"Research is the process of going up alleys to see if they're blind."

Marston Bates

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Doctor in de Biomedische Wetenschappen

Met dank aan het agentschap voor Innovatie door Wetenschap en Technologie (IWT) en het Limburg Clinical Research Program (LCRP), gesponsord door Limburg sterk Merk, de provincie Limburg, de Vlaamse overheid, de Universiteit Hasselt, het Jessa ziekenhuis en het ziekenhuis Oost-Limburg

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ALDH	Aldehyde dehydrogenase
aTL	Absolute telomere length
B2M	Beta-2-microglobulin
BMC	Bone-marrow derived cell
BMP	Bone morphogenetic protein
CAGB	Coronary artery bypass graft
CAM	Chorioallantoic membrane
CASC	Cardiac atrial appendage stem cell
CDC	Cardiosphere-derived cell
CM-CASC	Conditioned medium of CASCs
CM-MSC	Conditioned medium of MSCs
COL1A1	Procollagen type I alpha 1
COL3A1	Procollagen type III alpha 1
cJUN	Jun-proto-oncogene
cMYC	V-myc avian myelocytomatosis viral oncogene homolog
CSC	Cardiac stem cell
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
CX43	Connexin 43
DAPI	4’,6-diamidino-2-phenylidole
DMEM	Dulbecco’s modified eagle medium
DMEM-F12	Dulbecco’s modified eagle medium: nutrient mixture F-12
DPSCs	Dental pulpa stem cells
EA	Early apoptotic
ECM	Extracellular matrix
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell
ESC-CM	Embryonic stem cell derived cardiomyocyte
ET-1	Endothelin 1
FBS	Fetal bovine serum

FGF	Fibroblast growth factor
FHF	First heart field
FITC	Fluorescein isothiocyanate
FZD	Frizzled
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA4	GATA binding protein 4
GFP	Green fluorescent protein
GSK	Glycogen synthase kinase
HCN4	Hyperpolarization activated cyclic nucleotide gated potassium channel 4
HGF	Hepatocyte growth factor
HSC	Hematopoietic stem cell
hTERT	Human telomerase reverse transcriptase
IGF-1	Insulin-like growth factor 1
IGFBP-3	Insulin-like growth factor binding protein 3
IHD	Ischemic heart disease
IL	Interleukin
iPSC	Induced pluripotent stem cell
iPSC-CM	Induced pluripotent stem cell derived cardiomyocyte
IQR	Interquartile range
KDR	Kinase insert domain receptor
LA	Late apoptotic
LAA	Left atrial appendage
LRP	Lipoprotein receptor related protein
MI	Myocardial infarction
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
mtDNA	Mitochondrial DNA
MYL	Myosin light chain
NKX2.5	NK2 homeobox 5
NRCM	Neonatal rat cardiomyocytes
PD	Population doubling

PDGF	Platelet derived growth factor
PDGFRα	Platelet derived growth factor receptor α
PDL	Population doubling level
PDT	Population doubling time
PFA	Paraformaldehyde
PI	Propidium iodide
PL	Platelet lysate
POLR2A	Polymerase (RNA) II subunit A
PPS	Platelet plasma supernatant
PS	Penicillin/Streptomycin
PSC	Pluripotent stem cell
RAA	Right atrial appendage
ROS	Reactive oxygen species
SCG	Single copy gene
SDF-1	Stromal derived factor-1
SHF	Second heart field
siRNA	Silencing RNA
T	T brachyury transcription factor
TBX	T-box
TGF	Transforming growth factor
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF	Tumor necrosis factor
TNNT2	Troponin T2, cardiac type
VEGF	Vascular endothelial growth factor
WHO	World health organization
YWHAZ	Tyrosin 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

SUMMARY

Heart failure as a consequence of myocardial infarction (MI) remains the leading cause of morbidity and mortality worldwide, taking more lives than all cancers combined. Since the loss of functional cardiomyocytes underpins the pathophysiology of MI and subsequent development of heart failure, stem cell therapy has been widely investigated as a new therapeutic strategy. The success of a cardiac stem cell therapy largely depends on the selection of the appropriate stem cell type. Furthermore, the molecular mechanisms involved in cardiac repair, but also in progenitor cell proliferation and myocardial differentiation need to be further elucidated. Finally, the hostile microenvironment of the infarct, characterized by inflammation, ischemia and fibrosis needs to be tackled in order to improve stem cell survival, integration and differentiation.

Cardiac stem cells isolated based on an elevated aldehyde dehydrogenase (ALDH) activity, called cardiac atrial appendage stem cells (CASCs), are a promising candidate for myocardial regeneration. In the first part of this study, we demonstrated that CASC proliferation is not unlimited and accompanied by a minor but significant reduction in absolute telomere length due to the lack of telomerase activity. However, despite a decrease in the proliferative percentage of CASCs during culture, clinically relevant cell numbers were generated, equaling ranges used in previous clinical trials with cardiac stem cells. Furthermore, CASCs preserved their biological properties during culture, including their antigenic expression profile, ALDH expression and more importantly their myocardial differentiation potential as demonstrated by the sarcomeric organization of cardiac troponin T and I. Finally, CASCs were also successfully expanded in human platelet plasma supernatant while maintaining their biological properties, which is an important step towards the clinical application of CASCs.

In a second part of this study, we demonstrated that within the adult heart, CASCs are predominantly present in the atrial appendages and more abundant in the right than in the left atrial appendage. We showed that they express multiple early cardiac differentiation markers such as *NKX2.5*, *GATA4*, *TBX5* and *TBX18* and are committed towards myocardial differentiation as demonstrated by the expression of *TNNT2* and *MYL2*. These results also suggest a possible heterogeneous embryonic origin of CASCs since these early cardiac differentiation markers are

expressed in distinctive cardiac progenitor cell populations during cardiac development. Besides, the presence of several Frizzled receptors on CASCs suggested a role of Wnt signaling in self-renewal, proliferation and differentiation of the CASCs. However, despite an active role of Wnt signaling in CASCs as shown by the increase in total and active β-catenin levels, Wnt activation did not affect CASC proliferation or self-renewal. Furthermore, Wnt inhibition upregulated early cardiac markers without inducing mature myocardial differentiation. So, although Wnt signaling is functional in CASCs and has been described to play a crucial role in cardiogenesis and differentiation of pluripotent stem cells towards cardiac lineages, it has only limited effects on CASC proliferation and differentiation *in vitro*.

In a third part of this study, we aimed to improve CASC survival under oxygen-deprived conditions since the microenvironment of the infarct area is characterized by ischemia, inflammation and fibrosis and the survival of transplanted cells will most likely be negatively affected by the low oxygen levels in the targeted area. Here we showed that the declined CASC viability associated with hypoxia but not anoxia could be partly recovered by treating them with conditioned medium of mesenchymal stem cells (CM-MSC). The observed increase in CASC survival was also accompanied by an increase in CASC proliferation as shown by an increase in the number of Ki67 positive cells cultured in CM-MSC under hypoxic conditions. This paracrine effect was not mediated via VEGF or PDGF and the CM-MSC protection of CASCs against hypoxia induced cell death occurred in an Akt-independent manner. Instead, CM-MSC treatment of CASCs upregulated catalase expression levels under hypoxic conditions.

Finally, we developed an experimental approach to study cardiac fibrosis in an *in vitro* setting. Cardiac fibrosis does not only lead to the development of heart failure but is also an important limiting factor in the development of a successful cardiac regeneration therapy since CASCs that end up in the fibrotic tissue are not able to make contact with functional cardiomyocytes and therefore do not differentiate towards cardiomyocytes. This experimental approach can now be used to study the effect TGF-β1 and TGF-β3 on cardiac fibroblasts and to investigate whether the process of fetal wound healing can be mimicked in the heart. In contrast to adult wound healing, fetal wound healing is not accompanied by scar tissue

formation, partly mediated by the low TGF-β1 to TGF-β3 ratios and the absence of TGF-β1 induced collagen deposition.

In conclusion, the results described in this thesis provide important molecular and cellular insights in CASC proliferation, differentiation and survival. However, further research is essential to fully understand the underlying mechanisms and responsible factors which will improve CASC therapy for patients with heart failure by targeting CASC biology and the hostile microenvironment after an infarction.

Chapter 1

General introduction and aims

1.1. DEVELOPMENT OF THE HUMAN HEART

1.1.1.Cardiac development during mammalian embryogenesis

The heart is the first functional organ formed during mammalian embryogenesis. During cardiac development, three major sources of cardiac precursors, the cardiac mesoderm, the cardiac neural crest and proepicardial organ, give rise to distinct structures of the heart (Figure 1.1). The cardiac mesoderm forms the linear heart tube and ultimately the working myocardium in atria and ventricles. Hereby, the first heart field (FHF) progenitors contribute to the left ventricle and parts of both atria, while the second heart field (SHF) progenitors contribute to the right ventricle, parts of both atria and the outflow tract. The neural crest gives rise to the smooth muscle cells of the aortic arch, ductus arteriosus and the great vessels and is responsible for correct outflow tract septation during which the aorticopulmonary septum separates the distal outflow tract into the ascending aorta and pulmonary trunk. Furthermore, it contributes to essential components of the cardiac autonomic nervous system. Finally, the proepicardial organ generates the endothelium and smooth muscle of the coronary arteries and perivascular fibroblasts (Laugwitz et al. 2008).

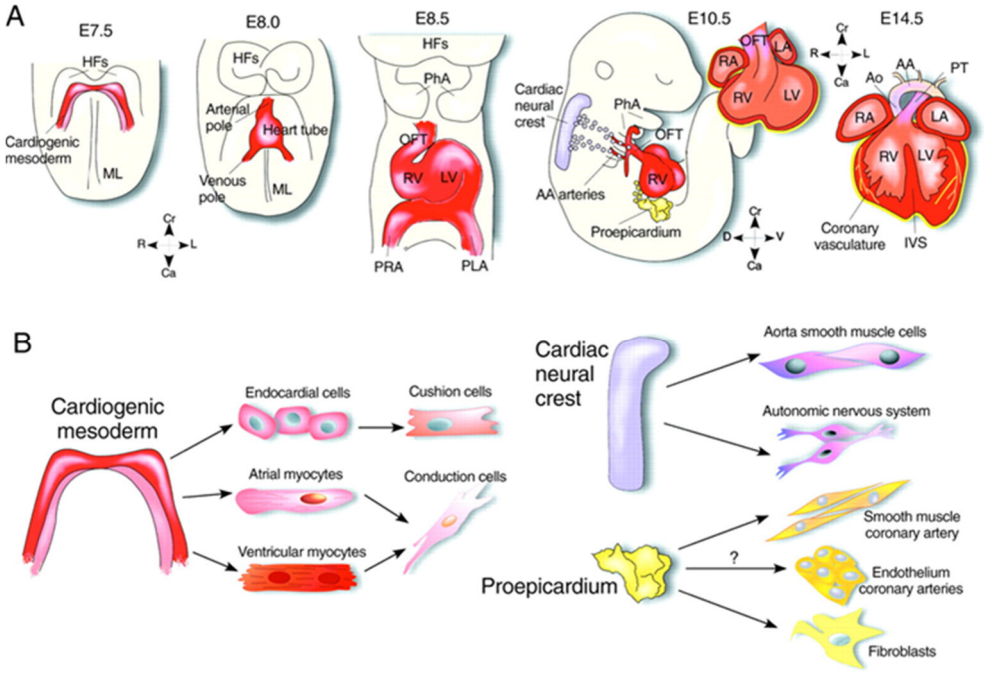

Figure 1.1: Origin and development of distinct structures in the mammalian heart. (A) Three populations of cardiac precursors, the cardiac mesoderm (red), cardiac neural crest (purple) and proepicardial organ (yellow), give rise to distinct structures of the heart. The cardiac mesoderm is subdivided in the first and second heart field (FHF and SHF) which form the linear heart tube and ultimately the four chambers of the heart. The cardiac neural crest will form the aortic arch arteries and the vascular smooth muscle tissue of the outflow tract (OFT). The proepicardial organ gives rise to the epicardium and the coronary arteries. (B) The three cardiac precursor pools, cardiac mesoderm, cardiac neural crest and proepicardium, give rise to specific cardiac cell types. AA = aortic arch; Ao = aorta; Ca = caudal; Cr = cranial; IVS = interventricular septum; L = left; LA = left atrium; LV = left ventricle; ML = midline; PhA = pharyngeal arches; PLA = primitive left atrium; PRA = primitive right atrium; PT = pulmonary trunk; R = right; RA = right atrium (Adapted from (Laugwitz et al. 2008))

1.1.2.Signaling pathways involved in cardiac development

The signaling pathways and transcriptional machineries that direct cardiac induction, differentiation, proliferation and cell fate of these cardiac precursors are tremendously complex. The relevant signaling pathways include (1) Wnt, (2) fibroblast growth factor (FGF), (3) transforming growth factor β (TGF-β), (4) hedgehog and (5) notch signaling (Figure 1.2). Furthermore, the transcriptional machineries that direct the specification and differentiation of these cardiac precursors are part of an evolutionarily conserved program that includes the *HAND*, *NKX*, *GATA*, *TBX* and *MEF2C* family of transcription factors. Finally, also epigenetic marks and chromatin remodeling subunits play an important role in cardiomyogenesis (Rana et al. 2013).

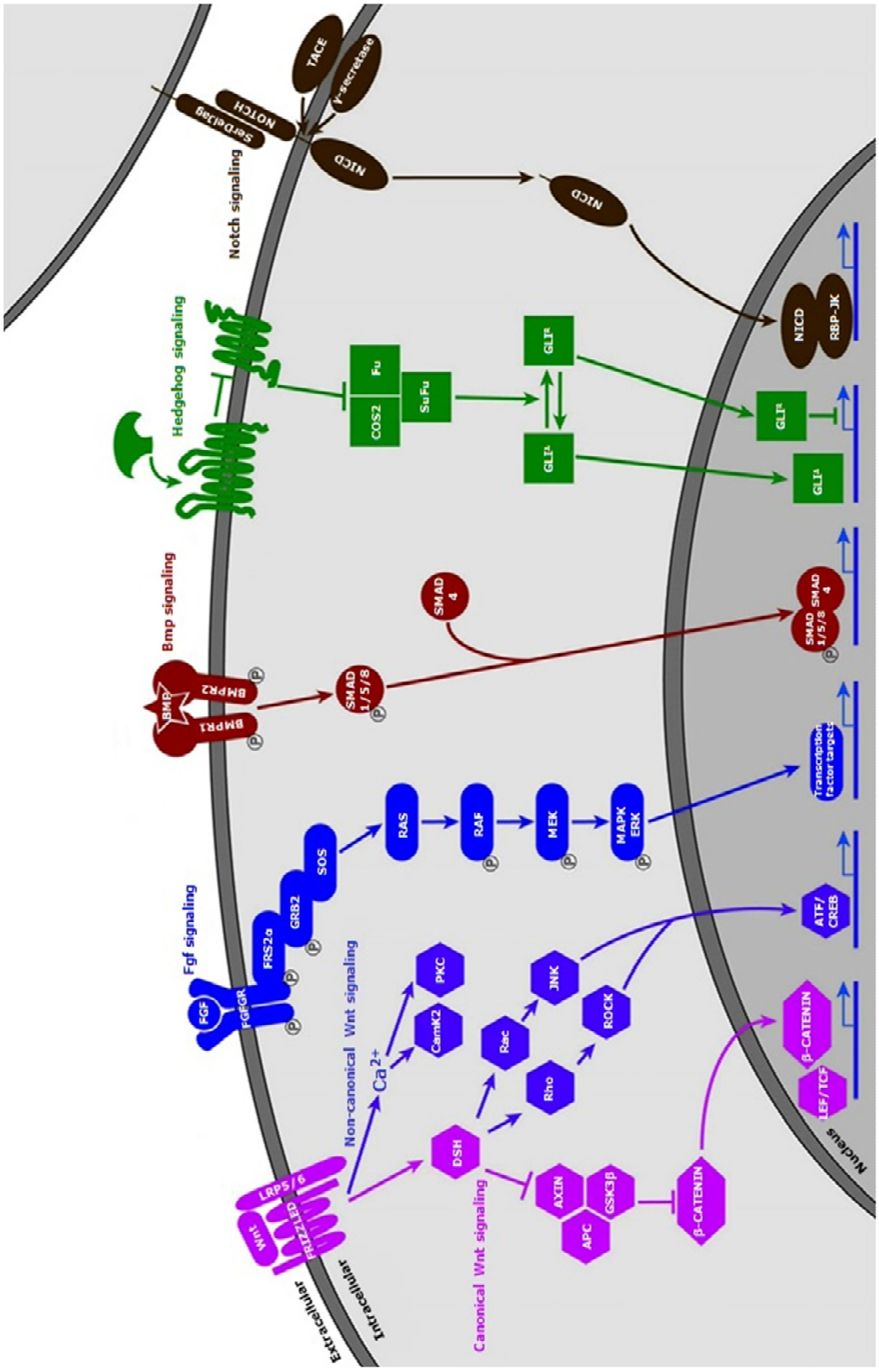
Extracellular
Intracellular
Nucleus
Wnt
LRP5/6
FRIZZLED
DSH
AXIN
APC
GSK3β
β-CATENIN
Canonical Wnt signaling
Non-canonical Wnt signaling
Ca2+
CamK2
PKC
Rac
Rho
JNK
ROCK
FGF
FGFR
FRS2α
GR82
SOS
RAS
RAF
MEK
MAPK ERK
Fgf signaling
BMP
BMPR2
BMPR1
SMAD 1/5/8
SMAD 4
Bmp signaling
Hedgehog signaling
Fu
COS2
SuFu
GLI
Notch signaling
Ligand
NOTCH
TACE
γ-secretase
NICD
RBP-JK
ATF/ CREB
LEF/TCF
β-CATENIN
Transcription factor targets

Figure 1.2: Core features of the Wnt, FGF, BMP, Hedgehog and Notch signaling pathways. (1a) In the canonical Wnt/β-catenin signaling pathway Wnt ligand binding to the frizzled-LRP5/6 receptor complex activates disheveled (DSH) which results in the inactivation of a multiprotein degradation complex composed of adenomatous polyposis coli (APC), axin and glycogen synthase kinase (GSK)3β. As a result, β-catenin accumulates in the cell and translocates to the nucleus where it binds to LEF/TCF transcription factors and activates target gene transcription. (1b) In non-canonical Wnt signaling, activated DSH promotes small G protein (Rac and Rho) activation which subsequently activates c-Jun N-terminal kinase (JNK) and Rho-associated kinase (ROCK). This eventuates in the activation of the ATF/CREB complex and subsequent target gene transcription. Alternatively, Wnt ligand binding results in intracellular Ca^{2+} release and activation of the Ca^{2+}/calmodulin-dependent kinase 2 (CamK2) and the protein kinase (PK)C pathways. (2) FGF signaling is initiated by ligand-dependent dimerization of the Fgf receptor (FGFR) which results in autophosphorylation of the Fgf receptor tyrosine kinase. This promotes the binding of the adaptor molecule FRS2α and the subsequent recruitment and activation of GRB2/SOS (growth factor receptor-bound protein 2/Son of sevenless) complex. SOS activates RAS which subsequently triggers a phosphorylation cascade leading to activation of RAF, MEK and ERK. Activated ERK phosphorylates target transcription factors which results in activated gene expression. (3) The Bmp signaling pathway is activated when Bmp, a member of the TGF-β family of signaling molecules, binds to a receptor complex composed of type 1 and type 2 Bmp receptors. This results in the formation of a Smad complex, composed of phosphorylated Smad1/5/8 and a co-activator Smad4, which enters the nucleus and activates target gene expression. (4) Hedgehog ligand binds to the Patched receptor which releases the inhibition of Smoothened (Smo). As a result, activated Smo blocks the COS2/FU/SuFu (Coastal-2/Fused/Suppressor of fused) complex, leading to the generation of the activator form of GLI (GLI^A) that translocates the nucleus and drives target gene transcription. In the absence of hedgehog ligand, the repressor form of GLI (GLI^R) is formed which inhibits target gene transcription. (5) Notch signaling is initiated when transmembrane ligand proteins (Ser, Serrate; Del, Delta; Jag, Jagged) on a neighboring cell interact with the Notch receptor on the target cell. This triggers the proteolytic cleavage of Notch by Tumor necrosis factor (TNF)α-converting enzyme (TACE) and γ-secretase and the release of the Notch intracellular domain (NICD). When NICD translocates to the nucleus, it forms a transcriptional complex with recombination signal binding protein (RBP)-Jκ to activate target gene transcription. (Adapted from (Rochais et al. 2009))

Wnt signaling

Three Wnt signaling pathways have been characterized: (1) the canonical Wnt pathway, (2) the non-canonical planar cell polarity or Wnt/jun N-terminal kinase (JNK) pathway and (3) the non-canonical Wnt/calcium pathway. The non-canonical Wnt signaling pathways promote cardiac differentiation via inhibition of the canonical Wnt pathway (Koyanagi et al. 2005, Cohen et al. 2012). The canonical Wnt signaling pathway regulates several target genes involved in cell proliferation and differentiation and influences cardiogenesis during four distinct phases. First, canonical Wnt signaling is upregulated to activate mesoderm formation. The subsequent inhibition of canonical Wnt signaling will induce cardiac

specification and cardiac progenitor cell formation. Afterwards, canonical Wnt signaling is re-upregulated again to stimulate cardiac progenitor cell proliferation. Finally, downregulation of the canonical Wnt signaling is required to obtain terminal cardiac differentiation of the progenitor cells (Gessert and Kuhl 2010).

Fibroblast growth factor signaling

The FGF signaling family, more particularly FGF8 and FGF10, is involved in SHF expansion. Furthermore, FGF8 is involved in early cardiac mesoderm specification and the formation of the ventricular myocardium through the formation of sufficient cardiomyocytes. In contrast, FGF10 is responsible for correct outflow tract septation and proper positioning of the heart in the thoracic cavity. Finally, FGF is also involved in myocardial specification and differentiation (Meganathan et al. 2015).

Transforming growth factor signaling

The TGF-β superfamily is involved in cardiac development through ligands like bone morphogenetic protein (BMP). TGF-β signaling also interacts with the other signaling pathways involved in cardiogenesis since BMP is also a downstream effector of the canonical Wnt pathway which negatively regulates FGF signaling. BMPs promote cardiogenesis by the induction of cardiac-specific genes and cardiomyocyte maturation (Rana et al. 2013).

Hedgehog signaling

In mammals, hedgehog signaling is induced by three different ligands, namely desert, indian and sonic hedgehog. The hedgehog pathway is required for proangiogenic gene expression and the maintenance of the adult coronary vasculature. Furthermore it is responsible for maintaining the size of the SHF population and plays important roles during outflow tract septation. Finally, overexpression of hedgehog also induces the expression of the early cardiac differentiation markers GATA4 and NKX2.5 and is therefore critical for early cardiogenesis (Clement et al. 2009).

Notch signaling

Notch signaling is involved in different stages of cardiac development, from mesoderm formation to cardiomyocyte maturation. Indeed, Notch signaling is involved in proper looping of the heart tube, the development of the atrioventricular canal, myocardial trabecular formation, outflow tract development, coronary vessel morphogenesis and it regulates the cardiac conduction system. Therefore, notch signaling is essential for proper myocardial function and abnormal notch signaling is associated with congenital heart defects (Zhou and Liu 2014).

1.2. HEART FAILURE AFTER MYOCARDIAL INFARCTION

1.2.1. Incidence

Ischemic heart disease (IHD), including myocardial infarction (MI), is one of the leading causes of death worldwide. According to the world health organization (WHO), it is responsible for 13.2% of all deaths (WHO, The top 10 causes of death, Fact sheet N°310). IHD is one of the major risk factors for the development of heart failure. Indeed, one third of the MI patients will develop heart failure. In Belgium, every year 15,615 patients are diagnosed with heart failure and the one year mortality is about 26% (Devroey and Van Casteren 2010). Furthermore, heart failure also imposes a major burden on society since 1-2% of the health care expenditures of developed countries are devoted to the treatment of heart failure (Liao et al. 2008).

1.2.2. Pathology

Any form of coronary artery occlusion can cause an MI, but in 98% of the cases the culprit is a thrombus caused by acute atherosclerotic plaque rupture or erosion and subsequent platelet aggregation (Figure 1.3A) (Arbustini et al. 1999). Severe ischemia downstream of the occluded artery causes massive loss of the affected cardiomyocytes within minutes. The subsequent exposure to reactive oxygen species (ROS) and toxic agents induces the upregulation of secretion of several cytokines and chemokines which trigger the infiltration of leukocytes, monocytes and macrophages into the ischemic core (Frangogiannis 2008). The immune cells

clear out the cellular debris at the site of injury and the infarct core is filled by granulation tissue. This tissue is formed by myofibroblasts which deposit collagen fibers and other extracellular matrix (ECM) components such as fibronectin and laminin (Frangogiannis 2008). A week after the initial insult, the granulation tissue starts to develop into dense scar tissue which secludes the lesion from the healthy tissue and prevents a cascade of uncontrolled deleterious events such as infarct expansion or cardiac rupture. However, it does not possess the physical and functional properties of the healthy heart muscle. To compensate for this loss in function, the remaining cardiomyocytes react by a mechanism of remodeling characterized by hypertrophy and changes in intrinsic contractility, electrophysiological properties and metabolism in order to counteract the increased cardiac load (Burke and Virmani 2007). These three elements, the cardiomyocyte loss, the increased interstitial fibrosis and the hypertrophy, are the hallmarks of myocardial remodeling. The loss of functional tissue and subsequent remodeling eventually leads to a reduced pump function and the development of heart failure and malignant arrhythmias (Figure 1.3B) (Azevedo et al. 2016).

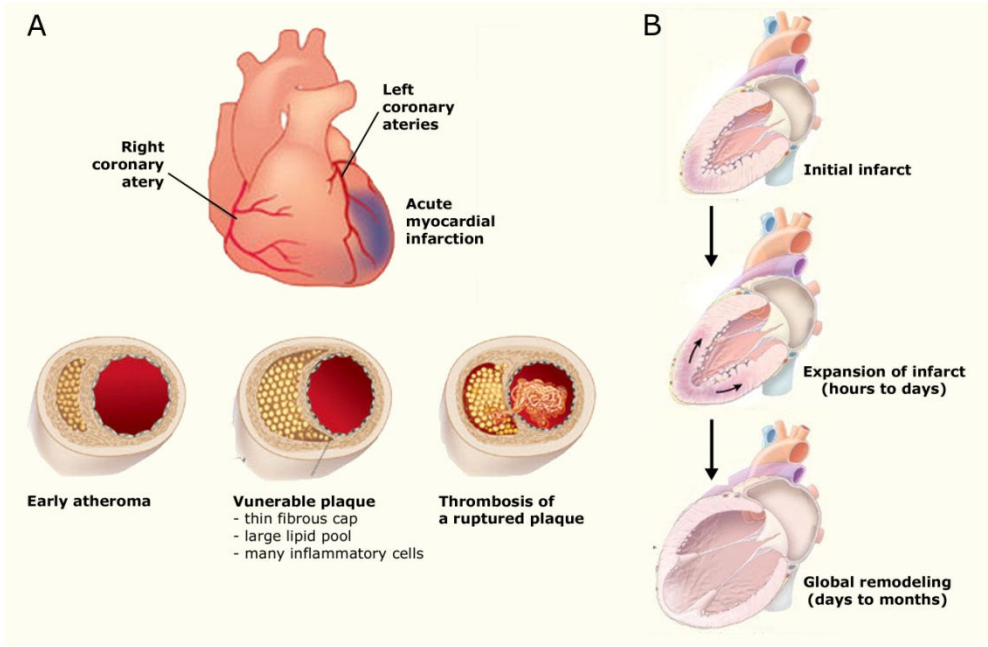

Figure 1.3: Pathophysiology of MI and heart failure. (A) Schematic overview of plaque formation, rupture and subsequent thrombosis which can result in an MI. (B) After the initial insult the infarct expands after which global remodeling occurs resulting in the dilatation of the ventricle and thinning of the ventricular wall. (Adapted from (Libby 2002, Jessup and Brozena 2003))

1.2.3.Therapy

The standard handling for IHD patients consists of pharmacological or surgical interventions to re-perfuse the occluded coronary arteries. These include anticoagulants, angioplasty with or without the introduction of a stent and coronary artery bypass graft (CABG) surgery. Afterwards, life-style changes and pharmacological therapies are introduced to counteract the deleterious effects of cardiac remodeling and to prevent or delay the development of heart failure. However, these therapies are not able to functionally restore the injured heart (American College of Emergency et al. 2013, Owens et al. 2016). The only currently available therapy that can achieve this goal is heart transplantation. However, this is not a feasible option for every patient since the number of heart failure patients is far greater than the actual availability of suitable donors and the potential rejection of the donor heart is an important area of concern (Korewicki 2009). Therefore, a lot of effort has been put in the development of new strategies to replace the lost cardiomyocytes with new, viable and functional cells.

1.3. STEM CELL THERAPY FOR CARDIAC REPAIR

Since the loss of functional cardiomyocytes underpins the pathophysiology of MI and subsequent development of heart failure, stem cell therapy has been widely investigated as a new therapeutic strategy. A wide variety of stem cell types are considered as candidates for myocardial regeneration, including pluripotent stem cells (PSCs), skeletal myoblasts, bone-marrow derived cells (BMCs) and endogenous cardiac stem cells (CSCs). These different cell types can improve cardiac function through a range of different mechanisms, including (1) heart muscle regeneration through myocardial differentiation, (2) stimulating angiogenesis via endothelial and smooth muscle cell differentiation and (3) paracrine mechanisms in which paracrine factors are secreted which stimulate cardiac recovery (Figure 1.4).

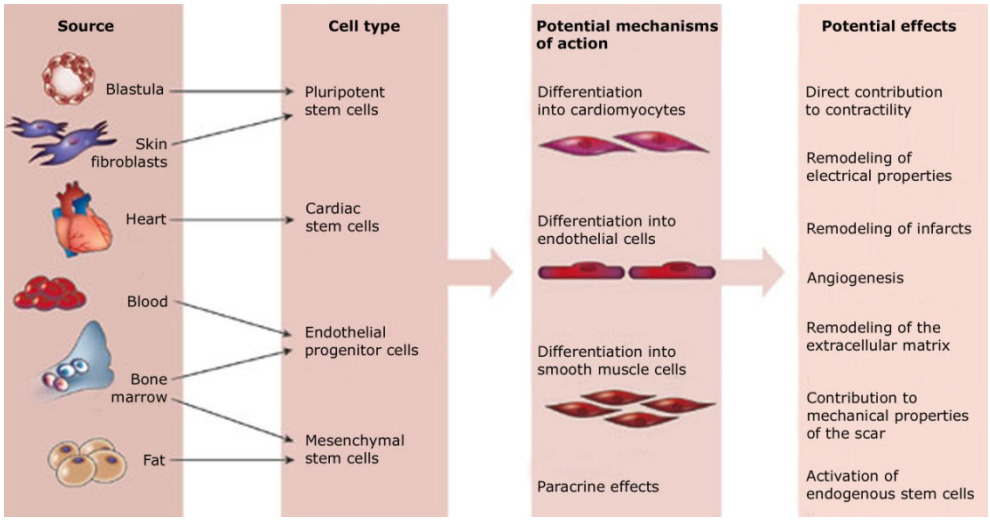

Figure 1.4: Cell types and mechanisms for cardiac regeneration. Stem cells and progenitor cells of different origin, can improve cardiac function through several mechanisms, including differentiation towards cardiomyocytes, endothelial cells and vascular smooth muscle cells, but also through paracrine effects. (Adapted from (Segers and Lee 2008))

1.3.1. Pluripotent stem cells

PSCs, including both embryonic stem cells (ESCs) and induced PSCs (iPSCs), can be propagated indefinitely and are able to differentiate into every cell type of the human body. ESCs are derived from the inner mass of the blastocyst while iPSCs can be derived from somatic cells by retroviral overexpression of the pluripotency-related transcription factors: *OCT4*, *SOX2*, *KLF4* and *MYC* (Takahashi et al. 2007). More recently, the reprogramming protocol was further optimized. This increases efficiency and makes the use of integrating viral vectors obsolete (Gonzalez et al. 2011).

Earlier studies with both mouse and human ESCs demonstrated that ESC-derived cardiomyocytes (ESC-CMs) are molecularly and functionally similar to cardiomyocytes *in vivo* (Kehat and Gepstein 2003, Sachinidis et al. 2003). This led to the transplantation of pre-differentiated human ESC-CMs in a primate model of MI which showed that intramyocardial transplantation of ESC-CMs generated extensive remuscularization of the infarcted heart (Chong et al. 2014). However, the use of ESCs has serious limitations including the risk of tumor formation, immune rejection (Nussbaum et al. 2007) and profound ethical concerns on the

isolation of ESCs from human embryos. Despite these limitations, Menasché *et al.* recently started a clinical study with ESC-derived cardiac progenitor cells for cardiac repair. To reduce the high risks associated with ESCs, the cells were pre-differentiated towards cardiac progenitor cells by using two compounds, namely BMP-2 and an FGF receptor inhibitor. The resulting population was purified based on the expression of Ils-1 and loss of SOX-2 and NANOG, with purity rates of 99%. Finally, the short duration and low dosing of the immunosuppressive regimen was chosen to further reduce the risk of tumor formation (Menasche et al. 2015). However, it is too early to assess the safety and therapeutic potential of this therapy.

Since iPSCs share numerous features with ESCs, but circumvent the ethical concerns associated with ESCs, they might be a suitable PSC alternative in cardiac repair. Like ESCs, iPSCs are able to differentiate into cardiomyocytes (Lewandowski et al. 2016). Although the efficiency of differentiation was initially lower than in ESCs, recent protocols are able to produce iPSC-derived cardiomyocytes (iPSC-CMs) at more than 95% purity (Burridge et al. 2014). However, these improvements in the efficiency of differentiation protocols were not accompanied by improvements in iPSC-CM maturation as they are not able to acquire the physiological structure, gene expression and function akin to mature cardiac tissue (Bedada et al. 2016). Therefore, one of the main challenges in the field is to improve iPSC-CM maturation which is essential for their functional integration and the prevention of arrhythmias after transplantation. So despite their promises, the *in vivo* safety and functionality need to be assured before testing them in a clinical setting.

1.3.2.Skeletal myoblasts

Skeletal myoblasts can be easily isolated from skeletal muscle and subsequently expanded and differentiated in contractile muscle cells. Furthermore, myoblast transplantation improved cardiac function in rabbits (Taylor et al. 1998). This led to a rapid translation into clinical trials. However, these showed no improvement in left ventricular function. Furthermore, transplanted cells failed to gain electromechanical coupling with the host myocardium resulting in a high prevalence of cardiac arrhythmias which further decreased the interest in these progenitor cells for cardiac repair (Menasche et al. 2008).

1.3.3. Bone-marrow derived cells

The bone marrow contains several stem cell types, including mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs). The study of Orlic *et al.* was the first to demonstrate myocardial differentiation of HSCs transplanted in mice (Orlic et al. 2001). However, this claim has been questioned by several other *in vivo* studies (Balsam et al. 2004, Murry et al. 2004). Similar controversy also exists with respect to the cardiomyogenic differentiation potential of MSCs (Gallo et al. 2007, Rose et al. 2008, Koninckx et al. 2009) and the same is also true for EPCs, (Gruh et al. 2006). Instead, EPCs are believed to contribute to angiogenesis (Young et al. 2007). However, the existence of EPCs remains controversial since there are no unique surface markers for their identification (Chao and Hirschi 2010, Fadini et al. 2012, Chong et al. 2016).

Despite the controversy surrounding the cardiac differentiation potential of BMCs, several clinical trials with BMCs for cardiac repair have been performed. A recent systematic review, including 29 randomized clinical trials and 7 systematic reviews and meta-analyses between January 2000 and July 2016, concluded that BMC therapy is safe but results in only modest benefits additionally to conventional therapy. Furthermore, the benefit of BMC therapy is most likely the result of paracrine effects, rather than *de novo* cardiomyogenesis (Nguyen et al. 2016). The effect of BMCs on mortality is currently examined in the prospective Phase 3 trial, "The Effect of Intracoronary Reinfusion of Bone Marrow-derived Mononuclear Cells (BM-MNC) on All-Cause Mortality in Acute Myocardial Infarction" (BAMI). This European study tests the hypothesis whether BMCs improve the survival after MI and the results of this study will determine whether a BMC transplantation can save lives in MI patients (NCT01569178).

1.3.4. Cardiac stem cells

Traditionally the heart is considered as a terminally differentiated organ that lacked regenerative capacity. However, about a decade ago, this changed due to the identification of cycling myocytes under both normal and pathological conditions (Beltrami et al. 2001, Bergmann et al. 2009). These findings introduced the hypothesis of resident CSCs in the adult heart which can contribute to cardiac

repair. Different methods have been reported to identify and isolate endogenous CSCs, including c-kit, sca-1, aldehyde dehydrogenase (ALDH), side population and cardiospheres (Bruyneel et al. 2016). Besides the typical stem cell properties such as long-term self-renewal, clonogenicity and multipotency, they are believed to be pre-programmed for cardiomyocyte differentiation since they most likely originate from the heart.

c-kit+ cardiac stem cells

The c-kit+ CSCs are the most studied, but recently also the most challenged CSC type. Beltrami *et al.* were the first to describe a c-kit+ CSC population in rat hearts which gave rise to cardiomyocytes, smooth muscle cells and endothelial cells and improved cardiac function after injection in a rat MI model (Beltrami et al. 2003). Later, this CSC type was also found in humans where they showed the typical characteristics of stem cells (Bearzi et al. 2007). Although other studies were not able to replicate these results, c-kit+ CSCs were used in the Stem Cell Infusion in Patients with Ischemic Cardiomyopathy (SCIPIO) phase I clinical trial. This resulted in a significant improvement in global and regional left ventricular function and a decrease in infarct size (Bolli et al. 2011). However, recently concerns have been raised concerning the integrity of certain data (The Lancet 2014). This investigation is still ongoing but meanwhile this research track might better not be pursued anymore until further details are available. Furthermore, Van Berlo *et al.* showed that 0.008% of the cardiomyocytes in the adult heart are derived from c-kit+ cells and these cells only minimally contributed to cardiac regeneration after MI in a mouse model (van Berlo et al. 2014). These results were also confirmed by Sultana *et al.* (Sultana et al. 2015). Altogether, this indicates that endogenous c-kit+ CSCs are most likely not the primary cell source for cardiomyocyte renewal in adults and might therefore not be the appropriate CSC type for future cardiac regeneration therapies.

Sca-1+ cardiac stem cells

Sca-1+ CSCs were first described in adult murine hearts by Oh *et al*. They also demonstrated that injection of these sca-1+ CSCs in a mouse model of MI resulted in a successful engraftment in the infarct border zone and subsequent myocardial differentiation. In contrast, spontaneous differentiation *in vitro* did not occur and

in vitro myocardial differentiation upon stimulation was only occasionally observed (Oh et al. 2003). Furthermore, several studies demonstrated that sca-1 depletion resulted in increased cardiac hypertrophy, fibrosis and dysfunction after cardiac injury which was accompanied by a decrease in resident CSC proliferation (Bailey et al. 2012, Rosenblatt-Velin et al. 2012, Zhou et al. 2012). Despite these promising results, sca-1 is absent in humans and although a "sca-1 like" population, able to differentiate towards contractile cardiomyocytes, can be isolated from the human heart by using the murine antibody, the existence of a "sca-1 like" CSC type in humans needs to be confirmed (van Vliet et al. 2008, Smits et al. 2009).

Side-population cardiac progenitors

Closely related to the sca-1+ CSCs are the cardiac side-population (SP) progenitors which possess the unique ability to efflux the DNA binding dye Hoechst 33342 using the ABSG2 transporter (Hierlihy et al. 2002). Remarkably, almost all SP cells are sca-1+, while only about 1% of the sca-1+ CSCs are SP cells (Unno et al. 2012). Although early reports demonstrated that SP cells differentiated towards cardiomyocytes *in vitro* and *in vivo* (Martin et al. 2004, Oyama et al. 2007), this was questioned by a recent study by Doyle *et al*. They indicated that although SP cells gave rise to cardiomyocytes, endothelial cells and vascular smooth muscle cells during cardiogenesis, in a mouse model of MI these cells only developed into endothelial cells and no longer into cardiomyocytes. (Doyle et al. 2016).

Isl-1+ cardiac stem cells

Another CSC type that is derived from the SHF and expresses Isl-1, has been identified in rat, mouse and human (Laugwitz et al. 2005). During development, the Isl-1+ SHF progenitor cells contribute to both atria and the right ventricle. Isl-1 expression is lost when these cells differentiate into cardiomyocytes (Cai et al. 2003). Interestingly, some Isl-1+ undifferentiated cells are found in postnatal mature human, rat and mouse hearts. Co-culturing these Isl-1+ CSCs with neonatal rat cardiomyocytes (NRCMs) induced cardiomyocyte differentiation, as shown by the expression of terminal differentiation markers, intact Ca^{2+} cycling and the generation of action potentials. However, these cells were isolated from

cardiac segments early after birth. Furthermore, there were no Isl-1+ cells found in a sample of a patient of 5 months old (Laugwitz et al. 2005). These findings suggest that the existence of Isl-1+ CSCs in the adult heart of older patients is highly unlikely. A more recent study of Weinberger *et al.* confirmed that Isl-1+ cells are rare in adult and in mice they are only present in the region of the sinoatrial node (Weinberger et al. 2012). Furthermore, no evidence of myocardial regeneration by Isl-1+ CSCs *in vivo* has been reported so far. Therefore, the use of Isl-1+ CSCs in cardiac regeneration therapies is probably limited.

Cardiosphere-derived cardiac stem cells

In 2004, Messina *et al.* described a CSC population that is spontaneously shed from human myocardial biopsies and murine heart samples (Messina et al. 2004). Injecting these cardiosphere-derived cells (CDCs) in a mouse model of MI improved cardiac function via differentiation into cardiomyocytes, endothelial cells and smooth muscle cells (Messina et al. 2004). Furthermore, a study of Li *et al.*, comparing CDCs with c-kit+ CSCs and BMCs, found that CDCs had more potency for myocardial repair compared to the other stem cell populations (Li et al. 2012). However, in our hands, cardiomyogenic differentiation of CDCs after co-culture with NRCMs was limited (Koninckx et al. 2011, Koninckx et al. 2013). The phase I CArdiosphere-Derived aUtologous stem CElls to reverse ventricULar dySfunction (CADUCEUS) trial, showed a reduction in infarct size and an increase in viable tissue, but no improvement in overall left ventricular function (Makkar et al. 2012). Nevertheless, a larger phase I/II clinical trial, the ALLogeneic heart STem cells to Achieve myocardial Regeneration (ALLSTAR) trial, was recently set up to investigate the safety and efficacy of intracoronary delivery of allogeneic CDCs (Chakravarty et al. 2016). Maybe, this will give more insights in the usability of this heterogeneous cell population for cardiac repair.

Cardiac atrial appendage stem cells

Finally, in 2013, yet another CSC type was identified based on elevated ALDH activity. These cardiac atrial appendage stem cells (CASCs) are typically ALDH bright ($ALDH^{br}$), $CD34^{+}$, $Isl1^{+}$, $CD45^{-}$ and $c\text{-}kit^{-}$, although CD34 expression is lost during cell culture (Koninckx et al. 2013). A similar $ALDH^{br}$ CSC population was also described in mice (Roehrich et al. 2013). In addition, CASCs express multiple

pluripotency associated genes, including *OCT-4*, *NANOG*, *CMYC* and *KLF4* and possess a clonogenicity of around 20%, confirming a stem cell phenotype. Co-culturing CASCs with NRCMs resulted in cardiomyocyte differentiation with a sarcomeric organization of cardiac troponin T (cTnT) and I (cTnI)(Koninckx et al. 2013). Their potential was further confirmed *in vivo* in a Göttingen minipig model of acute MI where autologous CASC transplantation preserved global and regional left ventricular function due to extensive engraftment and myocardial differentiation of the transplanted cells (Fanton et al. 2015). In contrast to the extensive myocardial differentiation potential, CASCs show limited differentiation towards endothelial cells, but stimulate angiogenesis via paracrine mechanisms (Fanton et al. 2015, Fanton et al. 2016). Indeed, not only the replacement of lost heart muscle by new functional cardiomyocytes, but also new blood vessel formation is essential to ensure full cardiac repair.

1.4. PARACRINE MECHANISMS FOR CARDIAC REGENERATION

Initially, *de novo* remuscularization and revascularization was considered essential for cardiac repair. This can be accomplished by stem cell transplantation in the infarct area or via activation of the endogenous cardiac repair mechanism. However, of the investigated stem cell types, only PSCs and some CSCs showed a profound cardiac differentiation potential, while this was not the case for BMCs. Therefore, functional improvements with BMCs are most likely the result of paracrine mechanism. Paracrine factors, including growth factors, cytokines and microRNAs (miRNAs) can promote cardiac repair via several mechanisms including (1) neovascularization, (2) cytoprotection, (3) limiting inflammation, fibrosis and remodeling and (4) importantly resident CSC activation.

1.4.1. Neovascularization

Therapeutic angiogenesis that aims at the formation of new blood vessels from preexisting blood vessels is important for the repair of the infarcted myocardium (Cochain et al. 2013). This complex and tightly regulated process involves several proteins such as vascular endothelial growth factor (VEGF), FGF, hepatocyte growth factor (HGF), TGF-β and angiopoietin. These proangiogenic factors are also

released by different adult stem cell types including BMCs, dental pulpa stem cells (DPSCs) and CSCs (Kinnaird et al. 2004, Li et al. 2012, Hilkens et al. 2014, Fanton et al. 2016).

Recently, we demonstrated that CASCs secrete numerous growth factors, including endothelin 1 (ET-1), insulin-like growth factor binding protein 3 (IGFBP-3) and VEGF, which promote endothelial cell proliferation, migration and tube formation which are essential steps in the angiogenesis process. Furthermore, CASCs and conditioned medium of CASCs (CM-CASC) promoted angiogenesis as demonstrated by extensive radial ingrowth of blood vessels in a chorioallantoic membrane (CAM) assay (Fanton et al. 2016). Therefore, cell therapy could possibly induce neovascularization at the site of injury through the paracrine release of several pro-angiogenic growth factors.

1.4.2.Cytoprotection

Adult stem cells can also promote cardiac function through the secretion of cytoprotective molecules which promote cardiomyocyte survival. Also here, VEGF and HGF but also platelet derived growth factor (PDGF) and insulin-like growth factor 1 (IGF-1) are important mediators (Takahashi et al. 2006, Gnecchi et al. 2008). In addition to these cytokines, also miRNAs are involved in the cytoprotective effects of BMCs and CSCs, by targeting numerous paracrine factors and pro-apoptotic and anti-apoptotic signaling molecules. Furthermore, these paracrine factors not only improve cardiomyocyte survival and contractility but also improve CSC survival upon transplantation in an oxygen-deprived environment (Gnecchi et al. 2005, Nakanishi et al. 2008, Jin et al. 2013). Typically, these cytoprotective effects are mediated via the activation of the PI3K/Akt and Ras-Raf-MEK-ERK pathways, which inhibit apoptosis and thereby stimulate cell survival (Awada et al. 2016).

1.4.3.Limiting inflammation, fibrosis and remodeling

After MI, inflammatory cells rush into the ischemic heart and trigger a strong inflammatory response. The lost cardiomyocytes are replaced by collagen deposition and other ECM proteins. This formation of non-contractile scar tissue starts the cardiac remodeling process which results in heart failure. By secreting

factors that limit inflammation and alter the ECM, cardiac remodeling can be reduced. Furthermore, this facilitates the functional integration of stem cells transplanted in the infarcted heart (Gallina et al. 2015, Awada et al. 2016, Hodgkinson et al. 2016, Khanabdali et al. 2016).

The inflammatory reaction after MI has both beneficial and detrimental effects. On one hand inflammatory cells induce angiogenesis by secreting proangiogenic factors and they clear out dead cells and cellular debris. However, these inflammatory cells also produce large amounts of ROS which cause cell death, negatively affect the remaining cardiomyocytes leading to remodeling and dysfunction and enhance matrix-degrading processes which lead to chamber dilatation. Therefore, an optimal spatiotemporal suppression of the inflammatory reaction is essential for effective cardiac repair (Frangogiannis 2014). Adult stem cells are able to suppress the inflammatory state by downregulating the expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 (Wen et al. 2011).

After the initial inflammatory reaction, a fibrotic process is activated which alters the structure and composition of the ECM. The ECM is composed of collagen and elastin and is regulated by matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs) (Graham et al. 2008). MSCs have been shown to decrease cardiac fibrosis via regulation of the MMP/TIMP ratio and via inhibiting the production of collagen type I and III (Xu et al. 2005). Therefore, paracrine factors released by adult stem cells can alter the structure of the ECM in order to prevent post-infarction remodeling and improve stem cell integration upon transplantation.

1.4.4.Activating resident cardiac stem cells

Finally, adult stem cells can also improve cardiac repair via the stimulation of resident CSCs. This can be achieved by inducing proliferation, mobilization and differentiation of these CSCs. Factors known to enhance CSC proliferation, mobilization, differentiation, survival and function include VEGF, HGF, IGF-1 and stromal derived factor-1 (SDF-1) (Gnecchi et al. 2008). Recently we demonstrated that conditioned medium of MSCs (CM-MSC) or specific factors released by MSCs

increase migration of CASCs from cardiac tissue. This migratory effect was partly mediated via the PDGF-AA/PDGFRα signaling pathway (Windmolders et al. 2014).

Compared to cell based cardiac regeneration therapies, *in situ* activation of resident CSCs has many advantages. First, it offers an "off-the-shelf" therapy which can be used at all times. Furthermore, it is preferred from a socioeconomic standpoint, but also for patients as it is easy to apply. However, the success of such a cell-free therapy requires a robust response that is able to regenerate enough functional cardiac muscle tissue to replace the lost heart tissue. This requires further insights in the molecular and cellular mechanisms involved in CSC proliferation, migration, survival and differentiation.

1.5. THE ROLE OF MIRNAS AND EXOSOMES IN CARDIAC REGENERATION

1.5.1. MiRNAs in cardiac regeneration

MiRNAs are short non-coding RNA molecules (20-25 nucleotides) that function as a post-transcriptional regulator of gene expression, by degradation or translation inhibition of the target RNA. In the nucleus, RNA polymerase II complex transcribes specific miRNA genes which leads to the formation of pri-miRNA. Afterwards, Drosha cleaves this pri-miRNA, leaving a hairpin-shaped pre-miRNA that is subsequently translocated to the cytoplasm. There, Dicer will cleave the pre-miRNA into a double-stranded miRNA molecule. One strand will eventually form the mature miRNA, while the other one is degraded. The mature miRNA will be incorporated in the RNA-induced silencing complex (RISC) and will interact with its target RNA (Figure 1.5) (Bartel 2004).

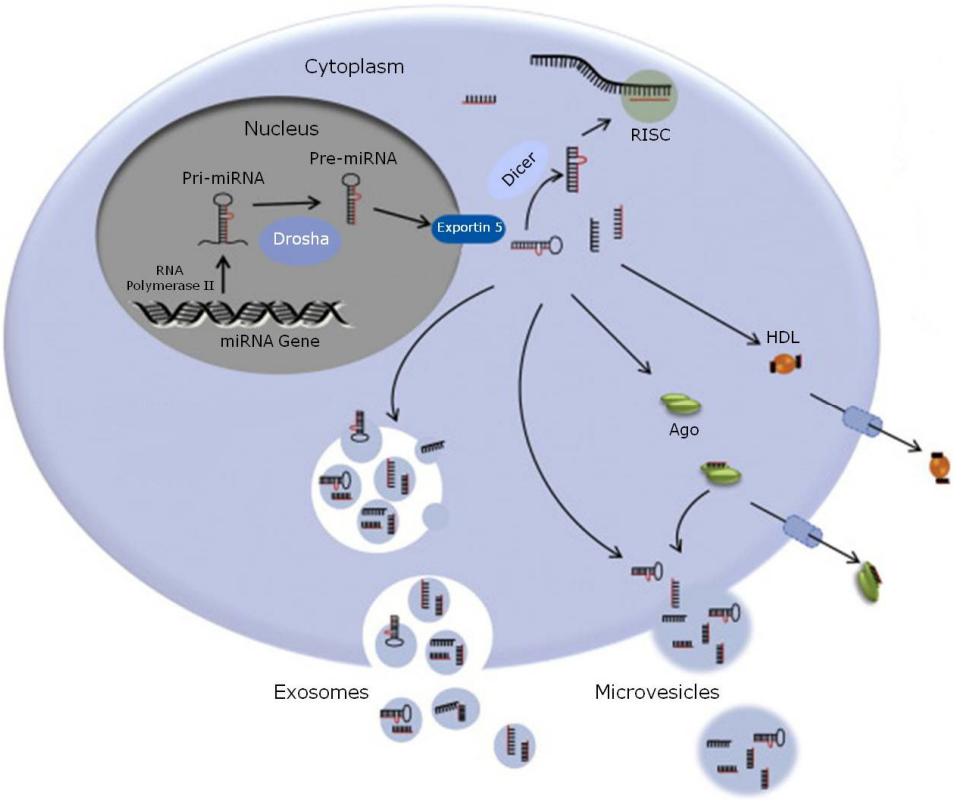

Figure 1.5: miRNA biogenesis, function and secretion. Pri-miRNAs are transcribed by RNA polymerase II and subsequently processed by Drosha to pre-miRNA in the nucleus. Exportin 5 transfers these pre-miRNAs to the cytoplasm where Dicer cleaves them into mature miRNAs. Once incorporated in the RISC complex, the mature miRNA will interact with its target RNA leading to either transcript degradation or translational repression. MiRNAs can also be released in the extracellular milieu in exosomes or microvesicles or coupled to Ago or HDL. Pri-miRNA = primary miRNA; Pre-miRNA = precursor miRNA; RISC = RNA-induced silencing complex; Ago = Argonaut; HDL = high density lipoprotein (Figure adapted from (Sohel 2016))

As cardiac development and homeostasis is tightly regulated by miRNAs (Thum et al. 2007), miRNA-based therapies might be of great use for cardiac (cell-based) regeneration therapies. Indeed, several miRNAs have been identified that regulate different processes after an MI, including cardiomyocyte death and survival, angiogenesis and cardiac fibrosis. Moreover, miRNAs have also been used to bring stem cell therapy to a higher level by improving proliferation, survival, integration and differentiation (Zhu et al. 2016).

MiR-21 and miR-24 serve as potential targets in the protection of cardiomyocytes against ischemia induced apoptosis. Dong *et al.* showed that miR-21 was significantly down-regulated in the infarcted area but upregulated in the border area. Interestingly, this down-regulation of miR-21 in the infarcted area was inhibited by ischemic preconditioning. The protective effect of miR-21 on ischemia-induced cell apoptosis was further confirmed in a rat model of acute MI, where overexpression of miR-21 resulted in less cell apoptosis in both the border and the infarcted areas of the heart (Dong et al. 2009). Also miR-24 overexpression in a mouse MI model inhibited cardiomyocyte apoptosis, reduced infarct size and improved cardiac function (Qian et al. 2011). This protective effect of specific miRNAs is also important in stem cell-based therapies for cardiac repair. Liu *et al.* showed that miR-155 overexpression in human cardiac progenitor cells augmented cell survival during oxidative-stress stimulation, independent of the Akt survival pathway (Liu et al. 2011).

In a mouse model of acute MI, inhibition of miR-92a stimulated blood vessel growth and functional recovery of the damaged tissue (Bonauer et al. 2009). This effect is caused by stimulating endothelial cell proliferation and migration through the regulation of proangiogenic proteins (Iaconetti et al. 2012). Also miR-24 may serve as a valuable target in improving ischemic heart recovery via influencing angiogenesis. Indeed, inhibition of miR-24 in a mouse model of MI, reduced endothelial cell apoptosis, improved vascularization and decreased infarct size, which resulted in a significant increase in survival rate (Fiedler et al. 2011).

Several miRNAs are known to play an important role in the development and progression of cardiac fibrosis, including miR-1, miR-15, miR-21, miR-29, miR-101 and miR-208. Overexpression of miR-101 suppressed proliferation and collagen production in cardiac fibroblasts and improved cardiac function in rat model of chronic MI (Pan et al. 2012). Therapeutic inhibition of miR-208 during hypertension-induced heart failure in Dahl hypertensive rats, prevented cardiac remodeling, enhanced cardiac function and improved survival (Montgomery et al. 2011). Also therapeutic inhibition of miR-15 in a mouse MI model reduced infarct size and cardiac remodeling and enhanced cardiac function (Hullinger et al. 2012). MiR-1 replacement therapy reduced pressure-overload-induced cardiac fibrosis and remodeling (Karakikes et al. 2013). A similar effect was also obtained by silencing miR-21 (Thum et al. 2008). Finally, down-regulation of miR-29 induced

collagen expression while over-expression of miR-29 reduced collagen expression in cardiac fibroblast cultures (van Rooij et al. 2008).

Besides influencing cardiac repair via modulating angiogenesis, cardiac fibrosis and protection, miRNAs can be used to reprogram cardiac fibroblasts into cardiomyocytes. Indeed, a combination of miR-1, miR-133, miR-208 and miR-499 proved to be effective in directly reprogramming cardiac fibroblasts into functional cardiomyocytes, both *in vitro* and *in vivo* (Jayawardena et al. 2012). Furthermore, *in vivo* reprogramming of cardiac fibroblasts significantly improved cardiac function after MI (Jayawardena et al. 2015). These results further validate the potential of such a cardiac reprogramming strategy for cardiac regeneration.

1.5.2.Stem cell-derived exosomes for cardiac repair

Exosomes have a size of the order of 30-100 nm and are produced through inward folding of the cell membrane which results in the formation of multivascular bodies. These multivascular bodies fuse with the plasma membrane which results in the release of their intraluminal vesicles in the form of exosomes. On their surface, exosomes carry some of the surface markers of their cell of origin and they are able to interact with the surface receptors on neighboring but also distant cells. In addition, due to their vesicular nature, they are able to carry cargo, including protein, mRNA and miRNA, and transfer this cargo to recipient cell which results in cell-to-cell communication (Emanueli et al. 2015).

As previously described, several stem cell types have shown positive paracrine effects on cardiac repair. Recent studies suggest that the involved paracrine factors are often released in exosomes and that the secreted factors/miRNAs are stem cell specific. Exosomes from different kinds of stem cells, such as ESCs, MSCs and CSCs have been studied for cardiac repair and the results indicate a positive effect due to several paracrine mechanisms earlier discussed including neovascularization, limiting fibrosis and activating endogenous cardiac repair (Figure 1.6) (Kishore and Khan 2016). For example, Whang *et al.* demonstrated that iPSC-derived exosomes promoted cardiomyocyte survival in a murine MI model via the release of miR-21 and miR-210 which are respectively regulated by Nanog and HIF-1α (Wang et al. 2015). Also MSC-derived exosomes had a cardio protective effect in a mouse MI model via restoration of bioenergetics and

reduction of oxidative stress, resulting in a reduced infarct size and a long-term preservation of cardiac function (Arslan et al. 2013). MSC-derived extracellular vesicles, including micro vesicles and exosomes, were taken up by endothelial cells and promoted angiogenesis, both *in vitro* and *in vivo* (Bian et al. 2014). Finally, Khan *et al.* showed that murine ESC-derived exosomes augmented cardiac function due to enhanced neovascularization, cardiomyocyte survival and reduced cardiac fibrosis in a mouse MI model (Khan et al. 2015).

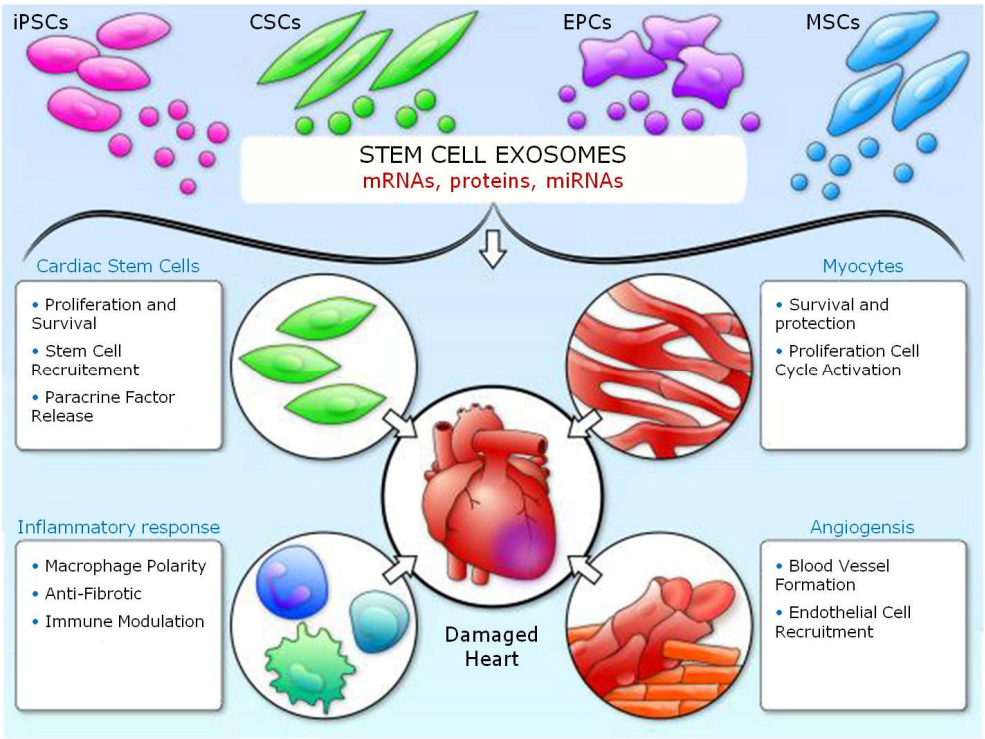

Figure 1.6: Stem cell-derived exosomes for cardiac repair. Exosomes from different stem cell types carry and deliver mRNAs, miRNAs and proteins which improve repair of the damaged heart via resident CSC activation, cardiomyocyte proliferation, neovascularization and modulating cardiac inflammation and fibrosis. CSC = cardiac stem cell; EPC = endothelial progenitor cell; iPSC = induced pluripotent stem cell; MSC = mesenchymal stem cell (Figure adapted from (Kishore and Khan 2016)

1.6. AIMS OF THE STUDY

Different stem cell populations such as PSCs, BMCs and CSCs are currently investigated for cardiac repair. However, success depends on the selection of the appropriate stem cell type for regeneration, the ability of producing enough cells and the ability of functional integration upon transplantation. Although previous results indicated that CASCs are an appropriate cell candidate for myocardial repair, additional knowledge about the mechanisms involved in CASC proliferation, survival and differentiation is essential to improve current CASC-based therapies for cardiac repair. Furthermore, current cardiac regeneration studies do not address the hostile micro-environment after infarction characterized by ischemia, inflammation and fibrosis which not only negatively impacts cardiac repair but also diminishes stem cell survival and integration.

In the first part of this study we evaluated the effect of long-term *in vitro* culture on CASC behavior by examining CASC proliferation, ageing, immunophenotype and differentiation characteristics at various time points during culture. This is essential to exclude any potential negative effects of long-term culture on CASC biology and to determine the feasibility and efficiency of obtaining clinically relevant cell numbers. In addition the suitability of an expansion method based on blood platelet-derived supplements was evaluated with the view to future clinical application of CASCs.

In a second part of this study, the CASC niches in the adult heart and the cardiac differentiation stadium of CASCs in culture was determined. This will hopefully give us more insights in the origin and nature of the CASCs, their differentiation behavior and the mechanisms involved in CASC biology. Until now, CASCs were isolated from both pig and human atrial appendages. However, little is known about other potential compartments in the adult heart and their exact origin and nature. To address this question, the expression of different early and late cardiac differentiation markers was studied in CASCs in culture. Furthermore, we selected the Wnt pathway to study CASC proliferation and cardiac maturation mechanisms. Within this study we focused on the canonical Wnt signaling pathway because of its crucial role in cardiogenesis and differentiation of PSCs towards cardiac lineages.

In a third part of this study the cytoprotective effect of MSCs on CASCs was evaluated. Even though, CASCs promote angiogenesis via paracrine effect, it is important that the transplanted cells survive the hostile environment of the infarct border zone, characterized by low oxygen levels, in order be able to functionally integrate, differentiate and achieve their paracrine effect. Earlier studies demonstrated that MSCs improve the survival of cardiomyocytes and other CSCs during oxygen deprivation (Nakanishi et al. 2008, Jin et al. 2013). The potential signaling pathways and paracrine factors responsible for this cytoprotective effect will therefore be studied in more detail.

Finally, we set up an experimental approach to study cardiac fibrosis since excessive collagen deposition not only leads to the development of heart failure but also negatively affects CASC integration. Indeed, previous results indicate that CASCs injected in the fibrotic infarct zone do not differentiate towards cardiomyocytes (Fanton et al. 2015). Interestingly, in contrast to adult wound healing, fetal wound healing is not accompanied by scar tissue formation due to the absence of TGF-β1 induced collagen deposition. Therefore, we have developed an experimental approach which can now be used to investigate whether the process of fetal wound healing can be mimicked in the heart.

The data obtained from the investigations described in this thesis will significantly contribute in the development of a promising CASC-based cell therapy for patients with IHD.

Chapter 2

Clinical-scale *in vitro* expansion of cardiac atrial appendage stem cells

Based on:

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This is a shared first author paper for which I developed the qPCR analysis for telomere length measurement, evaluated the growth properties of the $ALDH^{dim}$ and $ALDH^{br}$ subpopulation during culture and evaluated platelet plasma supernatant as an alternative for fetal bovine serum.

2.1 ABSTRACT

Recently, CASCs have emerged as an attractive candidate for cardiac regeneration after myocardial infarction. Like other cardiac stem cells, CASCs have to be ex vivo expanded to obtain clinically relevant cell numbers. However, fetal bovine serum (FBS), which is routinely used for cell culturing, is unsuitable for clinical purposes and the influence of long-term in vitro culture on CASC behavior is unknown. Therefore, we examined the effect of prolonged expansion on CASC biology and evaluated a culture protocol suitable for human use.

In FBS-supplemented medium, CASCs could be kept in culture for 52±15 days, before reaching senescence. Despite a small decrease in the number of proliferating CASCs and signs of progressive telomere shortening, their immunophenotype and myocardial differentiation potential remained unaffected during the entire culture period. Moreover, CASCs were successfully expanded in human platelet plasma supernatant (PPS), while maintaining their biological properties.

In conclusion, we developed a long-term cultivation protocol to obtain clinically relevant CASC numbers, while retaining the cardiogenic potential. These insights in CASC biology and the optimization of a humanized platelet-based culture method are an important step towards the clinical application of CASCs for cardiac regenerative medicine.

2.2 INTRODUCTION

Over the past decade, discovery of resident CSCs has raised high hopes for regenerating functional myocardium and restoring cardiac function after MI. CSCs can be found clustered in niches located in specific areas of the adult heart, such as the atria, apex and epicardium (Smart et al. 2007, Itzhaki-Alfia et al. 2009, Leinonen et al. 2013); they are self-renewing, clonogenic and multipotent (Laugwitz et al. 2005, Bearzi et al. 2007, Smith et al. 2007). Data from preclinical animal models have provided strong support that CSCs play a fundamental role in cardiac regeneration after ischemic insult (Johnston et al. 2009, Bolli et al. 2013). They are tissue-specific, pre-committed to cardiac fate and can be isolated from a target patient population making them suitable for autologous use. As a consequence, CSCs have recently been investigated in phase I clinical trials in which MI patients were transplanted with autologous CSCs to assess their safety, feasibility and efficacy (Chugh et al. 2012, Malliaras et al. 2014).

Very recently, our research group described the isolation of CASCs from IHD patients (Koninckx et al. 2013). The isolation procedure relies on elevated activity of ALDH, a well-known feature of several types of stem cell, such as HSCs, MSCs and neural stem cells (Corti et al. 2006, Gentry et al. 2007, Bell et al. 2012). In native tissue, CASCs are typically $ALDH^+$, $CD34^+$, $CD45^-$ and c-kit^-, but CD34 expression is lost during cell culture. The cells are clonogenic, express a number of pluripotency associated genes, and display functional cardiogenic differentiation in co-culture with NRCMs. In addition, autologous injection of expanded CASCs into the peri-infarct zone has resulted in successful engraftment and cardiac differentiation in a minipig MI model (Koninckx et al. 2013, Fanton et al. 2015). Results of a functional repair follow-up study in this preclinical animal model, have shown the potential of CASCs for clinical use in cardiac regenerative medicine (Fanton et al. 2015).

Many key questions regarding the biological properties of CASCs, which may directly affect their clinical success in IHD patients, remain unanswered. In addition, as for other CSCs, CASCs are found in relatively low frequency in the human heart. Thus, patient application of CASCs requires their isolation and subsequent large-scale *ex vivo* expansion, to obtain sufficient relevant cell quantities for clinical use. Moreover, CASCs have to be collected from elderly

patients, which might constrain their cardiac regeneration potential as they may already display signs of cell ageing, such as telomere shortening and dysfunction, causing cells to enter a crisis phase. In addition, long-term *in vitro* culture might exacerbate these ageing processes resulting in early senescence and loss of differentiation potential. Consequently, these concerns need to be addressed before CASCs can be applied in regenerative medicine.

Traditional cell culture protocols use FBS as a nutritional supplement. Considering clinical application, however, FBS is a less suitable additive as it has a number of disadvantages, such as potential risk of transmitting infectious agents, inducing xenogeneic immune reactions and high cost (Heiskanen et al. 2007, Sundin et al. 2007, Tekkatte et al. 2011). In this context, human-derived platelet lysate (PL) and PPS have been suggested to be adequate alternatives for FBS in large-scale cell culture for clinical settings (Doucet et al. 2005, Schallmoser et al. 2007, Bieback et al. 2009). PPS can be easily collected from common platelet units, while PL can be simply generated from the same units by freeze–thaw procedures.

The goal of the present study was to examine biological characteristics of CASCs over long-term *ex vivo* expansion. Thus, we evaluated CASC proliferation, ageing, immunophenotype and differentiation characteristics at various time points during culture. In addition, with a view to future transplantation studies, we evaluated suitability of a CASC culture method based on blood platelet-derived supplements.

2.3 MATERIAL AND METHODS

All procedures were carried out in accordance with the principles set forth in the Helsinki Declaration. Approval by the Jessa Institutional Review Board and informed consent from each patient were obtained. All animal studies were approved by the Hasselt University Institutional Animal Care and Use Committee.

2.2.1. Preparation of PPS and PL

Outdated platelet concentrates obtained by platelet aphaeresis were provided by the blood bank of the Jessa Hospital, maximum 1 week after collection. Platelet concentration was in the order of 1×10^9/ml. Platelet concentrates were centrifuged for 15 minutes at maximum speed (3600 g), PPS was collected and aliquots were stored at −20°C until use. The platelet pellet was suspended in X-Vivo 15 medium at $5.7x10^9$ platelets/ml and platelets were either snap frozen in liquid nitrogen or frozen at −80°C followed by rapid thawing at 37°C to lyse them. This freeze/thaw cycle was repeated twice. The suspension was centrifuged for 15 minutes at maximum speed (3600 g) and obtained PL suspension was collected and aliquots which were stored at −20°C until use.

2.2.2. Isolation and expansion of CASCs

CASCs were isolated from atrial appendages obtained from IHD patients undergoing routine cardiac surgery, as previously described by Koninckx *et al.* (Koninckx et al. 2013). Cells were seeded in fibronectin (8–24 µg/ml)-coated culture plates (Becton & Dickinson) and expanded at 37°C in humidified atmosphere containing 5% CO2. After the first cell passage, 20% FBS was reduced to 10% FBS or alternatively, replaced by 10% PL or different concentrations of PPS, in the presence of 2% penicillin–streptomycin–amphotericin B (Lonza) and 5 IU/ml heparin (Leo). For comparison of 10% FBS and 7.5% PPS, two different platelet units were tested for every CASC culture. Average values of these replicates were calculated and used for further analysis. Each time 80–85% confluence was reached, cells were replated at $5x10^3$ cells/cm². Medium was changed twice a week. CASCs were uninterruptedly maintained in long-term in vitro cultures until a state of proliferative arrest was reached. This was

characterized by population doubling (PD) of less than one, which leads to stabilization of the population doubling level (PDL) and cumulative cell number.

2.2.3. Determination of growth kinetics

Cells were counted, following trypan blue exclusion testing, at every passage. Total number of cells at each passage was calculated as ratio of total number of cells harvested at the current passage to total number of cells seeded at the previous passage, multiplied by total number of cells harvested at the previous passage. PDs were calculated using the formula: $PD = [\log(A/A_0)]/\log(2)$ where A_0 represents initial cell number and A represents cell harvest number (Cristofalo et al. 1998). To calculate cumulated PDL at a particular passage, calculated PD for this passage was added to PDs of previous passages. Population doubling time (PDT) was obtained from the formula: $PDT = \ln 2/[\ln(A/A_0)/t]$ where A_0 signifies initial cell number, A is cell number at time point 't' and t represents time (in days) since the last passage (Nekanti et al. 2010).

2.2.4. Cell cycle analysis

Cell cycle distribution was analyzed using the BD Cycletest™ Plus DNA Reagent Kit (Becton & Dickinson) according to the manufacturer's instructions. To this end, $2.5x10^5$ CASCs were cultured under standard culture conditions and 10 ng/ml KaryoMAX® Colcemid™ solution (Invitrogen) was added the following day, for 48 hours, to obtain mitotic arrest in metaphase. Cells were then harvested after trypsinization, frozen in buffer solution and stored until analysis. Cell DNA content was analyzed using FACSCanto® (Becton & Dickinson) apparatus. CASCs in S and G2M phase were defined as actively proliferating cells. DNA QC particles (Becton & Dickinson) were used for quality control according to the manufacturer's protocol. Normal peripheral blood cells collected from healthy individuals was utilized as diploid DNA reference for calibration and had <0.5% proliferating cells. Mitotic indices were calculated with ModFit LT software 3.0.

2.2.5. Immunophenotyping by flow cytometry

Antigenic expression profile of CASCs was determined by flow cytometry. $5x10^4$ CASCs/tube were incubated for 20 minutes in the dark with human monoclonal

antibodies as recommended by the manufacturer. Fluorescence minus one (FMO) combined with mouse IgG isotype control was used for correct gating and to identify non-specific staining. All antibodies (table 2.1) were purchased from Becton & Dickinson, except for CD105-FITC (Serotec).

Table 2.1: Antibodies used for immunophenotyping expanded CASCs.

	FITC	PE	PERCP	PE-Cy7	APC	APC-Cy7
Tube 1	CD105	CD13	CD14	CD10	CD29	CD45
Tube 2	CD106	CD44	CD117	CD34	CD55	
Tube 3		CD73			CD90	

FITC = fluorescein isothiocyanate; PE = phycoerythrin; PERCP = peridinyl chlorophylline-Cy5.5; PE-Cy7 = phycoerythrin-Cy7; APC = allophycocyanine; APC-Cy7 = allophycocyanine-Cy7

2.2.6. Analysis of ALDH expression

Flow cytometric analysis of ALDH expression in CASCs was performed using Aldefluor™ kit (Aldagen Inc.). For this, $4x10^4$ cells were incubated in 500 µl Aldefluor assay buffer containing 1.5 µm activated Aldefluor® reagents (Aldagen Inc). $ALDH^{br}$ and $ALDH^{dim}$ CASC populations were flow sorted and separately seeded in 96-well plates in X-Vivo 15 medium supplemented with 20% FBS and 2% penicillin/streptomycin (PS), to evaluate their continued growth. In addition, a fraction of both populations was flow sorted into FACS tubes containing Aldefluor assay buffer. Subsequently, cytospin centrifugation was performed for microscope visualization of the green fluorescent reaction product using an Axiovert 200M microscope (Zeiss). Alternatively, ALDH expression was directly analyzed on cultured CASCs by seeding $1x10^4$ cells/well in 24-well plates and performing similar incubation steps in the well plates. After incubation at 37°C for 30 minutes, cells were washed and kept in Aldefluor assay buffer for microscope visualization of green fluorescent reaction product. For all microscopy data acquisitions, exposure times were kept constant during each recording.

2.2.7. Production of GFP lentiviruses and transduction of CASCs

Prior to setting up co-culture differentiation assays, CASCs were labeled with green fluorescent protein (GFP). Production of GFP-containing lentiviruses and transduction of CASCs was performed as previously described (Koninckx et al. 2009).

2.2.8. Functional cardiomyogenic differentiation assays

To stimulate cardiomyogenic differentiation, co-culture systems were set up between GFP^+ CASCs and NRCMs as previously described. After 1 week, cardiac differentiation was evaluated by immunofluorescence for cTnT and cTnI (Koninckx et al. 2013).

2.2.9. Telomerase expression with real-time RT-PCR

Total RNA was extracted from CASCs using RNeasy Micro Kit Isolation System (Qiagen), according to the manufacturer's instructions. RNA quality and integrity were analyzed on an Agilent 2100 BioAnalyzer (Agilent Technologies). RNA was eluted in RNase-free H_2O and stored at −80°C. cDNA was synthesized using Superscript™ First-Strand cDNA Synthesis System (Invitrogen), according to the provider's protocol. Real-time RT-PCR reactions were performed in duplicate on Rotor-Gene Q (Qiagen). Reaction conditions consisted of 1X Absolute qPCR Sybr Green Mix (Thermo Scientific), 300 nm human telomerase reverse transcriptase (hTERT) primers or 450 nm Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers and 1 µl of cDNA. As previously described by Anedchenko *et al.*, hTERT primers were designed specifically to amplify the only functional full-length hTERT isoform 1: forward primer 5'-CTGTACTTTGTCAAGGTGGATGTGA-3' and reverse primer 5'-GTACGGCTGGAGGTCTGTCAAG-3' (Anedchenko et al. 2008). The single copy gene (scg) primers were designed in-house by primer express 3.0 with: GAPDH forward primer 5'-AGTCAACGGATTTGGTCGTATTG-3' and GAPDH reverse primer 5'-ATCTCGCTCCTGGAAGATGGT-3'. PCR reaction was performed as previously described: 5 minutes at 95°C, 50 cycles at 95°C for 15 seconds, 60 seconds at the corresponding annealing temperature (57°C for GAPDH and 59°C for hTERT) and 72 C for 20 seconds, which was followed by melt curve analysis (Anedchenko et al. 2008).

2.2.10. Telomere length measurement with real-time PCR

Genomic DNA was isolated from CASCs by ethanol precipitation or using QIAamp DNA mini kit (Qiagen), according to the manufacturer's instructions. The 1301 cell line (T-cell lymphoblastic leukemia), which has been reported to have long and constant telomeres, was used as positive control (Larsson et al. 1979).

Singleplex real-time PCR reactions were carried out in duplicate with Rotor-Gene Q (Qiagen), based on methods of Cawthon *et al.* (Cawthon 2009). Final reaction master mix composition consisted of 1X Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), 1 M Betaine (Sigma-Aldrich), 900 nm albumin or 450 nm telomere primer, respectively, and 20 ng DNA. PCR reaction consisted of the following steps: 95°C for 15 minutes, 2 cycles of 94°C for 15 seconds and 50°C for 15 seconds, 32 cycles of 94°C for 15 seconds, 62°C for 10 seconds, 74°C for 15 seconds (acquisition of telomere product fluorescence), 84°C for 10 seconds and 86°C for 15 seconds (acquisition of albumin product fluorescence), followed by melt curve analysis. To quantify absolute telomere length (aTL), standard curves of albumin and telomere plasmids were generated as previously described (O'Callaghan and Fenech 2011).

2.2.11. Statistical analysis

All quantitative results are presented as median ± IQR. To analyze whether starting number correlated to maximal cumulative number of CASCs, CASC populations were categorized into two groups: CASCs with $<4x10^4$ initial cells (A_0low; n=10 individual patients' CASC cultures) and CASCs $>4x10^4$ initial cells (A_0high; n=10 individual patients' CASC cultures). Comparisons between groups were performed in GraphPad version 5.01 using Mann–Whitney testing. The relationship between maximum CASC count and age or BMI was performed in GraphPad version 5.01 using linear correlation analysis. Linear mixed models were used for data measured at repeated time points during the follow-up period (patient evolution data). Linear mixed models were used to compare aTL per passage (random intercept) as well as number of proliferative CASCs per passage (random intercept and slope), after outlier detection and removal, with software 'R' version 2.10.1. For comparison of CASC aTL in FBS and PPS, a treatment–time interaction was included. All statistical analyses for linear mixed models were performed in SAS version 9.2. For each test, at least three patients per passage were analyzed, unless stated otherwise.

2.4 RESULTS

2.4.1 Isolation and long-term in vitro expansion of CASCs

Growth properties of CASC cultures from several IHD patients (n=21; Table 2.2) were evaluated over the entire expansion period. Cultures that did not grow beyond P5 were excluded from further analysis (n=4 individual patients' CASC cultures). In general, CASC cultures had an early, exponential growth phase (P2–P5), followed by a late phase (P6–P9) characterized by slow decay in growth rate. Cells remained in culture for 52±15 days on average and performed 9.66±4.95 PDs until ultimately reaching a state of proliferative arrest. This was accompanied by apparently enlarged volume morphology and formation of cell structures resembling apoptotic blebs and necrotic cell debris (data not shown). In terms of growth and in vitro lifespan, a wide inter-individual variability was observed among CASC cultures. This was clearly reflected in the maximum calculated cumulative number of cells, which ranged between $0.34x10^6$ and $11.07x10^9$ (Figure 2.2A). However, maximal cumulative number of cells did not correlate with specific patient characteristics (Figure 2.1) nor their starting number as initially harvested from tissue specimens (P=1.00, data not shown). Furthermore, early phase CASCs had an average of 2.06±0.95 PDs, which reduced slightly to 1.53±1.29 PDs for late phase cells. CASCs had average PDL of 5.83±3.27 at P5 and 12.91±4.95 at P9 (Figure 2.2B). According to PDT data, 52.9% of CASCs had high initial growth rate, slowing towards the end of the culture period, while 41.2% of the populations displayed variable growth rates, with alternating periods of slow and fast expansion. In 5.9% of CASC cultures, a rather slow expansion rate was observed during early phases which accelerated towards the end of the culture period, prior to reaching growth arrest. PDT measured in early phase CASCs was 2.60±2.11 days, increasing to 5.06±7.51 days in late phase CASCs. Average PDT commonly increased throughout all CASC cultures.

Table 2.2. Patient characteristics.

Age (years)	77±16
Gender: male/female (%)	76/24
Risk factors	
Weight (kg)	82±16[a]
Body mass index (kg/m²)	27±4[a]
Last creatinine level pre-operation (mg/dl)	1.0±0.3
Smoker: yes/no (%)	50/50
Family history of CAD (%)	25[a]
Diabetes (%)	25[a]
Hyperlipidemia (%)	70
Renal dysfunction (%)	20
Hypertension (%)	67
Chronic lung disease (%)	5
Peripheral vascular disease (%)	19
Cerebrovascular disease (%)	29
Pre-operative cardiac status	
Myocardial infarction (%)	35
Congestive heart failure (%)	20
Angina (%)	81
Arrhythmia (%)	19
Classification: NYHA I/II/III/IV (%)	0/55/20/25[a]
Pre-operative medicine	
Beta-blockers (%)	60
Nitrates PO (%)	50
Nitrates IV (%)	25
Diuretics (%)	10
ACE inhibitors (%)	30
Ca antagonists (%)	15
Anti-arrhythmias (%)	15
Lipid lowering (%)	75
Aspirin (%)	85
Other antiplatelets (%)	30
Number of vessels: 0/1/2/3 (%)	5/0/29/67
Surgical procedure	
CABG/valve/other (%)	95/20/0

ACE = angiotensin-converting enzyme; Ca = calcium; CABG = coronary artery bypass; CAD = coronary artery disease; CASC = cardiac atrial appendage stem cell; IV = intravenous; NYHA = New York Heart Association; PO = per oral. Values are expressed as median ± IQR or % of the total patient population. [a]n=20.

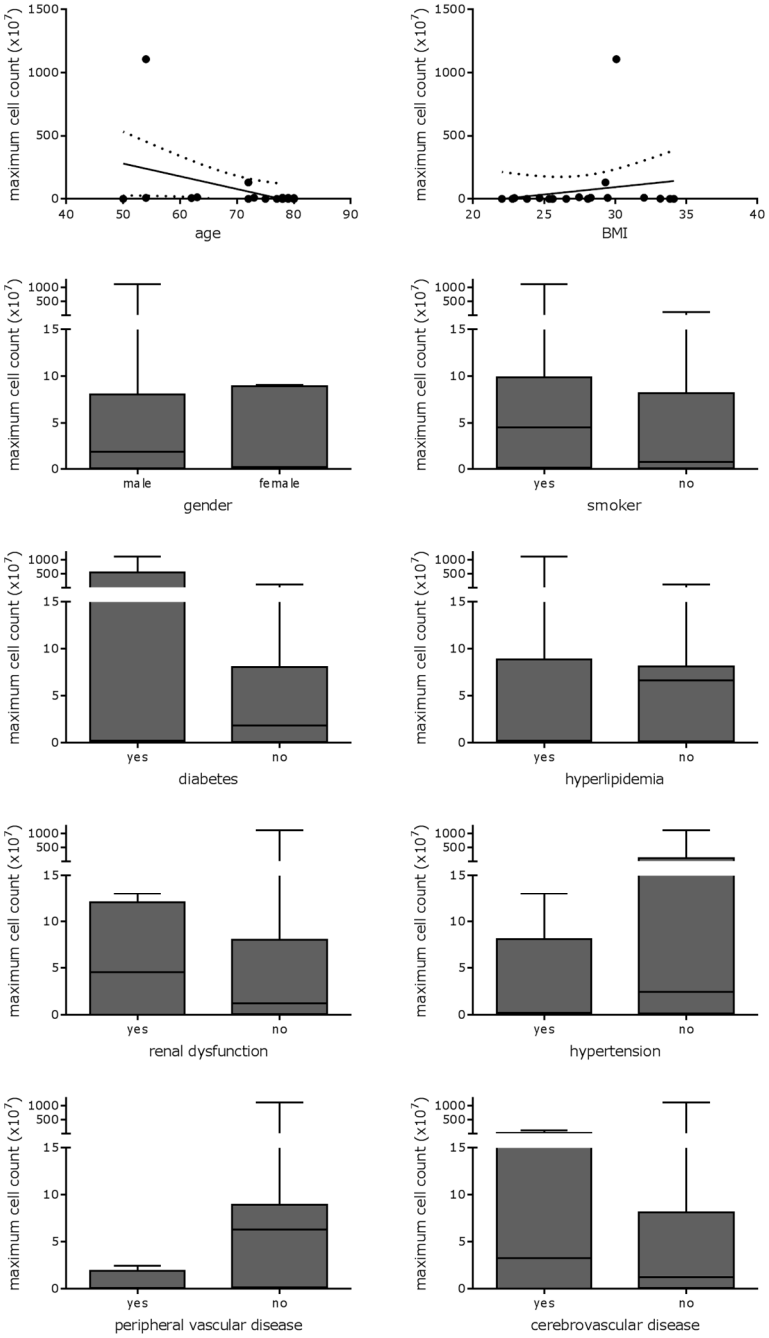

Figure 2.1: Studying correlation between specific patient characteristics and maximum CASC count after culture. For age and BMI, data are shown as individual data point with fitted linear regression line and corresponding 95% confidence band. For the other parameters, data are shown as median, 75th percentile, 25th percentile, minimum and maximum.

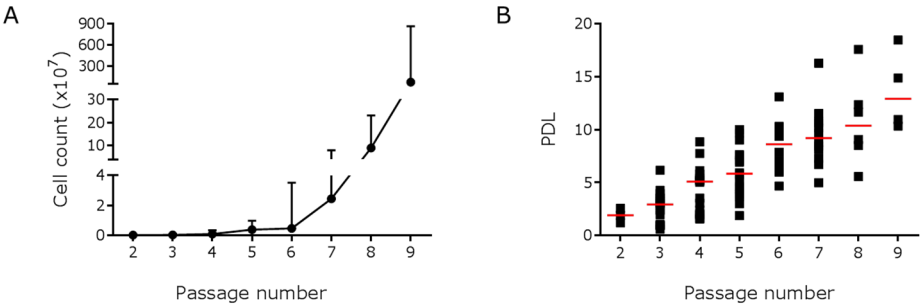

Figure 2.2: CASC growth kinetics over long-term expansion. Graphs represent cumulative cell number (A) and population doubling level (PDL, B). Graphs are displayed in function of passage number (P2–P9). Data are shown as median ± IQR (A). Individual data points with the red line representing the median (B). At each passage n≥4 individual patients' CASC cultures.

Additionally, proliferation assays were performed to follow cell cycle progression over long-term *in vitro* expansion (n=13 individual patients' CASC cultures; P4–P9). On average 18.82±6.52% of all CASCs were in mitosis (Figure 2.3A). This proliferative percentage ranged from maximum of 23.62±6.49% at P5 to 18.91±4.93% at P9. There was a slight but significant reduction in the number of proliferating CASCs at each passage ($P<0.05$; Figure 3.2B). This analysis revealed also that CASCs showed no signs of aneuploidy over the course of expansion, even at higher passages.

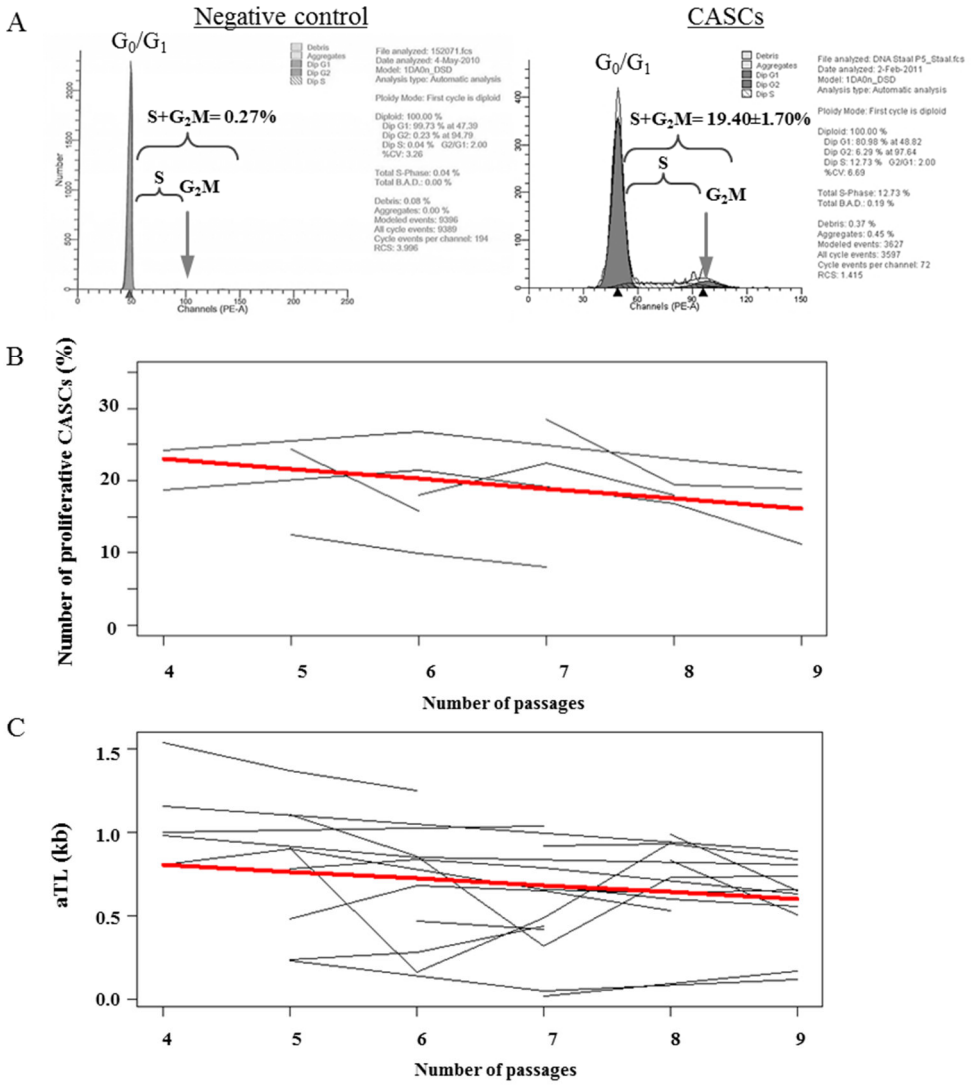

Figure 2.3: Proliferation and absolute telomere length (aTL) of expanded CASCs. (A) Representative cytometry output of cell cycle distribution of peripheral blood cells (negative control, left panel) and expanded CASCs (P4–P9; right panel). (B) Individual (grey lines) and average (bold red line) linear reduction in percentage of proliferating CASCs with increasing passages ($P<0.05$). (C) Individual (grey lines) and average (bold red line) inverse linear relation between aTL and passage number ($P<0.05$).

2.4.2 Ageing and cell senescence dynamics of CASCs

To assess the influence of concomitant serial passaging on cell ageing, aTL was determined in CASCs over their expansion period. Cells were collected and expanded from 19 different patients, subsequently harvested at consecutive passages (P4–P9) and aTL was measured with real-time PCR assay. Expanded CASCs had average aTL of 0.68±0.41 kb for the entire culture period, ranging from 1.00±0.18 kb at P4 to 0.64±0.24 kb at P9. Statistical analysis revealed that CASCs showed overall and significant reduction in aTL with increasing number of passages ($P<0.05$; Figure 2.3C). These results indicate that, despite inter-individual variation between patients, change of aTL in CASCs was consistent over time. In agreement with these observations, hTERT transcripts were not expressed in expanded CASC cultures up to P9 (n=2 individual patients' CASC cultures, data not shown).

2.4.3 Biological characteristics of expanded CASCs

The immunophenotype of CASCs (n=14 individual patients' CASC cultures, P4–P9) was continuously monitored at different passages throughout the culture period and retained a stable phenotype without any specific alteration. Expanded CASCs remained CD34, CD45 and CD117 negative. In contrast, the cells stably expressed CD29, $CD55^{dim}$, CD73, CD90 and CD105. For CD90 in particular, two subpopulations were demonstrated to be present, $CD90^{dim}$ and $CD90^{+}$. Data retrieved from distinct CASC populations are summarized in figure 2.4 (red line).

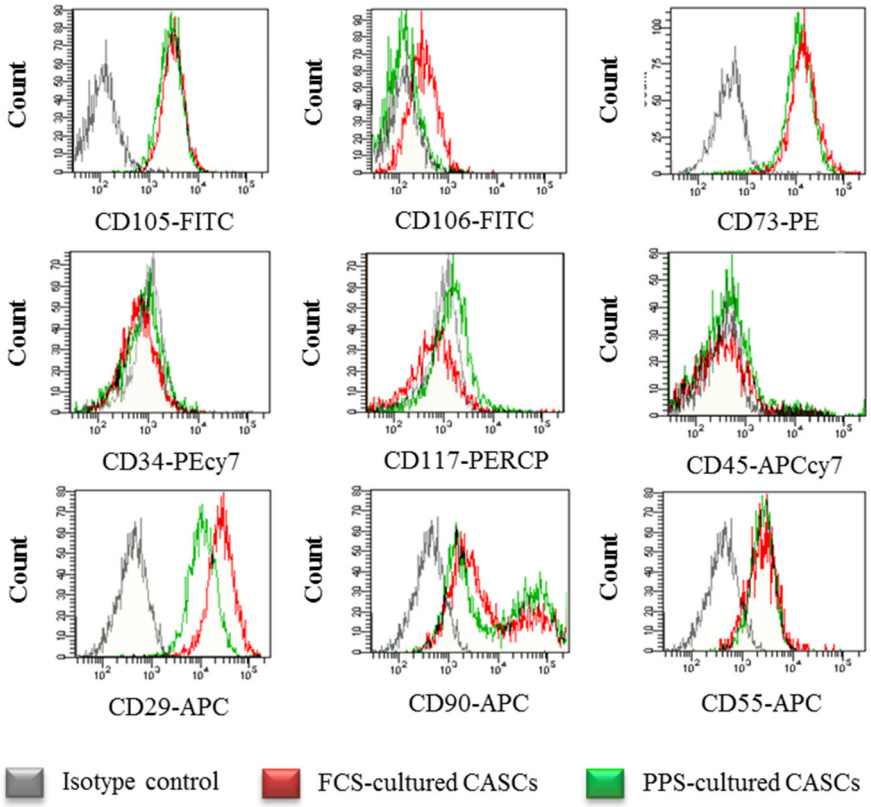

Figure 2.4: Immunophenotypic analysis of *ex vivo* expanded CASCs. Long-term *in vitro* expanded CASCs (P4-P9) displayed continuous stable phenotype with expression of CD105, CD73, CD29, CD90 and CD55, while lacking CD106, CD117, CD34 and CD45. n≥3 individual patients' CASC cultures at each passage for each antigen marker.

Furthermore, ALDH expression in the cells was monitored over their long-term expansion period (n=9 individual patients' CASC cultures). Our data indicate that ALDH activity tended to be constant, with average 50.10±16.45% of $ALDH^{br}$ CASCs during their expansion from P4 to P9. CASCs displayed no sign of decaying ALDH activity with increasing days in culture (Figure 2.5A). Interestingly, when $ALDH^{br}$ and $ALDH^{dim}$ CASC subpopulations were flow sorted and seeded in separate culture plates, only the $ALDH^{br}$ fraction attached to the culture plate and revealed continued growth and expansion (Figure 2.5B). In contrast, $ALDH^{dim}$ CASCs remained unattached, undergoing cell death.

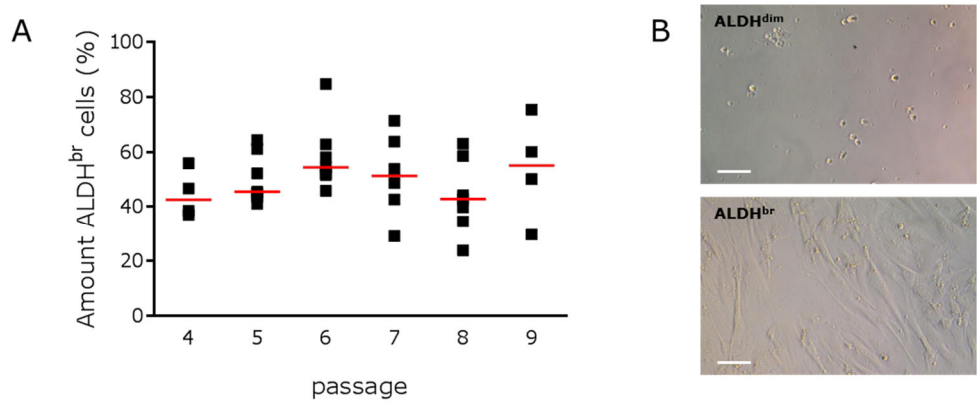

Figure 2.5: ALDH expression of expanded CASCs. (A) Flow cytometry analysis of ALDH activity in expanded CASCs (P4–P9), displayed by individual data points with the red line representing the median. (B) ALDH^{dim} (top panel) and ALDH^{br} cells (bottom panel) in culture. n≥4 individual patients' CASC cultures per passage. Scalebar = 100 µm.

To evaluate preservation of cardiogenic potential of CASCs (n=17 individual patients' CASC cultures) over long-term expansion, GFP^{+} cells were harvested at different time points and brought into co-culture with NRCMs. CASC cultures from P4 to P9 maintained their ability to differentiate towards cardiomyocytes, as demonstrated by expression of cardiac-specific proteins cTnT and cTnI (Figure 2.6A and 2.6B).

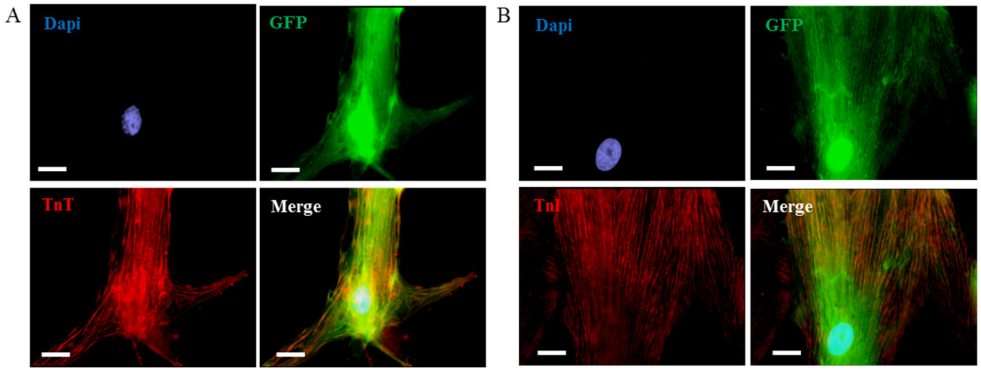

Figure 2.6: Cardiomyogenic differentiation of expanded CASCs. Immunofluorescence images illustrating cTnT (red, A) and cTnI staining (red, B) on GFP^{+} CASCs (green; P4–P9) after 1 week in co-culture with NRCMs. Nuclei stained with 4',6'-diamidino-2-phenylindole (DAPI; blue). n≥3 measurements at each passage. Scalebar = 20 µm.

2.4.4 PPS as a culture supplement for CASC expansion in a clinical setting

For the purpose of a future CASC cell therapy study, we aimed to establish a non-FBS-based culture method. To this end, we evaluated the use of human PL and PPS as potential alternative medium supplements for FBS for long-term *in vitro* culture of CASCs. Initial experiments showed that CASCs adhere to tissue culture plates within 24 hours of seeding, when cultured in the presence of 10% FBS or 10% PPS, but not in 10% PL (n=4 individual patients' CASC cultures/condition; data not shown). Further optimization experiments with 5%, 7.5% and 10% PPS revealed an optimal concentration of 7.5% PPS in the culture medium (n=4 individual patients' CASC cultures/condition; data not shown); this was therefore selected as the medium supplement for further experiments.

Subsequently, biological characteristics of CASCs expanded in medium enriched with 7.5% PPS were compared to those of CASCs cultured in 10% FBS (n=4 individual patients' CASC cultures/condition). In general, CASC growth kinetics in PPS were similar to those of cells cultured in FBS-based medium (Figure 2.7A and 2.6B). Maximal cumulative cell number ranged from $1.34x10^6$ to $2.56x10^8$ when the cells were cultured in medium with FBS, and maximal cumulative CASC numbers in PPS-supplemented medium ranged from $1.58x10^6$ to $6.65x10^7$. CASCs cultured in FBS- and PPS-supplemented medium displayed average maximal PDL of 9.01±3.72 and 8.95±2.09 respectively. Furthermore, average aTL of CASCs (P5–P9) cultured in PPS and FBS was similar with values of 0.65±0.36 kb and 0.53±0.14 kb, respectively. Indeed, no significant differences were detected between aTL of CASCs cultured in PPS- or FBS-enriched medium at the same passage (P=0.134; n=4 individual patients' CASC cultures/condition; Figure 2.7C). Flow cytometry analyses revealed the same antigenic profile for CASCs cultured in PPS- compared to FBS-enriched medium (P2–P9; n=3 individual patients' CASC cultures/condition; Figure 2.4 green line). Finally, in comparison with FBS, co-culturing GFP^+ CASCs expanded in PPS-enriched medium (P4–P9) with NRCMs revealed equally effective cardiomyogenic differentiation potential as demonstrated by expression of cTnT and cTnI (n=4 individual patients' CASC cultures/condition; Figure 2.8A and 2.8B).

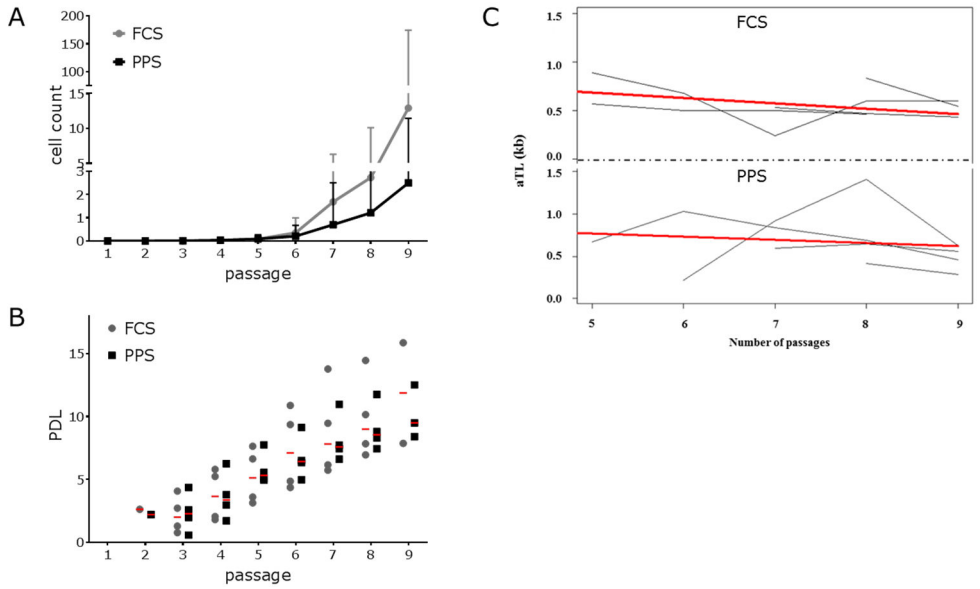

Figure 2.7: Comparison of growth kinetics and absolute telomere length (aTL) of CASCs expanded in medium enriched with PPS or FBS. Graphs illustrating cumulative cell count (A) and PDL (B) of expanded CASCs (P2–P9) in medium enriched with FBS and PPS. Data represent median ± IQR (A). Individual data points with the red line representing the median of each condition (B). In general, at each passage n=4 individual patients' CASC cultures, except for P2 n=1 and P9 n=2 or n=3 for respectively FBS and PPS. Graphs illustrative of individual (grey lines) and average (bold red line) aTL evolutions with increasing passages (C). No significant differences were detected between aTL of CASCs cultured in FBS- (top), PPS- (bottom) enriched medium of the same passage (P=0.134).

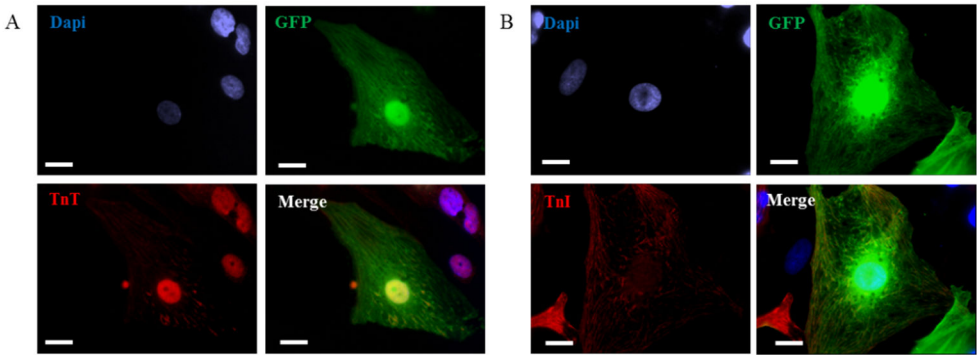

Figure 2.8: Cardiomyogenic differentiation of expanded CASCs in PPS-supplemented medium. Immunofluorescence images illustrating cTnT (red, A) and cTnI staining (red, B) on PPS-cultured GFP$^+$ CASCs (green; P4–P9) after 1 week in co-culture with NRCMs. Nuclei stained with 4',6'-diamidino-2-phenylindole (DAPI; blue). n≥3 measurements at each passage. Scalebar = 20 µm.

2.5 DISCUSSION

We have recently shown that CASCs may show great promise for cardiac regenerative therapies (Koninckx et al. 2013). As for other CSC-types, *ex vivo* expansion is an essential step in production of therapeutically meaningful cell numbers. However, long-term expansion protocols have been reported to negatively impact the differentiation capacity of cells (Bajpai et al. 2012). Therefore, the aim of this study was to investigate extensively the influence of prolonged culture, not only on differentiation potential of CASCs but also on their growth, phenotype and ageing processes.

In the present study, we have demonstrated that CASCs isolated from elderly IHD patients had good expansion capacity and could be maintained in culture for approximately 2 months. None of the CASC cultures bypassed the senescence phase. Using this expansion protocol, large quantities of cells were generated, equaling ranges used in previously conducted phase I clinical trials with CSCs (Bolli et al. 2011, Makkar et al. 2012). Although high inter-patient variability was observed in growth profiles and expansion potential of CASCs, this is a recurrent observation in most expansion evaluation protocols. This high variability could not be correlated to specific patient characteristics (e.g. age, gender…). A more profound study with more variables and a larger study population might reveal specific contributing factors to this high variability. The high variability in the observed growth profiles and expansion potential of CASCs might also be linked to de level of mitochondrial DNA (mtDNA) deletion in the isolated CASCs. Indeed, mDNA deletions have been implicated in mammalian aging and as mtDNA mutations are present in mature cardiomyocytes, it is not unlikely that they are also present in adult cardiac progenitor cells (Arai et al. 2003, Kujoth et al. 2007). A study of Lushaj *et al.* confirmed this hypothesis as it showed that mtDNA deletions are present in mouse SP cells, but more importantly they also demonstrated that cells with high levels of mtDNA deletions had slower proliferations rates (Lushaj et al. 2012). Therefore, screening CASC cultures for mtDNA deletions might be a good strategy for selecting high quality CASCs with a profound expansion potential. This is especially important since different hypotheses suggest that these mtDNA deletion could continually accumulate with increased passaging (Wallace 1992, Kowald and Kirkwood 2000). Furthermore,

RNA sequencing studies can be used to identify biomarkers for CASCs with the highest proliferation capacity which can further improve a future transplantation protocol by obtaining more cells. However, it is difficult to determine the correct minimal cell amount for treatment. The more the better is not necessary always true. Indeed, intramyocardial transplantation of as few as 1,150,000 cells in a Göttingen minipig model of acute MI resulted in a positive effect. In addition, no relationship between cell dose and functional improvement could be demonstrated, although the number of animals might be too low to make clear statements on this matter. In comparison, in the SCIPIO trial 500,000 or 1,000,000 c-kit+ CSCs were injected, while in both the CADUCEUS and the ALLSTAR trial 12,500,000 or 25,000,000 CDCs were injected (Bolli et al. 2011, Makkar et al. 2012, Chakravarty et al. 2016).

Despite observing rather stable growth up to P9, we noticed gradual reduction in proliferative percentage, accompanied by minor but consistent reduction in aTL. This is not remarkable as CASCs lack expression of telomerase catalytic subunits, and these findings are consistent with previous reports describing telomere kinetics in cardiac progenitor cells (Sussman and Anversa 2004, Anversa et al. 2005). As telomere shortening is a known trigger for cell senescence and one of the side effects associated with cell proliferation, excessive *ex vivo* expansion should be kept to a minimum to avoid detrimental effects on telomere maintenance and stem cell ageing. Nevertheless, despite progressive telomere shortening with an accumulating number of passages, cell cycle analysis demonstrated that CASCs did not display any genomic aneuploidy, suggesting the absence of major chromosomal aberrations, although this would have to be investigated in more detail in future experiments. The results here also demonstrate that CASCs can be expanded in the long-term while retaining their antigenic expression profile, ALDH expression and more importantly their cardiomyogenic differentiation capacity. Interestingly, ALDH expression remained constant during the entire culture period, with an average of 50% of CASCs displaying elevated ALDH levels. Separate flow sorting of $ALDH^{dim}$ and $ALDH^{br}$ subpopulations revealed that only $ALDH^{br}$ cells could be further expanded. This suggests that these two fractions most likely arose from asymmetrical cell division, in which loss of ALDH expression contributes to a more mature cell fate. Overall, despite a decline in growth rate and progressive telomere shortening at

higher passages, CASCs preserved their regenerative capacity through cardiac differentiation until the end of the culture period, indicating that their proliferative arrest over long-term expansion is most likely due to senescence. Nevertheless, ongoing research in a minipig model of MI will have to determine transplantation efficiency and functional impact of these cells.

FBS, which is traditionally used in cell expansion protocols, is less suitable for clinical use as it is a potential source of unknown xenogeneic antigens which might elicit immunological reactions in a recipient (Selvaggi et al. 1997). Moreover, animal pathogens might cause infection and lead to rejection of transplanted cells (Halme and Kessler 2006). In this regard, use of human platelet-derived growth factors as medium supplement offers great advantages, avoiding risks associated with FBS. Human PL has already been shown to represent an attractive medium supplement for large-scale expansion of bone marrow MSCs while they maintained stable phenotype and differentiation capacity (Doucet et al. 2005, Perez-Ilzarbe et al. 2009, Gottipamula et al. 2012). Noticeably, in this study, CASCs only demonstrated successful growth and expansion in PPS-supplemented but not PL-supplemented medium. A reasonable explanation might be that here, PL was prepared from expired platelets, which could no longer be used for platelet transfusion. Possibly, spontaneous activation of platelets during this prolonged storage period might have caused release of large contributors to their intragranular growth factor content (Zimmermann et al. 2001, Mirabet et al. 2008). Our results show that PPS was equally efficient for large-scale expansion of human CASCs compared to FBS. Moreover, no significant differences between aTL of CASCs cultured in PPS-, in comparison to FBS-enriched medium were observed. Furthermore, CASCs expanded in PPS preserved their antigenic expression profile and their ability to differentiate to cardiomyocytes, even after prolonged culture periods. To our knowledge, we are the first to provide detailed information on the influence of PPS on growth and differentiation of CSCs, in particular CASCs. Our findings are comparable to studies evaluating bone marrow MSCs cultured in platelet-derived medium supplements (Perez-Ilzarbe et al. 2009, Gottipamula et al. 2012). Thus, we suggest that replacement of FBS with human platelet-originating growth factors is very favorable for large-scale expansion of human CSCs in future clinical settings.

In conclusion, we have demonstrated that CASCs could be successfully isolated then expanded for a prolonged period of time, from elderly IHD patients, producing high cell numbers relevant to therapeutic application. Although serial passaging and concomitant ageing of CASCs resulted in progressive decline of telomere length, the cells cardiomyogenic differentiation potential remained unaffected. Despite this and absence of aneuploidy, more detailed examination on biosafety and tumor formation needs to be performed in a preclinical study to guarantee patient safety. Nevertheless, preservation of CASC biology, reported herein, and use of PPS as efficient replacement for FBS for large-scale clinical expansion of CSCs represent an important step towards clinical use of CASCs in cardiac regenerative medicine.

Chapter 3

The role of Wnt/β-catenin signaling in cardiac atrial appendage stem cells

Based on:

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(in preparation) **Cardiac Atrial Appendage Stem Cells for Cardiac Repair: No Win from Wnt**

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3.1. ABSTRACT

Over the past decade, the discovery of resident CSCs has raised high hopes for myocardial regeneration after myocardial infarction. The success of a cardiac stem cell therapy largely depends on the selection of the appropriate stem cell type. CSCs isolated based on an elevated aldehyde dehydrogenase activity, called CASCs, are good candidates for myocardial regeneration.

In this study, we demonstrate that in adult heart, CASCs are predominantly present in the atrial appendages and more so in the right than in the left atrial appendage. We show that CASCs express multiple early cardiac differentiation markers and are committed towards myocardial differentiation. Furthermore, CASCs express different Frizzled receptors, suggesting a role of Wnt signaling in clonogenicity, proliferation and differentiation of the CASCs. Wnt activation did indeed increase total and active β-catenin levels. Surprisingly, this did not affect CASC proliferation nor clonogenicity. Additionally, Wnt inhibition upregulated early cardiac markers but could not induce mature myocardial differentiation.

In conclusion, we confirmed that CASCs are committed towards myocardial differentiation, the Wnt pathway is active and can be modulated in CASCs. However, despite the crucial role of Wnt signaling in cardiogenesis and myocardial differentiation of pluripotent stem cell populations, our data indicate that Wnt signaling has limited effects on CASC clonogenicity, proliferation and differentiation.

3.2. INTRODUCTION

Heart failure after MI is the leading cause of mortality worldwide and results from the loss of cardiomyocytes and remodeling of the remote cardiac tissue due to coronary occlusion. While heart transplantation is currently the only available therapy to fully restore cardiac functionality after an MI, its major drawbacks such as the lifelong immunosuppressive therapy and the paucity of donor hearts, do not allow it to be a realistic option for every patient (Korewicki 2009). In that context, the development of cardiac regeneration strategies using various stem cell types including PSCs, skeletal myoblasts, BMCs and endogenous CSCs have raised a lot of interest. However, up to now, the results obtained in different clinical trials were rather disappointing because of the chosen cell type for transplantation and its characteristics upon differentiation (Gyongyosi et al. 2015).

Resident CSCs might offer better promises for cardiac regeneration as they are most-likely pre-programmed to become cardiomyocytes. Different approaches have been used to isolate and identify endogenous cardiac progenitor populations, including c-kit, Sca-1, ALDH, side population and cardiospheres (Bruyneel et al. 2016). Of these, CSCs isolated based on an elevated ALDH activity appear to be very promising candidates for myocardial regeneration (Koninckx et al. 2013, Fanton et al. 2015). Indeed, our previous studies have shown that these CASCs can be *ex-vivo* expanded towards clinically relevant cell numbers, comparable to previously conducted phase I clinical trials with other CSC types, while maintaining their biological properties including their cardiomyogenic differentiation potential (Windmolders et al. 2015). Their therapeutic potential was further confirmed *in vivo* in a Göttingen minipig model of acute MI where autologous CASC transplantation preserved global and regional left ventricle function due to extensive engraftment and myocardial differentiation of the transplanted cells (Fanton et al. 2015).

The molecular and cellular mechanisms involved in CSC proliferation and differentiation generating the promising results of some CSC populations remain largely unknown, but identifying these mechanisms could further enhance cardiac regeneration therapies. In that context, the Wnt/β-catenin pathway could be heavily involved since it is essential for vertebrate cardiogenesis and is re-

activated in response to cardiac injury by regulating transcription of target genes involved in cell differentiation and proliferation (Niehrs 2012). Therefore, it is of great importance to investigate its precise role in CASC proliferation and differentiation which can eventually lead to improved CASC-based cardiac therapies.

The canonical Wnt/β-catenin signaling pathway is activated when secreted Wnt ligands bind to the receptor complex, composed of a seven-transmembrane Frizzled (FZD) receptor and a low density lipoprotein receptor related protein (LRP)5/6 co-receptor. This interaction leads to the inhibition of glycogen synthase kinase (GSK)-3β and subsequently to a hypophosphorylation of β-catenin. As a result, accumulation of β-catenin in the nucleus activates transcription of specific target genes, including Cyclin D1, V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (cMYC) and Jun Proto-Oncogene (cJUN) (Nusse 2005). During cardiogenesis, the effects of canonical Wnt signaling occur in four distinct phases. First, canonical Wnt signaling is upregulated to induce mesoderm formation. Subsequently, inhibition of canonical Wnt signaling is essential for cardiac specification and cardiac progenitor cell formation. Thereafter, Wnt signaling is upregulated to enhance proliferation of these progenitor cells. Finally, Wnt signaling is inhibited again for terminal cardiac differentiation (Gessert and Kuhl 2010). The identification of these distinct phases has led to the development of new differentiation protocols for PSCs which promote cardiac differentiation by sequentially activating and inhibiting the canonical Wnt signaling pathway (Lian et al. 2013). We hypothesize that the same processes are involved in CASCs. We therefore aimed to identify whether Wnt activation is able to stimulate CASC proliferation and whether Wnt inhibition would induce cardiac maturation. Since Wnt signaling has distinct roles during the different phases of cardiac development we also studied the origin of CASCs by determining the localization of CASCs in adult heart and the cardiac differentiation stadium of CASCs in culture.

3.3. MATERIAL AND METHODS

All procedures were carried out in accordance with the principles set forth in the Helsinki Declaration. Approval by the Jessa Institutional Review Board and informed consent from each patient were obtained. The characteristics of the patients used to isolate and characterize CASCs are shown in table 3.1.

Table 3.1: Patient characteristics.

Age (years)	70±14[a]
Gender: male/female (%)	70/30[a]
Risk factors	
Weight (kg)	80±15[a]
Body mass index (kg/m²)	28±5[a]
Last creatinine level pre-operation (mg/dl)	1.0±0.3[a]
Smoking: current smoker/ex-smoker ≥1 month/never smoked (%)	15/36/48[a]
Diabetes (%)	33[a]
Hypercholesterolemia (%)	87[c]
Renal dysfunction (%)	9[b]
Hypertension (%)	61[c]
Chronic lung disease (%)	16[b]
Peripheral vascular disease (%)	13[b]
Cerebrovascular disease (%)	19[b]
Pre-operative cardiac status	
Myocardial infarction (%)	34[b]
Congestive heart failure (%)	16[c]
Angina: CCS 0/I/II/III/IV (%)	18/18/57/4/4[d]
Classification: NYHA I/II/III/IV (%)	91/18/14/0[d]
Surgical procedure	
Number of vessels: 0/1/2/3 (%)	9/3/27/61[a]
CABG/valve/other (%)	91/15/3[a,e]

CCS = Canadian Cardiovascular Society; NYHA = New York Heart Association. Values are expressed as median ± IQR or % of the total patient population. [a]n=33 [b]n=32 [c]n=31 [d]n=28 [e]3 patients underwent a double procedure

3.3.1. Cell culture

CASCs were isolated from atrial appendages and subsequently expanded as previously described by Koninckx *et al.* (Koninckx et al. 2013). In brief, tissue samples were minced and enzymatically dissociated with collagenase II (600

U/ml; Invitrogen). Single cells were subsequently stained with Aldefluor (Stem cell technologies) according to manufacturer's protocol. Cells were dissolved in aldefluor assay buffer in the presence of activated aldefluor. After 45 minutes $ALDH^{br}$ CASCs were flow sorted using a FACSAria (Beckton&Dickinson). Isolated CASCs were cultured in X-vivo 15 medium (Lonza) supplemented with 2% PS (Lonza) and 10% FBS (GE Healthcare HyClone). 293T and SW480 cell lines were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) medium (Life Technologies) supplemented with 2% PS and 10% FBS. All cell cultures were expanded at 37°C in humidified atmosphere containing 5% CO_2.

3.3.2. Flow cytometric analyses of $ALDH^{br}$ cells in different compartments of the heart

Equal amounts of tissue (3 gr) were taken from left and right atrial appendage (LAA and RAA), left and right atrium, left and right ventricle, septum and apex of slaughterhouse pig hearts (n=3). For human samples, LAA and RAA samples of the same patient were used (n=33). Besides the routinely isolated RAA, the LAA was obtained as a precaution to avoid thrombus formation in patients at risk of developing atrial fibrillation. Subsequently the percentage of $ALDH^{br}$ cells was determined by Aldefluor staining as previously described and expressed as percentage $ALDH^{br}$ cells of total cells.

3.3.3. Drugs and treatment

The following small-molecule inhibitors and stimulators of the Wnt signaling pathway, all dissolved in DMSO, were used at the indicated final concentrations: CHIR99021 (6 μM; Stemcell technologies; 72054), IWP2 (4 and 20 μM; Sigma Aldrich; I0536;), C59 (1 and 5 μM; Abcam; ab142216;), XAV939 (2 and 10 μM; Sigma Aldrich; X3004;) and IWR1-endo (4 and 20 μM; Stemcell technologies; 72562;). For all Wnt related experiments, except the serial clonogenic assays, low glucose DMEM (Life Technology) with 2% PS and 2% FBS was used. Equal concentrations of DMSO were used as control.

3.3.4. RNA isolation and cDNA synthesis

Total RNA was extracted using the RNeasy mini kit (Qiagen) and equal amounts of RNA were reverse transcribed with the SuperScript III Reverse Transcriptase Kit (Invitrogen) primed with a random hexamer primer according to manufacturer's instructions.

3.3.5. Conventional PCR

PCR reactions were performed on a C1000 Touch thermal cycler (Bio-RAD). The final reaction master mix (26 µl) was composed of 1x PCR buffer, 2.5 mM MgCl2, 250 µM dNTP's, 50 mU/µl Taq DNA Polymerase (all from applied biosystems), 500 nM primer (Eurogentec) and 1.5 µl of cDNA. Primer sequences with corresponding annealing temperature and expected fragment size are listed in table 3.2. Gene expression was visualized by gel electrophoresis. *β-actin* was used as internal control. For quantification, densitometry was performed with ImageJ software and normalized to *β-actin*.

Table 3.2: Primer sequence, annealing temperature and fragment size.

Primer	Primer sequence	Annealing temperature	Fragment size
B-actin	Forward: 5'-AGCGGGAAATCGTGCGTGACA-3' Reverse: 5'-CCTGTAACAATGCATCTCATATTTGG-3'	56°C	791 bp
FZD1	Forward: 5'-CCTTTCTTTCCTGGCTTGA-3' Reverse: 5'-CTCACCCTGTAACCAACTAAG-3'	56°C	285 bp
FZD2	Forward: 5'-CCCTACTCATTTGTCCTGTC-3' Reverse: 5'-TGAATAGACTGCAGGGAAAG-3'	56°C	405 bp
FZD3	Forward: 5'-CTCCTGAGGGATCCAAATAC-3' Reverse: 5'-GAGCCGATGAGAACTACTATG-3'	56°C	282 bp
FZD4	Forward: 5'-GAGAGAGAAGAGAGGAAATGG-3' Reverse: 5'-GGTCACTTAATTGTTGCTAGTT-3'	56°C	185 bp
FZD5	Forward: 5'-CCCAGAGCTAGGAAATGTAG-3' Reverse: 5'-GATGTGCTCTGTCCTGTT-3'	56°C	165 bp
FZD6	Forward: 5'-CATCAATGAGAGAGGTGAAAG-3' Reverse: 5'-GGGTGAACAAGCAGAGAT-3'	56°C	286 bp
FZD7	Forward: 5'-CATTTGGATCCTTTGAGGTAAA-3' Reverse: 5'-CTCTTCGTTCACTATGGTATCT-3'	56°C	203 bp
FZD8	Forward: 5'-CTCTTCCTACGTAAACTCCC-3' Reverse: 5'-GAGAGGGCAATGGTTAAATC-3'	56°C	356 bp

FZD9	Forward: 5'-CTCCAAGACTTTCCAGACC-3' Reverse: 5'-GTCCGTCTTAGTCATGTGC-3'	56°C	157 bp
FZD10	Forward: 5'-GATTCAGCCCTCAGAAGAA-3' Reverse: 5'-GCAGAGAGACTATTGGTGAA-3'	56°C	292 bp

FZD = Frizzled receptor

3.3.6. Quantitative PCR

Real time PCR reactions were carried out in duplicate with the Rotor-Gene Q (Qiagen) according to the MIQE guidelines (Bustin et al. 2009). The final reaction master mix composition was 1x Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), 2 mM extra MgCl2 (only for *TBX5* and *TBX18*; Invitrogen) and 2 µl of cDNA diluted 10-fold with DNase-RNase free water. Primer sequences with corresponding annealing temperature and concentration are listed in table 3.3. The PCR conditions were as follows: 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and annealing temperature for 30 seconds. For myosin light chain 7 (*MYL7*) an additional step of 80°C for 30 seconds was added to each cycle. SW480 cells were used as positive control for brachyury transcription factor (*T*). The most stable reference genes for each experimental set-up were identified with NormFinder.

Table 3.3: Primer sequence, annealing temperature and concentration.

Primer	Primer sequence	Annealing temperature	Concentration
B2M	Forward: 5'-AAGATGAGTATGCCTGCCGT-3' Reverse: 5'-TTCATCCAATCCAAATGCGGC-3'	60°C	300 nM
cJUN	Forward: 5'-AGGTGGAGTTGAAAGAGTTAAGA-3' Reverse: 5'-ACCATAGCATCAGGTACATCAG-3'	60°C	300 nM
cMYC	Forward: 5'-TTCTCTGAAAGGCTCTCCT-3' Reverse: 5'-GTGAAGCTAACGTTGAGGG-3'	60°C	300 nM
Cyclin D1	Forward: 5'-TCAAATGTGTGCAGAAGGAG-3' Reverse: 5'-TCTCCTTCATCTTAGAGGCC-3'	60°C	100 nM
GAPDH	Forward: 5'-AGTCAACGGATTTGGTCGTATTG-3' Reverse: 5'-ATCTCGCTCCTGGAAGATGGT-3'	60°C	300 nM
GATA4	Forward: 5'-ACCTGAATAAATCTAAGACACCAG-3' Reverse: 5'-CATCGCACTGACTGAGAAC-3'	60°C	100 nM
HCN4	Forward: 5'-AGTCGGCCGGATTTTGGATT-3' Reverse: 5'-AGGTGATGCCCACAGGAATG-3'	58°C	100 nM
KDR	Forward: 5'-CTAGGTAAGCCTCTTGGCCG-3' Reverse: 5'-CGATGCTCACTGTGTGTTGC-3'	66°C	300 nM

MYL2	Forward: 5'-TTGGGCGAGTGAACGTGAAA-3' Reverse: 5'-GGTCCGCTCCCTTAAGTTTCT-3'	60°C	300 nM
MYL7	Forward: 5'-GGAGTTCAAAGAAGCCTTCAGC-3' Reverse: 5'-GTCAGGGCGAACATCTGCT-3'	60°C	300 nM
NKX2.5	Forward: 5'-CAAGGACCCTAGAGCCGAAA-3' Reverse: 5'-CACCGACACGTCTCACTCAG-3'	58°C	450 nM
POLR2A	Forward: 5'-TCACAGCAGTGCGCAAATTC-3' Reverse: 5'-CCACGTCGACAGGAACATCA-3'	60°C	300 nM
T	Forward: 5'-ACTCCCAATCCTATTCTGACAACT-3' Reverse: 5'-CGTTGCTCACAGACCACAGG-3'	60°C	450 nM
TBX5	Forward: 5'-CAGGAGCATAGCCAAATTTACCA-3' Reverse: 5'-GGATAGCTAGAGCGGTAGAAGGA-3'	62°C	300 nM
TBX18	Forward: 5'-ATGCATTCTGGCGACCATCA-3' Reverse: 5'-ACGCCATTCCCAGTACCTTG-3'	62°C	300 nM
TNNT2	Forward: 5'-ACTTGGAGGCAGAGAAGTTCG-3' Reverse: 5'-CGGTGACTTTAGCCTTCCCG-3'	66°C	100 nM
YHWAZ	Forward: 5'-AGACGGAAGGTGCTGAGAAAA-3' Reverse: 5'-TGTGAAGCATTGGGGATCAAGA-3'	60°C	300 nM

B2M = Beta-2-Microglobulin; *cJUN* = Jun Proto-Oncogene; *cMYC* = V-Myc Avian Myelocytomatosis Viral Oncogene Homolog; *GAPDH* = Glyceraldehyde 3-phosphate dehydrogenase; *GATA4* = GATA Binding Protein 4; *HCN4* = Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 4; *KDR* = Kinase Insert Domain Receptor; *MYL2* = Myosin Light Chain 2; *MYL7* = Myosin Light Chain 7; *NKX2.5* = NK2 Homeobox 5; *POLR2A* = Polymerase (RNA) II Subunit A; *T* = T Brachyury Transcription Factor; *TBX5* = T-Box 5; *TBX18* = T-Box 18; *TNNT2* = Troponin T2, Cardiac Type; *YWHAZ* = Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

3.3.7. Western blot analysis

For total cellular protein, cells were immediately lysed with NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl, 137 mM NaCl, 10% Glycerol, 2 mM EDTA) supplemented with Halt Protease inhibitor cocktail (1X; Life Technologies) and 1 mM sodium orthovanadate. Equal amounts of protein, as measured by NanoDrop (Thermo Scientific), were electrophoresed on 4-15% Mini-PROTEAN TGX gels and transferred to 0.2 µm nitrocellulose membranes using the Trans-Blot Turbo Transfer System (all from Bio-Rad). Blots were blocked in Odyssey blocking buffer (Li-COR) for 1 hour and probed with primary and secondary antibodies for respectively 2 hours and 45 minutes. The primary antibodies used, were rabbit anti-β-catenin (D1048) mAb (1:1000; Cell signaling 8480), mouse anti-active β-catenin mAb clone 8E7 (1:1000; Millipore; 05-665), mouse anti-GAPDH mAb (1:2000; Abcam; ab8245) and rabbit anti-GAPDH mAb (1:5000; Abcam;

ab128915). The following infrared fluorescently-labeled secondary antibodies were used: goat anti-mouse IRDye® 800 CW (1:15000; LI-COR; 926-32210) and goat anti-rabbit IRDye® 680 RD (1:15000; LI-COR; 926-68071). Detection and quantification was accomplished using the Odyssey Infrared Imaging System and the LI-COR Odyssey Imaging software 2.1. Data are shown as ratios normalized to the loading control GAPDH and compared with their respective control condition.

3.3.8. Viability testing

CASC viability was measured with the Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Kit II (BD Pharmingen) according to the manufacturer's manual. Early apoptotic cells were defined as Annexin V positive and propidium iodide (PI) negative, while cells that stained positive for both Annexin V and PI were considered as late apoptotic cells. Viable cells were negative for both Annexin V and PI. The percentage of viable cells was measured by a FACSAria and expressed as the percentage Annexin V and PI negative cells of total cells.

3.3.9. Serial clonogenic assay

Self-renewal and proliferative potential of CASCs was assessed by serial cloning. Therefore, CASCs were cultured in X-vivo 15 medium with 2% PS and 20% FBS, supplemented with or without 6 µM CHIR99021 for respectively the treatment and control condition. Prior to assessing the clonogenic character, CASCs were labeled with green fluorescent protein (GFP). Production of GFP-containing lentiviruses and transduction of CASCs was performed as previously described (Koninckx et al. 2011). In brief, for viral production 293T cells were transfected with the cytomegalovirus GFP mammalian expression plasmid (pRRL-CMV-GFP) together with the packaging plasmids pMDLg-RRE, pRSV-REV and pCMV-VSVG, using EZLentifect (MellGen Laboratories nv). For GFP labeling, CASCS were incubated with viral supernatant containing replication-defective lentiviruses supplemented with 8 pg/ml polybrene (Sigma-Aldrich). Subsequently, GFP positive CASCs were flow sorted with a FACSAria in 96-well plates at a density of 1 cell per well. Single cell disposition was microscopically analyzed and wells with no cells or more than one cell were excluded. Medium exchange and scoring of the number and size of colonies was performed every 4 days. Clonogenicity (%) was defined as the

number of wells with > 1 cell; > 2 cells; > 5 cells; > 10 cells or > 50 cells to the total number of wells. At day 8 and 12, clones (> 50 cells) were harvested and reseeded at a density of 1 cell per well to generate secondary clones. Recloning of individual clones continued until cloning activity was exhausted.

3.3.10. Statistics

All quantitative results are presented as median ± IQR. Comparisons between two paired groups were performed in GraphPad version 6.01 using the non-parametric Wilcoxon signed rank test. To compare more than two paired groups, the non-parametric Friedman test with Dunn's post-hoc test was used. For the comparison of the percentage $ALDH^{br}$ cells in LAA and RAA of patients a paired parametric t-test was used. A value of $p<0.05$ was considered significant.

3.4. RESULTS

3.4.1. CASCs are predominantly present in the RAA

In order to optimize a clinically relevant harvest procedure and get some insights in the origin of the CASCs, their localization in the adult heart was determined. Within the same patient, the proportion of $ALDH^{br}$ cells was significantly higher in the RAA (5.1±4.4%) compared to the LAA (3.46±2.6%; $p<0.05$; n=33). To analyze the distribution of CASCs in other regions of the heart, the presence of $ALDH^{br}$ cells in different compartments was studied in adult pig hearts. As shown in table 3.4, $ALDH^{br}$ cells were predominantly present in LAA and RAA, corresponding to the data obtained from human atrial appendages. $ALDH^{br}$ cells were almost absent in the left ventricle and septum and could be found at low levels in the atria, the right ventricle and the apex. In general, $ALDH^{br}$ cells appeared to be more abundant in the right than in the left part of the heart, in both human and pig hearts.

Table 3.4: Percentages of $ALDH^{br}$ cells in different compartments of the pig heart.

	$ALDH^{br}$ cells (%)		
	Pig heart 1	Pig heart 2	Pig heart 3
LAA	4.4	4.9	3.5
RAA	10.5	5.6	8.7
Left atrium	0.4	1.5	1.5
Right atrium	2.8	1.5	3.9
Left ventricular free wall	0.5	0.4	0.2
Right ventricular free wall	1.1	1.9	1.9
Apex	3.1	1.6	0.2
Septum	0.3	0.4	0.2

LAA = left atrial appendage; RAA = right atrial appendage. Data are as individual datapoints. (n=3)

3.4.2. CASCs express early cardiac differentiation markers during expansion

To identify the cardiac differentiation stadium of human CASCs during expansion, a number of early and late stage cardiac specific markers were evaluated in $ALDH^{br}$ cells (Figure 3.1). For the pre-cardiac mesoderm markers, only Kinase insert domain receptor (*KDR*) was expressed in CASCs while Brachyury (*T*) was absent. Furthermore, the early cardiac transcription markers such as Gata binding protein

4 (*GATA4*), T-box 5 (*TBX5*), T-box 18 (*TBX18*) and NK2 homeobox 5 (*NKX2.5*) were expressed in human CASC cultures. In contrast, from the mature cardiac markers typically found in adult cardiomyocytes, only cardiac type troponin T2 (*TNNT2*) and myosin light chain 2 (*MYL2; MLC-2v*) were expressed in human CASC cultures. When comparing CASCs with adult atrial and ventricular heart muscle, a similar expression profile was detected, with exception of higher expression levels of *TBX18* and lower expression levels of *NKX2.5*, *TNNT2* and *MYL2*. In contrast to adult heart muscle, myosin light chain 7 (*MYL7; MLC-2a*) and hyperpolarization activated cyclic nucleotide gated potassium channel 4 (*HCN4*) were absent in CASCs. These data suggest that human CASCs display a cardiac progenitor-like phenotype rather than a mature cardiac phenotype during expansion.

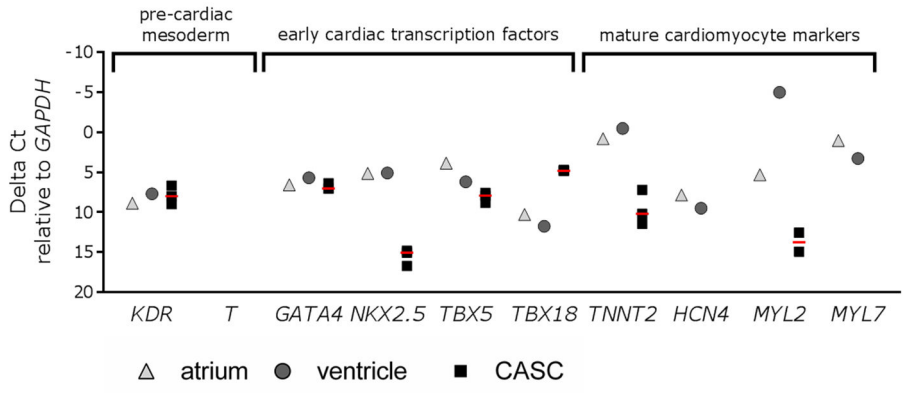

Figure 3.1: Early but no late cardiac differentiation markers are expressed in human CASCs during culture. mRNA expression of *KDR* and *T* (pre-cardiac mesoderm); *GATA4*, *NKX2.5*, *TBX5* and *TBX18* (early cardiac transcription factors); *TNNT2*, *HCN4*, *MYL2* and *MYL7* (mature cardiomyocyte markers). Individual data points with the red line representing the median for the CASC group. (n=1 for atrium and ventricle; n=3 individual patient cultures for CASCs)

3.4.3. Several FZD receptor subtypes are expressed in CASCs

As it would be beneficial for the clinical use of these cells to further promote CASC proliferation and differentiation, we investigated the role of Wnt signaling these processes. Since binding of the Wnt ligand to the FZD receptor is essential for the activation of the downstream Wnt/β-catenin pathway, we first analyzed the expression pattern of several FZD receptors in CASCs. As shown in figure 3.2, expression of *FZD1*, *2*, *4*, *6* and *7* was already detected after 25 cycli, indicating

high expression levels of these FZD subtypes. After 35 cycli, all subtypes, *FZD1* to *10,* were expressed in cultured CASCs (data not shown).

Figure 3.2: Several FZD receptors are expressed in human CASCs. Representative gel (left panel) and quantification (right panel) of *FZD1* to *FZD10* expression after 25 PCR cycles. Data are shown as median ± IQR. (n=5 individual patients' CASC cultures) au = arbitrary units

3.4.4. Wnt signaling can be modulated in CASCs by specific small-molecule activators and inhibitors

To test if the Wnt/β-catenin pathway could be modulated in CASCs, we investigated whether the levels of total and active β-catenin (dephosphorylated on Ser37 or Thr41) could be modified by CHIR99021 (small molecule Wnt activator) or C59, IWP2, XAV939 and IWR1-endo (small molecule Wnt inhibitors).

As shown in figure 3.3A, 6 μM CHIR99021 significantly increased the levels of total and active β-catenin up to respectively 2 fold and 5 fold in CASCs ($p<0.05$). 293T cells, used as a positive control, showed a 23 fold and 26 fold increase in respectively total and active β-catenin levels. As expected, CHIR99021 treatment did not upregulate total or active β-catenin levels in another control cell line, the SW480 cells, due to an adenomatous polyposis coli (APC) mutation which inhibits β-catenin ubiquitination (Yang et al. 2006). Finally, CHIR99021 treatment slightly but significantly reduced cell viability in both CASCs and control cell lines (Figure 3.3B).

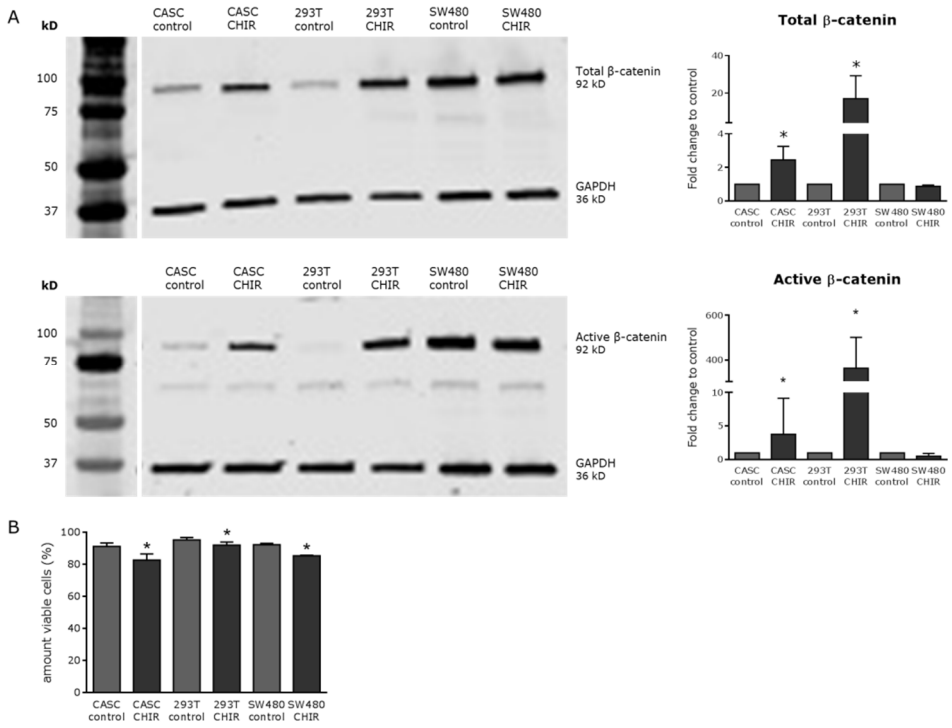

Figure 3.3: CHIR99021 is a potent Wnt activator in CASCs but slightly decreases its viability. (A) Representative western-blots (left panels) and subsequent quantification (right panels) of both total and active β-catenin after CHIR99021 treatment. (B) Cell viability of CASCs, 293T and SW480 cells treated with 6 μM CHIR99021. Data are shown as median ± IQR. (n=6 individual patients' CASC cultures/condition) *p<0.05 compared to respective control

To investigate whether Wnt/βcatenin signaling could be inhibited in CASCs, we applied several small-molecule inhibitors targeting different levels of the Wnt pathway. As shown in figure 3.4, 4 μM IWP2 or 1 μM C59, blocking Wnt ligand production and secretion, did not affect either total or active β-catenin levels, in both CASCs and SW480 cells. In contrast, treatment with 2 μM XAV939 or 4 μM IWR1-endo, stabilizing the APC/Axin/GSK-3β destruction complex of β-catenin, significantly reduced active β-catenin levels by 2 fold ($p<0.05$), indicating a potent inhibition of Wnt/β-catenin signaling in CASCs. Total β-catenin levels remained unchanged in CASCs (Figure 3.4A). In SW480 control cells total and active β-catenin levels decreased respectively 7 fold ($p=0.4016$) and 28 fold ($p<0.05$) after XAV939 treatment and respectively 8 fold and 62 fold ($p<0.05$) after IWR1-endo treatment (Figure 3.4B). Incubating the SW480 cells with a 5-fold higher

concentration of the small-molecule Wnt inhibitors (IWP2, C59, XAV939 and IWR1-endo) showed a similar effect (data not shown).

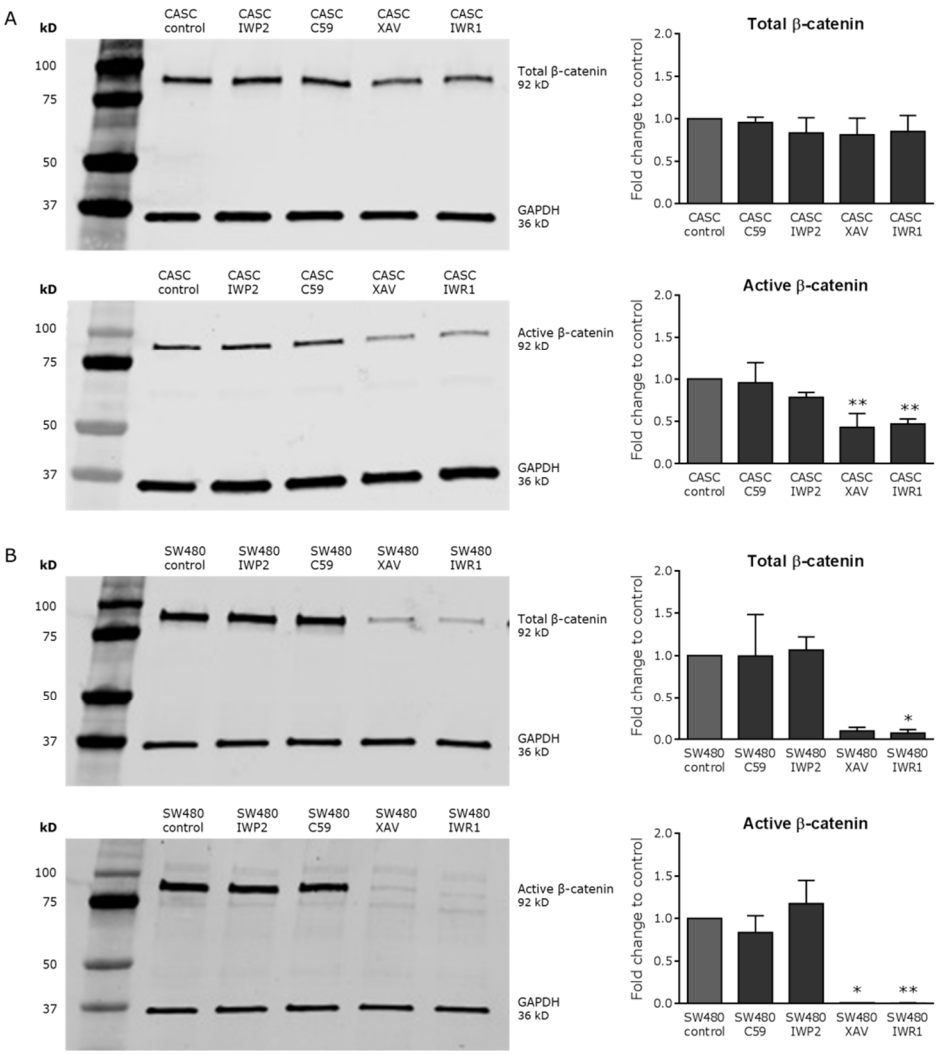

Figure 3.4: XAV939 and IWR1-endo decrease Wnt activity in CASCs. Representative western-blots (left panels) and subsequent quantification (right panels) of both total and active β-catenin after treatment with small molecule Wnt inhibitors in (A) CASCs and (B) SW480 cells. Data bars represent median ± IQR. (n=6 individual patients' CASC cultures/condition) *p<0.05 **p<0.01 compared to respective control

3.4.5. Modulating Wnt signaling does not influence neither CASC clonogenicity nor proliferation

Given that β-catenin levels could be modulated in CASCs, we investigated whether Wnt stimulation could influence clonogenicity and proliferation of CASCs in a serial cloning experiment. As shown in figure 3.5, CHIR99021 treatment had no effect on the number nor on the size of primary, secondary or tertiary clones suggesting that Wnt activation has little or no effect on CASC clonogenicity or proliferation.

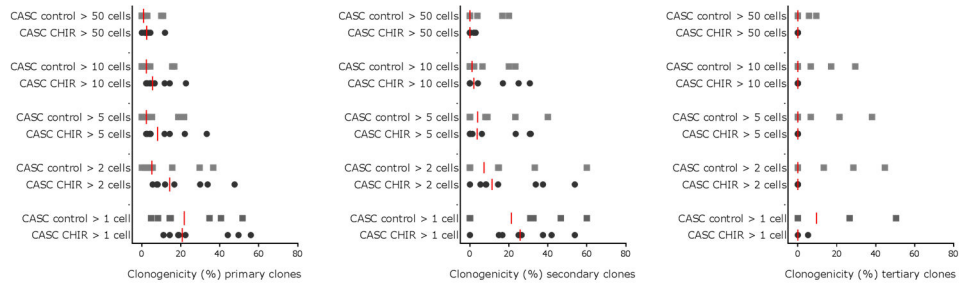

Figure 3.5: Wnt signaling does not influence neither CASC clonogenicity nor proliferation. The clonogenic potential of CASCs was studied by a serial cloning assay. Individual data points with the red line representing the median of each condition (n=8 individual patients' CASC cultures/condition)

To further analyze the lack of functional effect, we evaluated the expression levels of 3 proliferation associated Wnt target genes *cMYC*, *cJUN* and *Cyclin D1* (Figure 3.6). Indeed, activating Wnt signaling did not modify the mRNA levels of *Cyclin D1* and *cJUN* in CASCs. In 293T cells, CHIR99021 treatment induced a near 2 fold ($p<0.05$) increase in *Cyclin D1* and *cJUN* expression (Figure 3.6A and 3.6B left panels). When evaluating the effect of Wnt inhibition on proliferation associated genes, we found that none of the inhibitors tested changed the expression levels of *Cyclin D1* in CASCs (Figure 3.6A right panel). *cJUN* expression in CASCs was only decreased 1.3 fold ($p<0.05$) upon XAV939 treatment (Figure 3.6B right panel). In SW480 cells, there was a 1.5 fold decrease in *Cyclin D1* expression after XAV939 and IWR1-endo treatment (Figure 3.6B right panel). Furthermore, *cMYC* expression decreased 1.6 fold ($p<0.05$) in SW480 cells after IWR1-endo treatment (Figure 3.6C right panel). Interestingly, both Wnt stimulation and inhibition reduced *cMYC* levels in CASCs, respectively by 1.9 fold ($p<0.05$) after CHIR99021 treatment (Figure 3.6C left panel) and 1.5 fold ($p<0.05$) after XAV939 treatment (Figure 3.6C right panel). Our data indicate that Wnt inhibition or activation has

no or little effect on the expression of proliferation associated genes in CASCs, confirming the results of the serial cloning experiment.

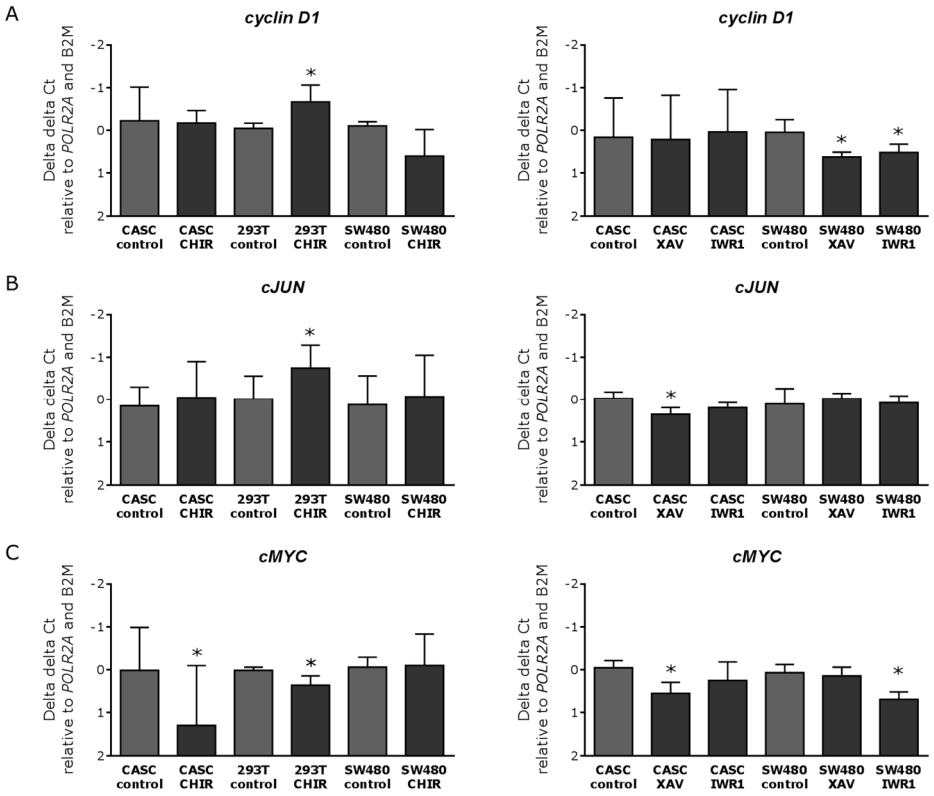

Figure 3.6: Wnt stimulation and inhibition in CASCs has no clear effect on the expression of proliferation associated genes. mRNA expression of the Wnt proliferation associated target genes (A) *Cyclin D1*, (B) *cJUN* and (C) *cMYC* after Wnt stimulation (left panels) and inhibition (right panels) in CASCs. Data are shown as median ± IQR (n=6 individual patients' CASC cultures/condition) *p<0.05 compared to respective control

3.4.6. Wnt inhibition in CASCs does not induce mature cardiomyocyte differentiation

Finally, a potential effect of Wnt inhibition on CASC differentiation was investigated. To this end, CASCs were treated with 2 µM XAV939 or 4 µM IWR1-endo which effectively inhibited canonical Wnt signaling as shown above. Subsequently, changes in mRNA expression of several cardiac differentiation markers (*GATA4*, *NKX2.5*, *TBX5*, *TBX18*, *TNNT2* and *MYL2*) were examined. As shown in figure 3.7, *GATA4* expression was 1.7 fold ($p<0.05$) increased after

XAV939 treatment. In addition, IWR1-endo significantly increased *TBX5* expression by 1.8 fold ($p<0.05$). However, treatment with IWR1-endo decreased the mature cardiomyocyte marker *TNNT2* by 2.9 fold ($p<0.05$). *NKX2.5*, *TBX18* and *MYL2* expression in CASCs was not affected by Wnt inhibition. These data demonstrate that Wnt inhibition in these conditions is insufficient to induce mature cardiomyocyte differentiation.

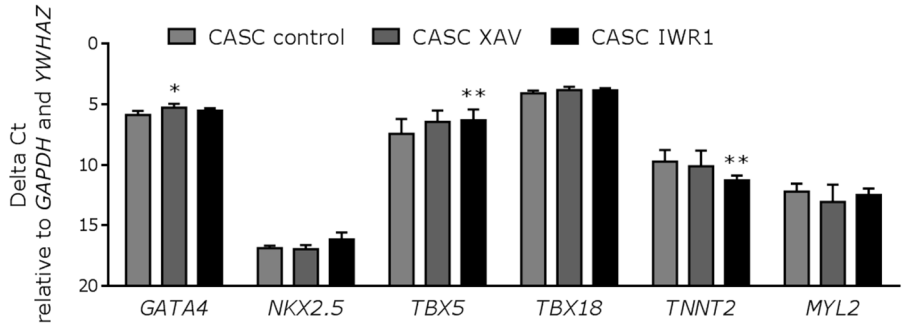

Figure 3.7: Wnt inhibition has limited effect on CASC differentiation. mRNA expression levels of the cardiac differentiation markers *GATA4*, *NKX2.5*, *TBX5*, *TBX18*, *TNNT2* and *MYL2* after treatment with XAV939 and IWR1-endo. Data are shown as median ± IQR. (n=6 individual patients' CASC cultures/condition) *p<0.05 **p<0.01 compared to respective control

3.5. DISCUSSION

In this study, we show that CASCs are predominantly present in atrial appendages and more in the right than in the left. Furthermore, studying the cardiac differentiation stadium of CASCs during expansion suggests that they are an intermediate already committed towards myocardial differentiation. Finally, despite the presence of canonical Wnt signaling and unlike other stem cell populations, modulating Wnt signaling has limited effect on CASC proliferation or differentiation.

In the last decade, a lot of effort has been put in to the development of cardiac regeneration therapies for heart failure patients. Although previous clinical studies indicated that BMC and CSC based therapies can be safely performed, functional outcomes are rather disappointing (Gyongyosi et al. 2015). Therefore, selecting the most appropriate stem cell type and understanding the mechanisms driving its proliferation and differentiation is fundamental. Previous studies already suggested CASCs as good candidates for cardiac regeneration therapies (Koninckx et al. 2013, Fanton et al. 2015). So far, CASCs have been routinely isolated from pig and human atrial appendages. However, whether this location is unique or whether they can also be found in other parts of the heart remained largely unknown up to now. In this study, we showed that CASCs are predominantly present in atrial appendages and more in the right than in the left, both in humans and in pigs. This precludes harvest via endomyocardial biopsies, but requires a surgical approach. However, the atrial appendages are relatively easy and safe to obtain via a mini-thoracotomy. Moreover, CASCs are more abundant in the atria rather than in the ventricles. Similar findings for $ALDH^{br}$ cells in 8-week old mouse hearts were obtained by Roehrich *et al.* (Roehrich et al. 2013).

When studying the cardiac differentiation stadium of CASCs during expansion, qPCR analysis demonstrated expression of *KDR*, *NKX2.5*, *GATA4*, *TBX5*, *TBX18*, *TNNT2* and *MYL2*, but not of *T*, *MYL7* and *HCN4*. *KDR* and *T* are both markers for early mesoderm and show similar expression levels for CASCs and adult heart. *TBX18* expression might confirm a possible pro-epicardial origin of CASCs (Cai et al. 2008). However, the early cardiac-specific transcription factors *NKX2.5, GATA4,* known to be expressed in first and second heart field (FHF and SHF) progenitors, are also expressed in CASCs. Recently, early expression of *HCN4* was

also reported as a marker for FHF progenitors (Liang et al. 2013). However, this marker was not expressed in CASCs, while another FHF marker *TBX5* was (Takeuchi et al. 2003). These results suggest a possible heterogeneous embryonic origin of CASCs, as also suspected for other CSC types (Santini et al. 2016). However, elaborated transcriptomics studies or lineage tracing experiments are needed to further unravel the precise origin and nature of CASCs. Although, the expression levels of the different markers were very similar amongst individual patient's CASC cultures, a broader screening in a larger patient population is necessary to identify inter-patient differences and whether this can be linked to differences in CASC proliferation, integration and differentiation, but also to differences in functional outcomes. To further investigate the heterogenic character of CASCs also single cell analyses, such as single cell sequencing, might be useful to reveal cellular differences within a patient-specific CASC population.

The expression of *NKX2.5*, although lower than in atrium and ventricle, might suggest a limited endothelial differentiation potential of CASCs. Wu *et al.* demonstrated that *NKX2.5* expression comprises endothelial differentiation in cardiac-specific *NKX2.5* positive cells from the developing mouse embryo (Wu et al. 2006). This was confirmed by the limited endothelial cell differentiation of less than 1% in the Göttingen minipig model for acute MI (Fanton et al. 2015). Despite this limited endothelial differentiation capacity, we recently demonstrated that CASCs stimulate angiogenesis via paracrine mechanisms through the secretion of numerous growth factors that promote important steps of new blood vessel formation (Fanton et al. 2016). The expression of *TNNT2* and *MYL2* in CASCs suggests that CASCs can be considered as an intermediate already committed towards myocardial differentiation. The profound myocardial differentiation potential is already confirmed both *in vitro* and *in vivo*. Indeed, co-culturing CASCs with neonatal rat cardiomyocytes induced sarcomeric organization of cTnT and cTnI while transplanting CASCs in the left ventricle of a Göttingen minipig model for acute MI induced sarcomere formation and protein expression of cTnT, cTnI, connexin 43 (CX43) and MLC-2v (Koninckx et al. 2013, Fanton et al. 2015).
Although CASCs can efficiently differentiate into cardiomyocytes, we observe a high variability between patients with respect to their proliferation potential. In addition, it is known that myocardial differentiation is only obtained when CASCs are in close contact with functional cardiomyocytes (Koninckx et al. 2013, Fanton

et al. 2015, Windmolders et al. 2015). Therefore, more insight in the molecular mechanisms involved in CASC proliferation and differentiation might be useful to amplify the proliferation potential of CASCs and to understand the essential cues that drive CASCs towards myocardial differentiation. Several factors, such as Wnt, FGF, BMPs, Notch, and Hedgehog are known to influence cardiac progenitor cell proliferation and differentiation (Freire et al. 2014). In this study, we focused on the role of canonical Wnt signaling in adult cardiac progenitor cells, more specifically its effect on CASC proliferation and differentiation. First, we show that CASCs express all ten FZD receptors, with highest expression of *FZD1*, *2*, *4*, *6* and *7*. According to human gene database GeneCards, these specific FZD receptors are also known to be expressed in adult heart. Furthermore, CHIR99021 treatment upregulated total and active β-catenin, while XAV939 and IWR1-endo reduced active β-catenin expression. However, CHIR99021 treatment resulted in a slight but significant decrease in cell viability. This is most likely caused by a toxic effect of the product and not the result of Wnt stimulation, since cell toxicity was also observed in SW480 cells where Wnt signaling was not further upregulated. This cytotoxic effect of CHIR99021 was also observed in mouse embryonic stem cells (Naujok et al. 2014). Since CHIR99021 treatment did not improve the number nor the size of the formed clones, Wnt activation had no or limited effect on CASC clonogenicity or proliferation. Furthermore, no upregulation in the proliferation associated genes *Cyclin D1*, *cMYC* and *cJUN* was detected in CASCs after Wnt activation. Instead, we observed a decrease in *cMYC* expression levels, both in CASCs and 293T cells. However, a similar decrease in *cMYC* expression was also seen after inhibiting canonical Wnt signaling in CASCs and SW480 cells. The reason why both Wnt upregulation and downregulation induce a decrease in *cMYC* expression is unknown, but a negative effect of *cMYC* on heart function by inducing cardiomyocyte hypertrophy has already been described (Xiao et al. 2001). Furthermore, Wnt activation seems to differently affect cell proliferation, depending on the CSC type (Qyang et al. 2007, Oikonomopoulos et al. 2011). Indeed, no effect of Wnt activation was observed on CASC proliferation, while it is known to enhance embryonic and postnatal Isl1 positive cardiovascular progenitor cell proliferation (Qyang et al. 2007) but decreases adult cardiac side population cell proliferation (Oikonomopoulos et al. 2011).

Finally, the effect of Wnt inhibition on CASC differentiation was examined. Current differentiation protocols for PSCs use a sequential incubation with small molecule Wnt activators and inhibitors to drive cardiomyocyte differentiation (Lian et al. 2013). In CASCs, Wnt inhibition only upregulated the early cardiac differentiation markers *GATA4* and *TBX5*, while downregulating the mature cardiac differentiation marker *TNNT2*. The upregulation of *GATA4* after Wnt inhibition is in line with the findings of Afouda *et al*. They showed that canonical Wnt signaling inhibits *GATA4* expression and that this restricts cardiogenesis in *Xenopus* animal cap and cardiac mesoderm explant assays (Afouda et al. 2008). A study of Zelarayan *et al.* also showed an increase in *GATA4* and *TBX5* expression after β-catenin depletion in mouse Sca-1 positive cardiac progenitor cells. However, this was also accompanied by an increase in the number cTnT positive cells, which was not the case in our study (Zelarayan et al. 2008). This difference is likely to be species and cell type related. Furthermore, Foley and Mercola showed that inhibition of the canonical Wnt pathway induces the expression the early cardiac differentiation genes *TBX5* and *NkX2.5*, but not the mature myocardial differentiation markers cTnI and myosin heavy chain (MHC)α (Foley and Mercola 2005). Finally, Burridge *et al.* also showed a decrease in cTnT positive cells after treatment with the Wnt inhibitor XAV939 during the early mesoderm differentiation of PSCs (Burridge et al. 2014). These findings suggest that Wnt inhibition upregulates the early cardiac markers but hinders differentiation towards mature cardiomyocytes.

In conclusion, these results indicate that CASCs are predominantly present in the atrial appendages, are committed towards myocardial differentiation and express several FZD receptors. However, despite the crucial role of Wnt signaling in cardiogenesis and differentiation of PSCs towards cardiac lineages, Wnt signaling is functional but has limited effects on CASCs. Further research is therefore necessary to identify the molecular mechanisms that are involved in CASC proliferation and differentiation. Furthermore, the role of Wnt signaling in CASCs remains to be elucidated. Once these questions are answered, it will become clear whether Wnt signaling is essential for cardiac regeneration or whether other pathways are responsible for the regenerating potential of CASCs.

Chapter 4

Mesenchymal stem cells protect cardiac atrial appendage stem cells against hypoxia induced cell death

Based on:

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(in preparation) **Mesenchymal stem cells protect cardiac atrial appendage stem cells against hypoxia induced cell death.**

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4.1 ABSTRACT

In the western world, cardiovascular disease remains the leading cause of morbidity and mortality. Despite the promising potential of CSCs, cardiac regeneration is still impaired by the detrimental microenvironment of the infarct area which affects stem cell survival and integration. Many positive paracrine effects of MSCs on cardiac repair have already been described, including mechanisms such as neovascularization, cytoprotection, and resident CSC activation. However, their direct protective effect on CSC survival has not yet been evaluated. Here, the cytoprotective effect of CM-MSCs on CASCs is investigated under both anoxic and hypoxic conditions.

Our data revealed that the declined CASC viability associated with hypoxia but not anoxia could be partly recovered by CM-MSC treatment. The observed increase in CASC survival was also accompanied by increased CASC proliferation as shown by an increased number of Ki67 positive cells cultured in CM-MSC under hypoxic conditions. This paracrine effect was however not mediated via VEGF or PDGF and CM-MSC protection of CASCs against hypoxia-induced cell death occurred in an Akt-independent manner. In contrast, CM-MSC treatment of CASCs upregulated catalase expression levels under hypoxic conditions.

In conclusion, our study revealed that MSCs can improve the survival of CASCs under hypoxic conditions most likely by protecting them against oxidative stress via the upregulation of catalase activity. These data suggest that CASC-based therapies can be improved by incorporating paracrine mediators from MSCs, although further research is necessary to fully exploit the potential.

4.2 INTRODUCTION

Cardiovascular disease, including MI, remains the leading cause of morbidity and mortality worldwide (WHO, Fact sheet cardiovascular disease). MI is characterized by cell death due to prolonged ischemia. The resulting damage is the result of both ischemia, the initial loss of blood flow, and reperfusion, the restoration of oxygenated blood flow. Although the heart can tolerate brief exposure to ischemia, persisted oxygen deprivation results in irreversible injury and cell death. Even though on long term, reperfusion is essential to ensure cardiac muscle survival, it leads to increased oxidative stress and massive ROS production that will directly injure the cell membrane and cause cell death. Furthermore, ROS also contribute to remodeling processes, such as hypertrophy and ECM reconfiguration, which will contribute to heart failure (Misra et al. 2009).

Despite the promising potential of CSCs, cardiac regeneration is still impaired by the detrimental microenvironment of the infarct area which affects stem cell survival and integration (Segers and Lee 2008). Apart from CSCs, MSCs are also extensively studied for cardiac repair. However, myocardial differentiation of MSCs is questioned and their beneficial effect on cardiac repair is mainly the result of the secretion of paracrine factors. Indeed, many positive paracrine effects of MSCs on cardiac repair have already been described, including reducing inflammation and fibrosis, promoting angiogenesis and preserving cardiac tissue (Gnecchi et al. 2008). Moreover, MSCs are able to stimulate endogenous cardiac repair by increasing CSC survival, integration, proliferation, migration and differentiation via paracrine mechanisms (Gallina et al. 2015).

CASCs are isolated from atrial appendages based on elevated ALDH activity. These cells can be *ex-vivo* expanded to clinically relevant cell numbers while preserving their antigenic expression profile and myocardial differentiation potential (Windmolders et al. 2015). Furthermore, CASCs are able to stimulate angiogenesis (Fanton et al. 2016) and intramyocardial autologous transplantation in a Göttingen minipig model of acute MI preserves cardiac function (Fanton et al. 2015). However, despite high cell retention compared to other stem cell types, CASC survival may still be impaired by the restricted oxygen supply of the infarct area. We hypothesize that MSCs could offer protection by improving CASC survival

under oxygen-deprived conditions. Therefore, the cytoprotective effect of CM-MSC is investigated on CASCs under both anoxic and hypoxic conditions.

4.3 MATERIAL AND METHODS

All procedures for the collection of human body material were performed conform the principles set forth in the Helsinki Declaration. Approval by the Medical Ethical Committee of the Jessa hospital and informed consent from each patient were obtained.

4.3.1 Cell culture

Cardiac atrial appendage stem cell isolation and culture

CASCs were isolated from atrial appendages and subsequently expanded as previously described by Koninckx *et al.* (Koninckx et al. 2013). In brief, tissue samples were minced and enzymatically dissociated with collagenase II (600 U/ml; Invitrogen). Single cells were subsequently stained with Aldefluor (Stem cell technologies) according to manufacturer's protocol. After 45 minutes $ALDH^{br}$ CASCs were flow sorted using a FACSAria (Beckton&Dickinson) and subsequently cultured in X-vivo 15 medium (Lonza) supplemented with 2% PS (Lonza) and 10% FBS (GE Healthcare HyClone) at 37°C in humidified atmosphere containing 5% CO_2.

Mesenchymal stem cell isolation and culture

MSC isolation, characterization and CM-MSC preparation was performed as described by Windmolders *et al.* (Windmolders et al. 2014). In brief, 20 ml bone marrow was aspirated at the level of the sternum after which the mononuclear cell fraction was separated via density-gradient centrifugation with Lymphoprep (Axis Shield). The isolated cells were rinsed and seeded at a density of $2x10^5$ cells/cm² in X-vivo 15 medium supplemented with 2% PS and 10% FBS. After 24 and 72 hours and subsequently every three days, medium was refreshed. After 14 days, cells were reseeded at a density of $6x10^3$ cells/cm² and subsequently cultured at 37°C in humidified atmosphere containing 5% CO_2.

4.3.2 CM-MSC preparation

Before CM-MSC preparation, MSC quality was confirmed by a phenotypical characterization. As described by Koninckx *et al.*, only cells positive for CD105, CD73, CD49c and CD90 and negative for CD45, CD34, CD184 and CD106 were

processed further (Koninckx et al. 2009). All antibodies (Table 4.1) were purchased from BD biosciences with the exception of CD105 (AbD Serotec) and at least $5x10^3$ cells were analyzed on a FACSAria (Beckton&Dickinson). FMO combined with isotype control was performed for correct gating and to identify any non-specific staining.

Table 4.1: Antibodies used for immunophenotyping expanded MSCs.

	FITC	PE	PERCP-Cy5.5	PE-Cy7	APC
Tube 1	CD105	CD73	CD45	CD34	CD184
Tube 2	CD106	CD49c	CD45	CD34	CD90

FITC = fluorescein isothiocyanate; PE = phycoerythrin; PERCP-Cy5.5 = peridinyl chlorophylline-Cy5.5; PE-Cy7 = phycoerythrin-Cy7; APC = allophycocyanine

CM-MSC was collected from passages 4 to 8 when 85-90% confluency was reached as previously described by Windmolders *et al.* (Windmolders et al. 2014). In brief, cells were washed and subsequently incubated with serum-free low glucose DMEM supplemented with 2% PS for 48 hours at 37°C. Cellular particles and debris were removed from harvested CM-MSC via a 0.2 µm filter (VWR). Next, the collected CM-MSC was concentrated by centrifugation at 3,600 g and 4°C during 1 hour using 3 kDa Amicon Ultra-15 centiprep tubes YM-10 (Merck Millipore). Serum-free low glucose DMEM was added to obtain 10x concentrates. Finally, 10x CM-MSC concentrates were sterile filtered, aliquoted and stored at -80°C until use.

4.3.3 Stimulated in vitro ischemic CASC culture

CASCs were seeded at a density of $5x10^3$ cells/cm^2 in X-vivo 15 medium supplemented with 2% PS and 10% FBS. The next day, the culture medium was replaced by CM-MSC, serum-free low glucose DMEM supplemented with 2% PS (control medium) or recombinant VEGF or PDGF-AA (R&D systems) dissolved in control medium at a concentration of 100 ng/ml. Alternatively, platelet derived growth factor receptor α (PDGFRα) was inhibited by adding neutralizing antibody (20 ng/ml; R&D systems) to CM-MSC. To mimic the MI-associated microenvironment, CASCs were incubated in an air-thigh chamber with an anoxic (0.2% O_2) or hypoxic (5% O_2) atmosphere created by AnoxomatTM (MART Microbiology) at 37°C during one, three or seven days.

4.3.4 Viability testing

CASC viability was measured with the FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen) according to manufacturer's protocol. Early apoptotic (EA) cells were defined as Annexin V positive and PI negative, while cells that stained positive for both dyes were considered as late apoptotic (LA) cells. Viable cells were negative for both Annexin V and PI. The percentage of viable, EA or LA cells was measured by a FACSAria and expressed as the percentage of respectively Annexin V and PI negative cells, Annexin V positive and PI negative cells or Annexin V and PI positive cells of total cells.

4.3.5 Proliferation assessment by Ki67 staining

After three days of hypoxic culture conditions, CASCs were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature. CASC proliferation was measured by an immunostaining for the proliferation marker Ki67 with 4',6-diaminidino-2-phenylindole (DAPI) counterstain as previously described by Fanton *et al.* (Fanton et al. 2016). In brief, cells were washed and permeabilized with 0.3% triton X-100 (Sigma Aldrich) and blocked with 10% goat serum (Abcam). Afterwards, cells were incubated overnight with rabbit monoclonal Ki67 ready-to-use antibody solution (1:2; Thermo Fisher Scientific; RM-9106) at 4°C followed by a 45 minutes incubation with goat anti-rabbit Alexa Fluor (1:500; Thermo Fisher Scientific; A11012). The percentage proliferating cells was expressed as the ratio of Ki67 positive cells to the total cell amount. Cells were counted blindly in 15 random microscopic fields.

4.3.6 Detection of apoptosis-related proteins after hypoxia

A human apoptosis array kit (ARY009; R&D systems) was used to detect the expression of 35 apoptosis-related proteins (listed in table 4.2) in CASCs cultured under hypoxic conditions. Therefore, CASCs were cultured in CM-MSC or control medium under hypoxic conditions for three days. Cells were lysed with the supplied lysis buffer supplemented with Halt Protease inhibitor cocktail (1X; Life Technology). Signal detection was performed using the LI-COR Odyssey Infrared Imaging System in combination with IRDye 800CW streptavidin (1:2000; LI-COR,

926-32230) and quantification was performed with LI-COR Odyssey Image Studio analyzer software 2.1 (Li-COR).

Table 4.2: List of apoptosis-related proteins detected with the apoptosis array kit.

Bad	TRAIL R1/DR4	PON2
Bax	TRAIL R2/DR5	p21/CIP1/CDNK1A
Bcl-2	FADD	p27/Kip1
Bcl-x	Fas/TNFSF6	Phospho-p53 (S15)
Pro-Caspase-3	HIF-1 alpha	Phospho-p53 (S46)
Cleaved Caspase-3	HO-1/HMOX1/HSP32	Phospho-p53 (S392)
Catalase	HO-2/HMOX2	Phospho-Rad17 (S635)
cIAP-1	HSP27	SMAC/Diablo
cIAP-2	HSP60	Survivin
Claspin	HSP70	TNF RI/TNFRSF1A
Clusterin	HTRA2/Omi	XIAP
Cytochrome c	Livin	

4.3.7 Western blot analysis

Lysates were prepared from CASCs cultured under hypoxic conditions for three days in control medium or CM-MSC. Cells were lysed with NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl, 137 mM NaCl, 10% Glycerol, 2 mM EDTA) supplemented with Halt Protease inhibitor cocktail (1X; Life Technology) and 1 mM sodium orthovanadate. Equal amounts of protein were electrophoresed on 4-15% Mini-PROTEAN TGX gels (Bio-Rad) and transferred to 0.2 µm nitrocellulose membranes using the Trans-Blot Turbo Transfer System. Blots were blocked in Odyssey blocking buffer (LI-COR) for 1 hour and subsequently probed with primary and secondary antibodies for respectively 2 hours and 45 minutes. The following primary antibodies were used: rabbit polyclonal anti-Akt (1:1000; Cell Signaling Technology; 9272), rabbit monoclonal anti-pAkt (1:2000; Cell Signaling Technology; 4060), rabbit polyclonal anti-Bad (1:1000; Cell Signaling Technology; 9292), rabbit monoclonal anti-pBad (1:1000; Cell Signaling Technology; 4366), mouse monoclonal anti-catalase (1:1000; R&D systems; MAB3398), mouse monoclonal anti-cleaved caspase 3/pro-caspase-3 (1:500; R&D systems; MAB707). Secondary antibodies utilized were: goat anti-mouse

IRDye® 800 CW (1:15000; LI-COR; 926-32210) and goat anti-rabbit IRDye® 680RD (1:15000; LI-COR; 926-68071). Membranes were scanned with the Odyssey Infrared Imaging System and quantified using the LI-COR Odyssey Imaging software 2.1. Relative protein expression was normalized to the loading control GAPDH and compared with their respective control condition.

4.3.8 Statistics

All quantitative results are presented as median ± IQR. Statistical analysis was performed with GraphPad version 6.01. For comparisons between two paired groups the non-parametric Wilcoxon signed rank test was used. For the comparison between anoxic and hypoxic conditions the non-parametric Mann Whitney test was used, since these observations were not paired. To compare more than two paired groups the non-parametric Friedman test with Dunn's post-hoc test was used.

4.4 RESULTS

4.4.1 CM-MSC protect CASCs under hypoxic but not under anoxic conditions

The paracrine effect of MSCs on CASC survival in the detrimental ischemic microenvironment was studied by demining the effect of CM-MSC on CASC viability under anoxic (0.2% O_2) and hypoxic (5% O_2) conditions. The optimal time point to study hypoxia- and anoxia-induced cell death of CASCs in control medium was first determined. As shown in figures 4.1A and 4.1B, cell death was limited after one day of anoxia or hypoxia. After seven days of anoxia, distinction between the different cell fractions was difficult and after seven days of hypoxia, cell survival was relatively low. Therefore, three days of anoxia or hypoxia was selected as an optimal time point for subsequent experiments. Subsequently, CASCs were cultured under anoxic or hypoxic conditions for three days with and without CM-MSC. Figure 4.1C shows that there was no protective effect of CM-MSC on CASC viability after three days of anoxia. In contrast, CM-MSC treatment significantly increased CASC viability from 41.8±21.1% (control) to 65.6±21.2% (CM-MSC) under hypoxic conditions. Accordingly, the percentage early and late apoptotic cells decreased from respectively 23.0±11.5% to 13.4±11.5% and from 29.0±17.4% to 15.4±10.9% after CM-MSC treatment (Figure 4.1D). Remarkably, cell death was higher after three days of hypoxia compared to anoxic conditions. CASC viability under normoxic conditions was 75.9±17.1% (n=9 individual patients' CASC cultures). Since, CM-MSC only protects CASCs under hypoxic but not under anoxic conditions, only hypoxic conditions were used for further investigation.

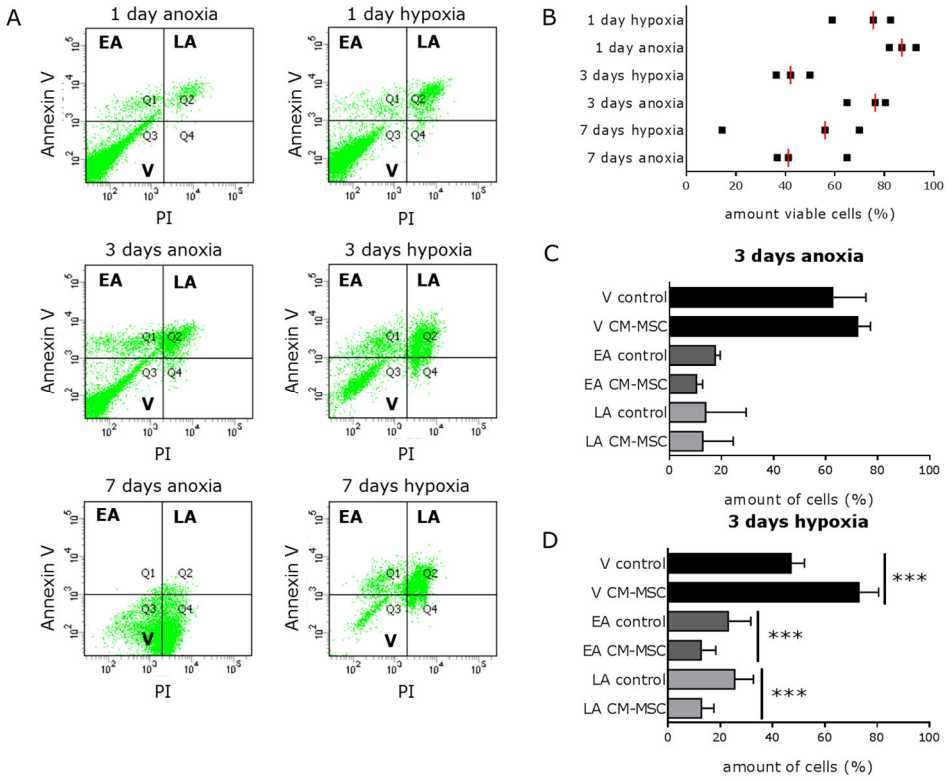

Figure 4.1: CM-MSC promotes CASC survival under hypoxic but not anoxic conditions. (A) Representative plots of CASC viability testing via FACS analysis and (B) percentage of viable cells after one, three or seven days of anoxia or hypoxia in control medium. Three days of hypoxia or anoxia was chosen as optimal time point for further testing. Individual data points with the red line representing the median of each condition (n=3 individual patients' CASC cultures/condition). (C) The percentage of viable (V), early apoptotic (EA) and late apoptotic (LA) CASCs was not influenced by CM-MSC treatment after three days anoxia (n=9 individual patients' CASC cultures/condition). (D) In contrast, CM-MSC treatment significantly increased the percentage of viable (V) CASCs and significantly decreased the percentage of early (EA) and late apoptotic (LA) CASCs under hypoxic conditions. PI = propidium iodide. Data bars represent median ± IQR. (n=17 individual patients' CASC cultures/condition) ****p<0.0001

4.4.2 CM-MSC stimulated CASC proliferation under hypoxic conditions

In addition to their cytoprotective properties, the effect of CM-MSC on CASC proliferation during hypoxia was studied by a Ki67 immunostaining. The fraction of proliferating CASCs, positive for the marker Ki67, was significantly higher in CM-MSC (7.1±9.6%) compared to control conditions (3.0±2.0%) after three days of hypoxia (Figure 4.2; $p<0.05$).

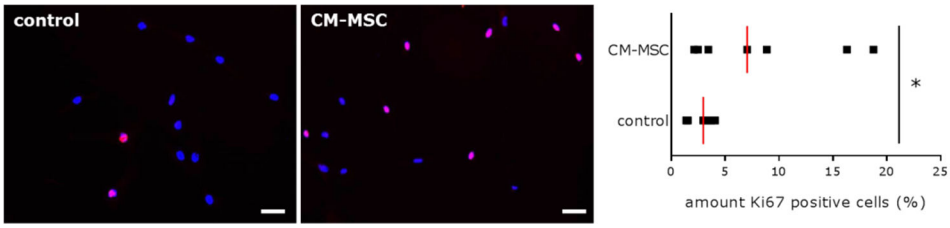

Figure 4.2: CM-MSC stimulates CASC proliferation under hypoxic conditions. Representative images of the Ki67 immunostaining (red nuclei) after three days. Nuclei were stained with 4',6-diaminidino-2-phenylindole (DAPI; blue). Quantification of the percentage Ki67 positive cells after three days of hypoxia revealed that CASC proliferation was significantly higher after CM-MSC treatment compared to control conditions. Scale bar corresponds with 50 µm. Individual data points with the red line representing the median of each condition (n=7 individual patients' CASC cultures/condition) *p<0.05

4.4.3 VEGF and PDGF or the Akt-pathway are not responsible for CASC protection under hypoxic conditions

It has been previously described that VEGF and PDGF are secreted by MSCs (De Boeck et al. 2013) and have a protective effect on the survival of endothelial and neural cells (Kilic et al. 2006, Lennartsson et al. 2010), so we evaluated their effect on CASC survival under hypoxic conditions. Adding recombinant PDGF or VEGF to the control medium had no effect on CASC survival after three days of hypoxia (Figure 4.3A). Also, inhibiting their common receptor, the PDGFRα, using a neutralizing antibody did not reduce the protective effect of CM-MSC on CASCs (Figure 4.3B). This suggests that these factors do not contribute to the protective effect of CM-MSC on CASC survival under hypoxic conditions

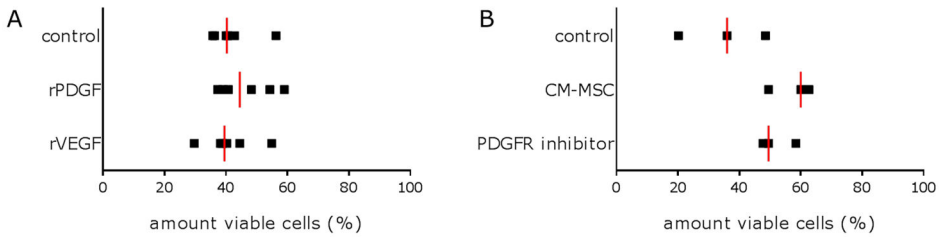

Figure 4.3: VEGF and PDGF are not responsible for CASC survival under hypoxic conditions. (A) Supplementing control medium with recombinant VEGF (100 ng/ml) or PDGF (100 ng/ml) did not alter CASC viability after three days of hypoxia. (n=6 individual patients' CASC cultures/condition) (B) Furthermore, adding inhibitory PDGFRα antibodies (20 ng/ml) to the CM-MSC did not decrease CASC survival after three days of hypoxia compared to the CM-MSC condition. Individual data points with the red line representing the median of each condition. (n=3 individual patients' CASC cultures/condition)

Subsequently, the molecular mechanisms underlying the observed cytoprotective effect of CM-MSC under hypoxic conditions were investigated. Since activation of the PI3K/Akt has been described to be responsible for the cytoprotective effects of MSCs, we investigated whether this is also the case for CASCs cultured in CM-MSC under hypoxic conditions. However, no differences in both Akt and pAkt expression could be detected between CM-MSC and control conditions after three days of hypoxia (Figure 4.4). In addition, the downstream pro-apoptotic protein Bad and its inactive phosphorylated form could not be detected after three days of hypoxia in both conditions (n=4 individual patients' CASC cultures/condition; data not shown).

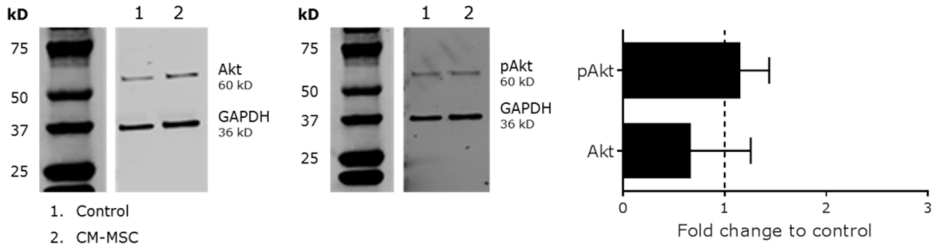

Figure 4.4: The Akt-pathway is not involved in the protective effect of CM-MSC under hypoxia. Representative western-blot analysis of Akt and pAkt. GAPDH served as loading control. Quantification revealed no altered Akt or pAkt expression levels after CM-MSC treatment. Data bars represent median ± IQR. (n=8 individual patients' CASC cultures)

4.4.4 Catalase is a promising mediator for CASC survival under hypoxic conditions

To further identify the underlying signaling pathways responsible for the protective effect of CM-MSC on CASC survival under hypoxic conditions, a global screening for 35 apoptosis-related factors was performed. After three days of hypoxia, only catalase and pro-caspase-3 were respectively 2 fold and 4 fold upregulated in CASCs cultured in CM-MSC compared to CASCs cultured in control medium (Figure 4.5A and Table 4.3). However, the upregulation of pro-caspase-3 could not be confirmed by western blot analysis (Figure 4.5B).

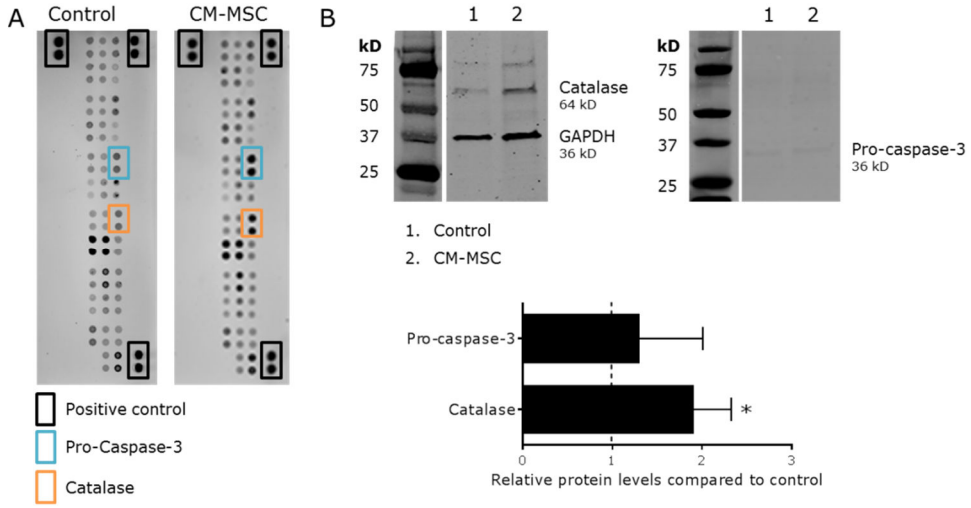

Figure 4.5: Catalase might mediate the protective effect of CM-MSC on CASC survival under hypoxia. (A) Representative blot showing the expression levels of 35 apoptosis-related proteins in control and CM-MSC conditions after three days of hypoxia. In the presence of CM-MSC, pro-caspase-3 and catalase expression is upregulated (n=2 individual patients' CASC cultures). (B) Representative western-blot analysis of catalase and pro-caspase-3. GAPDH served as loading control. Quantification revealed increased relative catalase expression levels after CM-MSC treatment, but no change in pro-caspase-3 levels. Data bars represent median ± IQR. (n=8 individual patients' CASC cultures) *p<0.05 compared to control

Table 4.3: Expression levels (au) and ratios of 35-apoptosis related factors.

	control	CM-MSC	CM-MSC/control
Bad	1.75±0.19	2.48±0.44	1.39±0.10
Bax	2.11±1.56	0.59±0.03	0.63±0.48
Bcl-2	2.06±0.18	2.36±0.04	1.15±0.12
Bcl-x	0.50±0.06	0.48±0.05	0.99±0.22
Pro-caspase-3	4.54±2.45	13.22±2.21	3.71±1.51
Cleaved Caspase -3	2.18±0.21	1.76±0.49	0.79±0.15
Catalase	2.44±0.54	5.09±0.03	2.19±0.50
cIAP-1	1.21±0.01	1.50±0.09	1.23±0.06
cIAP-2	1.33±0.01	1.33±0.04	0.99±0.03
Claspin	1.12±0.05	1.08±0.07	0.97±0.11
Clusterin	1.09±0.01	1.17±0.18	1.06±0.15
Cytochrome c	3.53±0.38	3.60±0.60	1.04±0.28
TRAIL R1/DR4	1.06±0.05	1.19±0.07	1.12±0.02
TRAIL R2/DR5	1.19±0.08	1.78±0.06	1.50±0.15
FADD	1.09±0.13	1.48±0.11	1.35±0.06
Fas/TNRSF6/CD95	1.24±0.20	1.90±0.02	1.57±0.27

HIF-1α	1.03±0.11	1.51±0.02	1.48±0.18
HO-1/HMOX1/HSP32	1.45±0.30	2.02±0.34	1.39±0.06
HO-2/HMOX2	1.04±0.06	1.50±0.05	1.44±0.13
HSP27	5.89±0.75	7.63±0.13	1.31±0.19
HSP60	2.72±0.06	3.13±0.21	1.15±0.10
HSP70	1.54±0.22	2.13±0.34	1.37±0.03
HTRA2/Omi	1.16±0.07	1.36±0.10	1.17±0.16
Livin	1.27±0.08	1.30±0.14	1.03±0.17
PON2	1.49±0.04	1.74±0.09	1.16±0.02
p21/CIP1/CDKN1A	1.22±0.08	1.78±0.50	1.43±0.31
p27/Kip1	1.10±0.01	1.09±0.07	0.99±0.08
Phospho-p53 (S15)	1.87±0.27	2.16±0.08	1.18±0.21
Phospho-p53 (S46)	1.24±0.02	1.41±0.02	1.13±0.03
Phospho-p53 (S392)	0.54±0.02	0.59±0.03	1.09±0.10
Phospho-p53 (S635)	1.08±0.01	1.34±0.12	1.23±0.11
SMAC/Diablo	5.54±0.17	6.72±0.14	1.21±0.01
Survivin	1.23±0.05	1.40±0.15	1.14±0.17
TNF RI/TNFRSF1A	0.85±0.02	1.05±0.08	1.22±0.07
XIAP	1.96±0.11	2.15±0.28	1.08±0.08

CM-MSC = conditioned medium of mesenchymal stem cells; au = arbitrary units Data are shown as median ± IQR. (n=2 individual patients' CASC cultures/condition)

4.5 DISCUSSION

CSC therapy offers great promise for cardiac regeneration and functional recovery after an MI. However, successful cardiac regeneration depends on the survival, integration and differentiation of the transplanted cells in the recipient's heart. Since the microenvironment of the infarct area is characterized by ischemia, inflammation and fibrosis, the survival of transplanted cells will most likely be negatively affected by the low oxygen levels in the targeted area. Therefore, the aim of this study was to improve CSC survival under oxygen-deprived conditions.

In the current study, the declined CASC viability associated with hypoxia could be partly recovered by CM-MSC treatment. These findings are in line with previous studies demonstrating positive effects of MSCs on the survival of cardiomyocytes and neonatal cardiac progenitor cells during oxygen deprivation. The study of Jin *et al.* demonstrated an increased rat cardiac myocyte survival when treated with conditioned medium of human umbilical cord mononuclear cells under hypoxic conditions (1% O_2). This was mediated by the upregulation of pAkt and Bcl-2 (Jin et al. 2013). Furthermore, treating neonatal rat cardiac progenitor cells with rat CM-MSC also improved cell survival under hypoxic conditions (1% O_2) (Nakanishi et al. 2008). The observed increase in CASC survival was also accompanied by an increase in CASC proliferation as shown by an increase in the number of Ki67 positive cells cultured in CM-MSC under hypoxic conditions.

Under anoxic conditions, CM-MSC treatment did not increase CASC survival. Remarkably, anoxia resulted only in limited cell death compared to hypoxia. According to Krijnen *et al.*, anoxia results in necrosis while hypoxia induces apoptosis through the mitochondrial pathway (Krijnen et al. 2002). Furthermore, during hypoxia ROS are formed which is not the case during anoxia since oxygen is central in the generation of ROS (Giordano 2005). This might explain the increased cell death in hypoxic conditions compared to anoxia, but needs further investigation.

Several factors have already been linked to the protective effect of MSCs against hypoxia-induced cell death, including VEGF and PDGF. Both factors are present in the CM-MSC as demonstrated by De Boeck *et al.* (De Boeck et al. 2013). Furthermore, these factors share a common receptor, the PDGFRα and an earlier

study of Windmolders *et al.* indicated that CM-MSC activates the PDGFRα in CASCs which leads to an increased CASC migration (Windmolders et al. 2014). However, adding VEGF or PDGF to the control medium did not increase CASC survival and inhibiting the shared PDGFRα did not reduce CM-MSC induced protection, suggesting these factors are not involved in the protective effect.

The most described survival pathway for the MSC mediated protective effects under ischemic conditions is the Akt pathway. Phosphorylation of Akt leads to its activation and subsequently inactivates Bad through phosphorylation which results in cell survival (Datta et al. 1997). Despite earlier results of *Jin et al*., indicating an important role for pAkt overexpression in the protective effects of MSCs on endothelial cells and cardiomyocytes (Jin et al. 2013), no upregulation of pAkt was detected in CM-MSC treated CASCs after three days of hypoxia. Furthermore, Bad or pBad could not be detected in CM-MSC treated CASCs after three days hypoxia. Instead, CM-MSC treatment of CASCs upregulated catalase expression levels under hypoxic conditions. This anti-oxidative enzyme catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). Similar findings have been described by *Khan et al.*, who showed that MSCs from umbilical cord Wharton's jelly induced the expression of catalase in adult neutrophils which protected them against cytotoxicity (Khan et al. 2015). Furthermore, a study of Zhang *et al.* indicated that catalase transduction blocks hypoxia/reperfusion induced cardiomyocyte apoptosis (Zhang et al. 2013). Therefore, CM-MSC might protect CASCs against hypoxia-induced cell death by protecting them against oxidative stress.

To conclude, our study revealed that CASC survival under hypoxic conditions is improved by paracrine effects of MSCs possibly by protecting them against oxidative stress via the upregulation of catalase activity. CASC therapy for patients with heart failure might therefore be improved through combination therapies, albeit that further study is needed to fully understand the mechanisms involved. Here, a global expression study and pathway analysis can be of great use to get better insights in how apoptosis genes in CASCs react upon treatment with CM-MSC, but this approach will also give a broad view on the different cellular processes positively influenced by CM-MSC treatment which can contribute to improved regeneration therapies.

Chapter 5

New approaches to study cardiac fibrosis

Contributing investigators:

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5.1. ABSTRACT

Cardiac fibrosis, which naturally occurs after an MI and is characterized by excessive collagen deposition, leads to a reduced pump function and will eventually contribute to the development of heart failure. Furthermore, cardiac fibrosis is an important limiting factor in the development of a successful cardiac regeneration therapy, since CASCs that end up in the fibrotic tissue are not able to make contact with functional cardiomyocytes and therefore do not differentiate towards cardiomyocytes.

In this study, we show that human cardiac fibroblasts, isolated as outgrowth cells from cardiac tissue explants, display a fibroblast phenotype as confirmed by co-staining of vimentin and collagen type I. Stimulation with TGF-β1 increased procollagen type I (COL1A1) expression which indicates that a pro-fibrotic expression profile could be induced in these cells. However, a pilot experiment using TGF-β3, a possible inhibitor of TGF-β1 induced collagen production, showed no effect on COL1A1 expressionlevels.

In conclusion, these results indicate that human cardiac fibroblasts can be easily isolated as outgrowth cells from cardiac tissue explants and a profibrotic expression profile can be induced in these cells by stimulation with TGF-β1. After further validation the presented experimental approach, might be suitable to test different anti-fibrotic strategies. However, preliminary results show no effect of TGF-β3 on TGF-β1 induced collagen expression.

5.2. INTRODUCTION

Cardiac fibrosis naturally occurs after an MI and is characterized by the excessive deposition of collagen in the infarcted area. While initially formed to protect the heart from rupture, the continuous increase in cardiac fibrosis leads to a decrease in pump function (Kong et al. 2014). While the initial collagen deposition is predominantly composed of thin elastic collagen type III fibers, in time they will be replaced by thick stretch resistant collagen type I fibers, eventually contributing to the development of heart failure (Pauschinger et al. 1999). Furthermore, cardiac fibrosis is an important limiting factor in the development of a successful cardiac regeneration therapy, which needs to be addressed.

A promising candidate for cardiac regeneration therapies are CSCs isolated from atrial appendages based on elevated ALDH activity, called CASCs (Koninckx et al. 2013). Intramyocardial autologous transplantation in a Göttingen minipig model of acute MI resulted in extensive engraftment and myocardial differentiation of the transplanted CASCs. However, CASCs that end up in the fibrotic tissue of a Göttingen minipig model of acute MI and are not able to make contact with functional cardiomyocytes, do not differentiate towards cardiomyocytes (Fanton et al. 2015)

One of the key mediators in the transition from inflammation to fibrosis is the anti-inflammatory cytokine TGF-β1, which promotes the differentiation from fibroblasts to myofibroblasts and the subsequent synthesis and deposition of collagen (Lijnen et al. 2000, Lijnen and Petrov 2002). While all 3 isoforms of TGF-β (TGF-β1, 2 and 3) are upregulated after an MI, the high TFG-β1/β2 to TGF-β3 ratios are responsible for compact collagen deposition (Lichtman et al. 2016). In contrast, in fetal wound healing there is no scar tissue formation, partly mediated by the low TFG-β1/β2 to TGF-β3 ratios and the absence of TGF-β1 induced collagen production (Namazi et al. 2011). Furthermore, TGF-β3 antagonizes TGF-β1/β2, thereby reducing fibroblast activation and collagen deposition in both dermal and vocal fold fibroblasts (Murata et al. 1997, Chang et al. 2014) and injection of TGF-β3 in human skin wounds reduced collagen deposition, which resulted in less scarring (McCollum et al. 2011).

In this study, we developed an experimental approach to study cardiac fibrosis and we investigated the effect of TGF-β3 treatment on TGF-β1 induced collagen expression.

5.3. MATERIAL AND METHODS

All procedures were carried out in accordance with the principles set forth in the Helsinki Declaration. Approval by the Jessa Institutional Review Board and informed consent from each patient were obtained.

5.3.1. Human cardiac fibroblast isolation and culture

Method 1: Fibroblast overgrowth from a single cell population
Cardiac atrial appendage samples were minced and enzymatically dissociated with collagenase II (600 U/ml; Invitrogen) for 90 minutes. The obtained single cell population was subsequently passed over a cell strainer (70 µm; Falcon) and seeded in tissue culture treated flasks at different cell densities ($2x10^4$ to $1x10^5$ cells/cm²) in different culture media (X-vivo 15 with 10% FBS and 2% PS or high-glucose DMEM with 10% FBS and 2% PS). Cells were cultured at 37°C in humidified atmosphere containing 5% CO_2 with medium changes twice a week.

Method 2: Cardiac explant outgrowth
Cardiac atrial appendage samples were minced and fragments of approximately 1 mm³ were placed in fibronectin-coated 24-well culture plates (Beckton&Dickinson) at a density of two fragments per well. These fragments were cultured in X-vivo 15 medium with 10% FBS and 2% PS. Medium changes were performed twice a week and after reaching a confluent monolayer, fragments were removed and cells were loosened with trypLE select (Gibco). Subsequently, cells were replated at a density of $5x10^3$ cells/cm² and cultured at 37°C in humidified atmosphere containing 5% CO_2.

5.3.2. Immunofluorescence of fibroblast markers

Cells cultured on sterile glass coverslips, were fixed with 4% PFA and blocked with 10% sheep serum for 30 minutes. Afterwards, cells were incubated overnight with rabbit anti-vimentin (1:500; abcam; ab92547) at 4°C followed by a 1 hour incubation with sheep anti-rabbit rhodamine (1:100; Merck Millipore; AQ301R). Afterwards, cells were incubated overnight with mouse anti-collagen type I (1:50; abcam; ab901395) followed by a 1 hour incubation with Goat anti-mouse fitc (1:10; Beckton&Dickinson; 349031).

5.3.3. qPCR analysis procollagen type I and III

Cells were treated with different concentrations of human recombinant TGF-β1 (R&D systems; 240-B-002), ranging from 1 pg/ml to 10 ng/ml and different combinations of TGF-β1 and TGF-β3, in serum-free low glucose DMEM with 2% PS for 48 hours. Recombinant TGF-β1 was reconstituted in sterile 4 mM HCl containing 0.1% bovine serum albumin at a stock concentration of 20 µg/ml. Recombinant TGF-β3 was reconstituted in sterile 4 mM HCl at a stock concentration of 50 µg/ml.

Total RNA was extracted using the RNeasy mini kit (Qiagen) and equal amounts of RNA were reverse transcribed with the SuperScript III Reverse Transcriptase Kit (Invitrogen) primed with a random hexamer primer. Real time PCR reactions were carried out in duplicate with the Rotor-Gene Q (Qiagen). The final reaction master mix composition was 1x Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) and 2 µl of cDNA diluted 10-fold with DNase-RNase free water. Primer sequences with corresponding annealing temperature and concentration are listed in table 5.1. The PCR conditions were as follows: 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and annealing temperature for 30 seconds.

Table 5.1: Primer sequence, annealing temperature and concentration.

Primer	Primer sequence	Annealing temperature	Concentration
GAPDH	Forward: 5'-AGTCAACGGATTTGGTCGTATTG-3' Reverse: 5'-ATCTCGCTCCTGGAAGATGGT-3'	60°C	300 nM
COL1A1	Forward: 5'-CCCTGGAAAGAATGGAGATG-3' Reverse: 5'-CACCATCCAAACCACTGAA-3'	60°C	300 nM
COL3A1	Forward: 5'-GGAAAGAGTGGTGACAGAG-3' Reverse: 5'-TACCAGGGAATCCTCGAT-3'	60°C	300 nM

GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; *COL1A1* = procollagen type I alpha 1 ; *COL3A1* = procollagen type III alpha 1

5.3.4. Statistics

All quantitative results are presented as median ± IQR. Comparisons between more than two paired groups were performed in GraphPad version 6.01 using the non-parametric Friedman test with Dunn's post-hoc test. A value of $p<0.05$ was

considered significant. *A priori* power analyses were performed with G*Power (Faul et al. 2007).

5.4. RESULTS

5.4.1. Isolation and characterization of human cardiac fibroblasts

Two different protocols were tested for the isolation of cardiac fibroblasts from human cardiac samples. The first method in which a single cell population was seeded and fibroblast overgrowth was anticipated, showed few to no attached cells and also no proliferation of the attached cells (Figure 5.1A). In contrast, the second method showed a clear outgrowth of cells from the cardiac explants. After approximately 2 weeks a monolayer was formed and cells could be isolated for further expansion (Figure 5.1B). A fibroblast phenotype was confirmed by a double staining for vimentin and collagen type I (Figure 5.1C).

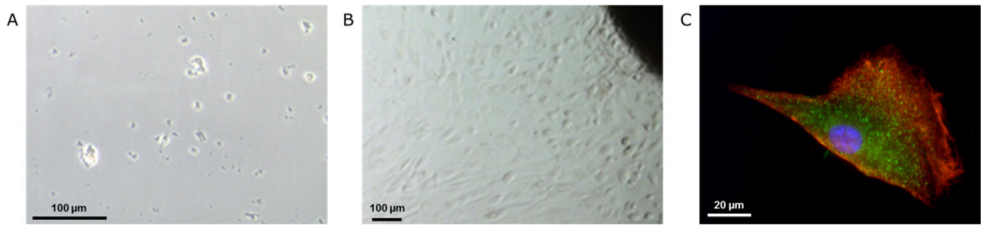

Figure 5.1: Isolation and characterization of cardiac fibroblasts from human atrial appendages. (A) Representative light microscopic image of the seeded single cell population showing few to no attached cells. (B) Representative light microscopic image of a tissue explant showing a monolayer of outgrowth cells. (C) Representative immunofluorescent image of cardiac fibroblast outgrowth cells confirming the fibroblast phenotype by co-staining of vimentin (red) and collagen type I (green). Nuclei were stained with 4',6-diamidino-2-phenylidole (DAPI; blue).

5.4.2. Functional testing of the isolated human cardiac fibroblasts

In order to test whether the isolated cardiac fibroblasts are functional, the effect of TGF-β1 on the production of collagen was determined. Figure 5.2. shows a dose responsive increase in the expression of *COL1A1* after a 48 hours treatment with increasing concentrations of human recombinant TGF-β1. This was not accompanied by an increase in the expression of procollagen type III (*COL3A1*), a precursor of the early thin elastic collagen fibers formed early after the infarction.

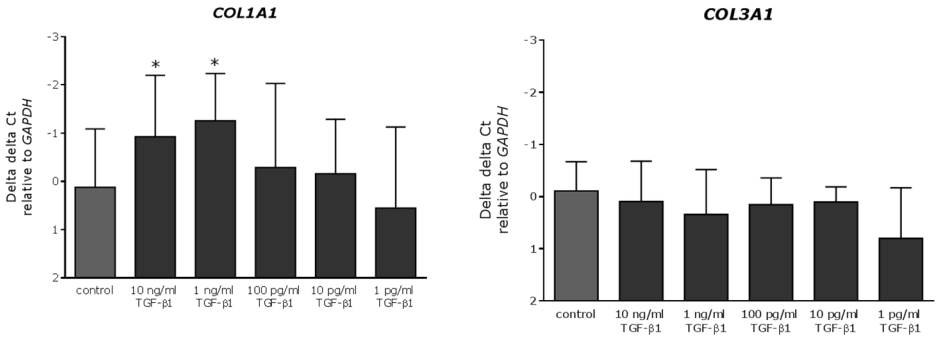

Figure 5.2: Treating the isolated human cardiac fibroblasts with TGF-β1 increases collagen type I production. mRNA expression of procollagen type I (*COL1A1*) and III (*COL3A1*) after TGF-β1 treatment in human cardiac fibroblasts (passage 2-4). Data are shown as median ± IQR. (n=5 individual patients' cardiac fibroblast cultures/condition) *p<0.05 compared to respective control and 1 pg/ml TGF-β1.

5.4.3. TGF-β3 treatment has no effect on TGF-β1 induced collagen production

TGF-β1 and TGF-β3 have similar effects on the expression levels of *COL1A1* and *COL3A1* (Figure 5.3). However, the effect on *COL1A*1 expression was not significant probably due to the smaller sample size. An *a priori* power analysis revealed that 5 samples should be sufficient to obtain a similar significant difference in *COL1A1* expression after TGF-β1 and TGF-β3 treatment.

Furthermore, the effect of TGF-β3 treatment on TGF-β1 induced collagen expression in cardiac fibroblasts was studied (Figure 5.4). TGF-β3 treatment could not lower the TGF-β1 induced *COL1A1* expression. This was independent of the ratio of TGF-β1 to TGF-β3 (Figure 5.4A). Additionally, no difference in the expression of *COL3A1* could be detected in the different conditions (Figure 5.4B)

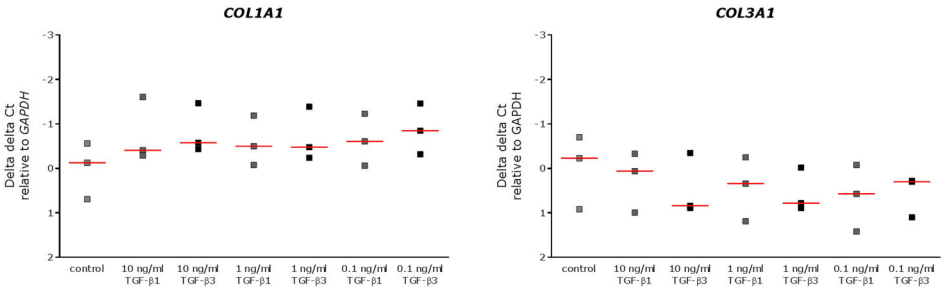

Figure 5.3: The effect of TGF-β1 and TGF-β3 on collagen production. mRNA expression of procollagen type I (*COL1A1*) and procollagen type III (*COL3A1*) after TGF-β1 and TGF-β3 treatment in human cardiac fibroblasts (passage 3-4). Individual data points with the red line representing the median of each condition. (n=3 individual patients' cardiac fibroblast cultures/condition)

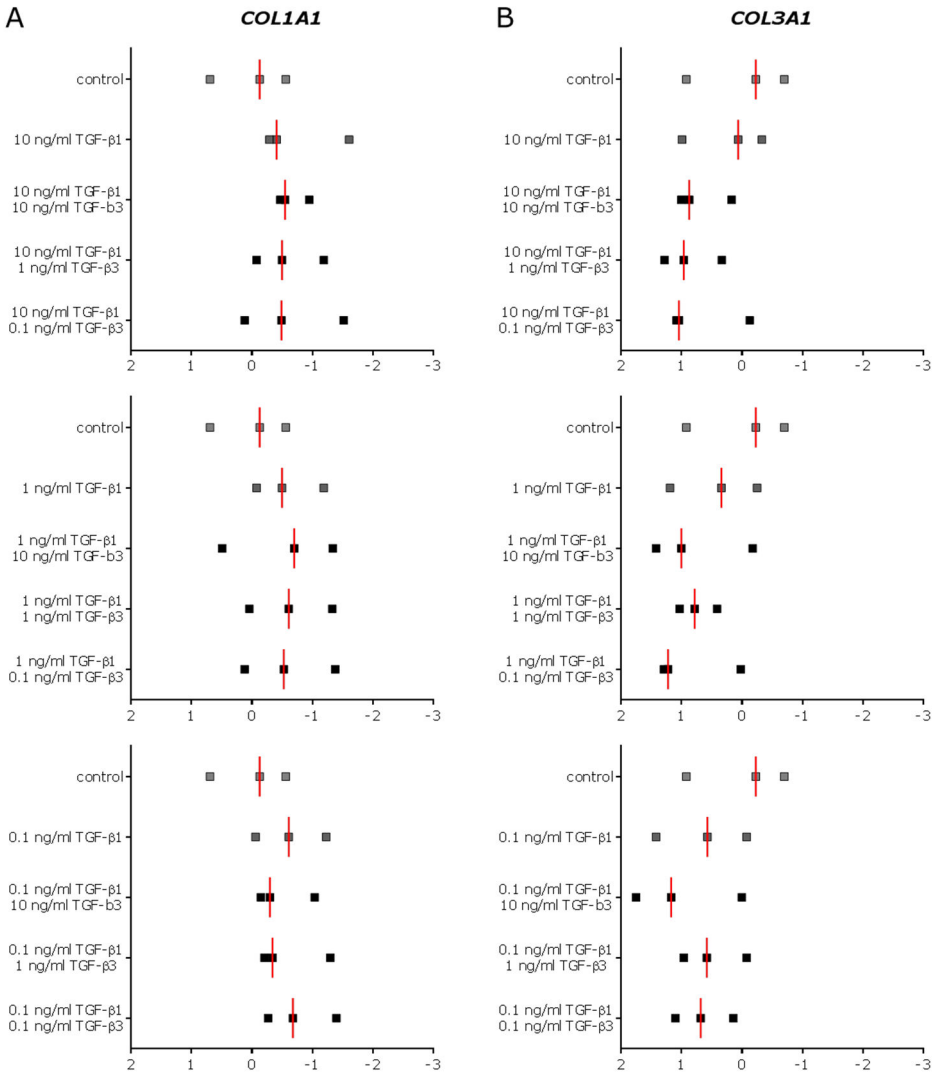

Figure 5.4: TGF-β3 treatment has no effect on TGF-β1 induced collagen production. mRNA expression of (A) procollagen type I (*COL1A1*) and (B) procollagen type III (*COL3A1*) after a combined TGF-β1 and TGF-β3 treatment in human cardiac fibroblasts (passage 3-4). Individual data points with the red line representing the median of each condition. (n=3 individual patients' cardiac fibroblast cultures/condition)

5.5. DISCUSSION

In this study, we show that human cardiac fibroblasts can easily be isolated as outgrowth cells from cardiac tissue explants. Their fibroblast phenotype was confirmed by co-staining of vimentin and collagen type I. Furthermore, a pro-fibrotic expression profile could be induced in these cardiac fibroblasts as demonstrated by the increase in collagen production upon stimulation with TGF-β1. This experimental approach for cardiac fibrosis can now be used to test different anti-fibrotic strategies in an *in vitro* setting.

Cardiac fibrosis, characterized by cardiac fibroblast activation and the excessive deposition of collagen type I as well as other ECM proteins is one of the main mechanisms responsible for cardiac remodeling that will eventuate in the development of heart failure (Burke and Virmani 2007). In our study cardiac fibroblasts were isolated by two different protocols: (1) cardiac fibroblast overgrowth after seeding a single cell population and (2) cardiac fibroblast outgrowth from cardiac tissue explants. However, only the second method generated cardiac fibroblasts, which could be used for further testing. Why method one failed repeatedly is unclear. While other studies were able to isolate cardiac fibroblasts by seeding a single cell population derived from enzymatic digestion of cardiac tissue, we were not able to reproduce these results (Neuss et al. 1994, Agocha et al. 1997, Kawano et al. 2000). Although these protocols were very similar, differences may be due to the used tissue sample (atrial appendage in our study versus atrium and ventricle in other studies).

One of the main mediators of cardiac fibrosis after an MI is the TGF-β signaling pathway (Dobaczewski et al. 2011). In mammals, 3 TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) have been identified, from which TGF-β1 is key for the activation of cardiac fibroblasts and subsequent increased collagen type I secretion (Lichtman et al. 2016). Therefore, upregulation of *COL1A1* expression after TGF-β treatment demonstrates that the isolated cardiac fibroblasts were functional (Pan et al. 2013). In contrast, *COL3A1* expression was not increased after TGF-β1 treatment.

Targeting the TGF-β signaling pathway could be an effective treatment strategy to prevent cardiac remodeling and the development of heart failure. However, correct timing seems crucial in this context since blockage of TGF-β during the

early phase could be harmful for infarct healing, while late inhibition of TGF-β suppresses remodeling and attenuates dilatation (Tan et al. 2010). In addition, it should be noted that TGF-β1 is a multifunctional cytokine, that also prevents the formation of atheromatous plaques. Inhibiting TGF-β can therefore result in increased plaque formation and higher risk for MI (Frutkin et al. 2009). Instead of direct inhibition of TGF-β, influencing the TGF-β1/TGF-β3 ratio is also an interesting therapeutic strategy. The injection of TGF-β3 in the infarct area might change this ratio towards a situation that can be found in fetal and oral mucosa wound healing. There, TGF-β1 to TGF-β3 ratios are much lower which prevents or reduces scar formation. Indeed, TGF-β3 antagonizes TGF-β1 and thereby reduces cardiac fibroblast activation and collagen deposition (Schrementi et al. 2008, Namazi et al. 2011, Chang et al. 2014) and injection of TGF-β3 in skin wounds reduced collagen deposition, which resulted in less scarring (McCollum et al. 2011). Treating cardiac fibroblasts with similar concentrations of recombinant TGF-β1 or TGF-β3 resulted in an equal increase in COL1A1 expression. These results are in line with the results described in a study of Murata *et al.* which showed that both isoforms increase procollagen type I expression in dermal fibroblasts. However, in contrast with this stimulatory effect, adding TGF-β3 in combination with TGF-β1 resulted in a downregulation of procollagen type I expression (Murata et al. 1997). Based on the detected RNA levels of *COL1A1* and *COL3A1*, the preliminary results in our study suggested no effect of TGF-β3 treatment on TGF-β1 induced collagen deposition, but more thorough research on protein level is essential to validate this observation. In addition, further research using different ratios of TGF-β3 to TGF-β1 is necessary to exclude a possible effect of TGF-β3 on TGF-β1 induced collagen expression. Furthermore, the TGF-β1 induced collagen deposition can also be targeted by endogenous or exogenous inhibitors of TGF-β or its receptor or by targeting upstream or downstream elements within the TGF-β signaling (Daskalopoulos et al. 2014).

In contrast to this presented two-dimensional approach, a three dimensional approach might be even more useful to study the process of cardiac fibrosis and potential anti-fibrotic therapeutic strategies. Very recently, Sadeghi *et al.* engineered a simplified but functional and physiologic-like heart tissue to study cardiac fibrosis. Here, rat cardiomyocytes and cardiac fibroblasts were encapsulated in a gelatin methacryloyl-based hydrogel. Subsequently, quiescent

cardiac fibroblasts were activated by the addition of TGF-β1, as shown by an upregulation of fibronectin, α-SMA and collagen type I, both on mRNA and protein level (Sadeghi et al. 2017).

In conclusion, these results indicate that human cardiac fibroblasts can be easily isolated as outgrowth cells from cardiac tissue explants and that TGF-β1 treatment increases *COL1A1* expression, which resembles the physiological process after an MI. Therefore, this experimental approach might be suitable to test different anti-fibrotic strategies since we hypothesize that suppressing cardiac fibrosis after an MI might be important, not only to limit cardiac remodeling but also to improve the integration and differentiation of stem cells upon injection into the infarct area. However, further validation of the model is essential and as preliminary results show no effect of TGF-β3 on TGF-β1 induced collagen expression, also other anti-fibrotic strategies should be considered.

Chapter 6

General conclusions and future perspectives

6.1. CARDIAC DISEASE REMAINS THE TOP KILLER

Heart failure as a consequence of MI remains the leading cause of morbidity and mortality worldwide, taking more lives than all cancers combined. Major improvements in the treatment of MI resulted in a decreased mortality rate. For instance, due to the improved reperfusion therapies for MI patients, more patients will survive but as a consequence these patients will suffer from substantial left ventricular damage and are at risk of developing major left ventricular dysfunction (Gerber et al. 2016). For this reason, future survival gains might only be obtained by addressing the root cause of the problem, namely by repairing the damaged tissue with new and functional cardiac tissue.

Approximately 15 years ago, when researchers discovered the presence of proliferating cardiomyocytes in the adult heart and male cells in female hearts transplanted in male recipients, they thought to have found a new therapeutic strategy for MI-associated heart failure, namely (cell-based) cardiac regeneration therapies (Quaini et al. 2002, Bergmann et al. 2009). Since then, various stem cell types have been studied for myocardial regeneration. ESCs were a very promising candidate for cardiac repair because of their profound myocardial differentiation potential (Kehat and Gepstein 2003, Sachinidis et al. 2003). However, because of the risks of rejection, teratoma formation and ethical concerns, this cell type was less suitable for clinical applications (Nussbaum et al. 2007). The discovery of iPSCs and the development of pre-differentiation protocols for PSCs might change that view, but at that time the focus shifted towards adult stem cell types which are suitable for autologous applications (Takahashi et al. 2007, Burridge et al. 2014, Menasche et al. 2015). The most studied adult stem cell populations for cardiac repair are those from the bone marrow. However, most of them showed a limited cardiomyogenic differentiation potential and the limited functional benefits are believed to be the result of paracrine effects (Nguyen et al. 2016). Furthermore, also transplanted skeletal myoblasts were no suitable candidates as they failed to gain electromechanical coupling with the host tissue (Menasche et al. 2008). Therefore, CSCs which are derived from the heart itself and were believed to be preprogrammed to replace the cardiac tissue were believed to be the ultimate solution. However, also here a lot of controversy has been raised surrounding the differentiation potential and

functional effect of several cardiac stem cells types (Koninckx et al. 2011, Weinberger et al. 2012, van Berlo et al. 2014, Sultana et al. 2015, Doyle et al. 2016). These results therefore suggest that we need to go back to the root of stem cell biology and the concept of regenerative medicine to improve stem cell efficacy in patients.

6.2. BACK TO THE ROOTS OF CELL-BASED CARDIAC REGENERATION

A lot of questions related to cell-based cardiac regeneration therapies remain unresolved: What is the ideal cell type to transplant? What is its origin? Which is the optimal expansion method? Do they need to be pre-differentiated or in any way treated before administration? When, where and how do they need to be administered? How to influence the hostile environment of the infarct zone? What are their characteristics upon differentiation?

All these issues will need to be addressed in an attempt to improve current stem cell strategies and to make way for their successful clinical translation. While some of these questions are addressed within this thesis, others still need to be investigated further.

6.2.1. CASCs as an appropriate stem cell type for transplantation

The majority of the clinical trials used BMCs for cardiac repair. However, the moderate therapeutic effects after transplantation are mainly obtained by paracrine actions (Nguyen et al. 2016). This led to an increased interest in resident CSCs, which are believed to be preprogrammed for cardiac differentiation. However, also here controversy has been raised due to the limited cardiomyogenic differentiation potential of c-kit+ cells and CDCs. Furthermore, claims about the integrity of certain data of the SCIPIO trial has left the scientific community sceptic about the potential of cell-based therapies for cardiac repair (The Lancet 2014).

CASCs isolated from atrial appendages based on their high ALDH activity, appear to be promising candidates for MI. In **chapter 2** we demonstrated that these cells can be successfully isolated from elderly patients and subsequently expanded for a prolonged period of time, producing relevant cell numbers comparable to other

clinical trials with CSCs while maintaining their biological characteristics such as phenotype, ALDH expression and most importantly their cardiac differentiation potential. Furthermore, in **chapter 3** we demonstrated that they are most abundant in the atrial appendages and more so in the right than in the left. This precludes harvest via endomyocardial biopsies, but requires a surgical approach. However, the atrial appendages are relatively easy and safe to obtain via a mini-thoracotomy. The reason why they are predominantly present in the atrial appendages remains unknown, but a possible explanation might be the low levels of electrical activity in this compartment. Furthermore, we demonstrated that CASCs are most likely intermediates already committed towards myocardial differentiation, as shown by the expression of *TNNT2* and *MYL2* during culture. This myocardial differentiation potential was further confirmed by an autologous CASC transplantation in a Göttingen minipig model of acute MI which showed cardiomyogenic differentiation in 98% of the transplanted cells. In contrast, endothelial differentiation was rarely observed upon transplantation (< 1%) (Fanton et al. 2015). Together with the limited expansion potential demonstrated in **chapter 2**, the decrease in the number of proliferating cells during culture and the absence of telomerase activity with corresponding decrease in telomere length, it is suggested that the term "progenitor cell" is more suitable for the CASCs.

When studying the origin and nature of the CASCs, the marker profile described in **chapter 3** suggests a possible heterogeneous embryonic origin, which is also expected for other CSC types. Therefore, it would be interesting to perform a lineage tracing experiment in order to define the origin of CASCs and their contribution during myocardial development. However, this requires a CASC specific marker and as the ALDH enzyme is expressed by multiple cells this is probably not a suitable candidate. Furthermore, as shown in **chapter 2**, only half of the CASCs maintain their elevated ALDH activity during culture and when sorting the $ALDH^{dim}$ and $ALDH^{br}$ cells only the $ALDH^{br}$ subpopulation could be further expanded. As transcriptomics studies can provide more insight in the genetic background of the cells, this might not only reveal a marker for future tracing studies but will also give us more insight in the different molecular pathways expressed in the CASCs. This leads us to the next challenge: improving

CASC expansion and unraveling the cues that drive them towards myocardial differentiation.

6.2.2. Mechanisms involved in CASC activation

During cardiac development, the working myocardium of the heart is formed by cardiac progenitor cells. Different signaling pathways, including Wnt, Hedgehog and notch, are responsible for progenitor cell proliferation, specification and differentiation during cardiogenesis (Rana et al. 2013). Of these, canonical Wnt signaling is involved in the induction of mesoderm formation, followed by a subsequent inhibition for cardiac specification and cardiac progenitor cell formation. Later on, proliferation of these progenitor cells is positively regulated by canonical Wnt signaling, while inhibition of this pathway is essential for terminal cardiac differentiation (Gessert and Kuhl 2010).

In **chapter 3** we demonstrated that canonical Wnt signaling is expressed and can be modulated in CASCs. However, we were not able to demonstrate that Wnt activation or inhibition had any effect on CASC proliferation or mature cardiomyocyte differentiation. Therefore, further research in the role of Wnt signaling in CASCs is necessary, but also the mechanisms controlling CASC proliferation and differentiation need to be studied to give us more insight into CASC-based cardiac regeneration therapy. These insights will contribute to the development of a suitable expansion protocol while still maintaining myocardial differentiation. The results described in **chapter 2** already indicate that PPS can be used as an efficient replacement for FBS for the large-scale expansion of CASCs in a clinical setting. However, also here total cell numbers are rather limited and there is high inter-patient variability in growth profiles and total cell numbers. Recently developed xeno- and serum-free culture media for human stem cells, such as mesencult or stemMACs, might therefore be more suitable. However, more detailed research in this topic is necessary. In addition, RNA sequencing studies can be used to identify biomarkers for the selection of CASCs with the highest proliferation capacity, as such further improving these expansion protocols. It could also be beneficial to combine this with a standard screening of CASC cultures for mtDNA deletions, since high levels of these mtDNA deletions are linked to slower proliferation rates (Lushaj et al. 2012).

Knowing the mechanisms involved in CASC proliferation and differentiation can also be used to boost the endogenous cardiac repair mechanism of the heart by activating resident CASCs. In comparison with an exogenous CASC-based therapy, this will offer an "off-the-shelf" therapy which is far more affordable and obviates the risks associated with the classical stem cell transplantation, such as early aging, genomic instability and the transfer of microorganisms during transplantation (Rubio et al. 2005). However, the spatiotemporal activation of the different mechanisms controlling CASC activation will be a huge challenge. Here, miRNA based therapies might be of great promise since a single miRNA can influence multiple pathways at once. Recent research already indicated the importance of miRNAs in cardiac progenitor proliferation and differentiation (Hodgkinson et al. 2015, Zhu et al. 2016). Although targeting specific miRNAs might be a rational strategy for cardiac repair, key issues still need to be addressed before miRNA based cardiac regeneration therapies can be taken into the clinic. The correct combination of miRNAs to induce cardiac regeneration needs to be determined and a safe but effective delivery system needs to be developed. Finally and most importantly, the expression profile and functional importance of miRNAs in CASCs are currently unknown. Therefore, additional research in the role of miRNAs in CASC activation is crucial if we want to pursue the road of miRNA-based therapies for cardiac regeneration.

6.2.3. Modulating the hostile microenvironment after an infarction

The main goal of cardiac regeneration is the formation of new cardiac tissue which is electrically and mechanically integrated into the native cardiac tissue. However, the hostile microenvironment after an infarction, characterized by ischemia, inflammation and fibrosis, might impair the survival and integration of the transplanted cells. Furthermore, it is also detrimental for resident cardiac (stem) cells and endogenous cardiac repair.

CASC transplantation in a Göttingen minipig model of acute MI resulted in a relatively high cell retention of 19% and a profound differentiation towards cardiomyocytes as shown by sarcomeric expression of cTnT and cTnI. Furthermore, electromechanical integration of CASC-derived cardiomyocytes was demonstrated by expression of the gap junction protein CX43. Together with the absence of malignant cardiac arrhythmias after transplantation, this is a first

indication that CASC transplantation is safe (Fanton et al. 2015). However, there is still room for improvement by tackling different limiting factors for cardiac repair, including ischemia, inflammation and fibrosis.

The infarct site is characterized by a restricted oxygen supply. In **chapter 4** we showed that CM-MSC protects CASCs against hypoxia-induced cell death, as shown by a higher percentage of viable cells and less early and late-apoptotic cells. However, the exact molecular mechanisms and paracrine factors responsible for this cytoprotective effect are not known. The upregulation of catalase in CASCs after CM-MSC treatment under hypoxic conditions might suggest that the observed positive effect of CM-MSC on CASC survival is associated with its protection against oxidative stress. Therefore, it would be interesting to further study oxidative stress levels under both conditions and further investigate the effect of catalase on the survival of CASC by inhibiting its function via e.g. silencing RNA (siRNA). Also here, transcriptomics studies may be of great interest in order to fully understand the responsible molecular mechanisms involved in this protective effect. This knowledge will lead to an improved clinical protocol in which CASCs can be co-administered with CM-MSC or its specific cytoprotective factors to augment the survival and integration of the transplanted cells.

The inflammatory reaction which occurs shortly after the infarction also negatively affects cell survival, integration and differentiation after transplantation. However, recent findings indicated that CASCs might have immunomodulatory properties and that CASC viability was not affected by physiologically relevant concentration of inflammatory cytokines (Fanton, personal communication). This suggests a low immunogenic profile of CASCs, both in non-inflammatory and inflammatory conditions. However, also here more insights in the molecular mechanisms responsible for these immunomodulatory effects of CASCs is essential to further improve a CASC-based therapy for cardiac repair.

Finally, cardiac fibrosis is also an important limiting factor for CASC-based functional repair since CASCs that ended up in the fibrotic infarct zone were not able to differentiate towards functional cardiomyocytes (Fanton et al. 2015). Furthermore, the excessive deposition of collagen type I as well as other ECM proteins is also one of the main mechanisms responsible for cardiac remodeling that will eventuate in the development of heart failure (Burke and Virmani 2007).

In **chapter 5** we described an experimental approach to study cardiac fibrosis which, after further validation, can be used to test different future anti-fibrotic strategies in an *in vitro* setting. Therefore, cardiac fibroblasts were isolated as outgrowth cells from cardiac explants and characterized by a double staining for vimentin and collagen type I. Their functionality was confirmed by the increased synthesis of collagen type I after treatment with human recombinant TGF-β1. Although preliminary results indicated no effect of TGF-β3 treatment on TGF-β1 induced collagen deposition, we hypothesize that limiting this TGF-β1 induced collagen synthesis might not only prevent cardiac remodeling but might also improve CASC integration upon transplantation into the infarct zone. Here, also other strategies should be considered as recent research indicated that specific miRNAs can reduce cardiac fibrosis. So stem cell-derived exosomes which contain specific paracrine factors and/or miRNAs might limit fibrosis (Montgomery et al. 2011, Shao et al. 2017).

In conclusion, treating the different elements of the hostile microenvironment upon infarction will further improve CASC survival, integration and differentiation. Eventually, this will lead to further functional repair beyond the levels obtained until now.

6.3. WHERE WILL THE FUTURE BRING US?

The translation of basic research and animal models into clinical practice has never been so rapid as in the field of cell-based cardiac regeneration therapies. However, despite the disappointing results of earlier studies with respect to cardiac differentiation and functional repair, these pioneering results should not be used to reject cell-based therapies for cardiac repair. Instead, in a first step they show that cell transplants in MI patients are safe to perform. However, more research is necessary to overcome the final hurdles and to prove clinical benefits for patients with this chronic disease. Furthermore, although the focus of most cardiac regeneration research has been ischemic heart disease, creative use of different cardiac regeneration approaches might be applied to solve the great need for therapeutic cardiac regeneration in congenital heart disease.

Currently, three main regenerative strategies are under investigation for cardiac regeneration: (1) non-myocyte cell fate reprogramming, (2) stem or progenitor cell transplantation (3) paracrine stimulation of cardiac repair. First, cardiac reprogramming of endogenous cardiac non-myocyte cells might be very promising as this approach reduces scar tissue while simultaneously generating new cardiomyocytes. Here small molecules or use of CRISPR technology is worth to investigate and might accelerate clinical translation of this reprogramming strategy for cardiac regeneration. However, this approach is still relatively new and requires more in depth research, both fundamental and in large animal models, especially for improvements in safety and efficiency. The second approach, stem or progenitor cell transplantation, is currently limited by the low viability and integration of the transplanted cells. In that perspective, cellular, molecular and genetic basic research in combination with preclinical and well-designed clinical studies will be key. Stem cell enhancement techniques currently under investigation include combinatory stem cells, preconditioning, pharmacological treatment and genetic modification. The third approach, paracrine stimulation of cardiac repair, focusses of enhancement of the cardiac microenvironment to stimulate endogenous cardiac regeneration. This includes stimulating cardioprotection and neovascularization and limiting fibrosis. A potential approach here is secretome byproduct injection, including stem cell-derived exosomes containing growth factors and miRNAs.

All these approaches have their specific benefits but also their shortcomings. Therefore, the ideal cardiac regeneration therapy will most likely be based on both cardiac differentiation of appropriate cells, resident or transplanted, for tissue restoration and a paracrine cocktail which limits fibrosis, improves the hostile microenvironment and stimulates functional integration of the attracted or transplanted cells. This approach is also used in the tissue engineering field which focusses on following three components: (1) the cell types being transplanted, (2) the type of scaffolds supporting the cells and (3) the type of small molecules, growth factors and other paracrine influencers. However, most critical aspect in this approach is the need for maintenance of cell viability and organization throughout the tissue and to match the mechanical properties to the native structure.

In conclusion, the future of cardiac regeneration therapies is still very promising and in my opinion, true repair of the damaged heart will be obtained in the years to come.

SAMENVATTING

Hartfalen ten gevolge van een hartinfarct blijft de belangrijkste oorzaak van ziekte en sterfte wereldwijd en veroorzaakt meer sterftegevallen dan alle kankers samen. Aangezien het verlies van functionele hartspiercellen aan de basis ligt van het ziektebeeld van een hartinfarct en de daaropvolgende ontwikkeling van hartfalen, wordt stamceltherapie wereldwijd onderzocht als een nieuwe behandelingsstrategie. Het succes van een stamceltherapie voor hartpatiënten wordt echter sterk bepaald door de selectie van het juiste stamceltype. Daarnaast moeten de moleculaire mechanismen betrokken bij herstel van het hart, maar ook bij de vermeerdering en differentiatie van stamcellen naar hartspiercellen verder opgehelderd worden. Tot slot moet de vijandige micro-omgeving van het infarct, gekenmerkt door ontsteking, zuurstofgebrek en littekenvorming aangepakt worden om de overleving, integratie en differentiatie van de stamcellen te bevorderen.

Cardiale stamcellen geïsoleerd om basis van een verhoogde aldehyde dehydrogenase (ALDH) activiteit, cardiale atriale appendage stamcellen (CASCs) genoemd, zijn een veelbelovende kandidaat voor de regeneratie (het volledig herstel) van het hart. In een eerste deel van deze studie tonen we aan dat de celvermeerdering van de CASCs niet oneindig is en gepaard gaat met een kleine maar significante afname in absolute telomeerlengte door het gebrek aan telomerase activiteit. Ondanks een afname in het aantal delende CASCs gedurende expansie, worden er klinisch relevante cel aantallen bekomen die overeenkomen met het bereik van eerdere klinische studies met cardiale stamcellen. Verder behouden de CASCs hun biologische eigenschappen tijdens expansie, inclusief hun antigen expressieprofiel, ALDH expressie en hun differentiatiepotentieel naar hartspierencellen als aangetoond door de sarcomerische organisatie van troponine T en I. Tot slot kunnen de CASCs ook succesvol opgegroeid worden in humaan bloedplaatjes plasma supernatant terwijl ze hun biologische eigenschappen behouden. Dit is dan ook een belangrijke stap in de richting van een klinische toepassing van de CASCs.

In een tweede deel van deze studie tonen we aan dat CASCs voornamelijk aanwezig zijn in hartoren en meer in het rechtse dan in het linkse hartoor. We tonen ook aan dat zij verschillende vroege hartdifferentiatie markers zoals

NKX2.5, *GATA4*, *TBX5* en *TBX18* tot expressie brengen. Daarnaast zijn zij ook geëngageerd om te differentiëren naar hartspiercellen als aangetoond door de expressie van *TNNT2* en *MYL2*. Deze resultaten suggereren een mogelijks heterogene embryonale oorsprong van de CASCs aangezien deze vroege hartdifferentiatiemarkers tot expressie komen in typische hart voorloper cel populaties tijdens hartontwikkeling. Verder doet de aanwezigheid van Frizzled receptoren op de CASCs vermoeden dat Wnt signalering een rol speelt bij zelfvernieuwing, vermeerdering en differentiatie van de CASCs. Ondanks een actieve rol van Wnt signalering in CASCs, als aangetoond door een toename in totale en actieve β-catenine niveaus, heeft Wnt activatie echter geen effect op CASC vermeerdering en zelfvernieuwing. Daarnaast zorgt Wnt onderdrukking wel voor een verhoogde expressie van de vroege hartdifferentiatie markers, maar introduceert het geen differentiatie naar mature hartspiercellen. Ondanks de cruciale rol van Wnt signalering in de vorming van het hart tijdens de embryonale ontwikkeling en bij de differentiatie van pluripotente stamcellen naar hartcellen, is Wnt signalering aanwezig in CASCs maar heeft het weinig effect op CASC vermeerdering en differentiatie *in vitro*.

In een derde deel van deze studie, trachten we de overleving van CASCs onder zuurstofarme condities te verbeteren aangezien het infarctgebied gekenmerkt wordt door zuurstofgebrek, ontsteking en littekenvorming en de overleving van de getransplanteerde cellen hoogstwaarschijnlijk negatief beïnvloed wordt door de lage zuurstofniveaus in het doelgebied. Binnen deze studie tonen we aan dat de verlaagde CASC overleving ten gevolge van hypoxie (lage zuurstofniveaus) maar niet anoxie (volledig zuurstofgebrek) gedeeltelijk hersteld wordt door de cellen te behandelen met geconditioneerd medium van mesenchymale stamcellen (CM-MSC). De geobserveerde toename in CASC overleving ging ook gepaard met een toename in CASC vermeerdering, aangetoond door een toename in het aantal Ki67 positieve cellen na behandeling met CM-MSC onder hypoxie. Dit paracrien effect werd niet gemedieerd door VEGF of PDGF en CM-MSC beschermt CASCs tegen hypoxie geïnduceerde celdood op een Akt-onafhankelijke wijze. In plaats hiervan verhoogt CM-MSC de katalase expressie in CASCs onder hypoxische condities.

Tot slot ontwikkelden we experimenteel ontwerp om littekenvorming in een *in vitro* situatie onderzoeken. Littekenvorming na een hartinfarct kan niet enkel

aanleiding geven tot de ontwikkeling van hartfalen, maar is mogelijks ook een belangrijke hinderpaal in de ontwikkeling van een succesvolle regeneratie therapie voor het hart aangezien CASCs die in het fibrotisch weefsel belanden geen contact kunnen maken met functionele hartspiercellen en daardoor niet zullen differentiëren naar hartspiercellen. Dit experimenteel ontwerp kan nu gebruikt worden om het effect van TGF-β1 en TGF-β3 op hartfibroblasten te onderzoeken en om na te gaan of het proces van foetale wondheling nagebootst kan worden in het hart. In tegenstelling tot adulte wondheling gaat foetale wondheling niet gepaard met littekenvorming. Dit is gedeeltelijk te wijten aan de lage TGF-β1 tot TGF-β3 ratio's en de afwezigheid van TGF-β1 geïnduceerde collageenafzetting.

Alles samen bieden de resultaten van deze thesis belangrijke inzichten in de moleculaire en cellulaire mechanismen betrokken bij CASC vermeerdering, differentiatie en overleving. Verder onderzoek is echter noodzakelijk om de onderliggende mechanismen en betrokken factoren volledig te begrijpen en zo de behandeling van hartpatiënten met CASCs te verbeteren door zich te richten op de biologie van de cellen alsook de vijandige micro-omgeving na een infarct.

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CURRICULUM VITAE AND ACADEMIC BIBLIOGRAPHY

Curriculum vitae

Leen Willems was born in Diest (Belgium) on January 27th, 1989. In 2012, she graduated magna cum laude as a Master in Biomedical Sciences from Hasselt University. During her last master year and for her master thesis she performed an internship at the Laboratory of Experimental Hematology at the Jessa Hospital in Hasselt. During this internship she studied the impact of platelet lysate on growth and differentiation of long-term expanded cardiac atrial appendage stem cells. After successfully finishing her master thesis, she received an IWT PhD scholarship to continue her research at the Laboratory of Experimental Hematology in cooperation with Hasselt University. During her PhD study, she investigated whether the endogenous repair mechanism of the heart can be stimulated by identifying the signaling pathways involved in survival, self-renewal, proliferation and differentiation of cardiac stem cells and by controlling the fibrotic process after a myocardial infarction.

Scientific publications

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Scientific posters

20 September 2015 "2nd Meeting of the Belgian Society for Stem Cell Research (BeSSCR)", Brussels, Belgium. **Willems L**, Windmolders S, Daniëls A, Linsen L, Fanton Y, Hendrikx M, Koninckx R1, Rummens JL and Hensen K. Clinical-scale *in*

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2-3 June 2015 "Biomedica", Genk, Belgium. **Willems L**, Windmolders S, Daniëls A, Linsen L, Fanton Y, Hendrikx M, Koninckx R1, Rummens JL and Hensen K. Clinical-scale *in vitro* expansion preserves biological characteristics of cardiac atrial appendage stem cells.

20 April 2015 "Interuniversity Stem Cell Meeting", Leuven, Belgium. **Willems L**, Azdud Y, Fanton Y, Daniëls A, Hendrikx M, Declercq J, Rummens JL and Hensen K. A role for Wnt signaling in cardiac atrial appendage stem cell biology.

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13 June 2014 "Recent Advances in Neuronal and Cardiac Tissue Engineering: From Lab to Clinic", Diepenbeek, Belgium. **Willems L**, Daniëls A, Fanton Y, Windmolders S, Hendrikx M, Rummens JL, Koninckx R and Hensen K. Wnt Signaling in cardiac atrial appendage stem cells, a target for regeneration strategies

8 May 2014 "Knowledge for growth", Ghent, Belgium. **Willems L**, Daniëls A, Fanton Y, Windmolders S, Hendrikx M, Rummens JL, Koninckx R and Hensen K. Wnt Signaling in cardiac atrial appendage stem cells, a target for regeneration strategies

Oral presentations

9 November 2013 "PhD Symposium: Medisch-wetenschappelijk onderzoek in de Limburgse ziekenhuizen: een blik op de toekomst", Hasselt, Belgium. Cardiale regeneratie versus fibrose: Op zoek naar het evenwicht voor functioneel herstel.

Courses and skills

General

- Good scientific conduct and lab book taking
- Biosafety

Techniques in molecular biology

- EMBO practical course: Analysis of small non-coding RNAs: From discovery to function
- Roche-VIB qPCR training

Statistics

- FLAMES summer school methodology & statistics 2013: Research methodology and basic statistical principles
- Linear mixed models: FLAMES
- Essential tools for R – introduction to R: FLAMES

GMP, GCP and quality control

- GCP training for investigator site teams and ethics committees: FormaliS
- Introduction to cleanroom: BMC consulting

Writing and presenting

- Presentation techniques: Vocational training and testing
- Effective scientific communication: Principiae
- Effective graphical displays: Principiae

Grant writing

- Strategic Basic Research (SBO): agency for innovation by science and technology (IWT): onderzoek naar de activatie van endogene herstelmechanismen van het hart en de onderdrukking van fibrose voor myocardiaal herstel na een infarct (2012; successful)

Business and management

- Project and time management: True colours
- Postgraduate Business Administration and Management: Hasselt university: School of Expert Education

Career development

- Early career development workshop: Developing your career: funding, fellowships & beyond: Prof. dr. Nicol Keith
- Business lunch Prof. Dr. Karen Hensen
- Grow your future career: Grow2Excel

Organizing symposia and events

- PhD symposium: Medisch-wetenschappelijk onderzoek in de Limburse ziekenhuizen: een blik op de toekomst (2013)
- Member of the BCF Ambassadors Network

Teaching and student coaching

- Lab training: Bachelor biomedical sciences
- Elective course on cardiology: Master biomedical sciences
- Genetics and genomics: Bachelor medicine and biomedical sciences
- Cell physiology: Bachelor medicine and biomedical sciences
- Literature study: Bachelor biomedical sciences
- Master training and thesis: Master biomedical sciences

DANKWOORD

Aan het einde van deze thesis neem ik nu graag even de tijd om stil te staan bij de weg die ik de afgelopen 5 jaar heb afgelegd, van het schrijven van mijn IWT aanvraag tot het schrijven van deze thesis. Gelukkig was dit steeds in goed gezelschap!

Allereerst wil ik graag mijn promotor **Prof. Dr. Karen Hensen** bedanken. Zonder jou was deze thesis er waarschijnlijk nooit gekomen want vanaf dag 1 stond je steeds voor mij klaar. Het begon allemaal met die geschreven, herschreven, verbeterde, nogmaals verbeterde en dan uiteindelijk finale IWT aanvraag en eindeloze drilsessies waardoor dat oh zo gevreesde kruisverhoor een mooie en positieve ervaring werd. Ook voor elk technisch probleem zocht je mee naar een oplossing en je verbeterijver kwam ook steeds van pas bij het schrijven van abstracts, rapporten, artikels en tenslotte deze thesis. Twee jaar geleden koos je helaas voor een nieuwe uitdaging in je carrière waardoor ik niet zomaar even op je deur kon komen kloppen. Toch vond je nog de tijd om af en toe eens samen te zitten om mijn werk mee op te volgen en mij te motiveren om zeker niet op te geven. Het zinnetje "tja, dat is onderzoek hé Leen" was ondertussen ook al zo in mijn hoofd gebrand waardoor het soms leek alsof je er toch nog steeds bij was.

Prof Dr. Jean-Luc Rummens, bedankt om mij, als copromotor, te begeleiden tijdens mijn doctoraatsonderzoek, maar ook om mij te introduceren in de werking van een klinisch labo. Ik heb steeds vol bewondering opgekeken naar hoe u zo een groot team begeleidt, maar hierbij toch de tijd neemt om iedereen persoonlijk te kennen en te weten wat er in hun leven speelt. Daarnaast wil ik u ook bedanken voor uw suggesties tijdens de vele maandagochtend vergaderingen, uw oppeppende woorden wanneer een artikel weeral werd afgewezen, maar ook voor de middelen en de ruimte om mijn onderzoek zo optimaal mogelijk uit voeren.

Dr. Remco Koninckx en Dr. Jeroen Declercq, jullie hebben elkaar afgewisseld in het co-promotorschap, elk met jullie eigen unieke talenten. **Remco**, jij bent een beetje "de peter" van de CASCs. Tijdens het schrijven van mijn IWT aanvraag en het begin van mijn doctoraat kon ik steeds bij jou terecht met al mijn vragen. Je stond altijd klaar om "even" naar mijn cellen te kijken, samen wat kleuringen te bekijken, maar ook om mij wegwijs te maken in het ziekenhuis. **Jeroen**, later

nam jij de plaats van Remco in als copromotor en ook al was jij nog niet zo thuis in het onderzoek, toch stond jij steeds klaar om mijn data en bijhorende conclusies, kritisch te evalueren en mij te begeleiden in het schrijven van mijn artikels, alsook deze thesis.

Vervolgens wil ik graag het woord richten tot mijn commissieleden **Prof. Dr. Marc Hendrikx en Prof. Dr. Virginie Bito**. Jullie hebben van bij de start mijn onderzoek nauwgezet opgevolgd en jullie input en suggesties, onder meer tijdens de jaarlijkse vergaderingen, hebben zeker hun effect op deze thesis niet gemist. **Dr. Hendrikx**, ik heb zelden iemand zo gedreven als u ontmoet. De manier waarop u het onderzoek bekijkt en de drive waarmee u dit aanpakt zijn volgens mij uiterst uniek. Verder wil ik u ook bedanken voor uw input tijdens de vele maandagochtendvergaderingen en uw kritische blik bij het schrijven van de artikels. **Virginie**, j'apprécie énormément votre avis concernant la qualité de la recherche scientifique, ainsi que la façon dont vous gérez votre équipe. De plus, je voudrais vous remercier pour votre aide pendant la rédaction de notre article et thèse.

Ik neem ook graag van de gelegenheid gebruik om de voorzitter en leden van de doctoraatsjury, **Prof. Dr. Sven Hendrix, Prof. Dr. Erik Biessen, Prof. Dr. Olivier De Wever en Prof. Dr. Bert Smeets**, te bedanken voor het nalezen mijn proefschrift en hun bijhorende constructieve suggesties en bemerkingen. **Prof. Dr. Erik Biessen**, u was er al bij van bij de start, aangezien u ook zetelde in mijn IWT jury. Ik heb dan ook mede dankzij u dit project kunnen aanvatten. **Prof. Dr. De Wever**, uw kleine maar daarom niet minder belangrijke suggestie in verband met het gebruik van TGF-β tijdens onze meeting zo'n twee jaar geleden, heeft ervoor gezorgd dat we toch van start zijn kunnen gaan met fibrose onderzoek. Uw suggesties zijn steeds van belang geweest voor het CASC onderzoek en ik wil u dan ook nog eens extra bedanken voor al uw input, niet enkel binnen mijn project. **Prof. Dr. Bert Smeet**s, tijdens onze meeting vorig jaar werd duidelijk dat uw expertise onder meer in transcriptomics, maar ook in het opstarten van klinische studies, een belangrijke meerwaarde kan zijn voor het CASC onderzoek. In dat kader, mag deze thesis hopelijk de start betekenen van een vruchtbare samenwerking.

Verder wil ik ook graag **alle collega's van het klinisch laboratorium van het Jessa Ziekenhuis** bedanken, de klinisch biologen (in opleiding), de wetenschappelijk medewerkers en assistenten, iedereen van de coördinatie cel, de laboranten, de verpleegkundigen van bloedafname, de secretariaatsmedewerkers en al het ondersteunend personeel. Jullie stonden steeds klaar om mij te helpen en de opmerkingen en suggesties tijdens de verschillende wetenschappelijke stafvergaderingen hebben zeker hun meerwaarde gekend in dit onderzoek. Daarnaast wil ik jullie ook bedanken voor de deugddoende adempauzes, denk maar aan de jaarlijkse kerstfeestjes en kermisactiviteiten. Om zeker niemand te vergeten, ga ik er mij niet aan wagen om al jullie namen hier op te sommen, maar ik beloof bij deze wel plechtig dat als ik nog eens koekjes te veel heb, ik zeker aan jullie zal denken!

Binnen het klinisch laboratorium, bevindt zich ook de afdeling *Experimentele Hematologie*, en ondanks dat de naam misschien iets verder van het huidige topic verwijderd is, was dit wel mijn thuis de afgelopen 5 jaar. In de tussentijd zijn er wel heel wat mensen gekomen en gegaan, maar **Karen H., Loes, Remco, Annick, Yanick en Severina**, deze thesis is er ook vooral dankzij jullie gekomen. Ondanks de meest uiteenlopende karakters, stonden de neuzen steeds in dezelfde richting en stonden jullie steeds klaar om mij te helpen waar nodig. Het was natuurlijk niet enkel hard werk, jullie zijn ook stuk voor stuk hele mooie mensen die steeds voor mij klaar staan en ik hoop dan ook dat onze "EXH-lab meetings" nog lang mogen blijven duren!

Verder wil ik ook **alle (ex-)inwoners van Huis 81** bedanken voor hun wetenschappelijk input, motiverende woorden, maar ook de lekkere ijsjes in de zomer en de kerstfeestjes in de winter. **Liliane, Sandra, Ingrid, Yati, Liene, Karen G., Toon, Lien, Jolien, Gitte, Charlotte, Valentino en Laura,** ik wens jullie heel veel succes in alles wat jullie doen! Velen van jullie weten wat een doctoraat juist inhoudt, sommigen hebben dit hoofdstuk al afgerond, anderen staan nog maar aan het begin, maar ik ben er zeker van dat het voor ieder van jullie een onvergetelijke ervaring is of was! **Lien en Toon**, voor jullie begint het weldra te korten, maar in zo een supergezellige, goedlachse en humorrijke bureau gaat dat zeker goedkomen! Ik vind het wel jammer dat ik daar maar een paar maandjes van heb kunnen genieten!

Ook **alle medewerkers van de dienst cardiothoracale heelkunde**, de chirurgen (in opleiding), de perfusionisten, de verpleegkundigen en de secretariaatsmedewerksters, verdienen een woordje van dank. Zonder jullie waren er immers geen stalen en had ik dit onderzoek ook niet kunnen uitvoeren. Hierbij wil ik nog een apart woord van dank richten aan **Dr. Hendrikx, Dr. Dubois, Dr. Jamaer, Dr. Bijnens, Nic en Boris** om mij te introduceren in het klinisch aspect van de CASC-studie en voor hun bijdrage aan de proefdierstudie. Ook al zijn deze resultaten niet opgenomen in deze thesis, toch was het een unieke ervaring om ook hier bij betrokken te zijn.

Graag bedank ik ook **alle studenten** die de afgelopen 5 jaar van dichtbij of wat verder af betrokken zijn geweest bij dit onderzoek. Hier wil ik vooral **Greet** nog eens extra bedanken voor al haar inspanningen en harde werk, welke van grote bijdrage zijn geweest voor hoofdstuk 4.

Sinds kort ben ik echter begonnen aan een nieuw, maar toch ook nog vertrouwd, hoofdstuk in mijn carrière en ik wil dan ook graag **alle collega's van BIOMED** bedanken om mij zo welkom te laten voelen binnen BIOMED, maar ook om mij te steunen in deze eindfase van mijn doctoraat. **Niels**, als mijn nieuwe leidinggevende wil ik je bedanken voor deze unieke kans. **Kim**, wat jij allemaal doet binnen BIOMED is niet in een boek te beschrijven. Dankzij jou heb ik snel mijn weg kunnen vinden binnen BIOMED en wil ik je dan ook bedanken voor al je hulp hierbij! Ook bedankt aan mijn twee bureaugenoten **Helene en Stefanie**, maar jullie zijn meer dan dat aangezien jullie beiden ook een belangrijke rol hebben gespeeld in mijn doctoraat als respectievelijk coördinator van het LCRP en de doctoral school. Tot slot wil ik ook graag **Veronique** nog bedanken voor haar hulp en begeleiding tijdens de eindfase van mijn doctoraat.

Uiteraard wil ik ook graag mijn familie en vrienden bedanken om er steeds voor mij te zijn, niet enkel tijdens mijn doctoraat maar ook daarbuiten.

Je hebt "**de vrienden van de unief**" die ondertussen elk hun eigen weg zijn ingeslagen, maar aangezien er deze zomer weer een aantal vrijgezellendagen en trouwfeesten gepland staan, gaan we tijd genoeg hebben om nog eens gezellig bij te praten. Dan heb je ook nog "**de vrienden van Paal (en omgeving)**" waarbij we zo van die vaste tradities hebben zoals nieuwjaar, spelletjesavond, spelletjes weekend en de nodige zomerse barbecues om de zinnen al eens te

verzetten, maar die ook steeds alle begrip hadden als ik er eens een keertje niet kon bij zijn. Verder heb je ook nog "**de collega vrienden**". Hier heb ik al eerder het clubje van EXH aangehaald, maar daarnaast heb je ook nog Eveline, Caroline, Veerle & co die mij niet enkel bijstonden in het labo, maar mij ook op tijd en stond eens naar buiten sleurden voor een cocktailfeestje of de jeneverfeesten. De laatste weken heb ik jullie soms een beetje verwaarloosd, maar ik beloof dit deze zomer goed te maken! Tot slot is er natuurlijk nog **ons Sara** (en Jonathan), ondertussen al 16 jaar, door dik en dun, staan we altijd klaar voor elkaar. Bedankt Sara, om er altijd te zijn, vooral op die momenten dat het echt nodig is!

Tot slot wil ik graag **mijn ouders, grootouders, zussen, schoonbroers en de rest van mijn familie** bedanken voor hun interesse in mijn doctoraat, maar vooral ook om er altijd voor mij te zijn (al is dat voor sommigen helaas enkel nog in mijn hart). **Mama en papa, Lies en Lotte**, ook al hadden jullie soms geen idee waarmee ik bezig was, toch waren jullie altijd oprecht bezorgd wanneer jullie zagen dat het allemaal niet liep zoals ik wou en vaak een beetje kort was in mijn antwoord. We zouden kunnen zeggen dat dit nu voorbij zal zijn, al veronderstel ik dat er snel wel weer een nieuwe uitdaging voor ons zal klaar staan, trouwen (niet ik hoor, maar toch), verhuizen (deze zomer minstens twee, oh help) en waarschijnlijk ook nog wel wat kleine "onverwachtheden" op de boerderij. Nu wat er ook gebeurt, zolang we maar samen zijn en een beetje geduld hebben met elkaar, komt dat zeker wel goed. Het verleden heeft dat immers al vaker bevestigd!

“When you have exhausted all possibilities, remember this: you haven't.”

Thomas Alva Edison