From the DEPARTMENT OF CELL AND MOLECULAR BIOLOGY
Karolinska Institutet, Stockholm, Sweden

STUDIES ON MYOCARDIAL REGENERATION

Olaf Ingmar Bergmann

Karolinska Institutet

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Front cover: Incorporation of the nucleotide analogue BrdU (red) into murine cardiomyocyte nuclei (Nkx2.5, white), indicating myocardial renewal after heart infarction. Cardiomyocytes are labelled with an antibody to cardiac myosin heavy chain (green).

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Don't worry about your heart, it will last you as long as you live.
W. C. Fields

ABSTRACT

Heart disease is one of the leading causes of adult and child morbidity and mortality. The underlying pathology leads typically to a loss of functional cardiomyocytes that causes heart failure. Because of the insufficient regenerative capacity of the human heart, cardiomyocytes have been thought to be incapable of renewing after the postnatal period.

In Paper I, we investigated the capacity of the human heart to generate cardiomycytes. We have taken advantage of the integration of the carbon isotope ^{14}C (carbon-14), generated by nuclear bomb tests during the Cold War, into DNA to establish the age of cardiomyocytes in humans. Using cardiac Troponin T and I and pericentriolar protein 1 (PCM-1) as a specific marker to isolate cardiomyocyte nuclei by flow cytometry (Paper I and II). We report that cardiomyocytes renew, with a gradual decrease from 1% turning over annually at the age of 25 to 0.45% at the age of 75. Fewer than 50% of cardiomyocytes are exchanged during a normal life span. The capacity to generate cardiomyocytes in the adult human heart suggests that it may be rational to work toward the development of therapeutic strategies aimed at stimulating this process in cardiac pathologies.

After cardiac infarction the formation of inappropriate scar tissue and cardiac remodeling further contribute to cardiac dysfunction. We provide evidence in Paper III, that inhibition of PDGF signalling reduces scar formation and an augmentation of cardiomyogenesis modulated by increased neoangiogenesis.

These findings points to the possibility to therapeutically exploit physiological cardiomyocyte renewal by better understanding processes that modulate cardiac regeneration after heart infarction.

LIST OF PUBLICATIONS

I. **Bergmann O***, Bhardwaj, RD*, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J. Evidence for cardiomyocyte renewal in humans. Science. 2009.324(5923):98-102.

II. **Bergmann O,** Zdunek S, Alkass K, Druid H, Bernard S, Frisén J. Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell turnover. Exp Cell Res. 2010 Sep 7. [Online ahead of print]

III. **Bergmann O**, Flores J, Röst P, Röll W, Jovinge S, Frisén J. Inhibiting Platelet-Derived Growth Factor (PDGF) signaling reduces scar formation and increases cardiomyogenesis after myocardial infarction in mice. Manuscript. 2010

Publications not included in this thesis:

I. Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, **Bergmann O**, Blomqvist L, Hoffstedt J, Näslund E, Britton T, Concha H, Hassan M, Rydén M, Frisén J, Arner P. Dynamics of fat cell turnover in humans. Nature. 2008 Jun 5;453(7196):783-7.

II. Das D, Lanner F, Main H, Andersson ER, **Bergmann O**, Sahlgren C, Heldring N, Hermanson O, Hansson EM, Lendahl U. Notch induces cyclin D1-dependent proliferation during a specific temporal window of neural differentiation in ES cells. Dev Biol. 2010 Sep 28. [Online ahead of print]

III. Barnabé-Heider F, Meletis K, Eriksson M, **Bergmann O**, Sabelström H, Harvey MA, Mikkers H, Frisén J. Genetic manipulation of adult mouse neurogenic niches by in vivo electroporation. Nat Methods. 2008 Feb;5(2):189-96.

*the authors contributed equally

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LIST OF ABBREVIATIONS

ACE	angiotensin-converting enzyme
AMS	accelerator mass spectrometry
Ang-1	angiopoietin 1
bFGF	basic fibroblast growth factor
BM-MC	bone marrow mononucleated cell
BrdU	5-bromo-2-deoxyuridine
Carbon-14	Radioactive isotope carbon-14
CPC	adult cardiac progenitor cell
cTroponin I	cardiac Troponin I
cTroponin T	cardiac Troponin T
EF	ejection fraction
EPC	endothelial progenitor cells
FHF	first heart field
hESC	human embryonic stem cell
hESC-CM	human embryonic stem cell-derived cardiomyocyte
IdU	5-iodo-2-deoxyuridine
IGF-1	insulin-like growth factor 1
Il-1β	interleukin-1 beta
iPS	induced pluripotent stem cell
LV	left ventricle
NC	cardiac neural crest
NCID	notch intracytoplasmatic domain
OFT	outflow tract
PCM-1	pericentriolar protein 1
PDGF	platelet derived growth factor
PDGFRα	platelet derived growth factor receptor alpha
PDGFRβ	platelet derived growth factor receptor beta
PDGFRβ mab	platelet derived growth factor receptor beta inhibiting monoclonal antibody
RV	right ventricle
SHF	second heart field
SMA	smooth muscle actin
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell

1 INTRODUCTION

Biology of Cardiomyocytes

The human heart is comprised of approximately 2-6 billion cardiomyocytes (Grajek et al., 1993; Olivetti et al., 1995; Olivetti et al., 1991), which contract synchronously between 60 and 90 times per minute to pump blood through the circulation system of our bodies. The final number of cardiomyocytes was shown to be already reached after the postnatal period (Mayhew et al., 1997). During normal aging the number of cardiomyocytes decreases in male hearts at a rate of 64 million per year which is accompanied by an increase in cardiomyocyte volume, whereas the female heart neither showed changes in cell number nor heart weight (Olivetti et al., 1995). This morphometric finding is supported by the detecting of rare events of cardiomyocyte apoptosis (Mallat et al., 2001; Olivetti et al., 1997). In contrast to most other cell types only 74% of all human cardiomyocytes are mononucleated, 25.5% are binucleated, and 0.5% contain even more than two nuclei, these ratios stay constant during adulthood and in hypertrophy (Olivetti et al., 1996). Human cardiomyocytes have the physiological property to synthesize DNA without undergoing cellular division. This phenomenon is called polyploidisation and occurs in a short time period between the age of 4 to 12 (Adler, 1991; Brodsky et al., 1994; Herget et al., 1997). Polyploidisation gives rise to cardiomyocyte nuclei that are in average 33.4% diploid, 55.8% tetraploid and 10.7% octaploid (Paper I and II). During pathological processes cardiomyocytes further increase the DNA content per nuclei up to 32n ploidy level (Adler and Friedburg, 1986; Meckert et al., 2005). A recent study suggested that this might be reversible after hemodynamic support of the left ventricle (ventricular assist device) in failing human hearts (Rivello et al., 2001; Wohlschlaeger et al., 2010).

For decades cardiomoycytes were seen as post-mitotic, not able to proliferate after the neonatal period in order to replace dead myocardium, or adapting to mechanical stress (Soonpaa and Field, 1997). This view was further supported by the insufficient regeneration capacity of myocardium after cardiac injuries. Contradictory to this opinion some investigations found an increase in the number of cardiomyocytes in hypertrophied hearts (Grajek et al., 1993; Olivetti et al., 1996). Mitotic figures in cardiomyocytes indicating replication of DNA were found in healthy hearts at a frequency of 0.0011% and at a frequency of 0.0131-0.052% after cardiac infarction and in end-stage cardiac insufficiency (Beltrami et al., 2001; Kajstura et al., 1998). Because

of the ability of human cardiomyocytes to ploidize these data need to be interpreted with caution. Mitotic figures or KI-67 positive cardiomyocyte nuclei might reflect endomitosis instead of cardiomyocyte duplication (Meckert et al., 2005). Furthermore, several authors reported chimerism in the human heart after sex-mismatch transplantations, indicating transdifferentation of circulating host cells into cardiomyocytes (Bayes-Genis et al., 2005; Laflamme et al., 2002; Quaini et al., 2002; Rupp et al., 2008; Thiele et al., 2004). However, transdifferentiation might not be the only mechanisms to explain chimersims, as bone marrow cells can fuse with cardiomyocytes to heterokaryons *in vivo* (Alvarez-Dolado et al., 2003; Nygren et al., 2004). Apart from these pieces of evidence, reports introducing the concept of resident stem cell niches in the heart challenged the concept of the non-regenerating heart (Bearzi et al., 2007; Beltrami et al., 2003).

A Brief Insight into Heart Development

The formation of the four-chambered heart requires diverse cell types with specialized functions. Cardiomyocyte precursor give rise either to atrial or ventricular cardiomyocytes or differentiate into cells of the conduction system with distinct phenotypes and functions (Mikawa, 1999).

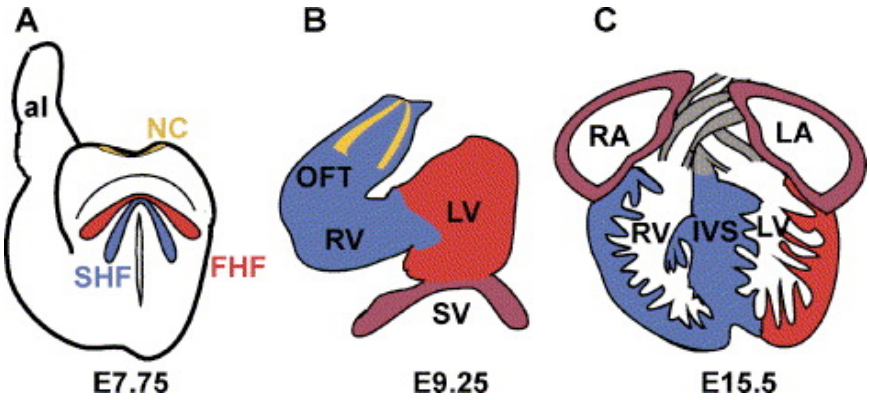

Figure1. Three stages of mouse heart development. (A) Three major populations of cells contribute to the developing heart and outflow tract. The first heart field (FHF) is depicted in red; the second heart field (SHF), is depicted in blue; and cardiac neural crest (NC) progenitors are shown at the dorsal neural tube in yellow. (B) Heart undergoing bulboventricular looping. The FHF (red) contributes to the left ventricle. The SHF (blue) contributes primarily to the right ventricle (RV) and outflow tract (OFT). Two streams of cardiac neural crest cells entering the outflow tract are depicted in yellow. (C) Almost mature embryonic heart. The free wall and majority of the left ventricle (LV) chamber is composed almost exclusively of cells derived from the FHF (red). The RV and interventricular septum (IVS) are composed primarily of cells descended from the SHF. The atria may be derived from a mixture of cells of FHF and SHF origins and the OFT is composed of cells of both NC and SHF origin. al, allantois; LA, left atrium; RA, right atrium. Reprinted with the permission from the publisher (Black, 2007). http://dx.crossref.org/10.1016%2Fj.semcdb.2007.01.001

Vascular progenitors give rise to endothelium and vascular smooth muscle cells.

Studies have demonstrated that two main progenitor cell population ("heart fields") in heart development exists in the mesoderm, which segregate from a common progenitor cell at gastrulation (Abu-Issa et al., 2004). The primary heart field located in the anterior splanchnic mesoderm is comprised of the earliest population of progenitor cells, which contributes to the left ventricle and atria after the bulboventricular looping (Figure 1). A second source of cardiac progenitors is referred to as secondary heart field derived from the pharyngeal mesoderm, which gives rise to right ventricle and outflow tract (Moretti et al., 2006) (Figure 1). Both progenitor populations show a typical expression pattern of transcription factors.

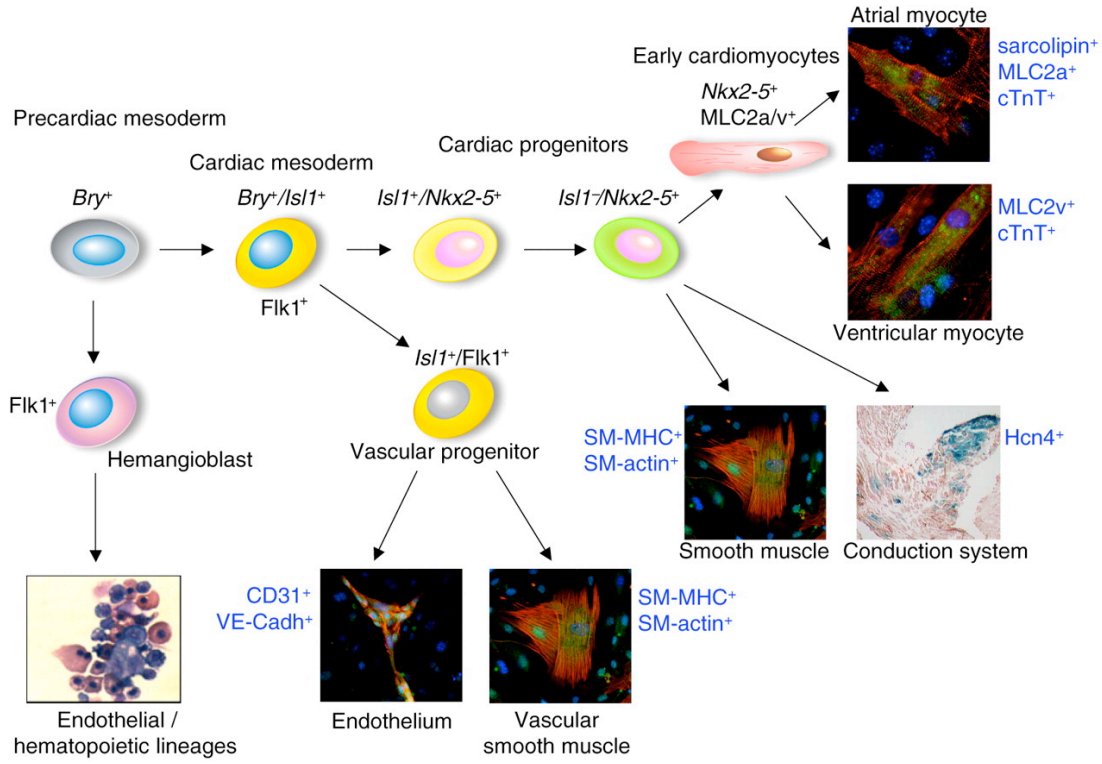

Figure 2. Proposed hierarchical progenitor model in the second heart field. Reprinted with the permission from the publisher (Laugwitz et al., 2008). http://dx.doi.org/10.1242/dev.001883

The primary heart field is defined by expression of T-box transcription factor Tbx5 and the bHLH transcription factor Hand1 and Nkx2.5, whereas Hand2, the LIM-homeodomain transciption factor Isl1, Fgf10 and Nkx2.5 mark the secondary field (Cai et al., 2003; Kelly et al., 2001). Recently, it was shown that the epicardium harbors Tbx18 expressing cardiac progenitor cells, which can give rise to cardiomyocytes, smooth muscle cells and cardiac fibroblasts in the ventricular septum and the atrial and ventricular walls (Cai et al., 2008). This finding was however questioned since it could be shown that Tbx18 is expressed also in the myocardium, indicating that the used

genetic fate mapping system does not allow any conclusions of an epicardial origin of cardiomyocytes *in vivo* (Christoffels et al., 2009). The transciption signature Isl1+/Bry+/flk1+ seems to mark progenitor cells, which are not only restricted to the myocyte lineage, but also generate smooth muscle and endothelial cells and therefore might act as multipotent primordial embryonic progenitor (Moretti et al., 2006) (Figure 2).

Adult Cardiac Progenitor Cells (CPCs)

In contrast to embryonic heart development the lineage of adult cardiac progenitors has not been determined. Isl1+ progenitor cells could be shown to be still present in neonatal heart where they act as multipotent progenitors in vitro and in vivo (Laugwitz et al., 2005). These cells populate the postnatal rodent and human heart at a low frequency. Although they are present in all parts of the postnatal heart, they reside preferentially in structures derived from the secondary heart field such as the outflow tract (198 per rat heart), atria (225 per rat heart) and right ventricle (67 per rat heart), indicating that these cells are developmental of the fetal Isl1+ progenitor (Laugwitz et al., 2005). Since Isl1+ progenitor cells disappear shortly after birth these cells cannot contribute to functional cardiomyocytes during adulthood. A recent report could show that Isl1+ cells might even be present in the outflow tract of adult rats (Genead et al., 2010).

Cardiac progenitor cell (CPC)	**Marker profile**	**Differentiation capacity (based on marker profile)**
Side population	ABCG2+, sca1+, c-kit+, CD34+, CD31-, CD45-, GATA4+, Mef2c+, Nkx2.5+	Cardiomyocytes Smooth muscles Endothelial cells
c-kit+	c-kit+, lin-, CD45-,GATA4+, GATA5+, MRF2c+,Nkx2.5+	Cardiomyocytes Smooth muscles Endothelial cells
Sca1+	Sca1+, ckit+, CD34+, CD45+, CD31+, Gata4+, MEf2c+, TEF-1+	Cardiomyocytes Smooth muscles Endothelial cells
cardiospheres	c-kit+, sca1+, CD34+, CD31+	Cardiomyocytes Endothelial cells
SSEA-1	SSEA+, neonatal Nkx2.5+ and GATA4+ Adult OCT3/4+	Cardiomyocytes Smooth muscles Endothelial cells

Table1. Characterization of adult resident cardiac progenitor cells

Adult cardiac progentior cells (CPCs) promise great therapeutic potential, however in order to exploit their capabilities, first one needs to understand their nature. Based on

established hematopoetic stem cell profiles, cell surface markers and properties were used to identify adult cardiac progenitors (CPCs): side population (Martin et al., 2004; Oyama et al., 2007), c-kit+ (Beltrami et al., 2003; Dawn et al., 2005), sca1+ (Oh et al., 2003; Tateishi et al., 2007), SSEA1+ (Ott et al., 2007) and "cardiopheres" derived CPCs (Messina et al., 2004; Smith et al., 2007) (Table1). CPCs populate the heart at a low frequency (1 per 40000 cardiac cells in mouse (Hosoda et al., 2009)). In order to replace dying myocardium CPCs need to be activated, expanded and directed to the site of injury. One critical question is whether in the course of disease the ability of CPCs to proliferate and differentiate is compromised in the course of disease. A study by Urbanek et al. could show that CPCs in chronic ischemic heart disease show shorter telomeres and higher fraction of CPCs express p16ink4 protein and undergo apoptosis (Urbanek et al., 2005). This response could possibly be mediated by activation of the insulin-like growth factor-1 (IGF-1) signalling pathway, which promotes cell proliferation and survival while counteracting aging and senescence. To counteract aging and senescence IGF-1 signalling pathway, which is involved in cell proliferation and survival seems to be a good candidate. It could be shown that an activation of the IGF-1 system preserves telomere length by increasing telomerase activity and promotes growth and survival in CPCs (Torella et al., 2004). Injection of CPCs along with IGF-1 bound to self-assembling peptide nanofibres, in the infarct border zone leads to higher cardiomyogenesis and neoangiogensis (Padin-Iruegas et al., 2009). Furthermore, Boni et al. could show that the Notch1 receptor is expressed in approx. 60% of freshly extracted c-kit+ cells (Boni et al., 2008). The Notch pathway is an evolutionary conserved intercellular and intracellular signalling system (Bray, 2006), which controls cell fate during embryogenesis and in adult stem cell niches in the brain (Carlén et al., 2009), skin (Estrach et al., 2006) and bone marrow (Calvi et al., 2003). Up to today four notch isoforms have been discovered. Upon binding of notch ligands the notch intracytoplasmatic domain (NCID) is cleaved and translocates to the nucleus where it forms a complex with the DNA recombinant binding protein RBP-Jk, which loses its repressor function and allows transcription of downstream targets including the Hes protein family (Iso et al., 2003). Treatment with the notch ligand Jagged1 led to an increase in CPC (c-kit+ cells) proliferation and increased the Nkx2.5 promotor activity by binding of RBP-Jk (Boni et al., 2008; Urbanek et al., 2010). Since Nkx2.5 is expressed in mature cardiomyocytes this indicates that Notch-signaling promotes cardiomyocyte differentiation. In contrast, injection of a γ-secretase inhibitor after cardiac infarction led to a reduction of CPCs in the border zone and of BrdU+

cardiomyocytes by 50% (Boni et al., 2008). Although CPCs have the potential to differentiate into cardiomyocyte and cardiomyocyte-like cells *in vitro* and *in vivo*, to this day, there is no consensus to what extent these cells can give rise to new cardiomyocytes in homeostasis and in cardiac injuries. A study using a transgenic c-kit:EGFP mouse suggested that ckit+ cells can give rise to all three main cell types in the postnatal heart, but failed to generate new cardiomyocytes after cryo-injury in adult mouse hearts (Tallini et al., 2009). A fate-mapping study utilizing a MHC-CreER transgenic mouse could show that new cardiomyocytes, derived from a progenitor cell pool, populate the heart after cardiac infarction and pressure overload, but could not be detected in uninjured adult hearts (Hsieh et al., 2007). This study design however does not rule out that cardiomyocytes can be renewed by self-duplication as shown by (Bersell et al., 2009). By carbon dating we could show that cardiomyocytes in humans are renewed throughout life (Paper I). However carbon-14 dating does not allow for discrimination between self-duplication and derivation from a CPC pool. Fate mapping studies are needed to characterize the lineage and potential of CPCs in the adult animal and human heart (Salipante and Horwitz, 2007).

Cell transplantation strategies

Myocardial infarction often leads to chronic heart failure due to loss and insufficient regeneration of cardiomyocytes. This has prompted efforts to restore cardiomyocyte mass by various cell transplantation strategies. Orlic et al. described in 2001 that bone marrow stem cells can transdifferentiate into mature cardiomyocytes after cardiac infarctions (Orlic et al., 2001). Although this finding was questioned by several studies soon after (Murry et al., 2004; Nygren et al., 2004), clinical trials were initiated to investigate the effect of bone marrow mononucleated cells (BM-MC) or endothelial progenitor cells (EPC) on myocardial regeneration after cardiac infarction and in chronic ischemic heart disease. Two randomized human trials with intracoronary injection of BM-MCs or EPCs indicated a positive effect on cardiac contractile function (Assmus et al., 2006; Schachinger et al., 2006), whereas another study failed to find any benefits (Lunde et al., 2006). Although the exact mechanism of action remains unclear (Dimmeler et al., 2008) a body of evidence suggest that BM-MC- and EPC-mediated improvement of neoangiogenesis modulates the positive clinical outcome after intracoronary infusion treatment. In addition to paracrine factors released by BM-MCs, e.g. basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), interleukin-1 beta (IL-1β), and tumor

necrosis factor-alpha (TNF-α) that stimulate angiogenesis (Kamihata et al., 2001), experimental studies could show that selective depletion of infused progenitor cells committed to the endothelial lineage also contributed to microvessel formation and improved clinical parameters (ejection fraction (EF)) (Yoon et al., 2010).

Another possible grafting strategy could be to isolate CPCs, expand them *in vitro* and re-infuse them into the myocardium or coronary vessels. The first pre-clinical studies were initiated using larger animal models to establish feasibility, safety and efficacy on the way to human CSC transplantation trials (Smith et al., 2008). Although several independent groups could show the existence of CPCs in different species including humans, it remains difficult to harvest them and only a subset might be capable of differentiating into mature cardiomyocytes.

Using Embryonic stem cells (ESCs) is also an option, which could provide a theoretical unlimited source of cells. Human ESC (hESC) can be differentiated into cardiomyocytes (hESC-CM) and survive several months after injection in rodent models of cardiac infarctions (Laflamme et al., 2007). Transplantation of hESC-CM exerts a beneficial effect on heart function, e.g. preservation of ejection fraction and fractional shortening and attenuation of adverse cardiac modeling (Laflamme et al., 2007; Leor et al., 2007). A major limitation in the clinical usability of hESCs is their potential to form tumors, including teratomas. Directed differentiation of hESCs in post-mitotic cells such as hESC-CM are promising first steps from bench-to-bedside. However long-term safety studies are still lacking.

In addition to the selection of cell type, an optimal timing for grafting procedures seems to be crucial. Delayed grafting (four weeks after cardiac infarction) of hESC-CM grafted in a rat model of cardiac infarction showed no beneficial effect on heart function, although the transplanted cells engrafted and survived in the scar tissue (Fernandes et al., 2010). This finding does not fit with the assumption that hESC-CM mediate their effect through grafted functional myocardium in the host heart. However, this data is in line with clinical BM-MCs grafting studies that showed a better cardiac function after acute and sub-acute BM-MCs treatment compared to late treatment (>4 weeks after infarction) (Ter Horst, 2010). Although the different cell sources are not comparable in terms of their differentiation potential, it seems that released paracrine factors that promote angiogenesis, prevent apoptosis and modulate the immune response do not find a responsive environment to elicit their effects in matured scar tissue (see chapter Myocardial Remodeling).

Induced pluripotent stem cells (iPS) with ES-like features can be generated by over-

expressing four defined factors (c-myc, Oct4, Klf4 and Sox2) in several cell types of choice, including fibroblasts (Takahashi and Yamanaka, 2006). Patient specific iPS generation could overcome cellular rejection issues of transplanted hES-CM (Zhang et al., 2009). The heart is comprised of over 50% fibroblasts (Snider et al., 2009) and after cardiac injuries these cells proliferate at a high rate and contribute to scar formation This makes fibroblasts an attractive source of cells for reprogramming. Ieda et al. have recently shown that Gata4, Mef2c and Tbx5 are sufficient to directly reprogram, circumventing a stem cell state, cardiac fibroblasts into cardiomyocytes (Ieda et al., 2010). These cells, called induced cardiomyoctes expressed mature myocyte markers, showed spontaneous contraction pattern and Ca^{2+} oscillations similar to neonatal cardiomyocytes. Although this study was not performed with human fibroblasts, it opens up the possibility to infuse intracoronary defined factors, inducing *in situ* reprogramming of scar tissue into functional myocardium after cardiac injury.

Myocardial Remodeling

Myocardial infarction induces a number of cellular and structural changes (cardiac remodeling). Cardiac remodeling after cardiac infarction is generally divided into three phases: inflammatory, proliferative and maturation phase (Dobaczewski et al., 2010; Frangogiannis, 2008). Coronary artery occlusion causes cardiomyocyte necrosis, the complement system is activated and free radicals are released. In mice, neutrophils, macrophages and other inflammatory cells are recruited to the infarction site within the first 24h after infarction, clearing the necrotic debris. Cytokines released during the inflammatory phase promote the transition to the proliferative phase (days 2 to 5 after infarction in rodents), in which the necrotic tissue is replaced by granulation tissue. Granulation tissue consists, not only of the remaining inflammatory cells, but also of other cell types responsible for angiogenesis and fibrous tissue deposition. This phase is characterized by a high degree of cellularity in the infarcted area, with myofibroblast and endothelial cell proliferation peaking at day 4 post-infarction (Virag and Murry, 2003). Myofibroblasts are the collagen-producing cells in the infarct. Several cell types, including resident fibroblasts, circulating fibrocytes, endothelial cells, pericytes and bone marrow cells (Bucala, 2008; Humphreys et al., 2010; Minami et al., 2005; Porter and Turner, 2009; Zeisberg et al., 2007), have been proposed to give rise to myofibroblasts in different tissues. They express contractile proteins such as smooth muscle actin (SMA), and are recruited upon injury. One hallmark of the maturation

phase is the collagen deposition, caused by myofibroblasts. The collagen, characterized by matrix cross-linking, makes up the mature scar (van den Borne et al., 2010). Fibrous tissue that forms at the site of cardiomyocyte loss has an ambiguous function. It preserves structural integrity but also creates patches that cannot contribute to contraction. It also contributes to cardiac dysfunction by inducing interstitial fibrosis of viable myocardium (Jugdutt, 2003). Limiting the scar formation after cardiac infarction by inhibiting the intracellular Wnt-signaling pathway have been shown to reduce apoptosis of cardiomyocytes and to promote survival of injected stem cells, leading to an improved functional outcome (Kobayashi et al., 2009). Due to the insufficient regeneration of functional myocardium after cardiac infarctions, late remodeling might occur, with an expansion of the scar into non-infarcted area, ventricular wall thinning and remote ventricular wall hypertrophy (Weisman et al., 1988). The consequence is a right and left ventricular dysfunction leading to congestive heart failure. Drug therapies aiming to prevent adverse remodeling already exist. Angiotensin-converting enzyme (ACE) and angiotensin type1 receptor blocker inhibiting the renin-angiotensin-aldosterone system, show a significant survival benefit in patients with ischemic heart disease (Pfeffer et al., 1988; Solomon et al., 2005).

PDGF signaling and pericytes in scar formation

The complex scar formation process involves several different cell types and even more diverse signaling pathways. Platelet-derived growth factor (PDGF) is a cytokine that plays an important role in the modulation of scar tissue in several organ systems including the heart (Ponten et al., 2005; Rajkumar et al., 2006; Simm et al., 1998; Tuuminen et al., 2009).

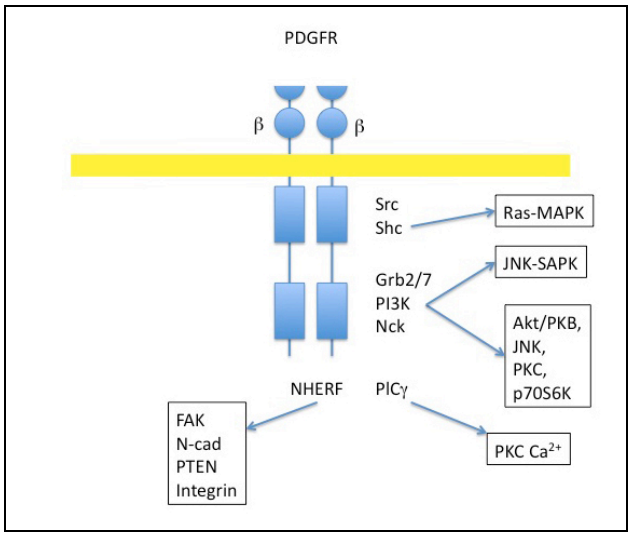

Figure 3. PDGFRβ signal transduction. PDGFRβ with intracellular domains and direct interactors. Arrows indicate interaction to major signaling systems; adopted from (Andrae et al., 2008).

PDGF exists in at least 5 isoforms, which bind to two structurally and functionally related receptors, PDGFRα and PDGFRβ, with different affinities. Dimerization and autophosphorylation occur upon ligand binding, resulting in activation of different intracellular signaling pathways (Ras-MAPK, PI3K or PLCγ), mainly through adaptor proteins (e.g. Src, Grb2/7), leading to cell growth, proliferation, chemotaxis and differentiation (Heldin and Westermark, 1999) (Figure 3).

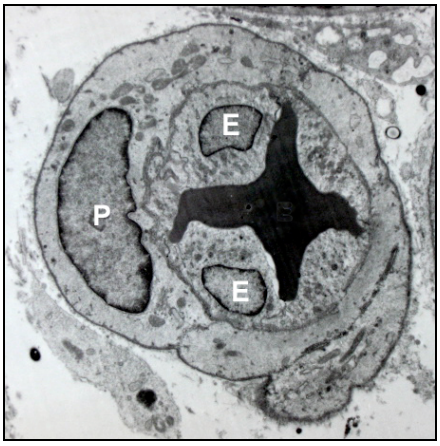

Figure 4. Electron microscopy image showing a microvessel with endothelial cells (E), pericyte (P) and lumen with an erythrocyte.

PDGF signaling is mediated via PDGF-alpha and -beta receptors. Zymek *et al.* observed an intense expression of PDGFRα and PDGFRβ in mouse infarct scars, within the first week after infarction. Inhibiting PDGFR signaling led to a decrease of collagen deposition in the scar and to an increase in capillary density accompanied by impaired vessel maturation (Zymek et al., 2006).

Notch signaling seems to directly interact with the PDGF signaling system. In the Notch3-deficient mouse, PDGFRβ expression is downregulated in vascular smooth muscle cells (VSMC) (Jin et al., 2008). *Vice versa* Notch activation leads to an overexpression of PDGFRβ in VSMC, indicating that Notch acts as an immediate upstream regulator of PDGFRβ. This finding establishes the involvement of Notch in PDGF signaling and suggests a possible implication in PDGFRβ mediated pericyte functions.

Pericytes specifically express PDGFRβ in the heart, which are branched cells covering endothelial cells being essential for modulating angiogenesis and vessel maturation. Pericytes are embedded within the microvascular basement membrane and partially enveloping endothelial cells (Figure 4). Pericytes represent a heterogeneous population of cells, which are developmentally derived from mesodermal origin, while in the CNS and the cardiac tract pericytes originate from neural crest. The diversity of pericytes is also reflected by various marker profiles, e.g. NG2 chondroitin sulfate proteoglycan, CD13, desmin, PDGFRβ and alkaline phosphatase (Díaz-Flores et al., 2009). Pericytes play a role in a number of physiological and pathological repair processes such as vessel stabilization, angiogenesis, regulation of vascular tone, synthesis of matrix proteins, macrophage-like properties and activity in immunologic defense, intervention in coagulation and mesenchymal progenitor potential (Crisan et al., 2008; Díaz-Flores et al., 2009). Pericytes closely interact with endothelial cells during angiogenesis. PDGF-B is released by the sprouting endothelium, while PDGFRβ expressing pericytes are recruited and lead to a maturation of the newly formed vessels. PDGFRβ or PDGF-B deficiency leads to perinatal lethality due to microvascular leakage and hemorrhage (Gerhardt and Betsholtz, 2003).

Recently, reports have indicated that pericytes seem to be also capable to contribute to scar formation by transforming into fibroblasts and myofibroblast, the main source of collagen in the infarcted scar tissue, in different organ systems including kidney and skin (Humphreys et al., 2010; Rajkumar et al., 2006). However, direct evidence provided by fate-mapping studies that pericytes can give rise to scar forming cells in the heart is still lacking.

Establishing Human Cardiac Renewal by Carbon-14 dating

Studying human cellular turnover in slowly dividing tissues is difficult. Detecting proteins expressed during the cell cycle such as KI-67 and PCNA only allows a quantification of dividing cells at the time of the staining and therefore it is not possible to draw any conclusions with regard to future fate or survival. Infusion and consecutive detection of nucleotide analogues such as 5-bromo-2-deoxyuridine (BrdU) or 5-iodo-2'-deoxyuridine (IdU) by specific antibodies allows determining turnover rates by applying mathematical modelling (Paper II). Since nucleotide analogues are toxic and potential cancerous only limited material from cancer patients is available for analysis. Most patients were treated with nucleotide analogues as radiosensitizer along with radiotherapy, which makes it difficult to study healthy subjects.

Therefore, we have developed a technique using carbon-14 from nuclear bomb tests in genomic DNA of humans to retrospective birth-date distinct cell populations in the human body (Paper I) (Spalding et al., 2008; Spalding et al., 2005a; Spalding et al., 2005b).

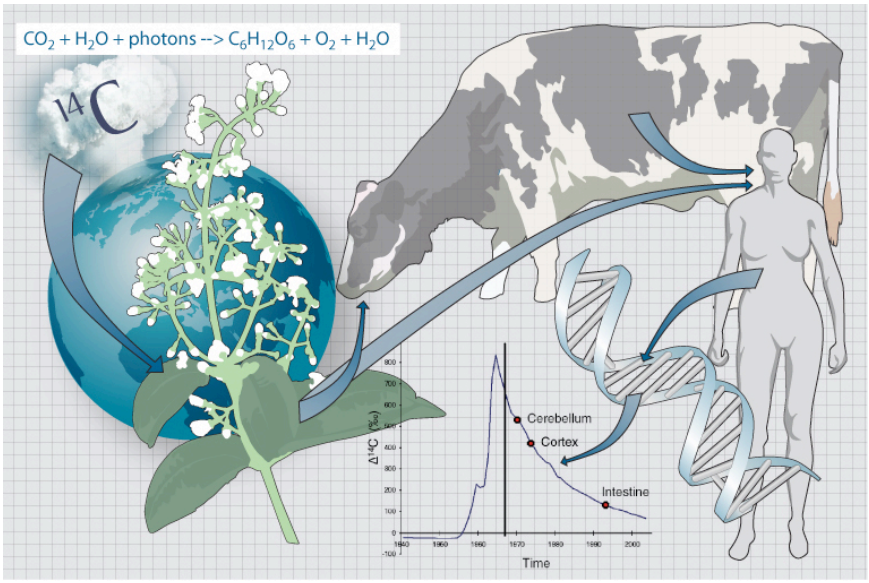

Figure 5. Schematic illustration of the carbon-14 cycle. ©Mattias Karlen

Carbon-14 exists in the atmosphere naturally produced through cosmic ray interactions with nitrogen. Normal atmospheric levels of the radioactive isotope carbon-14 are extremely low, however atmospheric thermonuclear-bomb testing in the late 1950s and early 1960s dramatically increased atmospheric levels of carbon-14 (Nydal and Lovseth, 1965). Atmospheric testing ended in 1963 (Limited Test Ban Treaty) and as a

result atmospheric levels of carbon-14 dropped exponentially since, not primarily because of radioactive decay (half-life of 5730 years), but by diffusion from the atmosphere (Levin and Kromer, 2004). Carbon-14 in the atmosphere reacts with oxygen and forms CO_2, which enters the biotope through photosynthesis. Our consumption of plants, and of animals that live off plants, results in carbon-14 levels in the human body paralleling those in the atmosphere. With the unique exception of genomic DNA, which is not exchanged after a cell has gone through its last division (Figure 5). Nucleotide exchange in postmitotic cells is minimal and is below the detection level of this technique (Bhardwaj et al., 2006). The level of carbon-14 integrated into genomic DNA thus reflects the level in the atmosphere at any given time and can be used to retrospectively establish the birth date of cells in the human body.

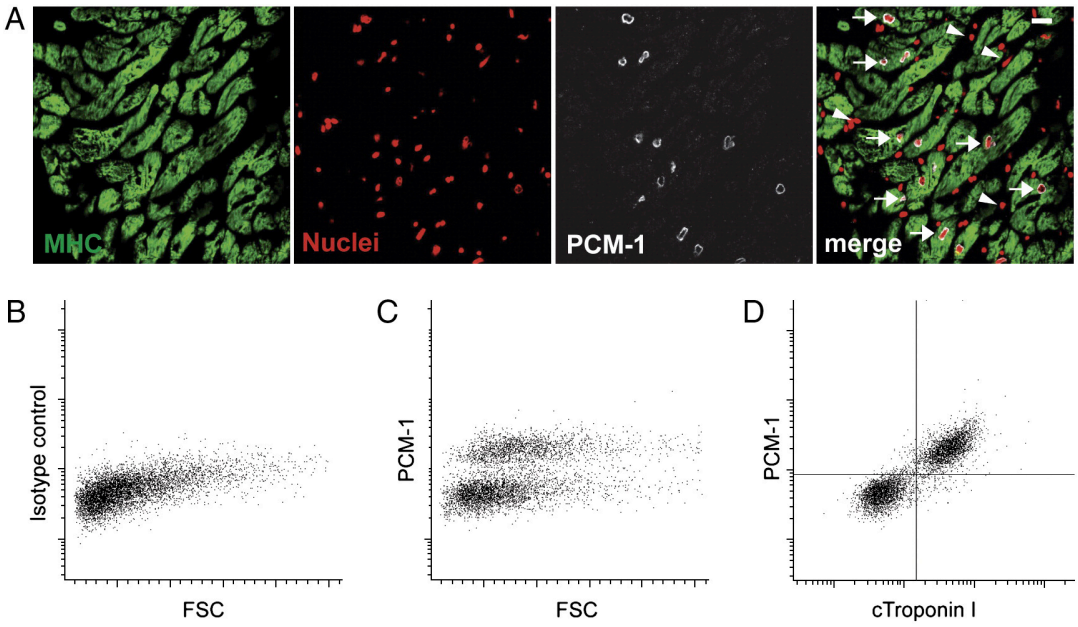

Figure 6. Cardiomyocyte nuclei sorting strategy.
(A) PCM-1 is localized to the nuclear membrane of cardiomyocytes (green). (B and C) Representative flow cytometry analysis of human cardiac nuclei with isotype control. (D) PCM-1 antibody labels nuclei which express cTroponin I, a specific marker for cardiomyocytes. Reprinted with the permission of the publisher (Paper II) http://dx.doi.org/10.1016/j.yexcr.2010.08.017

Given the fact that cardiomyocytes constitute only about 20% of all cells in the human myocardium, we have developed methodology to isolate cardiomyocyte nuclei by fluorescence-activated cell sorting using antibodies against cardiac specific troponins and PCM-1 (Figure 6). Since a substantial proportion of cardiomyocytes are

binucleated and polyploid sorting on nuclei rather than whole cells further allows us to separately analyze different ploidy levels (Paper I and II).

Accelerator mass spectrometry (AMS) is an ultra-sensitive technique for isotopic ratio measurements, which allows radiocarbon dating of biological samples in the range of micrograms (Salehpour et al., 2008). Once the DNA carbon-14 value has been measured by AMS, the value is read off a plot of known atmospheric carbon-14 values (as a function of time) to determine the age of the DNA (Figure 7).

The sensitivity for detecting cellular turnover depends on when the person was born in relation to when the new cells were generated. In an individual born just before the nuclear bomb tests, the relative difference in carbon-14 concentration is highest between the time of birth and the period of and just after the nuclear tests, and dropping thereafter. The sensitivity for detecting cell generation is therefore highest for the childhood period. A person born 20 years before the bomb pulse will instead have the largest relative difference in carbon-14 levels between the time up to adolescence and early adulthood.

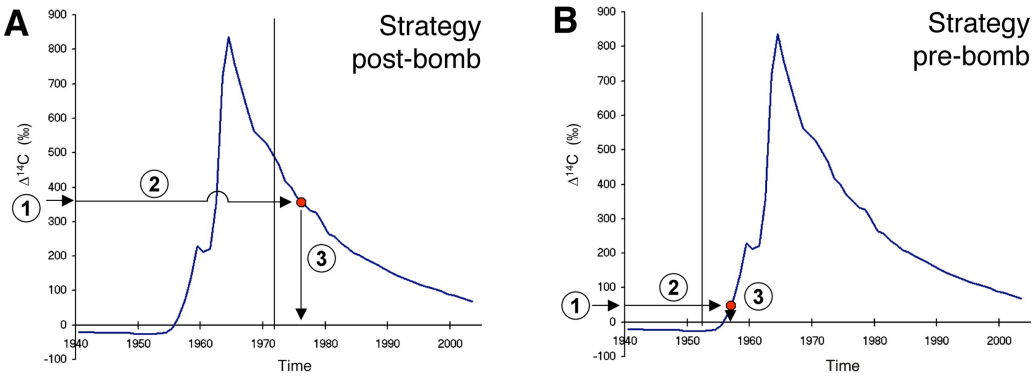

Figure 7. Schematic images depicting the strategy to establish the birth date of cells.
The individual in A is born after the period of nuclear bomb tests and the individual in B is born before. The time of birth of the person is indicated by a vertical line in each plot. First, the carbon-14 concentration in genomic DNA from the cell population of interest is established by AMS. Second, the measured carbon-14 value is related to the record atmospheric levels to establish at what time point they corresponded (indicated by red dot). Third, the year is read off the X-axis, giving the birth date of the cell population. Reprinted with permission from the publisher (Spalding et al., 2005b). http://dx.doi.org/10.1016/j.cell.2005.04.028

In contrast, analysis of cells from a person born after the bomb tests will have the highest sensitivity for the latest born cells. Taking these differences into account, the highest total sensitivity is reached by analysis of several individuals born at different time points in relation to the nuclear bomb tests. This allows for the detection of relatively small addition of new cells, and may also provide information as to when in

time a cell population is generated. Further, we have established a mathematical model integrating all dated carbon samples, which allows us to determine individual cell birth and death rates from all measured subjects (Paper I) (Spalding et al., 2008). This technique has the powerful advantage of being able to detect cell turnover in human hearts and to provide cumulative information about the total amount of cell turnover occurring over the whole lifespan of an individual.

2 AIMS OF THE THESIS

Listed according to publications

I. to establish whether adult cardiomyocytes can be renewed in the left ventricle of the human heart and to quantify the magnitude of this process

II. to validate cTroponins and PCM-1 as nuclear markers for cardiomyocytes for the analysis of cellular turnover and ploidy

III. to characterize the effect of PDGF signalling on scar formation and cardiomyogenesis in an mouse infarction model

3 SUMMARY OF RESULTS AND DISCUSSION

Paper I and II

We addressed the fundamental question whether cardiomyocytes renews in adult hearts in Paper I by utilizing atmospheric carbon-14 integrating into genomic DNA in cardiac cells at the time of the last cell division. We first carbon-dated left ventricle myocardial cells, including cardiomyocytes and other cell types. Carbon-14 concentrations from all individuals born around or after the nuclear bomb tests corresponded to atmospheric concentrations several years after the subjects' birth, indicating substantial postnatal DNA synthesis.

Because only about 20% of all cells within the human myocardium are cardiomyocytes, we could not draw any conclusions from our initial carbon-dating experiments to what extent myocytes are replaced. We were prompted to find a specific marker that labels mature cardiomyocyte nuclei and hence allows us to purify cardiomyocyte nuclei from other cell types by flow cytometry. Cardiac troponin I (cTroponin I) and cardiac troponin T (cTroponin T) have evolutionarily conserved nuclear localization signals and are partly localized to the nuclei of cardiomyocytes. Furthermore PCM-1 shows a perinuclear staining pattern specific to mature cardiomycytes. Western blot and quantitative reverse transcription polymerase chain reaction analysis of sorted nuclei demonstrated a high enrichment of cTroponin I and T, MHC and Nkx2.5 in the positive fraction and depletion in the negative. Conversely, cardiomyocyte markers were depleted in the cTroponin-negative fraction indicating that nearly all cardiomyocytes were isolated in the positive fraction. Thus, flow cytometry with antibodies against cTroponins and PCM-1 allows specific isolation of cardiomyocyte and non-cardiomyocyte nuclei. We further showed that the nuclear localization of cTroponins is not dependent on the age of the subjects and therefore most likely not associated with a senescence phenotype as Kajstura et al. suggested (Kajstura et al., 2010).

We next measured the carbon-14 concentration in genomic DNA of purified cardiomyocyte nuclei. In all individuals born before the onset of the nuclear bomb tests, the carbon-14 concentrations in cardiomyocyte genomic DNA were higher than the pre-bomb atmospheric concentrations, demonstrating DNA synthesis after 1955. Similarly, in all individuals born near or after the time of the nuclear bomb tests, the

carbon-14 concentrations in cardiomyocyte DNA corresponded to the concentrations several years after their birth, establishing postnatal cardiomyocyte DNA synthesis.

Data from previous studies indicate that there is no increase in the number of cardiomyocytes after the postnatal period but rather a slow, continuous decrease with age. About 25% of cardiomyocytes are binucleated in humans at birth, and this proportion stays constant throughout life (see Biology of Cardiomyocytes). Thus, the cardiomyocyte DNA synthesis detected by carbon-14 analysis cannot be explained by an increase in cardiomyocyte number or multinucleation. However, to meet the demands of contractile capacity during childhood, cardiomyocytes become hypertrophic and increase their DNA content per nucleus, also called polyploidisation. At birth almost all cardiomyocyte nuclei are diploid, but around the age of 10 most of them have become tetraploid. During the further life span no further increase in DNA content per nucleus occurs in healthy hearts. However, due to the DNA synthesis associated with polyploidization of cardiomyocyte, the measured carbon-14 concentrations do not only reflect cellular division. DNA synthesis in subjects born more than 10 years before the onset of nuclear bomb testing cannot be detected by carbon-14 dating until the beginning of nuclear bomb testing in 1955. Therefore, the elevation of genomic carbon-14 concentrations in three of our subjects born before 1945 cannot be explained by ploidisation, but indicates cardiomyocyte renewal after 1955. The stereotypical pattern of polyploidisation during childhood makes it possible to calculate its impact on carbon-14 concentrations in each subject. By subtracting the childhood polyploidization-associated carbon-14 incorporation from the measured carbon-14 concentration, we could calculate polyploidization-independent carbon-14 values. In all cases, the polyploidization-independent carbon-14 values corresponded to time points after birth for each subject, indicating cardiomyocyte renewal.

Several studies of sex-mismatched transplant recipients have indicated fusion of human cardiomyocytes with other cells (see Biology of Cardiomyocytes). However, cell fusion appears to mainly occur transiently after transplantation, associated with inflammation or organ rejection. The reported numbers, however, even in the acute phase are too low to explain the carbon-14 data. DNA damage and repair are very limited in differentiated cells and, at least in neurons, are well below the detection limit of the method used (Bhardwaj et al., 2006).

Mathematical modeling of carbon-14 data from subjects born both before and after the nuclear bomb tests, can provide an integrated view on cellular turnover (Spalding et al., 2008). We applied an analytical model that includes physiological polyploidization in

childhood to assess which one of several potential scenarios for cell birth and death best describes the data. The atmospheric carbon-14 values corresponding to DNA synthesis events are integrated to yield a calculated carbon-14 level, on the basis of each subject's birth date, age at death, and DNA content. The calculated carbon-14 levels were fitted to the purity-corrected values to find the best renewal rates for each scenario.

In a first step we computed what the annual turnover rate would be in each subject if the rate was constant throughout life. Based on this model there was a clear negative correlation to age, establishing that the turnover rate declines with age.

We next tested a series of different models allowing birth and death rates to change with age. The best fit was found with an inverse-linear declining turnover rate in which younger cardiomyocytes were more likely than older ones to be replaced. This model predicts that cardiomyocytes are renewed in a age-dependent manner, at a rate of 1% per year at the age of 25 and 0.45% at the age of 75. According to this model, most cardiomyocytes will never be exchanged during a normal life span. Only approx. 50% of adult cardiomyocytes will be replaced in a long life. The age of cardiomyocytes is on average 6 years younger than the individual. Our data further indicate a substantially higher exchange rate for non-cardiomyocytes, with a median annual turnover of 18% and a mean age of 4.0 years. As a limitation of our methodology, carbon-14 dating does not allow to distinguish whether new cardiomyocytes derive from cardiomyocyte duplication or from a progenitor cell pool, because both ways would result in similar carbon-14 integration in cardiomyocyte DNA.

Analysis of cell proliferation based on KI-67 and mitotic figures in the human myocardium has previously suggested a cardiomyocyte proliferation rate that could result in the exchange of all cardiomyocytes within 5 years (Beltrami et al., 2001), but the carbon-14 concentrations in DNA exclude such a high mitotic renewal rate. The five oldest subjects, who were born before the onset of nuclear bomb tests, showed lower genomic carbon-14 concentrations than contemporary carbon-14 values, establishing that not all cardiomyocytes had been exchanged after 1955 but that a substantial fraction remains from early in life.

A recently published study investigating iododeoxyuridin (IdU) incorporation into cardiomyocytes in cancer patients suggesting an annual cardiomyocyte cell turnover of approximately 22% (Kajstura et al., 2010), which is in average 20-fold higher than $^{carbon\text{-}14}$ dating and similarly much higher than previously reported nucleotide analogue incorporation rates in adult mice (Soonpaa and Field, 1997). However, these results should be interpreted with caution. First, all patients received IdU in a high dose

given as a radiosensitzer to augment the efficacy of radiation therapy. Both, radiation therapy as well as high dosages of IdU are known to cause DNA damage accompanied by DNA repair, which might lead to genomic IdU integration and false positive IdU labeling. Second, the authors did not apply a proper mathematical model to estimate cell turnover based on the IdU labeling. The reported high IdU-labeled cardiomyocyte fraction even after long chase periods (exceeding one year) is incompatible with the reported high renewal rates. A highly proliferative population generating new cardiomyocytes has to be matched by a rapid loss of cells in order to not increase the total number of cardiomyocytes. Labeled cardiomyocytes can only be generated during or shortly after IdU pulses. IdU is retained after the administration period in labeled cells and can be detected in cells generated up to five cell cycles after the last IdU pulse (Kiel et al., 2007). Kajstura et al., however assumed in their mathematical approach that labeled cardiomyocytes could be generated independent of the IdU administration throughout the whole observation period. In our re-analysis, we have therefore applied a model that describes a pulse-chase paradigm including the possibility of label retention in both precursor cells and dividing cardiomyocytes. Although we set up a range of models to describe cell turnover, the IdU data could not be fitted with reasonable assumptions on cell birth and death rates. By e.g. allowing a non-physiological high increase in cell number, the resulting turnover rates are even higher (up to 40% per day) than those reported by Kajstura et al. and therefore inconsistent with the long-term presence of the IdU label. Thus, the labeled fraction of cardiomyocytes cannot be explained only by turnover. One may speculate that the high IdU dose at the time of radiotherapy might have induced DNA repair resulting in IdU labeling not reflecting cardiomyogenesis.

Our data provide evidence for cardiomyocyte renewal in humans, albeit at a low annual rate. Careful analysis of our data and that from Kajstura et al. does not support a high turnover rate of cardiomyocytes in the left human ventricle. However, our studies open up the possibility to boost cardiomyogenesis pharmacologically as an alternative to cell replacement strategies.

Paper III

In this study, we explored the effect of the inhibition of PDGF signaling after cardiac infarction on changes in the composition of the infarct scar as well as on myocardiac regeneration. Scar formation after cardiac infarction is a consequence of the limited regenerative potential of the myocardium and necessary to maintain cardiac function. Extended scar formation, however causes ventricular remodeling and predicts a negative outcome in myocardial infarction. Platelet-derived growth factor (PDGF) signalling plays an important role in wound healing in several organ systems including the heart (Rajkumar et al., 2006; Zymek et al., 2006). PDGF signaling was inhibited by a specific function-blocking monoclonal antibody targeting PDGFRβ (PDGFRβ mab) (Sano et al., 2001). Antibody binding inhibits the dimerisation of PDGFRβ, which is critical for autophosphorylation and downstream signalling (Andrae et al., 2008). The effect of the treatment was evaluated at 3 and 21 days with regard to scar size, proliferation and density of different cell types involved in scar formation and cardiomyocyte proliferation.

In Paper III, we describe a pronounced reduction in scar size caused by the treatment with anti-PDGFRβ function-blocking antibodies to less than half of the average size observed in the control animals after 21 days post infarction. Cells expressing PDGFRβ+ in the uninjured heart are found almost exclusively in association with blood vessels and are therefore called pericytes (Díaz-Flores et al., 2009). However, after cardiac infarction PDGFRβ+ cells migrate away from vessels and a large population of cells expressing PDGFRβ can be found in the scar, 20 to 30% of them were non-associated with vessels and this population was specifically reduced in response to PDGFRβ mab treatment. Furthermore, we found that within these non-associated cells, the fraction expressing SMA (a common marker for myofibroblasts) was most affected by this treatment. The co-expression of PDGFRβ and SMA after cardiac infarction is in line with evidence supporting the hypothesis that pericytes can differentiate into myofibroblasts and thereby contributing to formation of fibrosis (Humphreys et al., 2010; Lin et al., 2008).

However, there is a body of evidence that several subtypes of fibroblasts are involved in scarring, which implies that more than one origin for collagen producing exists. Apart from pericytes, e.g. endothelial cells and circulating cells have been suggested to give rise to fibroblast subpopulations in the post infarcted heart (Minami et al., 2005; Zeisberg et al., 2007). Hence, our findings help to further understand the complexity of

the cardiac injury response. In establishing PDGFRβ+ cells as the origin of scar forming cells after cardiac infarction fate-mapping studies will be of importance.

Cardiomyocytes renew in homeostasis and after cardiac infarction at a higher rate (Paper I) (Hsieh et al., 2007). We found that cardiomyocyte renewal increased up to 50% in the PDGFRβ mab treated animals 21 days post injury, however, the amount of renewed cardiomyocytes (around 1%) that we found cannot account for the dramatic reduction in scar size in the treatment group. We therefore looked at early effects mediated by an inhibition of PDGFRβ, which could explain the salvage of the myocardium. Angiogenesis increases rapidly after cardiac injury (Virag and Murry, 2003) and provides thereby the basis for an efficient wound healing. Microvessel density was significantly increased by inhibiting PDGFRβ mediated signaling at 3 days post infarction, which has been shown to reduce infarction size and might improve the microenvironment for cardiac progenitor as well as cardiomyocyte survival after cardiac infarction (Yoon et al., 2010).

In conclusion, antibodies specifically inhibiting the function of PDGFRβ cause a significant reduction in infarct scar size, which we propose can be mediated by a decrease in the number of PDGFRβ expressing cells in the scar and increased early angiogenesis allowing a higher cardiomyocyte survival and renewal. Establishing the lineage of scar-forming cells by transgenic fate-mapping strategies will allow to investigate the impact of signalling systems involved in ischemia and wound healing on fate decision, proliferation and migration at different stages after cardiac injuries.

4 CONCLUDING REMARKS

This thesis addresses the important issue whether the human myocardium has the capacity to renew in homeostasis. We could show that human cardiomyocytes can be replaced at a low rate over the whole lifetime. Whether committed progenitor cells or dividing cardiomyocytes have to potential to give rise to new myocytes, however, cannot be answered by our technique. Studies in rodents indicate that both alternatives might be co-existing, both in healthy as well as in diseased hearts. Future studies need to establish the source of *de novo* cardiomyocyte formation in the adult human heart. The knowledge that human myocardium has the potential to be renewed opens up the possibility to augment this process as a therapeutic strategy for heart injuries.

Scar formation is one of the main hallmarks after cardiac infarction. Dead myocardium, with its limited capacity to regenerate, needs to be replaced by a scar comprised by cells with a contractile function and the capability to produce a robust extracellular matrix. Excessive scarring, however, is also associated with a negative outcome after cardiac infarction, since it leads to diastolic dysfunction and eventually to a global cardiac insufficiency. Furthermore, scarring might interfere with the homing and differentiation of activated progenitor cells in the infarction border zone. We could show that inhibiting the PDGF pathway, which is known to be involved in scar formation in several organ systems leads to reduction in infarction size. In line with this finding, the density of PDGFR-β expressing cells and the ratio of PDGFR-β expressing myofibroblasts were reduced after treatment. We further found that myocardiogenesis and angiogenesis were both increased after PDGFR-β mab treatment; both processes were shown to have a positive impact on functional recovery after cardiac infarction. In healthy hearts PDGFR-β is only expressed in pericytes being associated with blood vessels. Upon injury these cells start to detach from the vessels and start expressing SMA, a myofibroblast marker, indicating that pericytes are at least one source of myofibroblasts in post-infarction wound healing. Transgenic fate mapping studies that can provide direct evidence for this transition in cell fate, however, are still lacking and will be the next logical step to shed light on the formation of pericyte-mediated scarring. Our study suggests that PDGFR-β expressing cells (pericytes) modulate scar formation and post-infarction myocardial regeneration by a transition into scar forming myofibroblasts.

In conclusion, this thesis provides evidence that the regenerative capacity of the human myocardium is not restricted to mere adaptive processes to compensate for the massive

loss of functional cardiomyocytes upon injury and that modulating of scar formation by PDGF signalling shows new avenues to reduce infarction size after cardiac injury.

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