therapeutic efficacy of CASC transplantation after MI and provides important insights for the further development of the therapeutic application of CASCs in ischemic heart disease patients.

1. INTRODUCTION

1.1 Epidemiology

Cardiovascular disease (CVD) is the major cause of global morbidity and mortality, accounting for three in every ten deaths. In 2012, 17.5 million people died due to CVD. Of these, 7.4 million deaths were caused by ischemic heart disease (IHD) alone [1]. In Belgium, 28.8% of all deaths are caused by CVD, of which 7.8% specifically by IHD. Efforts on prevention and improvements in medical care have successfully reduced the incidence and increased the survival of IHD patients. Indeed, only 5.2% of the patients die within the first day after myocardial infarction (MI). However, these patients are strongly at risk for developing heart failure (HF) and the two year mortality rate of MI patients is 63.7% [2]. Improvements in medical care together with an aging population, have thus paradoxically resulted in a higher prevalence of HF with a lifetime risk of one in five. As much as 15,643 new HF patients are diagnosed in Belgium each year with an incidence of 0.2% [3]. In 2001, 19,398 hospital admissions were reported with HF as primary diagnosis, with an estimated cost of 94 million euros [4]. HF therefore not only constitutes an important medical challenge, but also has a high social and economic impact.

1.2 Pathophysiology

HF is the final stage of many heart diseases characterized by the inability of the heart's pump function to meet the body's needs. In general, it is a progressive condition, however, acute cases can present within 24h with occurrence of severe symptoms, including edema, dyspnea, fatigue and an increased heart

rate. Hereditary heart conditions or hemodynamic pressure and volume overloading, e.g. caused by high blood pressure, generally lead to a more progressive onset of disturbed contractility. Damage to the heart muscle, as seen after MI, on the other hand can cause a sudden demonstration of HF and is thought to be the most important risk factor for HF [5, 6]. Thrombus formation in an atherosclerotic lesion induces total or near total acute coronary occlusion, leading to irreversible cardiomyocyte loss in the area supplied by the coronary artery in question. The high metabolic need of the heart muscle makes it very susceptible to nutrient and oxygen depletion. Hence, a longer occlusion of the blood supply leads to a greater loss of heart muscle. Early reperfusion of the occluded coronary artery by catheter-based interventions and surgical bypass procedures can restore the blood supply to the infarcted area with a reduction of myocardial necrosis. Unfortunately, these procedures are not always successful and microvascular dysfunction in the ischemic area can still prevent efficient reperfusion. After the ischemic insult, an inflammatory phase is initiated with activation of tissue-resident immune cells. Both immune and non-immune cells produce pro-inflammatory cytokines and chemokines that lead to recruitment of circulating inflammatory leukocytes from the blood. Neutrophils and macrophages take care of the removal of dead cells and matrix debris. They also release cytokines and growth factors that lead to the formation of a highly vascularized granulation tissue by the proliferation of fibroblasts and endothelial cells (ECs) [7, 8]. Then, myofibroblasts are activated and the infarcted muscle is gradually replaced by non-contractile collagen-rich scar tissue. Apoptosis of ECs and fibroblasts leads to regression of micro-vessels and further maturation of

the scar. Complex pathophysiological changes, including EC dysfunction and suppressed angiogenesis, lead to further infarct expansion and wall thinning within hours to days after MI [8, 9]. These changes predispose the myocardium to additional ischemic insults and severely affect cardiac contractility, since interstitial fibrosis further impedes both myocardial contraction and relaxation. To compensate for the resulting impaired cardiac pump function and increased demands of the remaining cardiomyocytes, remodeling occurs. This process is characterized by cardiomyocyte hypertrophy, progressive wall thinning and ventricular chamber dilation. The resulting reduction in cardiac output and the increase in wall stress activate the adrenergic neuro-hormonal system, the renin angiotensin-aldosterone system (RAAS) and the hypothalamic-neurohypophyseal system. These systems serve to maintain arterial pressure and cardiac output by enhanced cardiac contractility, sodium and fluid retention and peripheral vasoconstriction [10, 11]. Initially, this remodeling process is able to preserve cardiac output and stroke volume, but over time it becomes detrimental with progressive deterioration of cardiac performance. Persistent activation of the adrenergic system increases ventricular afterload, contributes to an increase in heart rate and may cause hypertrophy, ischemia and tachy-arrhythmias. Indeed, patients presenting with ventricular remodeling are more likely to develop cardiac arrhythmias [12] and their ten-year mortality rate is increased from 50% to 71% [13].

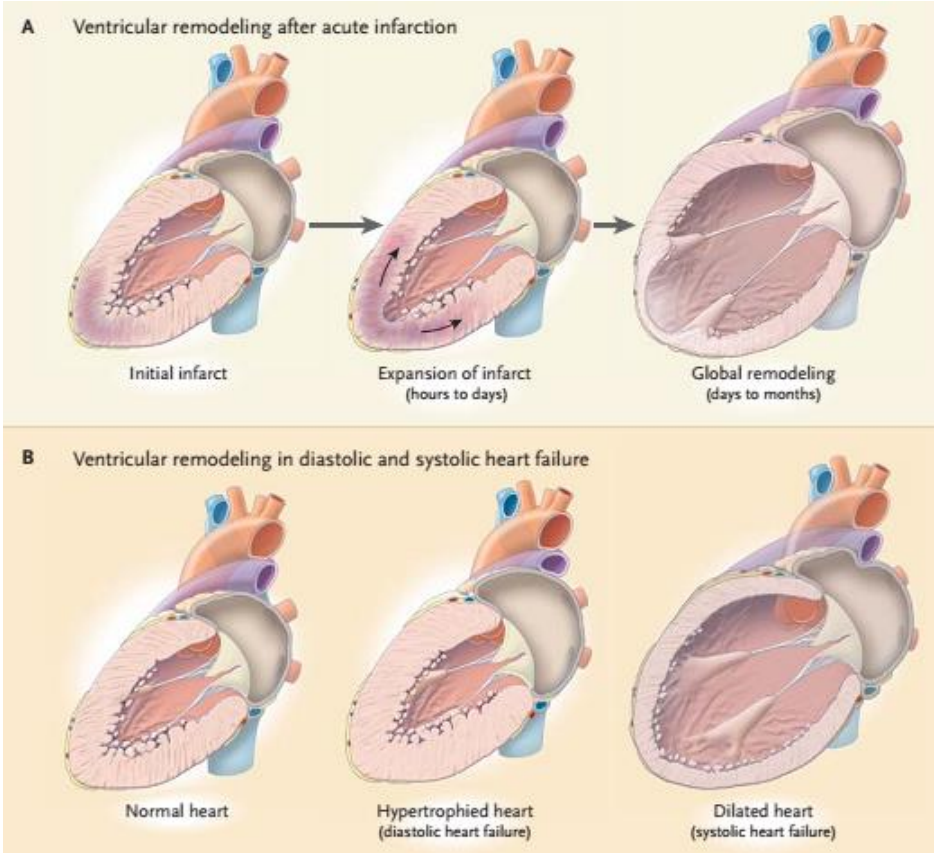


Figure 1: MI and ventricular remodeling.
A) A significant proportion of the heart muscle is lost after MI. Within hours to days, infarct expansion and wall thinning occur. Global remodeling takes place days to months after the ischemic event, which leads to ventricular dilatation and a decrease in contractile function**.**
B) Ventricular remodeling usually leads to myocardial hypertrophy with thickened ventricular walls, a normal-sized LV cavity and preserved systolic function (diastolic heart failure). Dilated cardiomyopathy on the other hand is characterized by thinning of the LV walls and enlarged heart chambers with a decrease in systolic pump function (systolic heart failure). Adapted from Jessup et al., 2003.

1.3 Treatment of myocardial infarction

After MI, the highest priority is to restore an adequate coronary blood flow as quickly as possible. This can be achieved by fibrinolysis, anti-platelet drugs or surgical revascularization procedures such as primary percutaneous transluminal coronary angioplasty and coronary artery bypass graft (CABG). After reperfusion, the MI patient is hospitalized and should be monitored continuously by electrocardiography (ECG) for adverse electrical or mechanical events since re-infarction and death occur most frequently within the first 24 hours. Aspirin, β-adrenoceptor blocking agents, nitrates or angiotensin converting enzyme (ACE) inhibitors are administered to reduce myocardial oxygen demand by lowering blood pressure and heart rate. The patient will have to continue drug treatment for an indefinite period of time. Additional medication and lifestyle changes are usually necessary to reduce risk factors such as high body weight, high cholesterol and high blood pressure [14]. Current treatment modalities aim at improving survival, slowing down disease progression and alleviating symptoms. Unfortunately they are unable to replace the lost heart tissue making them in a sense no more than palliative treatment. Heart transplantation is the only true cure, however, donor hearts are limited and the success of this procedures is restricted by immune rejection, infections and other complications [14, 15]. Therefore, development of new treatment modalities to restore the lost cardiac tissue and treat patients suffering from HF after MI is crucial.

1.4 Intrinsic myocardial repair mechanisms

Until a few decades ago, it was believed that the adult mammalian heart was a terminally differentiated organ without intrinsic capacity to regenerate after injury. Cardiomyocyte hypertrophy was thought to be the only mechanism of postnatal cardiac growth and repair, since cardiomyocytes are irreversibly withdrawn from the cell cycle soon after birth [16]. This view was changed by the presence of proliferating cardiomyocytes in both healthy and diseased hearts. Increased numbers of dividing cardiomyocytes in the hearts of ischemic or dilated cardiomyopathy patients compared to healthy controls suggested the ability of myocytes to proliferate and replace dying cells [17, 18]. This mitotic activity is however limited and seems to decrease with age as reported in a study by Bergmann et al., which shows that cardiomyocyte renewal gradually decreases from an annual turnover of 1% at the age of 20 to 0.3% at the age of 75 [19]. The presence of small numbers of amplifying cells [20] together with the detection of male cells in female hearts transplanted in male recipients [21] have suggested that the adult human heart contains a pool of cardiac stem cells (CSCs) [20]. These CSCs give rise to cardiomyocytes, smooth muscle cells and ECs and are capable of self-renewal [22]. Unfortunately, the regenerative capacity of the human myocardium is inadequate to compensate for the severe heart muscle loss after MI. Regenerative medicine aims to boost the limited self-renewal capacity of the human heart, mainly by focusing on the formation of new heart muscle and on angiogenesis to increase local blood supply. In this aspect, stem cell therapy has generated significant interest in clinical and basic scientific communities as promising strategy for a cure. Implantation of

exogenous stem cells may allow for replacement of damaged cardiac cells and compensate for the inadequate intrinsic repair mechanisms of the heart.

1.5 Stem Cells for Myocardial repair

Stem cells are promising candidates to replace the lost heart tissue after MI, as they can be expanded to large numbers by self-renewal and show potential to differentiate into specialized cell types. They can be isolated from embryonic or adult tissues and differ in their expression of surface markers, proliferation potential and their ability to differentiate into mature cell types. It was thought that stem cells would mainly regenerate the myocardium by differentiation into cardiomyocytes and vascular cells. Despite the original belief that transplanted cells would serve as building blocks to generate new heart muscle cells, cross-talk between delivered cells and the host myocardium also seems to be an important trigger for myocardial repair. Stem cells secrete paracrine and autocrine factors that influence cell survival, angiogenesis, cardiac remodeling and cardiomyocyte contractility and metabolism [23]. A growing number of preclinical studies and clinical trials have demonstrated the safety of various stem cell types with however conflicting improvements in cardiac function [24-26].

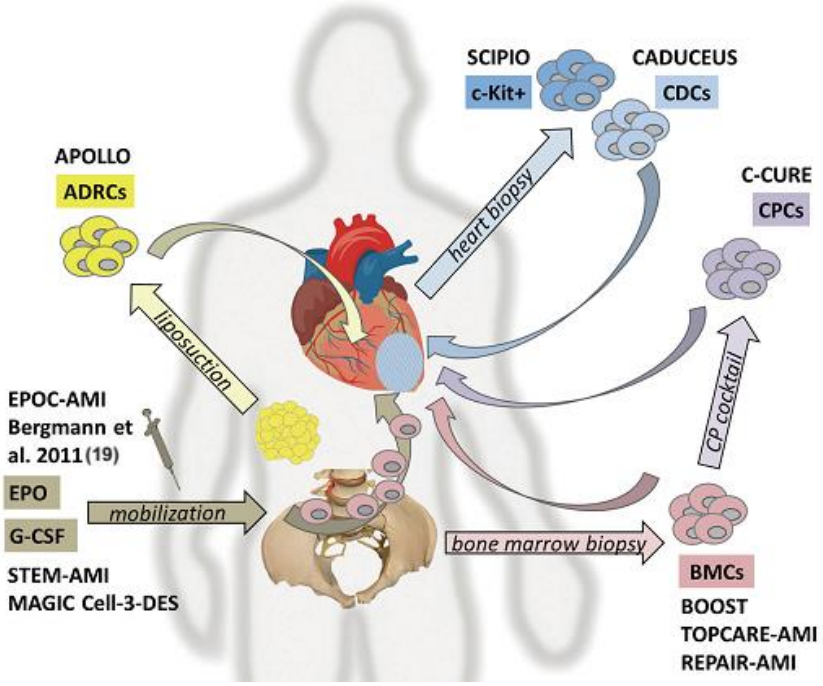


Figure 2: Stem cells administered in clinical trials with IHD patients. Clinical trials with BMCs, ADRCs, CDCs and c-kit+ stem cells have been performed to regenerate the lost myocardium after MI. In addition, cytokines such as EPO or G-CSF are administrated to mobilize progenitors from the bone marrow. BMCs bone marrow cells, ADRCs adipose tissue-derived regenerative cells, CPCs cardiopoietic stem cells, CP-cocktail cardiopoietic cocktail, CDCs cardiosphere-derived cells, G-CSF granulocyte colony stimulating factor, EPO erythropoietin. Adapted from Doppler et al., 2013.

1.5.1 Embryonic stem cells

Embryonic stem cells (ESCs) are isolated from the inner cell mass of the blastocyst. They possess a long replicative lifespan and their high plasticity allows them to differentiate into every cell type of the human body. Both *in vitro* and *in vivo* myocardial differentiation of ESCs has been reported [27, 28] In addition, undifferentiated ESCs positively influence contractility, infarct size, necrotic and apoptotic cell death, fibrosis and cardiac remodelling after MI by

paracrine mechanisms [29, 30]. Although no tumor formation was observed in murine MI models, risks of rejection and teratoma formation have been described and limit the use of ESCs. Moreover, ESCs can only be isolated from human embryos, raising ethical issues [31].

1.5.2 Induced pluripotent stem cells

Both ethical issues and problems with immune rejection of ESCs have been overcome by the discovery of induced pluripotent stem cells (iPSCs). Introducing a combination of pluripotency genes, including Oct-3/4, Sox-2, Klf-4, and c-Myc, into somatic cells makes them pluripotent with similar proliferation and differentiation properties as ESCs [32, 33]. In this way, patient's own somatic cells are reprogrammed to become pluripotent cells. Cardiomyogenic differentiation of iPSCs has been shown by numerous groups [34, 35] and iPSC treatment has been shown to restore cardiac function after acute MI [36]. Transplantation of iPSC-derived cardiomyocytes, ECs, and smooth muscle cells in a porcine model of acute MI was able to improved LV function and arteriole density, while reducing infarct size and ventricular wall stress without inducing ventricular arrhythmias [37]. Direct *in vivo* reprogramming of cardiac fibroblasts into induced cardiomyocytes has also been described as a promising strategy to regenerate the lost heart muscle after MI [38]. These *in vivo* induced cardiomyocytes appear to be more similar to endogenous cardiomyocytes compared to their *in vitro* counterparts. This indicates the importance of environmental cues such as extra-cellular matrix, signaling pathways and mechanical or electrical stimulation [39]. Despite these promising results, the reprogramming efficiency of somatic cells is still low and cannot be repeated in a standardized way because of

insufficient understanding of the mechanisms. Variations in reprogramming imply that only some iPSC lines derived from a single batch of somatic cells will be effective in myocardial repair. Juvenile cells and progenitor cells can be reprogrammed more efficiently towards iPSCs than terminally differentiated cells [40], suggesting that stem cells are a preferred reprogramming cell source for clinical applications. In addition, both iPSCs and their progenies have a high risk of tumor formation. Indeed, iPSCs contain abnormalities at the genetic and epigenetic level [34]. A better understanding of reprogramming and differentiation mechanisms is required to elucidate these problems. Despite their promises, the use of iPSCs is still limited. The safety and reproducibility of iPSC therapies need to be guaranteed before clinical applications are feasible.

1.5.3 Bone marrow stem cells

Bone marrow (BM) is an interesting source of progenitor cells as it can be easily accessed, is renewable and provides an autologous source for regenerative cells. The BM contains three main subpopulations of stem cells: hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs). BM harvest and the isolation of so-called BM mononuclear cells (MNCs) is routinely performed in BM transplantation for hematological malignancies. Despite the existence of controversy concerning the cardiomyogenic differentiation potential of BM MNCs [41, 42], a vast number of randomized controlled trials with BM MNCs in MI patients have been performed. The outcome of these studies is variable and meta-analysis suggest that if BM-derived cell therapy indeed truly has any beneficial effects, these are not clinically relevant to MI patients [43, 44]. This can be explained by the limited

cardiomyogenic differentiation potential of BM MNCs. However, paracrine mechanisms have been reported to increased neovascularization, cardioprotection and decreased cardiac remodeling [45]. Nevertheless, these effects did not seem powerful enough to substantially impact patient outcomes. Fine-tuning BM regenerative potential was therefore investigated by purification or enrichment of the specific stem cell subpopulations of HSCs, MSCs and EPCs.

Hematopoietic stem cells

HSCs give rise to all blood cell types through the process of hematopoiesis. As mature blood cells are short lived, HSCs continuously deliver more differentiated progenitor cells, while properly maintaining the HSC pool size by accurately balancing between self-renewal and differentiation. A major obstacle in HSC studies is the difficulty to control HSC self-renewal and differentiation in culture, leading to insufficient stem cell numbers for clinical applications [46]. Isolation usually occurs based on the absence of lineage differentiation markers (Lin-) combined with expression of surface markers c-kit, CD34, CD45, CD133, and CD90 [47] Although they primarily reside in BM, HSC can be isolated from the circulation at small numbers. The number of HSC in the peripheral blood can be enhanced by injections of certain cytokines, such as granulocyte colony stimulating factor (G-CSF) [48]. Interestingly, mobilized HSCs have been shown to respond to cytokine signaling from injured myocardium by homing to the damaged tissue [49]. Intracoronary infusion of G-CSF mobilized peripheral blood stem cells has been shown to promote angiogenesis and improve cardiac function after MI [50, 51]. Expression of early cardiac muscle, endothelial and stem

cell markers has been shown on circulating stem cells in the peripheral blood in the setting of MI [52]. Ischemic myocardium releases inflammatory and hematopoietic cytokines, such SDF-1 [53], which are known to stimulate the release of progenitor populations, including HSCs, MSCs and EPCs, from the BM niche [54, 55]. Nevertheless, controversy exists about the cardiomyogenic differentiation of HSCs. While some groups have reported the formation of *de novo* myocardium by extensive differentiation of HSCs into cardiomyocytes and vascular cells [56, 57], others reported only a low frequency of cardiomyogenic differentiation [58, 59] or adaptation of a cardiomyocyte phenotype by cell fusion-dependent mechanisms [60]. Although HSCs do not seem to readily adopt a cardiomyogenic fate after transplantation into the infarcted myocardium, paracrine effects on cardiac angiogenesis and ventricular remodeling have also been proposed. Still, because of the rather low plasticity of HSCs [58] and difficulties in their *in vitro* expansion [47], other progenitor populations in the BM have been explored.

Endothelial progenitor cells

Endothelial progenitor cells (EPCs) or angioblasts are the immediate precursors of EC during neovasculogenesis. As discovered by Asahara et al. in 1997, these cells reside in the BM and enter the blood in small numbers [61]. Classification into early and late EPCs can be made according to their time-dependent appearance after plating BM MNCs [62]. Early EPCs have an elongated morphology and a short lifespan in culture of three to four weeks, while late EPCs are cobblestone-shaped and show a long lifespan with rapid proliferation *in vitro*. In general EPCs

are reported to express the markers flk-1, VE-cadherin, CD34, CD31, CD133 and von Willebrand factor. The hematological marker CD45 is also expressed by early EPCs, while CD31 and KDR is only present on a subpopulation of them [62, 63]. Different roles for early and late EPCs in neovascularization have been suggested. Early EPCs mainly secrete angiogenic factors that stimulate ECs, whereas late EPCs serve as a high proliferative pool with vasculogenic differentiation potential [62, 63], suggesting both cell types might have beneficial effects in IHD. The number of circulating EPCs is increased in response to tissue ischemia [64] and they seem to incorporate into ischemic sites to actively promote neovascularization [61]. EPCs from IHD patients can transdifferentiate into functionally active cardiomyocytes *in vitro* after co-culture with rat cardiomyocytes based on cell contact mechanisms as shown by expression of α-sarcomeric actinin, cardiac troponin I, MEF2 and connexin 43 (Cx43) [65]. In 2001 it was shown by Orlic and coworkers, that injection of purified Lin-/c-kit+ EPCs into the infarcted myocardium reduces mortality and morbidity in a murine MI model [57]. Similar results were observed after EPC transplantation in a rat MI model with preservation of cardiac function based on increased neovascularization and a reduction in LV scarring [66]. Based on the promising results, clinical studies were carried out with hematopoietic progenitors as this population also contains a suitable number of EPCs. The TOPCARE-AMI study evaluated the delivery of EPCs or BM MNCs directly into coronary arteries in patients with reperfused acute MI. At four months, LV ejection fraction (LVEF) improved by 8.5% in both EPC and BMC patients, compared with 2.5% seen in the control group (patients matched for LVEF, infarct localization and infarct size

after acute reperfusion therapy with paired LV angiograms available acutely and at four month follow-up). Enhancement of regional wall motion, improvements in coronary blood flow and an increase in myocardial viability in the infarct zone were also observed. However, the control group of this study was not randomized with non-contemporary historical controls and no placebo control was included, making it impossible to attribute the persistent reduction to the application of cell therapy [67]. After a five year follow-up, LV end systolic volume remained stable (LVESV), but LV end diastolic volume (LVEDV) increased significantly [68]. In general, no or very modest clinical benefits were reported for hematopoietic progenitors, including EPCs [69].

Bone marrow mesenchymal stem cells

BM mesenchymal stem cells (MSCs) were first reported by Friendenstein et al. as fibroblast-like cells, who show rapid adherence to tissue culture vessels, pointing to an origin from the stromal compartment of the BM [70]. Later clonal expansion initiated by single cells (termed the colony-forming unit fibroblastic, CFU-Fs) was observed and bone, cartilage, adipose and fibrous tissue could be experimentally generated by the progeny of a single BM stromal cell. In 1991 the term MSC was proposed as an alternative to stromal or osteogenic stem cell [71]. Originally MSC specifically referred to cells in the BM, but the current notion has been extended to include cells from every postnatal connective tissue such as synovium, adipose tissue, dental pulp, umbilical cord blood, etc [72]. After organ injury, MSCs can mobilize to the peripheral blood and migrate to the site of injury. Indeed, the percentage of circulating CD45-/CD34-CD34-/CD90+

MSCs has been shown to be higher in patients with hypertrophic cardiomyopathy compared to healthy controls [73]. MSCs gained a lot of interest for the development of novel cellular therapies for numerous pathologies, since they are postnatal human stem cells with a broad differentiation potential. Moreover, these stem cells can be directly obtained from individual patients, eliminating complications associated with immune rejection, allogenic transplantation and ethical issues. Lack of uniformity concerning the isolation, identification and expansion of MSCs led to inconsistencies in the field [74]. Therefore, in 2006 the International Society for Cellular Therapy (ISCT) stated that 'multipotent mesenchymal stromal cells' (MSC) is the currently recommended designation for the plastic-adherent cells isolated from BM and other tissues that have often been labeled as MSCs. Three criteria were used to identify MSCs: 1) adherence to plastic in standard culture conditions, 2) a phenotype with >95% positive cells for CD73, CD90 and CD105 but negative (<2%) for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR and 3) *in vitro* differentiation into osteoblasts, adipocytes and chondroblasts (demonstrated by staining of *in vitro* cell culture) [75]. Makino et al., 1999 were the first to report the establishment of an adult BM MSC cell line that differentiated into cardiomyocytes by 5-azacytidine treatment *in vitro* [76]. About 30% of the cells began to beat spontaneously after two weeks, expressed cardiomyogenic markers (myosin, desmin, actinin), showed a cardiomyocyte-like ultrastructure and produced action potentials [76]. Others confirmed the expression of cardiomyogenic markers after *in vitro* differentiation of MSCs [59, 77-80], but functional *in vivo* cardiomyogenic differentiation was a rare event and, therefore, could not be the

main contributor for cardiac regeneration by MSCs [78]. To satisfy the clinical usage, the conditions of induction and the differentiation efficiency of MSCs needed to be improved. Several methods have been used to increase the cardiac differentiation of MSCs, including biochemical drugs (5-aza, BMP-2, Ang II, DMSO) and culture conditions that mimic the cardiac environment (cardiomyocyte lysate, co-cultures with cardiomyogenic cells or tissues) [81]. Although *in vitro* treatment with differentiation media leads to an enhanced expression of cardiomyogenic mRNA and proteins, also untreated MSCs show an intrinsic expression of cardiomyogenic mRNA and proteins [78, 82]. This indicates that the expression of cardiomyogenic mRNA and proteins might not exclusively be dedicated to cells of the cardiomyogenic lineage. In addition, differentiation of MSCs into cardiomyocytes could be established by co-culturing them with neonatal cardiomyocyte, but not in co-culture with adult cardiomyocytes, questioning the clinical significance of the cardiomyogenic potential of MSCs [83]. Even without extensive cardiomyogenic differentiation of MSCs after transplantation in infarcted heart tissue, significant functional improvements in the post-infarcted heart have clearly been observed [84-86]. However, an additional problem is the low cell retention of MSCs delivered to the heart by various methods (peripheral intravenous infusion, intracoronary infusion, catheter-based transendocardial injection, and direct surgical injection) [87]. MSCs are not retained in the myocardium and commonly are found in the lungs and spleen [79, 88-90]. These findings have suggested that not cardiovascular differentiation, but paracrine effects might account for most therapeutic effects of MSCs. Indeed, MSCs secrete cytokines, chemokines, growth factors, miRNAs and extracellular

vesicles that promote angiogenesis [91], prevent cardiomyocyte apoptosis [92], inhibit adverse remodeling [77] and activate resident CSCs [93]. Another interesting feature encouraging researchers to keep investigating MSCs for myocardial repair, is their unique immune phenotype together with their powerful immunosuppressive activity [87]. Human MSCs do not express major histocompatibility (MHC) complex class II or costimulatory molecules B7 and CD40 ligand and show low levels of human leukocyte antigen MHC class I [94, 95]. Cell-cell contact with immune cells and secretion of soluble factors, such as nitric oxide (NO), indoleamine 2,3-dioxygnease, hepatocyte growth factor, interleukin-2, interleukin-10 and heme-oxygenase render MSCs immunomodulatory properties [96-101], which allows allogeneic transplantation. Allogeneic MSC transplantation via intravenous [102], intramyocardial [90] or transendocardial [103] delivery were able to reduce scar formation and improve cardiac function in various animal models of MI.

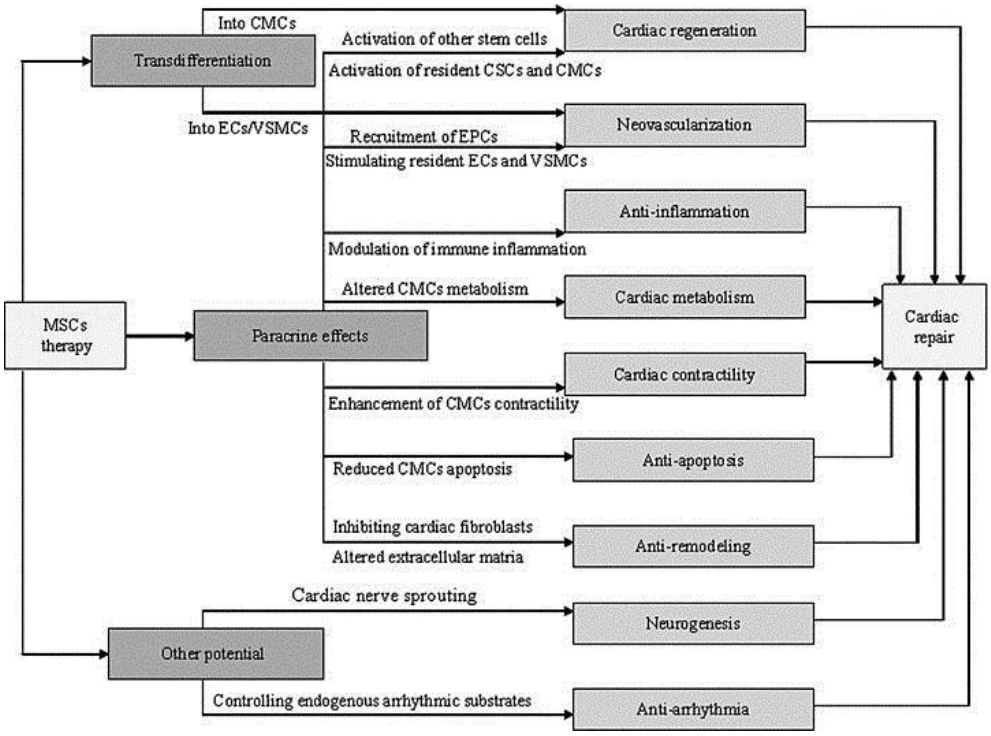


Figure 3: Mechanisms of MSC-mediated myocardial repair.
(Trans)differentiation of MSCs into cardiomyocytes and vascular cells promotes myogenesis and vasculogenesis. Paracrine effects promote endogenous cardiac regeneration, angiogenesis and have anti-inflammatory, anti-apoptotic and anti-remodeling effects together with modulation of cardiac contractility and metabolism. MSCs mesenchymal stem cells, CMCs cardiomyocytes, CSCs cardiac stem cells, EPCs endothelial progenitor cells, ECs endothelial cells, VSMCs vascular smooth muscle cells. Adapted from Wen et al., 2011.

Multipotent adult progenitor cells

Yet another BM progenitor cell could be distinguished from other BM MNCs, called the multipotent adult progenitor cell (MAPC) [104]. MAPCs derived from rats were originally described by Jiang et al. as adherent BM cultures exhibiting pluripotent lineage differentiation capacity for each of the three primitive germ

layers *in vitro* and *in vivo* [105]. These MAPCs are isolated by depleting CD45+ and glycophorin-A+ cells from BM followed by expansion in serum-low or serum-free culture conditions with EGF and PDGF-BB [106]. Human MAPCs can be expanded long-term for more than 70 population doublings and have long telomeres that do not shorten in culture. However, the differentiation potential of human MAPCs is more restricted than that of rat MAPCs. Human MAPCs can only differentiate into typical mesenchymal lineage cells [107] and ECs [108]. Although *in vitro* expression of Nkx2.5, GATA-4 and myosin heavy chain has been shown, differentiation of MAPC into cardiomyocytes with the typical functional characteristic, such as spontaneous rhythmic contractions, has not been established [109]. Autologous, allogeneic and syngeneic transplantation of MAPCs after MI resulted in an improvement in cardiac function based on paracrine effects on vascular density and potentially other trophic effects on injured cardiomyocytes [110-112]. A Good Manufacturing Practice human MAPC product was created, called Multistem, to guarantee the safety of these stem cells in clinical trials [113].

Despite the controversy concerning the cardiomyogenic differentiation of BM stem cell types, a vast number of clinical trials for BM stem cell transplantation in MI patients were performed over the past decades (Table 1). Meta-analysis reveals that BM cell transplantation is safe and feasible. Although significant improvements in LVEF have been found, these changes are not clinically relevant, suggesting that there is insufficient evidence for a beneficial effect of BM cell therapy [43, 114].

Table 1: Bone marrow stem cell trials (chronological order)

Study	Cell type	Delivery method	Follow-up	Outcome
Acute MI				
Strauer et al., 2002 [115]	MNCs	IC	3M	Reduction infarct size No improvement LV contractility
TOPCARE-AMI Assmus et al., 2002 [67]	MNCs CPCs	IC	12M	Preservation LVEF No difference MNCs or CPCs
BOOST Wollert et al., 2004 [116]	MNCs	IC	62M	No sustained improvement LVEF
ASTAMI Lunde et al., 2006 [117]	MNCs	IC	12M	No effect global LV function
Janssens et al., 2006 [118]	MNCs	IC	4M	No recovery global LV function Reduction scar size Improvement regional systolic function
REPAIR-AMI Schachinger et al., 2006 [119]	MNCs	IC	24M	Improved regional LV contractility Less major adverse cardiovascular events Improved LVEF
BALANCE Yousef et al., 2009 [120]	MNCs	IC	60M	Preservation LV function Reduced mortality
Penn et al., 2012 [121]	Multistem	IC	4M	Improved LVEF

Lee et al., 2014 [122]	MSCs	IC	6M	Modest improvement LVEF
Chronic MI				
IACT-STUDY Strauer et al., 2005 [123]	MNCs	IC	3M	Reduction infarct size Improved LVEF Improved wall movement Improved myocardial glucose uptake
Hendrikx et al., 2006 [124]	MNCs	IM	4M	Improvement regional LV contractility
STAR Strauer et al., 2010 [125]	MNCs	IC	62M	Improved hemodynamics Improved LV contractility Improved exercise capacity
FOCUS Perin et al., 2012 [126]	MNCs	TE	6M	No functional improvements
TAC-HFT Heldman et al., 2013 [127]	MNCs MSCs	TE	12M	MSCs superior to MNCs Scar reduction (MSCs) Improvement MLWHFQ (MSCs)
POSEIDON Hare et al., 2013 [128]	MSCs alloMSCs	TE	12M	Improvement 6-minute walk test (MSCs) Improvement MLWHFQ score (MSCs) Reduction infarct size (MSCs & alloMSC) Reduction LVEDV (alloMSCs) No immune response alloMSCs
HF				
Perin et al., 2004 [129]	MNCs	TE	12M	Improved exercise capacity

				Improved myocardial perfusion
C-CURE Bartunek et al., 2013 [130]	MSCs	TE	12M	Improvement 6-minute walk test Improved LVEF
Perin et al., 2015 [131]	alloMSCs	TE		No immune response Reduction HF-related MACE High-dose superiority (150 million cells)

alloMSCs allogeneic mesenchymal stem cells; CPCs circulating progenitor cells; IC intracoronary; IM intramyocardial; LV left ventricular; LVEDV left ventricular end diastolic volume; LVEF left ventricular ejection fraction; M months; MACE; major adverse clinical event; MI myocardial infarction; MLWHFQ Minnesota Living With Heart Failure Questionnaire ; MSCs mesenchymal stem cells; MNCs mononuclear cells; TE transendocardial

This lack of efficacy indicates that several questions still remain concerning the time frame, cell type, dose, delivery route, etc. For example, a meta-analysis by Liu et al., 2016 of randomized controlled trials in patients with AMI receiving BM MNC therapy revealed that administration four to seven days after MI leads to a better outcome than other time windows for improvement of LVEF or reduction of the incidence of major adverse cardiac events [132]. One of the most important methodological questions refers to the best delivery method of cells to the heart. Most studies have used intracoronary injection, however, homing of unselected BM MNCs to infarcted myocardium assessed by 18-fluorodeoxyglucose labeling, revealed a cardiac retention of only 1.3% to 2.6% [133]. Intramyocardial injection into the infarct border zone can be combined with CABG [134, 135] or can be performed via transendocardial catheter injection using the NOGA injection

system (Biosense Webster Ltd., Diamond Bar, California) [126]. The latter method is non-invasive and has been shown to yield higher cell retention levels [136]. Still, finding the right cell type for myocardial regeneration is key and the limited effects found in most clinical trials with BM stem cells is most likely due to the limited differentiation of these stem cells towards cardiomyocytes [23].

1.5.4 Cardiac stem cells

The adult mammalian myocardium is now known to harbor endogenous populations of progenitor cells that in certain circumstances can be stimulated to generate new cardiomyocytes. Because of their cardiac origin, these CSCs are thought to be pre-programmed to form cardiomyocytes, as well as other supporting cell types such as ECs and vascular smooth muscle cells [137]. Various CSC types have been identified based on c-kit, Sca-1 or islet-1 expression, efflux of Hoechst dye, the ability to form spheres and a high aldehyde dehydrogenase enzyme (ALDH) activity [138, 139]. Although the different CSC populations have long been regarded as separate entities, progenitor cells co-expressing various markers have been found in the adult heart, indicating that a relation between the populations might exist [140]. Furthermore, cardiosphere-derived cells (CDCs) from adult heart biopsies represent a mixed population, containing a low number of c-kit+ and islet-1+ cells [141]. In depth understanding should be gained on the phenotype of the various cardiac progenitor populations and their precise role in heart development and regeneration.

c-kit+ CSCs

Beltrami et al., 2003 first reported the discovery of a resident population of CSCs. These cells were relatively small and negative for the blood lineage markers CD34, CD45, CD20, CD45RO and CD8 (Lin-). They were positive for c-kit (receptor for stem cell factor) and showed self-renewal and clonogenicity together with multipotent characteristics by giving rise to cardiomyocytes, smooth muscle, and ECs [20]. When injected into the ischemic myocardium in rats, c-kit+ CSCs could reconstitute the ventricular wall with blood vessel-containing myocardium. At the preclinical level, many independent laboratories have documented beneficial effects of transplanted c-kit+ CSCs on ventricular function via myocardial regeneration in various animal models [142-146]. The preclinical success was confirmed with an improvement in cardiac function in the SCIPIO trial [147]. LV function did not improve in any of the control patients, while for most patients receiving c-kit+ CSCs an improved LV function and a reduced infarct size was observed. These effects were quite substantial with a 24% relative infarct size reduction and an 8.2% absolute improvement in LVEF after four months. The effects were even more pronounced in the remaining patients studied for twelve months [148].

Although c-kit has been proposed to be the most important marker for adult CSCs, it is not a unique CSC marker but is also expressed by HSCs and mast cells, which are present in the heart [149]. Moreover, a recent publication has shown that c-kit+ CSCs only minimally contribute to cardiomyogenesis, questioning their role in the replacement of lost cardiomyocytes [150]. Concern has recently been raised about the integrity of certain data generated for the 2011

Lancet SCIPIO paper, questioning the validity of c-kit+ CSCs for myocardial repair [151].

Cardiosphere-derived cells

Messina and colleagues [152] describe the isolation of CSCs from postnatal atrial or ventricular human biopsies and murine hearts that grow as self-adherent clusters, termed cardiospheres. These CDCs are clonogenic and are capable of long-term self-renewal and express stem cell (CD34, c-kit, sca-1) and vasculogenic markers (KDR, CD31). While mouse CDCs beat spontaneously, human CDCs only begin to beat when co-cultured with postnatal rat cardiomyocytes. Injection of human cardiospheres into viable MI border zones leads to a better preservation of wall thickness in the infarct area compared to PBS-injected mice [152]. Similar results were obtained when transplanting CDCs instead of complete spheres by Smith et al., 2007 [153]. They were also able to show cardiomyogenic differentiation of CDCs after co-culture with neonatal rat cardiomyocytes by expression of sarcomeric cardiac troponin I (cTnI), occurrence of calcium transients, spontaneous action potentials and fast inward sodium currents. However, in our hands cardiomyogenic differentiation after co-culture was limited [137, 154]. Later it was shown that the paracrine effects of CDCs on endogenous regeneration, angiogenesis and cardioprotection exceed their direct regeneration by differentiation into cardiomyocytes [155]. Although CDCs have been shown to be safe and effective in preserving ventricular function in a porcine model of chronic ischemia [156], their differentiation potential appears to be limited [157, 158]. This might explain the fact that LVEF and LV dimensions did

not improve by intracoronary administration of CDCs in a phase 1 clinical trial (CADUCEUS) [159]. Beneficial effects in preclinical animal models can be explained by the strong paracrine potential of CDCs based on a high secretion of various angiogenic, anti-fibrotic and anti-apoptotic factors [160, 161]. Intracoronary delivery of allogeneic CDCs in a porcine model of MI has been shown to be cardioprotective, reduces infarct size, prevents microvascular obstruction and attenuates adverse remodeling [162, 163].

Islet-1+ CSCs

The early cardiogenic marker islet-1 is thought to mark a more committed second heart field progenitor population [146]. However, lineage tracing studies have shown that islet-1 progenitors contribute to more than two-thirds of the cells in the embryonic heart, giving rise to cardiac muscle, parts of the conduction system, ECs and smooth muscle cells. Multipotent islet-1+ cardiovascular progenitors are marked by the transcriptional signature of islet-1, Nkx2.5 and Flk1/VEGFR2 [164]. After birth, few islet-1 precursors remain present in the adult heart, which reside in specific cardiac niches (atria, septum and right ventricle). Their distribution matches the contribution of islet-1 progenitors in embryonic heart development, indicating that these are remnants of the fetal progenitor population [165]. Laugwitz et al., 2005 were able to isolate islet-1 cardioblasts from postnatal mouse hearts based on reporter gene induction. These cells expressed Nkx2.5 and GATA-4, but were negative for Sca-1, c-kit and Hoechst dye exclusion. Co-cultures with neonatal rat cardiomyocytes induced organized expression of α-sarcomeric actinin, cardiac troponin T (cTnT)

and Cx43 together with contractile activity, periodic calcium oscillations and action potentials [165]. Their low numbers in the adult heart and the unsuitability of a nuclear marker for cell sorting or immunobead selection hamper the use of islet-1 progenitors for therapeutic applications [166].

Sca-1+ progenitors

The murine marker stem cell antigen 1 (Sca-1) has been used to isolate not only murine, but also human c-kit+ islet-1+ CSCs [167]. These cardiomyocyte progenitor cells (CMPCs) show a strong cardiomyogenic differentiation potential as they differentiate into immunologically mature cardiomyocytes *in vitro*, display excitation-contraction coupling involving L-type calcium channel activity and respond to β-adrenergic stimulation [168]. Moreover, endothelial and smooth muscle differentiation has also been reported with distinct differentiation preferences between fetal and adult CMPCs [169]. Adult CMPCs preferably give rise to smooth muscle cells and mature cardiomyocytes, while fetal CMPCs rather form ECs [169]. Intramyocardial injection of human CMPCs in a mouse model of acute MI showed improved cardiac function with an engraftment of 3.5%, differentiation of CMPCs towards coupled cardiomyocytes and an increased vascular density [170]. However, xenotransplantation of Sca-1+ CSCs in a porcine model of chronic ischemic HF did not improve cardiac function [171]. The antigen recognized by the mouse Sca-1 antibody on human cells is unknown and the human Sca-1 homologue still needs to be identified. Nevertheless, Sca-1 is an interesting marker for the isolation of CMPCs [168].

Side population cells

Multipotent progenitor cells have also been characterized by their ability to efflux metabolic markers, such as rhodamine and Hoechst 33342, based on a high expression of membrane pumps encoded by multiple drug resistance genes. Hierlihy et al., 2002 [172] reported the existence of a side population of progenitors in the adult mouse heart, which could exclude Hoechst dye and were verapamil-sensitive (±1% of the total cell number). The ability of SP cells to efflux rhodamine and Hoechst 33342 dye, seems to be mediated by Abcg2, a member of the family of adenosine triphosphate–binding cassette transporters. Cardiac SP cells are Sca-1^{high}, c-kit^{low}, CD34^{low}, and CD45^{low}. These progenitors give rise to cell colonies (±1 colony per 50,000 cells plated) and are capable of differentiating into the cardiomyocyte lineage in co-culture experiments with primary cardiomyocytes. The number of Abcg2-expressing cells in the border zone significantly increases after MI. More specific, CD31+/Sca-1+ cardiac SP cells show functional cardiomyogenic differentiation into mature cardiomyocytes, with not only expression of cardiomyocyte-specific transcription factors and contractile proteins, but also stimulated contraction and intracellular calcium transients similar to adult cardiomyocytes [173].

Cardiac atrial appendage stem cells

Our research group identified yet another CSC type in the adult human heart based on a high ALDH enzyme activity. These cardiac atrial appendage stem cells (CASCs) express CD90, CD73 and the second heart field marker islet-1, but lack CD45 and c-kit expression [154]. The marker CD34 is expressed on freshly

isolated CASCs, but is lost in culture. A high ALDH activity has also been shown for BM and peripheral blood, however, these ALDH+ stem cells are phenotypically different. ALDH+ cells from the BM show high levels of CD34 and CD45, but are negative for CD73. Blood ALDH+ cells are positive for CD45, but negative for CD34 and CD73. To confirm that CASCs are of cardiac origin and not mobilized from the BM or peripheral blood, typical functional HSC and MSC characteristics have been examined. CASCs do not show typical hematological colony formation in medium containing growth factors stimulating HSC growth and differentiation. Adipogenic or chondrogenic differentiation could not be induced, which is a typical MSC property. These results indicate that CASCs do not represent mobilized HSCs or MSCs [154]. Still, the origin of CASCs is unknown and it remains unclear whether these cells are already present in the fetal heart or if they are mobilized to the heart after birth.

Typical stem cell or progenitor characteristics were shown for CASCs. The clonogenicity of CASCs is much higher (17 ± 11%) than reported for c-kit+ CSCs or CDCs [154] and they express several pluripotency-associated genes such as Oct-4, Nanog, c-Myc, Klf-4, lin-28, DPPA and Tbx-3. Moreover, CASCs display a complete cardiac phenotype after co-culture with neonatal rat cardiomyocytes, which could not be obtained for CDCs or c-kit + CSCs [137]. This suggests that CASCs possess a superior *in vitro* myocardial differentiation potential compared to previously described stem cell types [137, 154]. Inward rectifying and voltage-dependent currents resembling those of action potentials and spontaneous contraction were also observed. Autologous CASCs were able to survive and engraft after intramyocardial injection in a pilot study of an acute porcine MI

model. Preliminary results of cardiomyogenic differentiation two weeks after transplantation were shown by a sarcomeric expression of cTnT and cTnI [154]. Although this pilot experiment showed that CASCs survived and differentiated in the hostile environment of acute MI, a longer follow-up or measurements of cardiac function were not performed. The ALDH enzyme used for the identification of CASCs is known to have a detoxifying role and cells expressing this enzyme are better protected against certain stress situations such as ischemia. It was recently shown that CASCs can be expanded with a humanized platelet-based culture method and long term expansion does not change CASC ALDH activity, surface antigen profile or *in vitro* cardiomyogenic differentiation [174], making CASCs suitable for human applications. In addition, factors secreted by MSCs have been shown to improve CASC migration from human cardiac tissue, raising the possibility of stimulating the recruitment of CASCs towards the site of myocardial injury [93].

An overview of all the different stem cells populations that have shown potential in myocardial regeneration, as described above, are listed in Table 2 together with important characteristics.

Table 2: Possible stem cell types for myocardial regeneration

	Markers	Proliferative capacity	CM differentiation	In vivo repair	Clinical Trial
ESCs	Positive: SSEA, AP, Oct-3/4, nanog, sox-2, tra-1 Negative: Lin	Yes	Yes Spontaneous in EBs	Yes	ND
iPSCs	Positive: SSEA, AP, Oct-3/4, nanog, sox-2, tra-1 Negative: Lin	Yes	Yes Spontaneous in EBs	Yes	ND
BM					
HSCs	Positive: c-kit, CD34, CD45, CD133, CD90 Negative: Lin	Difficulties *in vitro* culture	Limited	Yes	Variable results
EPCs	Positive: CD31, CD34,CD144, VEGFR2, CD133, vWF Negative: Lin	Yes	Limited	Yes	Variable results
MSCs	Positive: CD73, CD90, CD105 Negative: CD14, CD34, CD45, CD79a, HLA-DR	Yes	Limited	Yes	Variable results
MAPCs	Positive: CD13, CD44, CD73, CD90, CD105 Negative: CD34, CD45, c-kit, GYPA	Yes	Limited	Yes	Phase I: Safety Improved LVEF
Heart					
c-kit+	Positive: c-kit Negative: CD34, CD45, Lin	Yes	Limited	Yes	Phase I: Safety Improved LVEF Decrease infarct size
CDCs	Positive: c-kit, CD105, CD90, MHC, TnI, vWF, SMA	Yes	Limited	Yes	Phase I: Safety No improvement

					LVEF
	Negative: CD34, CD45, Lin				Decrease infarct size Improved regional contractility
islet-1	Positive: islet-1 Negative: c-kit, Sca-1 Hoechst exclusion	ND	Yes Co-culture NRCM		ND
Sca-1	Positive: ALDH, c-kit, islet-1 Negative: CD31, CD45, c-kit	Yes	Yes TGF-β supplementation	Yes	ND
SP cells	Positive: Hoechst exclusion Sca-1 Negative: c-kit^{low}, CD34^{low}, CD45^{low}	Yes	Yes Co-culture NRCM	Yes	ND
CASCs	Positive: ALDH, CD29, CD34, CD55, CD73, , CD90 islet-1 Negative: CD45, c-kit	Yes	Yes Co-culture NRCM	Yes	ND

AP alkaline phosphatase, ALDH aldehyde dehydrogenase, BM bone marrow, CASCs cardiac atrial appendage stem cells, CDCs cardiosphere-derived cells, EB embryoid body, EPCs endothelial progenitor cells, GYPA glycophorin A, HSCs hematopoietic stem cells, lin lineage markers, MAPCs multipotent adults progenitor cells, MSCs mesenchymal stem cells, ND not determined, NRCM neonatal rat cardiomyocytes, Sca-1 stem cell antigen 1, SP side population, SSEA-4 stage-specific embryonic antigen 4

1.6 Aims of the study

Various stem cell types such as iPSCs [175], MSCs [124] and CSCs [20, 176] have shown potential to improve heart function after MI by replacing the lost cardiomyocytes with functional healthy tissue. Only moderate therapeutic effects were observed in most clinical trials, which can be explained by the limited differentiation of these stem cells towards cardiomyocytes [23]. Only iPSCs show true cardiovascular differentiation, however, safety issues still limit their use. The recently discovered CASCs seem a better alternative as they possess a strong *in vitro* myocardial differentiation potential compared to previously described stem cell types [137, 154] and show cardiomyogenic differentiation *in vivo* [154]. Hence, further characterization of the myocardial regenerative properties of CASCs with respect to their safety and therapeutic efficacy together with elucidation of their repair mechanisms is necessary.

In a first part of this study, the contribution of CASCs in cardiac repair was studied in the Göttingen minipig infarct model [77]. The porcine animal model is preferential in this context because of the high similarities between human and porcine hearts [177]. It was assessed if CASCs improve cardiac function after MI in an ischemia-reperfusion model with intramyocardial injections in the acute setting. This proof of concept study also confirmed whether CASC engraftment and cardiomyogenic differentiation are the mechanism behind possible observed functional benefits. Secondly, a possible contribution of CASCs in myocardial angiogenesis was investigated. Previous experiments have shown that CASCs express several genes known to be involved in maintaining pluripotent features, including *OCT-4, DPPA-3*, *lin-28*, *C-MYC*, *Klf-4* and *Tbx-3*, indicating that CASCs could be multipotent [154]. This is supported by expression of the early cardiogenic

marker islet-1 by CASCs, as it has been shown that islet-1+ precursors can generate all three cardiac lineages [154, 164, 178]. Although CASCs can differentiate into functional cardiomyocytes *in vitro* and *in vivo*, little is known about their differentiation into the other major cell types of the heart. Hence, the second objective of this project examined the possible multipotent character of the CASCs by their differentiation into ECs and smooth muscle cells**.** Vasculogenic differentiation is not believed to be the main cause of the beneficial pro-angiogenic effects of stem cell therapy, since stem cell-derived ECs are rarely integrated in newly formed blood vessels *in vivo* [179]. Paracrine mechanisms are now considered to have more pronounced effects on the formation of new blood vessels [155, 160, 179]. Therefore, the role of CASCs in myocardial angiogenesis by paracrine mechanisms was also investigated. The third aim explored the effect of inflammation on CASC biology. Prolonged inflammation after MI, reduces the survival and contractility of the remaining cardiomyocytes and enhances cardiac remodelling and fibrosis [180]. Stem cells have been reported to reduce this harmful inflammation by the secretion of anti-inflammatory cytokines and cell-cell contact with immune cells [181]. In addition, a low immunogenic phenotype has also been reported for various progenitor types, which would allow allogeneic transplantation [182]. As reported for MSCs and CDCs, CASCs lack expression of HLA-DR, already suggesting a low immunogenic phenotype, which was further explored in this project.

This study thus further investigated to what extent CASCs contribute to myocardial regeneration, and thus if these CASCs would be suitable candidates for cell therapy in ischemic heart disease.

2. MATERIALS AND METHODS

All procedures were carried out in accordance with the principles set forth in the Helsinki Declaration. Approval by the institutional review board and informed consent from each patient were obtained. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (Eighth Edition, revised 2011) and the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. The research protocols of all animal experiments were approved by the Ethical Committee for Animal Experiments of Hasselt University, Belgium.

2.1 Stem cell isolation and cell culture

2.1.1 Isolation and expansion of human CASCs

CASCs derived from IHD patients were used to study the angiogenic and immune properties of CASCs. Atrial appendages were obtained from patients undergoing routine cardiac surgery, such as CABG. The extracted atrial appendage tissue was minced, washed and enzymatically dissociated, after which the resulting single cell suspension was stained with aldefluor®. ALDH+ cells were directly flow sorted in X-vivo 15 medium with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin (P/S). The isolated CASCs were seeded in fibronectin-coated culture plates and expanded in X-vivo 15 medium supplemented with 20% FBS and 2% P/S. The medium was changed twice weekly and cells were re-plated at a density of $5x10^3$cells/cm² when reaching 80-85% confluence. After the first passage, serum levels were reduced to 10%. At passage 1, some cultures were retrovirally transduced with green fluorescent

protein (GFP) under the control of the eukaryotic promoter (pRRL-pEF1a-GFP).

Patient characteristics of CASCs used for angiogenesis and immune property experiments are shown in Table 9 and in Table 3 respectively.

Table 3: Patient characteristics CASCs used for experiments immune properties

Patient	1	2	3	4	5	6	7	8	9	10
General patient history										
Age	56	53	77	61	59	72	80	57	45	65
Male	F	m	m	m	m	M	m	m	m	m
Risk factors										
Weight (kg)	89	110	95	75	74	83	80	87	85	94
BMI (kg/m^2)	36.6	34	32.9	25.1	21.6	27.1	26.1	26.9	29.1	29.3
Creatinine (mg/dl)	3.07	1.09	1.56	.	0.95	1.1	1.02	1.25	0.96	0.87
Smoker	.	y	y	.	n	.	y	.	y	n
Diabetes	n	n	n	.	n	n	n	n	.	n
Hyperlipidemia	.	y	.	.	y	y	.	.	y	y
RD	.	n	n	n	n	n	n	.	.	n
Hypertension	.	y	y	.	y	n	y	.	.	y
CLD	.	n	y	n	n	n	y	.	.	n
PVD	y	n	y	n	n	n	n	n	n	n
CVD	y	n	y	n	n	n	n	n	n	n
Pre-operative cardiac status										
MI	.	n	n	n	n	.	n	y	y	n
HF	.	n	.	n	n	.	n	.	y	n
Angina	.	ccs2	.	ccs2	ccs0	.	ccs2	ccs2	ccs4	ccs2
NYHA (I/II/III/IV)	2	1	.	1	1	.	1	1	3	1
Surgical procedure										
CABG/valve /other	valve	cabg	cabg	other	cabg	cabg	cabg	cabg	cabg	cabg
Vessel number (0/1/2/3)	0	3	3	.	.	3	3	3	1	3

(Table 3 continued) BMI body mass index, CCS Canadian Cardiovascular Society grading of Angina Pectoris, CABG coronary artery bypass graft, CLD chronic lung disease, CVD cerebrovascular disease, ET-1 endothelin 1, HF heart failure, IGFBP-3 insulin-like growth factor binding protein 3, MI myocardial infarction, NYHA New York Heart Association, PVD peripheral vascular disease, RD renal dysfunction, VEGF vascular endothelial growth factor

2.1.2 Isolation and expansion of porcine CASCs

Adult female Göttingen minipigs (n=21, weight= 19 ± 2 kg , age= 386 ± 50 days) were sedated by intramuscular injection of ketamine (12.5 mg/kg) and midazolam (0.25 mg/kg), followed by an intravenous injection of 5 mg propofol. General anesthesia was maintained with a continuous infusion of 1% propofol (10 ml/h) and remifentanyl (100 µg/ml, 5 ml/h). The right atrial appendage (1-2g) was exposed, clamped and removed through a right mini-thoracotomy in the 4^{th} intercostal space. After removal, the right atrium was closed. CASCs were isolated from the atrial appendage tissue based on a high ALDH activity as described above. At passage 1, cells were retrovirally transduced with GFP under control of the eukaryotic promoter (pRRL-pEF1a-GFP).

2.1.3 Isolation and expansion of human bone marrow mesenchymal stem cells

MSCs were isolated from human BM samples. A volume of 20ml BM aspirate was collected from the sternum of patients who underwent open cardiac surgery. The mononuclear cell fraction was separated via density gradient centrifugation based on Lymphoprep™ (Axis Shield, Oslo, Norway). The cell fraction was washed with DPBS and X-Vivo 15 medium, after which they were cultured in X-Vivo 15 medium supplemented with 10% FBS and 2% P/S and kept in a humidified environment at 37°C and 5% CO_2. Medium was replaced 24 and 72 hours after cell isolation and twice a week from then on. The MSCs were re-

plated at a density of $5x10^3$cells/cm² when reaching 80-85% confluence.

To confirm their MSC phenotype, characterization was performed by flow cytometry. MSC were labelled for the markers CD34-PE-Cy7, CD45-PerCP-Cy5.5, CD49c-PE, CD73-PE, CD90-APC, CD105-FITC, CD106-FITC and CD184-APC for 30 minutes at room temperature in the dark (BD Biosciences). The samples were washed and analyzed by a FACSAria® III. Recording of 10,000 events was performed and gates were set based on appropriate isotype controls.

2.1.4 Human microvascular endothelial cell line 1

A human microvascular endothelial cell line (HMEC-1) was used to study angiogenesis *in vitro* [179] and was obtained from the Centre for Disease Control and Prevention (Atlanta, GA). HMEC-1 were cultured in MCDB 131 medium (Invitrogen, Carlsbad, CA) supplemented with 2% P/S, 10 mM L-glutamine (Gibco, Paisley, UK), 10% FBS, 10 ng/ml human epidermal growth factor (hEGF, Gibco) and 1µg/ml hydrocortisone (HC, Sigma-Aldrich, Diegem, Belgium).

2.2 Lentiviral transduction with green fluorescent protein

Cells were lentivirally transduced with GFP under the control of the eukaryotic promoter (pRRL-pEF1a-GFP). For the viral production, pRRL-pEF1a-GFP was co-transfected with different plasmids (pMDLg-RRE, pRSV-REV, and pCMVa-VSVG) in HEK293-T cells using EZ lentifect (MellGen laboratories nv). Plasmids were kindly provided by Dr R.C. Hoeben (University Medical Center, Leiden, The Netherlands) [183]. Transduction efficiency was studied by flow cytometry. When the purity was less than 90%, flow sorting purification was performed. Lentiviral transduction did not affect CASC functionality [154].

2.3 Preparation of CASC conditioned medium and cell lysates

Conditioned medium (CM) was prepared from CASC cultures of passage 3 to 7 when reaching 85-90% confluence. Cells were washed three times with DPBS and cultured for 48h in serum-free low glucose Dulbecco's modified eagle medium (LG-DMEM). CM was harvested and passed through a 0.22µm filter to remove possible cell debris. Afterwards the medium was concentrated in 3kDa Amicon Ultra-15 centriprep tubes YM-10 (Amicon, Millipore Corp.) at 3600g and sterile filtered to obtain 10X concentrated CM (CASC CM). Batches of 20X concentrated CM were also prepared for the *in vivo* chorioallantoic membrane (CAM) assay. CASC viability was regularly checked after preparation of CM with an annexin V Kit by flow cytometry. CASC viability >80% was set as a cut-off to guarantee the quality of the CM. After preparation of CM, cell lysates were prepared by homogenization of CASCs in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1mM sodium orthovanadate and protease inhibitor cocktail (1:100; Thermo Fischer Scientific, Erembodegem, Belgium) and stored at -80°C until further analysis.

2.4 RNA extraction and RT-PCR

Total RNA was isolated using the RNeasy Micro kit (Qiagen). Cells were lysed by directly adding RLT-buffer to the tissue culture well. Quality of the RNA was determined using the Agilent bio-analyzer 2100 and the RNA 6000 Nano Chip Kit (both from Agilent Technologies Inc.). The protocol was performed as described by the manufacturer. The RNA-integrity (RIN) score for each sample had to be

at least 7 to be used for further analysis. cDNA was synthesized using Superscript III and random hexamers (Invitrogen).

2.4.1 Conventional RT-PCR

Reverse transcript polymerase chain reaction (RT-PCR) using Taq polymerase (Roche) was performed for 35 cycles consisting of 40 seconds at 95°C, 50 seconds at annealing temperature (AT) and 1 minute at 72°C with a final extension step of 10 minutes at 72°C. Primers were designed in house via NCBI Primer-BLAST and manufactured by Eurogentec. Primer sequances are listed below in Table 4.

Table 4: Primer sequences RT-PCR

Gene	Forward primer	Reverse primer	AT	bp
β-actin	AGCGGGAAATCGTGCGTGACA	CCTGTAACAATGCATCTCATATTTG G	56°C	791
MEF2C	GGGGACTATGGGGAGAAAA	TGATCAGCGCAATCTCAC	63°C	378
Nkx-2.5	GCAGGTCAAGATCTGGTTCCAGA	GAGTGAATGCAAAATCCAGGGGAC	56°C	551
VEGFR2	CGTGTCTTTGTGGTGCAC	GGTTTCCTGTGATCGTGG	62°C	320

AT annealing temperature, bp base pairs, PCR product, MEF2C myocyte enhancer factor 2C, RT-PCR reverse transcript polymerase chain reaction, VEGFR2 vascular endothelial growth factor receptor 2.

2.4.2 qPCR

Singleplex quantitative PCR (qPCR) reactions were carried out in duplicate with the Rotor-Gene Q (Qiagen) in a 25µl reaction using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). Primers were designed in house via NCBI Primer-BLAST and manufactured by Eurogentec (Table 5). The PCR

reaction was performed according to the following protocol: 2 min at 50°C, 2 min at 95°C followed by 40 cycles of 15s at 95°C, 30s at AT, with a melt curve analysis as final step.

Table 5: Primer sequences qPCR

Gene	Forward primer	Reverse primer	C	AT	bp
B2M	AAGATGAGTATGCCTGCCGT	TTCATCCAATCCAAATGCGGC	300nM	60	122
Calponin	GAAACAAGGTGAACGTGGG	GGCTGACATTGAGCGTGT	200nM	63	330
eNOS	GGCGACAATCCTGTATGG	CACCACGTCATACTCATCCA	200nM	63	121
GAPDH	AGTCAACGGATTTGGTCGTATTG	ATCTCGCTCCTGGAAGATGGT	300nM	60	223
PECAM-1	GTCGTATGTGAAATGCTCTCC	AGGCAAAGTTCCACTGATC	300nM	63	112
POLR2A	TCACAGCAGTGCGCAAATTC	CCACGTCGACAGGAACATCA	300nM	60	86
SMA	GCCTTGGTGTGTGACAAT	ACCCACGTAGCTGTCTTTT	200nM	63	147
VEGFR2	CGTGTCTTTGTGGTGCAC	GGTTTCCTGTGATCGTGG	200nM	63	320
VWF	CACAGTGACATGGAGGTGA	CCATCCCTCAGCATGAAGT	200nM	63	254
YWHAZ	AAGATGAGTATGCCTGCCGT	TTCATCCAATCCAAATGCGGC	300nM	60	122

AT annealing temperature, bp base pairs, PCR product, B2M beta-2-microglobulin, eNOS endothelial nitric oxide synthase, GAPDH Glyceraldehyde 3-phosphate dehydrogenase, PECAM-1 Platelet endothelial cell adhesion molecule 1, POLR2A polymerase II polypeptide A, SMA smooth muscle actin, VEGFR2 vascular endothelial growth factor receptor 2, VWF von willebrand factor, YWHAZ tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

2.5 Western blot

After heat denaturation, protein lysates were separated on 4-15% Protean® TGX™ Gels and transferred to 0.2µm Transblot® Turbo™ nitrocellulose membranes with the Transblot® Turbo™ Transfer System (all from Bio-rad, Temse, Belgium). The blots were blocked in Odyssey blocking buffer (Li-Cor) and primary antibodies used for western blot analysis included a rabbit ET-1 antibody (1:500; Abcam), a rabbit IGFBP-3 antibody (1:300; Santa Cruz Biotechnology, Heidelberg, Germany) and a rabbit VEGF antibody (1:300; Santa Cruz Biotechnology). Secondary antibodies were a goat polyclonal anti-rabbit IR-Dye 800CW and a goat polyclonal anti-mouse IRDye 680CW antibody (1:15000, Li-Cor). Detection was performed using an Odyssey Infrared Imaging System (Licor).

2.6 Enzyme-linked immunosorbent assay

The concentration of identified angiogenic factors was determined by means of ELISA for the Neg Contr, Pos Contr, CASC CM and CASC lysates. Quantikine ELISA kits against ET-1, IGFBP-3 and ET-1 (R&D Systems) were used according to the manufacturer's instructions.

2.7 Endothelial differentiation

CASCs or MSCs were grown until 80% confluence on Matrigel-coated well plates. Endothelial differentiation was induced by culturing the cells in EGM2 medium (Lonza) without FBS [184, 185] or in basal medium containing 100 ng/ml $VEGF_{165}$ (R&D Systems) for 7 and 10 days. Medium was replaced every 3 days.

2.8 Functional *in vitro* angiogenesis assays

2.8.1 Proliferation assay

The effect of CASC CM on HMEC-1 proliferation was explored in a 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay and by Ki67 immunofluorescence analysis.

For the MTT assay, HMEC-1 were seeded at a density of $5x10^3$ cells/cm^2 in standard HMEC-1 culture medium in 96-well plates, while for the Ki67 assay they were seeded on glass coverslips in 24-well plates. The following day, cells were washed three times with PBS and the medium was replaced by control medium or CASC CM. After 72h, HMEC-1 proliferation was assessed. For the MTT assay, HMEC-1 were incubated with 5 mg/ml MTT (Sigma-Aldrich, Diegem, Belgium) in Neg Contr medium. After an incubation period of 4 hours at 37°C, the MTT solution was removed and a mixture of 0.01 M glycine and DMSO was added to each well. The absorbance was measured at a wavelength of 540 nm with a FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany).

For the Ki67 analysis, immunofluorescence was performed as described above with a Rabbit Ki67 Ready-To-Use primary antibody solution (Thermo Fischer Scientific).

2.8.2 Migration assay

HMEC-1 migration was examined in a Transwell migration assay. The lower compartment consisted of 24-well plates containing Contr medium or CASC CM. Subsequently, HMEC-1 cells were seeded on 8-µm pore Thincert™ tissue culture inserts (Greiner Bio-One, Wemmel, Belgium) in Neg Contr medium at a density of $5x10^4$ cells/cm^2 (upper compartment). After 24h, the inserts were washed in

PBS, fixed with 4% PFA and stained with 0.1% crystal violet. Representative pictures were taken in using an Axiovert 200 M microscope (Carl Zeiss NV-SA, Zaventem, Belgium). Quantification was performed by Axiovision 4.8 software (Carl Zeiss).

2.8.3 Tube formation assay

HMEC-1 tube formation was investigated by culturing HMEC-1 on phenol red-free Matrigel™ (Beckton & Dickinson) in 15µ angiogenesis slides (Ibidi, München, Germany). HMEC-1 were seeded on Matrigel at a density of $4x10^5$ cells/cm^2 in CASC CM or Neg Contr medium. Standard HMEC-1 culture medium served as a Pos Contr as LG-DMEM 10% FBS only resulted in a limited tube formation response. Tube formation experiments were also set up for co-cultures of GFP+ CASCs and HMEC-1. The cells were seeded in monoculture or in co-culture (ratio CASCs:HMEC-1 1:10) at a total density of $4x10^5$ cells/cm^2 in LG-DMEM 0% FBS 2% P/S. To visualize HMEC-1 in the co-cultures, they were labeled by human plasma Acetylated Dil lipoprotein uptake according to the manufacturers' guidelines (DiI-Ac-LDL, Thermo Fisher Scientific, Erembodegem, Belgium). Overview pictures were taken after 24h with an Axiovert 200 M microscope. Total tube length was quantified using the angiogenesis analyzer tool for Image J.

2.9 Characterization CASC phenotype in inflammatory conditions

2.9.1 Licensing of CASCs with inflammatory cytokines

CASCs were seeded at a density of 5000 cells/cm² in X-Vivo 15 culture medium with 10% FBS and 2% P/S. Afterwards, the cells were incubated with the pro-

inflammatory cytokines IFN-γ and TNF-α (eBioscience, San Diego, USA) in RPMI 1640 medium (Lonza) supplemented with 10% FBS and 2% P/S at concentrations of 1 ng/ml, 10 ng/ml, and 100 ng/ml for 24h or 72h. RPMI with 10% FBS and 2% P/S was used as control medium.

2.9.2 Immune-related marker analysis by flow cytometry after inflammatory licensing

CASCs were cultured in medium supplemented with or without the pro-inflammatory cytokines IFN-γ and/or TNF-α during 24h and 72h as described above. Marker expression after serum-free medium culture or at a density of 10,000 cells/cm² was also tested. Afterwards, the cells were detached, washed and incubated with HLA-ABC-PE, CD80-FITC, CD86-PE-Cy7 and HLA-DR-APC antibodies or their corresponding isotype controls (BD Biosciences) during 20 minutes at room temperature in the dark. After another wash step, marker expression was determined by a FACSAria® III. Gates were placed based on the isotype controls for each antibody and the fluorescence minus one method.

2.9.3 CASCs cytokine secretion after inflammatory licensing

Nitric oxide (NO) concentrations were determined in CASC supernatant after inflammatory licensing by the Griess Reagent System (Promega Corporation, Madison, WI, USA). The assay was used according to the manufacturer's instructions. Absorbance was measured with the FLUOstar Omega Microplate reader (BMG Labtech, Ortenberg, Germany) at 530 nm. NO concentrations were calculated based on a reference curve.

2.10 Allogeneic and suppression assays

Allogeneic and suppression assays were set up with CASCs as stimulator cells and peripheral blood mononuclear cells (PBMCs) as responder cells. Stimulator CASCs were pre-plated in 24-well plates at different densities to form adherent monolayers before addition of responder PBMCs. PBMCs were isolated from whole blood of healthy donors by Lymphoprep density gradient centrifugation. Blood of 3 different donors was used. PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) according to the manufacturer's protocol with a final concentration of 4 μM. The labeled PBMCs were co-cultured with CASCs at a ratio of 10:1, 2:1, 1:1, 1:2, 1:5 respectively in RPMI 1640 medium supplemented with 1% sodium pyruvate, 1% nonessential amino, 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. Suppression assays were performed at a ratio of 1:1 of PBMCs and CASCs. Anti-CD3 antibody was added as polyclonal T-cell stimulus with a final concentration of 4 μg/ml. Co-stimulation with a CD28 antibody (eBioscience) and TNF-α was also tested. After 3 days, the PBMCs were harvested, stained for CD4 and CD8 and the CFSE signal of gated lymphocytes was analyzed by flow cytometry to assess proliferation.

2.11 *In vivo* chorioallantoic membrane angiogenesis assay

The angiogenic properties of CASCs were examined *in ovo* in the CAM assay. Fertilized white leghorn chicken eggs (Gallus gallus) were incubated at 37 °C in a humidified atmosphere. After 3 days (E3), 3–4 ml albumen was removed and a small opening was made in the shell. This opening was covered with

cellophane tape and the eggs were returned to the incubator. After 6 days (E9), droplets of phenol red-free growth factor-reduced Matrigel™ (Amsbio, Abingdon, U.K.) were placed on the CAM (30µl). Droplets containing 50,000 CASCs were compared with pure Matrigel droplets as a negative control. On the other hand, Matrigel mixed 1:1 with 20X CASC CM or 20X LG-DMEM 2% P/S 0% FBS were analyzed. On E12, the eggs were opened again, the CAM was carefully dissected out of the eggs and pictures were taken. In order to quantify angiogenesis, two concentric circles (radii 1.5 mm and 2 mm) were drawn and intersecting blood vessels were counted by 2 independent researchers in a double-blind fashion. The assay was performed 3 independent times on CASCs and CM from 6 different donors.

2.12 Minipig ischemia-reperfusion MI model and CASC delivery

2 months (2M) after right atrial appendage harvesting, ischemia-reperfusion MI was induced in the minipigs by a 2h left anterior descending coronary artery (LAD) snare occlusion, followed by reperfusion. The presence of MI was visually confirmed by dark discoloration of the cardiac muscle, ST-segment elevation on ECG measurements and increased troponin I (TnI) levels 12h after occlusion (MI-CASC TnI=91 ± 47 µg/l; MI TnI=97 ± 65 µg/l).

3D electromechanical mapping (EMM) with NOGA guidance (Biosense Webster, Johnson & Johnson, Irwindale, CA) was performed to assess electrophysiological tissue viability and wall motion. A NOGA mapping catheter (Biosense Webster) was inserted through a carotid sheath with an 8F introducer and moved across the aortic valve into the LV. The catheter was navigated along the endocardium

to record a local intracardiac ECG and wall motion properties, generating detailed 3D electromechanical maps of the LV. All points taken for analysis met the recommended stability criteria (cycle length <10%; loop stability <6 mm; location stability <4 mm). Segments with predominance of high unipolar or bipolar voltages (UPV or BPV) and high local linear shortening (LLS, violet, blue, green) were defined as normal myocardium. Segments with low UPV or BPV and low LLS (red, yellow) were defined as scarred myocardium. Segments with predominance of high UPV or BPV (violet, blue, green) and low LLS (red, yellow) were demarcated as infarct border zone and were selected for transendocardial CASC delivery. After LAD occlusion, the overall mortality rate of the 21 animals included in the study was 14%, resulting in 18 survivors. Animals were randomly assigned to the CASC transplanted (MI-CASC) or the non-transplanted (MI) group. Intramyocardial injection of $83x10^6$ ± $126x10^6$ CASCs was performed in volumes of 30µL using the MyoStar injection catheter (Biosense Webster, Diamond Bar, CA) for the MI-CASC group (n=10). MI animals received sham injections (n=8). 2 months after autologous transplantation, animals were sacrificed and hearts were excised for further analysis, *i.e*. cell retention and histological analysis, as described below.

2.13 Evaluation of porcine cardiac function

Cardiac MRI was performed in a 1.5-T scanner (Achieva; Philips Medical Systems) using a surface cardiac coil with ECG gating. Sequence parameters were as follows: balanced turbo field (b-TFE) gradient echo sequence with dynamic sequences in transversal plane, long axis, short axis, and 4-chamber

view; and 3-dimensional T1-weighted FFE gradient echo sequence with multishot and TFE pre-pulse with variable delay between 200 and 300 ms after intravenous contrast injection of Gadobutrol. The balanced fast field echo (FFE) gradient echo images were analyzed *in cine* view to detect dyskinetic or akinetic regions and wall thickness measurements were performed on short and long axis views.

Contrast-enhanced MRI was used to assess myocardial injury and to differentiate between necrotic and viable tissue by scanning 15 min after intravenous injection of 4 ml 1 mmol/ml Gadobutrol (Gadovist Bayer) (delayed enhancement).

Images were analyzed with CAAS MRV software (Pie Medical). Regional wall thickening was evaluated using a 17-segment model as proposed by the American Heart Association [186]. Pathologic segments were identified as segments with a marked decrease in wall thickness and without thickening during systole. Correlation was made with pathologic regions on delayed enhancement. Wall thickening was calculated as percent increase of wall thickness comparing end-systolic (ES) and end-diastolic (ED) wall thickness. MRI measurements and 3D EMM were performed before MI (PRE), immediately after infarct induction (POST-MI) and 2 months post-MI (2M).

2.14 Analysis of porcine heart rhythm

All animals had a cardiac loop recorder (Medtronic Reveal™ XT 9529) implanted in a left parasternal skin pocket in the period between the POST-MI MRI scan

until the final MRI scan at 2M. Recordings were analyzed using the Medtronic FullView SW007 Software Version 7.1©.

2.15 Quantification of cell retention

The extent of cell retention was analyzed by means of qPCR against the GFP gene. Tissue samples of 1 g were homogenized using the gentleMACS™ dissociator (Miltenyi Biotec) and gentleMACS™ M Tubes (Miltenyi Biotec). Genomic DNA was isolated from 25 mg of the homogenized tissue with the QIAamp DNA Mini Kit (Qiagen), according the manufacturer's guidelines. The DNA concentration of all samples was set to the range of 10 ng/µl based on Nanodrop ND-1000 Spectrophotometer (Saveen Werner, Malmö, Uppsala) measurements. GFP gene number quantification was performed via the absolute standard curve method. Singleplex qPCR reactions were carried out in duplicate with the Rotor-Gene Q (Qiagen) in a 25 µl reaction using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The final reaction mix consisted of 1X Platinum® SYBR® Green qPCR SuperMix-UDG, 300 nM GFP or GAPDH primers and 10 ng DNA. Primers were designed in house via NCBI Primer-BLAST and manufactured by Eurogentec (Table 6). The annealing temperature (AT) for GFP and GAPDH was 63°C. The PCR reaction was performed according to the following protocol: 2 min at 50°C, 2 min at 95°C followed by 40 cycles of 15s at 95°C, 30s at 63°C (AT), with a melt curve analysis as final step. Plasmids containing GFP and porcine GAPDH target sequences were used to create standards for the absolute quantification of the single gene copy number as described below.

Table 6: Primer sequences qPCR cell retention analysis

Gene	*Forward Primer*	*Reverse Primers*	*AT*	*C*	*Bp*
GAPDH	5'-ACATGAAGCAGCACGACTT-3'	5'-GTGCGCTCCTGGACGTA-3'	63°C	300nM	61
GFP	5'-CTACATGTTCCAGTATGATTCA-3'	5'-CTTTCCATTGATGACAAGCTCC-3'	63°C	300nM	85

AT annealing temperature, Bp base pairs, PCR product, C primer concentration, GAPDH glyceraldehyde 3-phosphate dehydrogenase, GFP green fluorescent protein

2.15.1 Generation of standard curves for GFP and GAPDH

To generate GAPDH and GFP standards, plasmid constructs were used. The GFP standard was created with the pRRL-EF1A-GFP plasmid used for the lentiviral production. For the GAPDH standard, the GAPDH target sequence containing an EcoRI and BamHI restriction overhang was annealed and subsequently cloned in the respective restriction sites of a pcDNA3 vector using T4 DNA ligase (Roche). The GAPDH target Oligo sequences (NM_001206359.1) were manufactured by Eurogentec and are shown in Table 7, with restriction overhang sites in bold.

Table 7: Oligo sequences GAPDH plasmid qPCR cell retention analysis

GAPDH Oligo	
Forward	5'-**AATTC**CTACATGTTCCAGTATGATTCCACCCACGGCAAGTTCCACGGCACAGTCAAGG CGGAGAACGGGAAGCTTGTCATCAATGGAAAG**G-**3'
Reverse	5'-**GATCC**CTTTCCATTGATGACAAGCTTCCCGTTCTCCGCCTTGACTGTGCCGTGGAACTT GCCGTGGGTGGAATCATACTGGAACATGTAG**G**-3

GAPDH glyceraldehyde 3-phosphate dehydrogenase; restriction overhang sites shown in bold

The plasmid construct was transformed in TOP10 competent Escherichia Coli (life technologies) by heath shock treatment. Successful cloning was checked by restriction digest analysis on plasmids purified with the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions.

2.15.2 Calculation absolute number GFP+ cells

The mass of one copy of each plasmid was calculated based on its base composition and the molecular weight of the nucleotides. One copy of the pRRL-EF1A-GFP plasmid has a mass of 9.14×10^{-18} g and 1 ng of plasmid contains approximately 1.09×10^{8} GFP copies. Each pcDNA-GAPDH plasmid has a mass of 5.65×10^{-18} g and 1 ng of plasmid contains approximately 1.77×10^{8} GAPDH copies. The number of GFP and GAPDH copies was calculated from the obtained cycle threshold (CT) value using the linear equation from the respective mean plasmid standard curve. The mean standard curve for GFP was $y = -3.21x + 36.56$ (n=6) and for GAPDH $y = -3.65x + 41.14$ (n=6). The number of GFP gene copies per diploid cell was first determined on DNA of GFP+ minipig CASCs in culture (n=2) by dividing the number of GFP copies by the number of GAPDH copies. It was found that each diploid cell contains on average 2 copies of GFP. The number of GFP copies per cell and possible sample dilution factors were taken into account for the calculation of the number of GFP+ cells per gram of tissue.

2.16 Tumorigenic potential of CASCs in immunocompromised mice

Possible tumorigenic properties of CASCs, isolated from human atrial appendages, were explored in immunocompromised mice. Female HsdCpb:NMRI-Foxn1nu mice were purchased from Harlan Laboratories. The mice were maintained in the Animal Research Facility of Hasselt University in individually ventilated cages and received disinfected food, water and bedding. CASCs were isolated from human atrial appendages as previously described and grown to clinically relevant cells numbers, mimicking future expansion protocols for human therapeutic applications (passage 5 to 7). A head and neck squamous cell cancer cell line developed at the University of Michigan (UM-SCC) was kindly provided by the research group of Morphology of the Biomedical Research Institute, Hasselt, Belgium and served as a positive control for tumor formation. The UM-SCCs were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 10 mM L-glutamine, 2% P/S and passaged at 80% confluence. For subcutaneous injections, one million UM-SCCs or CASCs were dissolved in 150 µl Matrigel-Collagen tissue construct [154] (mice UM-SCCs n=10; mice CASCs n=10). The cells and tissue constructs were subsequently injected subcutaneously in the flank region, while gently restraining the mouse. Tissue construct without cells was injected in the other flank of each mouse as a negative control. Mice were checked twice every week for possible tumor formation together was examination of their general health, with a follow-up of 6 months. At the time point for euthanasia and necropsy, mice were sacrificed by cervical dislocation and organs were collected for histological analysis.

2.17 Histological and immunofluorescence analysis

2.17.1 Immunofluorescence cultured cells

Prior to cell culture, glass coverslips were sterilized, placed in the culture wells and cells were seeded on top. At 80% confluence, the cells were fixed in 4% paraformaldehyde (PFA). CASCs were incubated overnight with a rabbit polyclonal VEGF (1:100; R&D systems), a goat IGFBP-3 (1:100; R&D systems) or a mouse ET-1 (1:100; Abcam) antibody at 4°C. An Alexa Fluor 594 goat anti-rabbit antibody and an Alexa Fluor 555 goat anti-mouse antibody (1:500; life technologies) were used as secondary antibodies. Nuclei were identified with 4',6-diamidino-2-phenylindole (DAPI). Pictures were taken with a Nikon Eclipse 80i fluorescent microscope and a Nikon DS-2MBWc digital camera.

2.17.2 Hematoxylin & eosin staining of mouse organs

Organs were fixed in 4% formalin and embedded in paraffin. Embedded tissues were cut into 5 µm-thick slices. Paraffin embedded tissue sections were deparaffinized by incubating the slides twice in xylene for 5 min followed by a gradual dehydration step consisting of a series of graded alcohol concentrations (100%, 96% and 70%). Hematoxylin & eosin (H&E) staining was performed using an automated Dako CoverStainer (Dako, Leuven, Belgium).

2.17.3 Immunohistochemical analysis of porcine heart tissue

The heart was cut into thin slices perpendicular to its longitudinal axis, fixed in 4% formalin and embedded in paraffin. Embedded heart tissue was cut into 5 µm-thick slices and deparaffinized. Immunohistochemistry was performed using various antibodies: CASCs were identified with a FITC-labeled goat polyclonal primary antibody against GFP (1:100; Abcam). Myocytes were characterized with a rabbit cardiac troponin T (cTnT; 1:200; Abcam), a rabbit cardiac troponin

I (cTnI; 1:200; Abcam) and a connexin43 antibody (Cx43; 1:50; Abcam). Ventricular cardiomyocytes were identified with a rabbit ventricular myosin light chain 2 antibody (MLC-2V; 1:200; Synaptic Systems) and atrial cardiomyocytes with a mouse atrial MLC 2 antibody (MLC-2A; 1:200, Synaptic Systems). Atrial and ventricular heart tissue served as a positive control for MLC-2V and MLC-2A expression. ECs were labeled with a rabbit von willebrand factor antibody (VWF; 1:100, Dako). A sheep anti-rabbit rhodamine-labeled (1:10; Millipore) and a goat anti-mouse AF555 (1:500; life technologies) were used as secondary antibodies. Nuclei were identified with 4',6-diamidino-2-phenylindole (DAPI). Co-localization of cell-specific markers with GFP identified cells originating from CASCs. Quantification of CASCs co-expressing cTnT was performed for 10 different image fields for 3 pigs. The cardiomyocyte differentiation potential of the CASCs was expressed as percentage of cells co-expressing cTnT and GFP of the total number of GFP+ cells found, as previously described [170]. Immunofluorescent stainings were visualized with the Axioplan 2 imaging fluorescence microscope (Carl Zeiss, Jena, Germany).

2.18 Second harmonic generation microscopy

CASCs were identified by immunohistochemical staining for GFP as described in the previous section. Second harmonic generation (SHG) imaging was performed using a Zeiss LSM510 META (Carl Zeiss, Jena, Germany) mounted on an Axiovert 200M and a 40x/1.1 water immersion objective (LD C-Apochromat 40x/1.1 W Korr UV-VIS-IR, Carl Zeiss). The excitation was provided by a femtosecond pulsed laser (MaiTai DeepSee, Spectra-Physics, CA, USA) tuned to

a central wavelength of 810 nm. An analogue photomultiplier tube, delivered by Zeiss, was used for forward detection of the signal which was collected by a 0.55 NA condenser and passed through a 10 nm wide band pass filter with a central wavelength of 405 nm [187].

The analysis was performed using the sarcomere model introduced by Rouède et al [188]. Several profiles (>30), spanning two to three sarcomeres, were selected from the raw SHG image data, both in the resident and CASC region. The theoretical profile, which is particularly defined by the sarcomere length (L), the A-band length (A) and M-band length (M), was fitted to the experimental data using the non-linear least squares regression method. The half width of the system point spread function in the radial (w_{xy}) and the axial direction (w_z) at the e^{-2} level are fixed in the analysis: w_{xy}=0.332µm, w_z=0.986µm. The refractive index at the fundamental and the harmonic was set to 1.33. The data selection and analysis algorithm was implemented in MATLAB® (R2013a, The Mathworks, Gouda, The Netherlands).

2.19 Statistical analysis

2.19.1 Statistical analysis minipig study

Data are reported as mean ± SD. All data sets were tested for normality by mean of a Shapiro-Wilk normality test (SPSS 9.0 for Windows and GraphPad Prism 5 Software). For data with a Gaussian distribution, paired data sets were compared by means of a paired student t-test and unrelated groups by an independent-samples student t-test (SPSS 9.0 for Windows). If no Gaussian distribution was present, comparison of serial measurements was performed

with the related-samples Wilcoxon signed ranks test (SPSS 9.0 for Windows). MI versus MI-CASC comparisons were performed using the Mann-Whitney U test (SPSS 9.0 for Windows). Comparison of SHG parameters was performed with a student t-test (MATLAB). Data with $p<0.05$ were considered significant.

2.19.2 Statistical analysis angiogenesis assays

Data are reported as mean ± SEM. Statistical analyses for the patient characteristics and growth factor correlations were assessed with SAS JMP PRO 12.1 (SAS Institute Inc.). Data sets were tested for normality by means of a Shapiro-Wilk normality test (SPSS 9.0 for Windows and GraphPad Prism 5 Software).Correlations between continuous data and angiogenic factors were assessed by multivariate analysis and the Spearman's ρ test. Categorical patient characteristics and growth factor secretion were explored by a Kruskal-Wallis Test or Wilcoxon pairwise comparisons (rank sums). Statistical analyses for the functional assays were performed with SAS version 9·3 software (SAS Institute Inc.). To take into account the different levels of dependency in the dataset, linear mixed models (proc MIXED in SAS) were fitted for all datasets with the different test conditions as fixed effects. For the functional *in vitro* assays with the HMEC-1, experiment and CM sample nested within experiment were fitted as random effects. Experiment was included as random effect to take variability between experiments into account. CM sample was included as random effect parameter since a paired relation exists between CM and CM with antibodies of the same patient sample. Total tube length of the 1X and 10X CASC CM was compared with the Wilcoxon Signed Rank Test. For the CAM assay, only experiment was modelled as random effect. The different test conditions are

compared using CONTRAST statements with a Bonferroni correction for multiple comparisons. The Bonferroni correction was calculated based on relevant comparisons between the test conditions of the functional assays. A global significance level of 5% is considered.

2.19.3 Statistical analysis immune properties CASCs

Data are reported as mean ± SEM. Statistical analyses were performed with SAS version 9·3 software (SAS Institute Inc.) or SPSS. To take into account the different levels of dependency in the dataset, linear mixed models (proc MIXED in SAS) were fitted for the viability datasets with the different test conditions as fixed effects. CASC donor was included as random effect parameter since a paired relation exists between different conditions of the same patient sample. The different test conditions are compared using CONTRAST statements with a Bonferroni correction for multiple comparisons. A global significance level of 5% is considered. Conditions of the marker experiments and the allogeneic and suppression assay were compared with a Kruskal-Wallis pairwise multiple Comparison test. A p-value of 0.05 was considered significant.

3. RESULTS

3.1 CASC transplantation in a minipig ischemia-reperfusion MI model

3.1.1 Introduction

Stem cell biology and regenerative medicine promise to replace the lost cardiac muscle with functional healthy tissue, dramatically improving the quality of life and survival in patients with numerous heart conditions. Various stem cell populations such as BM stem cells, EPCs and iPSCs are currently under investigation for myocardial regeneration. However, the first clinical studies were not able to reproduce the large benefits obtained in the preclinical experiments [24], which can be explained by the limited cardiomyogenic differentiation of the used stem cell types [189]. Resident CSCs are considered more suited for myocardial regeneration, since they are most likely 'pre-programmed' to become cardiomyocytes. Cardiac progenitors have been identified based on Sca-1 [168], c-kit [190] and islet-1 [166] expression or the ability to form cardiospheres, leading to so-called CDCs [153]. Although CDC administration in the CADUCEUS trial revealed a reduction in scar tissue and an increase in regional contractility, no significant improvement in global LV function was shown [159]. On the other hand, the SCIPIO trial using c-kit+ CSCs indicated that intracoronary infusion of autologous CSCs is effective in improving LV systolic function and reducing infarct size in MI patients [147, 191]. Although c-kit has been proposed to be the most important marker for adult CSCs, it is not a unique CSC marker but is also expressed by HSCs and mast cells, which are present in the heart [149]. Moreover, the contribution of c-kit+ CSCs to the formation of new cardiomyocytes has recently been questioned [150]. The early cardiogenic marker islet-1 is thought to mark a more committed second heart field progenitor population [146]. However,

lineage tracing studies have shown that islet-1 progenitors contribute to more than two-thirds of the cells in the embryonic heart, giving rise to cardiac muscle, parts of the conduction system, endothelial and smooth muscle cells. Their low numbers in the adult heart and the unsuitability of a nuclear marker for cell sorting or immunobead selection hamper the use of islet-1 progenitors for therapeutic applications [166]. The murine marker Sca-1 has been shown to be suited for the isolation of not only murine, but also human c-kit+ islet-1+ CSCs with a strong cardiomyogenic differentiation potential. The antigen recognized by the mouse Sca-1 antibody on human cells is unknown and the human Sca-1 homologue still needs to be identified. Still, it is an interesting marker for the isolation of cardiomyocyte progenitor cells [168]. Recently, our group identified a new CSC population based on a high ALDH enzyme activity, known as the cardiac atrial stem cell (CASC) [154]. Strikingly, these CASCs show expression of islet-1, suggesting that they represent a second heart field progenitor population present in the adult heart [164]. Furthermore, this ALDH+CD34+CD45- stem cell population displays exceptional cardiomyogenic differentiation properties, making them a promising candidate for myocardial regeneration [154].

The goal of the present study was to demonstrate the safety and effectiveness of CASC transplantation in a clinically relevant animal model. Intramyocardial administration of CASCs was performed to assess the role of CASCs in (1) the preservation of LV function in a porcine model of MI and (2) to confirm whether CASC engraftment and cardiomyogenic differentiation were responsible for the observed functional benefits. Finally, possible tumorigenic properties of CASCs were explored in immunocompromised mice.

3.1.2 Transendocardial CASC transplantation in a minipig ischemia-reperfusion MI model

A total of 21 pigs were used in this study, which were treated according to the protocol and timeline illustrated in Figure 4.

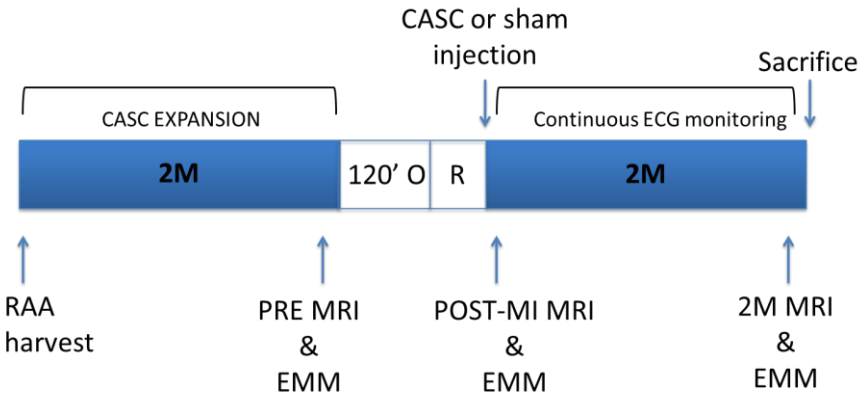


Figure 4: Experimental protocol minipig MI model.
Two months after right atrial appendage harvest and just before MI induction, animals underwent an MRI scan (PRE). MI was induced by a 120min LAD occlusion, followed by reperfusion. Minipigs received CASC transplantation (MI-CASC) or sham injection (MI). Within 48h, an MRI scan was performed (POST-MI) and a reveal unit implanted. Two months after MI induction (2M), final MRI was obtained. 2M two months after MI, POST-MI 48h after MI, EMM electromechanical mapping, M month, MI myocardial infarction, O occlusion, R reperfusion, RAA right atrial appendage, PRE before myocardial infarction.

Right atrial appendages were obtained from adult female Göttingen minipigs (19 ± 2kg and 386 ± 50 days), after which the pigs were randomly assigned to the control or CASC group. Porcine CASCs were isolated from single cell suspensions of the atrial appendages based on the ALDH reaction. An average of 0.8 ± 0.8% (median ± interquartile distance) of the total cell population showed high ALDH activity and 38,902 ± 34,871 (median ± interquartile distance) CASCs could be

isolated. At passage 1, cells were transduced with a lentivirus-expressing GFP under the control of the eukaryotic promoter (pRRL-pEF1a-GFP) and expanded for 2 months. MI was induced by snare ligation of the LAD. Of the 21 pigs, 2 died owing to ventricular fibrillation during LAD occlusion, and 1 control animal died five days after MI creation as a consequence of ventricular failure, giving an overall mortality rate of 14%. Of the 18 remaining pigs, 10 were part of the CASC transplanted group and 8 of the control group. Among these, 6 pigs (2 controls and 4 CASC-treated animals) developed ventricular fibrillation during LAD occlusion and were successfully cardioverted by internal defibrillation. Upon reperfusion, intramyocardial injection of $48x10^6$ ± $76x10^6$ (median ± interquartile distance; no normal distribution) CASCs was performed using the MyoStar injection catheter for the transplanted group. Controls received sham injections. A representative electromechanical map displaying the sites of CASC injection is shown in Figure 5. Segments with predominance of high unipolar or bipolar voltages (violet, blue, green) and low LLS (red, yellow) were demarcated as infarct border zones and were preferred for transendocardial CASC delivery.

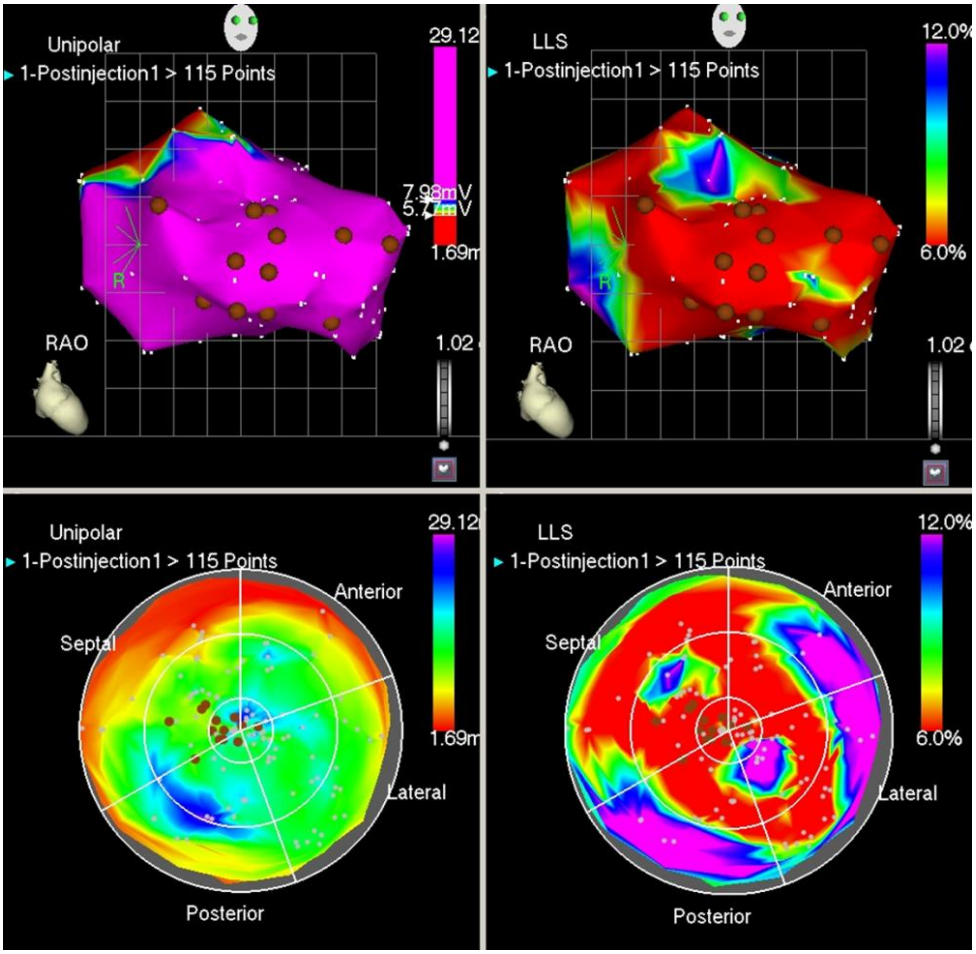


Figure 5: Electromechanical map of the LV with transendocardial CASC injection sites.
A representative example of 3D electromechanical maps of an MI-CASC animal showing UPV (left) and LLS (right). Segments with high UPV and high LLS (violet, blue, green) are defined as normal myocardium. Segments with low UPV and low LLS (red, yellow) are defined as scarred myocardium. Segments with predominance of high UPV (violet, blue, green) and low LLS (red, yellow) are defined as infarct border zone and represent target areas for transendocardial CASC delivery. Brown dots represent the sites of transendocardial CASC injections. White dots represent reference points used to create the 3D map. MI-CASC n=4. 3D three dimensional, LLS local linear shortening, UPV unipolar voltages.

3.1.3 Autologous CASC transplantation prevents worsening of global and regional cardiac function after MI

Progressive LV dilatation was observed in MI animals at 2M after the ischemia-reperfusion. LV end diastolic volume (LVEDV) significantly increased from 32 ± 6 ml to 45 ± 11 ml (Fig 6A, p=0.012) and LV end systolic volume (LVESV) from 15 ± 3 ml to 28 ± 8ml (Fig 6B, p=0.012), leading to a decrease in LVEF of 15 ± 3% (Fig 6C, p=0.012). CASC transplantation prevented LV dilation. Indeed, LV volumes were comparable to POST-MI values (Fig 6A,B), resulting in a preservation of LVEF at 2M (LVEF 52 ± 8% at POST-MI vs 50 ± 8% at 2M, Fig 6C, LVEF at 2M MI-CASC vs MI p=0.003).

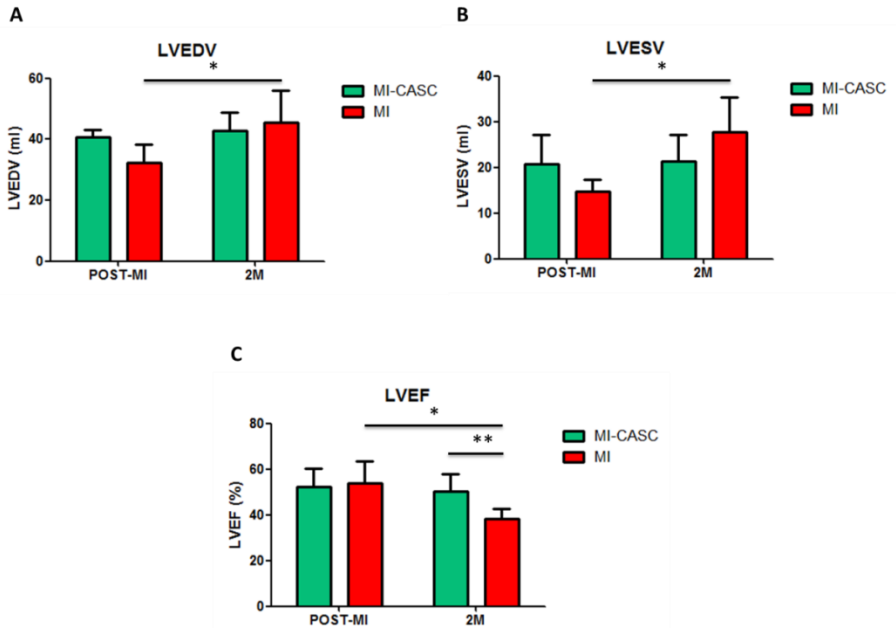


Figure 6: CASC transplantation improves global LV function.
LVEDV (A) and LVESV (B) increased in the MI group, while in the MI-CASC group LV dilation was prevented. LVEF significantly decreased in MI animals, but was preserved in the MI-CASC group. MI-CASC n=10; MI n=8. Data are shown as mean ± SD. * denotes p<0.05, ** denotes p<0.01. 2M two months, POST-MI 48h after myocardial infarction, LVEDV left ventricular end diastolic volume, LVEF left ventricular ejection fraction, LVESV left ventricular end systolic volume.

Figure 7A shows representative examples of MRI short axis views with contouring of the epi- and endocardial borders and delayed enhancement segments, which were used to determine the infarct mass. Infarct size POST-MI was similar between both groups with an infarct weight of 5.0 ± 1.6 g in the MI-CASC group and 5.5 ± 1.1 g in the MI group. However, at 2M, infarct weight was significantly reduced in the MI-CASC group compared to POST-MI (respectively 3.1 ± 1.9 g *vs* 5.0 ± 1.6 g, p=0.05), while it did not improve in the non-transplanted MI group (respectively 6.1 ± 1.8 g *vs* 5.5 ± 1.1g; Fig 7B; infarct mass at 2M MI-CASC vs MI group p=0.009).

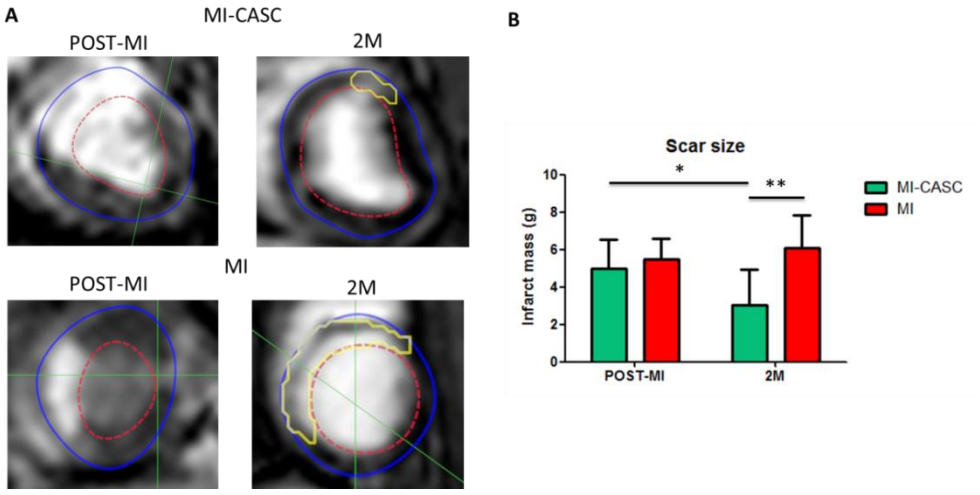


Figure 7: CASC transplantation reduces scar mass.
(A) Representative examples of MRI short axis views with contouring of the epi- and endocardial borders in blue and red respectively. Delayed enhancement segments are demarcated in yellow. (B) the average infarct mass significantly decreased in the MI-CASC group, but remained unchanged in MI animals. MI-CASC n=10, MI n=6. Data are shown as mean ± SD. * denotes p<0.05, ** denotes p<0.01. 2M two months, POST-MI 48h after myocardial infarction.

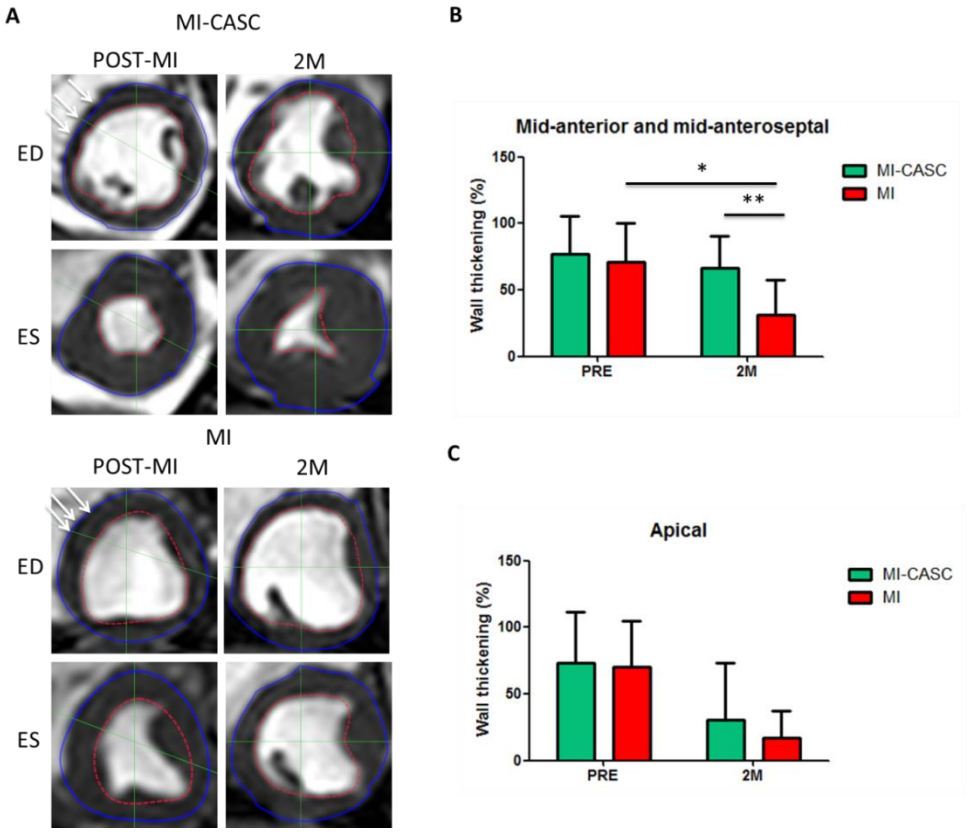


Figure 8: CASC transplantation improves regional wall thickening.
(A) Representative examples of mid-ventricular short-axis views in diastole and systole in both groups at POST-MI and 2M. Arrows indicate the infarct region. (B) Regional contractility significantly decreased in the MI group in the mid-anterior and mid-anteroseptal segments, while it was preserved in the MI-CASC group. (C) A significant decrease in wall thickening was observed for both groups in the apical segments. MI-CASC n=10; MI n=7. Data are shown as mean ± SD. * denotes $p<0.05$, ** denotes $p<0.01$. 2M two months, POST-MI 48h after myocardial infarction, ED end diastole, ES end systole, PRE before myocardial infarction.

As a measure of contractility, wall thickening was estimated in the different segments obtained from the MRI images (Fig 8A). The presence of MI was confirmed in mid-anteroseptal, mid-anterior, apico-septal and apical anterior

regions. Non-transplanted MI animals displayed a significant decrease in wall thickening in both mid-anterior and mid-anteroseptal segments from 71 ± 29% PRE to 31 ± 27% at 2M (Fig 8B, $p<0.001$). This decrease was limited by CASC transplantation with a wall thickening of 77 ± 29% before MI and 67 ± 24% at 2M (Fig 8B; wall thickening at 2M MI-CASC vs MI $p=0.001$). In contrast, the decrease in apical segment thickening was not rescued by CASC transplantation (Fig 8C). In all other segments (basal, inferior and lateral), contractility remained unchanged between POST-MI and 2M.

3D EMM was used to assess LV viability and regional wall motion in both groups. Reduced BPV and low LLS were present in the LAD perfusion areas for both groups (Fig 9A,B left panel), indicating ischemic areas with impaired local contraction respectively. At 2M, both BPV and LLS were clearly improved in the MI-CASC group (Fig 9A, right panel), while these parameters deteriorated in the non-transplanted MI group (Fig 9B, right panel).

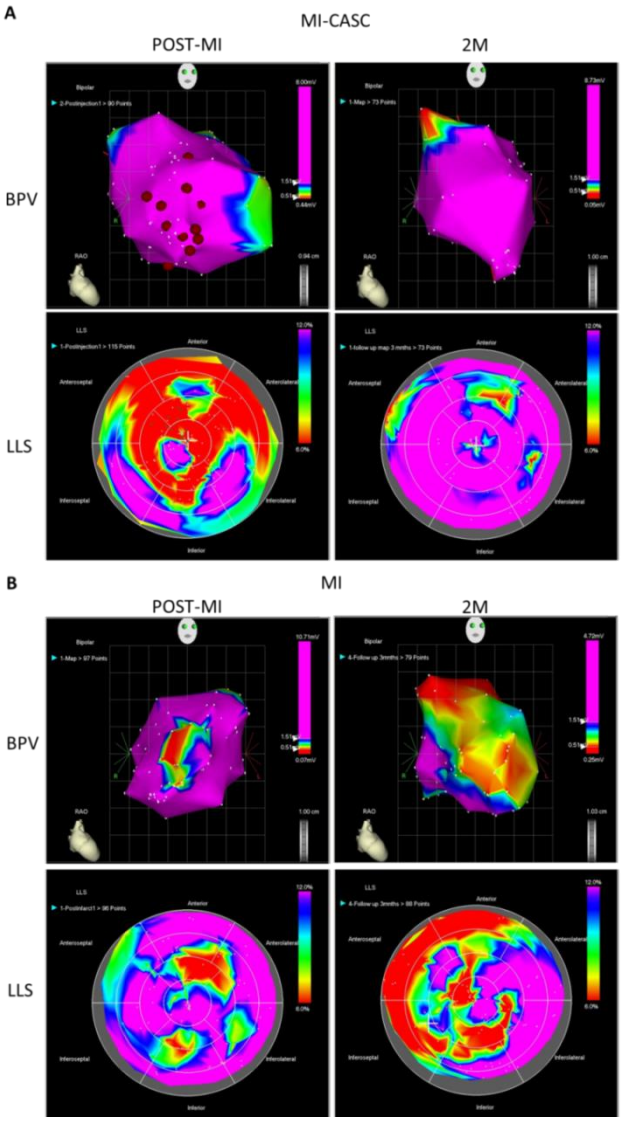


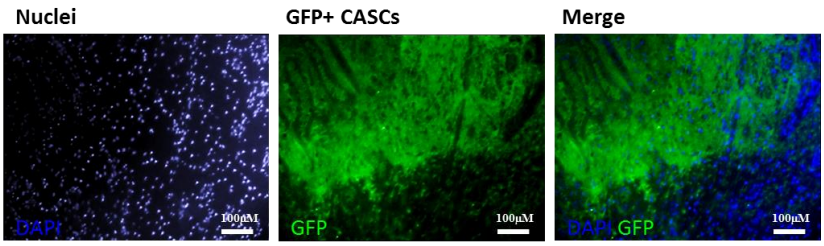
Figure 9: 3D EMM confirms improvement of regional contractility after CASC transplantation.
Representative examples of LV BPV and LLS measurements for the MI-CASC group (A) and MI animals (B). Low BPV and LLS (red and yellow) are visible in the LAD perfusion areas for both groups at POST-MI. At 2M, a clear improvement in BPV and LLS is visible for the MI-CASC group, but not for the MI group. MI-CASC n=4; MI n=1. 2M two months, POST-MI 48h after MI, BPV bipolar voltages, LLS local linear shortening.

Since stem cell transplantation has been associated with cardiac arrhythmias [192], continuous rhythm analysis and ECG monitoring was performed in both the MI-CASC and the MI group. No episodes of malignant ventricular tachyarrhythmia or atrial fibrillation were recorded in either group.

3.1.4 Heart function preservation *in vivo* is related to successful CASC engraftment and cardiomyogenic differentiation

2M after CASC transplantation, immunohistochemical examination of the harvested heart tissue revealed widespread regions of GFP+ cells in the border zone between viable and infarcted tissue (Fig 10A). Absolute quantification by qPCR confirmed the presence of high numbers of GFP+ cells in border zone areas, whereas no GFP signal was found in non-transplanted MI pigs nor the right ventricle of MI-CASC animals. From a subset of animals (n=4), estimation of the number of GFP+ cells per gram of tissue in representative border zone areas was performed as listed in Fig 10B. For each animal, the number of GFP+ cells was investigated in eight representative samples of the infarct border zone. Based on the results of these cell injection areas alone, an average cell retention of 19 ± 18% was found. This percentage is an underestimation, since other parts of the border zone tissue were used for immunohistochemical analysis and were hence not included in the cell retention analysis, although they also clearly contained GFP+ CASCs.

A



B

	Pig 1 (GFP+ cells/g)	Pig 2(GFP+ cells/g)	Pig 3 (GFP+ cells/g)	Pig 4(GFP+ cells/g)
Sample 1	0	619371	37492	7074581
Sample 2	74523	0	313042	133169
Sample 3	0	104304	0	14936
Sample 4	0	439048	71292	13503
Sample 5	35360	0	97851	0
Sample 6	20113	102521	0	168860
Sample 7	245278	23283	292435	109205
Sample 8	95508	19375	33773	100181
Total	**470781**	**1307902**	**845885**	**7614435**
Transplanted CASCs	1150000	72000000	3450000	95200000
Minimal retention (%)	**41**	**2**	**25**	**8**

Figure 10: GFP+ CASCs show a high cell retention in infarct border zones.
(A) Representative images at low magnification showing large regions of GFP+ cells in the infarct border zone of a CASC-treated heart at 2M. (B) Estimation of the number of GFP+ cells per gram of tissue in representative border zone areas was performed based on absolute qPCR quantification of the GFP gene, revealing an average cell retention of at least 19 ± 18%, ranging from 2 to 41%. Quantification was performed on hearts of 4 minipigs.

A sarcomeric organization, typical for striated muscle, of both cTnT and cTnI was observed in GFP+ CASCs (Fig 11A,B). Quantification revealed that 98 ± 1% of the GFP+ CASCs co-expressed cTnT. These newly formed cellular structures also expressed Cx43 (Fig 11C), specifically present between GFP+ CASCs and resident cardiomyocytes, indicating the integration of CASCs into the host myocardium. To determine whether CASCs, which originate from the atria, differentiate towards an atrial or a ventricular phenotype, immunofluorescent staining against MLC-2V (specific for the ventricle) and MLC-2A (specific for the atria), was performed. Transplanted CASCs were positive for MLC-2V (Fig 11D), but not for MLC-2A (Fig 11E), indicating differentiation of the CASCs towards a ventricular phenotype. CASCs in culture were negative for both MLC-2 types (Fig 12A), while atrial and ventricular tissue respectively served as a positive control with clear expression of these contractile proteins (Fig 12B).

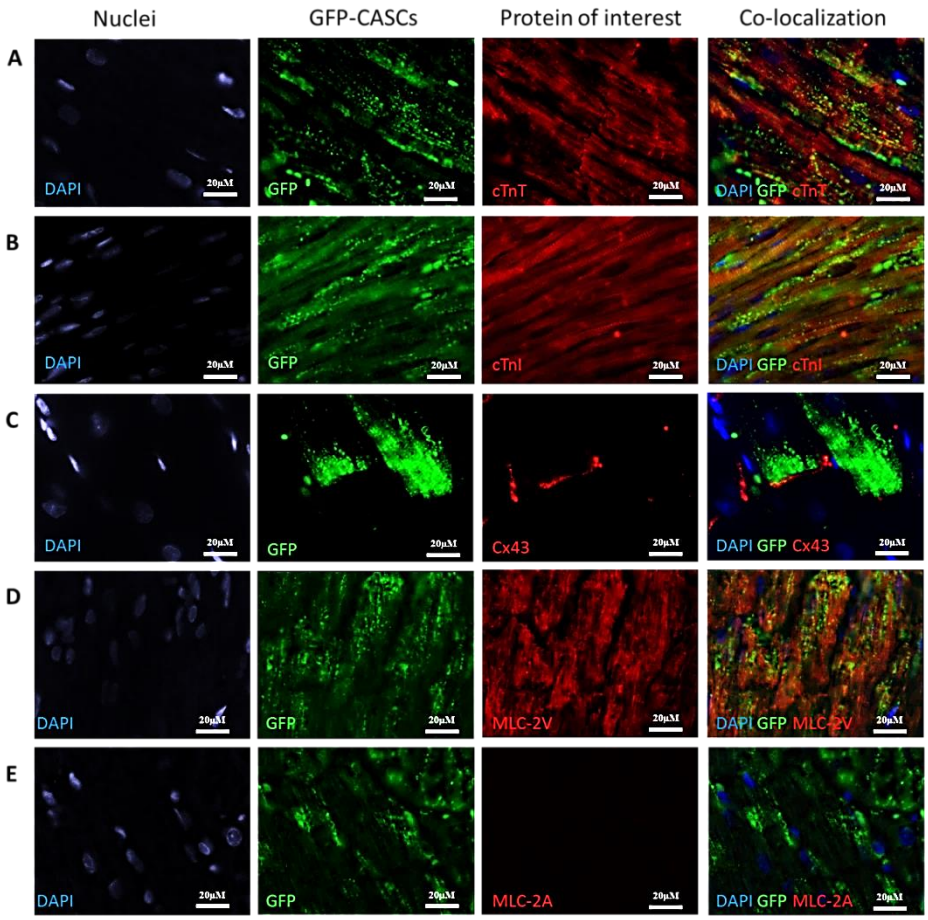


Figure 11: Immunofluorescence demonstrates differentiation of CASCs into cardiac tissue.
Immunofluorescence shows GFP+ CASCs at 2M in border zone areas co-expressing cTnT (A) and cTnI (B) in a sarcomeric pattern. Cx43 is expressed between CASCs and resident cardiomyocytes (C) and CASCs are positive for MLC-2V (D), but not for MLC-2A (E). MI-CASC n=3. cTnI cardiac troponin I, cTnT cardiac troponin T, Cx43 connexin 43, DAPI nuclear stain, GFP green fluorescent protein, MLC-2A atrial myosin light chain two, MLC-2V ventricular myosin light chain two.

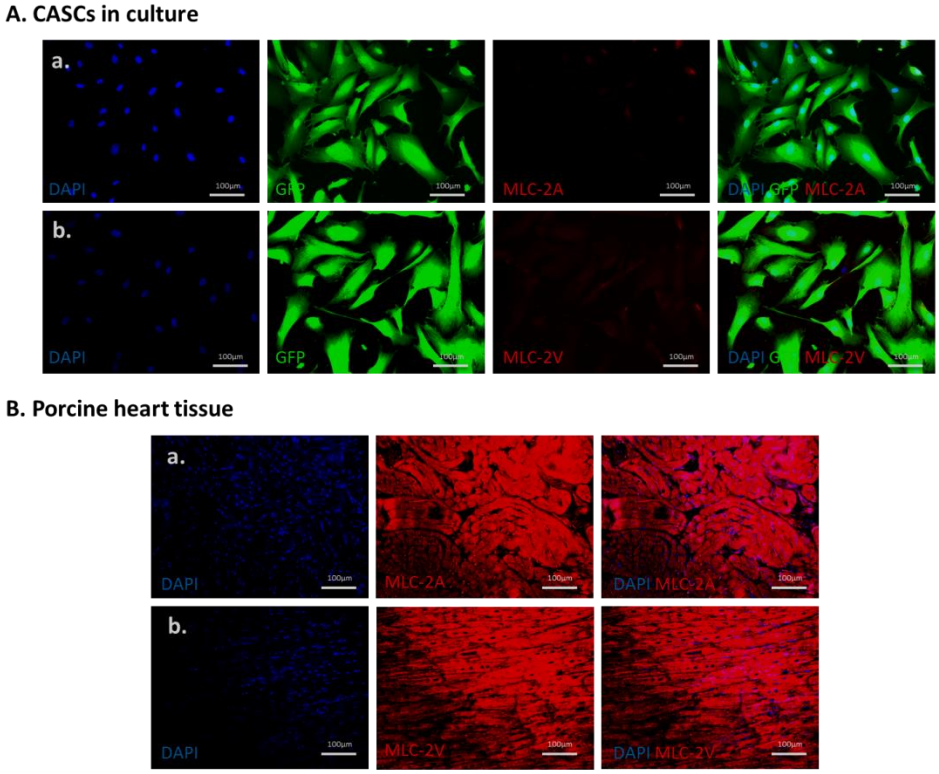


Figure 12: Immunofluorescence for MLC-2A and MLC-2V for CASCs and atrial or ventricular tissue.
Immunofluorescence shows that GFP+ CASCs in culture do not express MLC-2A or MLC-2V (A). MI-CASC n=3. Atrial and ventricular tissue was used as a positive control for MLC-2A and MLC-2V immunofluorescence respectively. DAPI nuclear stain, GFP green fluorescent protein, MLC-2A atrial myosin light chain two, MLC-2V ventricular myosin light chain two.

Cardiomyogenic differentiation of CASCs was confirmed by label-free SHG imaging (Fig 13). GFP+ CASCs (green) displayed organized sarcomeric structures (Fig 13A,C) resembling those of resident cardiomyocytes (Fig 13A,B). Analysis of the SHG profiles (Fig 13D,E) showed mature myosin assemblies and sarcomeric structures with sarcomere (L), A-band (A) and M-band (M) length comparable to resident cardiomyocytes of the viable remote area (Fig 13E).

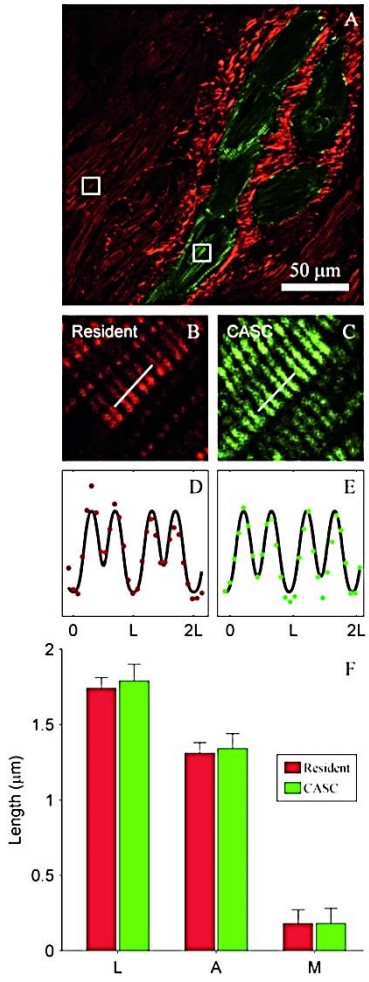


Figure 13: SHG microscopy confirms differentiation of CASCs towards cardiomyocytes.
(A) Typical SHG image of CASCs 2M after transplantation in infarcted tissue. Resident sarcomeres and collagen are visible in red, GFP+ CASCs are shown in green. More detailed images of the demarcated resident (B) and CASC region (C). Typical longitudinal profiles of the raw data of resident cardiomyocytes (D, red dots, from white line in B) and CASC (E, green dots, from white line in C) and the resulting fit with the theoretical model (dark solid line). The fit results in characteristic sarcomere properties such as sarcomere (L) length, A-band (A) length and M-band (M) length (F). There are no significant differences between CASC sarcomeres (n=35) compared to sarcomeres of resident cardiomyocytes (n=31). Data are shown as mean ± SD.

3.1.5 CASCs show paracrine angiogenic potential *in vivo*

Occasionally, GFP+ CASCs could also be identified in vessel walls, which were positive for the endothelial-specific marker VWF, indicating the differentiation of CASCs into an endothelial phenotype in this specific environment (Fig 14).

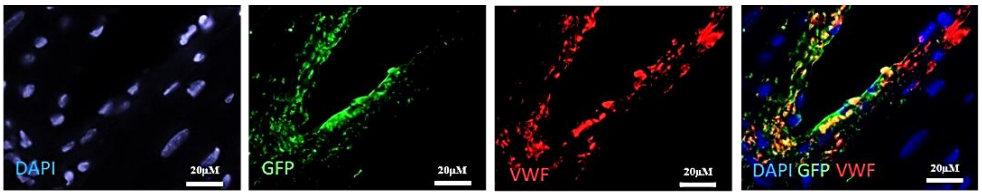


Figure 14: CASCs rarely differentiate into ECs.
Representative images showing co-localization of GFP with VWF in a blood vessel wall of a CASC-treated heart at 2M. MI-CASC n=3. DAPI nuclear stain, GFP green fluorescent protein, VWF von willebrand factor.

Possible paracrine effects of the CASCs on angiogenesis were investigated by quantifying the number of blood vessels in the infarct border zone in thee MI-CASC pigs and one MI animal. Immunofluorescent staining was performed for VWF to identify blood vessels (Fig 15A). The number of blood vessels per image field was significantly higher for one of the three MI-CASCs animals compared to the MI-heart ($p=0.039$), while no significant differences were observed for the other MI-CASC pigs (Fig 15B).

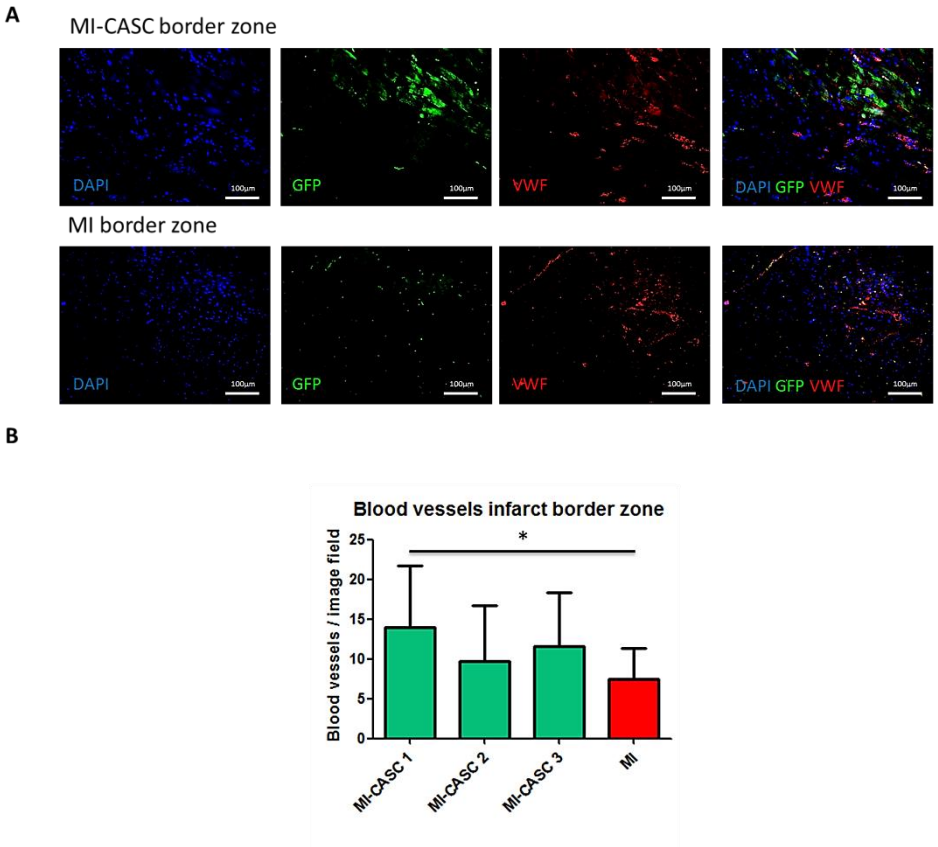


Figure 15: The number of blood vessels is higher in infarct border zone areas of CASC pigs.
(A) Immunofluorescence against VWF was performed to identify blood vessels in infarct border zones. (B) The number of blood vessels in the infarct region was significantly higher for one MI-CASC animal compared to the MI-heart ($p=0.039$), but not for the other MI-CASC pigs. MI-CASC $n_{animals}=3$, MI $n_{animals}=1$. MI-CASC 1,2,3 and MI $n_{image\ fields}=15$. Data are shown as mean ± SD. * denotes $p<0.05$. DAPI nuclear stain, GFP green fluorescent protein, VWF von willebrand factor.

3.1.6 CASCs do not show tumorigenic effects after subcutaneous transplantation in immunocompromised mice

Possible tumorigenic effect of CASCs were investigated after injection in immunocompromised mice. A head and neck cancer cell line (UM-SCC) was used as a positive control. All mice injected with UM-SCC tumor cells had to be sacrificed with large subcutaneous tumors after 3 weeks (Fig 16A,B). Three CASC-injected mice had to be sacrificed before the six months follow-up (Fig 16A). The mice suffered from severe weight loss, enlarged lymph nodes and inflated organs (Fig 16B). One mouse showed an enlarged liver, spleen, uterus and swollen ovaries. The second mouse showed emaciation with a lack of fat between the organs and enlarged glands in the groin area. Both animals had developed lymphomas, predominantly in abdominal regions. The third mouse was also emaciated with no clear cause of death. The remaining seven CASC mice were sacrificed after six months as described in the protocol. Macroscopic evaluation revealed enlarged organs and development of lymphomas in all mice, except one. Histological examination demonstrated immune cell infiltration in the lungs, kidneys, liver, spleen, pancreas and lymph nodes (Fig16C). However, untreated negative control mice (n=2) of another experiment also showed lymphoma formation, suggesting a predisposition of this mouse strain for this pathology. None of the CASC mice showed signs of subcutaneous tumors, indicating that CASCS themselves do not show any tumorigenic potential.

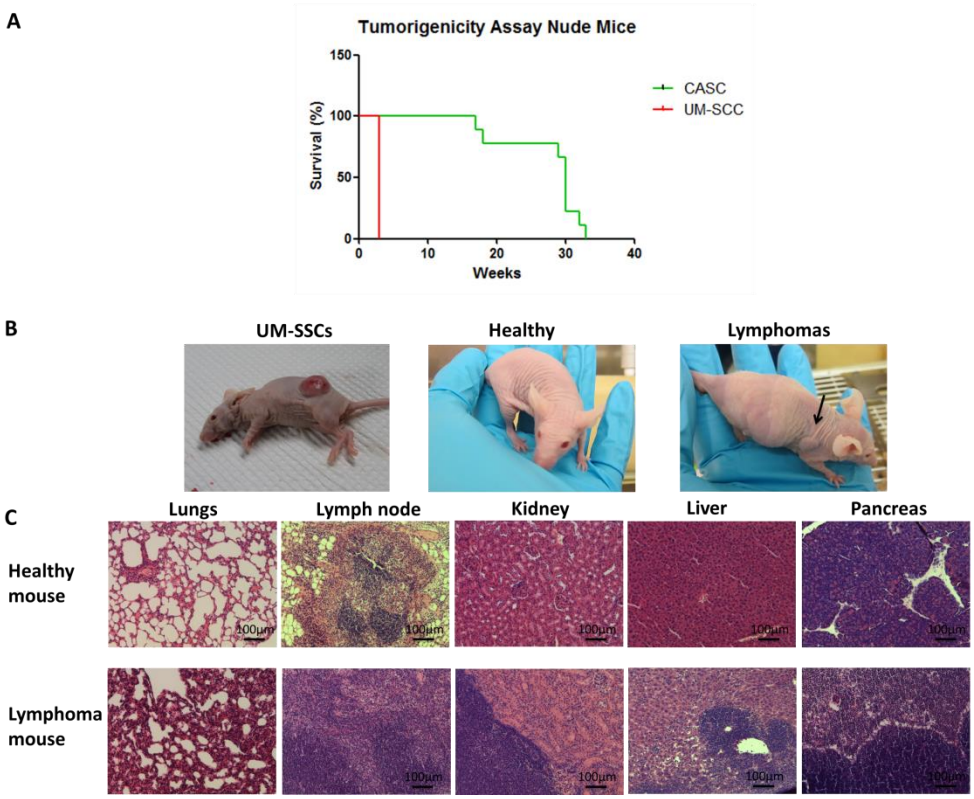


Figure 16: CASCs do not form subcutaneous tumors after transplantation in nude mice.
Possible tumorigenic properties of CASCs, isolated from human atrial appendages, were explored in immunocompromised mice. One million CASCs or UM-SSC tumor cells dissolved in Matrigel were injected in Foxn1nu mice. After 6 months none of the CASC mice developed subcutaneous tumors, while all mice injected with UM-SCCs had to be sacrificed with large tumors after 3 weeks (A, n=10 for each group). In total 9 out of 10 CASC mice did, however, develop spontaneous lymphomas. (B) H&E staining revealed immune cell infiltrates in organs of the mice with lymphomas (C). Scale bar=100µm.

3.1.7 Discussion

Various stem and progenitor cells from different sources show potential to promote cardiac repair after MI [24]. Unfortunately, the majority of the transplanted progenitors does not develop into cardiomyocytes and the observed improvement of cardiac function is mainly based on the activation of endogenous repair processes [193]. Stem cells from the heart itself, such as c-kit+ cells or CDCs, were thought to be more suited for myocardial repair by the direct formation of heart muscle cells, but also their cardiomyogenic regenerative potential is now under debate [150, 155].

Our research group recently reported the isolation of a CSC population from human and porcine atrial appendages based on a high ALDH enzyme activity. These CASCs homogeneously express the second heart field marker islet-1, but lack CD45 and c-kit expression [154]. Not only is their clonogenicity much higher (17 ± 11%) than reported for c-kit+ stem cells [190] or CDCs [153], but CASCs also display a complete cardiac phenotype after co-culture with neonatal rat cardiomyocytes, which could not be obtained for CDCs or c-kit+ CSCs [137]. It was recently shown that CASCs can be expanded with a humanized platelet-based culture method. Moreover, long term expansion does not change CASC ALDH activity, surface antigen profile or *in vitro* cardiomyogenic differentiation [194]. These new findings make CASCs suitable for human applications. The effectiveness of autologous CASC transplantation in a clinically relevant animal model was explored here. CASCs were administered immediately upon reperfusion, analogous to patients treated with revascularization in the context of an acute coronary syndrome. Intramyocardial administration was preferred over intracoronary delivery, due to the issues of embolization at the capillary

level [195] and limited retention after intracoronary administration [133]. In addition, the feasibility of CASC transplantation via transendocardial catheter-based injection in combination with EMM was established, allowing targeted cell delivery into the viable border zone of the infarct area, which has been shown to yield superior myocardial cell retention with a better clinical response [136, 196].

CASC transplantation resulted in an improvement of both regional and global LV function, associated with extensive cell engraftment and the formation of new, fully differentiated cardiomyocytes. Cardiac progenitor delivery in the setting of an acute MI creates a hostile environment for cell survival, engraftment and differentiation. Nevertheless, we could obtain substantial improvement in LVEF in the MI-CASC group, attributed to extensive CASC engraftment and cardiomyogenic differentiation. Cell retention was at least 19% on average, which is much higher than reported for other stem cell types [24, 37, 197]. Similar cell retention results have been documented for transendocardial injections in patients with non-ischemic dilated cardiomyopathy, suggesting this delivery method to be highly effective [136]. The use of an ALDH+ stem cell population might be advantageous in this respect, since the ALDH enzyme has been shown to promote the survival of multiple cell types in stress situations, such as ischemic conditions [198, 199]. Moreover, as much as 98% of the transplanted CASCs differentiated towards cardiomyocytes as shown by a sarcomeric expression of cardiac-specific proteins. Label-free SHG imaging confirmed that the characteristic alternating dark I- and bright A-bands of differentiated CASCs were identical to those of mature sarcomeres in resident cardiomyocytes. Interestingly, CASCs expressed the ventricular MLC-2V, suggesting that despite

their atrial origin, they differentiated towards a ventricular contractile profile. This adaptation of a fully cardiomyogenic phenotype suggests that CASCs are an efficient progenitor population with strong cardiomyogenic regenerative properties. Indeed, although CDCs have been shown to be safe and effective in preserving ventricular function in a porcine model of chronic ischemia [156], their differentiation potential appears to be limited [157, 200]. C-kit+ stem cells have been shown to improve ventricular function, based on differentiation into all cardiac lineages [143, 147]. However, a recent publication has shown that c-kit+ CSCs only minimally contribute to cardiomyogenesis, questioning their direct role in the replacement of lost cardiomyocytes [150]. Sca-1+ c-kit+ cardiomyocyte progenitor cells on the other hand do show a strong cardiomyogenic differentiation potential [168]. These cells also express islet-1 and surprisingly a high ALDH enzyme activity has been shown for a subfraction of these progenitors [201]. Although the different CSCs have long been regarded as separate entities, co-expression of their characteristic markers has been reported, indicating a relation between these stem cell populations. In depth understanding should be gained on the phenotype of the various cardiac progenitor populations and their precise role in heart development and regeneration. Nevertheless, it was shown here that CASCs are reliable progenitors for myocardial repair because of their strong cardiomyogenic potential [164, 166]. The gap junction protein Cx43 was present at the surface of differentiated CASCs aligned with surviving cardiomyocytes, as shown for c-kit+ and CDC-derived CSCs [143, 202]. This suggests electromechanical integration of CASC-derived cardiomyocytes into the existing cardiac muscle, which is essential

for the functional behavior of the newly integrated CASC-derived cardiomyocytes. Since the development of arrhythmogenic events is one of the major safety concerns in stem cell-based therapies after MI [192, 203], additional rhythm analysis and ECG monitoring was performed for both MI and MI-CASC animals. CASC transplantation did not induce any malignant cardiac arrhythmias. This is a first indication of the safety of CASC transplantation, consistent with the finding of Cx43 expression between CASCs and resident cardiomyocytes. Comparing the contraction properties and electrophysiological behavior of isolated CASCs with resident cardiomyocytes would be interesting to further analyze the electromechanical behavior of CASC-derived cardiomyocytes.

Occasionally, GFP+ CASCs could be observed in vessel walls, which were positive for VWF. This indicates that CASCs might also be able to differentiate into ECs in a vascular microenvironment. However, this was a very rare event, suggesting that CASCs adopt a predominant myogenic phenotype. Preliminary results of blood vessel counts in one control and three MI-CASC animals indicate potential paracrine effects on CASCs on angiogenesis. The number of blood vessels in infarct border zone of one CASC-MI animal was higher compared to the MI pig. However, further studies need to be performed to confirm these preliminary data. Only one control animal was included and the tissue construct used for CASC delivery might have influenced vascularization in CASC areas, which could have biased the results. Moreover, blood vessel quantification of the central infarct, the border zone and the non-affected remote area would allow a more accurate interpretation of revascularization.

Although subcutaneous transplantation of CASCs in nude mice did not lead to tumor formation at the injection sites, 90% of the injected mice developed spontaneous lymphomas. A limitation in this study is the lack of vehicle-treated only mice to rule out any effects of the Matrigel-collagen construct on lymphoma formation. However, untreated mice of the same strain used in another experiment also developed this condition and spontaneous lymphoma formation has been reported at old age of Foxn1nu mice [204]. Some human lymphomas are thought to be an abnormal responses to persistent antigenic stimulation and arise from situations related to immune deficiency states [205]. Although the mice were kept germ free, they were not necessarily antigen free. Antigen stimulation either by subcutaneous CASCs transplantation or other factors might have induced lymphoma formation. The experiments should therefore be repeated with inclusion of a non-treated and a vehicle-treated group to rule out the induction of the observed lymphomas by CASCs.

3.2 The role of CASCs in myocardial angiogenesis

3.2.1 Introduction

Various stem cell types such as iPSCs [175], MSCs [124] and CSCs [20, 176] have shown potential to improve heart function after MI. Most beneficial effects observed so far were mediated by paracrine actions, as stem cells secrete cytokines, growth factors and miRNAs that promote cardioprotection, angiogenesis and activate resident CSCs. However, only moderate therapeutic effects were observed in clinical trials, explained by limited differentiation of these stem cells towards cardiomyocytes [23].

The recently described CASCs [154], on the other hand, preserve left ventricular function in a Göttingen minipig MI model based on extensive cardiomyogenic differentiation and functional integration [154, 206]. Moreover, CASCs can be expanded to clinically relevant cell numbers [194], making them a perfect candidate for myocardial regeneration. However, to fully restore cardiac function, revascularization of the infarcted tissue is essential. Current catheter-based interventions and surgical bypass procedures are often not successful in re-establishing myocardial blood flow in MI patients, leading to an increased mortality and a poor clinical outcome [207]. In addition, cells transplanted in the infarct area end up in an ischemic environment, implicating that new blood vessel formation is necessary for their successful survival, engraftment and differentiation [208]. Ideally, cell-based therapies should therefore focus on both cardiomyogenesis and angiogenesis to induce optimal repair of the heart muscle. This study explored a possible contribution of CASCs in myocardial angiogenesis by investigating expression of pro- and anti-angiogenic factors and

their role in three consecutive steps of the angiogenesis process (EC proliferation, migration, tube formation). Stimulation of angiogenesis would further strengthen the therapeutic potential of the CASCs in ischemic heart conditions.

3.2.2 CASCs do not show multipotent or vasculogenic differentiation properties *in vitro*

First, important cardiovascular lineage markers were analyzed in CASCs, including the myocardial markers NK2 homeobox 5 (Nkx2.5) and myocyte enhancer factor 2C (MEF2C) and the vascular lineage marker vascular endothelial growth factor receptor 2 (VEGFR2). These markers are shown to be related to multipotent properties of other CSCs [164, 209-211] and hence might already give insights in the lineage commitment of CASCs. CASCs showed a low expression of the early cardiomyogenic transcription factor Nkx2.5 at mRNA level (Fig 17A). In addition, a nuclear expression of MEF2C was found at mRNA and protein level (Fig 17B,C). On the other hand, CASCs show a low expression of the VEGFR2 at protein level (Fig 17D). The absence of clear VEGFR2 expression at protein level together with a homogeneous MEF2C expression, might suggest a more cardiomyogenic commitment of CASCs.

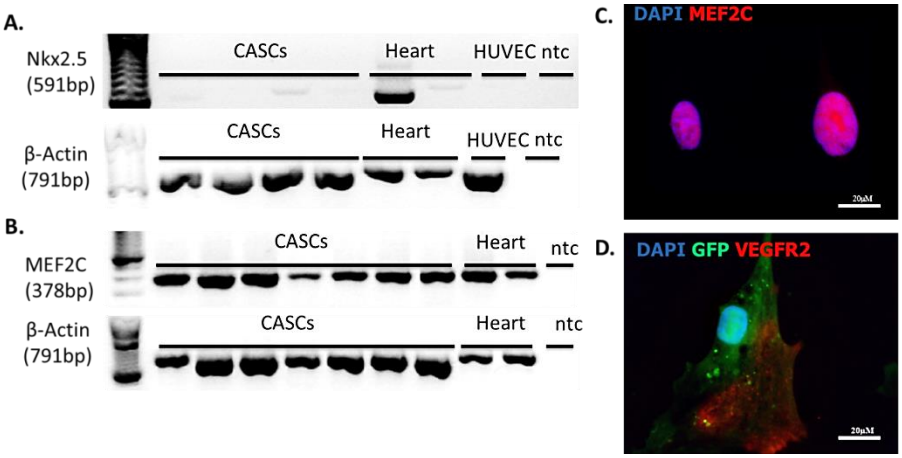


Figure 17: CASCs express important cardiovascular lineage markers.
(A) Nkx2.5 was found at mRNA levels in CASCs, albeit at very low levels. Heart tissue was used as a positive control, but also gave variable results. (B) MEF2C was expressed at mRNA level in CASCs and heart tissue. (C) Immunofluorescence revealed a homogeneous expression of MEFC in the nuclei of CASCs (merge DAPI and MEF2C = purple). (D) At protein level a low expression of the VEGFR2 (red) was detected in GFP+ CASCs (green).
β-Actin was used as a house hold gene in A and B. Nuclei were stained with DAPI (blue) in C and D. MEF2C myocyte enhancer factor 2C, Nkx2.5 NK2 homeobox 5, VEGFR2 vascular endothelial growth factor receptor 2

The multipotent character of CASCs was further assessed via their endothelial differentiation potential *in vitro*. MSCs served as positive control for the differentiation protocol. CASCs and MSCs were grown in medium supplemented with 100 ng/ml VEGF or serum-free EGM2-MV medium. After 7 and 10 days, vascular marker expression was analyzed. The VEGFR2 was expressed by CASCs at levels of the detection limit of the qPCR reaction (no signal until cycle 20-25, no plateau phase). Consequently, instability of the signal for the VEGFR2 yielded a lot of variability between the different patients and samples, hence, the data of CASC for VEGFR2 expression were excluded from the results. Although changes in

markers expression could be observed for both MSCs (Figure 18A) and CASCs (Figure 18B) in the tested differentiation media, the results were not uniform. Differences between patient samples and between the two time points were found, suggesting that MSCs nor CASCs differentiated into mature ECs.

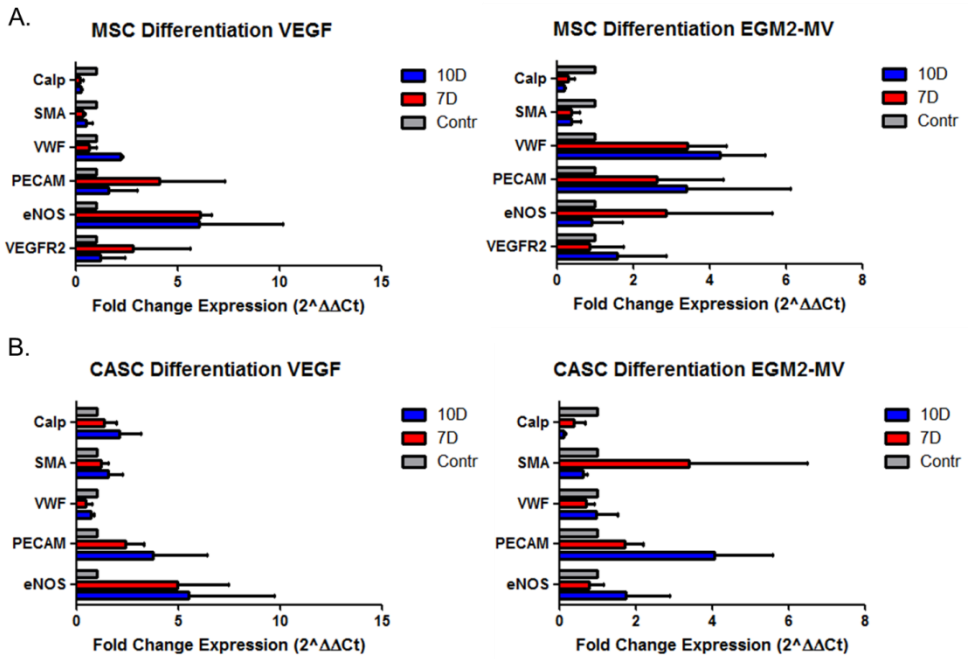


Figure 18: CASCs show limited vasculogenic *in vitro.* MSCs and CASCs were cultured in medium supplemented with 100 ng/ml VEGF or serum-free EGM2-MV medium. Expression of endothelial and smooth muscle markers was analyzed after 7 and 10 days. No clear changes in marker expression were observed for MSCs (A) or CASCs (B). Quantification was performed with the ΔΔCT method. Relative expression is shown as fold change compared to control MSCs or CASCs. Data are expressed as mean ± SEM. MSC n=2, CASC n=3. Calp calponin, eNOS endothelial nitric oxide synthase, PECAM platelet endothelial cell adhesion molecule, SMA smooth muscle actin, VWF von willebrand factor.

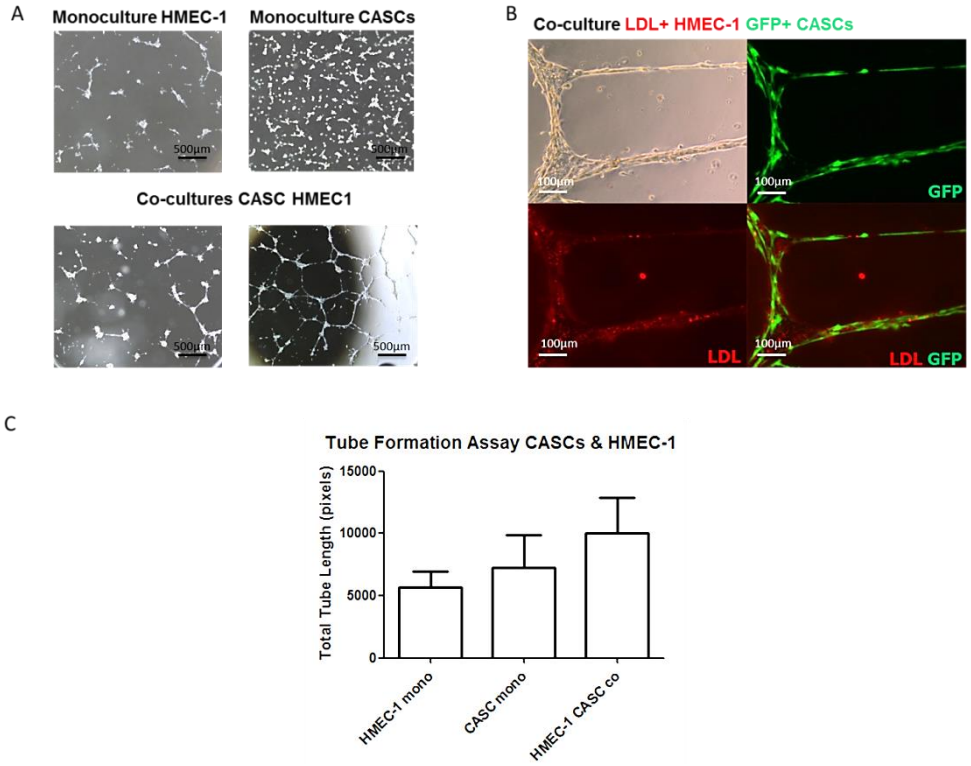


Figure 19: Matrigel co-cultures with HMEC-1 suggest a supportive role for CASCs.
HMEC-1 and CASCs were seeded on Matrigel (ratio 10:1) in LG-DMEM 0% FBS 2% P/S. (A) Network formation of mono- or co-cultured CASCs and HMEC-1 was evaluated after 24h. (B) Alignment of GFP+ CASCs (green) with LDL+ HMEC-1 (red) was observed. (C) No differences in total tube length were observed between HMEC-1 monocultures and co-cultured CASCs and HMEC-1. HMEC-1 mono n=5, CASC mono n=4, Co-culture n=7; Data are expressed as mean ± SEM. GFP green fluorescent protein, LDL low density lipoprotein.

Attachment of pericytes or smooth muscle cells is an essential step for the stabilization of newly formed blood vessels. A supportive role has been ascribed to MSCs and, therefore, it was investigated if CASCs are also capable of vascular stabilization. Mixtures of GFP+ CASCs and HMEC-1 were seeded on Matrigel and

tube formation was analyzed. CASCs seem to surround EC tubes (Fig 19A,B; CASCs in green and HMEC-1 in red), suggesting a supportive function. However, no significant increase in network formation was found for HMEC-1 in monoculture compared to co-cultures with CASCs (Fig 19C).

3.2.3 CASCs show paracrine angiogenic properties

CASCs secrete numerous angiogenic growth factors

To investigate a possible paracrine effect of CASCs on angiogenesis, we first determined their expression and secretion of important angiogenic growth factors. Numerous anti- (red) and pro-angiogenic (green) factors were identified in CASC CM by a human angiogenesis array, whereas no growth factors were detected in the Neg Contr (Fig 20A). Based on consistent high relative expression levels, the pro-angiogenic growth factors ET-1, IGFBP-3 and VEGF were further investigated. Expression of ET-1, IGFBP-3 and VEGF in CASCs was confirmed by immunofluorescence on cultured cells (Fig 20B) and Western blot on CASC lysates (Fig 20C). ELISA revealed high concentrations of all three factors in CASC CM, while the levels for the Neg Contr were below detection limit (Table 8). CASC lysates contained substantially lower growth factor concentrations compared to CM. CASCS thus express and secrete factors that could positively influence the angiogenesis process.

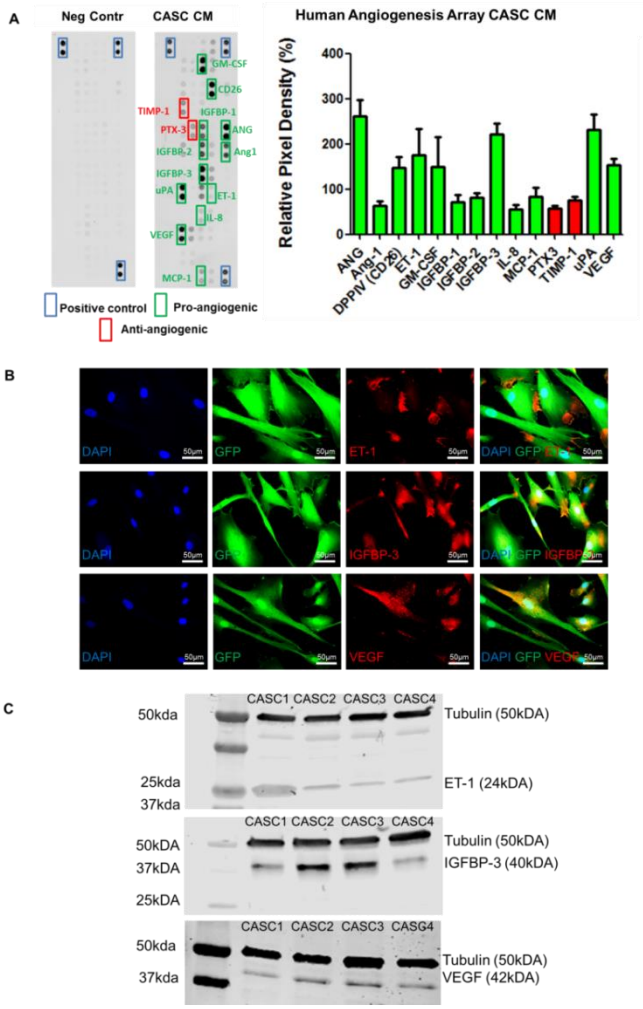


Figure 20: CASCs secrete numerous angiogenic growth factors.
(A) Both anti-angiogenic (red) and pro-angiogenic (green) growth factors were identified in CASC CM, but not in the Neg Contr (Neg Contr n=1, CASC CM n=3). Positive control spots were considered as 100% (blue). Expression of ET-1, IGFBP-3 and VEGF was confirmed in CASCs by immunofluorescence (B; n=3) and western blot (C; n=4). Data are expressed as mean ± SEM. ANG angiogenin, Ang-1 angiopoietin 1, DPPIV dipeptidyl peptidase 4, GM-CSF granulocyte-monocyte colony stimulating factor, ET-1 endothelin 1, IGFBP-1/2/3, insulin-like growth factor binding protein 1/2/3, IL-8 interleukin 8, MCP-1 monocyte chemotactic protein 1, PTX-3 pentraxin 3, TIMP-1 tissue inhibitor of metalloproteinase 1, uPA urokinase plasminogen activator, VEGF vascular endothelial growth factor.

Table 8: CASC angiogenic growth factor concentrations

	Neg contr	CASC CM	CASC lysate
ET-1 (ng/ml)	<<	32.4±9.8	1.2±0.4
IGFBP-3 (ng/ml)	<<	885.5±158.2	99.1±43.9
VEGF (ng/ml)	<<	64.8±18.2	0.8±0.1

<< below detection limit, Data are expressed as mean ± SEM, Neg Contr n=1, CASC CM n=10, CASC lysates n=4, ET-1 endothelin 1, IGFBP-3, insulin-like growth factor binding protein 3, VEGF vascular endothelial growth factor

A possible relationship between patient information and growth factor secretion was investigated. Patient characteristics and concentrations of ET-1, IGFBP-3 and VEGF in CASC CM of ten patients are shown in Table 9. No clear correlation could be observed between general patient history (Fig 21), risk factors (Fig 22) or relevant co-morbidities (Fig 23) and secreted growth factor concentrations. For the pre-operative cardiac status (Fig 24), a significant increase in ET-1 secretion was observed for patients suffering from HF compared to non-HF subjects. As expected, a positive correlation was found for BMI and weight. Some trends seemed to be present, for example, weight might negatively influence VEGF secretion, however, the number of analyzed samples is too small to draw any conclusions.

Table 9: Patient characteristics and angiogenic growth factor concentrations in CASC CM

Patient	1	2	3	4	5	6	7	8	9	10
General patient history										
Age	77	57	53	72	86	67	60	55	68	68
Male	f	F	m	f	m	m	f	m	m	m
Risk factors										
Weight (kg)	51	72	81	66	67	104	73	67	82	76
BMI (kg/m^2)	18.5	25.5	24.2	23.7	24.9	36.85	30.4	25.22	30.5	26
Creatinine (mg/dl)	0.72	0.71	0.91	0.87	1.59	1.5	0.91	.	2.08	1.1
Smoker	n	Y	y	n	.	y	n	n	n	n
Diabetes	n	N	n	n	n	y	n	y	n	n
Hyperlipidemia	y	Y	n	y	y	y	y	y	y	y
RD	n	N	n	n	n	y	n	n	n	n
Hypertension	y	N	n	y	.	y	n	y	y	y
CLD	n	N	n	n	y	y	n	n	n	n
PVD	y	Y	n	n	n	n	n	n	n	n
CVD	n	N	n	n	n	n	n	y	n	n
Pre-operative cardiac status										
MI	y	Y	y	n	n	n	y	n	y	y
HF	n	N	n	n	y	y	n	y	n	n
Angina	ccs0	ccs3	ccs2	ccs2	ccs1	ccs0	ccs3	ccs0	ccs2	ccs2
NYHA (I/II/III/IV)	3	3	4	1	3	3	1	4	1	1
Surgical procedure										
CABG/valve	cabg	Cabg	cabg	cabg	cabg	valve	cabg	cabg	cabg	cabg
Vessel number (0/1/2/3)	3	3	1	3	3	0	2	3	2	2
Angiogenic factors CM										
ET-1	4409	513	2153	2885	68999	55046	64331	76979	24035	24311
IGFBP-3	655	1026	527	325	1624	1257	1474	418	1257	292
VEGF	47530	63752	21011	30567	212285	6870	86015	80768	60053	39116
Lysates										
ET-1					1423		138	1019		614
IGFBP-3					146		30	73		15
VEGF					342		712	607		364

(Table 9 continued) BMI body mass index, CABG coronary artery bypass graft, CCS Canadian Cardiovascular Society grading of Angina Pectoris, CLD chronic lung disease, CVD cerebrovascular disease, ET-1 endothelin 1, HF heart failure, IGFBP-3 insulin-like growth factor binding protein 3, MI myocardial infarction, NYHA New York Heart Association, PVD peripheral vascular disease, RD renal dysfunction, VEGF vascular endothelial growth factor

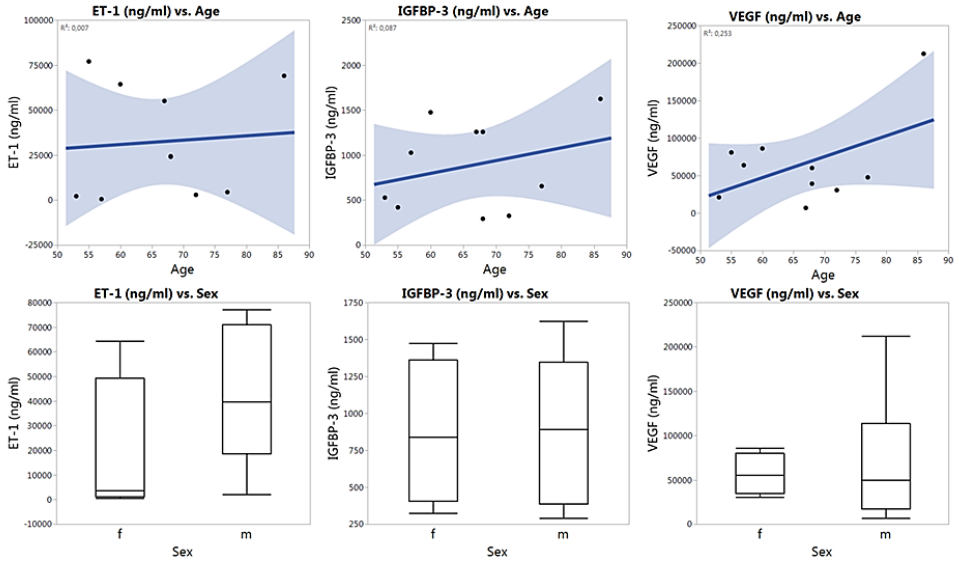


Figure 21: No correlation exists between general patient characteristics and CASC secretion of ET-1, IGFBP-3 and VEGF.
Concentrations of ET-1, IGFBP-3 and VEGF in CASC CM, as measured by ELISA, did not correlate with age or sex. Data are expressed as mean ± SEM. ET-1 endothelin 1, f female, IGFBP-3, insulin-like growth factor binding protein 3, m male, VEGF vascular endothelial growth factor.

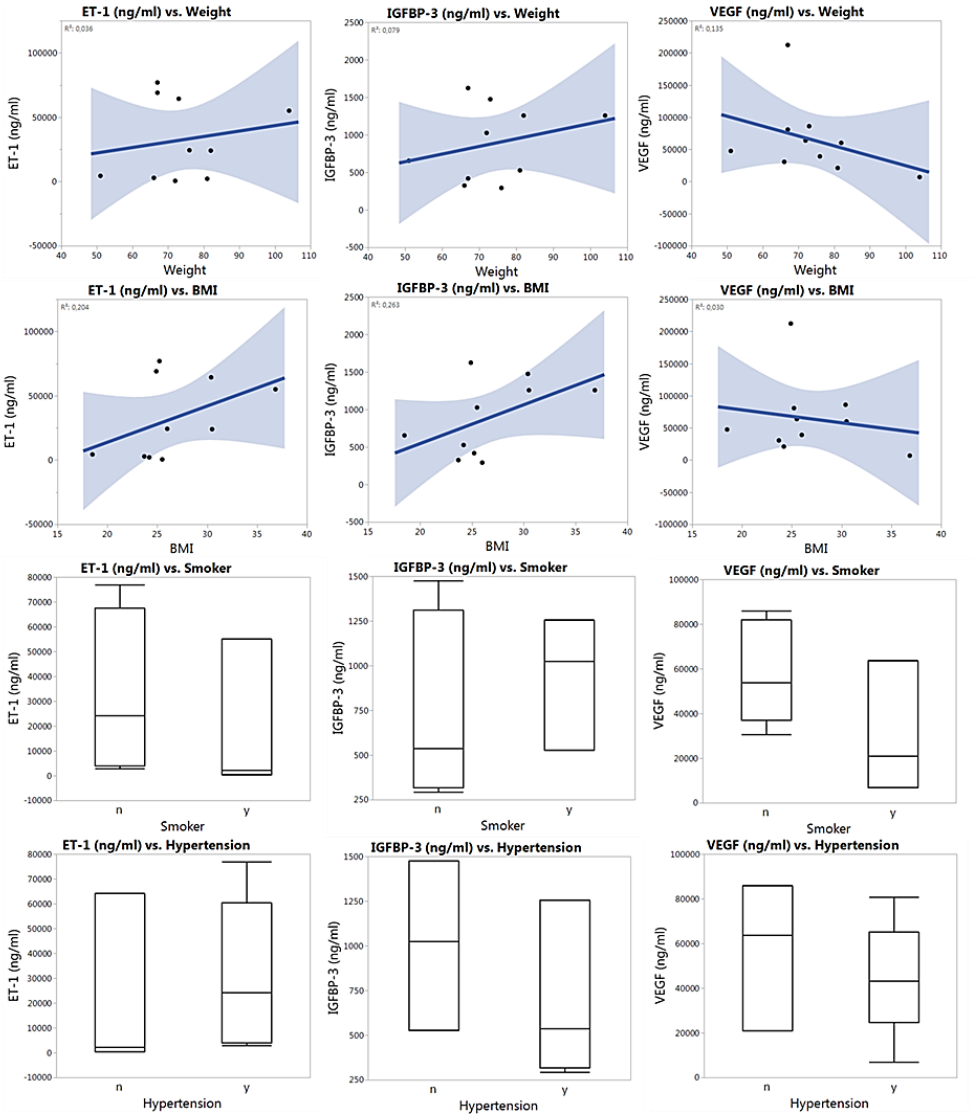


Figure 22: No correlation exists between risk factors and CASC secretion of ET-1, IGFBP-3 and VEGF.
Concentrations of ET-1, IGFBP-3 and VEGF in CASC CM, as measured by ELISA, did not correlate with weight, BMI, smoking or hypertension. Data are expressed as mean ± SEM. ET-1 endothelin 1, IGFBP-3, insulin-like growth factor binding protein 3, n no, VEGF vascular endothelial growth factor, y yes.

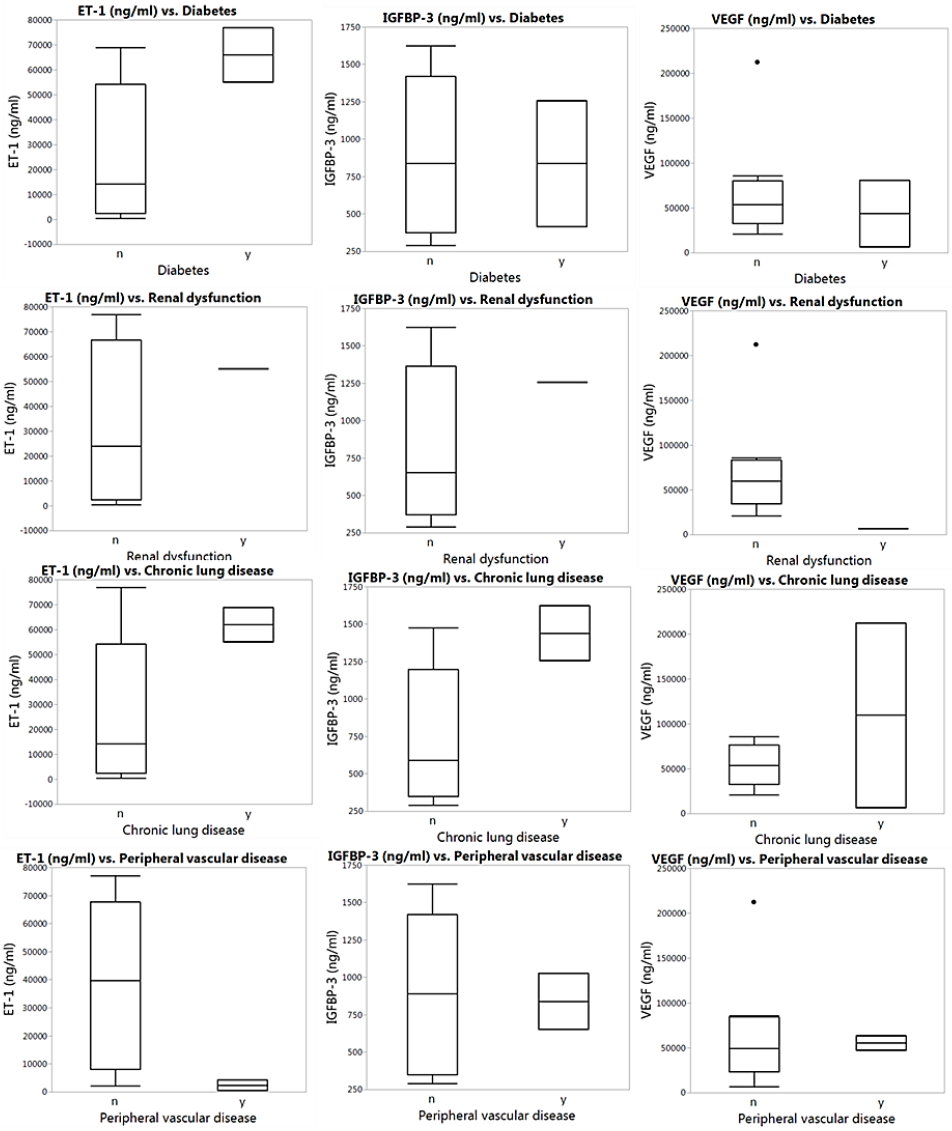


Figure 23: No correlation exists between patient co-morbidities and CASC secretion of ET-1, IGFBP-3 and VEGF.
Concentrations of ET-1, IGFBP-3 and VEGF in CASC CM, as measured by ELISA, did not correlate with diabetes, renal dysfunction, chronic lung disease or peripheral vascular disease. Data are expressed as mean ± SEM. ET-1 endothelin 1, IGFBP-3, insulin-like growth factor binding protein 3, n no, VEGF vascular endothelial growth factor, y yes.

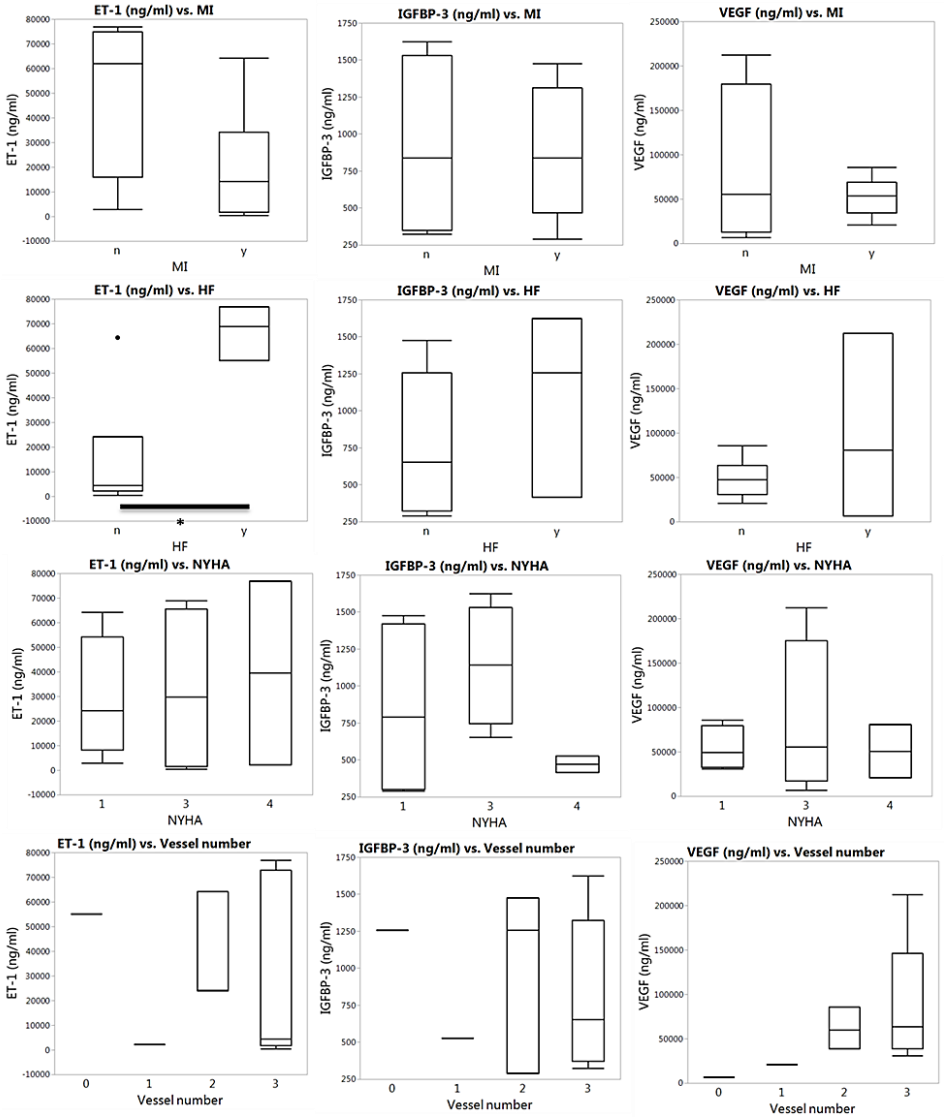


Figure 24: No correlation exists between patient pre-operative cardiac status and CASC secretion of ET-1, IGFBP-3 and VEGF, except for a higher ET-1 secretion in patients with HF.
Concentrations of ET-1, IGFBP-3 and VEGF in CASC CM, as measured by ELISA, did not correlate with the presence of MI, NYHA status or the number of affected blood vessels in case of coronary artery disease. Higher levels of ET-1 were observed in HF patients. Data are expressed as mean ± SEM. ET-1 endothelin 1, IGFBP-3, insulin-like growth factor binding protein 3, n no, VEGF vascular endothelial growth factor, y yes.

CASCs promote HMEC-1 angiogenesis in vitro by the secretion of ET-1, IGFBP-3 and VEGF

Next we explored the effects of CASC CM on main steps of the angiogenesis process, being EC proliferation, migration and tube formation. Involvement of the individual factors ET-1, IGFBP-3 and VEGF in these different steps was assessed by pre-incubating CASC CM with inhibitory antibodies against these factors. CM incubated with isotype control antibodies was included to rule out any non-specific influences.

It was demonstrated that the different test conditions did not affect HMEC-1 viability (Fig 25), confirming that effects on HMEC-1 survival did not negatively influence the results.

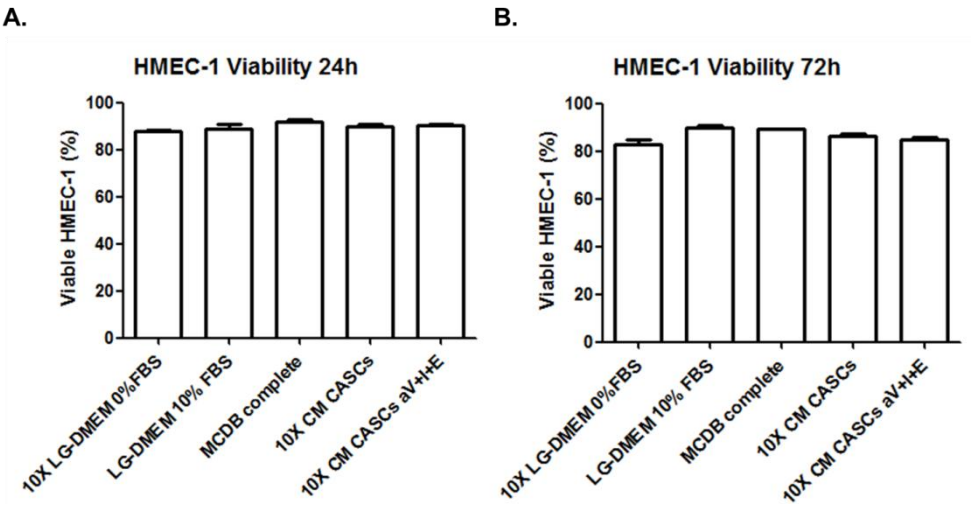


Figure 25: Experimental conditions do not affect HMEC-1 viability.
No differences in HMEC-1 viability were observed after culture in the different medium conditions after 24h (A) or 72h (B). 10X LG-DMEM 0% FBS and MCDB complete culture medium HMEC-1 n=6, LG-DMEM 10% FBS n=5, 10X CASC CM n=7, 10X CASC CM aV+I+E n=3. Data are expressed as mean ± SEM. aV+I+E antibodies against ET-1, IGFBP-3 and VEGF. FBS fetal bovine serum.

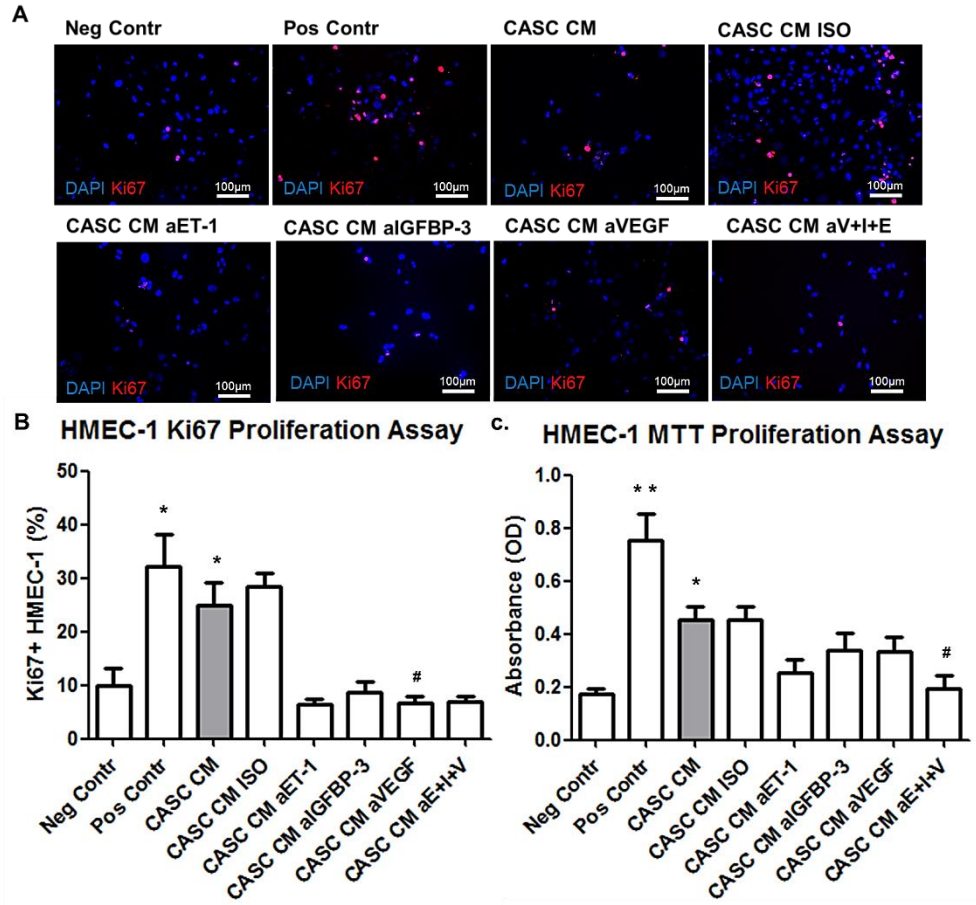


Figure 26: CASCs promote HMEC-1 proliferation in an MTT and a Ki67 assay.

HMEC-1 were cultured in Contr and CASC CM for 72h, after which proliferation was examined. Representative pictures of the Ki67 assay are displayed in a with nuclei in blue (DAPI) and Ki67 in red (A). Both the Ki67 and the MTT assay revealed a significant increase in HMEC-1 proliferation for the Pos Contr and CASC CM compared to the Neg Contr (B,C). This effect was significantly inhibited by an anti-VEGF antibody in the Ki67 assay (B) and by a combination of an antibody against ET-1, IGFBP-3 and VEGF in the MTT test (C). For the MTT assay, Contr media n=11, CASC CM n=18, ISO n=9, aET-1 n=6, aIGFBP-3 n=8, aVEGF n=8, aE+I+V n=6. For the Ki67 assay, Contr media n=10, CASC CM n=15, ISO n=8, aET-1 n=6, aIGFBP-3 n=6, aVEGF n=6, aE+I+V n=6. Data are expressed as mean ± SEM. * significance compared to Neg Contr; # compared to CASC CM. * or # p-value <0.004; ** p-value < 0.0008. ET-1 endothelin 1, IGFBP-3, insulin-like growth factor binding protein 3, ISO isotype control antibodies, Neg Contr unconditioned serum-free medium, Pos Contr medium with 10% FBS, VEGF vascular endothelial growth factor.

EC proliferation was investigated in an MTT assay and by Ki67 immunofluorescence. HMEC-1 proliferation was increased after incubation with the Pos Contr and CASC CM compared to the Neg Contr in both the Ki67 (Fig 26A,B; p-value 0.0004 and 0.0006 respectively) and the MTT assay (Fig 26C; p-value <0.0001 and 0.0038 respectively). Inhibition of VEGF reduced the effect of CASC CM in the Ki67 assay (Fig 26B; p-value <0.0001), while for the MTT assay only combined inhibition of ET-1, IGFBP-3 and VEGF led to a significant decrease (Fig 26C; p-value 0.0032). However, a clear trend was observed for all inhibitory antibodies in both assays.

To study the role of CASC CM in EC migration, a transwell migration assay was performed (Fig 27). Our results showed that HMEC-1 migration was 4.3 times higher for CASC CM compared to the Neg Contr (p-value <0.0001), although the response was not that strong as the Pos Contr. Incubation of the CM with antibodies against ET-1, IGFBP-3 or VEGF alone significantly diminished these effects (p-value <0.0001 for all antibodies), with an even stronger response when all three inhibitory antibodies were combined, reducing the migration effect of CASC CM by 2.7 fold (p-value <0.0001).

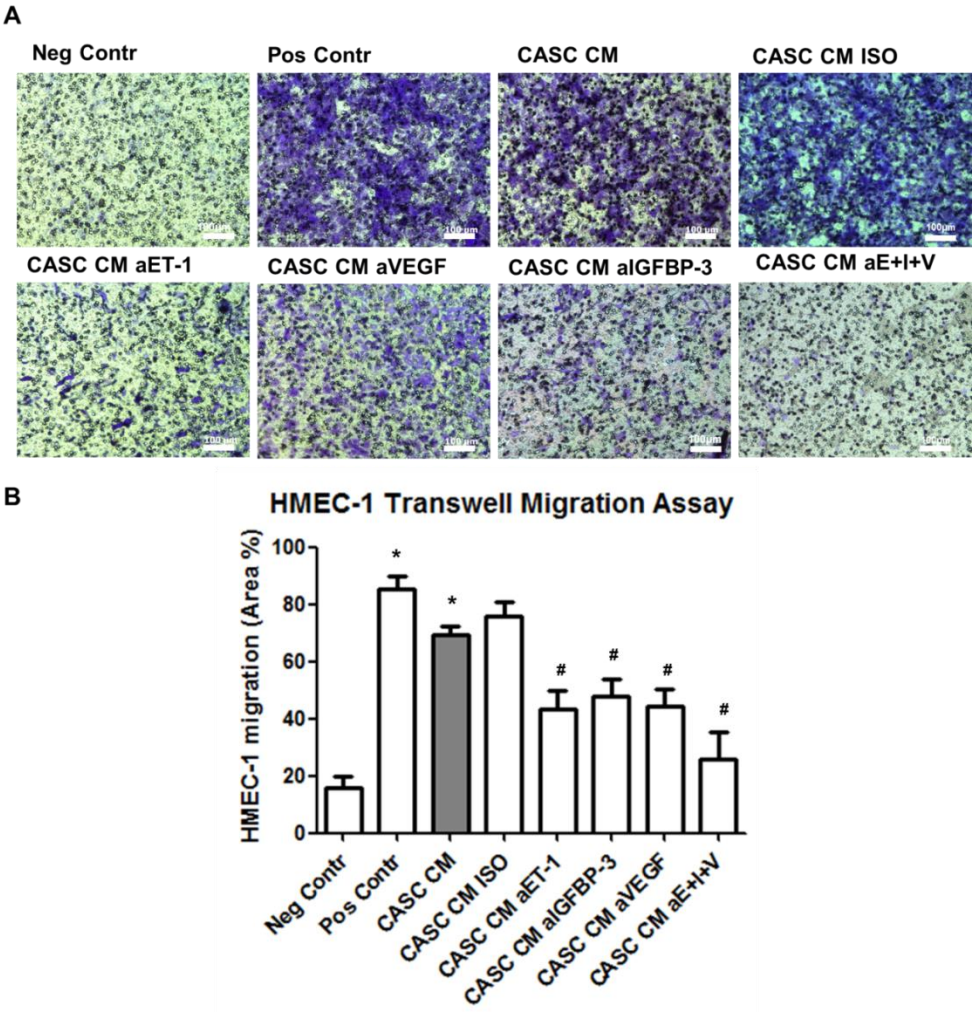


Figure 27: CASCs promote HMEC-1 migration in a transwell assay.
(A) Representative pictures show HMEC-1 migrating through the transwell membrane (purple). (B) A significant increase in HMEC-1 migration was observed for the Pos Contr and CASC CM compared to the Neg Contr. CM-induced migration could be reduced by addition of antibodies against ET-1, IGFBP-3 and VEGF. Neg Contr n=17, Pos Contr n=20, CASC CM n=28, ISO n=3, aET-1 n=20, aIGFBP-3 n=18, aVEGF n=15, aE+I+V n=9. Data are expressed as mean ± SEM. * significance compared to Neg Contr; # compared to CASC CM .* or # p-value <0.004. ET-1 endothelin 1, IGFBP-3, insulin-like growth factor binding protein 3, ISO isotype control antibodies, Neg Contr unconditioned serum-free medium, Pos Contr medium with 10% FBS, VEGF vascular endothelial growth factor.

EC assembly into tube-like structures, was explored by plating HMEC-1 on Matrigel in Contr media or CASC CM (Fig 28). HMEC-1 network formation, expressed as the total tube length, was significantly higher after culturing the cells in the Pos Contr and CASC CM compared to the Neg Contr (p-value 0.0001 and 0.0018 respectively). Pre-incubation of CASC CM with a combination of inhibitory antibodies against ET-1, IGFBP-3 and VEGF significantly reduced HMEC-1 network formation (p-value <0.0001), which could not be accomplished by blocking these growth factors individually. In addition, a dose-dependent effect of CASC CM on HMEC-1 tube formation was observed, by incubation with 1X and 10X concentrated CM (Fig 29; p=0.007).

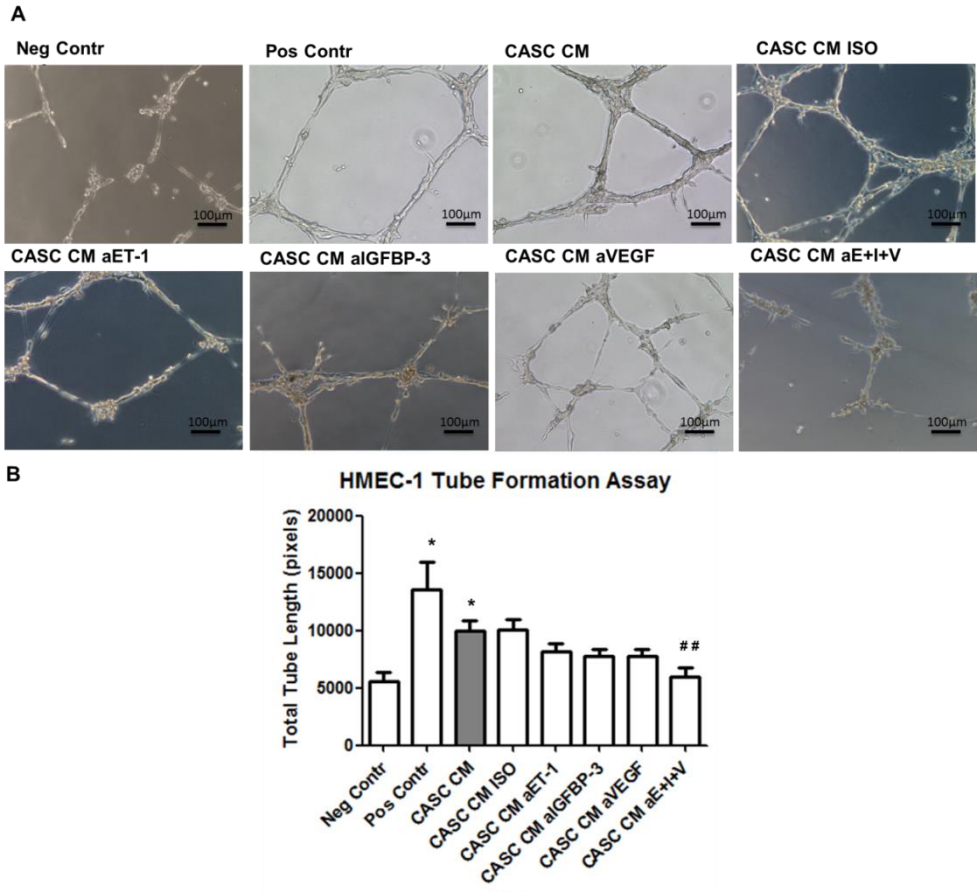


Figure 28: CASCs promote HMEC-1 tube formation.
(A) Representative pictures of HMEC-1 tubular networks after 24h. (B) A significant increase in total tube length was observed for the Pos Contr and CASC CM compared to the Neg Contr. Combined inhibition of ET-1, IGFBP-3 and VEGF significantly reduced these effects. Neg Contr n=18, Pos Contr n=14, CASC CM n=25, ISO n=6, aET-1 n=17, aIGFBP-3 n=20, aVEGF n=20, aE+I+V n=13. Data are expressed as mean ± SEM. * significance compared to Neg Contr; # compared to CASC CM.* or # p-value <0.004; ## p-value < 0.0008. ET-1 endothelin 1, IGFBP-3, insulin-like growth factor binding protein 3, ISO isotype control antibodies, Neg Contr unconditioned serum-free medium, Pos Contr HMEC-1 culture medium with 10% FBS L-Glut hEGF and HC, VEGF vascular endothelial growth factor.

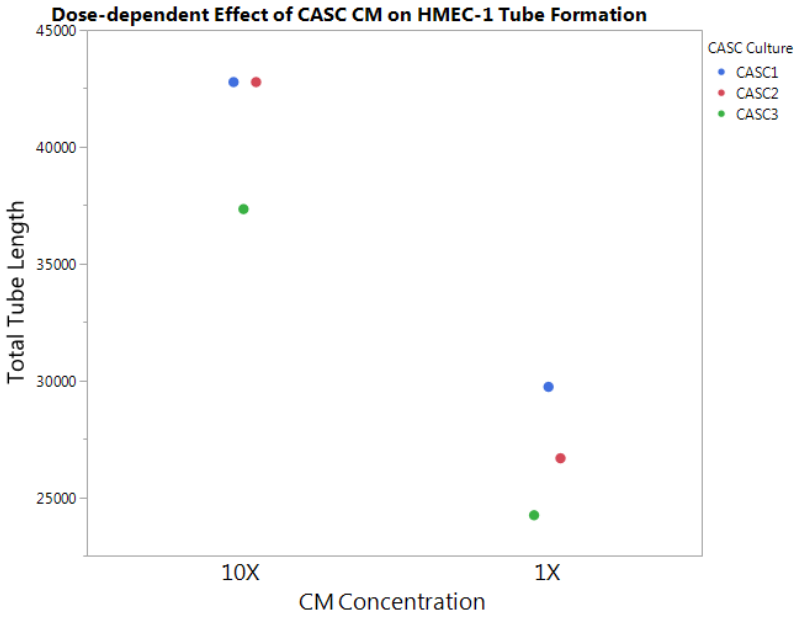


Figure 29: CASC CM promotes HMEC-1 tube formation in a dose-dependent way.
A tube formation assay was performed by seeding HMEC-1 in 1X and 10X concentrated CASC CM of the same CASC culture (CASC from each donor are shown in a different color). Network formation was significantly higher in 10X CASC CM (left) compared to 1X CASC CM (right) (p-value 0.007). n=3. Data are expressed as mean ± SEM.

These *in vitro* experiments show that CASCs promote all important steps of the angiogenesis process. The effect seems to be mainly mediated by ET-1, IGFBP-3 and VEGF secretion, although other factors are likely also involved.

CASCs promote angiogenesis in vivo in the chorioallantoic membrane assay

To confirm the angiogenic effect, an *in vivo* CAM assay was performed by incubating the CAM with CASCs, CASC CM or Neg Contr samples (Fig 30A). After 72h, radial growth of vessels in a spoke wheel pattern towards the droplets was

visible for all conditions (Fig 30B,C). Quantification of the number of blood vessels, based on intersection with two concentric circles (Fig 30B), revealed a significant increase for both CASCs and CASC CM compared to their Neg Contr (Fig 30D). The number of blood vessels increased from 22.4 to 26.3 (inner circle; p-value 0.0364) and from 29.2 to 37.0 (outer circle; p-value 0.0011) for the Neg Contr compared to CASCs respectively. For CASC CM an augmentation was observed from 18.9 to 23.3 (inner circle; p-value 0.0102) and from 29.1 to 32.5 (outer circle; p-value 0.0202) respectively. These results indicate that both CASCs and CASC CM promote blood vessel formation *in vivo*.

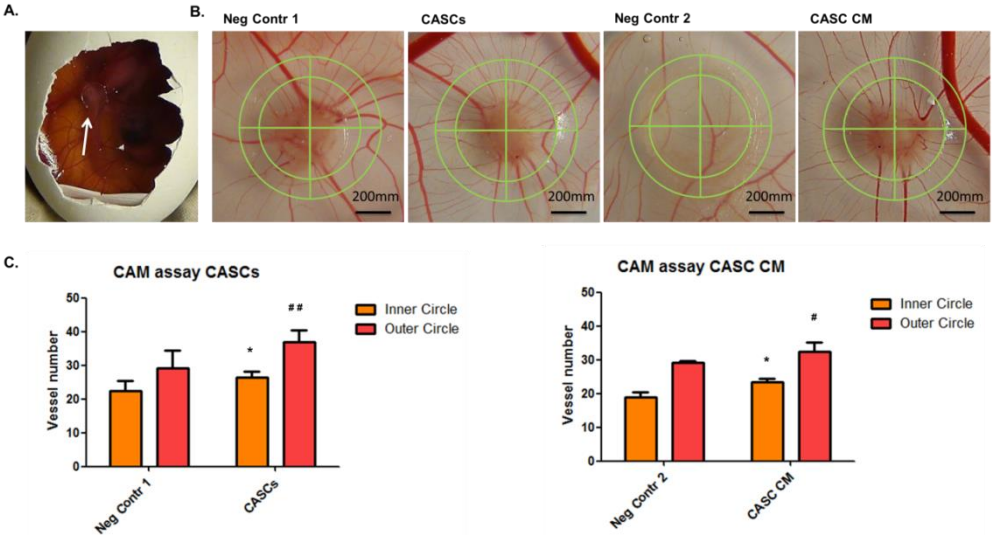


Figure 30: CASCs promote angiogenesis *in vivo* in the CAM assay.
The CAM membrane of fertilized chicken eggs was incubated at E9 with Matrigel droplets containing different test conditions (A). Representative pictures of each condition show the radial ingrowth of blood vessels with a typical spoke wheel pattern (B). A significant increase in blood vessel number was observed for CASCs (C, left panel) and CASC CM (C, right panel) compared to their Neg Contr for the inner and outer circle. The CAM assay was repeated 3 independent times with 6 CASC and CASC CM donor samples. Data are expressed as mean ± SEM. * p-value <0.05; ** p-value < 0.01. Neg Contr 1 = Matrigel; Neg Contr 2 = Matrigel mixed 1:1 with serum-free medium.

3.2.3 Discussion

We have previously shown that CASCs preserve cardiac function in a minipig MI model based on extensive engraftment and cardiomyogenic differentiation [206]. However, not only replacement of the lost heart muscle, but also new blood vessel formation is essential to restore cardiac function. This ensures the supply of critical oxygen and nutrients to the ischemic heart tissue, and guarantees the survival and engraftment of the transplanted progenitors [207]. Vasculogenesis and angiogenesis are the main mechanisms of new blood vessel formation, while arteriogenesis enhances blood flow by increasing the luminal diameter of existing arteries [212]. Vasculogenesis by EC differentiation and vascular integration has been described for e.g. EPCs [213], MSCs [42] and CSCs [20]. In contrast, EC differentiation was only rarely observed (<1%) after CASC transplantation in a minipig MI model, in accordance with a strong cardiomyocyte differentiation of 98% as described in chapter 3.1. CASCs show a limited expression of the vascular lineage marker VEGR2, but are positive for the early cardiomyogenic marker MEF2C at protein level. Variable expression was observed for Nkx2.5 at mRNA level in cultured CASCs and positive control atrial appendage tissue, while Nkx2.5 is normally expressed in the atrial appendage. Further optimization of the PCR protocol might be necessary for Nkx2.5. The observed expression of important early cardiomyogenic markers, limited *in vivo* EC differentiation and absence of vasculogenic differentiation *in vitro*, suggests a predominant myogenic commitment of CASCs.

Despite the limited EC differentiation of CASCs, a trend towards an increase in blood vessel density in border zone areas with transplanted CASC was observed in preliminary results of the minipig MI model, suggesting a role for CASCs in

myocardial angiogenesis [206]. Hence, in line with the emerging paracrine hypothesis for stem cell-induced myocardial repair, possible paracrine angiogenic effects of CASCs were explored in this study.

Angiogenesis, characterized by the formation of new blood vessels via the branching or elongation of preexisting vessels, is tightly regulated by a balance of both pro- and anti-angiogenic mediators [212]. Numerous angiogenic inducers could be identified in CASC CM after an initial screening, which are listed in Table 10.

Table 10: Function of identified angiogenic growth factors in CASC CM

Pro-angiogenic factors		
Angiogenin (ANG)	EC proliferation, tube formation	214, 215
Angiopoietin 1 (Ang-1)	EC migration, survival Vessel stabilization	214, 216-218
Dipeptidyl peptidase IV (DPPIV) / CD26	Activation neuropeptide Y (EC proliferation, migration and tube formation)	219
Endothelin 1 (ET-1)	VEGF induction EC proliferation, migration	220-223
Granulocyte monocyte colony stimulating factor (GM-CSF)	EC proliferation, migration	224
Insulin-like growth factor binding protein 1,2,3 (IGFBP-1,-2,-3)	EC migration, tube formation	214, 225-227
Interleukin-8 (IL-8)	Activation VEGF-VEGFR2 signaling EC proliferation, migration, differentiation	228-230
Monocyte chemotactic protein 1 (MCP-1/CCL2)	EC migration and monocyte recruitment	231
Urokinase plasminogen activator (uPA)	EC migration via ECM degradation	232
Vascular endothelial growth factor (VEGF)	Increase vessel permeability, ECM degradation EC proliferation, migration, survival, tube formation	214, 233-235

Anti-angiogenic factors		
IGFBP-3	EC apoptosis	[236]
Pentraxin 3 (PTX3)	Inhibition FGF-2 angiogenesis	[237, 238]
Tissue inhibitor of metalloproteinase 1, 4 (TIMP-1,4)	Inhibition MMPs	[239, 240]

ET-1 [221], IGFBP-3 [225] and VEGF [241, 242] were selected for further analysis, because of their high and uniform expression level in CASC CM. Their concentration in CASC lysates was consistently lower compared to the CM, as previously reported for MSCs [243]. This might already suggest a strong paracrine angiogenic potential for CASCs by the secretion of high levels of angiogenic proteins. The concentration of VEGF, as most widely studied angiogenic growth factor, is markedly higher in CASC CM compared to other stem cells types [160, 244]. However, direct comparison of growth factor concentrations in stem cell CM is not feasible, because of differences in the preparation methods.

It is known that patient characteristics can influence stem cells potency, including their angiogenic properties. Age and diabetes have been shown to reduce the angiogenic potential of adipose tissue-derived stem cells [245, 246]. Patient characteristic have also been reported to influence the number of isolated cardiac stem cells by Itzhaki-Alfia et al., 2009 [247]. Their study revealed the right atrium as superior cell source and demonstrated an association of female sex with a higher number of c-kit+ CSCs. Hypertension is known to influence EPCs, as angiotensin II dramatically reduces the activity of the

telomerase enzyme, which accelerates stem cell senescence and inhibits their proliferation [248]. In contrast, in combination with VEGF, angiotensin increases the proliferation of EPCs [249]. Furthermore, nicotine can cause adverse effects on the chondrogenic differentiation of BM MSCs, suggesting that smoking has a negative effect on stem cell potency [250]. Comparing patient characteristics with angiogenic growth factor secretion by CASCs is therefore very interesting. Important aspects of the patients history were correlated with the concentration of ET-1, IGFBP-3 and VEGF in CASC CM. Unfortunately, no clear correlations could be found between patient characteristics and growth factor concentrations in CASC CM, although some trends were definitely observed. CASCs obtained from HF patients secreted higher amounts of ET-1 compared to patients without HF. Increased ET-1 plasma levels have been shown for HF patients [251] and seem to correlate with disease severity and a poor prognosis. This suggests that HF might have an effect on CASC growth factor secretion, although more repeats and detailed analysis are necessary to confirm this. A trend of lower VEGF secretion for CASCs derived from patients with hypertension might be suggested, but the number of analyzed samples is too small to draw any conclusions.

Nevertheless, the importance of VEGF, IGFBP-3 and ET-1 in neovascularization further confirms a likely involvement of these mediators in CASC-induced myocardial angiogenesis. VEGF is one of the most studied angiogenic factors and is involved in almost all steps of the angiogenesis process [241, 242]. IGFBP-3 has been reported to have inhibitory effects in tumor angiogenesis [252], while pro-angiogenic effects have been shown in human umbilical vein ECs [225]. ET-1 is a

mitogen for vascular cells and promotes EC migration and tube formation [221]. Despite high relative expression levels of ANG and urokinase plasminogen activator (uPA), no suitable inhibitory antibodies were available for investigation in our cell culture assays. The role of all identified growth factors in angiogenesis can be found in Table 10. A wide variety of angiogenic growth factors are known to be secreted by various stem cell types, such as fibroblast growth factor, hepatocyte growth factor, interleukins and VEGF [155, 241, 253, 254]. Other reparative processes have also been shown to be influenced by these secreted growth factors, which also seem likely for the identified CASC secreted mediators. Indeed, VEGF reduces inflammation and promotes cardioprotection [241], while MCP-1 attracts monocytes and macrophages, which clear the infarct area from dead cells and debris leading to activation of reparative pathways [255].

Undoubtedly, the identified factors are involved in important steps of angiogenesis and therefore *in vitro* assays with HMEC-1 were performed to establish the functional pro-angiogenic effects of CASCs. EC proliferation is one of the first important steps in neovascularization and can best be investigated by a combination of cell number quantification and cell cycle analysis [256]. Both an MTT and a Ki67 assay indicated that CASC CM stimulates an increase in HMEC-1 proliferation. Neutralization of VEGF reduced the effect of CASC CM in the Ki67 assay, while for the MTT assay only combined inhibition of ET-1, IGFBP-3 and VEGF led to a significant decrease. Discrepancy between the observed results can be explained by the different approach of the assays to assess proliferation. The Ki67 assay quantifies the number of actively proliferating cells, while the MTT test is a measure of the total number of cells and their metabolic activity.

Still, a clear trend towards a reduction in HMEC-1 proliferation was observed for all inhibitory antibodies in both assays and significance could be reached for all antibody conditions without Bonferroni correction. Bonferroni correction is known to reduce the statistical power of the test significantly [257]. Our data hence suggest ET-1, IGFBP-3 and VEGF to be important mediators of CASC-induced HMEC-1 proliferation. The proliferative effect of CASC CM was reduced to baseline levels in most antibody conditions. This clearly demonstrates the importance of these specific mediators in the delicate balance of pro- and anti-angiogenic proteins. Still, ANG, IL-8 and GM-CSF were also identified in CASC CM in the angiogenesis array, which are also known to be involved in EC proliferation (Table 10). Similar effects on EC proliferation have been reported for CM obtained from various MSC types *in vitro* [258-260] and fetal aorta–derived CD133+ progenitors *in vivo* [261], while no proliferative effect was observed for dental stem cells (DSCs) [244, 262].

After the proliferation phase, ECs migrate towards chemotactic stimuli. In a transwell system, we were able to show that CASC CM induced similar HMEC-1 migration as the Pos Contr. Individual inhibition of ET-1, IGFBP-3 and VEGF reduced this process, which was enhanced when combining the three inhibitory antibodies. This demonstrates that ET-1, IGFBP-3 and VEGF are all important mediators of CASC-induced EC chemotaxis. Despite this promising result, the effect of CASC CM was not completely reduced. This suggests that other factors are also involved in EC chemotaxis as shown in migration assays for EPCs [263], MSCs [259, 260], DSCs [244, 262] and other CSC types [264]. The presence of Ang-1, MCP-1, IL-8 and GM-CSF in CASC-CM is probably responsible for this effect.

As a last step in the angiogenesis process, migrating ECs assemble into tube-like structures that form the base of the newly formed blood vessels, which was assessed in a Matrigel tube formation assay. Our results indicated that CASC CM improves HMEC-1 network formation as previously reported for MSCs [265], DSCs [244, 262] and other CSCs [158]. Only combined inhibition of ET-1, IGFBP-3 and VEGF reduced HMEC-1 tube formation to baseline levels, pointing towards a synergistic effect. This complete inhibition occurred despite high relative levels of ANG, which is also known to promote EC tube formation. Moreover, a dose-dependent effect on HMEC-1 tube formation was demonstrated for 1X and 10X concentrated CASC CM.

Although CASCs stimulate the main phases of neovascularization, the formation of functional blood vessels requires successful occurrence of all subsequent steps of the angiogenesis process with complex interactions between ECs, pericytes or smooth muscle cells and stromal cells. Therefore, a CAM assay was performed to confirm the angiogenic effects of the CASCs *in vivo*. The CAM is a highly vascularized extraembryonic membrane which serves as a gas exchange surface of the chicken embryo. As the chick immune system is not fully established until later stages of development, the embryo serves as a naturally immunodeficient host capable of sustaining grafted mediators and cells without species-specific restrictions [266]. Moreover, the CAM can be easily manipulated and observed, making it an ideal model to study angiogenesis *in vivo* [267]. Both CASCs and CASC CM promoted angiogenesis, as observed by extensive radial ingrowth of blood vessels. Although CASC CM was concentrated 10X, similar effects were observed for CASCs and CASC CM. This can be explained by the

continuous production of angiogenic growth factors by CASCs, while CM or single protein administrations require higher doses because of a short protein half-life [268]. Similar results were observed in CAM assays with DSCs [244, 262] BM stromal cells [265] and placental MSCs [269].

In conclusion, CASCs, isolated from atrial appendages of MI patients, stimulate angiogenesis *in vitro* and *in vivo*. In accordance with the rare EC differentiation of CASCs *in vitro* and *in vivo*, this effect was mediated by paracrine mechanisms and not by direct vascular differentiation. Indeed, CASCs secrete numerous growth factors that promote important steps of blood vessel formation. Together with their strong cardiomyogenic differentiation potential, these newly identified angiogenic properties distinguish CASCs from other stem cells types. This unique therapeutic combination makes CASCs a promising candidate for the treatment of ischemic heart disease.

3.3 Immune properties of CASCs

3.3.1 Introduction

Myocardial ischemia leads to a substantial loss of cardiac tissue. Although restoration of blood flow to the ischemic area is essential to preserve cardiac function, it also leads to the formation of reactive oxygen species, causing more cell death [270, 271]. An inflammatory reaction is initiated with activation of local immune cells, such as macrophages, together with infiltration of neutrophils, monocytes and lymphocytes from the peripheral blood. Infiltrated immune cells clear the infarct area from dead cells and debris, which activates reparative processes [8]. However, extensive or long-term inflammation further damages the surviving cardiomyocytes and has a negative impact on cardiac homeostasis and ventricular remodeling [255]. Indeed, the extent and duration of the inflammation are closely linked to post-infarct remodeling and cardiac dysfunction. Excessive inflammation may lead to the formation of a scar with reduced tensile strength, activation of apoptotic pathways, infarct expansion and even cardiac rupture [255, 272-274]. Inflammatory cytokines also cause endothelial dysfunction, which further leads to disturbance of myocardial function [275].

Controlling the inflammatory reaction after MI is thus necessary for the formation of a supportive scar in the infarct area and restoration of cardiac homeostasis. Although animal models and clinical trials with anti-inflammatory drug treatment have shown a restriction of immune cell infiltration in the ischemic heart, no improvement in clinical outcome was found. Early inhibition of the immune reaction even seems to increase the inflammatory process because of reduced wound clearance and inhibition of inflammation-related

reparative pathways [276-278]. Selective targeting of the inflammatory response instead of global suppression thus seems to be a more suited approach.

MSCs are known to home to sites of injury [279, 280] and have strong immunomodulatory properties [281], making them an interesting candidate to target inflammation after MI. They are immune-privileged as shown by a lack of allogeneic T-cell activation *in vitro*, which can be explained by intermediate MHC I molecule expression and lack of MHC II and costimulatory molecules CD80 and CD86 [282, 283]. As a consequence the host immune system will not attack MSCs. Immunomodulatory properties have been described for MSCs by direct cell contact with immune cells and via the secretion of a wide variety of growth factors, cytokines and microvesicles [284]. Because of these properties, MSCs are able to steer the immune reaction towards an anti-inflammatory response in a more gentle and controlled manner [272, 285]. A low immunogenic profile also allows allogeneic stem cell transplantation, which would allow development of an 'off-the-shelf' cell-based therapy. This would resolve the time needed for patient-specific tissue harvesting, cell processing and reduce possible variations in donor cell potency. MSC transplantation has been shown to improve cardiac function after MI by modulating the inflammatory response leading to a cardioprotective effect [281, 286, 287]. Unfortunately, MSC transplantation does not induce clinically relevant therapeutic effects after MI [126, 136], because of their limited cardiomyogenic differentiation [137]. Similar immunomodulatory effects have been shown for CDCs, which are thought to be predestined to form cardiac muscle. CDCs show a similar surface marker profile as MSCs (MHC I+, MHC II-, CD80-, CD86-) and they inhibit the proliferation of alloreactive T-cells. Indeed,

cardiospheres express factors known to dampen local immune reactions such as interleukins 6, 7 and 8, transforming growth factor-β, macrophage colony-stimulating factor and VEGF [288]. Allogeneic cardiospheres have been shown to diminish ventricular remodeling and reduce infarct scar in a rat MI model [289]. CDC transplantation in the CADUCEUS trial revealed a reduction in scar tissue and an increase in regional contractility, but no significant improvement in global LV function was found [159]. The cardiomyogenic differentiation potential of CDCs also appears to be restricted [157, 200], explaining their limited functional improvement after MI.

The recently described CASCs [154] are able to preserve cardiac function in a Göttingen minipig infarction model based on extensive cardiomyogenic differentiation [154, 206]. Like MSCs, CASCs lack expression of MHC II and it was shown in chapter 3.2 that they secrete IL-8 and VEGF, suggesting that CASCs could have additional positive effects by reducing inflammation and preventing deleterious cardiac remodeling and scar formation in addition to their cardiomyogenic differentiation and angiogenic potential. This study aims to investigate the immunogenic profile of CASCs together with possible immunomodulatory properties.

3.3.2 High concentrations of IFN-γ and TNF-α minimally reduce CASC viability

The effect of inflammatory conditions on CASC viability was explored by incubating them with IFN-γ and TNF-α *in vitro* (Fig 31). Even high concentrations of IFN-γ or TNF-α alone were not able to decrease CASCs

viability. Combined IFN-γ and TNF-α did significantly reduce CASCs survival at a concentration of 10 and 100 ng/ml compared to the Contr ($p<0.0001$), while this decrease was not observed at a concentration of 1 ng/ml. The effect of combined IFN-γ and TNF-α treatment on CASC survival seemed to be dose-dependent with a significant difference between 1 and 10 ng/ml ($p=0.0007$).

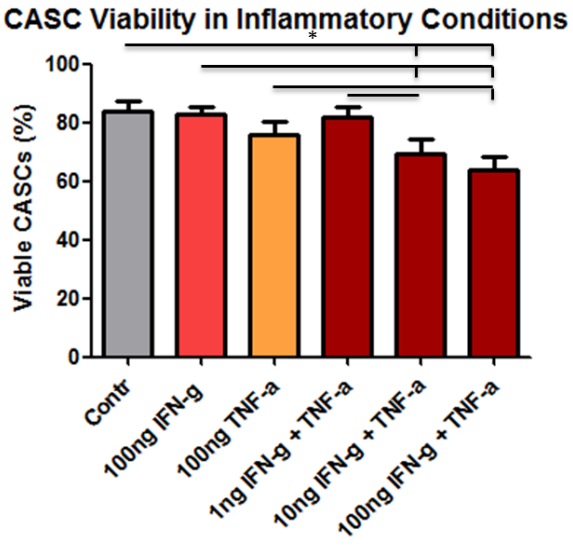


Figure 31: Inflammatory cytokines minimally reduce CASC viability at high concentrations.
CASC viability was assessed after a 72h exposure to IFN-γ and TNF-α. Only combined treatment with IFN-γ and TNF-α significantly reduced CASC survival compared to the Contr at a concentration of 10 and 100 ng/ml. Viability was significantly lower for combined IFN-γ and TNF-α compared to either cytokine alone. A dose-response effect of combined IFN-γ and TNF-α on CASC viability was observed. Contr, IFN-γ, TNF-α n=8, IFN-γ and TNF-α n=6. Data are expressed as mean ± SEM. * p-value <0.0033. IFN-γ interferon γ, TNF-α tumor necrosis factor α.

3.3.3 Licensing with inflammatory cytokines changes immune-related marker expression of CASC

To gain insights in the immune properties of CASCs, expression of important immune-related surface markers was investigated. As inflammatory conditions are known to influence the immune marker profile of stem cells, the marker profile of CASCs was analyzed in both control conditions and after inflammatory cytokine treatment for 24h and 72h (Fig 32). In control culture conditions, CASCs were negative for CD80 and MHCII, a small percentage was positive for CD86 and the majority of the population was positive for MHCI. No significant changes in marker expression were observed for CD80 after cytokine treatment (Fig 32A). IFN-γ alone or combined with TNF-α significantly reduced CD86 expression compared to the control condition after 72h (Fig 32B; IFN-γ $p=0.0003$; IFN-γ and TNF-α $p=0.0011$ respectively). However, a trend towards an increased CD86 expression was also seen for the control conditions at 72h compared to 24h. MHCI expression was significantly higher after exposure to pro-inflammatory cytokines for 72h compared to the control (Fig 32C; IFN-γ and IFN-γ+TNF-α $p<0.0001$, TNF-α $p=0.0002$). However, MHCI decreased in the control condition over time ($p<0.0001$).

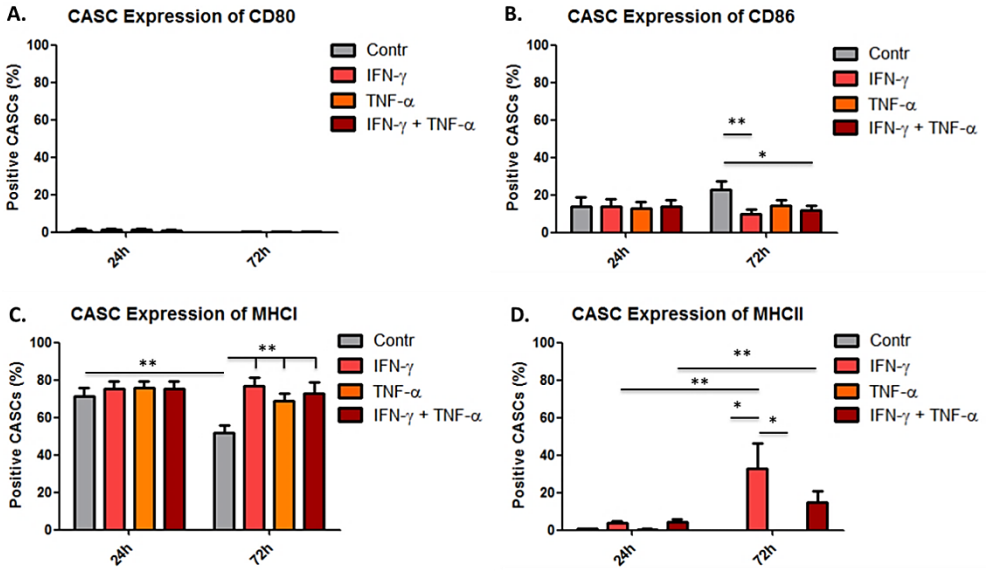


Figure 32: CASCs show a low immunogenic marker profile.
CASCs marker expression was investigated in control conditions or after IFN-γ and TNF-α treatment for 24h and 72h. CASCs are negative for CD80 (A). A minority of the cells is positive for CD86 and a majority is positive for MHC I in control and inflammatory conditions (B,C). No expression is observed for MHC II in control conditions, but an upregulation of this marker is observed after 72h of IFN-γ exposure (D). Contr, IFN-γ, TNF-α and IFN-γ+TNF-α n=6. Data are expressed as mean ± SEM. * $p < 0.0083$, ** $p < 0.00017$. IFN-γ interferon γ, MHC major histocompatibility complex, TNF-α tumor necrosis factor α.

IFN-γ alone or in combination with TNF-α increased MHCII expression compared to the associated condition at 24h (Fig 32D; $p<0.0001$ and $p=0.0067$ respectively). After 72h, MHCII was significantly increased for IFN-γ treatment compared to the control condition ($p=0.0012$) and TNF-α ($p=0.0012$).

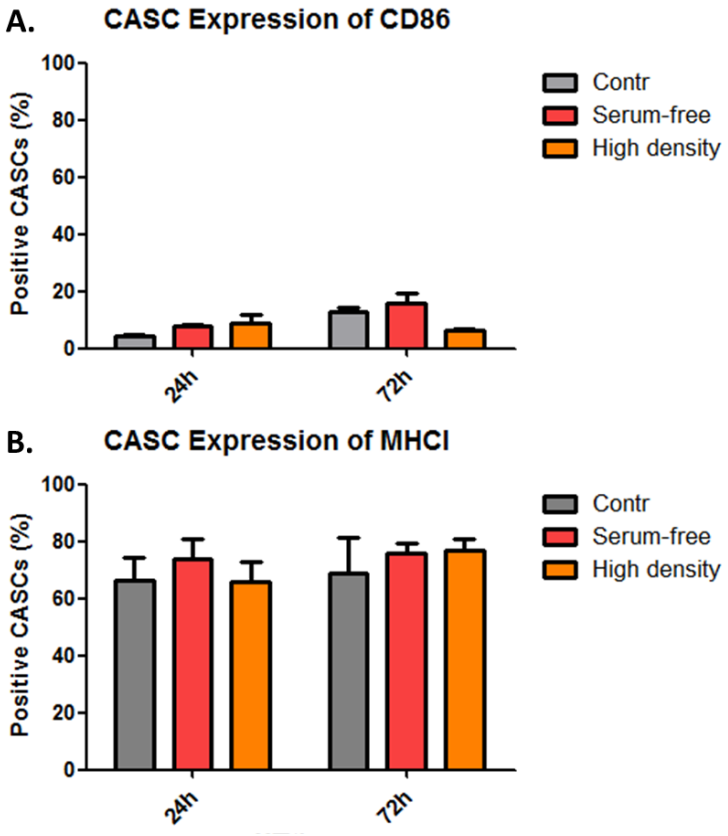


Figure 33: Serum or cell density do not affect CASC CD86 or MHC I expression.
CASCs marker expression was investigated in control conditions, in serum-free medium or at a high cell density for 24h and 72h. A minority of the cells is positive for CD86 (A), while most cells are positive for MHC I (B). No clear changes in expression could be induced by the different conditions. n=2. Data are expressed as mean ± SEM. MHC major histocompatibility complex.

Expression of MHCI and CD86 under control conditions was not stable, indicating that no conclusions can be made for these markers under control or inflammatory conditions. As culture conditions can influence surface marker expression, the effect of serum-free medium or a high cell density on CASC

CD86 and MHCI expression was investigated (Fig 33). However, no clear changes in CD86 or MHCI expression by CASCs could be observed after 24h or 27h in serum-free medium or after culture at a high cell density.

3.3.4 CASC show low immunogenic and immunomodulatory properties *in vitro*

The effect of CASCs on immune cell activation was examined to assess a possible low immunogenic profile for CASCs. Allogeneic assays were set up by co-culturing CASCs with peripheral blood mononuclear cells (PBMCs) at various ratios (Figure 34A). A significant increase in PBMC activation was observed for the positive control of PBMCs mixed from 2 different donors for CD4 T-cells ($p=0.022$) and CD8 T-cells ($p=0.019$). In general CASCs did not activate CD4 or CD8 T-cells, although a small but statistically significant increase was observed in CD4 activation for PBMCs in monoculture compared to the CASC-PBMC co-cultures at a ratio of 1:5 ($p=0.016$).

Immunomodulatory properties were shown for CASCs in suppression assays with anti-CD3 activated PBMCs (Fig 34B). A significant increase in CD4 and CD8 T-cell activation was observed after anti-CD3 stimulation ($p=0.016$ and 0.007 respectively). In co-culture with CASCs, CD3 stimulation could not increase CD4 T-cell activation compared to the PBMC control ($p=0.159$ and 0.166 respectively). The decrease in CD3 PBMC activation in CASC co-cultures was not significant hence more repeats are necessary.

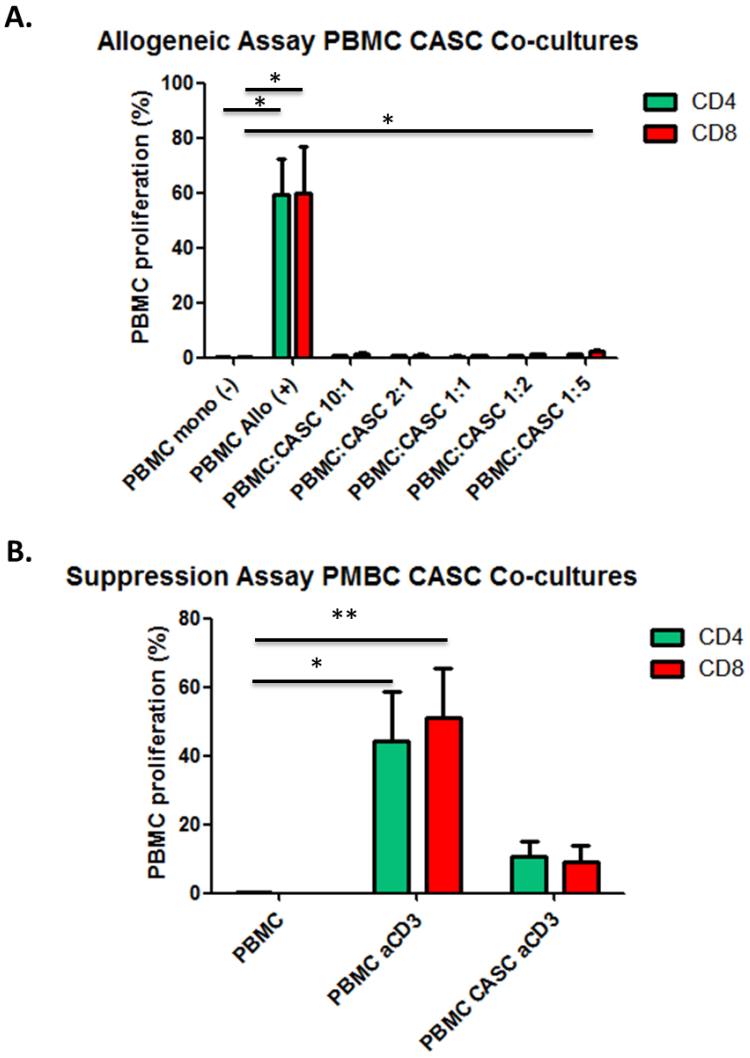


Figure 34: CASCs show low immunogenic and immunomodulatory properties *in vitro.*
(A) Co-cultures of CFSE-labeled PBMCs and CASCs at different ratios. CASCs did not elicit an allogeneic proliferative reaction of CD4 or CD8 T-cells, except for CD4 T-cells in monoculture compared to the CASC PBMC co-culture at a ratio of 1:5. Mixed PBMC cultures of different donors were included as positive control. (B) Co-cultures of activated PBMCs and CASCs (ratio 1:1). CASCs reduce CD4 and CD8 T-cell proliferation. Allogeneic assay: PBMC & PBMC:CASC n=6, PBMC Mixed n=2. Suppression assay: PBMC & aCD3 n=3, PBMC CASC aCD3 n=7. * $p<0.05$, ** $p<0.01$.

However, because of problems with the PBMC activation, the assay could not be repeated after these preliminary experiments. Combinations of CD3, CD28 and TNF-α (Fig 35B,C) were not able to induce a strong activation of CD4 and CD8 T-cells compared to unstimulated PBMCs (Fig 35A), with almost no activation of CD8 T-cells. A mixed PBMC culture of immune cells from two different donors was included as positive control and shows a strong CD4 and CD8 T-cell activation of about 50% (Fig 35D).

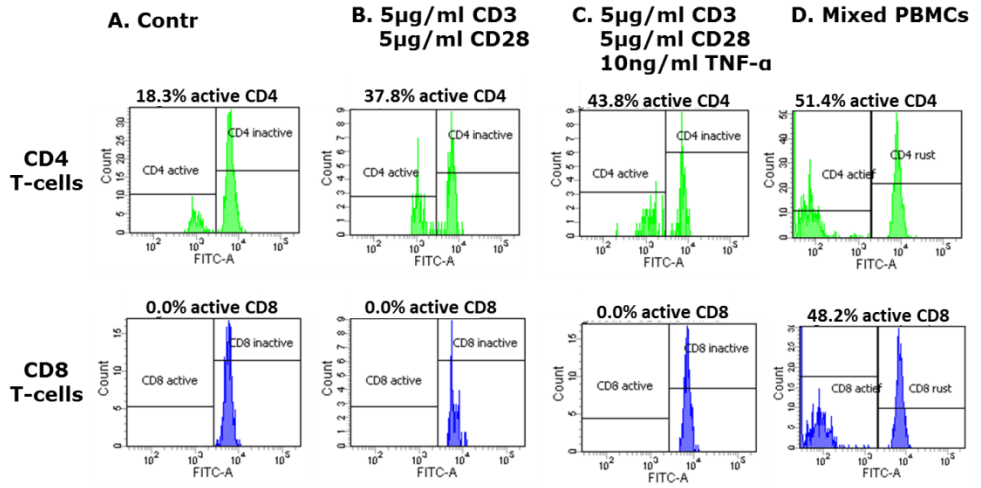


Figure 35: PBMC stimulation does not lead to T-cell activation *in vitro.* PBMCs from healthy donors were cultured with or without the presence of stimulatory antibodies and cytokines for 72h. Combinations of CD3, CD28 and TNF-α (B,C) were not able to induce substantial activation of CD4 and CD8 T-cells compared to unstimulated PBMCs (A). A mixed PBMC culture of two different donors was included as positive control and shows a substantial T-cell activation (D). PBMC peripheral blood mononuclear cell. TNF-α tumor necrosis factor α.

3.4.5 Discussion

Stem cells transplanted after MI end up in the infarct area, characterized by ischemia and inflammation. This hostile environment has a negative effect on stem cell survival and differentiation. Nevertheless, CASC transplanted in the acute MI phase in a minipig model were able to preserve LV function, based on extensive engraftment and cardiomyogenic differentiation [206]. Lack of MHC II expression and secretion of IL-8 and VEGF suggest that CASCs could have additional anti-inflammatory effects, which would shield them against the inflammatory infarct environment and would have additional cardioprotective effects. Therefore, the immune properties of CASCs together with possible immunomodulatory effects were investigated.

First, the effect of inflammatory conditions on CASC viability were investigated by incubating them with pro-inflammatory cytokines *in vitro*. IFN-γ or TNF-α alone did not affect CASCs viability. Combined IFN-γ and TNF-α did significantly reduce CASCs survival compared to control conditions, but only at high concentrations (10 and 100 ng/ml). A concentration of 1 ng/ml of IFN-γ and TNF-α no longer affected CASC viability, while the physiologically concentration of these cytokines immediately after MI or in chronic MI always remains in the pg/ml range [290-294]. These results are in accordance with the high cell retention levels after CASC transplantation in the acute infarct setting in the minipig MI model [206]. The high survival under inflammatory conditions might partly be explained by the detoxification role of the ALDH enzyme, which is known to promote the survival of progenitors in stress situations, such as inflammation or ischemia [198, 199]. Indeed, CASC seem to be more resilient against inflammatory conditions compared to other stem cell types. Liu et al., 2011 reported almost

complete cell death of BM MSCs after treatment with similar IFN-γ or TNF-α cytokine concentrations [295]. Human umbilical cord MSCs showed TNF-α induced apoptosis and this effect was amplified by IFN-γ, both at lower concentrations as described in our study [296]. On the other hand, survival of MAPCs remained unaltered after exposure to 100 ng/ml IFN-γ and TNF-α for one day, however, these cells were derived from rats [297]. TNF-α, but not IFN-γ has been shown to be toxic to neural stem cells [298]. Moreover, under inflammatory conditions neuronal stem cells failed to generate neurospheres, while under differentiating conditions IFN-γ enhanced neuronal and inhibited astrocyte differentiation. The influence of inflammatory cytokines on CASC proliferation and differentiation should also be investigated, as variable effects on MSCs have been shown [299, 300].

The immune phenotype of CASCs was also investigated in control and inflammatory conditions to gain insight in possible allogeneic transplantation possibilities. CASCs did not show the co-stimulatory molecule CD80 and had a low expression of CD86. The majority of the cells did express MHC class I antigens, which seemed to be upregulated after IFN-γ and TNF-α treatment. However, MHC I expression in the control condition did not remain stable over time. A decrease in MHC I was observed in the control condition at 72h compared to 24h, which could explain the higher expression levels of this marker after 72h of IFN-γ and TNF-α treatment. Cell density and serum components have been shown to influence MHC II expression [301]. In a limited set of experiments (n=2), however, we could not find a link between serum concentrations or cell density and MHC I expression for the CASCs. Variability in

marker expression suggests interpreting changes in immune marker levels after cytokine treatment with some caution. Nevertheless, MHC I expression makes CASCs less vulnerable for natural killer cell-mediated apoptosis, although other surface markers might cause natural killer cell activation, such as MHC class I-related chain A or nectin-2 [302]. MHC class II antigens were absent in control conditions, allowing CASCs to escape recognition from CD4+ T helper cells. Inflammatory cytokine treatment did induce an upregulation of MHC II, which together with the low expression of CD86 might induce T-cell activation. The expression profile of the CASCs confirms the general concept that stem cells express MHC I and can be induced to express MHC II when exposed to inflammatory cytokines or other microenvironment factors [303]. Indeed, a similar hypoimmunogenic profile has been demonstrated for MSCs [304], MAPCs [305] and CDCs [182]. In contrast to MSCs [95] and CDCs [182], CD86 is expressed on CASCs in both control and inflammatory conditions, albeit at low levels. This might lead to activation of the host immune response after allogeneic CASC transplantation. Still, an upregulation of MHC I and MHC II has also been shown after treatment with inflammatory cytokines for MSCs , CDCs and MAPCs, while these cells still show a low immunogenic profile and exert immunomodulatory effects. Licensing of MSCs and MAPCS with inflammatory cytokines has even been shown to potentiate their immunomodulatory properties [306, 307], despite the observed upregulation of MHC surface markers. Requirement of a licensing step suggests that MSCs actively and decisively respond to inflammatory signaling, allowing targeted amelioration of local immune reactions. It would be interesting to examine HLA-G expression by CASC, as this is an MHC-like protein known to

protect the fetal allograft against natural killer cell-mediated rejection [308] and plays a role in MSC-mediated immunomodulation [309]. Furthermore, it should be established if CASCs keep their hypoimmunogenic marker profile after cardiomyogenic differentiation, as MHC I is increased in ESCs after differentiation [310].

To functionally assess the immunogenic properties of CASCs, co-cultures were set up with PBMCs. CASCs did not elicit activation of CD4 or CD8 T-cells in allogeneic assays with unactivated PBMCs, confirming their *in vitro* hypoimmunogenicity, despite the observed expression of MHC I and CD86. Moreover, immunomodulatory effects were suggested by reduced activation of CD4 and CD8 T-cells in co-culture with anti-CD3 activated PBMCs. However, these preliminary results could not be confirmed because of suboptimal T-cell activation in a second batch of experiments. Activation of T-cells by mixed PBMC cultures of two different donors did yield the expected results, suggesting that the problem lies in the antibody activation step. Changes in the original PBMC activation protocol by immobilization of the anti-CD3 antibody and by additional anti-CD28 antibody or TNF-α supplementation could not increase T-cell activation to the original level and the protocol thus requires further optimization and validation. Additional supplementation of IL-1β [311], IL-2 [312] or the use of mixed lymphocyte reactions has also been performed, however, care should be taken not to overstimulate the cells as this causes apoptosis [313]. Nevertheless, non-immunogenic and immunomodulatory effects in co-culture with PBMCs have also been reported for MAPCs [305], MSCs [314] and CDCs [182].

Still, *in vivo* confirmation of anti-inflammatory effects for CASCs and the associated reduction in fibrosis and ventricular remodeling should be confirmed in a MI model. In a study by Malliaras et al., 2012 allogeneic CDC transplantation was shown to be safe and improved heart function in a rat MI model [182]. However, the beneficial effects of allogeneic CDCs were mainly mediated by stimulation of endogenous repair, while for CASCs functional integration and differentiation is the main repair mechanism. Few allogeneic CDCs could be detected after three weeks, however, successful homing of CASCs is important for their therapeutic effects, making allogeneic CASCs transplantation more challenging. In a swine model, allogeneic c-kit+ CSC administration after acute MI could ameliorate adverse remodeling, however, none of the injected cells could be detected ten weeks after administration, suggesting that again paracrine effects were responsible for the functional benefit. In addition, BM MNC transplantation has been shown to restore the inflammatory cytokine balance in MI patients [287]. However, no significant differences in LVEF were observed between the treated and the placebo group.

More experiments are required before allogeneic CASC transplantation can be implemented. The immunomodulatory properties of CASCs in co-culture with PBMCs should be confirmed. Mechanisms responsible for the immunomodulatory properties of CASCs should be identified. Nitric oxide is a mediator of MSC [315] and MAPC [297] immunomodulation. However, in a Griess reagent assay no NO secretion could be shown for control CASCs or CASCs treated with IFN-γ or TNF-α (all below detection limit), suggesting that this factor does not play a role in CASC-mediated immunomodulation. Confirmation of these negative results with

another method such as ELISA should be performed. Many other cytokines have been demonstrated to be responsible for stem cell-mediated anti-inflammatory effects, such as IL-4, IL-6, IL-8, IL-10, IL-13 and prostaglandin E2. Moreover, indoleamine 2,3-dioxygenase and HLA-G are known to be important mediators as well [302].

In conclusion, preliminary results suggest a low immunogenic profile and immunomodulatory properties for CASCs. As excessive inflammation contributes to infarct expansion and has a negative impact on cardiac homeostasis and ventricular remodeling [255], the possible anti-inflammatory effects of CASCs would be an interesting additional therapeutic mechanism. A low immunogenic profile opens the possibility for allogeneic CASCs transplantation, circumventing the problem of time needed for patient-specific tissue harvesting and cell expansion and eliminates possible variations in donor cell potency. If feasible, allogeneic CASC transplantation would increase the patient population that could be treated and allows the development of an 'off-the-shelf' cell therapy. The optimal timing for stem cell injection is said to be one week after MI [316], which is not feasible in autologous transplantation protocols. Allogeneic transplantation might therefore also increase the therapeutic efficacy of CASC administration. However, more research is necessary before allogeneic CASC transplantation can be considered. The low immunogenic profile of CASCs and the mechanisms of their possible immunomodulatory effects should be elucidated further. Moreover, a proof of concept study in a clinically relevant animal model should be performed as a final step before moving to the clinic.

4. General conclusions and future perspectives

Despite major improvements in medical care, IHD is still one of the major causes of global morbidity and mortality. Stem cells are promising candidates for the treatment of this condition as they should be able to replace the lost cardiac muscle with functional healthy tissue. Various stem cell types have shown potential for myocardial regeneration. However, the first clinical studies showed only minor improvements in cardiac function, which can be explained by the limited cardiomyogenic differentiation of most stem cells types. The recently described CASCs demonstrate exceptional cardiomyogenic differentiation properties, making them a promising candidate for myocardial regeneration. The goal of this study was to gain further insights in the therapeutic potential and safety of CASC transplantation after MI.

A proof of concept study was performed in a clinically relevant animal model to assure successful translation of preclinical results to the clinical situation. CASCs were obtained from atrial appendages of adult minipigs and expanded to clinically relevant cell numbers. MI was induced by ligation of the LAD and CASC transplantation was performed via transendocardial catheter-based injection in combination with EMM. This allows targeted cell delivery into the viable border zone of the infarct area, which has been shown to yield superior myocardial cell retention with a better clinical response. CASC transplantation resulted in an improvement of both regional and global LV function together with a reduction in scar mass. These functional benefits were associated with extensive cell engraftment and the formation of new, fully differentiated cardiomyocytes. SHG

microscopy demonstrated that CASC sarcomeres were identical to those of resident pig cardiomyocytes, indicating the development of a mature contractile profile. Moreover, CASCs showed electromechanical integration into the host myocardium and adaptation of a ventricular phenotype, despite the atrial origin of CASC. In accordance with these findings, no cardiac arrhythmias were observed during ECG monitoring. Functional integration and adaptation of a fully cardiomyogenic phenotype indicates that CASCs show remarkable cardiomyogenic regenerative properties. Although CASC transplantation significantly reduced the amount of scar tissue, substantial areas of dead tissue remain and regional contractility in apical segments of the LV largely did not benefit from CASC administration. Close contact between progenitor cells and viable cardiac tissue seems to be mandatory for adequate differentiation. In depth understanding of the molecular pathways involved in CASCs differentiation might allow steering of CASCs differentiation without the need of cell contact. Further investigation of functionality at cellular level would also be interesting. Electrophysiology and electrical field stimulation would be of great value to confirm the correct excitation contraction coupling in CASCs after transplantation. Moreover, the presence of t-tubuli, SERCA pumps and ryanodine receptors would give additional information on the cellular properties of CASC-derived cardiomyocytes, such as calcium handling. Although sarcomere properties of CASC were identical to those of resident cardiomyocytes, this does not give an indication on the development of hypertrophy in CASC cardiomyocytes, hence, comparison with healthy heart muscle cells would be of additional value to understand long term functional effects. Variable cell

numbers were transplanted in this study, as growth characteristics were diverse between the different pigs. This reflects the human conditions since variable cell amounts are also obtained after expansion of human CASCs. No relationship between cell dose and functional improvement could be demonstrated here as the number of animals was too small. Variability in expansion properties, however, needs further research as currently not all CASC cultures can be expanded to clinically relevant cell numbers. Insights in CASC biology together with optimization of the expansion and transplantation protocol will help to accomplish optimal myocardial regeneration by CASC. Although no tumors were observed in the minipig study, additional studies confirming the long-term safety of CASC transplantation are necessary. Possible tumorigenic effects of CASCs were investigated by subcutaneous injections of CASCs in immunocompromised mice. After 6 months, none of the CASC mice developed subcutaneous tumors. However, spontaneous lymphomas were observed in both CASC and untreated mice, suggesting that this condition is not related to CASC injection. This finding was confirmed by the occurrence of similar problem in the same mouse strain in other research groups. Indeed, the mouse strain used in this study is known to be vulnerable for spontaneous lymphoma formation and the experiments should therefore be repeated with another immunocompromised mouse or rat strain with inclusion of untreated and vehicle-treated mice. Long-term follow-up of minipigs after CASCs transplantation with pathological examination of all organs might also be of value in this respect. A Matrigel-collagen matrix was used as a vehicle to inject CASCs, which contains animal-derived components and is thus not suited for human applications. Suitable delivery vehicles are available for

stem cell transplantation in a clinical setting, but they should be validated to confirm CASC viability, engraftment and differentiation. With respect to the angiogenic properties of CASCs, injection was performed in a Matrigel-collagen construct. This vehicle contains growth factors that already induce blood vessel formation and might have biased the effects of CASCs on angiogenesis in the minipig study. Still, stimulatory effects of CASCs on blood vessel formation were shown in the CAM assay. Moreover, the Matrigel-collagen vehicle might be important for the survival and integration of the CASCs after transplantation. This should be taken into account when choosing a suitable matrix for clinical cell transplantation.

Although endothelial differentiation has been widely shown for other CSCs, this event for CASCs was very rare after transplantation in the minipig MI model and could not be induced *in vitro*. These results suggest that CASCs are already committed to the myogenic lineage, in accordance with their exceptional cardiomyogenic differentiation properties. One might argue concerning the terminology of CASC as a cardiac stem cell population in this respect, since the terminology of myogenic progenitor population might be more suited. It would be interesting to perform a lineage tracing study to investigate to which cell types the CASCs contribute during myocardial development. Finding a CASC specific marker for this purpose would be of great value. As the ALDH enzyme is expressed by multiple cell types including cardiomyocytes is does not seems suitable. However, multiple ALDH isozymes exist with various functions. Some of these isozymes are expressed in the nucleus and are important for cell proliferation. The combination of a specific CASC marker with early

cardiomyogenic and vascular markers might give an indication on the origin and identity of CASCs, as it is currently not known if they originate from the neural crest, the first or second heart field,.

A trend towards a higher vascularity in the infarct area was observed for CASC-treated animals compared to a control animal in the minipig MI model. Because of the limited endothelial differentiation capacities of CASCs, possible effects of CASCs on angiogenesis via paracrine mechanisms were therefore investigated. It was shown that CASCs secrete numerous angiogenic growth factors, which stimulate the most important steps of the angiogenesis process, being EC proliferation, migration and tube formation. The angiogenic potential of CASCs was confirmed *in vivo* in a chorioallantoic memebrane assay. Although the paracrine angiogenic properties of CASCs were proven by these findings, confirmation of CASC-induced neovascularization in a MI model should still be established. For example, by assessing perfusion in the infarct area by MRI in the minipig model. Still, these newly identified angiogenic properties strongly increase the therapeutic potential of CASCs as myogenesis and angiogenesis are two essential processes in myocardial repair. The combination of strong cardiomyogenic differentiation properties and paracrine stimulation of blood vessel formation by CASCs thus opens new perspectives for regeneration of the infarcted heart.

The inflammatory reaction after MI is yet another aspect that should be taken into account when developing an innovative cell therapy for myocardial regeneration. Extensive or long-term inflammation has a negative impact on cardiac homeostasis and ventricular remodeling and negatively influences stem

cell survival, integration and differentiation after transplantation. However, immunomodulatory properties are suggested for CASCs as they lack expression of MHC II and secrete IL-8 and VEGF. CASC viability was not affected by physiologically relevant concentrations of inflammatory cytokines. This is in accordance with the high cell retention of CASCs after transplantation in the acute setting of ischemia-reperfusion in the minipig MI model. CASCs show a low immunogenic profile in both non-inflammatory and inflammatory conditions and show signs of immunomodulation *in vitro*. These results indicate that allogeneic CASC transplantation might be possible, which would allow development of an 'off-the-shelf' cell-based therapy. This would eliminate the time required for patient-specific cell expansion and solves the problem of variations in donor cell potency. Immunomodulatory effects could have additional therapeutic effects by reducing inflammation and fibrosis after MI. However, these results are very preliminary and need further investigation. Understanding the mechanisms of possible immunomodulatory effects of CASCs can be performed by looking at secreted cytokines or receptor-mediated pathways. Confirmation of the feasibility and the therapeutic efficacy of allogeneic CASCs transplantation in a clinically relevant animal model would be of importance. Allogeneic use of CASCs would increase the patient population that could benefit from CASC transplantation. However, caution should be taken here as long-term cell retention is usually absent in the allogeneic setting, while this is essential for the therapeutic effect of CASCs.

In depth characterization of CASCs is essential for future clinical applications of CASCs. The origin of CASCs is still unknown with no knowledge on the

contribution of CASCs in heart development. In depth analysis of the gene expression profile of CASCs will definitely give more insights into CASC biology and their therapeutic mechanisms. The function of the ALDH enzyme in CASC is currently not identified, but a high activity of this enzyme seems to be essential for their expansion and is possibly implicated in other important aspects such as their survival in the ischemic infarct area after transplantation. Although the results of this thesis confirm the potential of CASC in myocardial regenerative medicine, efforts should be made to fully characterize and understand the therapeutic mechanisms behind CASC transplantation, before moving on from bench to bedside.

Many different stem cell subpopulations have been identified in the heart and all claim to be superior concerning their cardiac regenerative properties. For an organ that was long thought to be post-mitotic, the occurrence of a wide variety of different stem cell types seems very unlikely. Indeed, overlapping marker expression has been reported for most cardiac stem cell subtypes, suggesting that differences in phenotype or potential are induced by isolation and culture conditions or that they are part of one general stem cell population with slightly different maturation stages. Although strong cardiomyogenic differentiation properties were shown for CASCs in this study, defining them as superior compared to other CSC types would require direct comparison in an MI model. Positioning the various stem cell populations, including CASCs, against one another might provide in depth understanding of cardiac development and elucidate new mechanisms for the regeneration of the human heart.

This study provided proof of concept for the safety and therapeutic efficacy of CASCs transplantation after MI. However, the safety and long-term therapeutic effect of CASCs in a chronic MI model needs to be confirmed. Development of a humanized isolation and expansion protocol is another issue that needs to be addressed before the implementation of CASCs in clinical studies. The manufacturing process of CASCs should exclude all animal components or possible contaminating factors. Moreover, in depth characterization would be of great value to truly understand CASC biology. These additional steps are essential to guarantee the safety and quality of our stem cell product.

SAMENVATTING

Hartfalen ten gevolge van een hartinfarct is wereldwijd nog steeds één van de grootste oorzaken van morbiditeit en mortaliteit. De huidige behandelingen focussen vooral op het bestrijden van de symptomen. Ze kunnen de achteruitgang van de hartfunctie wel vertragen, maar ze zijn niet in staat het hart te herstellen. Stamcellen zijn veelbelovend om patiënten met een hartinfarct te genezen. Zij zouden de verloren hartspier namelijk kunnen vervangen door nieuw functioneel weefsel en zo het hart weer herstellen. De eerste klinische studies toonden echter weinig verbetering aan in de functie van het hart na stamceltransplantaties, hetgeen waarschijnlijk te wijten is aan de beperkte hartspiervormende eigenschappen van deze stamceltypes. Recent werd er een nieuw cardiaal stamceltype ontdekt, genaamd de cardiale atriumstamcel (CASC). Deze CASCs vertonen typische stamcelkenmerken en differentiëren uitzonderlijk goed in hartspiercellen. Bijgevolg onderzocht deze studie CASC-transplantatie als mogelijke therapie voor patiënten met een hartinfarct.

In het eerste deel van de studie werden CASC getransplanteerd in een klinisch relevant diermodel om de veiligheid en de therapeutische werking van de stamcelbehandeling na te gaan. CASCs werden geïsoleerd uit de hartoortjes van minivarkens en deze werden opgekweekt tot klinisch relevante aantallen. Een hartinfarct werd geïnduceerd bij de varkens door het afbinden van de linker kransslagader gedurende 2 uur. Meteen na de reperfusie werden CASCs toegediend via transendocardiale injecties in de infarctgrenszones op basis van

elektromechanische mapping. CASC-behandeling zorgde voor een verbeterde pompfunctie en een vermindering in de hoeveelheid littekenweefsel. De verbeterde hartfunctie ging gepaard met een hoge celretentie en een uitgesproken differentiatie van CASCs naar hartspiercellen. De sarcomeren van hartspiercellen afkomstig van CASC waren identiek aan sarcomeren van het resident hartspierweefsel, hetgeen wijst op de vorming van een volledig ontwikkeld contractieapparaat. Verder vertoonden CASCs tekenen van elektromechanische integratie en ontwikkelden ze een ventriculair fenotype, ondanks hun atriale afkomst. Continue elektrocardiogramopvolging toonde inderdaad aan dat er geen hartritmestoornissen opgewekt waren, hetgeen een belangrijk eerste bewijs is voor de veiligheid van CASC transplantatie na een hartinfarct. Een ander belangrijk veiligheidsrisico bij stamceltransplantaties is de vorming van tumoren. Om tumorvorming uit te sluiten, werden CASCs subcutaan geïnjecteerd bij immuungecomprimeerde muizen. Er werden geen subcutane tumoren teruggevonden na 6 maanden, maar spontane lymfomen waren echter wel aanwezig bij zowel CASC-geïnjecteerde dieren als onbehandelde muizen. Het optreden van spontane lymfomen is beschreven voor het gebruikte type muis, maar bevestiging van dit veiligheidsaspect in een andere muis- of ratsoort is nodig.

Aangezien de vorming van nieuwe bloedvaten van groot belang is voor het functioneel herstel van het hart na een hartinfarct, werden de bloedvatvormende eigenschappen van de CASCs bestudeerd in het tweede deel van dit project. Er werd aangetoond dat CASCs niet substantieel bijdragen aan de vorming van nieuwe bloedvaten door zelf te differentiëren naar bloedvatcellen, maar wel door

de secretie van belangrijke angiogene groeifactoren. Via paracriene mechanismen promoten CASCs de belangrijkste stappen van het angiogenese proces, namelijk endotheelceldeling, -migratie en -buisjesvorming. Deze *in vitro* resultaten werden ook bevestigd *in vivo* in kippenembryo's. Een trend tot een verhoogd aantal bloedvaten in de infarctgrenszones van CASC-behandelde minivarkens sluit aan met deze resultaten, maar verdere bevestiging in een myocardinfarctmodel dient nog uitgevoerd te worden. De stimulatie van bloedvatvorming samen met de uitzonderlijke hartspiervormende eigenschappen van CASCs maken hun ideaal voor het behandelen van ischemische hartziekten.

In het laatste deel van dit project werd het effect van inflammatie op de CASCs onderzocht. Aanhoudende inflammatie na een hartinfarct leidt niet alleen tot infarctexpansie en remodelleringsprocessen, maar heeft ook een negatieve impact de overleving en de differentiatie van getransplanteerde stamcellen. Inflammatoire condities hadden slechts in zeer beperkte mate een effect op de overleving van CASCs *in vitro*. Verder vertoonden CASCs eerder een laag immunogeen oppervlaktemarkerprofiel in controle en inflammatoire condities. Dit werd bevestigd door het gebrek aan immuuncelactivatie in co-cultuur experimenten. CASCs vertoonden zelfs eventuele immuunmodulerende effecten na co-cultuur met geactiveerde immuuncellen. Bijgevolg zouden CASCs dus niet alleen gunstige anti-inflammatoire eigenschappen hebben, maar deze bevindingen suggereren ook dat allogene CASC-transplantatie mogelijk zou zijn in de toekomst. De patiënt zou op deze manier niet langer afhankelijk zijn van de isolatie en opkweek van zijn eigen stamcellen, hetgeen toelaat om meer

patiënten te behandelen zonder verlies van tijd om voldoende autologe cellen te verkrijgen.

Deze studie bracht belangrijke nieuwe inzichten tot stand betreffende de CASC als celtherapie voor het genezen van een hartinfarct. De eerste bewijzen omtrent de veiligheid en de therapeutische efficiëntie van CASC-transplantatie na een hartinfarct werden aangetoond. CASCs hebben sterke hartspiervormende eigenschappen, bevorderen bloedvatvorming en oefenen mogelijk anti-inflammatoire effecten uit. De veiligheid en therapeutische voordelen van CASCs op lange termijn dienen echter nog aangetoond te worden. De celtherapie dient nog verder op punt gezet te worden om het productie- en transplantatieproces geschikt te maken voor toepassingen op patiënten. Deze laatste stappen zullen ervoor zorgen dat de kwaliteit en de veiligheid van CASC-transplantatie in toekomstige klinische toepassingen gegarandeerd kan worden.

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CURRICULUM VITAE

Skills

Competencies: flexibility, good clinical practice, perseverance, problem solving, project writing, standard operating procedures, teaching and student training, team player, time management and project planning, verbal and written communication skills, working conform quality control guidelines (ISO), will to learn

Laboratory techniques: cell culture, clean room basic knowledge, ELISA, flow cytometry, fluorescence activated cell sorting, hypoxia assays, immunofluorescence, *in vitro* models, lentivirus production, microscopy, quantitative polymerase chain reaction, working with small animal models (mice and rats), working with large animal models (minipigs: MRI, Noga mapping, anesthesia, basic surgery techniques), peripheral blood mononuclear cell isolation and immunomodulation assays, statistical analysis, stem cell isolation and differentiation, western blot

Working experience

PhD Hasselt University – Jessa Hospital **October 2012 - to be completed in September 2016**

The Study of the Myocardial Regenerative Capacities of Cardiac Atrial Appendage Stem Cells.
Jessa Hospital-Hasselt University, Experimental Hematology: Prof. dr. Karen Hensen, Prof. dr. Marc Hendrikx.

- Contribution of cardiac stem cells in myocardial angiogenesis
- Effects of the ischemic infarct area on cardiac stem cells after transplantation
- Stem cell transplantation in the Göttingen minipig infarction model
- Planning Advanced Therapeutic Medicinal Product CASC therapy for clinical trial phase I

Working experience during Internships

Exploring and comparing the angiogenic properties of different dental stem cell populations. Biomed, Functional morphology group: Petra Hilkens, Prof. dr. Ivo Lambrichts .
November 2011 -June 2012.

- Effects of isolation methods on dental stem cell properties
- Angiogenic properties of dental stem cell subtypes derived from the dental pulp, the dental follicle, the apical papilla and the periodontal ligament

Publications:

- P Hilkens, Y Fanton, W Martens, P Gervois, T Struys, C Politis, I Lambrichts, A Bronckaers. (2014) Pro-angiogenic impact of dental stem cells in vitro and in vivo. Stem Cell Research; 12(3):778-790.
- P Hilkens, P Gervois, Y Fanton, J Vanormelingen, W Martens, T Struys, C Politis, I Lambrichts, A Bronckaers. (2013). Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. Cell and Tissue Research;353(1):65-78.

Human umbilical cord matrix-derived stem cells as potential therapy for Multiple Sclerosis. Biomed, Stem cells-immunology group: Raf Donders, Prof. dr. Niels Hellings.
January 2011-April 2011.

- Potential of umbilical cord mesenchymal stem cells as treatment in multiple sclerosis
- Optimization of human and rat umbilical cord mesenchymal stem cell isolation and culture
- Clonogenicity and differentiation assays
- Assessment of immunogenicity and alloresponse of umbilical cord stem cells

Role of the glycine receptor in the proliferation and migration of cortical neurons in the embryo.
Biomed, Physiology group: Ariel Avila Macaya, Prof. dr. Jean-Michel Rigo.
April 2010-June 2010

- Effect of the glycine receptor on the proliferation and migration of cortical neurons
- *Ex vivo* slice cultures of embryonic mouse brains

Education

- **Business management**
  2015-2016
  Hasselt University – School of Expert Education
  Modules Strategy, Business HR, Marketing

- **Master Clinical and Molecular Sciences**
  2010-2012
  Hasselt University

  Additional courses during first Master year:

  - *Laboratory animal sciences:*
    Handling of laboratory animals in accordance with the Belgian and European legislation.
    FELASA C certificate: authorization independent animal research.
  - *Radiation protection:*
    Legislation and risks concerning radiation protection, implications in the medical sector. Radiation protection certificate: competence to work with responsibility in a radiological environment.
  - *Stem cell biology and clinical applications:*
    Basic principles of stem cell biology, treatment and valorisation possibilities.
  - *Electrophysiology and microfluorimetry:*
    Insight on ion channel properties and electrophysiological recordings, basic knowledge on fluorescence microscopy and laser scanning confocal microscopy with some important applications (FRET, FRAP, FLIM).

- **Bachelor Biomedical Sciences**
  2007-2010
  University Hasselt
  Obtained degree: *magna cum laude*

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Scientific publications

Windmolders S, Willems L, Daniëls A, Linsen L, Fanton Y, Hendrikx M, Koninckx R1, Rummens JL, Hensen K. Clinical-scale *in vitro* expansion preserves biological characteristics of cardiac atrial appendage stem cells. Cell Prolif. 2015; 48: 175–186.

Fanton Y, Robic B, Rummens JL, Daniels A, Windmolders S, Willems L, Jamaer L, Dubois J, Bijnens E, Heuts N, Notelaers K, Paesen R, Ameloot M, Mees U, Bito V, Declercq J, Hensen K, Koninckx R and Hendrikx M. Cardiac atrial appendage stem cells engraft and differentiate into cardiomyocytes in vivo: A new tool for cardiac repair after MI. International journal of cardiology. 2015;201:10-19.

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Hendrikx, M., Fanton, Y., Willems, L., Daniels, A., Declercq, J., Windmolens, S., Hensen, K., Koninckx, R., Jamaer, L., Dubois, J., Dilling-Boer, D., Vandekerkhof, J., Hendrikx, F., Bijnens, E., Heuts, N., Robic, B., Bito, V., Ameloot, M., Steels, P., Rummens, J. L. From bone marrow to cardiac atrial appendage stem cells for cardiac repair: a review. Current Medicinal Chemistry. 2016, 23

Published abstracts

Fanton Y, Robic B, Daniëls A, Windmolders S, Willems L, Jamaer L, Dubois J, Bijnens E, Heuts N, Notelaers K, Paesen R, Ameloot M, Mees U, Rummens JL, Hendrikx M, Hensen K, Koninckx R. Cardiac atrial appendage stem cells preserve cardiac function in a minipig acute myocardial infarction model. Circulation, 2013; 128:A13580

Scientific Posters

8 may 2014 "Knowledge for growth, Ghent, Belgium" Fanton Y, Houbrechts C, Willems L, Windmolders S, Koninckx R, Daniëls A, Rummens JL, Hendrikx M, Hensen K. Cardiac Atrial Appendage Stem Cells Promote Angiogenesis After Myocardial Infarction.

13 June 2014 "Recent Advances in Neuronal and Cardiac Tissue Engineering: From Lab to Clinic, Diepenbeek, Belgium" Fanton Y, Houbrechts C, Willems L, Windmolders S, Koninckx R, Daniëls A, Rummens JL, Hendrikx M, Hensen K. Cardiac Atrial Appendage Stem Cells Promote Angiogenesis After Myocardial Infarction.

20 April 2015 "Interuniversity Stem Cell Meeting, Leuven, Belgium" Fanton Y, Houbrechts C, Willems L, Windmolders S, Koninckx R, Daniëls A, Declercq J, Rummens JL, Hendrikx M, Hensen K. Cardial Atrial Appendage Stem Cells Promote Angiogenesis After Myocardial Infarction.

25 april 2016. "The Stem Cell Summit 2016 Conference, April 25-27, 2016 in Boston, MA." Fanton, Y, Daniëls, A, Robic, B, Rummens, J-L, Windmolders, S, Willems, L, Jamaer, L, Dubois, J, Bijnens, E, Heuts, N, Notelaers, K, Paesen, R, Ameloot, M, Mees, U, Bito, V, Declercq, J, Hensen, K, Koninckx, R, Hendrikx, M Cardiac Atrial Appendage Stem Cells Engraft and Differentiate into Cardiomyocytes in Vivo: a New Tool for Cardiac Repair after MI.

Oral Presentations

Yanick Fanton. Cardial stamcellen voor hartspierregeneratie: een 'bypass' voor de progressie naar hartfalen. PhD Symposium: Medisch-wetenschappelijk onderzoek in de Limburgse ziekenhuizen: een blik op de toekomst, Hasselt, Belgium, 9 november 2013.

Yanick Fanton. Cardiac Atrial Appendage Stem Cells as a Promising Candidate for Myocardial Regeneration: 'Bypass' the Progression Towards Heart Failure. Masterclass Franqui Leerstoel, Hasselt, Belgium, 20 juni 2014.

Yanick Fanton. Cardial Atrial Appendage Stem Cells Promote Angiogenesis After Myocardial Infarction. Recent Advances in Neuronal and Cardiac Tissue Engineering: From Lab to Clinic. Diepenbeek, Belgium , 13 juni 2014.

Yanick Fanton. Cardial Atrial Appendage Stem Cells Promote Angiogenesis After Myocardial Infarction. Interuniversity Stem Cell Meeting. Leuven, Belgium, 20 april 2015.

Yanick Fanton. Cardiac Atrial Appendage Stem Cells Engraft and Differentiate into Cardiomyocytes in Vivo: a New Tool for Cardiac Repair after MI. The Stem Cell Summit 2016 Conference, April 25-27, 2016 in Boston, MA. 25 april 2016.

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