Table of contents

List of Figures

List of tables

List of abbreviations

5-aza: 5-azacytidin

ABCG-2: ATP-binding cassette superfamily of membrane transporters

Ac-DiI-LDL: acetylated-DiI-LDL

ALDH: aldehyde dehydrogenase

AMI: acute myocardial infarction

APC: allophycocyanine

APC-Cy7: Allophycocyanin-Cy7

AS: autologous serum

AT: annealings temperature

bFGF: basic fibroblast growth factor

BFU-E: burst-forming unit, erythrocyte

CABG: coronary artery bypass graft

CASC: cardiac atrial appendage stem cell

CDB: cell dissociation buffer

CDC: cardiosphere derived cell

CFU-GEMM: colony-forming unit, granulocyte-erythrocyte-monocyte-megakaryocyte

CFU-GM: colony-forming unit, granulocyte-macrophage/monocyte

CGM: cardiosphere growth medium

CHD: Coronary heart disease

CM: cardiomyocyte

CMFDA: 5-chloromethyl diacetate

CMTMR:5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine

CSC: cardiac stem cell

cTnI: cardiac Troponin I

cTnT: cardiac troponin T

Cx43: connexin 43

DAPI: 4,6-diamino-2-phenylindole

DEAB: diethylamino benzaldehyde

DEPC : diethyl pyrocarbonate

DMSO: dimethylsulfoxide

DPBS: Dulbecco's phosphate buffered solultion

EF: ejection fractiion

EGF: epidermal growth factor

EPC: endothelial progenitor cell

FABP-4: fatty acid binding protein-4

FCS: fetal calf serum

FITC: fluorescin isothiocyanate

FSC: forward scatter

GFP: green fluorescent protein

hCM: human cardiomyocyte

HNA: human nuclear antigen

HSC: hematopoietic stem cell

I_{Ca}: Inward calcium current

I_{Na}: Inward sodium current

$I_{to,f}$: Fast transient outward current

Isl-1: Lim-homeo-domain transcription factor islet 1

LDL: low-density lipoprotein

Lin: lineage commitment marker

LVEF: left ventricular ejection fraction

MHC: myosin heavy chain

MI: myocardial infarction

MNC: mononuclear cell

MSC: mesenchymal stem cell

NRCM: neonatal rat cardiomyocyte

PCI : percutaneous coronary intervention

PDGFRbeta: platelet derived growth factor, beta polypeptide

PE: phycoerythrin

PE-Cy7: phycoerythrin-Cy7

Pen-strep: penicillin-streptomycin

Percp-Cy5: peridinyl chlorofylline-Cy5

PETt : positron emission tomography

rNkx2.5: rat Nkx2.5

Sca-1: stem cell antigen-1

SP: side population

SSC: side scatter

TEM: transmission electron microscopy

TGF-β: transforming growth factor-β

TnI: troponin I

TnT: troponin T

VEGFR-2: vascular endothelial growth factor receptor-2

vWF: von Willebrand factor

1. Introduction

1.1 Myocardial infarction: epidemiology and treatments

In Belgium, the incidence of heart failure is 0.1-0.5% each year with a prevalence of 200.000 (IHE; Instituut voor Hygiëne en Epidemiologie). In 2007 approximately 6.000 people died as a consequence of coronary heart disease (CHD) or myocardial infarction (MI). CHD is the most important cause of death in the Western world with an annual mortality of 50% for class IV patients (Muirhead et al., 1992; Lloyd-Jones et al., 2009). Worldwide around 7.5 million people died from CHD in 2005, accounting for 30% of all deaths. Several pharmacological drugs, e.g. digoxin, diuretics, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, β-blockers, and surgical therapies, e.g. coronary artery bypass graft (CABG), ventricular restoration procedures or implantation of a ventricular assist device were developed over the years. These therapies and treatments moderately improved the quality of life and increased life expectancy, but are no cure for heart failure. Heart transplantation restores the patient's heart function but several adverse events can occur due to the transplant or immunosuppressive treatment necessary to prevent rejection of the donor heart e.g. failure of the donor heart, complications from medicines, infections and even cancer.

MI and cardiomyopathy are characterized by a considerable loss of cardiomyocytes (CMs) due to severe apoptosis and necrosis. As a consequence, the infarcted area is infiltrated by fibroblasts, resulting in the formation of non-contractile scar tissue. In addition, since adult CMs only display a very low mitotic index, the surviving CMs compensate for this loss of function by hypertrophy which will eventually lead to the development of heart failure (Kajstura et al, 1998).

1.2 The heart, a dynamic organ with an intrinsic regeneration capacity

Traditionally mammalian CMs are considered to withdraw from the cell cycle after birth, hereby maintaining the dogma of the heart being a post-mitotic, terminally differentiated and static organ. As a consequence, the number of CMs is established at the time of birth and will not be replaced during a life-time. This implies that the heart is incapable of repairing any form of damage. Indeed, the heart responds to damage induced by a MI by remodeling the ventricle. Ventricular remodeling is characterized by the expansion of the infarct zone in the first 72h. Hereafter the entire ventricle remodels and dilates in a time-dependent manner hereby changing the ventricular shape. Due to this morphological modification, the viable CMs hypertrophy with an increase to 70% in cell volume (Anversa et al., 1985; Nadal-Ginard et al., 2003). However, in 1998 Kajstura et al. described the presence of proliferating CMs in healthy and diseased hearts (Kajstura et al., 1998). They showed that in ischemic or dilated cardiomyopathy an average of 140 proliferating myocytes per million cells could be detected versus only 14 per million in normal ventricles. In 2001 Beltrami et al. measured a mitotic index of 0.015% in explanted hearts from patients in end-stage heart failure. In patients who died within days after infarction, a subpopulation of CMs had reentered the cell cycle and underwent nuclear mitotic division. This mitotic activity of CMs has recently also been reported by Bergmann et al. (Bergmann et al., 2009). Not only was the existence of a subpopulation of highly proliferative CMs confirmed, they also showed a decline of turnover rate with age. Besides proliferating CMs, several immature cell types have been documented to reside in the heart. These cardiac stem cells (CSCs) are clonogenic, self-renewing and able to differentiate into mature cell types (Beltrami et al., 2003; Oh et al., 2003; Messina et al., 2004), suggesting that,

as a reaction to stress, the heart is capable of regeneration in order to replace malfunctioning CMs (Anversa et al., 2006).

As a consequence of a MI the endogenous repair mechanism is overwhelmed and the regeneration capacity of the heart is unable to restore heart function since not only CMs but also the residing CSCs are lost. Although CSCs residing in the non infarcted areas of the heart are not able to spontaneously migrate into the infarcted area, a number of differentiating cells could be detected in the border zone after infarction (Urbanek et al., 2005). The heart clearly possesses some intrinsic regeneration capacity, however these processes fall short to restore cardiac function. Hence a promising approach to repair the injured heart could be the transplantation of stem cells.

1.3 Scar replacement by the transplantation of stem cells

Several types of stem cells, e.g. skeletal myoblasts and bone marrow-derived stem cells, have already been used in clinical trials.

1.3.1 Skeletal myoblasts

Skeletal myoblasts are progenitor cells present in striated muscles in the body. Since these cells are resistant against ischemia and are already committed towards the myogenic lineage they were thought to be suitable candidates for cardiac regeneration.

Recently the results of the first phase II study have been published by Menasché et al. (Menasché et al., 2008). The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial involved 30 centra where 300 patients would be treated. However, only 97 patients were enrolled. The data showed no significant improvement when comparing the transplant group with the placebo.

Furthermore, a higher number of arrhythmic events in the myoblast-treated group was reported. This recurrent event of ventricular arrhythmia occurring after myoblasts transplantation could be explained by the fact that these cells are not able to electro-mechanically couple with the surrounding CMs. Reinecke et al. reported no expression of connexin 43 (Cx43) in transplanted myoblasts in rats. Therefore the differentiated myoblasts do generate action potentials on their own but do not synchronize with the surrounding CMs (Reinecke et al., 2000). Furthermore, there is a clear difference in an action potential of skeletal muscle cells and CM. The action potential of a myocyte is very short (5ms) while the action potential of a CM is much longer (350ms). As a consequence, the refractory period, the time necessary for an excitable membrane to be ready for a second stimulus, is much shorter in skeletal myocytes compared to CM (Katz, 2006). This difference in refractory period between these cells can give rise to arrhythmias.

The minimal functional improvement, not persisting in time, and the recurrent events of ventricular tachycardia make the future of this cell type as a therapy for the treatment of heart failure very uncertain.

1.3.2 Bone marrow-derived stem cells

The bone marrow is one of the richest stem cell sources in the body. It has been reported that bone marrow-derived cells are able to differentiate into adult CMs (Hruban et al., 1993; Murry et al., 2002; Deb et al., 2003). Furthermore, bone marrow-derived mononuclear cells (MNCs) are easily accessible and, like skeletal myoblasts, can be transplanted in an autologous manner.

1.3.2.1 Transplantation of MNC after an acute MI

The first study about transplanting the MNC fraction after an acute MI was published by Strauer et al. (Strauer et al., 2002). Ten patients received standard therapy whereas 10 others underwent intracoronary cell transplantation. After three months no significant increase of ejection fraction (EF) was reported in the treatment group as well as in the control, 5% and 4% respectively. However, the wall movement velocity increased with 2cm/s in the transplant group and only with 0.5cm/s in the control. Furthermore, the infarct size decreased significantly in the transplant group (18%) but only slightly in the standard therapy group (5%). In the same year, Assmus et al. published their first findings of the Transplantation Of Progenitor Cells And Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) trial (Assmus et al., 2002). Two years later these researchers expanded their patient group and reported the 1 year follow-up results (Schächinger et al., 2004). A total of 59 patients was studied, of which 30 received circulating blood progenitor cells and 29 patients received bone marrow cells. In both groups left ventricular EF (LVEF) increased with 8% at 4 months and 9.3% at 12 months. These trials showed that the intracoronary injection of bone marrow-derived MNCs is safe and can improve cardiac function. However, larger trials with longer follow-up were needed. In 2004 Wollert et al. published their results of the intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomized controlled clinical trial (Wollert et al., 2004). Sixty patients were, after a percutaneous coronary intervention (PCI), randomized to either a control group receiving optimal drug treatment or a transplant group receiving transfer of autologous bone marrow cells on top of the optimal drug treatment. After 6 months LVEF increased 6% more in the bone marrow treatment group than in

the control. However, after 18 months the difference in increase of LVEF between the transplant group (5.9%) and the control group (3.1%) was no longer statistically significant (Schaefer et al., 2006). After 5 years, LVEF decreased in the control as well as in the treated group, leading to the conclusion that in this study, a single administration of bone marrow cells did not preserve long-term benefits (Meyer et al., 2009). The ASTAMI (Autologous Stem cell Transplantation in Acute Myocardial Infarction) trial included 50 patients in the treatment group and 50 patients in the control group (PCI + optimal medical treatment). No significant differences in LVEF were observed, nor at short term (Lunde et al., 2005), nor at longer (3 years) follow-up (Beitnes et al., 2009). Janssens et al. also reported these findings in 2006. Within 24h after PCI, patients were either assigned to a control group (infusion of placebo; n=34) or a transplant group (infusion of MNCs; n=33). After 4 months, global LVEF was not significant different between the two groups. However, in the treatment group infarct size decreased with 28% in comparison to the control. The researchers concluded that the transplantation of MNCs within 24h after PCI does not improve global LVEF but could have a favorable effect on heart remodeling (Janssens et al., 2006). The largest trial performed to date, treating acute MI (AMI) with the intracoronary infusion of bone marrow cells is the REPAIR-AMI trial (Reinfusion of Enriched Progenitor Cells And Infarct Remodeling in Acute Myocardial Infarction; Schächinger et al., 2006). This multi-center trial enrolled 204 patients 103 of which were assigned to the control group (placebo medium), and 101 to the treatment (MNCs) group. After 4 months LVEF increased in the treatment group as well as in controls, 5.5% and 3% respectively (p=0.02). This significant difference reported by these authors is probably due to the large number of patients recruited in this trial

since the overall difference in LVEF between the transplant and the control group is not different from earlier reported trials. The researchers concluded that the administration of bone marrow cells slightly enhances left ventricular contractile recovery.

Besides these 4 landmark trials, more than 20 smaller trials have been performed. Several meta-analysis have been performed. The most comprehensive of which comprises 811 patients (Martin-Rendon et al., 2008). This study revealed that stem cell therapy improved left ventricular function by 2.99% and a decrease in infarct size by 3.51% in comparison to controls.

So far, all clinical trials reported no or only a limited improvement in cardiac function. A possible reason for this marginal effect could be the time of the injection. At the onset of a cardiac ischemic insult, aerobic glycolysis stops and CMs reduce their contractility (Kumar et al., 2005). When the ischemia persists for more than 20 minutes, cellular damage occurs and cells will become apoptotic or necrotic (Trump et al., 1998). This cellular apoptosis attracts macrophages and neutrophils to the infarcted region. The necrotic tissue is removed and scar tissue is formed by the infiltration of non-contractile fibroblasts. This process of wound healing takes several weeks. All clinical trials, described above, transplanted bone marrow cells within 1 week after the MI. The huge amount of inflammatory cells, present in the infarcted region, could have an unfavorable effect on the cardiomyogenic differentiation of the transplanted cells. Therefore, one approach to augment the effect of stem cell injection could be to transplant the cells after the inflammatory response has subsided.

1.3.2.2 Transplantation of MNC in chronic heart failure

Six weeks after the onset of an infarction wound healing is completed by fibrosis and the inflammatory process is reduced. The cells now present in the scar tissue or the peri-infarct region will therefore secrete different cytokines, perhaps favoring stem cell differentiation down the cardiomyogenic lineage. To test this hypothesis, several clinical trials have been performed to assess the cardiac improvement potential of bone marrow stem cells in chronic infarcted hearts.

In 2006 Assmus et al. performed a controlled crossover study which enrolled 92 patients with a MI older than 3 months (Assmus et al., 2006). Of these, 35 patients received bone marrow-derived cells, 34 were administrated circulating blood progenitors and 23 received no intracoronary injection (control). After 3 months the transplantation of bone marrow-derived cells induced an increase in LVEF (from 41% to 43%) when compared to patients who received circulating blood progenitors (no change 39%) or the control group (from 43% to 42%). Hendrikx et al. carried out a randomized controlled clinical trial in which 20 patients with a chronic MI were enrolled (Hendrikx et al., 2006). An average of $60x10^6$ bone marrow-derived MNCs were injected intramyocardially at the end of CABG surgery. At 4 months follow-up, LVEF increased 3.6% in the control group (n=10) and 6% in the transplant group (n=10; p = n.s.). However, the transplant group could be divided into a responder and a non-responder group. The responder group was characterized by a significantly higher percentage and absolute number of CD34+ cells compared to the non-responder group (3.1% versus 0.9%; p = 0.03). Using fluorodeoxyglucose to monitor cell homing by positron emission tomography (PET-scan), Hofmann et al. reported that 14-39%

of a CD34-enriched population homed into the infarcted myocardium after coronary administration (Hofmann et al., 2005). This is significantly higher than an unselected MNC fraction (1.3-2.6%). These observations suggested that the transplantation of a selected stem cell subpopulation could be more beneficial to regenerate the infarcted heart after acute inflammatory processes have faded out.

1.4 Enhancing cell therapy for cardiac repair

The limited improvement in cardiac function reported in the trials mentioned above have sent researchers back to the bench in order to augment the regenerative effect of stem cell transplantation. The limited improvement and the sometimes conflicting data reported, may be related to differences in cell type, cell preparation, methods and timing of cell delivery and cell dosage.

Trials performed at present try to find a variable or a combination of variables in order to enhance the outcome of cell therapy for cardiac repair. Recently the results of the MYSTAR (Myocardial stem cell administration after acute myocardial infarction study) trial were published (Gyöngyösi et al., 2009). This trial compared the clinical outcome of patients (n=60) receiving stem cell transplantation 3-6 weeks (early) or 3-4 months (late) after AMI. These data confirmed previous studies and reported a limited improvement of LVEF (3.5±5.6% (early) and 3.4±7.0% (late)) and no difference between early and late stem cell administration. Indicating that when inflammation reduces, time is probably not a crucial factor in cardiac regeneration.

Since almost all studies used the total bone marrow-derived MNC population, no study was able to reveal whether a particular cell type was responsible for

improvement in cardiac function. In the recently published REGENT (Regeneration by intracoronary infusion of selected population of stem cells in acute myocardial infarction) trial, the cardiac function of patients receiving intracoronary transplantation of unselected bone marrow MNCs or $CD34^+CXCR4^+$ selected cells after PCI was compared with that of patients after PCI only receiving medical treatment (Tendera et al., 2009). In both treatment groups, LVEF increased with 3% after 6 months, indicating that $CD34^+CXCR4^+$ cells did not further augment the effect of stem cell transplantation compared to an unselected stem cell population. Not only cells of hematopoietic origin are in use in clinical trials. In 2004 Chen et al. reported the first intracoronary transplantation of autologous bone marrow-derived mesenchymal stem cells (MSCs) (Chen et al., 2004). $48\text{-}60x10^9$ MSCs were transplanted in 34 patients 10 days after PCI. After 6 months LVEF of the transplant patients increased 18% compared to only 6% in the control group ($p = 0.01$). However, some questions were raised about the purity of the transplanted stem cell population, since 10 days is a very short period to obtain a large homogenous population of MSCs. Recently the first randomized, double-blind, placebo-controlled, dose-escalation study intravenously transplanting allogeneic MSCs was published (Hare et al., 2009). This study enrolled 53 patients, 34 of which received MSCs and 19 were assigned to the placebo group. After 6 months an increase in LVEF of 6.5% and 7.4% could be detected in the treatment versus the placebo group, indicating that intravenous injection of MSCs does not contribute to cardiac regeneration. In the PROMETHEUS (Prospective RandOmized study of MEsenchymal stem cell Therapy in patients Undergoing cardiac Surgery; www.clinicaltrials.gov/ NCT00587990) trial, patients will receive an intramyocardial transplantation of either a low ($2x10^7$) or high dose ($2x10^8$) of MSCs. This phase I/II randomized,

double-blinded placebo controlled trial will evaluate the safety and effectiveness of injecting MSCs into the heart to repair and restore heart function in people after a MI and CABG surgery.

Also the use of non bone marrow-derived stem cells, e.g. adipose-derived stem cells or CSCs, is entering the clinical arena. Adipose tissue, which is available after liposuction, is a source of adipose tissue-derived stromal cells. These cells effectively improve left ventricular function in animal models of both acute and chronic myocardial infarction (Miyahara et al., 2006; Schenke-Layland et al., 2009). Based on these positive results, 2 clinical trials referred to as the APOLLO (Randomized clinical trail of Adipose-derived stem cells in the treatment of patients with ST-elevation myocardial infarction) trial (www.clinicaltrials.gov/NCT00442806) and the PRECISE (Randomized clinical trial of adipose-derived stem cells in treatment of non revascularizable ischemic myocardium) trial (www.clinicaltrials.gov/NCT00426868) are ongoing. At this time also 3 clinical trials, transplanting CSCs, started with the recruitment of patients. The SCIPIO (Cardiac Stem Cell Infusion in Patients with Ischemic cardiOmyopathy) trial will enroll 40 patients receiving intracoronary infusion of CSCs (www.clinicaltrials.gov/NCT00474461). This trial will provide more insight in the safety and feasibility of CSC transplantation to restore cardiac function. The ALCADIA (AutoLogous human CArdiac-Derived stem cell to treat Ischemic cArdiomyopathy) trial will enroll 6 patients. They will receive an autologous CSCs transplantation and the implantation of a gelatin hydrogel sheet releasing basic fibroblast growth factor (bFGF). This pilot trial will study the safety and efficacy on the use of autologous CSCs with the controlled release of bFGF (www.clinicaltrials.gov/NCT00981006). The third trial is referred to as the

CADUCEUS (CArdiosphere-Derived aUtologous Stem CElls to reverse ventricULar dysfunction) trial (www.clinicaltrials.gov/NCT0089336). This phase I randomized, dose escalation study will assess the safety and efficacy of intracoronary delivered cardiosphere-derived stem cells (CDCs) in patients after MI.

Not only selecting the proper subtype of cells will enhance the outcome of cell therapy for cardiac repair. Other clinical trials try to detect other variables important for the enhancement of cell therapy for cardiac regeneration. The TIME study (www.clinicaltrials.gov/NCT00684021) is a phase II, randomized double-blind, placebo-controlled trial that evaluates when stem cells should be administered after a MI. Bone marrow MNCs will be administered 3 or 7 days after AMI. At the same time, in a different trial referred to as the late TIME study, researchers will assess the safety and effectiveness of adult stem cell transplantation 2-3 weeks after a heart attack for improving cardiac function (www.clinicaltrials.gov/NCT00684060). These studies will provide better insight into the appropriate timing of transplanting autologous bone marrow MNCs in high risk patients after AMI.

1.5 From bench to bedside and back

Although most data derived from clinical trials report an albeit limited clinical benefit from stem cell therapy, the mechanisms of action remain uncertain and may be multi-factorial. The clinical outcome may be the result of a paracrine effect (Yoon et al., 2005) or the formation of functional CMs and/or vascular structures (Rota et al., 2007). Understanding these processes may further optimize the beneficial effect of stem cell transplantation. However tracking cellular differentiation after transplantation, particularly of human stem cells, is

difficult and not very reliable. Therefore it is appropriate to investigate these mechanisms in a controlled *in vitro* environment.

1.6 Aims of the study

In this thesis, we will investigate the myocardial differentiation capacity of 2 types of human bone marrow-derived stem cells, MSCs (Chapter 3) and hematopoietic stem cells (HSCs; Chapter 4). Furthermore, the heart probably contains several resident cardiac progenitor cell populations. In chapter 5 we examine the cardiomyogenic differentiation potential of 2 well described human CSC populations. The first type of CSCs was isolated based on the expression of the antigen c-kit. The other CSC population is referred to as CDCs and are isolated based on their ability to form spheres. Furthermore, we compare the phenotypical and functional properties of these c-kit$^+$ CSCs and CDCs with those of bone marrow-derived MSCs. Moreover, we describe the isolation, *ex vivo* expansion and myocardial differentiation of a new endogenous CSC named Cardiac Atrial appendage Stem Cells (CASCs). These cells are isolated based on their enzymatic aldehyde dehydrogenase (ALDH) activity (chapter 6). The cardiomyogenic differentiation of these different stem cell population was stimulated by culturing them in the presence of neonatal rat cardiomyocytes (NRCMs).

2. Material & 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.

2.1 Stem cell isolation and characterization

2.1.1 Mesenchymal stem cells

Human MSCs were isolated as previously described by Pittenger et al. (Pittenger et al., 1999). Briefly, 20ml bone marrow was aspirated from the patients' sternum before cardiac surgery. The MNC fraction was isolated by gradient-density-centrifugation using Percoll (Axis shield). Cells were rinsed twice with Dulbecco's phosphate buffered solution (DPBS) (Lonza), seeded at $2x10^5$ cells/cm^2 in X-Vivo 15 medium (Lonza) supplemented with 10% fetal calf serum (FCS) (Hyclone) and 2% penicillin-streptomycin (pen-strep) (Lonza) and cultured at 37°C and 5% CO_2 air atmosphere. The medium was refreshed 24h and 72h after seeding and subsequently every 3 days. After 14 days cells were re-plated at a density of $5\text{-}6x10^3$/cm^2 and each time they reached 80-85% confluence.

Cells were flow cytometrically analyzed at the time of isolation and at every passage. For immunophenotyping, the following mouse anti-human monoclonal antibodies and similarly conjugated isotype-matched control antibodies (all from Becton&Dickinson unless stated otherwise) were used: CD105- (AbD Serotec) and CD106-fluorescein isothiocyanate (FITC), CD73- and CD49c-phycoerythrin (PE), CD45-peridinyl chlorofylline-Cy5 (PerCP-Cy5), CD184- and CD90-allophycocyanine (APC) and CD34-phycoerythrin-Cy7 (PE-Cy7). Cells were

analyzed on a FACSAria® (Becton&Dickinson) (Pittenger et al., 1999; Colter et al., 2001; Boiret et al., 2005).

2.1.2 Hematopoietic stem cells

Just before cardiac surgery 45ml bone marrow was aspirated from the patients' sternum. The MNC fraction was isolated as described above. Cells were incubated with mouse anti-human CD34-Pe-Cy7 and CD133-PE (Miltenyi Biotec) for 30 minutes in the dark at room temperature and washed with DPBS. Cells positive for both CD133 and CD34 were isolated by a two step flow sorting method. First the CD34+ population was enriched, followed by a sorting step of CD133+/CD34+ (HSCs) cells under stringent purity conditions. To assess the percentage of contaminating endothelial progenitor cells (EPCs) present in this CD133+/CD34+ cell population, a more thorough characterization was performed. As described by Vasa et al. EPCs do also express CD133 and CD34 but can be distinguished from HSCs by their capacity of low-density lipoprotein (LDL) uptake (Vasa et al., 2001). Therefore, bone marrow-derived MNCs were incubated for 1h at 37°C with acetylated-DiI-LDL (ac-DiI-LDL) (5µg/ml) (Biomedical Technologies). Hereafter cells were incubated with the following antibodies (all from Becton&Dickinson unless stated otherwise): CD45-FITC, c-kit-PE (CD117) (Miltenyi Biotech), CD14-PerCP, CD133-APC (Miltenyi Biotech), CD34-Pe-Cy7. Cells positive for CD133/CD34/ac-DiI-LDL and negative for CD14 were judged as EPCs. This thorough flow cytometric analysis resulted in the isolation of a pure population of HSCs. Flow cytometric analysis and cell sorting were performed on a FACSAria®.

2.1.3 Cardiosphere-derived cells

CDC isolation was performed as previously described but with minor modifications (Messina et al., 2004; Smith et al., 2007). Human right atrial appendages were removed during routine cardiac surgery. The heart tissue was minced, washed with DPBS and digested 3 times for 5 minutes with Tryple LE select (Gibco). Fragments were placed on fibronectin coated plates in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep. After 1.5 to 2 weeks cardiosphere forming cells were isolated by pooling two washes of DPBS, one with Versene (Gibco) and one mild trypsinisation with Tryple LE Select (Gibco). Isolated cells were seeded in poly-D-lysine coated culture plates (Beckton&Dickinson) with a density of $3x10^4$ cells/ml in cardiosphere growth medium (CGM: 35% IMDM / 65% DMEM:F12 (Lonza) supplemented with 3.5% FCS, 2% B-27 (Gibco), 100µM dithiothreitol (DTT; Invitrogen), 10ng/ml epidermal growth factor (EGF; Tebu-Bio), 20ng/ml bFGF (Tebu-Bio), 40nM cardiotrophin-1 (Tebu-Bio), 40nM thrombin (Tebu-Bio) and 2% pen-strep. After 5-7 days spontaneously detached cardiospheres were isolated and replated in fibronectin coated culture flasks. After attachment of the cardiospheres, mono-layers of CDCs were generated in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep.

2.1.4 c-kit$^+$ CSCs

Fragments of human right atrial appendages were removed during routine cardiac surgery and minced into 1 – 2 mm^3 cubes. Hereafter, tissue pieces were plated in fibronectin coated plates in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep. After 2 weeks, outgrowth cells were detached from the bottom of the culture plates by incubation with cell dissociation buffer (CDB;

Gibco). Cells were incubated with a PE-labeled mouse anti-human c-kit antibody (Miltenyi Biotec) for 30 minutes at 4°C. C-kit$^+$ cells were isolated by flow sorting with a FACSAria® under stringent purity conditions. Purified c-kit$^+$ cells were expanded on fibronectin coated plates in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep. To assess whether these c-kit$^+$ cells are indeed stem cells, the clonogenic potential of these cells was tested. For this, c-kit$^+$ cells were sorted at a density of 1 cell/well with a FACSAria. Single cell disposition was microscopically analyzed and wells with more than one cell were excluded.

2.1.5 Cardiac atrial appendage stem cells

Right atrial appendages, removed during routine cardiac surgery, were minced and washed with DPBS. Tissue fragments were dissociated by collagenase treatment (600U/ml; Invitrogen). The single cell suspension was stained with Aldefluor® (Aldagen Inc) according to the manufacturers' instructions. Briefly, cells were dissolved in 1ml Aldefluor assay buffer containing 5µl of activated Aldefluor® reagent and incubated for 45 minutes at 37°C. After a washing step, cells were dissolved in Aldefluor assay buffer and incubated for 30 minutes at 4°C with CD34-Pe-Cy-7 and CD45-APC-Cy7. Cells were analyzed and isolated by a FACSAria®. ALDH$^+$ cells expressing CD34 but negative for CD45 were directly flow sorted in FCS. Afterwards cells were centrifuged and dissolved in X-Vivo 15 medium supplemented with 20%FCS and 2% pen-strep. Cells were *ex vivo* expanded on fibronectin coated culture plates. The medium was changed twice a week and cells were replated with a density of $5x10^3$ cells/cm² each time they reached 80-85% confluence.

2.2 Isolation of NRCMs

NRCMs were isolated from 3 to 4-day-old Whistar rats using the Worthington Neonatal Cardiomyocyte Isolation System Kit (Worthington biochemical corporation). After centrifugation, the pellet was dissolved in X-Vivo 15 medium supplemented with SmGM-2 SingleQuots® (insulin, human fibroblast growth factor B, GA-1000, human endothelial growth factor and 5% FCS) (Lonza) and plated at a cell density of $7.5x10^4/cm^2$. Cells were maintained at 37°C in a humidified atmosphere of 5%CO_2. To prevent overgrowth of fibroblasts 48h after seeding, contractile NRCMs were treated with 2µg/ml mitomycin C (Sigma-Aldrich) during 24h (Condorelli et al., 2001).

2.3 Flow cytometric characterization

2.3.1 Flow cytometric characterization of ex vivo expanded stem cells

At passage 4 a small aliquot of expanded cells were incubated for 30 minutes in the dark at room temperature with the following human monoclonal antibodies (Table 1) and similarly conjugated isotype-matched control antibodies. (All antibodies were purchased from Becton&Dickinson except CD133-APC and CD133-Pe (Miltenyi Biotec), CD105-FITC (Serotec), TIE-2, VEGFR-2 and VEGFR-3 (R&D systems). Cells were analyzed on a FACSAria® (Becton&Dickinson).

Table 1: Antibodies with conjugated fluorochrome used for flowcytometric characterization

	FITC	PE	PerCP	APC	Pe-Cy7	APC-Cy7
Tube 1	CD2	CD13	CD19	CD5	CD10	CD45
Tube 2	CD4	CD38	CD3	CD29	-	CD45
Tube 3	CD15	CD44	-	CD55	CD56	CD45
Tube 4	CD16	CD33	CD117	HLA-DR	CD34	CD45
Tube 5	CD50	CD11b	CD117	VEGFR-3	CD34	CD45
Tube 6	CD71	CD109	CD14	CD90	CD34	CD45
Tube 7	CD90	CD140b	CD117	CD133	CD34	CD45
Tube 8	CD105	CD73	CD14	CD184	CD34	CD45
Tube 9	CD106	CD49c	CD117	CD90	CD34	CD45
Tube 10	CD31	CD133	CD14	TIE-2	CD34	CD45
Tube 11	CD144	CD133	CD14	VEGFR-2	CD34	CD45

2.3.2 Flow cytometric analysis of freshly isolated ALDH+ cells derived from bone marrow, peripheral blood and heart tissue

Twenty milliliter of bone marrow was aspirated from patients' sternum before cardiac surgery and the MNC fraction was isolated as previously described (see 2.1). For the flow cytometric analysis of peripheral blood ALDH+ cells, first a red blood cell lysis was performed by incubating the samples with ammonium chloride (NH_4Cl) in a ratio of 1:4 (blood:NH_4Cl) for 10 minutes on ice. Both samples were dissolved in 1ml Aldefluor assay buffer/$5x10^6$ cells and incubated as described above (see 2.5). Subsequently the cells were incubated with CD34-Pe-Cy7, CD45-APC-Cy7 and CD73-PE for 30 minutes in the dark at 4°C.

2.4 Multilineage differentiation assay

To assess whether the *ex vivo* expanded stem cells kept their multipotent characteristics, a multilineage differentiation assay was performed. We used the "Human Mesenchymal Stem Cell Functional Identification Kit" (R&D systems) to

stimulate adipogenic, osteogenic and chondrogenic differentiation. Protocols were performed according to manufacturers' recommendations.

2.5 Proliferation assay

$12.5x10^4$ MSCs, CDCs or c-kit$^+$ CSCs were incubated with CD140b and plated in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep. After 48h cells were detached and counted. The number of proliferative cells was assessed by incubating the cells with a ki-67-PE conjugated antibody (Beckton&Dickinson) according to the manufacturer's instructions.

2.6 Lentiviral transfection with green fluorescent protein

Cells were infected with a lentivirus expressing green fluorescent protein (GFP) under the control of a cytomegalovirus promoter (pRRL-CMV-GFP; kindly provided by dr. R.C. Hoeben, University Medical Center, Leiden, The Netherlands) (Carlotti et al., 2004).

2.6.1 Transformation of E.coli and isolation of plasmids

Plasmids were diluted till a final concentration of 100pg/µl. Plasmid solution was added to TOP10F' competent E.coli (Invitrogen) and treated as described by the manufacturer. Plasmids were isolated from of the bacteria using the Qiagen midi prep kit (Qiagen).

2.6.2 Transfection of 293-T cells for the production of GFP-expressing lentiviruses

The day before transfection, $2x10^3$ 293-T cells/mm² were seeded and incubated overnight. The plasmids, pRRL-CMV-GFP, pMDLg-RRE, pRSV-REV and pCMV-VSVG were mixed with EZ lentifect (MellGen Laboratories nv). Viral supernatant

was harvested after 48h, 60h and 72h and snap-shot frozen in liquid nitrogen. The titer of each viral stock was determined in 293-T cells.

2.6.3 Transduction of cells with lentiviruses expressing GFP

For lentiviral transduction, 10^4 cells were seeded in a 24-well plate. After overnight incubation the medium was changed by adding equal amounts of viral supernatants and fresh culture medium supplemented with 16µg/ml polybrene (Sigma). After 5h, fresh culture medium was added to the cells and incubated overnight. To augment the transduction efficiency cells underwent a second cycle of transduction. When passaged, transduction efficiency was analyzed by flow cytometry.

2.7 Cardiomyogenic differentiation

The purpose of this study was to examine the myocardial differentiation capacity of these different types of stem/progenitor cells *in vitro*. Therefore, a cardiac micro-environment was set-up by co-culturing the stem/progenitor cells with NRCMs. The co-culture system is able to stimulate differentiation through interaction of hormones and cytokines secreted by the NRCMs and the corresponding human receptors. Furthermore, the stem cells are also subjected to the mechanical stress exerted by the contraction of the NRCMs. This model also allows to investigate the influence of several differentiation inducing factors like 5-azacytidin (5-aza), dimethylsulfoxide (DMSO) and transforming growth factor-β (TGF-β). It has been reported that the demethylating agent 5-aza is able to induce myogenic differentiation in embryonic mice cells (Konieczny et al., 1984) and that these data can be extrapolated to human MSCs (Xu et al., 2004). P19 embryonic mice cells stimulated with DMSO differentiated into CMs (Skerjanc et al., 1999; Paquin et al., 2002). Whether these data can be

extrapolated to human adult stem cells remains to be determined. TGF-β up-regulates the expression of cardiac specific transcription factors such as *Nkx2.5* and *GATA-4* (Behfar et al., 2002; Li et al., 2005).

2.7.1 Cardiomyogenic differentiation potential of bone marrow-derived MSCs and HSCs

Before co-culture set-up, MSCs and HSCs were labeled with 20µM 5-chloromethylfluorescein diacetate (CMFDA) (invitrogen) cell tracker green and NRCMs were labeled with 40µM 5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethyl rhodamine (CMTMR) cell tracker red (Invitrogen) for 45 minutes.

Co-cultures with MSCs were performed at different cell ratios, MSCs:NRCMs (1:6, 1:3 and 1:1). While co-cultures with HSCs were set-up in a 1:4 ratio (stem cells:NRCMs).

Co-cultures were grown in X-Vivo 15 medium supplemented with 2% autologous serum (AS) or 2% FCS. Twenty-four hours after seeding, myogenic differentiation was stimulated by adding 1% DMSO for 48h or 3µM 5-aza for 24h. As negative control, culture conditions without additives were used. The medium was changed twice a week. After 3 weeks, cell populations were separated by flow sorting based on their specific fluorescence using a FACSAria®. To prevent RNA-degradation, cells were directly sorted in 350µl RLT-buffer (Qiagen).

To assess whether direct contact between NRCMs and MSCs is important in myocardial differentiation, MSCs were mono-cultured in conditioned medium (X-Vivo 15 medium supplemented with 2% FCS) obtained from NRCMs cultured

under normoxic (21% O_2; 5% CO_2 – 48h) or hypoxic (0.2% O_2 – 48h) conditions.

To control if cell fusion occurred during long term co-culture, green fluorescent MSCs were sorted out and spun down on microscope slides. Nuclei were stained with DAPI and cells were analyzed under the Axioplan 2 imaging fluorescence microscope (Zeiss).

2.7.2 Cardiomyogenic differentiation potential of bone marrow-derived MSCs compared to that of c-kit⁺ CSCs and CDCs

Mono-cultures

To test the myocardial differentiation potential in mono-culture, stem cells were seeded with a density of $12.5x10^3/cm^2$ in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep. After 24h the myogenic differentiation was stimulated by reducing serum contents to 2% or 0% and 0, 1 or 3 ng/ml TGF-β was added throughout the entire culture time.

Co-cultures with NRCMs

To test whether the cardiomyogenic differentiation potential of the stem cells was stimulated by a cardiac micro-environment, cells were co-cultured in the presence of NRCMs at a 1:3 ratio (stem cells:NRCMs) in X-vivo 15 medium with (2%) or without FCS and supplemented with 1 ng/ml TGF-β. Prior to the co-culture set-up, stem cells were labelled with GFP by lentiviral infection as described above.

2.7.3 Cardiomyogenic differentiation of CASCs

To induce differentiation, GFP expressing CASCs were co-cultured in a 1:3 (CASCs:NRCMs) ratio for 1-3 weeks in X-vivo 15 medium supplemented with 1

ng/ml TGF- β1 and 2% pen-strep. As negative control, co-cultures without additives were used. After 1 week CASCs were isolated out of the co-culture using a FACSAria®. To prevent RNA-degradation, the flow sorter was cleaned with bleach, diethyl pyrocarbonate (Fluka; DEPC) treated solutions were used and isolated cells were directly sorted in 350µl cooled (4°C) RLT-buffer.

2.8 RNA extraction and RT-PCR

Total RNA was isolated using the RNeasy Mini (MSCs and HSCs) or micro (MSCs, CDCs, c-kit$^+$ CSCs and CASCs) kit (Qiagen). cDNA was synthesized using Superscript III and random hexamers (Invitrogen) according to manufactures' description. PCR was performed with *Taq* polymerase (Roche) 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. RT-PCR was performed to detect expression of the following genes: *α-actinin*, *troponin T (TnT), troponin I (TnI), myosin heavy chain (MHC), Cx43, Kv4.3, α1c, GATA-4* and *Nkx2.5* (table 2). We chose to study the expression of *α-actinin, TnT, TnI* and *MHC* because the proteins encoded by these genes form the molecular structure of the sliding filament mechanism of the CM.

Cx protein subunits form gap-junctions which allow direct intercellular communication between adjacent cells. The expression of Cx40, Cx43 and Cx45 is found in CMs (Gros et al., 1996). Cx40 and Cx45 are mainly expressed in the atria and the conduction system, respectively. Cx43 on the other hand is predominantly expressed in the ventricles (van Veen et al., 2006). When the Cxs of two adjacent cells are opposed, a pore is formed which is important for the electrical cell coupling. Therefore, expression of Cx43 is indispensable for electric integration of transplanted stem cells with host tissue.

The expression of ion channels like the potassium voltage-gated channel *Kv4.3* and the L-type calcium channel *α1c* are important for the generation of action potentials. In human CMs Kv4.3 appears to be solely responsible as the pore-forming subunit for the fast transient outward current ($I_{to,f}$) (Bertaso et al., 2002). This current is responsible for the rapid repolarization phase in human CMs (Sanguinetti, 2002). The cardiac L-type Ca^{2+} channels are composed of 4 polypeptide subunits (α1, β, α_2/δ) and form a hetero-tetrameric complex (Wang et al., 2004; Bodi et al., 2005). The α1 subunit is a hydrophobic polypeptide fixed in the membrane and is responsible for the voltage dependent Ca^{2+} channel opening and channel selectivity for calcium ions (Bodi et al., 2005). To date 10 different α1 subunits are described but only α1c is expressed at high levels in cardiac muscle.

The transcription factors GATA-4 and Nkx2.5 are both expressed in human CMs. The family of GATA transcription factors consists of six proteins (GATA-1-6) (Patient et al., 2002). Interestingly, GATA-4 is one of the earliest transcription factors expressed in developing CMs (Heikinheimo et al., 1994). The Nkx2.5 gene promoter contains two GATA elements, which bind GATA-4 and are necessary for the promoter activity in developing CMs (Lien et al., 1999). Nkx2.5 targets cardiac specific genes encoding for ANP (Durocher et al., 1996), α-actinin (Chen et al., 1996) and the transcription factor Myocyte Enhancer Factor-2C (von Both et al., 2004).

Table 2: **RT-PCR primer sequences for genes**

Gene	Forward primer	Reverse primer	AT	bp
Cardiac genes				
β-actin	*5'-AGCGGGAAATCGTGCGTGACA-3'*	*5'-CCTGTAACAATGCATCTCATATTTGG-3'*	56°C	*791*
α-actinin	*5'-GGCGTGCAGTACAACTACGTG-3'*	*5'-AGTCAATGAGGTCAGGCCGGT-3'*	*56°C*	*579*
TnT	*5'-AGAGGTGGTGGAAGAGTACGAG-3'*	*5'-GACGTCTCTCGATCCTGTCTTT-3'*	*56°C*	*406*
MHC	*5'-GGAGGAGGACAGGAAAAACCT-3'*	*5'-CGGCTTCAAGGAAAATTGC-3'*	*53°C*	*360*
TnI	*5'-CCCTGCACCAGCCCCAATCAGA-3'*	*5'-CGAAGCCCAGCCCGGCCAACT-3'*	*60°C*	*232*
Cx43	*5'-CTTGGCGTGACTTCACTACTTTT-3'*	*5'-GCATTTTCACCTTACCATGCTCT-3'*	*53°C*	*490*
Kv4.3	*5'-CTTAAGACGATTGCAGGGAAGAT-3'*	*5'-CTTCTTGTGGATGGGTAGTTCTG-3'*	*53°C*	*473*
α1c	*5'-CTGGACAAGAACCAGCGACAGTGCG-3'*	*5'-ATCACGATCAGGAGGGCCACATAGGG-3'*	*60°C*	*562*
Nkx2.5	*5'-GCAGGTCAAGATCTGGTTCCAGA-3'*	*5'-GAGTGAATGCAAAATCCAGGGGAC-3'*	*56°C*	*550*
GATA-4	*5'-CCCCAATCTCGATATGTTTG-3'*	*5'-AGGAGCTGCTGGTGTCTTAG-3'*	*50°C*	*396*
α-actinin (2)	5'*CCTGCCTTCATGCCCTCCGA-3'*	*5'-TGCTCCACGCGGTCCTGGTG-3'*	*56°C*	*307*
MHC (2)	*5'-GAACACCAGCCTCATCAACC-3'*	*5'-AGGTTGGTGTTGGCTTGCTC-3'*	*53°C*	*522*
Endothelial genes				
vWF	*5'-CTCGTCTCACTCAGGTGTCAGTGCTG-3'*	*5'-CTGGTCTGGCAAGGTCCAGACGTC-3'*	*58°C*	*520*
VEGFR-2	*5'-CGGTCAACAAAGTCGGGAGAGGAGAG-3'*	*5'-CCATGACGATGGACAAGTAGCCTGTCTTC-3'*	*58°C*	*825*
e-NOS	*5'-GCTGCACAGTTACCAGCTAGCCAAAGTC-3'*	*5'-CAAATGTGCTGGTTACCACCAGCACCAG-3'*	*58°C*	*506*
GATA-2	*5'-CACCTACCCCTCCTATGTGCCGG-3'*	*5'-CCTCGACGTCCATCTGTTCCCTAGC-3'*	*58°C*	*722*
Pluripotency genes				
OCT-4	*5'-TCAGCCAAACGACCATCTGCCGCT-3'*	*5'-GAAGTGAGGGCTCCATAGCCTGG-3'*	*58°C*	*455*
DPPA3	*5'-GTTACTGGGCGGAGTTCGTA-3'*	*5'-TGAAGTGGCTTGGTGTCTTG-3'*	*50°C*	*167*
Lin-28	*5'-CCTTGTTCCCAACCTCCTAAG-3'*	*5'-CAGGTACAGGCTTTCCTACCC-3'*	*50°C*	*994*
c-myc	*5'-GTGCGTAAGGAAAAGTAAGG-3'*	*5'-AAGACTCAGCCAAGGTTG-3'*	*45°C*	*116*
Klf-4	*5'-CTGTTATGCACTGTGGTT-3'*	*5'-GTATGCAAAATACAAACTCC-3'*	*45°C*	*201*
Tbx-3	*5'-TTCCTACCTCACCGGGCG-3'*	*5'-CCGTTGGGAGGCAGCGT-3'*	*50°C*	*94*
Nanog	*5'-CGTTCTGCTGGACTGAGCTGGTTG-3'*	*5'-CCAGGAGTGGTTGCTCCAGGACTG-3'*	*58°C*	*848*
c-kit	*5'-AAGACAACGACACGCTGGTCCGCT-3'*	*5'-GGACACAGACACAACAGGCACAGG-3'*	*58°c*	*268*
Rat gene				
rNkx2.5	*5'-GACATCCTGAACCTGGAGCAGCAG-3'*	*5'-CGTTGTAGCCGTAGGCGTTGAGAC-3'*	*58°C*	*768*

2.9 Immunofluorescence and Transmission Electron Microscopy

RNA interference controls which genes are active and how active they are. This implies that even if a certain gene is expressed at the RNA-level it is not necessary that the protein encoded by this RNA is formed or folded in an organized manner. Therefore, one has to investigate the presence of the protein by e.g. immunofluorescence. In this thesis expression of cardiac TnT (cTnT) and cardiac TnI (cTnI) are used to determine the cardiac differentiation of the stem cells at the protein level. Furthermore, we also studied the cellular morphology by transmission electron microscopy (TEM). This allowed us to investigate the internal cellular organization.

2.9.1 Immunofluorescence on cultured cells

Prior to the culture, glass cover-slips were sterilized and used as culture surface. After the (co-)culture, cells were fixed in 4% paraformaldehyde for 20 minutes and subsequently permeabilized with 0.3% Triton X (Sigma-Aldrich) at room temperature. Cells were incubated overnight with unconjugated primary antibodies at 4°C. The next day, cells were incubated with a conjugated secondary antibody for 1h at roomtemperature. When fluorochrome-conjugated antibodies were used cells were incubated for 1h at room temperature. Cells were visualized with the Axioplan 2 imaging fluorescence microscope (Zeiss). Detailed overview of antibodies with their corresponding incubation time and temperature is described in table 3.

Table 3: **Antibodies for immunofluorescence**

	Incubation time	Incubation t°	Dilution	compagny
Unconjugated primary antibody				
Rabbit-anti-human Cardiac troponin I (cTnI)	Overnight	4°C	1:500	Chemicon
Rabbit-anti-human Cardiac troponin T (cTnT)	Overnight	4°C	1:500	Abcam plc
Mouse-anti-human-CD34	Overnight	4°C	1:100	Beckton&Dickinson
Mouse-anti-human Human nuclear antigen (HNA)	Overnight	4°C	1:100	Millipore corporation
Conjugated primary antibody				
Mouse-anti-human CD45-FITC	1h	Room temperature		Beckton&Dickinson
Mouse-anti-human CD105-FITC	1h	Room temperature		AbD Serotec
Mouse-anti-human c-kit-PE	1h	Room temperature		Miltenyi Biotec
Conjugated secondary antibody				
Goat-anti-mouse FITC (for *HNA)*	1h	Room temperature	1:200	Sigma
Goat-anti-mouse FITC *(for CD34)*	1h	Room temperatire	1:10	Beckton&Dickinson
Sheep-anti-rabbit Rhodamine	1h	Room temperature	1:10	Millipore corporation

2.9.2 Immunofluorescence of paraffin embedded heart slices

After paraffin embedding of right atrial appendages, 5µm slices were prepared. Tissues slices were deparaffinized by incubating them twice for 5 minutes in

xylene and gradually dehydrated by a series of graded acetone concentrations. Antigen retreavel was perfomed by incubating them for 10 minutes in 10mM citrate buffer at 99°C. Hereafter, cells were incubated with the primary antibodies CD34 and cTnT and their matching secondary antibodies as described in table 3.

2.9.3 Transmission Electron Microscopy

MSCs and NRCMs were seeded directly onto Thermanox®-slides (Nunc A/S). For freshly isolated HSCs, cells were spun down onto Thermanox®-slides by cytospin-centrifugation. Initially, cells were fixed with 2% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) at 4°C. The fixative was gently aspirated with a glass pipette and specimens were postfixed in 2% osmium tetroxide for 1h, followed by staining with 2% uranyl-acetate in 10% acetone for 20 minutes. Samples were gradually dehydrated by a series of graded acetone concentrations and embedded in epoxy resin (Araldite). For light microscopy semi-thin sections (0.5 µm) were stained with a solution of thionin and methylene blue (0.1% aqueous solution). For transmission electron microscopy (TEM), ultra-thin sections (0.06 µm) (Ultracut E ultramicrotoom; Reichert-Jung) were mounted on 0.7% formvar-coated grids, contrasted with 5% uranyl-acetate followed by lead citrate, and examined in a Philips EM 208 transmission electron microscope operated at 80 kV.

2.10 Cardiosphere forming assay

MSCs, c-kit$^+$ CSCs, primary cultures of adult human dermal mesenchymal cells and primary cultures of myofibroblasts isolated from colon tissue (both kindly provided by Prof. M. Bracke, MD, University of Ghent, Belgium) were plated with

a density of $3x10^4$cells/ml in CGM (see 2.1.3) on poly-D-lysine coated plates. After 5-7 days the number of spheres was scored.

2.11 Functional comparison between bone marrow-derived ALDH+ cells and CASCs

HSC cultures were set up by isolating ALDH+ cells from bone marrow MNCs. Thereafter both ALDH+ cells and CASCs were seeded in MethoCult® GF H4434 (Stem cell Technologies). Cells were incubated for 2 weeks at 37°C and 5%CO_2 air atmosphere. After 14 days the number of burst-forming unit, erythrocyte (BFU-E), colony-forming unit, granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM) and colony-forming unit, granulocyte-macrophage/monocyte (CFU-GM) were counted.

2.12 Electrophysiology of cultured CASCs

Mono and co-cultures for electrophysiological recordings were set-up as described above. GFP expressing CASCs were seeded onto contractile NRCMs and co-cultured for 1 to 3 weeks prior to the recordings. Electorphysiology experiments used the whole-cell patch clamp technique (Hamill et al., 1981) and were performed at room temperature with borosilicate glass patch electrodes. Patch pipettes were pulled from capillary glass (GB 200-8P; Science Products) on a DMZ-Universal Puller (Zeitz-Instrumente), which fire-polished the electrodes to a final resistance of 2-3MW when filled with internal solution. The electrodes were connected to an Axoclamp-2B amplifier (Axon Instruments), and command pulses were given and data acquired using pCLAMP software (Axon Instruments). The voltage-clamp protocols consisted of either steps (for 1s) from the holding potential of -80mV to various levels (to measure inward sodium and calcium currents) or 4s symmetrical ramps from -120mV to +80 mV and

back to -120mV, applied every 10 seconds. During the ascending limb of the ramp, the slow rate of depolarization ($0.1V.s^{-1}$) allowed activation and inactivation of the voltage dependent Na^+ current. The potassium currents were measured during the descending limb of the voltage ramp, between +80mV and -120mV. Data were analyzed with Clampfit 8.2 (Axon Instruments) and Origin 6.1 (Microcal). Inward sodium (I_{Na}) and/or inward calcium currents (I_{CaL}) were measured as the difference between the peak inward and the steady state current. Values are given as mean±S.E.M and were compared using ANOVA and/or two tailed *t*-test.

The extracellular solution during measurements was a Tyrode solution with the following composition (in mM): 135 NaCl, 5.4 KCl, 0.9 $MgCl_2$, 1.8 $CaCl_2$, 0.33 NaH_2PO_4, 10 HEPES and 10 glucose (pH 7.4; titrated with NaOH). The composition of the pipette solution contained (in mM): 130 KCl or K-glutamate, 25 KCl, 1$MgCl_2$, 5 Na_2ATP, 1 EGTA, 0.1 Na_2GTP and 5 HEPES (pH 7.25; adjusted with KOH).

2.13 Statistics

The data were analysed using the SPSS® 15.0 software package (IBM, New York, USA). Continuous variables are displayed as mean±SD. Categorical data are presented as counts. Comparisons between groups were performed with the Kruskal-Wallis test, the Pearson's χ^2 test or the Fisher's Exact test as appropriate. Statistical significance was set at a p-value < 0.05.

3. Mesenchymal stem cells

3.1 Introduction

3.1.1 Mesenchymal stem cells

MSCs can be found in the human bone marrow. There they represent around 0.001% of the total MNC fraction. The term MSCs was introduced in 1961 by Friedenstein et al. (Friedenstein, 1961). They discovered that the marrow stromal cell population did contain osteogenic progenitors. After this observation, the *ex vivo* expansion and differentiation of these progenitor cells was vigorously investigated. In the early 90s Caplan et al. discovered that MSCs could be retrieved from the bone marrow by first isolating the MNC fraction by Percoll density centrifugation (Caplan et al., 1991). The MSCs were isolated based on their ability to adhere to the bottom of the culture plate. After 1 week colonies of this adherent cell population became visible. However, MSCs in culture become homogenous within time. There is no real consensus about the antigen expression profile of these cells at the moment of isolation. It is described that MSCs do express the following membrane antigens: CD13, CD29, CD73, CD90, CD105,… Furthermore, these cells are negative for a lot of markers present on cells committed to the hematopoietic lineage like CD3, CD4, CD14, CD34, CD45, CD117,… (Colter et al., 2001; Boiret et al., 2005). Despite this difficult determination of the antigen expression profile of these cells, MSCs are very attractive to be used in different clinical settings. First of all, MSCs can be isolated in a reproducible manner (plastic adherence) and can be extensively expanded (Colter et al., 2001). Secondly, it is described that MSCs lack the expression of the major histocompatibility complex II (le Blanc, 2003). This makes it possible to transplant these cells in an allogenic setting and create an off-the-shelve therapy for different kinds of diseases. Thirdly, it has been well

described that MSCs are able to differentiate into various kinds of cell types like adipocytes, osteocytes and chondrocytes, proving the multipotent character of these cells (Pittenger et al., 1999; Colter et al., 2001). All these cell types are known to be of mesodermal origin, therefore it might be that these MSCs are able to differentiate into CMs. The cardiomyogenic differentiation of MSCs is stimulated by exposing these cells to a variety of physiologic or nonphysiologic stimuli (Rangappa et al., 2003; Xu et al., 2004).

3.1.2 Cardiomyoplasticity of MSCs

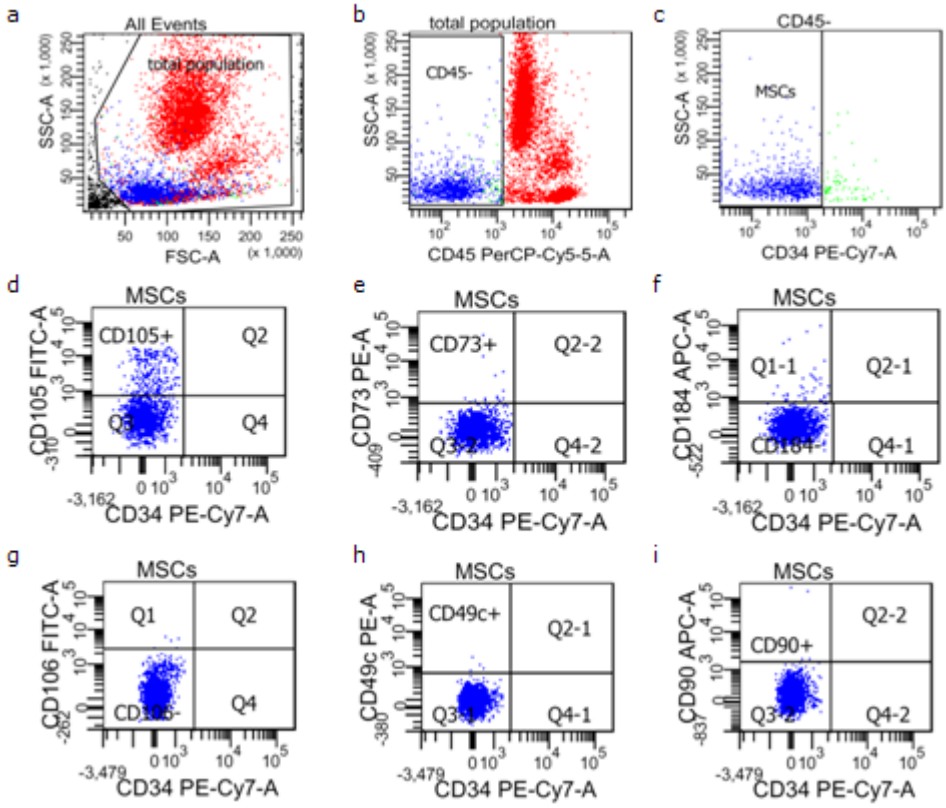
MSCs have some intrinsic features that make them good candidate cells for cardiomyoplasty. They can be isolated from the patient's bone marrow and allow an autologous transplantation. Tomita et al. reported that rat bone marrow-derived MSCs transplanted in the infarcted rat heart, differentiated down the cardiomyogenic lineage (Tomita et al., 1999). Cardiac-like muscle cells stained positive for MHC and TnI. Moreover, animals that received MSCs treated with the DNA-demethylating agent 5-aza generated also higher diastolic and systolic pressures. This study indicated that bone marrow MSCs should be considered as a cell source to repair the damaged myocardium. They also noticed a greater vascular density in the transplant region. Only occasional MSCs were integrated in newly formed vascular structures. Enhanced vascularisation however could not be explaind by transdifferentiation of MSCs into vascular endothelial cells alone but was mainly the result of a paracrine effect. In the same year Makino et al. showed that a cardiomyogenic cell line could be generated by treating murine stromal cells with 5-aza (Makino et al., 1999). Not only displayed these cells typical sarcomeres, they also showed spontaneous beating. These positive results have encouraged several laboratories to investigate the myocardial

differentiation potential of human bone marrow-derived MSCs (Rangappa et al., 2003; Fukuhara et al., 2003). Our group has examined this potential of MSCs by co-culturing these cells in the presence or absence of differentiation stimulating factors like DMSO or 5-aza.

3.2 Results

3.2.1 Isolation and characterization of MSCs

For isolation of MSCs a MNC fraction was prepared from bone marrow of 15 cardiac surgical patients. Before starting the culture, MNCs were analyzed by flow cytometry but no cells with a mesenchymal phenotype could be detected (Figure 1).



<u>Figure 1:</u> Flow cytometric analysis of bone marrow-derived MNC fraction. No cells with a MSC-phenotype could be detected. CD45- cells (b) are negative for CD34 (c), CD105 (d), CD73 (e), CD184 (f), CD106 (g), CD49c (h), CD90 (i).

MNCs were seeded and 24h later a small percentage of these cells attached to the bottom of the culture flask. After 3 days, some attached cells formed spindle

like structures giving rise to MSC colonies, while other cells stayed loosely adherent (Figure 2).

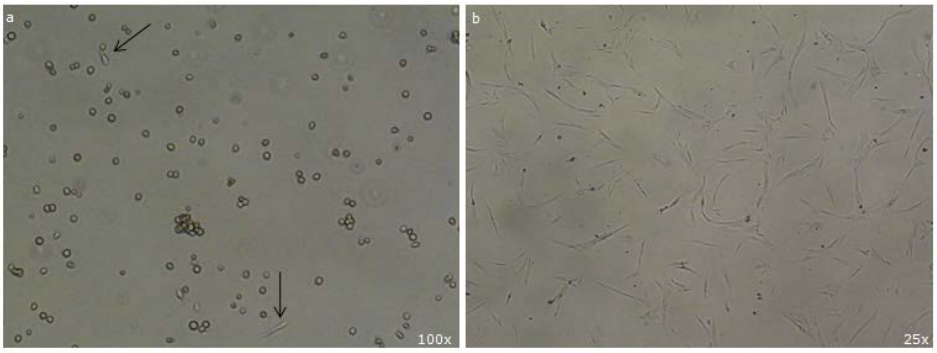
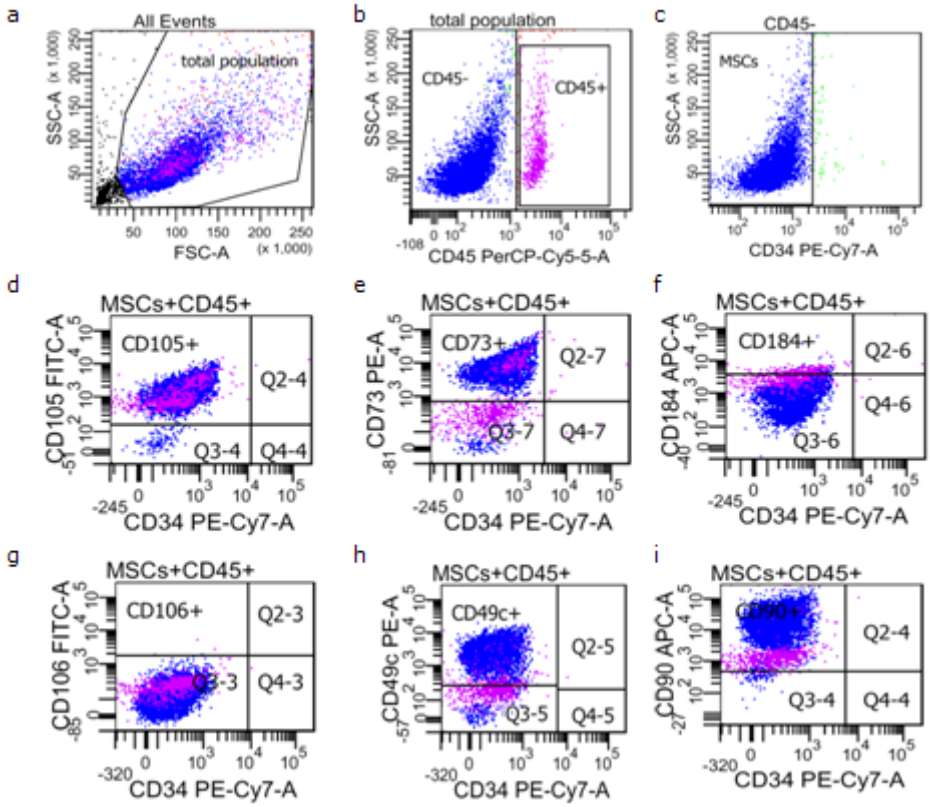


Figure 2: Light microscopic analysis of *ex vivo* expanded MSCs. Twenty-four hours after seeding the MNC fraction, cells attached to the bottom of the culture flask and started to elongate (arrows) (a). After 1 week, these cells started to give rise to MSC colonies (b).

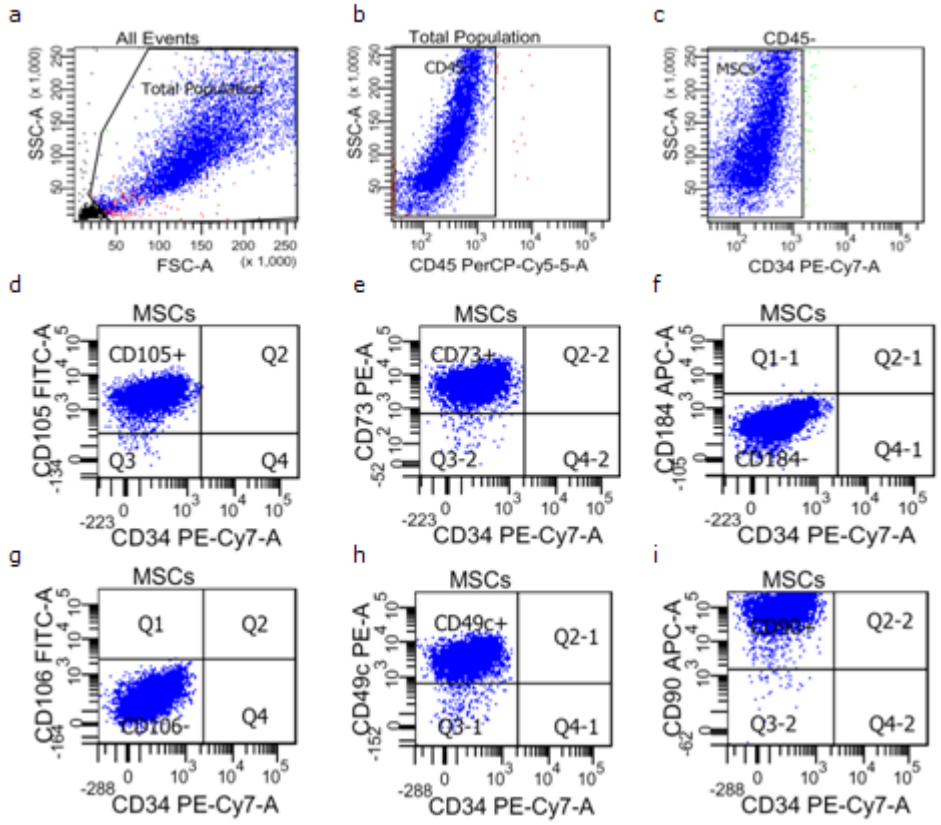
Indeed, cells of passage 1 represented a heterogeneous population containing 40±29% CD45+ cells (Figure 3b). These CD45+ cells are CD105+ (Figure 3d), CD184+ (Figure 3f), CD90+ (Figure 3i), CD34- (Figure 3c) and CD106- (Figure 3g) but only 25±19% of these CD45+ cells express CD73 (Figure 3e) and 13±10% express CD49c (Figure 3h).



<u>Figure 3:</u> Flow cytometric characterization of cultured MSCs at passage 1. Forward scatter/side scatter of total culture population (FSC/SSC) (a). Analysis of CD45 expression in the total population (b). At passage 1, cells formed a heterogeneous population with 40±29% CD45+ cells (pink population) and CD45- cells (blue population). The CD45- cells showed a mesenchymal phenotype (CD49c+ (h), CD73+ (e), CD90+ (i) CD105+ (d) CD34- (c), CD106- (g) and CD184- (f)). The CD45+ cells are CD105+ (d), CD184+ (f), CD90+ (i), CD34- (c) and CD106- (g). 13±10% of these cells were also positive for CD49c (h) and 25±19% expressed CD73 (e).

Further culturing of these MSCs resulted in the loss of all loosely adherent cells. Flow cytometrical analysis of cells at passage 2 and 3 revealed a gradual loss of CD45+ cells (data not shown). After 4 passages all loosely adherent cells had disappeared and no CD45+ cells could be detected, indicating that these loosely adherent cells were CD45+. MSCs of passage 4 represented a phenotypically homogeneous population positive for CD105 (Figure 4d), CD73 (Figure 4e),

CD49c (Figure 4h) and CD90 (Figure 4i) and negative for CD45 (Figure 4b), CD34 (Figure 4c), CD184 (Figure 4f) and CD106 (Figure 4g).



<u>Figure 4:</u> Flow cytometric characterization of cultured MSCs at passage 4. The total cell population (FSC/SSC (a)) shows a homogeneous population positive for CD105 (d), CD73 (e), CD49c (h) and CD90 (i) but negative for CD45 (b), CD34 (c), CD184 (f) and CD106 (g).

After *ex vivo* expansion MSCs were still able to differentiate into adipocytes (Figure 5a,b), osteocytes (Figure 5c,d) and chondrocytes (Figure 5e,f) (n=5). Labeling of MSCs with cell tracker green had no negative impact on their differentiation capacity (data not shown).

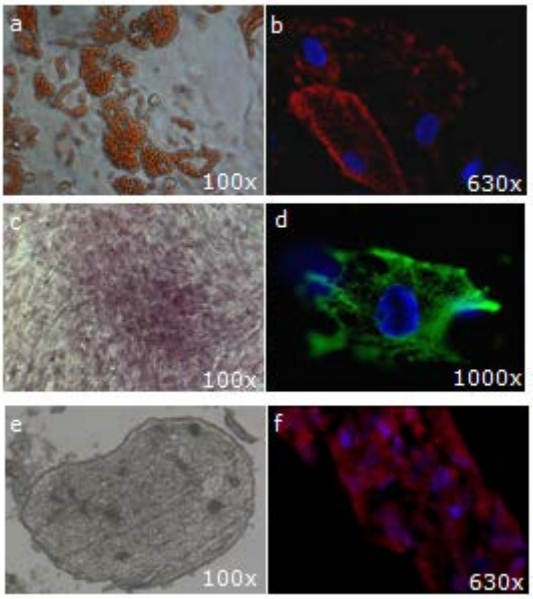


Figure 5: Quality control on multipotency of *ex vivo* expanded MSCs. Cells were stimulated to differentiate into adipocytes (a,b), osteocytes (c,d) and chondrocytes (e,f). Oil-red-O staining showing lipid droplets (a) and by immunofluorescence the prescence of fatty acid binding protein 4 (FABP-4) (b) could be detected. Alizarin-red staining of Ca^{2+}-deposits (c) and positive immunofluorescence for osteocalcin (d). Light microscopic analysis of cryosections shows structural and cellular organization (e). Chondrogenic differentiation was shown by positive immunofluorescent staining for aggrecan (f). Nuclei are stained with 4,6-diamino-2-phenylindole (DAPI: blue).

3.2.2 Co-culturing MSCs induces TnT and GATA-4 expression only at the RNA level

During the co-culture period no apparent change in cell morphology of the MSCs could be observed (n=10). *Ex vivo* expanded MSCs already expressed genes for *β-actin, Cx43, Kv4.3* and *α1c* while *Nkx2.5* was only expressed when differentiation was induced both in mono- and co-cultured MSCs (Figure 6A, B, C, D, E). Expression of *GATA-4* and *TnT* was induced in co-cultured MSCs (Figure 6B, C, D). Addition of either DMSO or 5-aza to the mono- or co-cultured MSCs did not influence gene-expression (Figure 6A, C, D). *GATA-4* expression was also

induced in mono-cultured MSCs using normoxic conditioned medium (n=3), while hypoxic conditioned medium (n=3) had no effect (Figure 6E). No expression of *α-actinin*, *MHC* or *TnI* could be detected in any condition (Figure 6A, B, C, D, E).

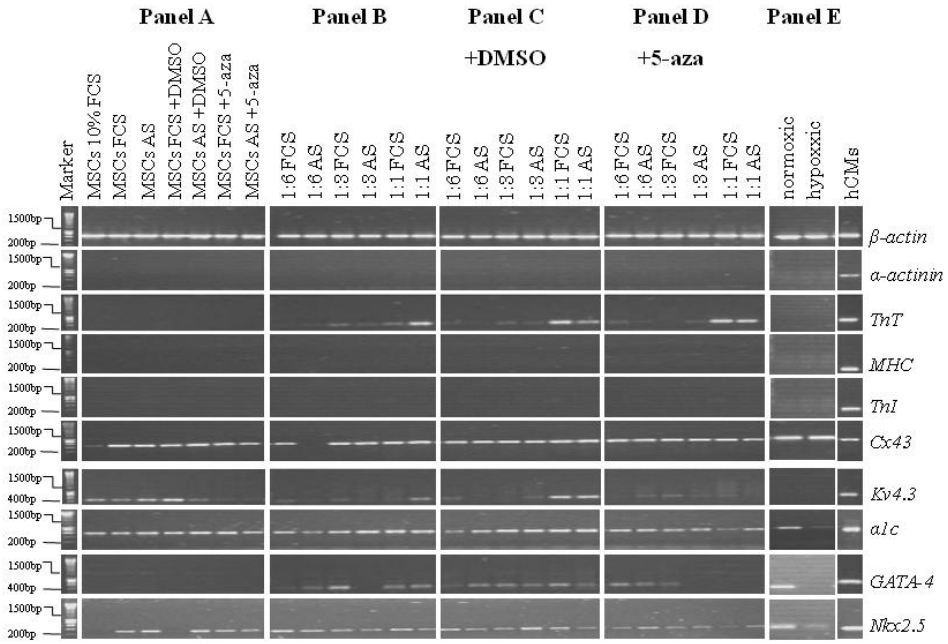


Figure 6: Analysis of cardiac gene expression in MSCs by RT-PCR. *Panel A*: Gene expression of *ex vivo* expanded MSCs (10% FCS) and mono-cultured MSCs, in the absence or presence of DMSO or 5-aza in either 2% FCS or 2% AS. *β-actin* (internal control), *Cx43*, *Kv4.3* and *α1c* are expressed. *Nkx2.5* is only expressed in mono-cultured MSCs. *Panel B*: Effect of co-culturing MSCs with NRCMs. Different cell ratios (MSCs:NRCMs) were used as indicated by cell numbers. Co-culturing MSCs results in the expression of *GATA-4* and *TnT* in most conditions. No expression of *α-actinin*, *MHC* or *TnI* was observed. *Panel C-D*: Same conditions as panel B. Effect of DMSO (C) or 5-aza (D) on co-cultured MSCs. Same result as panel B. *Panel E*: Use of normoxic or hypoxic conditioned DM (X-Vivo 15 medium + 2% FCS) did influence the gene expression of mono-cultured MSCs. MSCs treated with normoxic DM expressed *GATA-4* whereas MSCs treated with hypoxic DM did not. Furthermore, no expression of *TnT* could be detected. Human CMs (hCMs) are used as positive control.

To assess whether the RT-PCR was performed on a pure human cell population and no fusion occurred during the co-culture, a PCR to detect *rat Nkx2.5 (rNkx2.*5*)* was performed. This revealed that no expression of *rNkx2.5* could be detected in any condition (Figure 6F).

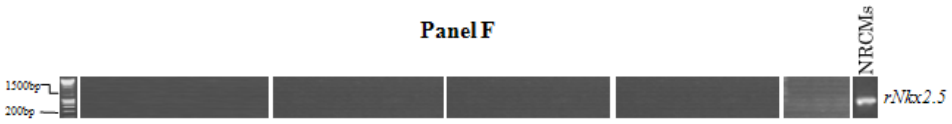


Figure 6 panel F: Control RT-PCR reaction. No *rNkx2.5* could be detected in any condition. NRCMs were used as positive control.

To further exclude the possibility of cell fusion the cytospins of sorted cells were analyzed. This showed that all cells from the sort gate were mononuclear and labeled with green fluorescent cell tracker (Figure 7a,b).

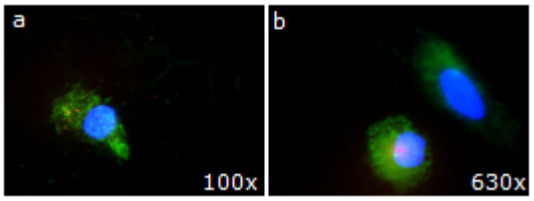


Figure 7: Fluorescent microscopy analysis. Cells giving a positive signal in the FITC-channel were sorted out and analyzed under a fluorescence microscope. This revealed that all cells from the sort gate were mononuclear and labeled with the green fluorescent cell tracker (a, b).

For confirmation of our RT-PCR results immunofluorescence for CD105 and cTnT was performed (n=3). E*x vivo* expanded (Figure 8a), mono- (Figure 8b) and co-cultured MSCs (Figure 9b,d) are CD105+ (green) but do not express the protein cTnT (red).

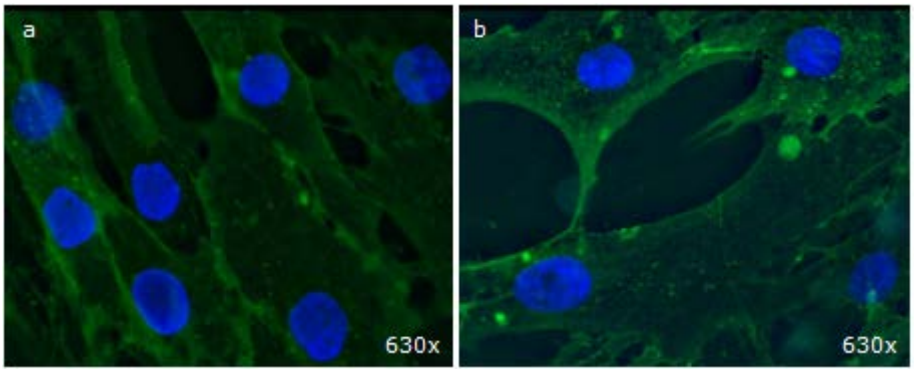


Figure 8: Protein analysis by immunofluorescence on MSCs. a: *Ex vivo* expanded MSCs express the membrane antigen CD105 (green) but no expression of cTnT (red) could be observed. Nuclei are stained with DAPI. b: Mono-cultured MSCs still express the membrane antigen CD105 (green). Again no expression of cTnT (red) could be detected. Nuclei are stained with DAPI.

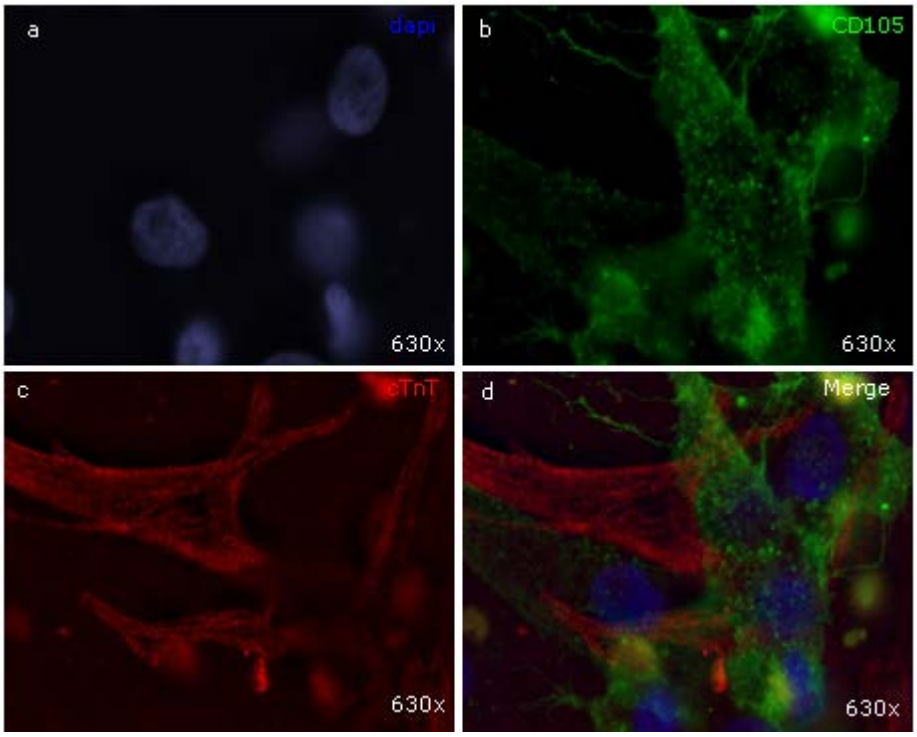


Figure 9: Protein analysis by immunofluorescence on co-cultured MSCs. Co-cultured MSCs express CD105 (green) (b,d). Expression of cTnT (c,d) could be detected in NRCMs (CD105-) (c,d). However, no cTnT expression could be detected in co-cultured human MSCs (d). Nuclei are stained with DAPI (a,d).

3.2.3 Transmission electron microscopy

TEM-analysis of *ex vivo* expanded MSCs revealed that these cells were characterized by large euchromatic nuclei. Few organelles were present within the cell cytoplasm. Some fibril-like stress-fibers consisting of actin were visible close to the cell membrane (Figure 10a). In co-cultured MSCs, fibrils appeared to be more prominent. However, no sarcomeric organization was observed after co-culture (Figure 10b). Figure 10c shows the sarcomeric organization detected in NRCMs.

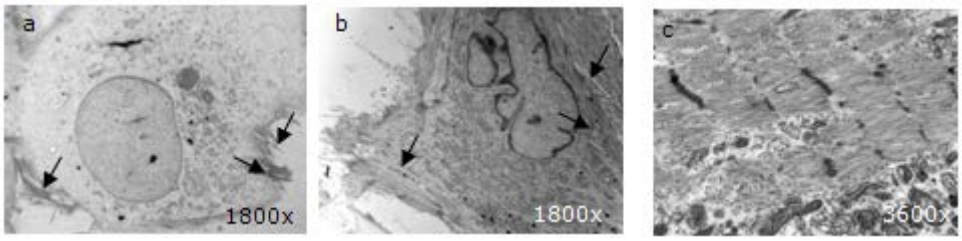


Figure 10: Transmission electron microscopy analysis of *ex vivo* expanded and co-cultured MSCs. TEM-analysis of *ex vivo* expanded MSCs showed that these cells were characterized by large euchromatic nuclei. Few organelles were present within the cell cytoplasm. Some fibril-like stress-fibers (arrows) were observed close to the cell membrane (a). TEM analysis of co-cultured MSCs showed that these cells did not have a contractile apparatus. The fibrils were still present and seemed to be more prominent. However, no sarcomeric organization was detected in the cells (b). Myofibrils exhibited organized striation in NRCMs (c).

3.3 Discussion

In this chapter we investigated the plasticity of human bone marrow-derived MSCs by seeding them on a monolayer of beating NRCMs for three weeks. By adding DMSO or 5-aza cardiac differentiation was further stimulated. A co-culture system between MSCs and NRCMs allows differentiation through gap-junctions (Valiunas et al., 2004; Rose et al., 2008).

MSCs were isolated by adherence to the bottom of the culture flask as described by Pittenger et al. (Pittenger et al., 1999). Although no cells with a MSC phenotype could be detected in the freshly isolated MNC-fraction, flow cytometric analysis of MSCs at passage 4 clearly showed a homogeneous population, negative for markers of the hematopoietic lineage, including leukocyte common antigen CD45 and CD34 but positive for CD49c, CD73 (SH4), CD90 and CD105 (SH2) (Pittenger et al., 1999; Grinnemo et al., 2006). Co-culturing these MSCs induced the expression of both *TnT* and *GATA-4*. While mono-culturing MSCs in normoxic conditioned medium only induced the expression of *GATA-4* but not of *TnT*. This observation suggests that besides soluble factors, physical interaction with NRCMs is a trigger for differentiation. Interestingly, mono-cultured MSCs in hypoxic medium did not express *TnT* nor *GATA-4*. Furthermore, mono- and co-cultured MSCs expressed *Cx43*, the potassium voltage-gated channel *Kv4.3*, the L-type calcium channel *α1c* subunit and the transcription factor *Nkx2.5*. However, no expression of important structural cardiac proteins like *α-actinin, MHC* or *TnI* could be detected by RT-PCR. Despite the fact we detected *TnT* at the RNA level, no expression of cTnT was observed at the protein level by immunofluorescence. This lack of sarcomeric organization was confirmed by TEM analysis of co-cultured MSCs.

The presence of fibril-like stress fibers accounted for the systematic expression of *β-actin* in all culture conditions. Furthermore, the co-cultured MSCs did not express *rNkx2.5*, indicating that the RT-PCR analysis was performed on a pure cell population of human origin and expression of *TnT* is not the consequence of contamination by NRCMs or fusion between MSCs and NRCMs. It cannot be excluded that the *ex vivo* expansion of the MSCs could have influenced their multipotent characteristics in a negative way. However, MSCs of passage 4 were still able to differentiate into adipocytes, osteocytes and chondrocytes, proving that these *ex vivo* expanded MSCs kept their multipotent characteristics. The present results are in accordance with the ones published by Rangappa et al. (Rangappa et al., 2003). They co-cultured MSCs with human CMs and after 48h their MSCs expressed *TnT* and *MHC*, but no expression of *TnI* could be observed. Our results indicate that even after three weeks of co-culture, differentiation could not be enhanced. Despite the fact that this and other reports showed positive immunofluorescent staining of cTnT, the protein itself was not organized or folded in a structural manner as one would expect in functional CMs (Rangappa et al., 2003; Li et al., 2007). In contrast to other reports, incubating our mono- or co-cultured human MSCs with DMSO or 5-aza did not influence the molecular or morphological characteristics of these cells. Ding et al. and Paquin et al. described the differentiation of mouse embryonic stem cells, after incubation with DMSO, into beating CMs (Paquin et al., 2002; Ding et al., 2006). Both papers show that the oxytocin/oxytocin receptor system plays an important role in the cardiomyogenic differentiation of these mouse embryonic stem cells. DMSO stimulates the formation of p204 which triggered the transcription of GATA-4 and Nkx2.5 (Ding et al., 2006). Our data suggest that the function of this system present in murine embryonic stem cells cannot be extrapolated to

human MSCs. Another factor inducing differentiation towards the cardiomyogenic lineage is 5-aza (Xu et al., 2004; Anatonitsis et al., 2007). However, the reported increase in [Ca^{2+}] following stimulation with low [K^{+}] may not be the result of cardiac differentiation (Xu et al., 2004). Our *ex vivo* expanded MSCs already expressed the L-type calcium channel *α1c* and the potassium voltage-gated channel *Kv4.3* without induction of 5-aza. Makino et al. showed that a contractile cardiomyogenic cell line could be generated by incubating immortalized murine bone marrow stromal cells with 5-aza (Makino et al., 1999). Furthermore, their TEM analysis showed well organized sarcomeres and upon transplantation in mice, cardiomyogenic cells fully integrated in the myocardium (Hattan et al., 2005). Several other differentiation protocols also used non-human derived MSCs which expressed *MHC* and *TnI* and even contracted synchronously with NRCMs (Fukuhara et al., 2003; Yoon et al., 2005; Li et al., 2007). These positive differentiation protocols can be explained by species-dependent stem cell plasticity. Indeed this species-dependent phenomenon is not only observed with MSCs but also with CSCs. Messina et al. isolated murine and human CSCs. Murine cardiospheres showed spontaneous beating, whereas human derived cardiospheres only contracted when co-cultured with rat CMs (Messina et al., 2004). Pijnappels et al. reported that after co-culture with NRCMs, neonatal rat MSCs showed a typical cardiac cross striated pattern of *α-actinin* and *TnI* (Pijnappels et al., 2008). Furthermore, these cardiomyogenic differentiated MSCs exhibited spontaneous action potentials. A similar co-culture study with rat embryonic CMs was performed by Rose et al. showing that mice MSCs adopted a cardiomyogenic phenotype, expression of *α-actinin* and *TnI*, but did not generate action potentials (Rose et al., 2008). Although these cells expressed cardiac specific genes they still

retained their MSC phenotype after co-culture, i.e. antigen-expression of CD73, CD90 and CD105. Our results also demonstrated that co-cultured MSCs did express CD105 and therefore still have a stromal phenotype. A second explanation for the limited differentiation capacity of the MSCs in this study could be the fact that the cells are derived from ischemic heart disease patients. Indeed, when human MSCs from healthy volunteers were injected in immunodeficient adult mice, Toma et al. reported expression of several cardiac markers including *TnT*, *β-MHC* and *α-actinin* (Toma et al., 2002). In contrast, Grauss et al. transplanted MSCs derived from ischemic heart disease patients in NOD/scid mice and showed that the engrafted cells differentiated to a more endothelial fate (Grauss et al., 2007). These MSCs did not stain positive for the cardiac markers TnI, α-actinin or MHC. Additionally, several other studies also reported limited cardiomyogenic differentiation capacity of bone marrow stem cells when derived from old or infarcted mice or rats (Wang et al., 2008; Van Tuyn et al., 2008).

In conclusion, the cardiomyogenic differentiation capacity of bone marrow-derived MSCs from patients suffering from ischemic heart disease is limited. After 3 weeks in co-culture with NRCMs, human MSCs express *TnT* and *GATA-4*. However, important genes for the translation of contractile proteins like *MHC*, *α-actinin* and *TnI* were never expressed. These observations were confirmed by immunofluorescence and TEM, showing the lack of sarcomeric organization in these co-cultured MSCs. Therefore, there is no convincing evidence of transdifferentiation of MSCs into functional CMs.

4. Hematopoietic stem cells

4.1 Introduction

4.1.1 Hematopoietic stem cells

In 1963 Becker et al. described the existence of a clonogenic bone marrow cell that gave rise to hematopoietic colonies in the spleen (Becker et al., 1963). It was generally assumed that the continuous supply of both red and white blood cells had to come from one or more progenitor cells. Not only did these progenitors have to differentiate into at least one type of descendant, they also have, in order to preserve their own population, to produce new undifferentiated cells (Lajtha et al., 1962). These two definitions are better known as multipotency and self-renewal. Over the years, HSCs are still defined as cells that are able to differentiate into mature blood cell types and to self-renew. First in 1979 Hodgson et al. and later in 1988 Spangrude et al. purified and characterized mouse HSCs by the use of a variety of phenotypic markers like c-kit and stem cell antigen-1 (Sca-1). Human HSCs are also c-kit positive but also express CD34, CD45 and CD133 (Hodgson et al., 1979; Spangrude et al., 1988). Furthermore, these HSCs are negative for several lineage commitment markers (lin^-). Hao et al. reported that these $CD34^+Lin^-$ cells can differentiate into a variety of cell types like: natural killer cells, T-cells and dendritic cells (Hao et al., 2008). The expression of CD34 is rapidly down-regulated when these cells differentiate into a more mature cell population. This makes that indeed CD34 is a marker to isolate cells with a hematopoietic potency. However, the functional role of CD34 in hematopoiesis is still unclear. Some studies have described a cell adhesion and homing function (Healy et al., 1995). This was also reported in 2005 by Hofmann et al. They showed a better cell retaining in the heart when an enriched CD34+ cell population was injected intracoronary in

comparison to the total MNC fraction (14-39% versus 2%) (Hofmann et al., 2005). However, recently it has been shown that the CD34 antigen also plays a role in myeloid differentiation. Salati et al. showed that the silencing of CD34 resulted in a diminished erythroid differentiation and a more pronounced differentiation towards the granulocyte and megakaryocyte lineage (Salati et al., 2008). Besides CD34, HSCs also express CD133, a 5-transmembrane glycoprotein. This antigen is down-regulated before CD34, indicating that the co-expression of CD34 and CD133 represents a more immature cell type than the $CD34^{+}CD133^{-}$ cells. However, not only HSCs do express these membrane antigens, EPCs are also characterized by the expression of CD34 and CD133. But, EPCs also express vascular endothelial growth factor receptor-2 (VEGFR-2) and are able to take up ac-DiI-LDL (Vasa et al., 2001). Due to the overlap of some markers (CD34, CD133) between these two cell types it is difficult to isolate and study a pure population of HSCs.

4.1.2 Cardiomyoplasticity of HSCs

The idea of using HSCs in order to repair heart function was primarily due to the pro-angiogenic capacity of these cells. This was shown in the rat hind limb ischaemia model (Hamano et al., 2001). Therefore it was thought to be helpful to transplant these cells into the ischemic heart in order to induce angiogenesis and improve blood flow. In 2001, Kocher et al. transplanted human CD34+ in a myocardial infarction rat model (Kocher et al., 2001). They reported the formation of new blood vessels in the infracted area and the proliferation of already existing vasculature. This new vessel formation resulted in a decreased apoptosis in the peri-infarct region and an increased survival of the viable myocardium. The cardiomyogenic differentiation potential of HSCs is relatively

unknown. However, the first publication that clearly demonstrated the multi-lineage transdifferentiation of HSCs transplanted in the infarcted mouse model was published in 2001 (Orlic et al., 2001). $Lin^{-}c\text{-}kit^{+}$ bone marrow cells were isolated and transplanted in the peri-infarct region. After 9 days, 70% of the infarcted area consisted of newly formed CMs. Despite these encouraging results, 2 other reports failed to reproduce these results. Both papers suggested that $lin^{-}c\text{-}kit^{+}$ bone marrow cells adopt only traditional hematopoietic fates (Balsam et al., 2004; Murry et al., 2004). However, in 2005 Patel et al. published one of the first randomized clinical trials transplanting a purified CD34+ bone marrow cell population in patients with ischemic heart failure (Patel et al., 2005). After 6 months the LVEF in the treatment group increased by 15% compared to 6.5% in the control group. Also in 2006 Hendrikx et al. published their findings of a randomized controlled clinical trial (Hendrikx et al., 2006). In this study a subdivision could be made between responders and non-responders in the bone marrow treatment group. The injected MNC fraction of the responder population (increased wall thickening, decreased thallium defect score and better improvement of LVEF) was characterized by a significantly higher percentage of CD34+ cells. Furthermore, Stamm et al. reported that the transplantation of CD133 isolated bone marrow cells was safe and resulted in a significantly improved LVEF from 37.4% to 47.1% (treatment) and from 37.9% to 41.3% (control) (stamm et al., 2007). Furthermore, the left ventricular end diastolic volume decreased in the treament group from 57.1mm to 54.5mm and from 58.9mm to 57.0mm in the control group. These results were all very promising, making it interesting to investigate the cardiomyogenic differentiation potential of human bone marrow-derived HSCs ($CD34^{+}/CD133^{+}$) in order to

design a protocol whereby these cells are isolated and transplanted into the patient's heart.

4.2 Results

4.2.1 Isolation of a pure population of HSCs

In the MNC fraction, 1.1±0.4% of the cells expressed CD34 as assessed by flow cytometry (Figure 11a). About 60±6% of these cells co-expressed CD133 (Figure 11b). Flow cytometric analysis of these CD133/CD34 double positive cells showed co-expression of the panhematopoietic marker CD45 (n=11). Furthermore, more than 90% of these cells also express the stem cell factor receptor c-kit (Figure 11c). To investigate whether a significant fraction of this CD34+CD133+ cells addressed to an EPC-phenotype, a multi-step-gating strategy was performed. Analyzing the LDL uptake capacity of these CD45+CD34+CD133+ cells showed a small sub-fraction of cells that were LDL+CD14- (EPCs) (Figure 11d), 0.03±0.02% within the total MNC fraction and 1.71±0.75% within the CD45+CD34+CD133+ population (n=4).

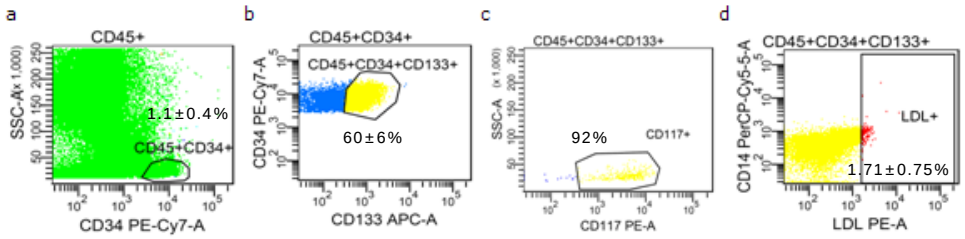


Figure 11: Flow cytometric analysis and isolation of human HSCs (CD34+/CD133+). 1.1±0.4% (n=11) of the mononuclear cells isolated from bone marrow samples expressed CD34 (a). Of this CD34+ population 60±6% co-expressed CD133 (b). More than 90% of these CD34+CD133+ cells were positive for c-kit (c). In this CD45+CD34+CD133+ cell population only 1.17±0.75% cells did correspond to an EPC phenotype (LDL+CD14-) (d) (n=4).

In order to combine the highest purity with the maximum yield of our HSCs isolation, a two step flow sorting method was performed. The enrichment of CD34+ cells resulted in a cell population of which 44±14% expressed CD34. The subsequent purification step of CD34/CD133 double-positive cells from this

enriched CD34+ cell fraction resulted in a 96±1% pure cell population (Figure 12a-e).

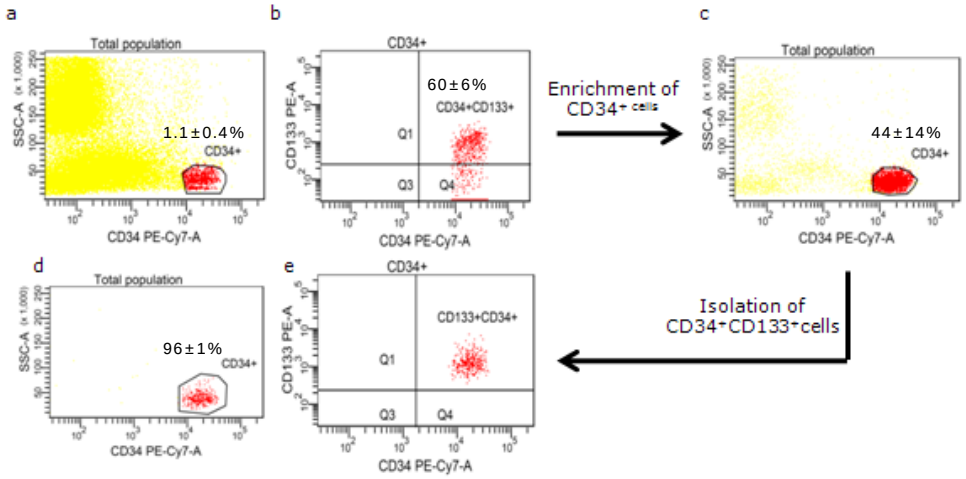


Figure 12: Flow cytometric isolation of human HSCs (CD34+/CD133+). 1.1±0.4% (n=11) of the mononuclear cells expressed CD34 (a). Of this CD34+ population 60±6% co-expressed CD133 (b). After the first isolation procedure, 44±14% of the cells were CD34+ (c). Subsequently, isolation of CD34+CD133+ cells resulted in a 96±1% pure population (d,e).

4.2.2 Co-culturing a pure population of HSCs results in the induction of TnT

To prevent differentiation of stem cells induced by *in vitro* expansion, freshly isolated HSCs were stained with cell tracker green and immediately added to the co-cultures (n=5). After 24h when HSCs attached to the mono-layer of NRCMs, cultures were treated with DMSO or 5-aza. Three weeks later, HSCs were still round in shape. In freshly isolated HSCs, RT-PCR revealed that these cells expressed *Cx43*, *Nkx2.5*, *GATA-2* and *vWF*. After 3 weeks of co-culture expression of *TnT* was induced but *GATA-2* expression was lost while *vWF* remained expressed. However, no expression of the cardiac genes *α-actinin*, *MHC*, *TnI*, *Kv4.3*, *α1c* and *GATA-4* and the endothelial genes, *VEGFR-2* and *e-NOS* could be detected in any condition. In addition, adding DMSO or 5-aza to

the co-cultures did not influence the gene-expression profile of HSCs. Figure 13a and b display RT-PCR results of freshly isolated and co-cultured HSCs of respectively cardiac and endothelial gene expression.

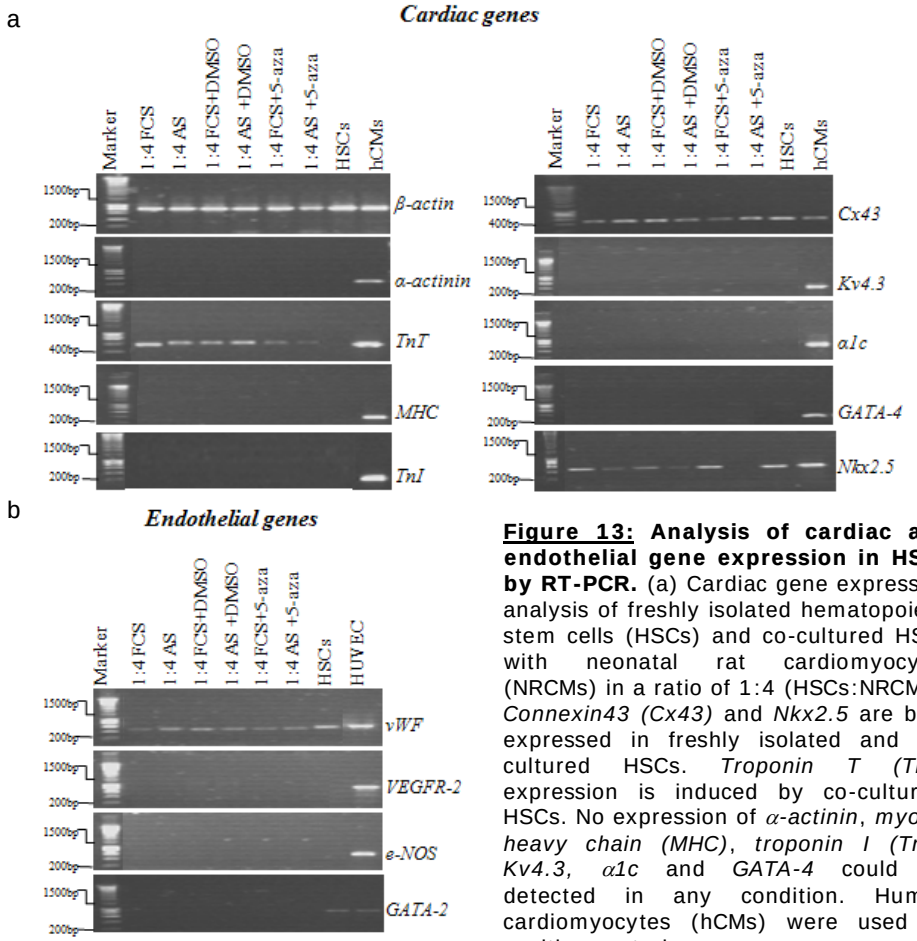


Figure 13: Analysis of cardiac and endothelial gene expression in HSCs by RT-PCR. (a) Cardiac gene expression analysis of freshly isolated hematopoietic stem cells (HSCs) and co-cultured HSCs with neonatal rat cardiomyocytes (NRCMs) in a ratio of 1:4 (HSCs:NRCMs). *Connexin43 (Cx43)* and *Nkx2.5* are both expressed in freshly isolated and co-cultured HSCs. *Troponin T (TnT)* expression is induced by co-culturing HSCs. No expression of *α-actinin*, *myosin heavy chain (MHC)*, *troponin I (TnI)*, *Kv4.3*, *α1c* and *GATA-4* could be detected in any condition. Human cardiomyocytes (hCMs) were used as positive control. (b) Endothelial gene expression analysis of freshly isolated and co-cultured HSCs. *Von Willebrand factor (vWF)* is expressed in freshly isolated and co-cultured HSCs. GATA-2 is only expressed by the freshly isolated HSCs. No expression of *e-NOS* and *vascular endothelial growth factor receptor-2* (*VEGFR-2*) could be detected. Human umbilical vein endothelial cells (HUVEC) were used as positive control.

In order to assess whether HSCs also express the protein cTnT, immunofluorescence was performed on co-cultured HSCs. This staining showed

that HSCs (green nuclei) do not express cTnT on the protein level (Figure 14a, b, c, d) (n=3).

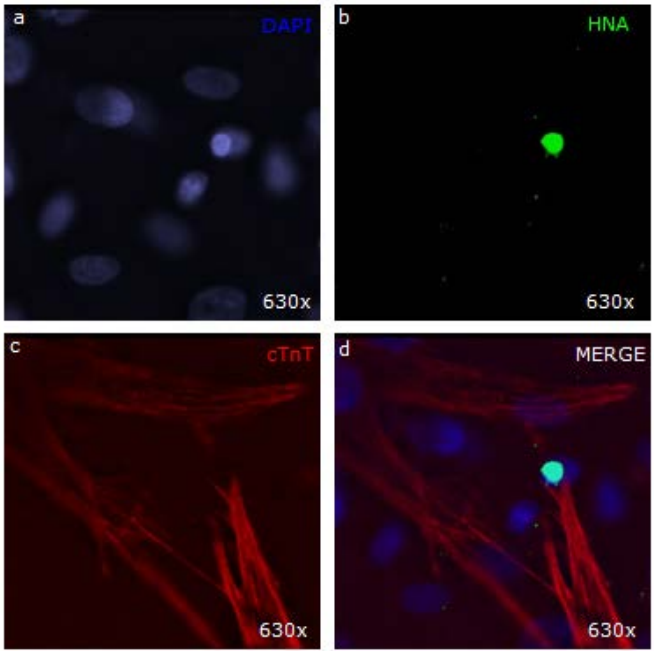


Figure 14: Protein analysis by immunofluorescence on co-cultured HSC. To discriminate human cells from NRCMs, hematopoietic stem cells (HSCs) were specifically stained with human nuclear antigen (HNA: green) (b,d (overlay)). These HSCs stained negative for cardiac troponin T (cTnT) (c,d). NRCMs stained positive for cTnT (c,d). Nuclei were stained with DAPI (a,d).

4.2.3 Transmission electron microscopy of co-cultured HSCs

After co-culture, HSCs are characterized by large euchromatic nuclei with one or more prominent nucleoli (Figure 15a, b). The perinuclear zone contains prominent rough and smooth endoplasmatic reticulum cisternae. In addition, wide collections of clear blisters, vacuoles, and vesicles are observed giving this area a rather multilocular appearance (Figure 15a-e). Numerous mitochondria surround the perinuclear compartment, thereby clearly separating this region from the periphery (Figure 15e). The entire periphery of the cell is poor in

organelles and exhibits a rather electron-lucent appearance. The cell surface often shows undulations and filopodia (Figure 15a, d, e).

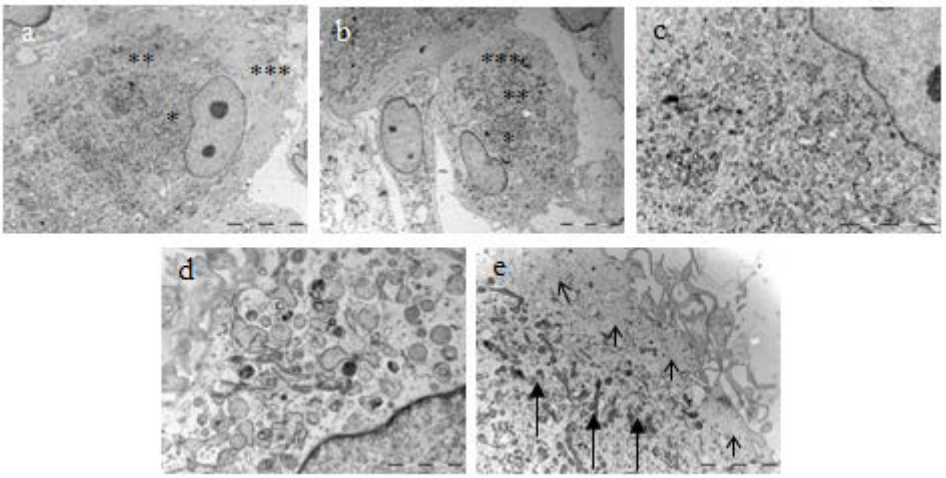


Figure 15: Transmission electron microscopy analysis of co-cultured HSCs. TEM-micrographs of hematopoietic stem cells (HSCs) in co-culture with neonatal rat cardiomyocytes. HSCs show euchromatic nuclei with prominent nucleoli and a thin rim of heterochromatin beneath the nuclear envelope. Distinct subcellular compartments can be observed: a perinuclear region rich in organelles (*), a region containing numerous mitochondria surrounding the perinuclear area (**), and the cell periphery (***) (a, b). Most cell organelles are located at the perinuclear cytoplasm (c). The perinuclear region contains well developed smooth and rough endoplasmatic reticulum and numerous vacuoles (d). Mitochondria are concentrated primarily surrounding the perinuclear space (large arrows). The entire cell periphery is poor in organelles (small arrows). The cell membrane often shows undulations and filopodia (e).

4.3 Discussion

Hematopoietic stem cells are already in clinical use and experimental work has documented that an enriched $CD34^+$ cell population resulted in superior neovascularisation compared to the total MNC fraction (Kawamoto et al., 2006). Hendrikx et al. transplanted the MNC-fraction by intramyocardial injections in the peri-infarct zone of the heart (Hendrikx et al., 2006). Statistical analysis made it possible to distinguish between a responder and a nonresponder population. The responder population was characterized by a significantly higher number and percentage of injected CD34+ cells when compared to the nonresponder population. Despite these results the cardiomyogenic differentiation capacity of bone marrow-derived HSCs (CD34+/CD133+) remains unclear. In this study a pure population of HSCs was co-cultured with NRCMs. A thorough flow cytometric characterization of these HSCs revealed that they were positive for CD34, the panhematopoietic marker CD45, CD133 and c-kit. Moreover only a very small fraction of our CD34+CD133+ cells (<2%) was able to take up LDL indicating a pure HSCs population was used in our experiments. Co-culturing these purified HSCs induced *TnT* but no expression of *TnI, MHC, α-actinin, GATA-4, Kv4.3* or *α1c* could be observed. Immunofluorescent staining for cTnT revealed that this protein could not be detected in the co-cultured HSCs. These findings were in accordance with our TEM-results where no sarcomeric organization could be detected in these co-cultured HSCs. Therefore we can conclude that HSCs, in an *in vitro* co-culture model with NRCMs, do not differentiate into CMs. Despite initial reports suggesting that HSCs could adopt a cardiomyogenic phenotype (Orlic et al., 2001), these findings could not be reproduced by others (Balsam et al., 2004; Murry et al., 2004). Balsam et al. described that in mice, $lin^-c\text{-}kit^+$ cells injected in the border zone of infarcted

hearts, did not express cardiac markers, but instead expressed typical hematopoietic markers like CD45 and Gr-1 (myeloid neutrophil marker) (Balsam et al., 2004). Additionally Murry et al. described only small round cells 1-4 weeks after injection, all staining negative for several cardiac markers and Sondergaard et al. could not detect any human CD34+ cells 96h after transplantation into the infarct border of athymic nude mice (Murry et al., 2004; Sondergaard et al., 2008). Because of the lack of expression of cardiac genes, we investigated whether HSCs differentiated to a more endothelial phenotype. There was no indication that HSCs showed endothelial differentiation. *vWF* could be detected in both co-cultured and freshly isolated HSCs while no expression of *VEGFR-2* and *eNOS* was observed. Interestingly, *GATA-2*, a transcription factor present in EPCs, could only be identified in freshly isolated HSCs but expression was lost during co-culture suggesting that the HSCs progressed to a more mature cell type. The fact that endothelial genes were expressed in the population of freshly isolated HSCs is not surprising since a very small subfraction (<2%) of these CD34+CD133+ cells addressed to an EPC phenotype.

In conclusion, the cardiomyogenic differentiation potential of human bone marrow derived HSCs is very low and only a few cardiac genes (*TnT* and *Nkx2.5*) are expressed after three weeks of co-culture. However, immunofluorescence showed that cTnT is not expressed at the protein level. Furthermore, no endothelial differentiation could be detected as shown by the lack of expression of *e-NOS* or *VEGFR-2*. This indicates, as stated by other authors, that HSCs in a cardiac environment differentiate into an adult hematopoietic phenotype (Balsam et al., 2004; Murry et al., 2004). Therefore, the overall improvement,

reported by the transplantation of the bone marrow-derived MNC-fraction or a purified CD34+ population is probably due to a paracrine effect on the surviving CMs and resident CSCs rather than transdifferentiation of the transplanted progenitors into new CMs.

5. Cardiac stem cells

5.1 Introduction

5.1.1 The heart, a postmitotic organ?

As discussed above, the heart is no longer seen as a post-mitotic and static organ. Several studies have reported the existence of a proliferating subpopulation of adult CMs (Kajstura et al., 1998; Beltrami et al., 2001; Bergmann et al., 2009). These findings triggered the search for an organ specific stem cell present in the heart. If present, this endogenous CSC population could explain the origin of dividing myocytes and their increase in response to cardiac damage.

5.1.2 Cardiac stem cells

C-kit$^+$ cells infiltrate the yolk sack, liver and other organs. All infiltrated organs produce stem cell factor, the ligand for the c-kit receptor (Teyssier-Le Discorde et al., 1999). Similarly, the heart produces stem cell factor as a response to myocardial ischemia (Frangogiannis et al., 1998). Therefore, one could assume that if the heart possesses an endogenous stem cell population, it would express the stem cell factor receptor c-kit. Indeed, Beltrami et al. described the existence of c-kit$^+$ CSC population present in rat hearts (Beltrami et al., 2003). The adult rat myocardium showed the presence of cells that were negative for the expression of blood lineage markers and positive for c-kit, Sca-1 and MDR-1. These cells were distributed throughout the myocardium with a higher density in the atria and the ventricular apex. These c-kit$^+$ CSCs are self-renewing, multipotent and clonogenic. They can differentiate into CMs, smooth muscle cells and endothelial cells *in vitro*, although they fail to contract spontaneously. Furthermore, transplantation of these CSCs in the infarcted ventricle led to engraftment, migration, proliferation and differentiation of CSCs, resulting in the

replacement of dead tissue with new functional myocardium (Beltrami et al., 2003). This group of CSCs can also be isolated from different adult mammalian species like mice, dogs and humans. Indeed, in 2007 Bearzi et al. described the isolation and characterization of c-kit$^+$ CSCs in humans (Bearzi et al., 2007). Flow cytometric analysis of these cells showed no expression of blood lineage markers like CD34, CD45, VEGFR-2, CD31 and CD8. Thus, c-kit$^+$ CSCs can be isolated, expanded and, when administered in a clinically relevant manner, integrate in the adult myocardium.

Despite the fact that the c-kit$^+$ CSCs are the most extensively characterized CSC population, several other types of CSCs are described all expressing a variety of cell membrane antigens. The identification of a c-kit$^-$ CSC was reported by Oh et al. (Oh et al., 2003). In 2003, they reported the existence of a Sca-1$^+$ population in mice that co-expressed CD31 but lacked the expression of c-kit and blood lineage markers. The incubation of these cells with 5-aza resulted in the expression of *α-* and *β-MHC* and *Nkx2.5*. Immunofluorescence showed the presence of sarcomeric actin and TnI. Hereafter, several other studies have confirmed the existence of a Sca-1$^+$ population in adult mice hearts (Matsuura et al., 2004; Pfister et al., 2005). The Sca-1 antigen is considered to only be expressed on rodent cells. However, Goumans et al. described the isolation, expansion and differentiation of human Sca-1$^+$ CSCs (Goumans et al., 2007). Cultured cells were forced to differentiate in the myocardial direction by incubating the cells with TGF-β. These cells spontaneously differentiated into beating CMs with an efficiency of nearly 100%.

In 2005 Laugwitz et al. reported the expression of the lim-homeo transcription factor islet 1 (Isl-1) on c-kit$^-$, Sca-1$^-$ CSCs isolated from newborn mice, rats and

humans (Laugwitz et al., 2005). In co-culture with NRCMs, these cells display a mature cardiac phenotype with stable expression of myocytic markers and also the electrophysiologic characteristics of fully differentiated CMs. Despite these very convincing and hopeful data, Isl-1 cells are only detected in neonatal species. Until now there is no evidence that these cells can be detected and isolated from the adult human heart, limiting their usefulness as a candidate cell in regenerative therapy for heart failure.

In 2004, Messina et al. isolated a new CSC type not based on the expression of a membrane antigen but on the functional properties of CSCs to generate cardiospheres (Messina et al., 2004). These spheres consisted of proliferating c-kit$^+$ cells and more differentiated cells, expressing MHC, TnI and vascular proteins e.g. KDR and CD31. Myocardial differentiation was convincingly shown by spontaneous beating of mouse derived cardiospheres. Cardiospheres derived from human cardiac tissue did not contract spontaneously. However, when co-cultured with NRCMs these human spheres started to beat. In 2007, Smith et al. modified the original cardiosphere culture protocol allowing to generate more cells with less starting material (Smith et al., 2007). The generated cardiospheres were isolated and replated resulting in the generation of a mono-layer of CDCs. When cardiomyogenic differentiation was stimulated by co-culturing these CDCs with NRCMs, they showed spontaneous intracellular calcium transients (I_{Ca}) and expression of TnI. After transplantation in an immunodeficient mouse model of MI, the differentiation of CDCs forming islands or continuous bands of human tissue was shown.

A variety of CSCs has been described, all expressing different antigens. Despite these differences all CSCs described above are able to differentiate down the

cardiomyogenic lineage *in vivo* or *in vitro* as shown by the expression of several cardiac specific markers by RT-PCR or immunofluorescence. The purpose of this study was to examine the phenotype and myocardial differentiation potential of human c-kit$^+$ CSCs and CDCs and compare these results with the differentiation potential of human MSCs under the same controlled conditions. Hereby, we wanted to evaluate which of these cell types would be the best candidate to be used in a clinical setting.

5.2 Results

5.2.1 Patient characteristics

Table 4 shows the patient characteristics of the different stem cell groups. No significant differences could be detected among the three groups.

Table 4: Patient characteristics

	MSC Group (n=5)	CDC Group (n=5)	c-kit Group (n=5)	*P*
Age	65.8±9.6	69±5.7	58.4±10.3	0.21
Male	3	2	5	0.12
Risk factors				
Weight	79.8±21.5	77.6±12.3	88.8±10.7	0.33
Body mass index	29.2±4.6	27.3±3.1	27.8±2.6	0.93
Last creatinin level preop	0.9±0.2	1±0.2	1.1±0.3	0.27
Smoker	1	1	3	0.30
Family history of CAD	3	3	2	0.76
Diabetes	1	0	1	0.56
Hyperlipidemia	5	3	3	0.25
Renal dysfunction	0	2	1	0.28
Hypertension	5	4	2	0.09
Chronic lung disease	1	0	0	0.34
Peripheral vascular disease	0	1	3	0.09
Cerebrovascular disease	0	1	2	0.28
Pre-operative cardiac status				
Myocardial infarction	1	0	2	0.28
Congestive heart failure	1	1	0	0.56
Angina	4	2	4	0.30
Arrhythmia	1	1	1	1
Classification NYHA (I/II/III)	(0/3/2)	(0/4/1)	(1/0/4)	0.10
Pre-operative medicine				
Beta-blockers	4	5	5	0.34
Nitrates PO	1	2	2	0.74
Nitrates IV	1	0	2	0.28
Diuretics	1	1	0	0.56
ACE-inhibitors	3	2	1	0.43
Ca-Antagonists	1	1	0	0.56
Antiarrhythmias	0	0	1	0.34
Lipid lowering	4	2	2	0.34
Aspirin	5	4	2	0.09
Other anti platelets	1	0	2	0.28
N vessel (0/1/2/3)	0/1/2/2	1/0/2/2	1/0/1/3	0.72
Surgical Procedure				
CABG/Valve/Ablation/DOR	4/0/0/1	4/1/0/0	4/0/1/0	

5.2.2 Isolation of CDCs

For isolation of CDCs, small fragments of right atrial appendages removed during routine cardiac surgery were placed in fibronectin coated plates resulting in an outgrowth of cells (n=5) (Figure 16a). On top of this cell monolayer, clear phase bright cells appeared that have been described to be sphere forming progenitor cells (Messina et al., 2004; Fig 16b; arrows). In order to isolate these cells, a mild trypsinisation was performed and the obtained cells formed cardiospheres after 5-7 days when cultured in CGM (Fig 16c). These spheres were harvested, replated (Fig 16d) and when *ex vivo* expanded in X-vivo 15 medium, resulted in a mono-layer of CDCs (Fig 16e).

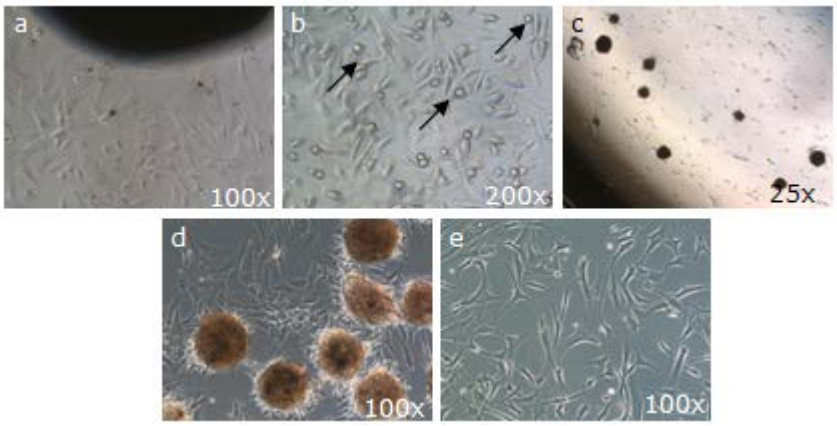


Figure 16: Light microscopic analysis to obtain cardiosphere derived cells (CDCs). After 1.5-2 weeks a monolayer of outgrowth cells was generated (a). Small phase bright cells migrated over this monolayer (b; arrows) and generated cardiospheres after isolation (c). These spheres reattached and outgrowth of CDCs started after 2-3 days (d). After passage 1 no spheres could be detected and a monolayer of CDCs was obtained (e).

5.2.3 Characterization and isolation of c-kit⁺ CSCs in cardiac explants outgrowth

A monolayer of cells grown out of the cardiac explants was generated after 1.5-2 weeks. In this outgrowth a small percentage of CD45⁺ cells could be detected (Figure 17 b,d). These CD45⁺ cells did not stain positive for c-kit (Figure 17 c,d) (n=3).

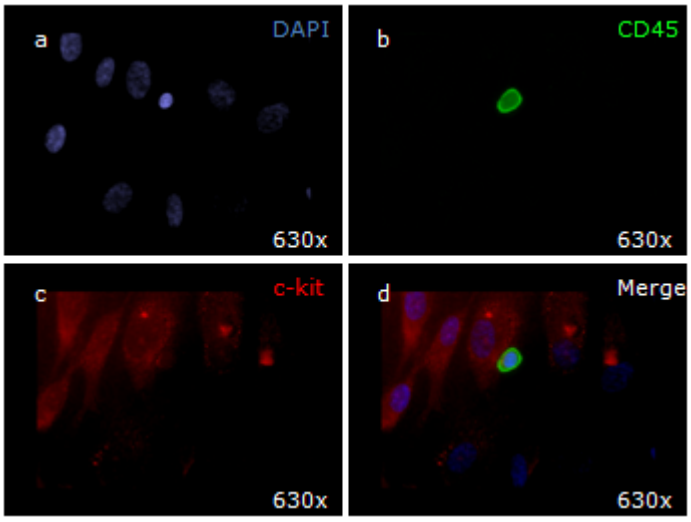


Figure 17: Immunofluorescent staining on cells grown out of cardiac tissue (c-kit; CD45). Cells incubated with CD45-FITC (b,d) and c-kit-PE (c,d). DAPI was used to stain nuclei (a,d). Outgrown c-kit⁺ cells do not coexpress CD45 and c-kit.

Double staining with CD105 (FITC) and c-kit (PE) revealed that almost all cells in the outgrowth are CD105⁺ and that a subpopulation of these cells co-expresses c-kit (Figure 18 a-d) (n=3).

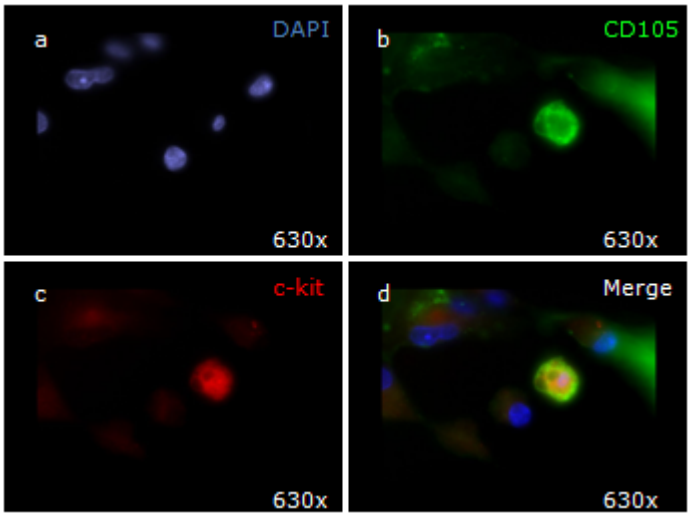


Figure 18: Immunofluorescent staining on cells grown out of cardiac tissue (c-kit; CD105). Cells are incubated with CD105-FITC (b,d) and c-kit-PE (c,d). 95% of the cells do express CD105 and a subpopulation coexpresses c-kit (d). Nuclei were stained with DAPI (a,d).

To obtain a pure culture of c-kit$^+$ CSCs, the explant outgrowth cells were harvested and c-kit$^+$ cells (0.2%±0.1%) (Figure 19a), negative for CD45 and CD34 (Figure 19b), were purified by flow sorting and subsequently *ex vivo* expanded (Figure 19c) (n=5).

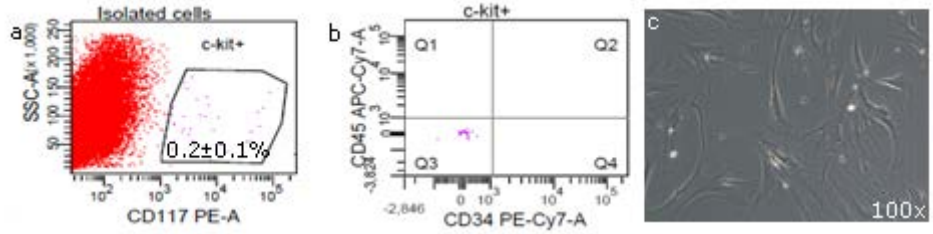


Figure 19: Isolation of c-kit$^+$ CSCs from cardiac explant culture. Of the total population, only 0.2±0.1% of the cells expressed CD117 (c-kit) (a). All these cells were negative for CD45 and CD34 (b). After isolation these cells were *ex vivo* expanded (c).

In order to assess whether these freshly isolated c-kit$^+$ CSCs were indeed stem cells, the clonogenic potential of these cells was analyzed. In total, 960 cells were sorted out and 18 colonies were formed after 2.5 weeks, resulting in a

cloning efficiency of 1.87% (Figure 20a-c). However, 9 colonies stopped proliferating after 4 weeks. After 2 months 9 colonies were still in culture, resulting in a clonogenicity of 0.9%.

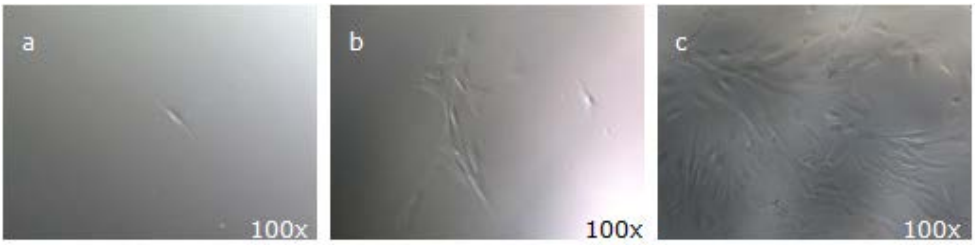


Figure 20: Clonogenicity of freshly isolated c-kit⁺ CSCs. In total, 960 cells were isolated. Single cell disposition was examined after 24h (a). After 2.5 weeks 18 clones were generated (b). After 2 months 9 clones were still in culture, resulting in a clonogenicity of 0.9% (c).

5.2.4 Phenotypical and functional comparison between cultured MSCs, CDCs and c-kit⁺ CSCs

Light microscopic examination of *ex vivo* expanded MSCs (Figure 21a), CDCs (Figure 21b) and c-kit⁺ CSCs (Figure 21c) showed that all three cell types displayed a very similar fibroblastic morphology.

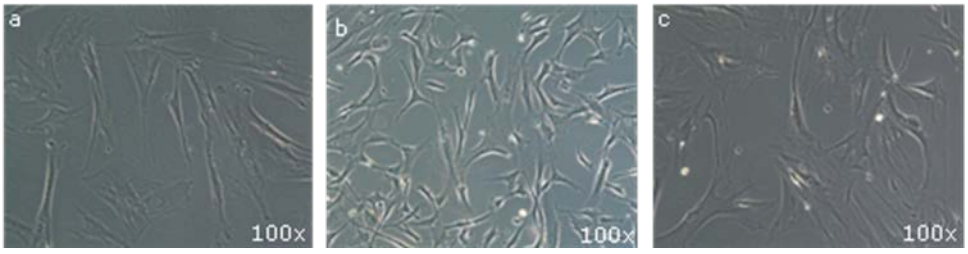
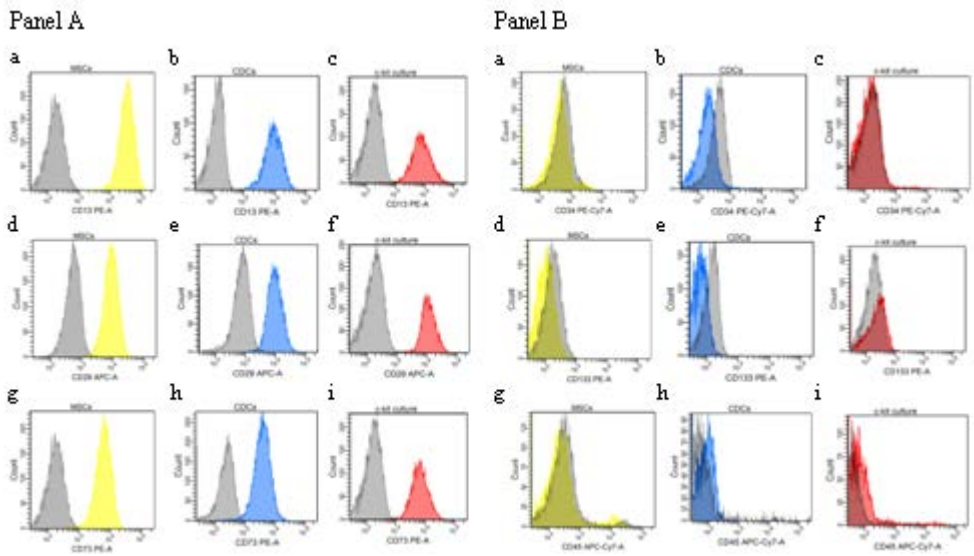


Figure 21: Morphological comparison between MSCs, CDCs and c-kit⁺ CSCs. Light microscopic analysis of cultured MSCs (a), CDCs (b) and c-kit⁺ CSCs (c) shows that these cells all display a very similar fibroblastic morphology.

In order to identify a parameter to distinguish between the three cell types, the antigen expression profile of these *ex vivo* expanded cells was assessed by flow cytometry, using a large series of antibodies (Table 1) (n=3 for each stem cell type). These results revealed that the three *ex vivo* expanded cell types display only limited phenotypical differences. For instance, all cell types expressed the membrane antigens CD13 (Figure 22Aa-c), CD29 (Figure 22Ad-f) and CD73 (Figure 22Ag-i) and were uniformly negative for CD45 (Fig 22Bg-i) and the hematopoietic stem cell markers CD34 (Figure 22Ba-c) and CD133 (Figure 22Bd-f) Several other antigens (Table 1), mostly markers of the hematopoietic lineage, could not be detected on the membrane surface of the three cell types (data not shown).



<u>Figure 22:</u> Flow cytometric analysis of cultured MSCs, CDCs and c-kit$^+$ CSCs
Panel A: MSCs, as well as CDCs and c-kit$^+$ CSCs express CD13 (a-c), CD29 (d-f) and CD73 (g-i). Panel B: All three cell types are uniformly negative for the hematopoietic markers CD34 (a-c) and CD133 (d-f), the leukocyte common antigen CD45 (g-i). grey histogram = isotype control.

Strikingly, although we started from a pure c-kit$^+$ cell population to obtain CSCs, *ex vivo* expansion of these cells resulted in the complete loss of c-kit expression (Figure 23c).

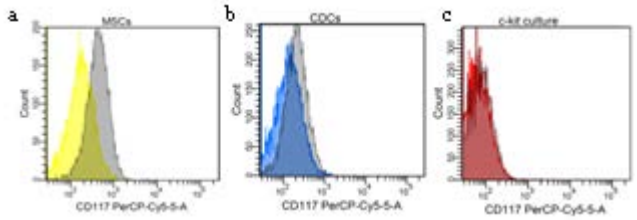


Figure 23: Flow cytometric analysis of the c-kit expression of *ex vivo* expanded MSCs, CDCs and c-kit$^+$ CSCs. All three cell types, MSCs (a), CDCs (b) and c-kit$^+$ CSCs (c), were negative for CD117 after *ex vivo* expansion.

Despite these large phenotypic similarities between MSCs and cardiac progenitors we were able to detect a membrane antigen in which these cells differ from each other. While MSCs presented CD140b on their membrane, this antigen could not be detected on CDCs or c-kit$^+$ CSCs (Figure 24a-c).

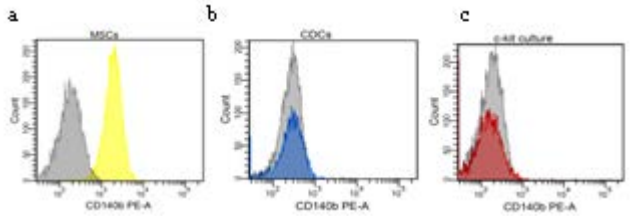


Figure 24: Flow cytometric analysis of the CD140b expression of *ex vivo* expanded MSCs, CDCs and c-kit$^+$ CSCs. The only phenotypic difference between MSCs and both types of CSCs is shown by the expression of CD140b. MSCs express this antigen on their membrane (a) while CDCs (b) and c-kit$^+$ CSCs (c) do not.

This phenotypic difference was confirmed by the fact that antibody-blocking of the CD140b receptor inhibited the proliferation of MSCs. After blocking, the percentage of proliferative MSCs (n=3), as assessed by Ki-67 expression, decreased considerably from 34.3±10.4% to 13.4±5.2%. On the other hand, the percentage of proliferative CDCs (n=2) and c-kit$^+$ CSCs (n=2) was not

affected by the antibody, 28.9±8.7% vs 29.1±5.5% and 29.4±9.5% vs 28.3±5.7% respectively (Figure 25).

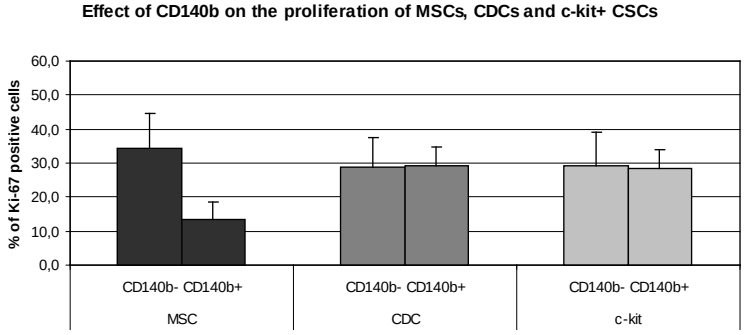


Figure 25: Analysis of proliferation after incubation with CD140b. After the incubation with CD140b the percentage of proliferative MSCs decreased significantly from 34.3±10.4% to 13.4±5.2%. However, the proliferation of CDCs and c-kit$^+$ CSCs was not effected (28.9±8.7% vs 29.1±5.5% and 29.4±9.5% vs 28.3±5.7% respectively).

Furthermore, we compared the adipogenic and osteogenic differentiation capacity of these cardiac progenitors and MSCs. It is well known that *ex vivo* expanded MSCs are able to differentiate into adipocytes, osteocytes and chondrocytes (Pittenger et al., 1999; Prockop et al., 2001). In contrast, when c-kit$^+$ CSCs (n=3) or CDCs (n=3) were stimulated to differentiate down to the adipogenic and osteogenic lineage, no signs toward differentiation could be observed (Figure 26). Only negative background staining was obtained in the histochemical staining oil-red O (Figure 26a) and Alizarine Red (Figure 26b). Immunofluoresent staining for FABP-4 (Figure 26c) and osteocalcin (Figure 26d) were also negative.

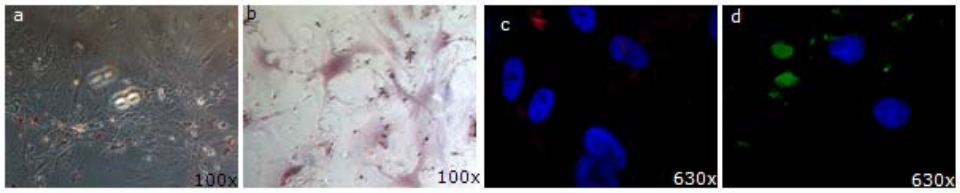


Figure 26: Multilineage differentiation assay. As opposed to MSCs, CSCs failed to differentiate either into adipocytes or osteocytes and cells had a senescent-like morphology. Only negative background staining of Oil-red-O (a) and Alizarin-red (b) was obtained. Immunofluorescent analysis for FABP-4 (c) and osteocalcin (d) showed a similar staining pattern as in the negative control (only secondary antibody) and therefore judged negative.

5.2.5 Sphere formation is not a unique stem cell characteristic

Sphere formation has not only been described as a way for isolating cardiac progenitors, it is also considered to be a marker for stemness and has previously been reported for rat, dog and human (Smith et al., 2007; Bartosh et al., 2008; Kawaguchi et al., 2008). Therefore, we wanted to compare the stem cell characteristics of human c-kit$^+$ CSCs and MSCs in their ability to form cardiospheres. As a negative control, primary cultures of adult human dermal mesenchymal cells and myofibroblasts from colon were cultured in CGM. Surprisingly, all cell types were able to form spheres in this medium (Fig 27a-d). Furthermore, the number of spheres formed by these cells was comparable. Out of $3x10^4$ cells, MSCs formed 147±10 spheres (Figure 27a; n=3), while c-kit$^+$ CSCs formed 102±60 spheres (Figure 27b; n=3). Similar results were obtained by the negative control cells, approximately 135 spheres were generated by human dermal mesenchymal cells (Figure 27c) and 257 spheres by myofibroblasts (Figure 27d).

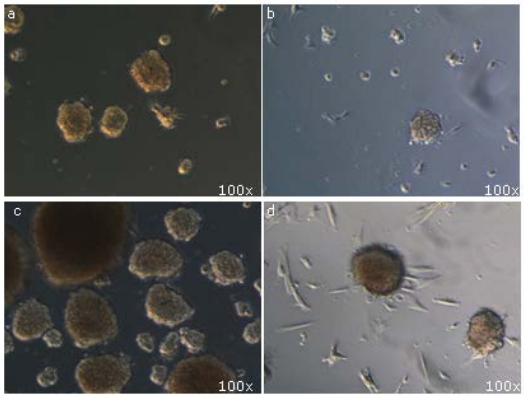


Figure 27: Cardiosphere forming assay. All four cell types, MSCs (a), c-kit$^+$ CSCs (b), cultured dermal mesenchymal cells (c) and myofibroblasts (d) were able to form spheres. The amount of spheres formed and the morphology of these spheres were comparable between the different cell types.

5.2.6 CDCs and c-kit$^+$ CSCs display a greater myocardial differentiation potential compared to MSCs

To determine which cell type has the best capability to differentiate into CMs, the cardiomyogenic differentiation potential of all three cell types was tested by setting-up mono-cultures in medium with low serum concentration (0% or 2%). Since the cardiomyogenic inducing characteristics of TGF-β have been described in some reports (Goumans et al., 2007; Gwak et al., 2009), different concentrations of TGF-β were used as additional cardiomyogenic stimulus (n=3 for each stem cell type). Differentiation was assessed by analyzing the expression of cardiac-specific genes by RT-PCR (Figure 28). Regardless of the differentiation medium used, all three stem cell types expressed *α-actinin*, the gap-junction protein *Cx43*, the voltage-gated potassium channel *Kv4.3* and the L-type Ca^{2+}-channel *α1c* subunit. In contrast to MSCs, both types of CSCs expressed *TnT* and *GATA-4*, a transcription factor activating the expression of sarcomeric genes like *troponin C* and *α-* and *β-MHC,* in all conditions (Ip et al.,

1994; Charron et al., 1999). TGF-β only influenced the myocardial differentiation of MSCs by inducing *TnT* expression. However it failed to activate the expression of *GATA-4*. Despite these promising results, all three stem cell types failed to express *Nkx2.5* and some important structural genes like *MHC* and *TnI* (Figure 28). *β-actin* was used as an internal control.

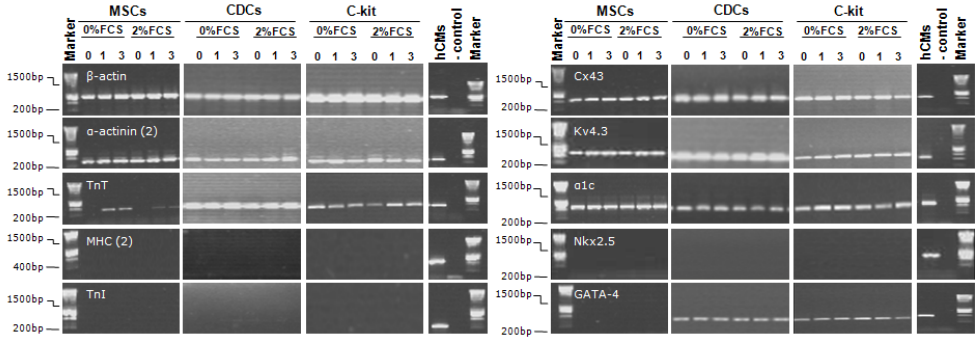


Figure 28: Myocardial differentiation of mono-cultured MSCs, CDCs and c-kit⁺ CSCs. Gene expression of mono-cultured MSCs, CDCs and c-kit⁺ CSCs in absence or presence (2%) of FCS and TGF-β (concentrations are indicated as 0, 1 or 3 ng/ml). Expression of *β-actin* (internal control), *α-actinin*, *Cx43*, *Kv4.3* and *α1c* could be detected in all conditions. Regardless of the differentiation medium used, CDCs and c-kit⁺ CSCs also expressed *TnT* and *GATA-4* while MSCs only expressed *TnT* under the influence of TGF-β. However, no expression of *GATA-4* could be detected in these mono-cultured MSCs. The expression of *MHC*, *TnI* and *Nkx2.5* could not be detected in any condition. Human CMs (hCMs) were used as positive control.

To investigate if a cardiac micro-environment is required for an enhanced CM differentiation, the three types of stem cells were cultured on a layer of contractile NRCMs in low serum concentrations (n=3 for each stem cell type). Since TGF-β improved cardiac differentiation of MSCs in mono-culture, this agent was also used in the co-cultures. All types of co-cultured stem cells showed an identical gene expression profile as shown by RT-PCR (Figure 29). Like in the mono-cultures, cells expressed *α-actinin, TnT, Cx43, Kv4.3, α1c* and *β-actin* (internal control). The presence of NRCMs stimulated the expression of *MHC* in all three cell types. Furthermore, expression of *GATA-4* was induced in

MSCs after 1 week. Although culturing stem cells in a heart micro-environment improved myocardial differentiation, no expression of *Nkx2.5* or *TnI* could be detected.

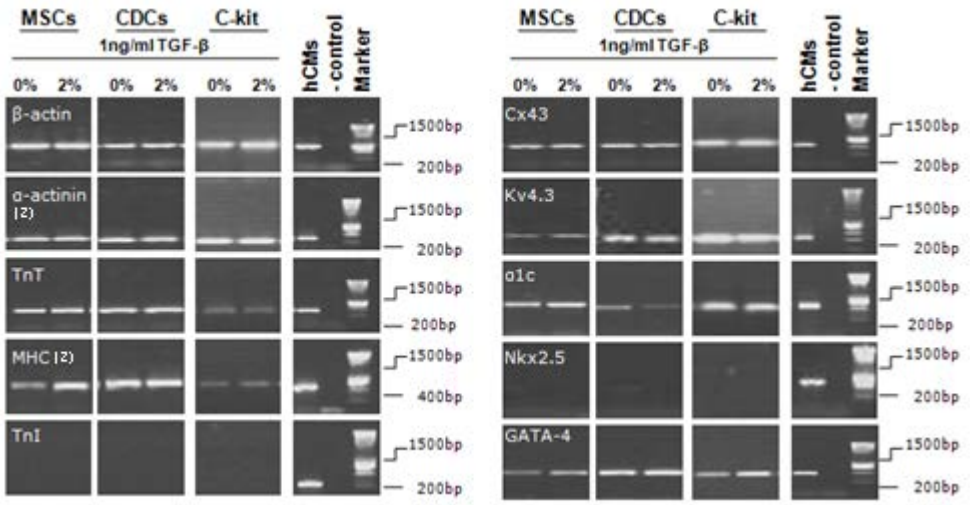


Figure 29: Myocardial differentiation of co-cultured MSCs, CDCs and c-kit⁺ CSCs. All cell types expressed *β-actin*, *α-actinin*, *TnT, MHC*, *Cx43*, *Kv4.3*, *α1c* and *GATA-4*. No expression of *TnI* or *Nkx2.5* could be detected in any condition. hCMs were used as positive control.

Since the transcription of genes is no guarantee that the corresponding protein is present in an organized fashion or even expressed at all, immunofluorescence was performed to detect cTnT and cTnI (n=3 for each stem cell type). This revealed that despite the expression of *TnT* on the RNA level no cTnT could be detected in any condition of mono-cultured stem cells (data not shown). In co-culture conditions however, both types of CSCs expressed this protein in an organized manner showing sarcomeric structures already after 1 week (Figure 30e-l). In accordance with previous results, MSCs still failed to express cTnT in co-culture (Figure 30a-d).

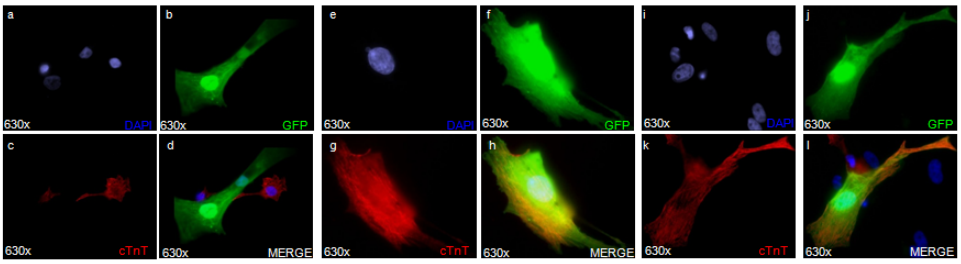


Figure 30: Analysis of cTnT expression in co-cultured stem cells by immunofluorescence. Co-cultured GFP-transfected MSCs (green) (b,d) do not express cTnT (red). Both types of CSCs, CDCs (f,h) and c-kit$^+$ CSCs (j,l) do express cTnT. Cell nuclei are stained with dapi (blue).

Concurrent with the RT-PCR results all three cell types failed to express cTnI in mono-culture (data not shown) as well as in co-culture (Figure 31a-l).

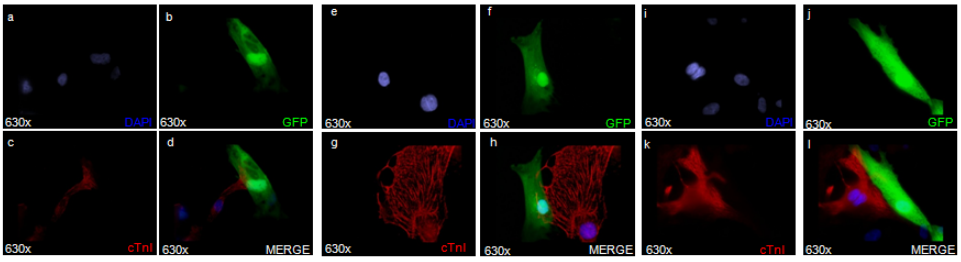


Figure 31: Analysis of cTnI expression in co-cultured stem cells by immunofluorescence. All three cell types, MSCs (a-d), CDCs (e-h) and c-kit$^+$ CSCs (i-l) failed to express cTnI. Nuclei are stained with DAPI (blue).

5.3 Discussion

In this chapter the phenotypical and functional characteristics of bone marrow-derived MSCs are compared with those of two types of well defined CSC populations namely CDCs and c-kit$^+$ CSCs. The comparison was based on an extended flow cytometric as well as a functional analysis on the molecular and the protein level.

After an AMI, the patient's cardiac function can be severely diminished. At present a range of pharmacological and surgical treatments are used to treat these patients. However, these therapies only delay remodeling of the heart and eventually heart failure will develop. Regenerative stem cell therapy is a promising approach to repair the injured heart after a MI. Over the last years a variety of clinical trials, transplanting the bone marrow-derived MNC fraction in acute or chronic infarcted patients, have been performed (Wollert et al., 2004; Janssens et al., 2006; Hendrikx et al., 2006; Schächinger et al., 2006). All these studies reported no or only a limited improvement in left ventricular function. One possible explanation could be the very low percentage of stem cells present in the bone marrow-derived MNC fraction. In order to augment the effect of stem cell therapy, one approach could be to transplant a more enriched stem cell population. However, the ideal stem cell to be used in this setting has still to be determined. For example, CD133$^+$ purified HSCs have already been used in clinical trials but showed only limited improvement of cardiac function (Manginas et al., 2007; Klein et al., 2007). Moreover, others and our group have previously shown that bone marrow-derived MSCs do not differentiate into functional CMs after co-culture with NRCMs (Rose et al., 2008; Koninckx et al., 2009). The inability of these cells to differentiate down the cardiomyogenic lineage was

shown by the failure to express *MHC, TnI* and *α-actinin* by RT-PCR. Also no sarcomeric organization could be detected by immunofluorescence or electron microscopy. In the present study we showed that TGF-β enhances the myocardial differentiation of bone marrow-derived MSCs by the expression of *TnT* in mono-culture and *MHC* in co-culture. Furthermore, co-cultured MSCs expressed the transcription factor *GATA-4* but no expression of *Nkx2.5* could be detected, which is normally activated by binding of *GATA-4* at the Nkx2.5 promoter region (Lien et al., 1999). The failure to induce this expression could be either due to the short co-culture time or simply by the fact that no functional protein is present. Indeed, the improved cardiac differentiation of MSCs on RNA-level was not associated with the corresponding proteins. For instance, cTnT could not be detected by immunofluorescence. Based on these results we can conclude that bone marrow-derived MSCs still only display a limited cardiomyogenic differentiation potential and are probably not the ideal stem cell type to regenerate the injured heart. Therefore, the myocardial differentiation capacity of other cell types are currently under investigation.

Increasing evidence emerges that the heart is not a static organ but contains some intrinsic regenerative capacity through the proliferation of adult CMs and differentiating CSCs (Kajstura et al., 1998; Beltrami et al., 2003; Messina et al., 2004; Bearzi et al., 2007; Bergmann et al., 2009). These CSCs can be isolated, *ex vivo* expanded and are able to integrate in the myocardium to improve cardiac function (Smith et al., 2007; Bearzi et al., 2007). The isolation of the adult human CSCs is either based on the expression of the membrane antigen c-kit or by their ability to form cardiospheres. To date, no clear evidence exists if these cells are indeed intrinsic stem cells from the heart. Several studies ascribe

MSC properties to human CSCs and vice versa (Tateishi et al., 2007; Itzhaki-Alfia et al., 2009) but phenotypical and functional comparison between these two CSC types and bone marrow-derived MSCs had not yet been reported. In our study, phenotypic characterisation showed that all three cell types were uniformly positive for CD13, CD29 and CD73 and negative for CD34, CD45 and CD133, indicating that the markers normally used to characterize MSCs are also present on human CSCs. Although upon isolation CSCs were uniformly positive for c-kit, during *ex vivo* expansion they lost their c-kit expression. These data are in contradiction with earlier reports stating that after expansion these cells still express the stem cell factor receptor c-kit (Smith et al., 2007; Bearzi et al., 2007). On the other hand, Tateishi et al. also reported a low c-kit expression in clonally derived CSCs (Tateishi et al., 2007). The reason for this discrepancy is unclear but it is possible that during the culture time the c-kit receptor is internalized, a phenomenon also described by Jahn et al. (Jahn et al., 2002). Hendrikx et al. also noticed that overnight incubation of bone marrow-derived MNCs in Teflon Bags was accompanied by a decrease in CD34 expression (personal communication). In addition, it is well known that the c-kit receptor is susceptible for trypsinisation. Although several products and protocols have been tested for harvesting the cells, we were never able to detect the c-kit receptor on the membrane.

Strikingly, thorough flow cytometric analysis revealed that MSCs can be discriminated from cardiac progenitor cells by the expression of CD140b on their membrane. CD140b is a tyrosine kinase receptor for platelet-derived growth factor-beta polypeptide (PDGFRbeta), and is mostly used as a marker for cells from mesenchymal origin (Vogel et al., 2003; Gargett et al., 2009). PDGFRbeta

activates PI-3 which in turn is part of the AKT pathway, known to be involved in MSC proliferation (Fierro et al., 2007). Tateishi et al. reported that proliferation of human CSCs was also mediated via the AKT pathway and therefore resembled MSCs (Tateishi et al., 2007). However, the receptor mediating this activation had not been determined. Our results showed that CD140b antibody inhibited only the proliferation of MSCs but did not have any influence on human CSC proliferation. The AKT pathway can be activated by all kinds of receptors like insuline like growth factor-1 receptor or EGF receptor (Urbanek et al., 2005; Fierro et al., 2007). Indeed, Tateishi et al. stimulated CSC proliferation using EGF/bFGF, indicating that the AKT pathway in CSCs is probably activated by the EGF/bFGF receptors (Tateishi et al., 2007).

Besides this phenotypic difference a clear functional distinction could be made between the MSCs and both types of CSCs. In accordance to previously published data, *ex vivo* expanded MSCs were able to differentiate into adipocytes and osteocytes (Pittenger et al., 1999; Prockop et al., 2001), while both populations of CSCs could not. The failure of CSCs to differentiate into adipocytes and osteocytes is in contradiction with recently published data by Itzhaki-Alfia et al. (Itzhaki-Alfia et al., 2009). These apparently conflicting results can be explained by the heterogeneous population that was used for their multilineage differentiation assay. Only 22% of their total cell population expressed c-kit. Therefore it can not be excluded that contaminating cells like MSCs are responsible for the positive differentiation results. In the present study, adipogenic and osteogenic differentiation was induced on a purified homogeneous c-kit$^+$ CSC population. Our results indicate that cardiac progenitor cells behave differently from MSCs and therefore suggestively are a different cell

population. However, a true specific cardiac progenitor marker still has to be identified.

It is known that CDCs as well as c-kit$^+$ CSCs are able to differentiate into functional CMs (Smith et al., 2007; Bearzi et al., 2007). Indeed, in the present study CSCs clearly displayed a greater myocardial differentiation capacity compared to MSCs. As opposed to MSCs, CDCs as well as c-kit$^+$ CSCs already expressed *TnT* and *GATA-4* in mono-culture although these differences in gene expression profile disappeared after co-culture. Moreover, at the protein level, MSCs failed to express cTnT after three weeks of co-culture, while CDCs and c-kit$^+$ CSCs expressed this protein in an organized sarcomeric fashion. These data indicate that both types of CSCs have a better cardiomyogenic differentiation capacity and are therefore better candidates for cardiac regenerative therapy. However, these data are based on *in vitro* experiments only. The *in vivo* behaviour of these cells should be studied in the larger animal model because in rodents, the hydrdynamics as well as the heart rate is totally different when compared to humans. Most of the positive myocardial differentiation data involving MSCs are obtained after transplantation of these cells in rats or mice (Hattan et al., 2005; Yoon et al., 2005). However, when MSCs were transplanted in the chronic infarction swine model, the cells differentiated into vascular smooth muscle cells or endothelial cells but, as in accordance with the results presented here, not into CMs (Zhou et al., 2009).

Despite the fact that CDCs and c-kit$^+$ CSCs were obtained by two different isolation protocols, no phenotypical or functional distinction could be made between these two types of CSCs. Both cell types expressed the same membrane antigens as assessed by flow cytometry and they both failed to

differentiate into adipocytes and osteocytes. Furthermore, in mono- as well as in co-culture these cells displayed the same gene and protein expression profile. Based on these data we hypothesize that CDCs and c-kit$^+$ CSCs are derived from the same ancestor cell or even just are the same cells. As shown by Andersen et al. the phase bright cells, assumed to be cardiosphere-forming cells (Messina et al., 2004), are actually CD45+ and unable to form cardiospheres (Andersen et al., 2009). We have also shown that CD45+ cells present in outgrowth cells from heart fragments are negative for c-kit (Koninckx et al., 2008). Isolation of these phase bright cells is based on a method of mild trypsinisation which can not exclude accidental contamination of fibroblast like cells. All these data support the theory that cardiospheres are probably generated by *"contaminating"* c-kit$^+$ fibroblast-like cells from the explant outgrowth. As a consequence, the cardiosphere forming cells and c-kit$^+$ CSCs could be similar or at least originate from the same precursor. Furthermore, one should be careful using sphere formation as a functional assay to discriminate between cell types. In this study we showed that not only cardiac progenitor cells are able to form spheres. Heart tissue unrelated cells like bone marrow-derived MSCs and even primary differentiated cells like human dermal mesenchymal cells and myofibroblasts from colon were able to grow into spheres with a similar morphology when cultured in cardiosphere specific medium. Accordingly, in a recent publication Andersen et al. showed that murine cardiospheres are not a source of cardiac progenitor cells but arise by aggregation of multiple cells instead of clonal cells (Andersen et al., 2009). These findings strongly challenge the use of this assay to characterize the stemness of a certain cell type and caution has to be taken to isolate stem cells based on this functional assay. As a matter of fact several reports describe cardiospheres as a heterogeneous cell population consisting of

CSCs, MSCs and fibroblast like cells (Smith et al., 2007; Andersen et al., 2009). The data presented here suggest that a pool of stem cells is present in the human adult heart. This CSC population is phenotypically and functionally distinct compared to MSCs. CSCs display a greater cardiomyogenic differentiation potential compared to bone marrow-derived MSCs. In case cardiomyogenic differentiation is aimed for in regenerative cardiac therapy, CSCs would be the preferred choice. However, researchers should decide based on their discretion which isolation protocol best fits their expertise and infrastructure. We have shown that despite a difference in isolation protocol two well described types of CSCs are identical in phenotype and functional properties. This could imply that these cells, irrespectively of the isolation method used, are generated by the same ancestor cell or even are the same cells. We suggest that the pool of stem cells present in the human adult heart is further characterized and that its phenotypical and functional properties are well documented before these cells are used in future clinical trials.

6. Cardiac Atrial appendage

Stem Cells

6.1 Introduction

In the previous chapter we have compared the cardiomyogenic differentiation potential of MSCs, c-kit$^+$ CSCs and CDCs. The results showed that both types of CSCs display a greater cardiomyogenic differentiation potential. However, some hurdles had to be taken in order to obtain a homogenous stem cell population. First, the isolation of c-kit$^+$ CSCs is based on antigen-antibody interaction and is susceptible to non-specific binding. This could result in a possible risk to isolate contaminating non-stem cell like cells. Second, the isolation of CDCs is hampered by a laborious protocol with different culture conditions. Moreover, sphere formation is not a unique stem cell characteristic and spheres arise by aggregation of cells and not by clonal expansion (Andersen et al., 2009). To overcome these hurdles we investigated whether a homogenous CSC population could be isolated based on their ALDH activity.

ALDH is an enzyme involved in metabolizing aldehydes to their corresponding carboxylic acids *(Cheung et al., 2007)* and in mechanisms of resistance to alkylating agents e.g. cyclophosphamide *(Storms et al.; 1999; Mirabelli et al., 2008)*. Hence a more detoxifying role is ascribed to ALDH and cells expressing this enzyme are better protected against certain cytotoxic effects *(Storms et al., 1999; Fallon et al., 2003)*. The ALDH substrate, BAAA (BODIPY®-aminoacetaldehyde), diffuses freely into the cells and is converted into a green fluorescent product, BAA (BODIPY®-aminoacetate), which is trapped inside cells dissolved in the assay buffer *(Storms et al., 1999)*. Other protocols also use enzymatic activity to isolate stem cells. Side population (SP) cells are known to transport the dye Hoechst33342 out of the cell by the ATP-binding cassette superfamily of membrane transporters (ABCG-2) (*Wolf et al., 1993; Hierlihy et*

al., 2002; Montanaro et al., 2004). However, recently it has been reported that pluripotent stem cells, which do not express ABCG-2, actually retain this dye and therefore are not located in the SP-gate *(Zeng et al., 2009)*. Furthermore, Kallestad and coworkers demonstrated the cellular toxicity of Hoechst33342 since untreated bone marrow cells contained 11.1±1.2% dead cells, whereas after exposure to the Hoechst dye, the percentage of dead cells increased to 37.3±2.0% *(Kallestad et al., 2010)*. Furthermore, the technique reported in this chapter is based on staining only viable stem cells, which do not have to be co-stained with propidiumiodide or 7-AAD to exclude apoptotic or dead cells.

To obtain a homogenous CSC population, we have optimized a new isolation protocol based on the ALDH activity of cells in human right atrial appendages. These isolated CASCs are an intrinsic CSC population that is able to differentiate into functional CMs.

6.2 Results

6.2.1 Isolation and characterization of CASCs

After enzymatic treatment the heart tissue samples were incubated with Aldefluor® and CD34- and CD45-antibodies (n=90). The cell suspension was then flow cytometrically analyzed (Figure 32a). First, a control sample was prepared in which diethylaminobenzaldehyde (DEAB), an inhibitor of ALDH, was dissolved. This was used as a negative control in order to set the ALDH+ gate (Figure 32b). In the human right atrium, an average of 0.9±0.8% of the total cell population expressed high levels of ALDH (Figure 32c). Within this $ALDH^+$ population 83.8±13.4% expressed CD34 but were uniformly negative for CD45 (Figure 32d). Cells displaying this $ALDH^+C34^+CD45^-$ phenotype were judged as CASCs.

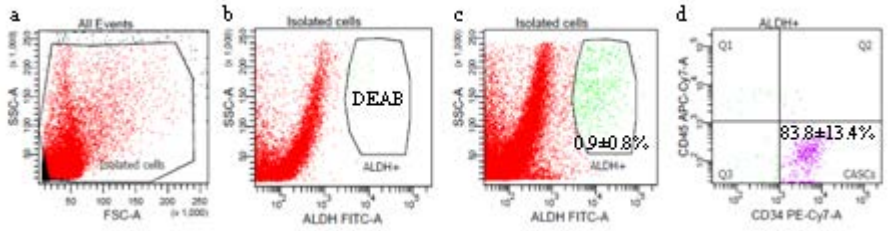


Figure 32: Flow cytometric analysis of ALDH expressing cells in the atrial cell population. Total cell population obtained after enzymatic digestion (a). No $ALDH^+$ cells could be detected in the total cell population when the activity of this enzyme was blocked by diethylaminobenzaldehyde (DEAB; negative control; b). When the ALDH activity was not blocked around 0.9±0.8% of the total cell population was $ALDH^+$ (c). Within this $ALDH^+$ population 83.8±13.4% of the cells expressed CD34 but were uniformly negative for CD45 (d).

In order to investigate whether our CASCs were a phenotypically distinct population from the earlier described human CSCs a more thorough flow cytometric characterization was performed. Incubation of the total atrial cell suspension with the antibodies listed in table 1 revealed that within the total cell population, 0.1±0.1% of the cells were c-kit$^+$ (Figure 33a). This c-kit$^+$ cell population was a heterogeneous population of which almost half of the cells expressed CD45 (Figure 33b). All these c-kit$^+$CD45$^+$ cells were uniformly positive for CD34 and were therefore judged as HSCs (Figure 31c). On the other hand, all c-kit$^+$CD45$^-$ cells were all negative for CD34 and were judged as CSCs (Figure 33c).

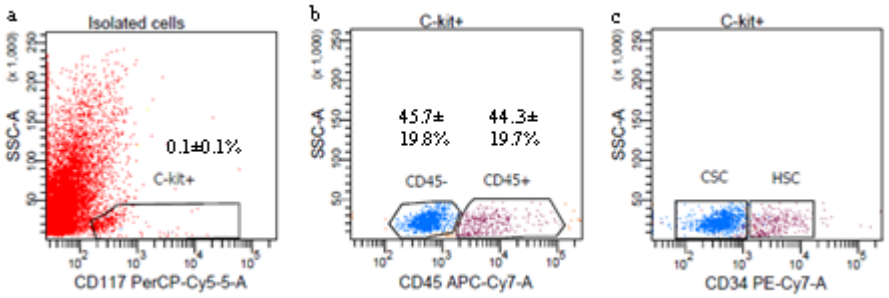


Figure 33: Flow cytometric analysis of c-kit$^+$ cells in the total atrial cell population. A small percentage, 0.1±0.1%, of the isolated cell population expressed CD117 (c-kit) (a). Of this c-kit$^+$ cell population 44.3±19.7% were also CD45 positive while 45.7±19.8% were CD45 negative (b). The c-kit$^+$CD45$^+$ cells were uniformly positive for CD34 and therefore judged as hematopoietic stem cells (HSC; c). On the other hand c-kit$^+$CD45$^-$ cells were all negative for CD34 and were judged as cardiac stem cells (CSC; c).

When the antigen expression profile of these c-kit$^+$ CSCs was compared with that of the freshly isolated CASCs it became clear that these two populations were phenotypically different. First, these populations differed from each other in their ALDH (Figure 34a) and CD34 expression. Furthermore, the c-kit$^+$ CSCs

were negative for CD29, CD55 and CD73 while the CASCs expressed these markers (Figure 34 b-d).

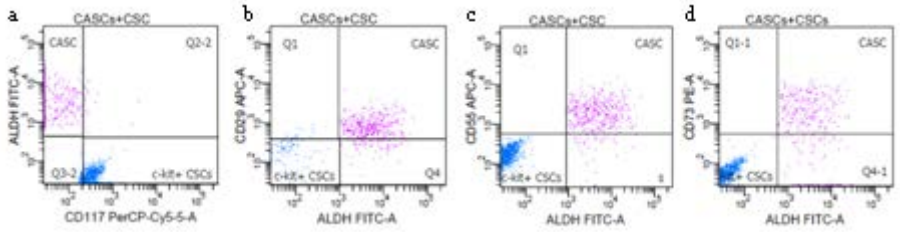


Figure 34: Flow cytometric comparison between freshly isolated CASCs and c-kit$^+$ CSCs. CASCs express high levels of ALDH and are negative for c-kit while the c-kit$^+$ CSCs show no ALDH activity but are positive for c-kit (a). Furthermore, c-kit$^+$ CSCs were negative for CD29 (b), CD55 (c) and CD73 (d) while CASCs expressed all the aforementioned markers

Nevertheless, the CASCs are, just like the c-kit$^+$ CSCs, not randomly distributed throughout the atrium but are found in so-called stem cell niches. Immunofluorescence for the detection of CD34 on paraffin embedded heart tissue slices shows that CASCs are present in the atria (Figure 35) (n=5).

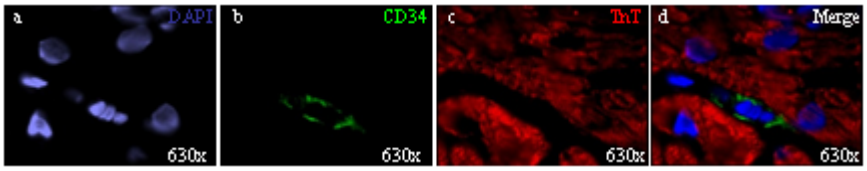


Figure 35: Identification of CASCs in right atrial heart tissue. Immunofluorescent staining of CASCs present in parafine embedded heart tissue slices. (b-d) CD34$^+$cells (green) were not randomly distributed throughout the atrium but are localized in so-called stem cell niches surrounded by adult cardiomyocytes expressing troponin T (TnT). (a,d) Nuclei are stained with DAPI.

6.2.2 Flow cytometrical analysis of freshly isolated and ex vivo expanded CASCs

Since this is a new CSC population a thorough flow cytometric analysis was performed to characterize the membrane antigen expression profile of these cells. It is known that *ex vivo* expansion can alter the phenotype of cells. Indeed, culturing CASCs induced the expression of several antigens like CD13 and CD44. On the other hand the expression of CD34 was down-regulated. Despite the fact that these cultured CASCs lost the expression of this stem cell marker, the ALDH activity of these cells was not influenced as shown by flow cytometry (Figure 36A) and fluorescent microscopy (Figure 36B) (n=3). A complete phenotypic comparison between freshly isolated and *ex vivo* expanded CASCs can be found in Table 5 (n=3).

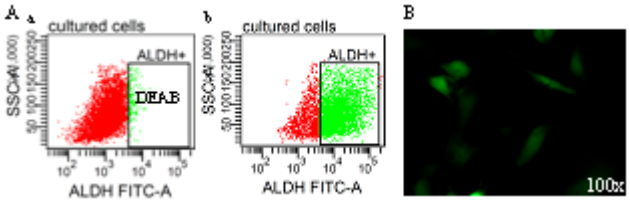


Figure 36: ALDH activity of *ex vivo* expanded CASCs. (A) *Ex vivo* expanded CASCs still displayed high levels of ALDH as shown by flow cytometry. When the ALDH inhibitor was added no $ALDH^+$ cells could be detected (a). However, when the inhibitor was removed, almost the entire population is $ALDH^+$. (B) These results were confirmed by fluorescent microscopy showing a green fluorescent signal after cells were incubated with Aldefluor®.

Table 5: Antigen expression profile of freshly isolated and *ex vivo* expanded CASCs

Antigen	Fresh CASCs	cultured CASCs	Antigen	Fresh CASCs	cultured CASCs
CD2	-	Negative	*CD55*	Positive	Positive
CD3	Negative	Negative	*CD56*	Negative	Negative
CD4	-	Negative	*CD59*	Negative	Negative
CD5	Negative	Negative	*CD71*	-	Negative
CD10	Negative	Negative	*CD73*	Positive	Positive
CD11b	Negative	Negative	*CD90*	Positive	Positive
CD13	Negative	Positive	*CD105*	-	Positive
CD14	Negative	Positive	*CD106*	-	Negative
CD15	-	Negative	*CD109*	Negative	Negative
CD16	-	Negative	*CD117*	Negative	Negative
CD19	Negative	Negative	*CD133*	Negative	Negative
CD29	Positive	Positive	*CD138*	Negative	Negative
CD31	-	Negative	*CD140b*	Negative	Negative
CD33	Negative	Negative	*CD144*	-	Negative
CD34	Positive	Negative	*CD184*	Negative	Negative
CD38	Negative	Negative	*HLA-DR*	Negative	Negative
CD44	Negative	Positive	*TIE-2*	Negative	Negative
CD45	Negative	Negative	*VEGFR-2*	Negative	Negative
CD49c	Negative	Positive	*VEGFR-3*	Negative	Negative
CD50	-	Negative	*Aldefluor®*	Positive	Positive

6.2.3 CASCs are not mobilized bone marrow cells

Bone marrow stem cells and in particular bone marrow-derived HSCs express high levels of ALDH and are $CD34^+$ (Storms et al., 2005; Gentry et al., 2007; Mirabelli et al., 2008). Therefore, one could argue that the isolated CASCs ($ALDH^+CD34^+$) are not an intrinsic CSC population but actually mobilized bone marrow cells. In order to refute this theorem, we have first compared the flow cytometric characteristics of bone marrow-derived and peripheral blood-derived $ALDH^+$ cells with those of isolated CASCs. Bone marrow (n=10) as well as peripheral blood $ALDH^+$ cells (n=6) display a different phenotype from the CASCs. In bone marrow around 1.2±0.4% of the cells were $ALDH^+$. Within this $ALDH^+$ cell population 85.2±10.1% of the cells expressed CD34. These $ALDH^+CD34^+$ cells were uniformly positive for CD45 but negative for CD73 (Figure 37A). In peripheral blood 0.8±0.8% $ALDH^+$ cells could be detected. Non of these cells expressed CD34 but all were positive for CD45 and negative for CD73 (Figure 37B). In contrast the freshly isolated CASCs, $ALDH^+CD34^+$, were negative for CD45 but positive for CD73 (Figure 37C).

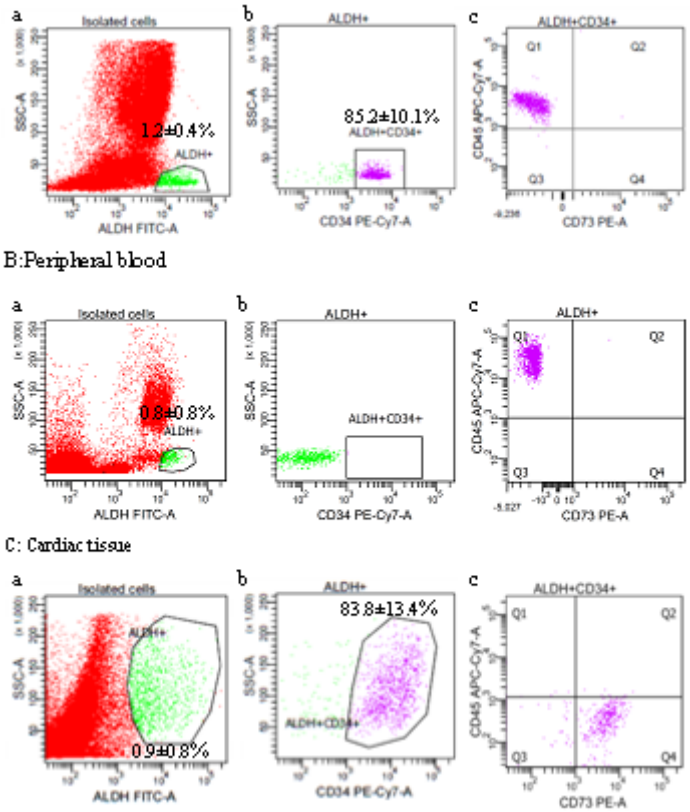


Figure 37: Flow cytometric comparison between ALDH$^+$ cells derived from bone marrow, peripheral blood and cardiac tissue. (A) Within the total bone marrow-derived cell population 1.2±0.4% of the cells expressed ALDH (a). 85.2±10.1% of these cells co-expressed CD34 (b). This double positive population was positive for CD45 but did not express CD73 (c). (B) In peripheral blood around 0.8±0.8% of the cells expressed ALDH (a) but were negative for CD34 (b). Still the ALDH$^+$ cells expressed CD45 but were negative for CD73 (c). (C) In cardiac tissue, 0.9±0.8% of the cells were ALDH$^+$ (a). Within this ALDH$^+$ population 83.8±13.4% of the cells expressed CD34 and were judged as CASCs (b). As opposed to bone marrow and peripheral blood-derived ALDH$^+$ also these CASCs were negative for CD45 but expressed CD73 (c).

Assuming that these CASCs originate from bone marrow, they could display a different phenotype since they are no longer located in their normal stem cell niche. Therefore we compared the functional properties of bone marrow-derived ALDH$^+$ cells with those of the CASCs. Bone marrow-derived ALDH$^+$ cells and heart tissue-derived ALDH$^+$ were isolated by flow sorting and seeded in HSC

medium (n=2). After two weeks, bone marrow-derived ALDH$^+$ cells formed burst-forming units, erythrocyte (BFU-E; *), colony-forming units, granulocyte-macrophage/monocyte (CFU-GM, **) and colony-forming units, granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM; ***) (Figure 38Aa), while the isolated CASCs did not (Figure 38Ab). Also the functional characteristics of *ex vivo* expanded bone marrow-derived and heart tissue-derived ALDH$^+$ cells were compared. First, both ALDH$^+$ cell populations were isolated and *ex vivo* expanded under identical culture conditions. When sufficient cell numbers were obtained, cells were forced to differentiate down the adipogenic and osteogenic lineage (n=3). Cells cultured in adipogenic culture medium displayed a more round morphology and numerous vacuoles were present in the cells. After an oil-red O staining these vacuoles stained red indicating that they were fat droplets (Figure 38Ba). After three weeks in osteogenic inducing medium the bone marrow-derived ALDH$^+$ cells formed Ca^{2+}-deposits as shown by a positive alizarine-red staining (Figure 38Ca). As opposed to bone marrow-derived ALDH$^+$ cells, CASCs showed no signs of differentiation after histological stainings, indicating that these cells did not transform into adipocytes or osteocytes (Figure 38Bb, Cb).

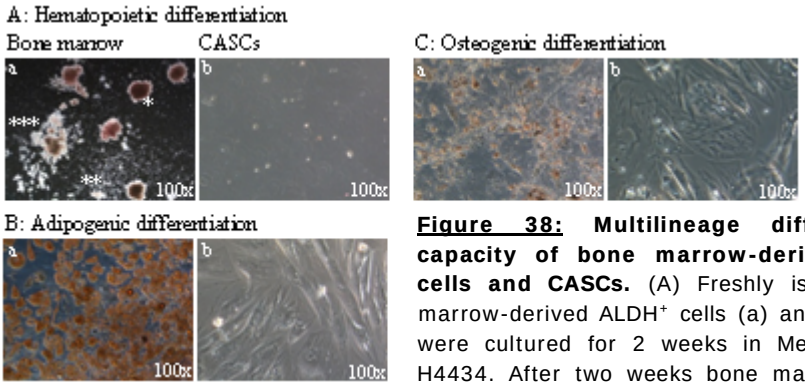


Figure 38: Multilineage differentiation capacity of bone marrow-derived ALDH$^+$ cells and CASCs. (A) Freshly isolated bone marrow-derived ALDH$^+$ cells (a) and CASCs (b) were cultured for 2 weeks in MethoCult® GF H4434. After two weeks bone marrow-derived formed BFU-E (*), CFU- GM (**) and CFU-GEMM (***) while CASCs did not shown any colony formation. (B, C) *Ex vivo* expanded ALDH$^-$ bone marrow cells and CASCs were stimulated to differentiate down the adipogenic (B) and osteogenic lineage (C). After three weeks bone marrow cells showed clear presence of lipid vacuoles after oil-red O staining (a), while no signs of adipogenic differentiation could be detected in the CASCs (b). Similar results were obtained from cells incubated for three weeks in osteogenic differentiation medium. The bone marrow-derived ALDH$^+$ cells showed the formation of Ca^{2+}-deposits after an alizarin-red staining while the *ex vivo* expanded CASCs did not.

6.2.4 Cloning assay and expression of pluripotency associated genes

To assess whether CASCs are indeed stem cells, the clonogenic potential of these cells was tested (n=6). A total of 3456 GFP+ cells was sorted with a density of 1 cell/well. After 5 days 582 clones were formed (Figure 39 a-d) resulting in a cloning efficiency of 16.8±11.4%.

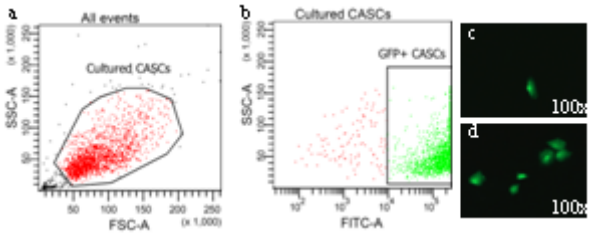


Figure 39: Clonogenicity of *ex vivo* expanded CASCs. (a) Forward/Side scatter (FSC/SSC) of cultured CASCs. (b) GFP$^+$ CASCs were sorted with a density of 1 cell/well under stringent purity conditions. A total of 3456 cells were sorted. (c) Single cell disposition was confirmed by fluorescent microscopy. (d) After 5 days 582 clones were collected resulting in a clonogenicity of 16.8±11.4%.

Furthermore, the expression of several pluripotency genes was analyzed (n=3). The freshly isolated CASCs expressed several pluripotent associated genes, like *OCT-4*, *DPPA3*, *Lin 28*, *c-myc*, *Klf-4* and *Tbx-3*. Also, the *ex vivo* expansion of these CASCs did not influence the expression of these genes (Figure 40).

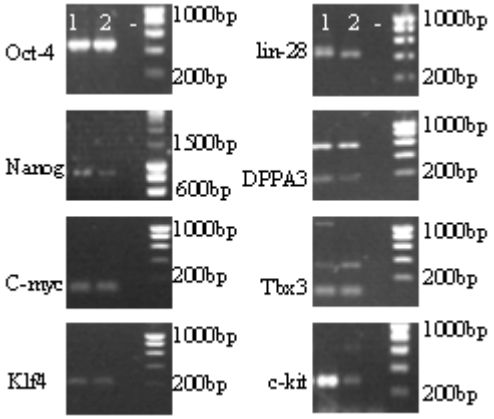


Figure 40: RT-PCR analysis of the expression of pluripotency associated genes. Lane 1: Freshly isolated CASCs Lane 2: *ex vivo* expanded CASCs. CASCs uniformly expressed *Oct-4*, *NANOG*, *C-myc*, *Klf4*, *lin-28*, *DPPA3*, *Tbx3* and *c-kit*.

The commitment of these cells towards the cardiomyogenic lineage was shown by the expression of several cardiac specific genes like *α-actinin*, the voltage gated potassium channel *Kv4.3* and *GATA-4* (Figure 41).

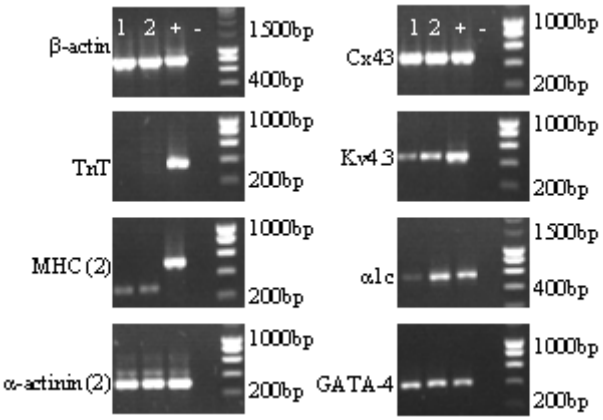
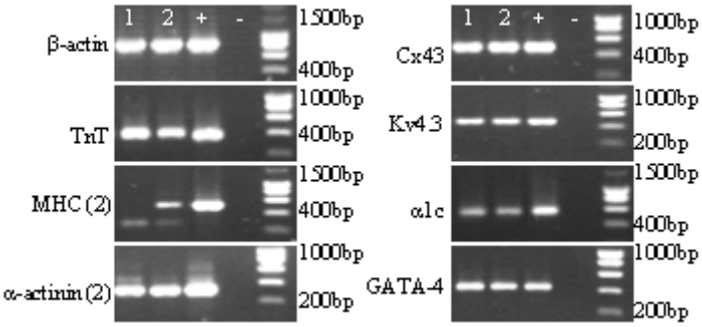


Figure 41: RT-PCR on RNA from freshly isolated and *ex vivo* expanded CASCs to determine cardiomyogenic gene expression. Lane 1: Freshly isolated CASCs Lane 2: *ex vivo* expanded CASCs. The cardiac commitment of these CASCs was shown by the expression of several cardiac specific genes e.g. *α-actinin*, *Cx43*, *Kv4.3*, *α1c* and *GATA-4*. β-actin is used as internal control, hCMs were used as positive control (+).

6.2.5 CASCs do differentiate into functional cardiomyocytes

To restore cardiac function the transplanted cells should be able to differentiate into functional CMs. Therefore, we examined the cardiomyogenic differentiation potential of the *ex vivo* expanded CASCs. In mono-culture these cells uniformly expressed *TnT*, *α-actinin*, *Cx43*, *Kv4.3*, *α1c* and *GATA-4*. However, the differentiation of the *ex vivo* expanded CASCs was not influenced by the presence of TGF-β. Nevertheless, no expression of *MHC* could be detected in any condition (Figure 42). On the contrary, when these cells were co-cultured with NRCMs, expression of *MHC* was induced (Figure 42).



<u>Figure 42:</u> Cardiomyogenic differentiation potential of *ex vivo* expanded CASCs assessed by RT-PCR. Cardiomyogenic differentiation potential of *ex vivo* expanded CASCs. (A) Lane 1: mono-cultured CASCs; Lane 2: co-cultured CASCs; +: hCMs (positive control); -: H_2O (negative control). Mono-cultured CASCs expressed *TnT, α-actinin*, *Cx43*, *Kv4.3*, *α1c* and *GATA-4*. Co-cultured CASCs express all the aforementioned markers. Furthermore, co-culturing CASCs induced the expression of *MHC*.

More complete cardiomyogenic differentiation was also shown by immunofluorescence: despite the fact that mono-cultured CASCs expressed *TnT* in all conditions, the protein itself could not be detected in these cells. Furthermore, no expression of cTnI could be detected. However, after 1 week of co-culture, GFP-transfected CASCs expressed cTnT (Figure 43Aa-d) and cTnI (Figure 43Ba-d) in an organized fashion as one would expect in adult CMs. These results indicate that a cardiac micro-environment influences the differentiation of these cells.

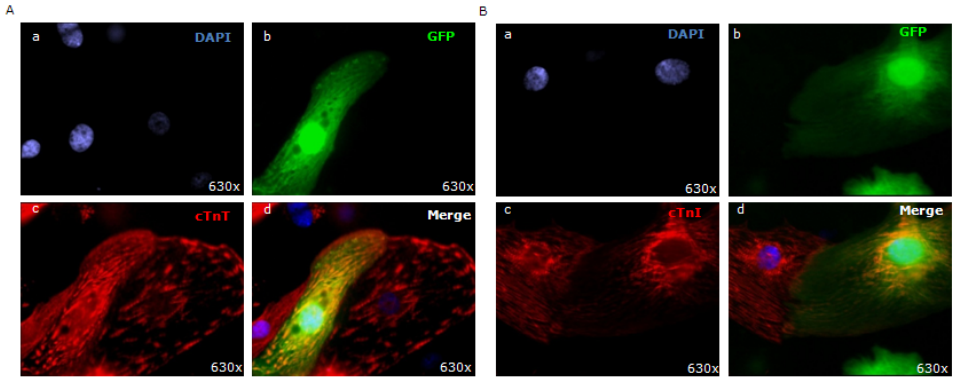
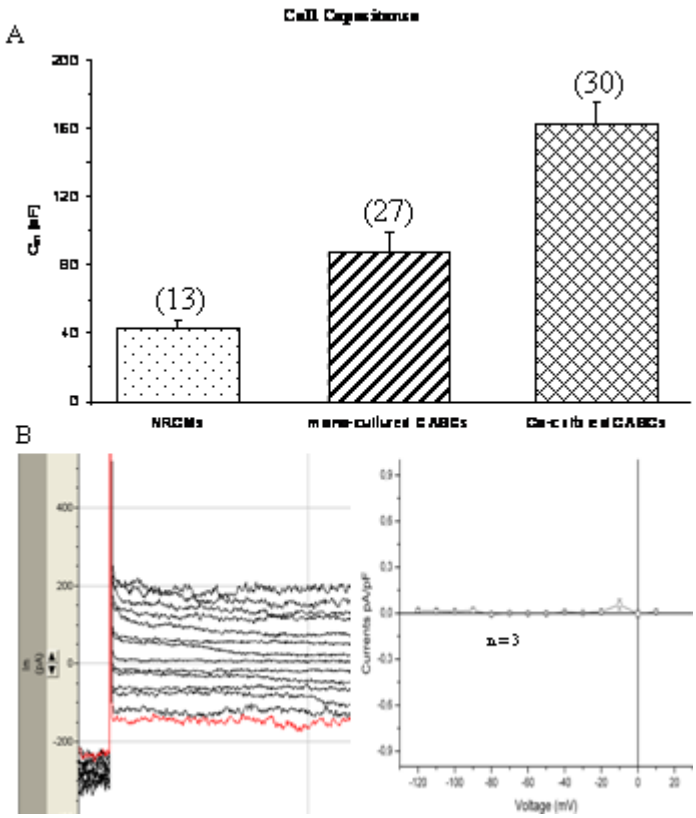


Figure 43: Cardiomyogenic differentiation of co-cultured CASCs assessed by immunofluorescence. Immunofluorescent staining of contractile proteins cTnT and cTnI on GFP expressing CASCs after 1 week of co-culture with NRCMs. A: Positive staining for the cardiomyocyte specific protein cTnT (c,d) in GFP+ CASCs (b,d) and NRCMs (c,d). B: Expression of the cardiomyocyte specific protein cTnI (c,d) in GFP+ CASCs (b,d) and NRCMs (c,d). Nuclei were stained with DAPI. GFP-positive cTnT or cTnI positive cells show clearly sarcomeric organization and are newly formed cardiomyocytes.

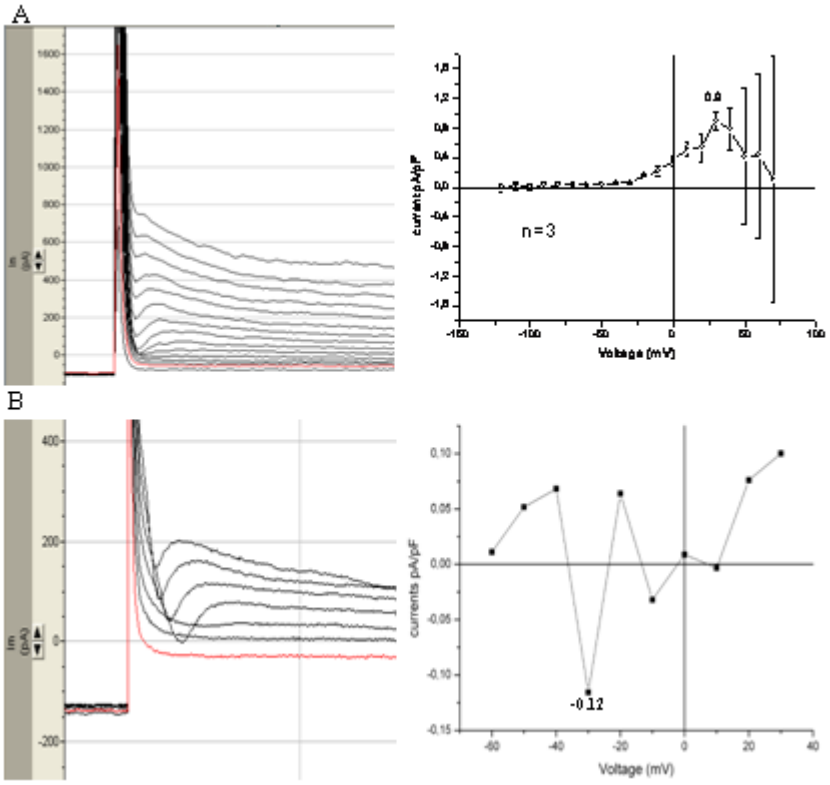
Electrophysiological measurements were performed on mono and co-cultured lentiviral transduced CASCs. The capacitance data showed that CASCs increase in size in co-culture, compared to mono-cultured CASCs (Figure 44A). Despite the fact that co-cultured CASCs expressed TnT and TnI in an organized fashion after 1 week, electrophysiology revealed that cells displayed no I_{Na} or I_{Ca} currents and no outward potassium (Figure 44B).

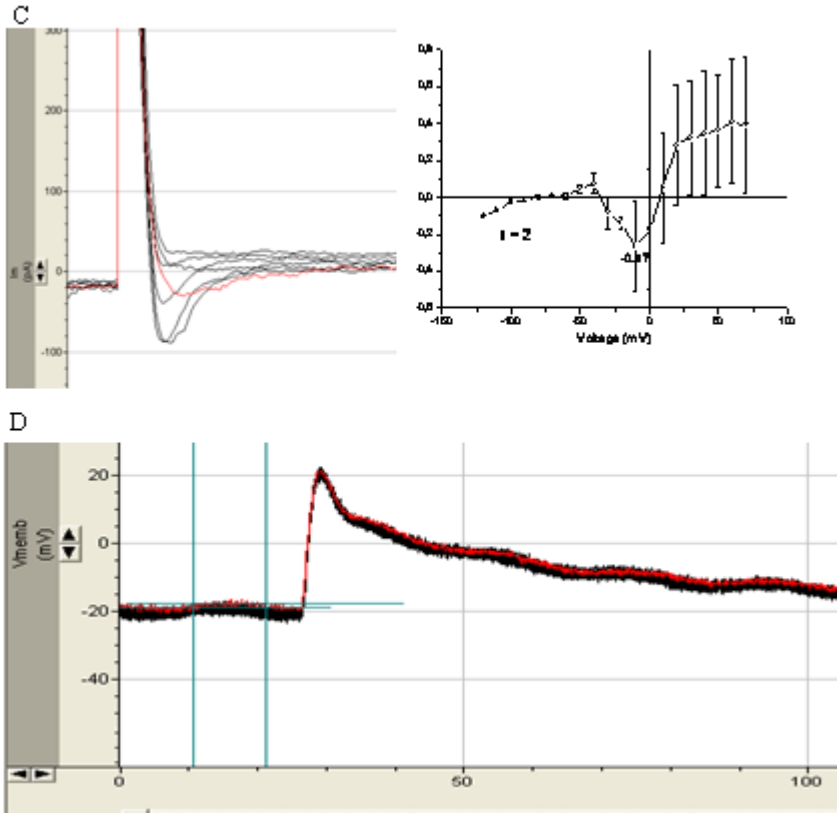


<u>Figure 44:</u> Electrophysiological measurements in order to assess the functionality of CASCs after 1 week of co-culture. (A) As shown co-cultured CASCs are enlarged when compared to the mono-cultured cells. Neonatal rat cardiomyocytes (NRCMs) are readily smaller while co-cultured CASCs resemble a more ventricular size. (B) After 1 week in co-culture, no I_{Ca}, I_{Na} or outward potassium currents were present in the CASCs.

However, after 8-10 days these cells started to display transient outward potassium currents (Figure 45A) indicating that they were differentiating further down the cardiomyogenic lineage. Indeed, after 14 day these cells displayed I_{Ca} currents, proving that the pore-forming α-subunit was present (Figure 45B). These data is in accordance with our RT-PCR results showing the expression of *α1c*. Also fast I_{Na} currents were recorded in co-cultured CASCs (Figure 45C). Despite the presence of these currents and the expression of *Kv4.3*, a voltage gated potassium channel involved in the $I_{to,f}$ repolarisation current, CASCs

showed only slow action potentials (Figure 45D) which are probably mediated by $I_{to,\ slow}$ regulated by kv1.4 (Kääb et al., 1998). The failure to generate ventricular like action potentials is also due to the fact that most cells displayed a more depolarized membrane potential (-40 - 0mV). However, these results indicate that these CASCs have the potential to differentiate into adult functional CMs.





<u>Figure 45:</u> Evidence for cardiomyogenic differentiation of co-cultured CASCs. (A) After 8-10 days CASCs started to show some transient outward potassium currents. (B) I_{na} and outward potassium currents recorded in CASCs after 2 weeks of co-culture. (C) These CASCs also exhibited clear I_{ca} currents. (D) An example of slow action potential recorded in CASCs.

6.3 Discussion

In this chapter we describe the existence of new intrinsic CSCs population isolated on its ALDH activity. Since we are the first to describe these CASCs, an extended flow cytometric characterization and functional analysis were performed. Furthermore, the myocardial differentiation capacity of these cells was investigated.

To date, two main human adult CSC populations have been described, both expressing the stem cell factor receptor c-kit (Smith et al., 2007; Bearzi et al., 2007). The c-kit receptor is a tyrosine kinase receptor which plays a role in cell survival, proliferation and differentiation. As discussed earlier, the heart produces stem cell factor as a response to myocardial ischemia (Frangogiannis et al., 1998), indicating that an intrinsic CSCs would express the stem cell factor receptor c-kit. However, flow cytometric analysis of our freshly isolated CASCs showed that these cells do not express the c-kit antigen. Furthermore, a thorough flow cytometric comparison between freshly isolated CASCs and c-kit$^+$ CSCs revealed that these cells not only differ in their c-kit or ALDH expression. These cells also differ by the expression of several markers like CD29 and CD55. Therefore it can be concluded that the CASCs do not represent the earlier described c-kit$^+$ CSCs (Bearzi et al., 2007). Despite the absence of c-kit on the membrane of the freshly isolated CASCs, these cells do express express CD34, an antigen commonly used for the characterisation of bone marrow-derived HSCs. However, more recently this antigen has also been used to identify other tissue specific stem cells like muscle satellite cells (Sacco et al., 2008; Negroni et al., 2009; Kallestad et al., 2010). Despite extensive studies, the functions of this membrane antigen remain very uncertain. It is described that CD34 plays a

role in enhancing proliferation and blocking differentiation of stem cells. Indeed, CD34 expression is progressively down-regulated on maturing cells (Fackler et al., 1995). Recently new functions of the CD34-family were described implying chemokine-mediated trafficking and regulation of asymmetric cell division. Nielsen and coworkers suggested that a combination of stem cell factor and stromal derived factor, both produced following myocardial ischemia, is a chemo-attractant for CD34 expressing cells (Hu et al., 1998; Nielsen et al., 2008). This chemotactic gradient could explain why a large percentage of $ALDH^+CD34^+$ cells are present in the infarcted human heart. However, the phenotype of these cells still raises some questions about their origin. Since the expression of ALDH and CD34 is commonly used to isolate HSCs (Storms et al., 2005; Gentry et al., 2007; Mirabelli et al., 2008), one could argue that these CASCs are not an endogenous CSC population but are actually mobilized bone marrow stem cells. In order to proof that these CASCs are an intrinsic CSC population we phenotypically and functionally compared them with $ALDH^+$ bone marrow cells. These experiments clearly showed that first of all there was a clear phenotypical difference between the CASCs ($CD45^-CD73^+$) and the bone marrow $ALDH^+$ cells ($CD45^+CD73^-$). Furthermore, CASCs differ functionally from bone marrow since, in contrast to $ALDH^+$ bone marrow cells, they show no hematopoietic colony-forming activity and are not able to differentiate into adipocytes or osteocytes. However, based on the data presented here we can not determine whether the CASCs are a resident stem cell population already present in the fetal heart or are actually cells mobilized to the heart after birth. Although, most cells of hematopoietic origin, e.g, HSCs and lymphocytes, express CD45 while the CASCs are negative for this common leukocyte antigen. Furthermore, the expression of CD73, a 5'-nucleotidase, on the freshly isolated

CASCs allows us to assume that these cells play a protective role in the heart. As the oxygen supply is inadequate for the given work load of the heart, the energy status of the heart is imbalanced and free cytosolic ADP increases. As a consequence of the myokinase equilibrium, this ADP is converted to free AMP, a substrate for 5'-nucleotidases. These enzymes rapidly convert the free AMP into adenosine (Decking et al., 1997). This nucleoside plays an important role in restoring the balance between energy supply and energy demand by its vasodilatory (Stepp et al., 1996) and anti-adrenergic (Neumann et al., 1995) effect. Based on the phenotypical and functional differences, it is possible that the CASCs are indeed an intrinsic CSC population or that they have resided in the heart long enough to adopt a more cardiac committed phenotype. This commitment to the cardiomyogenic lineage was shown by the expression of several cardiac specific genes like *α-actinin, Cx43* and *GATA-4*. However, these cells show some lineage specific commitment they were true stem cells. The CASCs displayed a clonogenicity of 16% which is much higher than the c-kit$^+$ CSCs (0.8%; Bearzi et al., 2007). Furthermore, these cells express several important pluripotency associated genes like *Oct-4, Nanog, c-Myc* and *Klf4*. Somatic cells can be reprogrammed into a more embryonic stem cell-like state by simultaneously introducing 4 factors, Oct-3/4, Sox2, c-Myc and Klf4 (Takahashi et al., 2006). Furthermore, transient transfection of Oct-4 only is sufficient to transform mouse interfollicular epidermal basal keratinocytes to a more embryonic stem cell-like state (Grinnell et al., 2007). Therefore Oct-4 might be the most important regulator of the pluripotent state in mammalian cells. Next to *Oct-4* CASCs also expressed *Nanog, c-Myc* and *Klf4*. Nanog is a homeobox transcription factor that is shown to be downregulated upon differentiation. Furthermore, the expression of Nanog allows embryonic stem

cells to grow in feeder-free conditions C-Myc is associated with histone acetyltransferase complexes e.g. p300 and CREB binding protein (Vervoorts et al., 2003). Therefore, c-Myc could be responsible for a global histone acetylation allowing Oct-4 to bind to its specific target locus (Fernandez et al., 2003). *Klf4* has an anti-apoptotic function by the repression of p53 (Rowland et al., 2005) and inhibits cell proliferation by the activation of $p21^{cip1}$ (Zhang et al., 2000). However, this inhibition is counteracted by the expression of c-Myc which suppresses the expression of $p21^{cip1}$ (Seoane et al., 2002). Therefore it is suggested that the delicate balance between c-Myc and Klf4 expression is important for maintaining the pluripotent state and proliferation capacity of stem cells or induced pluripotent cells (Takahashi et al., 2006). These cells also expressed *Lin-28*, *DPPA3*, *Tbx3* and *c-kit*. Lin-28 blocks the maturation of the precursors of let-7 by binding to the terminal loops of this miRNA family thereby keeping the cells in their undifferentiated pluripotent state (Heo et al., 2008). Just like lin-28, tbx3 and DPPA3 are involved in sustaining cellular pluripotency and differentiation (Ivanova et al., 2006; Wiese et al., 2009). Although, CASCs expressed *c-kit* at the RNA-level, flow cytometry showed that these cells do not express this antigen on their membrane. Combining the flow cytometric data with the clonogenic character and expression of pluripotency associated genes of the CASCs, we can postulate that these cells are indeed stem cells.

Since cardiomyogenic differentiation of stem cells is aimed for in this research we also examined this characteristic of the CASCs. When these cells were placed in a cardiac micro-environment of contracting NRCMs they showed the expression of *TnT* and *MHC.* Furthermore, these cells expressed cTnT and cTnI in a sarcomeric fashion as expected in adult CMs. The differentiation of these

cells towards functional CMs was shown by the electrophysiologic recordings of I_{Na} and I_{Ca} currents. Furthermore the generation of slow action potentials showed that these cells are able to differentiate into functional CMs.

In conclusion, we have described a new CSC population that can be isolated based on its specific ALDH activity. Phenotypically and functionally these CASCs are not mobilized bone marrow cells but actual stem cells residing in the heart. The stem cell characteristics of these cells were shown by the expression of several pluripotency associated genes and a high clonogenicity. Furthermore, these cells can be *ex vivo* expanded without losing these characteristics. When co-cultured with NRCMs they differentiated into functional CMs as shown by the expression of cTnT and cTnI. Furthermore, the fact that these CASCs indeed differentiated into functional CMs was also shown by the presence of I_{Ca}, I_{Na}, transient outward potassium and slow action potentials. The discovery of this new endogenous CSC population could open new perspectives in the search to regenerate the infarcted heart.

7. General Conclusions

After a MI the heart function is severely diminished due to an irreversible loss of CMs. The heart is no longer considered a static organ but contains some intrinsic regenerative capacity which however is not sufficient to restore function after MI. Regenerative stem cell therapy is a promising approach to improve cardiac function.

7.1 Bone marrow-derived stem cells

Since Bone marrow-derived stem cells can be transplanted in a autologous setting and are easy accessible, they were seen as a potential source of stem cells to be transplanted in the infarcted heart. In chapter 3 we described the isolation, characterization, functionality and myocardial differentiation potential of bone marrow-derived MSCs. RT-PCR showed that after 3 weeks of culture in a cardiac micro-environment only the expression of *TnT* and *GATA-4* was induced. The limited cardiac differentiation capacity of these MSCs was shown by immunofluorescence, no cTnT expression at the protein level, and TEM, no sarcomeric organization. In conclusion, these data suggest that MSCs are not able to differentiate in functional CMs.

In chapter 4 we investigated the transdifferentiation potential of HSCs. These HSCs were isolated by flow sorting based on the membrane antigen expression of CD34 and CD133 which resulted in a 96±1% pure population. After co-culture these cells only expressed *TnT* at the RNA-level since no expression of this protein could be detected by immunofluorescence. These results were confirmed by TEM.

The results presented here imply that the functional improvement shown by the different clinical trials, all transplanting the MNC fraction derived from ischemic

heart disease patients, is probably due to a paracrine effect exerted by the transplanted cells. This theory is supported by the data published by Noiseux et al. (Noiseux et al., 2006). They transplanted MSCs in the infarct mouse model. These cells stimulated stem cell mobilization towards the infarcted region and improved cardiac function due to enhanced angiogenesis. Therefore, it was necessary to continue the search for a stem cell population with a greater myocardial differentiation capacity.

7.2 cardiac stem cells

In 2001 the dogma of the heart being a post-mitotic organ was proven wrong (Beltrami et al., 2001). Not only was there proof of dividing CMs, also the presence of an endogenous CSC was described (Beltrami et al., 2003). Since that discovery several types of CSCs are described. In chapter 5 we compared the phenotype, functionality and cardiac differentiation capacity of two well described CSC populations, c-kit$^+$ CSCs and CDCs, with bone marrow-derived MSCs. Phenotypically these cells only show a difference in the expression of CD140b. MSCs express this antigen while both types of CSCs did not. A subsequent proliferation assay confirmed the flow cytometry and showed that this tyrosine kinase receptor is important for MSC proliferation. Furthermore, these CSCs did not differentiate down the adipogenic and osteogenic lineage while MSCs can as described in chapter 3. The RT-PCR results show that TGF-β1 influences the differentiation potential of mono-cultured MSCs by the induction of *TnT*. The expression of *TnT* and *GATA-4* was detected in every condition in both types of CSCs. Co-culture induced the expression of *MHC* in all conditions in all three cell types. Despite this similarity, a greater differentiation potential for

the CSCs was shown by a positive staining for cTnT in a sarcomeric organization after 1 week of co-culture.

Despite these positive results some difficulties had to be faced when these CSCs were isolated and cultured. The isolation of c-kit$^+$ CSCs was hampered by a very small percentage (0.1%) of cells present in the human heart. Furthermore, the isolation was impeded by the fact that the isolated population could be contaminated with other cells also expressing c-kit. A second problem was the limited proliferation capacity of these c-kit$^+$ CSCs. This problem did not occurred during the *ex vivo* expansion of CDCs. However, other issues have to be faced when translating this culture protocol to a clinical useable protocol. Several culture media and culture surfaces have to be used in order to generate these CDCs. These changes in culture environment create a more unstable culture environment which could influence the cell characteristics. Furthermore as shown in chapter 5, the formation of spheres is not a unique stem cell characteristic. These findings strongly challenge the use of this assay to characterize the stemness of a certain cell type and caution has to be taken to isolate stem cells based on this functional assay.

In chapter 6 we describe the isolation, characterization and myocardial differentiation of a new endogenous CSC population named CASCs. We succeeded to isolate these CASCs based on their ALDH activity. These CASCs showed a different antigen expression profile than the earlier described c-kit$^+$ CSCs. Phenotypical and functional comparison between CASCs and ALDH$^+$ bone marrow stem cells show that these CASCs were not mobilized bone marrow stem cells but actual stem cells residing in the heart. Furthermore, the stem cell properties of these CASCs were shown by the expression of several pluripotency

associated genes e.g. *OCT-4, lin-28*, … and a clonogenicity 16%. After co-culture these cells also expressed *TnT, α-actinin, MHC* and *GATA-4*. However, the greater myocardial differentiation potential of these CASCs as compared to other described CSC types was shown by the expression of cTnT and cTnI assessed by immunofluorescence. Furthermore, Electrophysiological measurements showed the presence of I_{Na}, I_{Ca}, transient outward potassium currents and the recordings of slow action potentials. The discovery of this new endogenous CSC population could open new perspectives in the search to regenerate the infarcted heart.

7.3 Future perspectives

In the near future, it will be interesting to further investigate the properties of the CASCs *in vivo*. These should be investigated in the large animal model, better fitted to extrapolate the obtained data to humans when compared to the murine or rat model. It would be useful to study the functional improvement of the heart after the transplantation of these CASCs measured by echo and magnetic resonance imaging. Furthermore, the differentiation, distribution and homing of these cells can be evaluated. These data will give information about the transplantation method that has to be used in order to have the highest homing efficiency in the peri-infarct zone.

A second area that should be subjected to further research is the paracrine effect exerted by bone marrow-derived MSCs. The transplantation of these MSCs does improve the heart function by the mobilization and recruitment of stem cells towards the infarcted region. The identification of the secreted factors responsible for this recruitment would be very promising in the development of an off-the-shelve treatment for patients suffering from IHD.

8. Nederlandse samenvatting

Een hartaanval is de belangrijkste doodsoorzaak in Vlaanderen en de rest van de Westerse Wereld. De wereldwijde prevalentie van coronaire hartziekte wordt geschat op 16.000.000 mensen waarvan 54% mannen zijn. Een hartinfarct wordt veroorzaakt door de obstructie van een coronaire arterie waardoor het weefsel bevloeid door deze arterie te weinig of geen nutriënten en zuurstof krijgt. Hierdoor sterven een massaal aantal cardiomyocyten af, waardoor het hart een deel van haar contractiele functie verliest. Als reactie op dit verlies ondergaat het hart een conformatieverandering wat leidt tot een inefficiënte pompfunctie en uiteindelijk uitmondt in hartfalen.

Over de jaren heen zijn er verschillende farmacologische en chirurgische therapieën ontwikkeld die de voortgang van het hartfalen vertragen. Een nadeel van deze behandelingen is dat ze het verloren hartweefsel niet vervangen waardoor de patiënt nog steeds een verminderde pompfunctie heeft. Een mogelijkheid om dit verlies te compenseren is door stamcellen te transplanteren in het hart. Deze stamcellen zouden differentiëren in cardiomyocyten die zich integreren in het hartweefsel en op deze manier de pompfunctie van het hart herstellen.

In het eerste deel van **Hoofdstuk 1** wordt er een overzicht gegeven van de epidemiologie van een hartinfarct in België en de rest van de Westerse Wereld. In het tweede deel wordt er ingegaan op de verschillende klinische trials die reeds werden uitgevoerd om de hartfunctie te verbeteren door de transplantatie van stamcellen. Op het einde van dit hoofdstuk worden de doelen van deze thesis aangehaald.

Hoofdstuk 2 beschrijft de verschillende methodes die werden gebruikt om de verschillende stamceltypes te isoleren en te karakteriseren. Ook worden de gebruikte methodes en merkers verklaard.

Hoofdstuk 3 handelt over de isolatie, karakterisatie en myocardiale differentiatie van mesenchymale stamcellen geïsoleerd uit humaan beenmerg. Er wordt in dit hoofdstuk aangetoond dat het percentage mesenchymale stamcellen aanwezig in het beenmerg zeer laag is. Het kweken van deze cellen leidt uiteindelijk tot een homogene celpopulatie die haar multipotente eigenschappen nog steeds bezit. Verder wordt in dit hoofdstuk de differentiatie van deze cellen naar cardiomyocyten bestudeerd. Ondanks het feit dat de mesenchymale stamcellen enkele cardiaal specifieke genen zoals *troponine T*, *GATA-4* en *Nkx2.5* tot expressie brengen, differentieerden deze cellen niet tot cardiomyocyten. Immunofluorescentie toonde immers aan dat troponine T niet op eiwitniveau aanwezig was. Ook werd via electronenmicroscopie aangetoond dat in deze cellen geen sarcomerische structuren aanwezig waren. Er kan dus gesteld worden dat mesenchymale stamcellen niet in staat zijn om te differentiëren tot functionele cardiomyocyten.

In **hoofdstuk 4** wordt de isolatie en de karakterisatie van uit beenmerg geïsoleerde hematopoietische stamcellen beschreven. Deze cellen werden geïsoleerd met behulp van flow-sorting, wat resulteerde in een 96% zuivere stamcelpopulatie. Na de co-cultuur met neonatale rat cardiomyocyten brachten deze cellen enkel *troponine T* tot expressie. Maar via immunofluorescentie analyse kon het eiwit niet gedetecteerd worden. Verder werd er ook via electronenmicroscopie aangetoond dat deze cellen geen sarcomerische

structuren bevatten. Deze data doet ons besluiten dat hematopoietische stamcellen niet kunnen differentiëren in de cardiale richting.

Tijdens dit onderzoek werd het in de literatuur meer en meer duidelijk dat het hart zelf ook een endogene stamcelpopulatie bezit.

In **Hoofdstuk 5** werden twee types van cardiale stamcellen geïsoleerd, namelijk c-kit$^+$ cardiale stamcellen en cardiosphere-derived cells. Het fenotype, de functionaliteit alsook de myocardiale differentiatiecapaciteit van deze twee celtypes werd vergeleken met die van de mesenchymale stamcellen. De data tonen aan dat er fenotypisch slechts kleine verschillen aantoonbaar zijn tussen de mesenchymale (CD140b+) en de cardiale stamcellen (CD140b-). De myocardiale differentiatiecapaciteit van deze cellen werd geanalyseerd na mono- en co-cultuur met neonatale rat cardiomyocyten en transforming growth factor-β1. Deze data tonen aan dat de myocardiale differentiatie van de mesenchymale stamcellen wordt beïnvloed door TGF-β1, terwijl dit niet het geval is voor de cardiale stamcellen. Na de co-cultuur brachten alle celtypes *MHC* tot expressie. Toch kon er een verschil in differentiatiecapaciteit worden aangetoond door middel van fluorescentieanalyse van de gecocultiveerde stamcellen. Ondanks het feit dat mesenchymale stamcellen *troponine T* tot expressie brachten op RNA niveau, kon het eiwit niet worden aangetoond. Dit was wel het geval in beide types van gecocultiveerde cardiale stamcellen. Deze cellen brachten het eiwit tot expressie in een duidelijke sarcomerische organisatie. Op basis van deze data kan er dus besloten worden dat het hart een endogene cardiale stamcelpopulatie bezit die geïsoleerd en *ex vivo* geëxpandeerd kan worden. Verder is deze populatie in staat om te differentiëren in de myocardiale richting.

De cardiale celtypes beschreven in hoofdstuk 5 waren niet altijd even gemakkelijk te isoleren en te expanderen. **Hoofdstuk 6** beschrijft de isolatie, karakterisatie, expansie en myocardiale differentiatie van een nieuw endogeen cardiaal stamceltype, namelijk Cardiac Atrial appendage Stem Cells (CASCs). Deze cellen werden geïsoleerd op basis van hun aldehyde dehydrogenase (ALDH) activiteit aanwezig in de cel. Flowcytometrische karakterisatie toont aan dat deze cellen fenotypisch verschillend zijn van de c-kit$^+$ cardiale stamcellen die gebruikt werden in hoofdstuk 5. Tevens tonen de data ook aan dat deze cellen zowel phenotypisch als functioneel verschillend zijn van de ALDH+ cellen geïsoleerd uit het beenmerg. Na de co-cultuur brengen deze cellen ook verschillende cardiaal specifieke genen tot expressie zoals *troponine T, myosin heavy chain* en *GATA-4*. Immunofluorescentie toont aan dat na de co-cultuur deze cellen niet enkel troponine T maar ook troponine I tot expressie brengen. Dit laat ons toe te besluiten dat een nieuw endogeen cardiaal stamceltype geïsoleerd kan worden op basis hun ALDH activiteit. Deze cellen kunnen *ex vivo* geëxpandeerd worden waarna ze nog in staat zijn om te differentiëren in de myocardiale richting.

Tot slot werd in **hoofdstuk 7** een besluit geformuleerd op basis van een kort overzicht van de resultaten van deze thesis. De bevindingen van deze studie dragen bij tot een beter inzicht over de myocardiale differentiatiecapaciteit van zowel beenmergstamcellen als de verschillende types cardiale stamcellen. De positieve resultaten van de cardiale stamcellen zullen nu in een groot proefdiermodel bevestigd moeten worden. De resultaten van deze thesis zullen dus bijgedragen hebben aan de ontwikkeling voor een betere therapie voor patiënten die een hartinfarct hebben gehad.

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Curriculum Vitae

Remco Koninckx werd geboren op 28 januari 1984 te Bilzen. In 2001 behaalde hij het diploma Algemeen Secundair Onderwijs (ASO) Latijn-Wiskunde aan het Koninklijk Atheneum in Tongeren. In september van hetzelfde jaar begon hij aan zijn opleiding biomedische wetenschappen aan de faculteit Geneeskunde van het Limburgs Universitair Centrum (LUC). In november 2004 vatte hij zijn licentiaatsstage aan in het laboratorium “Experimentele Hematologie” van het Jessa ziekenhuis in Hasselt. Voor zijn proefschrift, getiteld “Opsporen van genetische afwijkingen in gesorteerde plasmacellen bij de ziekte van Kahler” kreeg hij de thesisprijs. In juli 2005 studeerde hij met grote onderscheiding af als licentiaat biomedische wetenschappen aan de Universiteit Hasselt (UHasselt). Later dat jaar behaalde hij zijn IWT doctoraatsbeurs die in oktober 2007 werd omgezet naar een BOF-beurs waardoor hij voor 2 jaar werd aangesteld als doctoraatsstudent aan de UHasselt. Ter voorbereiding van dit doctoraatsproefschrift verrichtte hij in het laboratorium “Experimentele Hematologie” van het Jessa ziekenhuis 4 jaar lang onderzoek naar de myocardiale differentiatiecapaciteit van verschillende stamceltypes. Tijdens deze periode volgde hij een proefdiercursus aan de UHasselt, een proefdiercursus aan de KU Leuven, een cursus over het gebruik van Göttingen minipigs en een doctoraatsopleiding die in 2009 succesvol werd beëindigd.

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Oral presentation

7 mei 2009

"FlandersBio: Knowledge for Growth 2009 Ghent, Belgium"

Annick Daniëls, **Remco Koninckx**, Paul Steels; Marc Hendrikx; Jean-Luc Rummens; Karen Hensen.

Cardiac stem cells: a new potential tool for cardiac repair?

Posters

23 maart 2005
"9th Maastricht Medical Students Research Conference 2005, Maastricht, the Netherlands"
Koninckx R, Franke S**,** Hensen K, Stinissen P, Rummens J-L.
Fluorescence activated cell sorting of plasma cells in multiple myeloma

2-5 juni 2005: "EHA 10th Annual Congress 2005, Stockholm, Sweden"
J.L. Rummens, **R. Koninckx**, H. Jongen, S. Franke, B. Maes, V. Peeters, R. Cartuyvels, P. Declercq, K. Magerman, A. Mewis, K. Hensen

"Fluorescence activated cell sorting of plasma cells for further cytogenetic analysis"

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13-16 november 2005: "AHA Scientific sessions 2005, Dallas, Texas, USA"

Hendrikx M, Hensen K, Clijsters C, Jongen H, **Koninckx R**, Bijnens E, Ingels M, Jacobs A, Geukens R, Dendale P, Vijgen J, Dilling D, Steels P, Mees U, Rummens JL.

A randomized controlled clinical trial of autologous bone-marrow transplantation by direct intramyocardiel injection for myocardial regeneration.

18 november 2005 "10th Annual Symposium BVC/ABC, Brussels, Belgium"

J.L. Rummens, **R. Koninckx**, H. Jongen, S. Franke, B. Maes, V. Peeters, R. Cartuyvels, P. Declercq, K. Magerman, A. Mewis, K. Hensen

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27-28 januari 2006 "21th General Meeting of The Belgian Hematological Society. Genval, Belgium"

Franke S, Hensen K, Maes B, **Koninckx R**, Peeters V, Rummens JL

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6-8 oktober 2006 "EHA Scientific Workshop on Biology and Clinical Applications of Mesenchymal Stem Cells, Mandelieu, France"

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Characterisation of human mesenchymal stem cells in monoculture and in coculture with neonatal rat cardiomyocytes.

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Koninckx R, Hensen K, Schurgers E, Jongen H, Clijsters C, Steels P, Hendrikx M, Rummens J-L

Neonatale rat cardiomyocyten induceren de expressie van hart-specifieke genen bij humane mesenchymale stamcellen.

30 juli – 2 augustus 2007 "4th Annual Symposium of the American Heart Association Council on Basic Cardiovascular Sciences: Cardiovascular Repair and Regeneration: Structural and Molecular Approaches in the Cellular Era Keystone, CO, USA"

Remco Koninckx, Karen Hensen, Evelien Schurgers, Christel Clijsters, Hanne Jongen, Paul Steels, Jean-Luc Rummens, Marc Hendrikx

Co-culture with neonatal rat cardiomyocytes induces expression of myosin heavy chain in human mesenchymal stem cells.

26 oktober 2007 "12th BVAC-meeting, Mons, Belgium"

Remco Koninckx, Hanne Jongen, Paul Steels, Marc Hendrikx, Paul Dendale, Dominique Hansen, Jean-Luc Rummens, Karen Hensen

A correct gating strategy plays an important role in rare event detection of endothelial progenitor cells.

6 juni 2008 "FlandersBio: Knowledge for Growth 2008, Ghent, Belgium"

Remco Koninckx, Hanne Jongen, Paul Steels, Marc Hendrikx, Paul Dendale, Dominique Hansen, Jean-Luc Rummens, Karen Hensen

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A correct gating strategy plays an important role in rare event detection of endothelial progenitor cells.

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Cardiac stem cells : a new potential tool for cardiac repair?

7 mei 2009 "FlandersBio: Knowledge for Growth 2009 Ghent, Belgium"

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Cardiac stem cells : a new potential tool for cardiac repair?

26-29 mei 2009 "ISHR North American Section Meeting: New discoveries for prevention and treatment of heart disease, Baltimore, MA, USA"

Annick Daniëls, **Remco Koninckx**, Paul Steels, Marc Hendrikx, Jean-Luc Rummens, Karen Hensen

Cardiac stem cells : a new potential tool for cardiac repair?

9-12 september 2009 "38th Annua Scientific Meeting of the ISEH-Society for Hematology and Stem Cells, with the Hellenic Society of Heamtology, Athens, Greece"

K Hensen, **R Koninckx**, S Windmolders, A Daniels, M Hendrikx, J-L Rummens

The human heart contains a CD34+ non-haematopoietic stem cell population.

Cardiac Stem cells: a new potential tool for cardiac repair ?

17-18 March 2010 “The life Science Summit” Aachen, Germany

K Hensen, **R Koninckx**, S Windmolders, A Daniels, M Hendrikx, J-L Rummens

The human heart contains a CD34+ non-haematopoietic stem cell population.

S Windmolders, A De Boeck, K Hensen, O De Wever, **R Koninckx**, A Daniëls, M Hendrikx, M Bracke, J-L Rummens

Mesenchymal stem cells exert migratory effects on cardiac (stem) cells

Dankwoord

Ondertussen al meer dan 4 jaar geleden werd het voorstel gedaan om te doctoreren in het Jessa ziekenhuis. Zonder veel aarzelen zeg je dan direct ja. Het is een unieke kans die ik kreeg om me te ontplooien in een onderzoeksveld waar ik persoonlijk al interesse in had. Maar waar begin je aan? Het enige dat ik met 100% zekerheid kan zeggen is dat ik niet alleen deze thesis tot stand heb doen komen. Ook al staat het dankwoord helemaal achteraan in deze thesis, ik denk dat absoluut niet mag vergeten worden wat de mensen die hieronder volgen zowel voor mijn doctoraat alsook voor mij persoonlijk hebben betekend en nog steeds betekenen.

In de eerste plaats zou ik mijn promotor willen bedanken, Prof. dr. Marc Hendrikx. Vanaf dag 1 hebt u uw deur voor mij opengezet. Ondanks uw drukke schema was ik steeds welkom in uw kantoor waar we vaak ***uren*** hebben gepraat en nagedacht over de resultaten die ik dan ***snel*** even kwam laten zien. Uw kritische bemerkingen, tijdens deze gesprekken maar ook tijdens de door iedereen geliefde maandagochtend vergaderingen, kwamen steeds opbouwend over waardoor de motivatie om een experiment nog eens en nog eens en nog eens te herhalen altijd bleef bestaan. Ook na de niet-verlenging van mijn IWT-beurs stelde u me direct gerust. U zou een oplossing vinden waardoor ik mijn doctoraatsproject tot een goed einde zou kunnen brengen. En inderdaad u vond een oplossing zonder dat ik me ergens zorgen diende over te maken. Ik wil u dan ook bedanken dat u me 2 jaar geleden overtuigd hebt om dit onderzoek verder te zetten. Natuurlijk moet ik u ook bedanken voor het veelvuldig nalezen van al mijn abstracts, posters, papers en niet te vergeten deze doctoraatsthesis.

Ook mijn co-promoter, Prof. Dr. Paul Steels, verdient een woord van dank voor zijn steeds kritische opmerkingen die hij formuleerde bij de verschillende vergaderingen die hij ondanks het vroege uur (7u.30) steeds bijwoonde. Tevens wil ik u ook bedanken voor het herhaaldelijk nalezen van deze thesis die mede dankzij u tot dit geheel is gevormd.

Dr. Rummens, als hoofd van “Experimentele Hematologie” (EXH) hebt u mij de kans gegeven om na mijn licentiaatsthesis onderzoek te kunnen doen in een stabiele omgeving waar een onderzoeker zich geen zorgen hoeft te maken over de producten die hij bestelt. Steeds weer als ik aan uw deur stond te kloppen via Josepha voor een nieuw antilichaam of een nieuwe buffer kreeg ik een goedkeurende handtekening. Dit maakte het voor mij makkelijker om bepaalde experimenten te kunnen uitvoeren. Ook wil ik u bedanken voor uw kritische en wetenschappelijke blik op dit doctoraatsonderzoek en voor het herhaaldelijk nalezen van de verschillende abstracts, papers en posters.

Ik denk dat er nu een dame bedankt moet worden wiens deur ik het meest heb platgelopen de afgelopen 4 jaar, dr. Karen Hensen. Karen, steeds opnieuw stond je voor me klaar. Niet enkel voor al mijn wetenschappelijke vragen maar ook voor een gewone simpele babbel als ik me weer eens eenzaam ☹ voelde op EXH. Jouw deur is voor niemand ooit gesloten. Er zijn dagen geweest en er zullen er nog komen waar je als psycholoog *ad interim* werkt. Daarom stel ik nog steeds voor je grote kast te vervangen door een mooie longchair waar we af en toe eens even onze geest kunnen komen leeg praten tegen jou. Ik ben er zeker van dat zonder jou mijn motivatie om steeds te blijven verder doen vaker onder nul had gezeten dan nu het geval was. Steeds weer wist je me te kalmeren en te overtuigen dat ik (lees we) goed bezig was(ren). Je vertelde me

steeds opnieuw: de resultaten komen wel; Geen paniek; Dat is bij alle onderzoeken zo; (en natuurlijk de dooddoener) Tja, dat is onderzoek hé. Liefste Karen, ik kan je alleen nog maar eens opnieuw uitdrukkelijk bedanken voor alles wat je voor mij gedaan hebt. Bij deze: DANK JE WEL!

Verder moet ik ook een zeer speciale dank je wel richten aan mijn 3 (ex)-collega's, Hanne, Christel en Annick van het labo EXH. Hanne, jij hebt jouw passie en inzicht in de flowcytometrie en specifieker de FACSAria overgebracht op mij. Uren hebben we samen aan dit toestel gespendeerd om nog meer eens opnieuw een compensatie in te zetten of een nieuwe isotypecontrole te analyseren. Tevens zal ik de babbels die we hadden over huizen, inrichtingen, skiën en snowboarden,… nooit vergeten. Bedankt Hanne om op alle mogelijke tijdstippen voor me klaar te staan. Christel, ook jij was in een niet zo ver verleden een medewerkster van EXH. Je hebt me met veel geduld de technieken van steriele celkweek aangeleerd, iets van cruciaal belang in het vervolledigen van deze thesis. En toen kwam Annick mij uit mijn eenzaamheid verlossen na het vertrek van Hanne en Christel. Na een ietwat stil begin zijn we een volledig complementair team geworden. Je stond altijd voor me klaar om, als ik weer eens te veel hooi op mijn vork had genomen, een kweekje te verversen of een hartoortje op en neer te pipeteren of een PCR-tje in te zetten. Voor al deze zaken wil ik jullie, Hanne, Christel en Annick, van harte bedanken. Volgens mij dient er in deze paragraaf nog een prachtige dame bedankt te worden. Want eigenlijk als je EXH zegt weten we allemaal wat er volgt; BIO. Loes, bedankt voor de vele soms-op-niets-uitdraaiende-en-Annick-op-stangjagende discussies. Maar ook voor de wetenschappelijke discussies en je kritische blik op het

onderzoek. Je aanwezigheid en enorme energie doet iedereen deugd. Hou nooit op met je enthousiaste JIJ te zijn ☺.

Dan zijn er ook nog de studenten die met hun jeugdig enthousiasme ieder jaar opnieuw een frisse wind lieten waaien door het labo. Evelien, Ellen, Lindsey en Severina bedankt voor de maanden dat jullie rechtstreeks of onrechtstreeks hebben meegewerkt aan dit doctoraat. Verder wil ik bij deze Severina nog eens een dikke proficiat wensen met het behalen van haar IWT-beurs en haar ook héél veel succes wensen met haar doctoraat. Ik ben er zeker van dat je de komende 4 jaar allerlei boeiende uitdagingen gaat voorgeschoteld krijgen zoals het verzorgen van minivarkentjes ☺.

Tevens wil ik alle mollekes bedanken voor het altijd klaar staan voor mij. Rita, Lieve, An, Miet, Ilse en Jonathan het was altijd wel een gevecht voor het minste aantal stalen om cDNA te maken, maar ook als ik er meer had dan jullie waren jullie altijd bereid om mijn staaltjes ook mee te nemen. Een keertje RNA isoleren in een overvolle isolatieruimte, geen probleem iedereen zat wat dichter op elkaar en dan ging het ook wel. Bedankt voor de heel fijne tijd op gang G0.

Ook de laboranten van het labo anatome-pathologie wil ik bedanken voor het snijden van de cryosecties en parafinecoupes. Sabine en Dorien, jullie lezen nu dat ik me aan mijn woord heb gehouden hé ☺.

Ook wil ik alle klinisch biologen, wetenschappelijk medewerksters en laboranten van het klinisch labo bedanken. Iedereen stond altijd klaar om me met raad en daad bij staan. Ik besef heel goed in wat voor een luxepositie ik zat om dit doctoraat af te werken. Iedereen Bedankt.

Een volgend bedankje gaat uit naar de mensen van operatiezaal 7. Nooit (tenzij we echt te laat waren) maakten zij er een probleem van als we nog maar eens beenmerg, bloedbuizen of een hartoortje nodig hadden. Mede dankzij hen was het plezant om de dag met een nieuw staal te beginnen. Allemaal heel fel bedankt.

Voor mijn andere bedankjes moet ik het een beetje verder gaan zoeken. Ik wil deze paragraaf dan ook wijden aan de mensen van de UHasselt. Eerst en vooral wil ik Ivo Lambrichts en Marjan Moreels bedanken voor de onophoudelijke inzet om alle kweken die ik langs bracht electronenmicroscopisch te analyseren. Wendy, Katherine en Inge bedankt voor de pups die ik van jullie kon gebruiken maar ook om me altijd op de hoogte te houden van de veranderingen die er op til stonden in BIOMED. Wendy, ook bedankt voor de lekkere napoleonekes die ik bij ieder bezoek kreeg ☺.

Een tweede stop die ik buitenshuis moet maken is in Leuven. Hierbij wil ik professor Kanigula Mubagwa bedanken voor het openstellen van zijn labo voor de electrofysiologie experimenten. De data die met deze experimenten werden gegenereerd zorgden voor een enorme bijdrage aan de het laatste hoofdstuk. Ook Regina mag zeker niet vergeten worden in dit dankwoord. Regina, you introduced me into the world of sodium and potassium currents. You thought me the basics in electrophysiology and helped me with all my questions. I could ask everything and you explained it always very calmly. Thank you for all your help with my experiments. I hope you have a good time now you are united with your family in Lithuania.

Dit lijkt me ook het aangewezen moment om mijn familie en vrienden te bedanken. Eerst en vooral, mama en papa dank je wel om me 4 jaar geleden te steunen in mijn beslissing om een IWT-beurs aan te vragen en te starten met doctoreren. Het was soms misschien niet allemaal even duidelijk waar ik allemaal mee bezig was, maar na het lezen van dit boekje is het zo klaar als een klontje hé ☺. Bedankt om er altijd voor mij en Debby en binnenkort onze zoon/dochter te zijn. Het is fijn om te weten dat er altijd een warme thuis is waar we af en toe eens kunnen komen uitblazen en ons buikje rond eten ☺. Ook Kris, Marleen, Jonas, Vincent, Betty, Théo en Davy allemaal heel fel bedankt om me te steunen en te begrijpen waarom ik soms later op een feestje aankwam.

En last but certainly not least, Debby. Kleine muis, er is in de afgelopen 4 jaar veel veranderd in ons leven. We zijn gaan samenwonen, hebben een huis gekocht en nu verwachten we ons eerste kindje. Maar ondanks alle veranderingen heb jij altijd voor een stabiele en warme thuis gezorgd. Daarom denk ik dat jij dit doctoraat misschien evenveel verdient als ik. Nooit heb je je geduld verloren als ik weer eens later thuis was, nogmaals “eventjes” ’s avonds ging werken, of een zaterdagvoormiddagje wat celkweek ging doen in het ziekenhuis. Het was fijn om te weten dat ik mij voor 100% kon concentreren op mijn doctoraat zonder me zorgen te maken over bepaalde huiselijke zaken. Bedankt voor alles kleine muis!