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ANALYSIS OF CARDIAC CELL TURNOVER IN HUMANS BY RADIOCARBON DATING AND MATHEMATICAL MODELING

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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That some planets are uninhabited could very likely be because their nuclear scientists were more successful than ours.

- *Torvald Gahlin*

ABSTRACT

Cardiovascular disease is the largest cause of morbidity and mortality in the Western World. Disease progression often involves a loss of contracting cells, cardiomyocytes, which leads to cardiac failure and the need for heart transplantation with time. However the shortage of donor hearts is a large problem and a strong motivator for finding alternative solutions; this is the focus of regenerative heart medicine. For new treatment strategies to be effective we first need to better understand the potential and capacity of the heart and its cells. This thesis addresses two questions specifically: 1) Do cardiomyocytes renew in human hearts during healthy aging? 2) How does cardiac disease affect cardiomyocyte renewal? Studies in experimental animals and to a small extent in humans had previously not been able to resolve these questions, mainly because limitations in methods and ethical restrictions. We employed primarily two methodologies, ^{14}C birth dating and mathematical modeling. ^{14}C birth dating is a method developed within the Frisén group that exploits the changes in atmospheric ^{14}C levels due to testing of nuclear weapons during the Cold War. The ^{14}C concentration in the genomic DNA of a cell reflects when the cell was born, and hence the level of renewal. The core part of the mathematical model is a first order partial differential equation (PDE). It describes cells according to their age and how the distribution of ages changes as the individual grows older. We found that human cardiomyocytes in healthy hearts indeed renew throughout life, with a declining turnover not exceeding 1% per year in adult life, and that the cell number is established already at birth. Endothelial and mesenchymal cardiac cells are more dynamic, both in terms of changes in cell number and baseline turnover (**Paper II and IV**). Preliminary results indicate that ischemic heart disease and dilated cardiomyopathy can increase the renewal rate to 2.7% per year; however it is likely that individual turnover estimates differ from this, which may reflect the differences in disease etiology and patient specific manifestation (**Paper I**). In order to reach these conclusions we developed a method to isolate cardiomyocyte nuclei, based on the molecular markers, PCM-1, cTroponin T, and cTroponin I (**Paper III**). This work shows that adult cardiomyocytes in healthy and diseased hearts have a measurable regenerative capacity, suggesting that it can be exploited for developing new therapeutic strategies to treat heart disease.

LIST OF PUBLICATIONS

I. **Zdunek S.**, Bergmann O., Jashari R., Andrä M., Salehpour M., Alkass K., Bernard S., Felker A., Possnert G., Druid H., Jovinge S., Frisén J. Regeneration of cardiomyocytes in human heart disease. *Manuscript*

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III. Bergmann O., **Zdunek S.**, Alkass K., Druid H., Bernard S., Frisén J. (2011). Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell turnover. Experimental Cell Research *317*, 188-194.

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*These authors contributed equally.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND MEDICAL TERMS

^{12}C,^{13}C,^{14}C	Carbon isotopes ^{12}C, ^{13}C, ^{14}C
^{15}N	Nitrogen isotope ^{15}N
AGTR1/2	Angiotensin receptor 1/2
ALMS1	Alström protein 1
AMS	Accelerator mass spectrometry
BrdU	Bromodeoxyuridine
c-kit	Tyrosine-protein kinase kit
CPC	Cardiac progenitor cell
Cre	Cre recombinase
DCM	Dilated cardiomyopathy
DNA	Deoxynucleic acid
dUTP	Deoxyuridine triphosphate
eGFP/GFP	Enhanced green fluorescent protein/green fluorescent protein
ErbB4	Erb-B2 receptor tyrosine kinase 4
hESC-CM	Human embryonic-stem-cell-derived cardiomyocytes
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
Flk-1	Kinase insert domain receptor
FSC	Forward side scatter
G1,S,G2	Cell-cycle: Gap 1, Synthesis, Gap 2
GATA4	GATA binding protein 4
GDF-11	Growth differentiation factor 11
Has-mir 590/199a	Micro RNA 590/199a
HCM	Hypertrophic cardiomyopathy
IdU	Iododeoxyuridine
IHD	Ischemic heart disease
IL-10	Interleukin-10
iPSC	Induced pluripotent stem cell

Isl-1	Insulin gene enhancer protein ISL-1
lin	Lineage
loxP	Locus of X(cross)-over in P1
MEIS1	Meis homeobox 1
MHC	Myosin heavy chain
MIMS	Multi-isotope imaging mass spectrometry
MYH6	Myosin heavy chain 6
Nkx2.5	Homeobox protein Nkx-2.5
PCM-1	Pericentriolar material 1
PDE	Partial differential equation
PHH3	Phosphorylated histone H3
PPR	Pattern recognition receptors
Sca-1	Stem cell antigen-1
SSEA	Stage-specific embryonic antigen-1
Tbx18	T-box 18
TGF- β	Transforming growth factor beta
Wt-1	Wilms tumor 1

PERSONAL REFLECTIONS ON MY THESIS

This thesis is part of my doctorate in Medicine, with a special focus on regenerative heart medicine. Regenerative heart medicine is the scientific investigation of myocardial regeneration, and aims to restore tissue damage and function caused by heart disease. It is a large field, reflecting the current and expected need for better treatments of this debilitating condition. Not much in biology is simple, but there is essentially just three ways in how to think about the perspective of completely curing a patient with heart failure; either you give the patient a new heart, or you give him new heart cells, or you help the heart to make its own new cells. The first one does not actually concern regenerative medicine; however, of the three options, it is the only one available today. When I started my PhD it was not known whether the muscle cells in the human heart (cardiomyocytes) naturally exchange in adult life, or we die with the same cells that we were born with. To a “knowledge-greedy” scientist this is of course a very intriguing question, but for the future of heart medicine the answer is of fundamental value. If the cardiomyocytes retain some capacity to renew it suggests that the third option, to help the heart help itself, is both rational and realistic. This is also the main question for my studies; does it occur in health and disease and to what extent? Although my studies deal with regeneration in the human heart, I have included some words about heart regeneration in other species as well as developmental heart biology, since they serve important roles as scientific inspiration and provide experimental models. Developmental biology can teach us about the mechanisms of cardiomyocyte proliferation when it occurs on an impressive level, such as in the embryonic and neonatal heart. Other species can provide similar insight, and experimental animals allow for hypotheses testing and manipulations that are not possible in humans.

INTRODUCTION

The heart – it beats as long as we live

The human heart is built up of four chambers, the left and right ventricles and the left and right atria. This makes it a highly compartmentalized organ in which oxygen-rich and oxygen-poor blood is separated for efficient oxygenation of the body via the circulatory systems. The two circulation systems are the pulmonary circulation, connecting the lungs to the heart, and the systemic circulation that serves all tissues and organs. The blood supply to the cells of the heart itself is part of the systemic circulation through the coronary vessels. In addition to oxygenation, the blood circulation is also important for transportation of nutrients, water, and waste products. During one round of circulation, deoxygenated blood from the body enters the right atria through two large vessels called the superior and inferior vena cava (Figure 1). It then travels to the right ventricle, from where it is pumped out through the pulmonary arteries to the lungs. Here it takes up oxygen and releases carbon dioxide. The oxygenated blood then returns to the heart via the pulmonary veins, through the left atria, to reach the left ventricle. Subsequently the blood is pumped out through the aorta to the rest of the body. The pressure created in the arteries by the contraction of the left ventricle is called the systolic blood pressure; this is the higher blood pressure in the system and has an approximate value of 120 mmHg. After the contraction the ventricle begins to relax while the left chambers refill with blood from the pulmonary veins, during which the pressure is approximately 80 mmHg. During one minute at rest the heart makes 60-100 such contractions/relaxations. The cells responsible for the contractions are called cardiomyocytes. They comprise 20% of the cardiac cells, but in volume they take up more space than the other cells (endothelial cells, smooth muscle cells and cardiac fibroblasts). This is due to their large cytosols filled with contractile fibers (myofibrils), which are organized in sarcomeres, the basic unit of contraction. The sarcomeres make it possible for the myofibrils to slide across each other to change the length of the myocyte. The two main proteins of myofibrils are myosin (thick filament) that works as the motor in driving the contraction, and actin (thin filament) that binds to the boundaries (Z-lines) of the sarcomere and anchors the myofibrils. Cardiomyocytes are in majority polyploid cells, which mean that their

nuclei have more than two copies of each chromosome. Approximately 35% are diploid, 65% are tetraploid, and 5% are octaploid. These proportions are established during adolescence but can change during heart disease. Around 20% of the cardiomyocytes are also multinucleated, having more than one nucleus per cell. Endothelial and smooth muscle cells are diploid and make up the walls of the vessels in the myocardium. Cardiac fibroblasts are also diploid; they produce the extracellular matrix that gives structure and mechanical stability to the heart tissue.

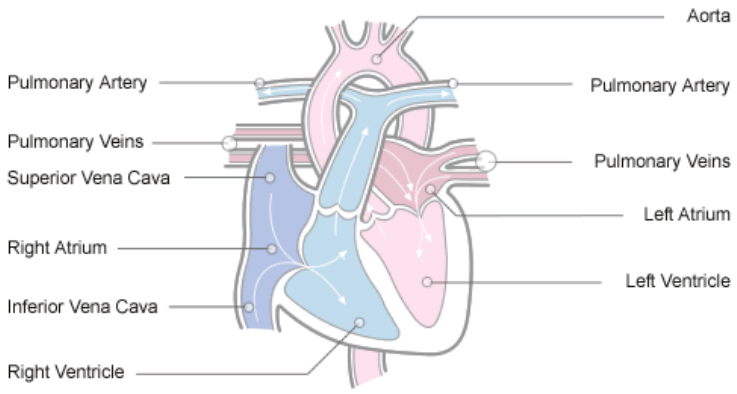

Figure 1: The human heart and the blood flow. Red and blue indicate oxygenated and de-oxygenated blood respectively.

A brief insight into heart development

The heart is the first organ to become functional during fetal development, and already at week 3 the primitive heart starts beating spontaneously. First the heart is just a tubular structure, called the primitive heart tube. This is a short-lived structure that is followed by morphogenesis at day 23, a process of stretching and looping that gives the heart its correct shape and its cells the correct spatial distribution. The main walls of the heart are formed during the fourth and fifth week, and by the ninth week the valves are completed. However, it is not until birth the heart fully separates the arterial and venous blood, and the pressure differences of the left and right side is established (closing of foramen ovale). Along with the heart's structural development, the cells within it develop as well. The cardiac cells in the adult heart, atrial/ventricular cardiomyocytes, vascular smooth muscle cells, endothelial cells, and

cardiac fibroblasts, are generated from three sources of embryonic precursors: (1) the cardiogenic mesoderm, (2) the cardiac neural crest, and (3) the proepicardial organ. The cardiogenic mesoderm (mesoderm is one of the three embryonic tissues) is located to the cranial end of the embryo, anterior to the future brain, and can be divided into the first and second heart field (FHF and SHF respectively) (Kelly and Buckingham, 2002). The FHF and SHF precursor cells have acquired their name because of the timing of their contribution to the developing heart; the FHF provides cells already to the heart tube while the SHF adds cells during the looping stage. That this timing is crucial is evidenced by severe defects in the out-flow tract as a result of too early or too late differentiation of the SHF. The FHF primarily gives rise to the left ventricle and partly to the atria while the SHF develops into the right ventricle, ventricular septum, and the outflow tract. The outflow tract is a transient embryonic structure that connects the embryonic ventricles to the aortic sac, which gives rise to the ascending aorta and the pulmonary trunk (the base of the two pulmonary arteries). The cardiac neural crest migrates into the heart at later stages of development and is important for outflow-tract septation and proper differentiation of the early myocardium. The majority of the cardiac vessels are derived from the cardiac neural crest. The proepicardial organ develops into the mesenchyme portion (cardiac fibroblasts and smooth muscle) of the heart and the majority of the cells in the epicardium (outer layer of the ventricular wall) (Laugwitz et al., 2008).

Since the advent of molecular biology in 1970, much knowledge has been attained about the different embryological cell types and the molecular signaling involved in cardiac development. This has provided a more detailed view of the developmental process, but also somewhat complimentary as distinct morphological structures not necessarily correspond to distinct cell types. For instance, to date there is no known set of molecular markers that define the first and the second heart field. However, the common progenitor has been shown to be marked by the transcription factors Mesp1/2 and Fgf8, which are expressed in the early cardiac mesoderm during the existence of the primitive streak (a region of cell movement that separates the mesoderm from the endoderm). A subsequent progenitor giving rise to all the cardiac cells has been shown to express the factors, Nkx2.5+/Isl1+/Flk-1+, and then by loss of either Nkx2.5 or Flk-1 muscle and vascular progenitors arise respectively (Bu et al., 2009; Lam et al., 2009; Moretti et al., 2006). Cardiac fibroblasts may arise from the vascular progenitors through endothelial-to-mesenchymal transition. A potentially

distinct proepicardial progenitor, marked by Tbx18+ (T-box transcription factor) may also exist. This progenitor was shown to contribute to coronary smooth muscle, cardiac fibroblasts, and some atrial and ventricular cardiomyocytes (Cai et al., 2008). However, this finding has been questioned on the basis of the specificity of the genetic lineage tracing used (Christoffels et al., 2009). Recently, it was published that the earliest committed cardiomyocyte progenitor, referred to as the cardiomyoblast, has been identified (Jain et al., 2015). This cardiomyoblast was defined by the Hopx (homeobox gene) expression in cardiac progenitors from the SHF (Wnt-activated Isl+ progenitors) that are localized to the outflow-tract and start expressing Hopx after down-regulating Wnt. The hope is that furthering the understanding of the developmental mechanisms, and of the identity of progenitors committed to the myocyte lineage, will advance cardiac regenerative therapies.

What goes wrong in heart disease?

Heart disease is any disease that involves the heart and the cardiac blood vessels. It is a large class of diseases that can be separated into cardiomyopathies and non-cardiomyopathies. The former is also referred to as primary heart disease and the latter secondary heart disease. According to the latest classification from the American Heart Association cardiomyopathy is defined as: "*Cardiomyopathies are a heterogeneous group of diseases of the myocardium associated with mechanical and/or electrical dysfunction that usually (but not invariably) exhibit inappropriate ventricular hypertrophy or dilation and are due to a variety of causes that frequently are genetic. Cardiomyopathies either are confined to the heart or are part of generalized systemic disorders, often leading to cardiovascular death or progressive heart failure-related disability*". The non-cardiomyopathies include pathological myocardial processes and dysfunction that are direct consequences of other cardiovascular abnormalities such as valvular heart disease, systemic hypertension, congenital heart disease, and atherosclerotic coronary artery disease/ischemic heart disease (IHD) (Maron et al., 2006).

Dilated cardiomyopathy (DCM) is characterized by ventricular chamber enlargement with maintained normal left ventricle wall thickness in the absence of coronary sclerosis, and systolic dysfunction. It is the third most common cause of

heart failure and the most common cause for heart transplantation. The cause can be genetic or non-genetic, including specific infectious agents and toxins; it has been reported that 20-35% of DCM are hereditary with association to more than 20 loci. The predominant mode of inheritance is autosomal dominant. Left ventricle chamber dilation can also occur as a secondary response to other cardiac conditions but should be distinguished from DCM, which is a primary heart disease. For instance, left ventricle myocardial hypertrophy can with time lead to chamber dilation, if the hypertrophy is so-called eccentric. This condition involves a thickening of the ventricle wall and is a consequence of cardiac volume or pressure overload, or to stimuli that are not yet completely understood (Borer, 2004). Hypertrophic cardiomyopathy (HCM) is, despite the similar name, not equal to left ventricle myocardial hypertrophy. HCM is an autosomal dominant genetic heart disease, which is the most common cause of sudden cardiac death (in the United States). HCM is assessed morphologically as an enlarged but non-dilated left ventricle in the absence of other systemic or cardiac disease related conditions such as systemic hypertension or aortic valve stenosis, which would instead lead to the diagnosis cardiac hypertrophy (non-cardiomyopathy that can occur with age for instance). HCM is caused by a variety of mutations that frequently involve contractile proteins (e.g. beta-myosin heavy chain and myosin-binding protein C), mitochondrial genes, or proteins associated with metabolic pathways. Takotsubo cardiomyopathy, or more commonly known as broken heart syndrome, is an acquired cardiomyopathy that results from strong physiological stress (e.g. as in Paper I in which a mother lost her baby). Given the right medical treatment this condition has a good prognosis (Maron et al., 2006).

Heart disease is also reflected on the cellular and extracellular level in various ways, collectively referred to as pathological myocardial remodeling. This process involves increased myocardial mass associated with hypotrophy of individual cardiomyocytes, alterations in gene expression, and changes in both the quantity and quality of the extracellular matrix. Depending on the nature of the pathological stimulus these alterations may take different forms. For instance systolic overload (e.g. from aortic stenosis) results in ventricular wall stress that promotes addition of sarcomeres in parallel, making the cardiomyocytes wider (concentric hypertrophy). If the wall stress is caused by an increase in ventricular diastolic volume instead (e.g. mitral regurgitation) the cardiomyocytes grow in length, as sarcomeres are added in series (eccentric hypertrophy). One prominent feature of ventricular remodeling is

pathological polyploidization of cardiomyocytes. This is a process in which the genome is multiplied, either through the creation of polyploid nuclei (e.g. tetraploid cells become octaploid) or by multinucleation. It is not known if pathological polyploidization is reversible but a study in patients with left ventricular assist devices suggests it is possible under such circumstances (Wohlschlaeger et al., 2010). Another feature is the re-expression of fetal genes (genes expressed during cardiac development) coding for atrial natriuretic peptide, brain natriuretic peptide, α-skeletal actin, fibroblast growth factor (FGF), and transforming growth factor β (TGF-β) (Colucci, 1997). The TGF-β is a large family of growth factors and individual members might exert different roles in disease; one of its growth hormone members (GDF11) is for instance implicated in reversal of age-related cardiac hypertrophy (Loffredo et al., 2013). FGF and TGF-β are important factors for the composition of the extracellular matrix. The extracellular matrix consists of structural proteins (e.g. collagen and fibronectin) that give structural support to the myocardium, and non-structural proteins that have immune modulatory functions. During a myocardial infarction up to 1 billion cardiomyocytes might be lost, depending on the size of the infarction, and the vast majority of these will not be replaced (Laflamme and Murry, 2011). Without the reparative actions of cardiac fibroblasts and fibrosis, the myocardium would be left with tissue ruptures (Dobaczewski et al., 2010; Savvatis et al., 2014). On the other hand the sealing of the ruptures may prevent the formation of new cardiomyocytes, which naturally would be the preferred new tissue together with new blood vessels. Studies in mice indicate that new cardiomyocytes can be produced after myocardial infarction; up to 4% of cardiomyocytes in the proximity to an infarct showed molecular evidence of proliferation (Bersell et al., 2009; Kimura et al., 2015; Malliaras et al., 2013; Senyo et al., 2013). However, whether new cardiomyocytes contribute during human heart disease is currently not known, but is the focus of Paper I.

In recent years it has been increasingly appreciated that the immune system plays an important role in cardiac injury. It is involved both in the acute inflammatory response and the regenerative response. Much of its actions are believed to be a consequence of the primary function of the immune system – to maintain homeostasis following injury and infections, and that the cost of this is destructive inflammatory damage (Epelman et al., 2015). Resident cardiac immune cells are triggered by damage-associated molecular patterns, DAMPs, (or pathogen-associated molecular

patterns in myocarditis for instance) by a number of pattern recognition receptors (PPRs). The PPRs can for instance recognize the molecular patterns of dying or injured myocardial cells. The time course of the inflammatory response is remarkably consistent, irrespective of the cause of the injury, and entails a rapid influx of neutrophils followed by monocytes into the injured area. The involvement of the immune system in heart disease is best characterized in acute ischemic injury. Adaptive immune cells, like CD8+ T-cells, are known to act anti-inflammatory and reduce infarct size through their angiotensin receptor type II (AGTR2) and production of IL-10 (pro-fibrotic cytokine). Angiotensin II is part of the renin-angiotensin system, which has long been known to be a crucial pathological pathway in heart disease (Cohn and Tognoni, 2001; Dickstein et al., 2002). Angiotensin II is thought to primarily induce cardiomyocyte hypertrophy and increase vascular tone by signaling through AGTR1 and modulated by AGTR2 (Curato et al., 2010). The transition from acute inflammation to tissue fibrosis is mediated largely by macrophages through their production of anti-inflammatory and pro-fibrotic cytokines. The ensuing maturation of the fibrotic area leads to scar formation, which permanently limits the function of the heart.

165 years of heart work

From 1850 to the first quarter of the 20^{th} century the common view among cardiologists was that cardiomyocytes in adult hearts are able to multiply (proliferate). How would it otherwise be possible to observe patients with larger than normal (hypertrophied) hearts (Carvalho and de Carvalho, 2010). The idea that this might not be true was suggested in 1925 (Karsner et al., 1925), based on the failure to detect mitotic figures in microscopic examinations of human cardiomyocytes collected from hypertrophied hearts. This led to the long-held view of the heart being a post-mitotic organ in which cardiomyocytes can only grow in size but not in numbers. It was not until the late 1990s that the first indications of cardiomyocyte proliferation started to appear (Beltrami et al., 2001; Kajstura et al., 1998). Kajstura et al. reported in 1998 cardiomyocyte mitotic indexes in the range of 10^{-5} and 10^{-4} in healthy and diseased hearts respectively. The extrapolated turnover suggested that all cardiomyocytes are exchanged within a few years. This made cardiac researchers open up for the

possibility that cardiomyocytes might be able to divide after all. However, data on cardiomyocyte DNA synthesis in other mammals such as mouse and rat, which often are used as models for human hearts, was highly variable and raised questions about the actual magnitude of cardiomyocyte renewal (Soonpaa and Field, 1998). It is likely that at least part of the variability can be explained by the different methods used to detect DNA synthesis and identify cardiomyocyte nuclei. The importance of accurate identification of cardiomyocytes and their ploidy prompted us to publish a method paper on this topic (Paper III).

This was around the time when the field of stem cells was growing fast and the beginning of the present stage, from which regenerative medicine has grown. The first stem cells to be identified were those of the blood system (hematopoietic stem cells), residing in the bone-marrow and expressing the factors, c-kit and Sca-1 (Lkuto et al., 1991). It had been suggested that these cells not only contributed to the blood lineage but also to other tissues (Krause et al., 2001), which led to the claim that this was true for the heart as well (Orlic et al., 2001). The idea that the heart could be renewed by stem cells outside the heart, referred to as extracardiac stem cells, was also reinforced by studies in humans. These were based on gender-mismatched transplantations in which female hearts were found to harbor Y-chromosome-positive cardiomyocytes (Deb et al., 2003; Laflamme et al., 2002; Muller et al., 2002). However, it was soon after shown that the cells from the bone marrow were not stem/progenitor cells, nor did they differentiate/transdifferentiate, but were instead fusing with the cardiomyocytes in the donor hearts or adopted a mature hematopoietic fate (Alvarez-Dolado et al., 2003; Balsam et al., 2004; Murry et al., 2004; Nygren et al., 2004). Nevertheless, it was suggested that the heart does contain proliferating immature cells (c-kit+/Sca-1+/lin-), and these cells were coined resident cardiac stem/progenitor cells (CPCs) (Beltrami et al., 2003). In trying to characterize the CPCs a plethora of marker profiles and properties that would identify them were generated. When using the term CPCs today it usually refers to one of the following populations: side population (Martin et al., 2004), c-kit+ (Beltrami et al., 2003; Dawn et al., 2005), Sca1+ (Oh et al., 2003; Tateishi et al., 2007; Uchida et al., 2013), SSEA1+ (Ott et al., 2007), and cardiosphere-derived CPCs (Messina et al., 2004; Smith et al., 2007). Among these, the c-kit+ population has got the most attention by far, which is linked to Anversa and his colleagues being strong proponents of their potential (Leri et al., 2014). It should be noted that the CPC populations are not mutually exclusive; for

instance cells can be both c-kit+ and Sca1+. It has been shown that CPCs populate the heart at a low frequency with an estimate of 1 per 40,000 cardiac cells in mouse (Hosoda et al., 2009). According to the classical definition of a stem cell the CPCs should be able to give rise to all the cell types of the adult heart, including cardiomyocytes. However, since it was recognized that it is very difficult to know the fate and functional role of CPCs, only based on immunohistological identification, there was a need to address this issue in another way. Today this is commonly performed by advanced genetic fate mapping strategies in transgenic mice (most often), in which recombination can be controlled (see section Techniques for studying cardiomyocyte renewal). However, early efforts to trace the fate of c-kit+ cells were performed using non-inducible tracing strategies. They were instructive in studying c-kit+ expression during development and after injury, but could not address if the c-kit+ cells differentiated into cardiomyocytes (Ferreira-Martins et al., 2012; Fransioli et al., 2008; Tallini et al., 2009). The first important contribution to study cardiomyocyte renewal using a conditional genetic fate-mapping approach was made by Prof. Richard Lee and his group at Harvard (Hsieh et al., 2007). In an elegant approach using transgenic mice they traced cardiomyocytes instead of CPCs (which has proven to be very difficult). They labeled cardiomyocytes with GFP (green fluorescent protein) under the control of *Myh6* (α-myosin heavy chain) promotor, recorded the labeling frequency, waited one year to reflect normal aging or performed an ischemic injury, and then analyzed the recombination frequency again. With this strategy they detected a dilution of GFP labeled cardiomyocytes bordering the infarction, indicating that cardiomyocytes were renewed by unlabeled (GFP negative) progenitors. It was also shown that these progenitors could be stimulated by transplantation of bone-marrow derived c-kit+ cells (Loffredo et al., 2011). However, during normal aging no cardiomyocytes were renewed (Hsieh et al., 2007). This was in stark contrast to the magnitude of renewal argued by Anversa and his colleagues, who had published turnover rates of 50-80% per year in mice and 7-40% per year in humans (Hosoda et al., 2009; Kajstura et al., 2010a; Kajstura et al., 2010b). It was however consistent with our estimates of less than 1% per year in adulthood (Paper II and IV).

Heart regeneration is also studied in lower vertebrates, and in zebrafish cardiomyocytes regenerate primarily by means of epicardial cardiomyocytes (*gata4+*) that dedifferentiate, proliferate, and relocate to sites of injury (Jopling et al., 2010;

Kikuchi et al., 2010; Poss et al., 2002). In fact the epicardium (and sub-epicardial layer) might be a region with higher regenerative potential, as also suggested in (Smart et al., 2011), by harboring cells able to re-express Wt1 (Wilm's tumor 1) upon thymosin β4 priming. This inspired us to investigate regional differences of cardiomyocyte turnover in humans (Paper II). In a recent study, applying advanced multi-isotope imaging mass spectrometry (MIMS) technology, Richard Lee's group showed that also cardiomyocytes in the mouse heart regenerate mainly through self-duplication (Senyo et al., 2013). The apparent discrepancy with their previous study probably reflects the difficulties in studying biological processes without interfering with them (see section Techniques to study cardiomyocyte renewal). Consequently, the researchers in the field could not agree on a unified view on the regenerative capacity of the adult mammalian heart, or whether new cardiomyocytes come from self-duplication or progenitor cells. Anversa and colleagues are still strong proponents of the potential of the c-kit+ cells (Ellison et al., 2013), while many other investigators in the field are less convinced (van Berlo and Molkentin, 2014). Although there is evidence for c-kit+ cells contributing to new cardiomyocytes after injury in the neonate, they fail to do so even after favorable inductive conditions in the adult heart (Jesty et al., 2012). The c-kit+ cells have now also been investigated by means of conditional fate-mapping (during development, aging, and after injury in adulthood) and it was then shown that c-kit+ cells do not contribute meaningfully to cardiomyocyte regeneration (van Berlo et al., 2014). In general new transgenic tools have made it easier to trace the progeny of potential stem cells. Recently it was shown, by an inducible Cre-labeling approach, that Sca-1+ CPCs are able to generate cardiomyocytes during normal aging and after injury (Uchida et al., 2013). In this study individual Sca-1+ cells were however biased towards a specific cell fate, which could be concluded from using the so called R26R-Confetti reporter mouse.

Working along the lines of cardiomyocyte self-duplication the neonatal period and young individuals have been studied to get insight into which factors are in play when the cardiomyocytes have, or might have, a robust regenerative capacity (Walsh et al., 2010). In mice it was determined that a proliferative window exists during the first 7 days in the neonatal mouse, after which cardiomyocytes lose their ability to divide (Porrello et al., 2011). Both in humans and mice it was suggested that the cardiomyocyte number increases postnatally (Mollova et al., 2013; Naqvi et al., 2014), but earlier studies did not find this (Mayhew et al., 1997), and we have

disputed it based on both design-based stereology and radiocarbon measurements (Paper II and (Alkass et al., submitted)).

Perhaps because of the controversies regarding the mode and magnitude of cardiomyocyte renewal, efforts in recent time have a strong focus on mechanistic evidence. For instance, the oxygen level in the heart is emerging as an important factor in negatively regulating proliferation. The metabolic transition from anaerobic glycolysis to oxidative phosphorylation at birth has been shown to induce cell-cycle arrest in the majority of cardiomyocytes via the DNA-damage response (Puente et al., 2014). Building on this, the same group showed earlier this year that a small subpopulation, constituting 0.05% of the total cardiomyocytes, remain proliferative and are characterized by being hypoxic (Kimura et al., 2015). Since accumulating evidence indicates that the main mode of renewal is self-duplication efforts have been made in trying to control the cardiomyocyte cell cycle *in vivo*. For instance, it has been shown that cardiomyocytes in mouse hearts can be induced to proliferate by supplying the growth factor neuregulin1, a ligand to the cardiomyocyte tyrosine kinase receptor ErbB4 (in this study undifferentiated progenitors did not contribute to the proliferative response) (Bersell et al., 2009). The transcriptional co-factor Yap, which acts in the hippo signaling pathway, has been found to not only regulate proliferation in embryonic cardiomyocytes but also activate the proliferative program in adult cardiomyocytes upon forced expression (Xin et al., 2013). Genetic deletion of *Meis1* in mouse cardiomyocytes is sufficient for reactivating the proliferative program, without any deleterious effects in the adult heart (Mahmoud et al., 2013). Meis1 is a homeodomain transcription factor required for normal heart development, and a negative regulator of cardiomyocyte proliferation. The Alström protein *Alms1* has been shown to act in the same direction, as evidenced by a mutation in the ALMS1 gene leading to increased cardiomyocyte proliferation in affected infants (Shenje et al., 2014). Recently two microRNAs (miRNA), hsa-miR-590 and hsa-miR-199a, were found to promote cell cycle re-entry of cardiomyocytes and restoration of cardiac function after myocardial infarction in adult mice (Eulalio et al., 2012). miRNAs are small non-coding RNA molecules that have emerged as important factors in RNA silencing and post-transcriptional regulation of gene expression. The miRNAs can play diverse regulatory roles and a member of the miR-15 family has for instance been shown to repress cardiac regenerative capacity in the neonate (Porrello et al., 2013). By learning from studying different life stages from the embryo to the

adult, considering different species, and employing different techniques, the knowledge about cardiac regeneration seems to converge to one picture; the annual turnover of cardiomyocytes in adult mammals is most likely below 2% and the main source for the regeneration is the cardiomyocytes themselves (van Berlo and Molkentin, 2014).

A few words on transplantation strategies

This thesis has focused on the heart's potential to regenerate itself, also called endogenous regeneration. In regenerative heart medicine there are two main strategies, one is based on promoting and enhancing the endogenous regenerative potential therapeutically (Bergmann and Jovinge, 2014; Bersell et al., 2009; Kuhn et al., 2007; Lorts et al., 2009), and the other is to transplant exogenous cells in some context to the weakened heart. The main disadvantage with the latter is that life-long immunosuppression is often needed following the transplantation, which makes the former an attractive alternative. With the transplantation strategy, many different types of cells have already been tried in pre-clinical and clinical settings, including skeletal myoblasts, bone marrow-derived hematopoietic cells, mesenchymal stem cells, adipose-derived cells, endothelial progenitor cells, and cardiac-derived cells (e.g. c-kit+ cells and cardiospheres), for a review see (Behfar et al., 2014; Gerbin and Murry, 2015). If the goal is to provide the diseased heart with new cardiomyocytes one may wonder why cardiomyocytes is not on this list. In fact transplantation of cardiomyocytes has been tried, but was not successful at the experimental stage in rats (Reinecke et al., 1999). Although cardiomyocytes from fetal and neonatal rat hearts survived in the recipient hearts the adult cardiomyocytes did not (Reinecke et al., 1999).

There are many requirements that transplanted cells must fulfill in order to be beneficial to the heart. In addition to surviving, they also need to integrate, possibly differentiate, and electrically couple and beat synchronously with the host myocardium. The latter is one of the most challenging issues to resolve; too mature cells, like terminally differentiated cardiomyocytes, are not plastic enough to adjust to the new environment and therefore die, and too immature cells, including stem or progenitor cells, need complex guiding cues to be of any benefit or not be harmful

(e.g. proarrhythmic). Attempts on guiding include *in-vitro* differentiation, addition of small molecules (e.g. cytokines, peptides) to the transplantation cocktail, addition of other cells (that secrete paracrine factors), and placing the cells in engineered sheets that provide structural support (Zimmermann et al., 2006).

Most transplantation strategies must thus involve both finding a good cell source and finding out how to properly guide the cells. Human embryonic-stem-cell-derived cardiomyocytes (hESC-CMs) is one example, which has shown promising results in a study using an infarction model in monkeys (Chong et al., 2014). These cardiomyocytes integrated and survived in the infarcted myocardium (up to 3 months), and electrically coupled to the host cardiomyocytes. Nonfatal arrhythmias were observed however, which needs to be managed for safe translation to the clinic. Another example is a preclinical study on pigs, in which induced pluripotent stem cells (iPSC) were differentiated into a desirable maturation stage of cardiomyocytes (Kawamura et al., 2012) before transplantation. iPSC are engineered cells obtained by reprogramming somatic cells using exogenous transcription factor cocktails (Takahashi and Yamanaka, 2006). The two main advantages with iPSC are that they allow for autologous transplantation, circumventing immunosuppression, and can be produced in sufficient numbers from the patient's own dermal fibroblasts for instance. In fact the first clinical trial using iPSC to treat a degenerative eye disease was recently launched in Japan (Li et al., 2014). However, iPSC are still mainly used as scientific tools in experimental animal research and disease modeling *in vitro*. The field of reprogramming cells has grown rapidly after the introduction of iPSC, and has for instance been used to reprogram cardiac fibroblasts directly (not via iPSC) (Ieda et al., 2010). Clinical heart trials in humans using CPCs have proven to be safe in general but only moderately improve clinical parameters such as LV ejection fraction (Bolli et al., 2011; Makkar et al., 2012; Makkar et al., 2014). The effect is thought to be mediated by paracrine factors, which the transplanted cells secrete (or promote the host tissue to secrete). However, they have not been successful in providing new cardiomyocytes in any meaningful quantities.

Techniques for studying cardiomyocyte renewal

Cell proliferation and apoptosis (cell death) is often studied on tissue sections by visualization of molecular markers present in various stages of the cell cycle. The most common markers for proliferation are the protein Ki-67, and the phosphorylated form of histone-H3 (PHH3). Ki-67 is expressed during all active phases of the cell cycle (G1, S, M, G2), and histone-H3 is phosphorylated during the M-phase (prophase, metaphase, anaphase, telophase). The S-phase and the M-phase are the stages in the cell cycle where DNA is synthesized and the nucleus divides respectively, which are commonly followed by cytokinesis/cellular division. Polyploidization and multinucleation are exceptions to this and are examples of abortive cytokinesis. Apoptosis is studied with the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) technique, which detects fragmentation of DNA (and also to some extent other forms of cell death (Kraupp et al., 1995)). Detecting and counting cells positive for these proliferation and apoptosis markers gives a snapshot of the process and thereby some information on which cells are being renewed and to what extent at the time of analysis. If then the duration of the proliferation/apoptosis phase is considered it is possible to calculate how many cells are added/die per day, per year, and so on. However, there is no consensus on the length of the apoptotic phase and estimates range from a few hours to days, which makes the extrapolated value unreliable. Also the extrapolation from fractions per day to fractions per year implicitly assumes a constant death rate, which might not be the case. Usually the reason for performing such analyses is to gain more knowledge about the regenerative capacity of a tissue and its cells. With this aim there are additional limitations using cell cycle markers; one is that the cells might be renewed by a progenitor/stem-cell source and then it is difficult to assess the fate of the proliferating cells, or the proliferating cells may constitute a subpopulation among otherwise quiescent cells, which would then overestimate the turnover. These limitations probably reflect the large variation seen in the reports on mammalian cardiomyocyte turnover. Some studies failed to detect any renewal (Bersell et al., 2009) while others found that cardiomyocytes renew several times during a lifetime (Kajstura et al., 2010a; Kajstura et al., 1998).

Using experimental animals it is possible to overcome some of these limitations. One frequently used method is to use BrdU (Bromodeoxiuridine) and

label proliferating cells continuously over some time, thereby avoiding extensive extrapolation of the data. BrdU is a modified nucleic acid base with bromine attached to it, which makes it possible to distinguish the cells that recently synthesized DNA. However, the compound is toxic during prolonged administration, so it is not possible to track proliferation over extended time periods. In fact both BrdU and its analogue IdU are sometimes used as radiosensitizers in treatments of cancer patients (Kajstura et al., 2010b). The MIMS technology can overcome this limitation by using non-radioactive tracers such as ^{15}N-thymidine (Lechene et al., 2006). ^{15}N-thymedine, like BrdU, is incorporated during DNA synthesis when administered to the subject (experimental animal or human). The labeled cells are subsequently visualized in an atomic mass image created by ionizing a fraction of the tissue sample. The image is created by the combined use of ion microscopy and ion mass spectrometry, which allows both qualitative and quantitative information to be recorded. The MIMS technology was recently used to visualize cardiomyocyte division (Senyo et al., 2013).

Another method for tracing cell fates in transgenic experimental animals is the Cre-loxP technique. In this technique the genetic construct for the enzyme, Cre recombinase (Cre), is inserted under the control of a cell specific promotor (controls gene expression) and the loxP recognition sites are placed flanking a stop-codon upstream of a gene coding for a reporter protein (e.g. EGFP, enhanced green fluorescent protein, originally from the jellyfish *Aequorea victoria*). Upon activation of the cell specific promotor Cre is expressed, which leads to the excision of the stop codon via the loxP sites (the recombination event), thereby allowing also expression of the reporter. This requires breeding of two transgenic mice, one containing Cre and the other the reporter protein (e.g. the Rosa26 reporter mouse). The advantage of the Cre-loxP technique is that both the cells of interest and its progeny will be permanently labeled through the general expression of the ROSA26 locus. There is also a tamoxifen-inducible form of Cre that can be used to control the timing of the recombination event and hence when the cells are labeled. However, limitations to the Cre-loxP technique include possibly low recombination efficiency (fraction of recombined/labeled cells), which can make it difficult to draw conclusions on the whole cell population. In addition there is potential toxicity associated with tamoxifen (Bersell et al., 2013). This technique has for instance been used to trace cardiomyocytes (Hsieh et al., 2007). In light of the above discussion, and the limited

alternatives for studying cell renewal in humans, our method of ^{14}C birth dating has become a very powerful tool in investigating cardiomyocyte turnover. The main advantage is that turnover can be studied retrospectively, without interfering with biological processes, and even very small annual turnover rates can be detected due to the cumulative recording of ^{14}C in the genomic DNA over time.

^{14}C birth dating

^{14}C is the heaviest of the three carbon isotopes (^{12}C, ^{13}C, ^{14}C) and exists naturally in the atmosphere due to cosmic ray interactions with nitrogen. Normal atmospheric levels of ^{14}C are very low but testing of nuclear weapons during The Cold War resulted in a dramatic increase, reaching its maximum in 1964 (Figure 2). Since then the levels are decreasing, which is attributed to diffusion and interaction mainly with the oceans. The radioactive decay contributes only minimally since the half-life of ^{14}C is 5730 years (Levin and Kromer, 2004). ^{14}C in the atmosphere reacts with oxygen to produce carbon dioxide, which enters the biotope through photosynthesis, and is subsequently taken up by humans through the intake of vegetables and animals that live off plants. The transfer of ^{14}C from carbon dioxide to living material is fast as documented by carbon dating of tree rings (Figure 2), and specimens from subjects that died from sudden infant death syndrome (Spalding et al., 2005).

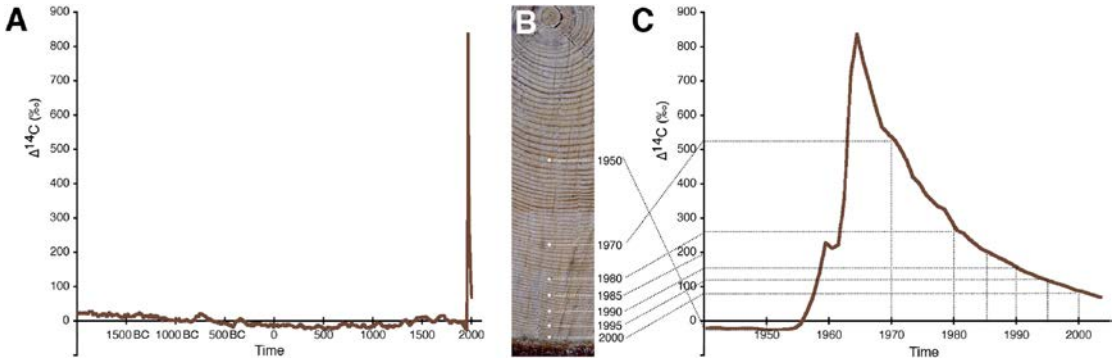

Figure 2: (A) Atmospheric ^{14}C levels were low and constant for thousands of years until 1955 when it spiked due to testing of nuclear weapons. (B-C) Validation of the transfer of ^{14}C from the atmosphere to the biotope, here exemplified by tree rings. (C) Magnification of the spike in (A).

This is because all living organisms frequently exchange carbon via metabolism. However, on a molecular level the exchange can vary greatly depending on the specific molecule. DNA is the most stable biological molecule and exchange carbon

essentially only during cell division, which means that changes in the carbon composition reflects the cell's division history. We have taken advantage of this and developed a method to retrospectively date cells in the human body, including the heart (Paper I, II and IV). We refer to this method as the ^{14}C birth dating technique. Nucleotide exchange in post-mitotic cells is minimal and below the detection limit of the technique (Bergmann et al., 2012; Bhardwaj et al., 2006; Huttner et al., 2014). The sensitivity for detecting cell turnover depends on: 1) when the individual was born in relation to when the cells were born; the larger the ^{14}C difference the larger the sensitivity, 2) the proportion of cells in the population that turns over; under optimal conditions we can detect a subpopulation making up 1% of the total population (Spalding et al., 2005) and 3) the number of individuals analyzed. However, it will never be possible to reach single cell resolution since the atmospheric ^{14}C concentrations are too low for every cell's genome to contain even one ^{14}C atom. To achieve the highest sensitivity we have developed a sorting strategy to isolate cardiomyocyte nuclei from other cardiac cell nuclei. This entails antibody labeling of the cardiomyocyte nuclear specific antigens PCM1 (pericentriolar material-1) and cardiac troponin-I and T, and fluorescence-activated cell sorting (FACS) of these labeled nuclei (Figure 3).

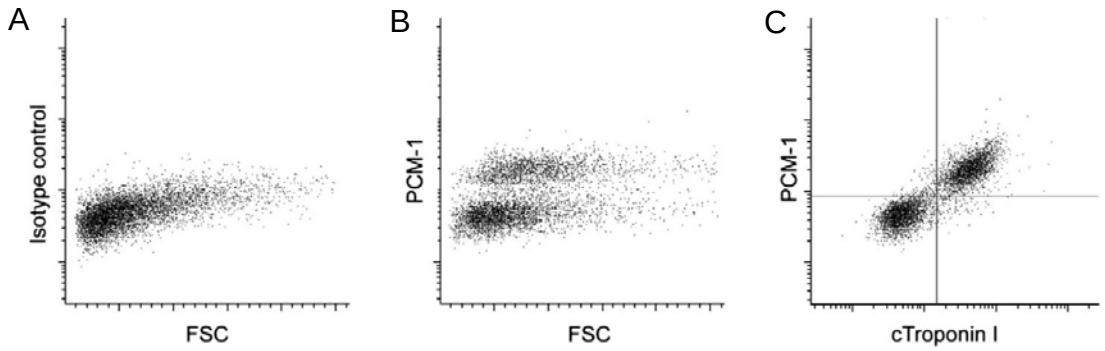

Figure 3: FACS analysis and sorting of isolated cardiac nuclei based on PCM-1 labeling to identify cardiomyocyte nuclei. (A) Nuclei labeled with an isotype control to the anti-PCM-1antibody (rabbit IgG), to control for unspecific labeling. (B) Nuclei labeled with anti-PCM-1 clearly separates cardiomyocyte (PCM-1+) from non-cardiomyocyte nuclei on forward scatter (FSC). (C) PCM-1 and cTroponin I co-labeling shows that both markers label the same population (cardiomyocyte nuclei). Lines indicate FACS gates for the sorting of cardiomyocyte and non-cardiomyocyte nuclei.

High-purity sorted nuclei are subsequently processed for DNA extraction, and the ^{14}C concentration in the DNA is measured with Accelerator Mass Spectrometry (AMS). The AMS measurement includes a preprocessing step in which the sample is combusted to CO_2 and then graphitized. During this step the mass of the DNA sample

is also determined, which serves as an additional control to make certain that no contamination has entered the sample (Salehpour et al., 2008).

The ^{14}C concentration of the DNA is reported as $\Delta^{14}C$, which is a relative unit normalized to an international standard and corrected for isotope fractionation (lighter isotopes are more reactive in chemical reactions) and decay. The first step in interpreting a ^{14}C value is to visualize it by plotting the atmospheric bomb curve, mark the birth date of the individual with a vertical line, and the measured ^{14}C value with a circle on the line. This gives an indication of the level of turnover in the population; for instance circles that display large deviations from the bomb curve indicate high turnover while circles located on the curve indicate no turnover. However, to be able to gain a more detailed description of the renewal, such as yearly turnover rates, a mathematical model is needed. The model presented here (see section Mathematical modeling of cell turnover, and Paper I, II, and IV) provides this by predicting the measured ^{14}C concentration in the population through formulation of birth, death, and polyploidization of cells.

Mathematical modeling of cell turnover

Although there are several quite diverse strategies when analyzing cell turnover, I will in this section limit the description to our core model, the so-called age-structured partial differential equation (PDE).

The measured ^{14}C value of a cell population is an average of the ^{14}C concentrations of the individual cells making up the population. This implies that the cells should be described by a cell specific feature, reasonably their age. This is because if we know how old a cell is we also know when it was born, and consequently what ^{14}C it incorporated. The cell age is denoted *a*. As mentioned above (section ^{14}C birth dating) the possible ^{14}C values that can occur in an individual depend on when the individual lived; for the same reason as we keep track of the cell age we should also keep track of the individual's age, denoted *t*. Here we define the time of birth of the individual to be $t = 0$ (i.e. not the actual calendar birth year). Furthermore, we want to describe the cell density, *n*, in order to use a conservation law for the derivation of the PDE. The cell density is the number of cells within an age class $(a, a + \Delta a)$ at time *t*, and is therefore described as $n(t, a)$. A conservation

law is one of the fundamental laws of nature, stating that that some quantity in a closed system is conserved. In this case we choose to conserve the density of cells, i.e. the density is the same within all age classes. Consequently, we are only considering aging of cells; however, we will shortly also include cell death and birth. The rate of change of the cell density can be thought of as a flux, *J*, a process commonly used in physics that refers to a flow rate (per unit area) of a quantity, for instance mass or charge. Here it relates to cells that flow or get transported through different age classes as the individual growths older, and the unit is cells per year instead. Hence, the rate of entry of cells, aged *a*, into an age class, minus the rate of their departure at age $a+\Delta a$, can be formulated as

$$\frac{\partial n(t,a)}{\partial t}\Delta a = J(t,a) - J(t,a+\Delta a)$$

$$\frac{\partial n(t,a)}{\partial t} = \frac{J(t,a) - J(t,a+\Delta a)}{\Delta a},$$

and in the limit $\Delta a \rightarrow 0$,

$$\frac{\partial n(t,a)}{\partial t} = -\frac{\partial J(t,a)}{\partial a}. \quad (1)$$

For an in depth treatment of population dynamics see for instance Perthame's Transport Equations in Biology (Perthame, 2006). To express the flux in a meaningful way we note that all cells become older in the same way. Hence we can assume that the flux is proportional to the cell density, i.e.

$$J(t,a) = v \cdot n(t,a), \quad (2)$$

where *v* is the rate of aging. Furthermore, aging corresponds one-to-one to the passage of time through

$$v = \frac{\partial a}{\partial t} = 1. \quad (3)$$

Using equations 1-3 and moving the terms to the left side we obtain a homogenous PDE,

$$\frac{\partial n(t,a)}{\partial t} + \frac{\partial n(t,a)}{\partial a} = 0. \quad (4)$$

We have not yet included that cells may die and that new cells can be born. Hence, we include cell death, μ (unit: 1/year), on the right side of (4)

$$\frac{\partial n(t,a)}{\partial t} + \frac{\partial n(t,a)}{\partial a} = -\mu(t,a)n(t,a) \quad (5)$$

Equation (5) states that the change in cell density with time and with cell age equals the fraction of cells lost by cell death. This is a linear partial differential equation that was first developed by McKendrick to describe infectious spread (McKendrick, 1925). In addition to our application of it (Bergmann et al., 2009; Bergmann et al., 2015; Bernard et al., 2010; Yeung et al., 2014), there are applications in demography and ecology, which also take advantage of its age-structure (Mohr et al., 2014). To include birth of new cells we add another equation that serves as a boundary condition, i.e. on the boundary $a = 0$

$$n(t,0) = \beta(t). \quad (6)$$

To have cell birth on the boundary is natural since birth only concerns cells with age zero (unit of β: cells/year). For the initial distribution (initial condition) of cell ages we have the generalized Dirac delta function, which is non-zero only when $a = 0$

$$n(0,a) = N_0\delta(a). \quad (7)$$

This assumes that all cells are newborn, i.e. have age zero, when the individual is born. The solution to (5), together with the side conditions, (6) and (7), is found by the method of characteristics (Figure 4 and Paper II and IV). For $a \leq t$ it has the closed form solution

$$n(t,a) = N_0\delta(a-t)\mathrm{e}^{-\int_0^a \gamma(t-a+s,s)\mathrm{d}s} + \beta(t-a)\mathrm{e}^{-\int_0^a \gamma(t-a+s,s)\mathrm{d}s}. \quad (8)$$

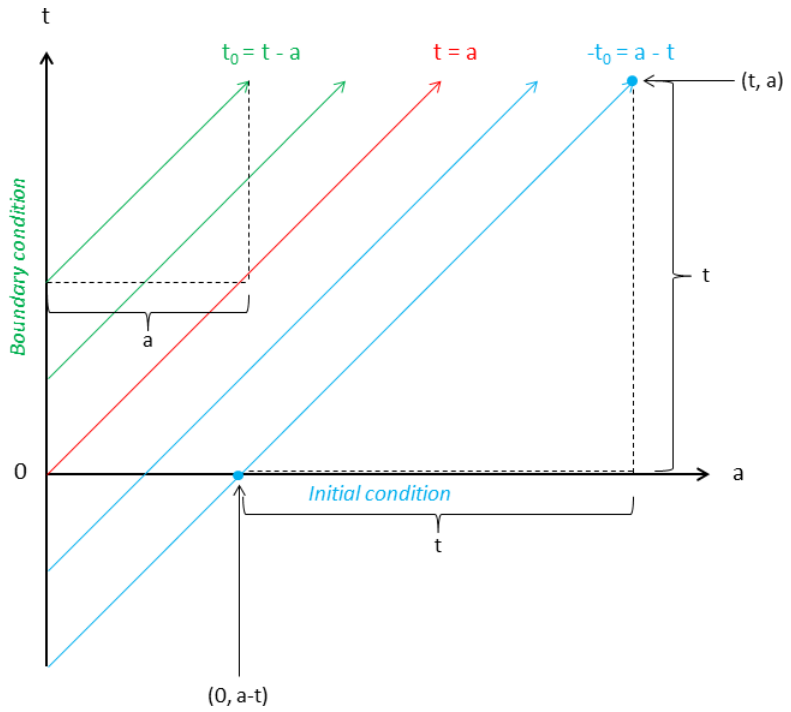

Figure 4: Method of characteristics for solving the PDE. The characteristics are straight lines with slope 1 and the solution is the sum of the solutions on all the characteristics (blue, red, and green). In the standard version of the PDE, concerning the red and green characteristics, the subject is older or have the same age as the cells ($t \geq a$). In the two-phase PDE (see Paper II) blue characteristics are also used. Cells grow older along the characteristics and the blue dots represent the probability of cells surviving from age a-t to age a. The initial distribution of cell ages (initial condition) is defined where the blue and red characteristics cross the a-axis. Cell birth is defined where the green characteristics cross the t-axis.

Hence the solution is a cell age distribution at time t during the life of the individual. However, we are most interested in the solution at the time point when the individual died, or the heart was collected ($t = T$). This distribution specifies when the cells, still present at heart collection, incorporated carbon. The average ^{14}C concentration in the population, $C_{population}$, is found by integrating over the solution, using the part of the bomb curve relevant to the individual, $C(a)$. Then we have

$$C_{population} = \frac{\int_0^T n(t,a)C(a)\mathrm{d}a}{N(T)}, \tag{9}$$

where $N(T)$ is the total cell number. $N(T)$ is found by integrating (8) over the cell ages 0 to T. The interpretation of (9) is that the differently aged cells are weighted with different ^{14}C concentrations, according to the bomb curve, and then normalized to the total cell number. It should be pointed out that the above $C_{population}$ refers to the non-cardiomyocytes in the heart (diploid cells). Since cardiomyocytes polyploidize during adolescence, part of the carbon incorporation is not associated with cell turnover; we

have taken this into account by adjusting the ^{14}C weights ($C_{adjusted}(a)$, see Paper IV). Depending on the birth and death rates of cells, different age distributions are obtained and, consequently, different values on the estimated ^{14}C in the population. The modeling of cell turnover consists of finding these rates so that the measured ^{14}C can be reproduced.

SUMMARY AND DISCUSSION

We have addressed two fundamental questions in regenerative heart medicine: do human cardiomyocytes turn over in homeostasis (Paper II and IV), and do cardiomyocytes contribute to the regenerative response in human heart disease (Paper I). To support the reached conclusions we published a method paper on how to accurately identify cardiomyocyte nuclei and their ploidy (Paper III).

In Paper IV we investigated cardiomyocyte turnover in the left ventricle of the healthy adult human heart. By ^{14}C birth dating and mathematical modeling we found that cardiomyocytes turn over with an annual rate of 1% in young adults, declining to 0.45% in advanced age. This was the first study to show that cardiomyocytes in the human heart turn over in a life-long process and that the rate is much lower than was previously suggested (Anversa and Nadal-Ginard, 2002; Kajstura et al., 1998). Since the turnover we reported was low, it was claimed that our use of cTroponin I only targeted old and senescent cardiomyocytes and therefore did not represent the whole cardiomyocyte population (Kajstura et al., 2010b). However, it is very unlikely that this is true, because the cardiac nuclei that were negative for these markers did not express the cardiomyocyte genes Nkx2.5, Gata-4, or MHC. This shows that we did not exclude any cardiomyocytes, at least not any defined by the established characteristics. The cTroponin I negative cells instead expressed von Willebrand factor, vimentin, ACTA2 and CD45, consistent with being fibroblasts, endothelial cells, and smooth muscle cells. This makes us convinced that the magnitude of cardiomyocyte turnover we have reported reflects the true turnover well.

In Paper III we showed that the nuclear cardiomyocyte markers cTroponin I/T and PCM-1 are accurate and sufficient markers to isolate the full complement of cardiomyocyte nuclei. We performed this study to strengthen the evidence for the validity of our markers. We also wanted to investigate and confirm the mainly polyploid status of cardiomyocytes, which Anversa et al. had been arguing against to support their high cardiomyocyte turnover level. All investigations of cardiomyocyte renewal need to accurately account for polyploidization since the DNA synthesis it entails can easily be mistaken for proliferation if care is not taken. Therefore we assessed ploidy both on isolated cardiomyocyte nuclei and in tissue sections. From this analysis it was clear that the majority of cardiomyocytes in healthy hearts are tetraploid. Anversa et al. used thin sections in their analysis and it is likely that many

of the large polyploid nuclei, which we found to have a diameter of >4um, were cut and therefore not counted. We also reanalyzed data from a previous study (Kajstura et al., 2010b) that used the presence of high levels of iododeoxyuridine (IdU) in cardiomyocytes as evidence for high turnover (in average 20-fold higher than indicated by ^{14}C birth dating). IdU is a radiosensitizer, sometimes used in cancer treatments, and labels cells during DNA synthesis. Therefore if many cardiomyocytes are positive for IdU soon after the IdU treatment it may indicate high turnover. However, high turnover will also dilute the IdU-label with time, so the time between treatment and death is important when drawing conclusions on the turnover level. By taking this into account we showed that the number of IdU positive cardiomyocytes and the turnover reported in the study were not compatible with each other. The inconsistency could probably be explained by DNA repair during the cancer treatment.

In Paper II we extended the analysis to include several aspects of cardiac renewal to provide a comprehensive view of the regenerative capacity of the healthy heart. We found that the total number of cardiomyocytes stays constant over lifetime, while endothelial and mesenchymal cell numbers increase during the first decades in life. Cardiomyocytes in different regions of the heart (left and right ventricles, apex, base, endocardium, and epicardium) as well as different subpopulations (diploid, tetraploid, and octaploid) turn over to the same degree, which is consistent with the dynamics found in Paper IV. An independent study that also looked at cardiomyocyte numbers in growing human hearts, using design-based stereology, a 3.4-fold increase during the first 20 years in life was found, in contrast to us (Mollova et al., 2013). In stereology, cell nuclei are used as landmarks for cells and without a marker for cardiomyocyte nuclei it is difficult to appreciate which nuclei belong to which cell. This in combination with the fact that cardiomyocytes are small in young ages, and that the nuclei are closely positioned, could possibly explain the differences between our studies.

In Paper I we investigated cardiomyocyte turnover in human heart disease (ischemic heart disease and dilated cardiomyopathy). We found that cardiomyocytes in diseased hearts have increased DNA synthesis and that this is mainly due to polyploidization. Preliminary results on turnover indicate that it increases to 2.7% per year during the disease, but that individual turnover rates probably deviate from this. Data on diploid and polyploid cardiomyocytes suggest heterogeneity in the

population, such that postnatally born cardiomyocytes are more likely to ploidize and die than the original neonatal population. However, it will be important to analyze these findings further for statistical significance. The idea of cardiomyocyte heterogeneity is exciting as it could bring us closer to discerning the phenotype and mechanisms of cardiomyocytes that can regenerate. Interestingly, it has recently been suggested that in mice a subset of cardiomyocytes are hypoxic and that these cells have higher proliferative capacity (Kimura et al., 2015).

The discrepancies between our findings and the opposing results, mainly reported by Prof. Anversa and colleagues, are most likely due to the different experimental methods used and their approach to data interpretation. Not all scientific findings will withstand the test of time and it is inevitable that some turn out to be pieces of the puzzle that did not fit. In this respect we can note that during the last couple of years evidence is accumulating that supports our view on the regenerative capacity of the heart (van Berlo and Molkentin, 2014).

CONCLUDING REMARKS

Our investigation into the regenerative capacity of the human heart has provided promising evidence that cardiomyocytes have the capacity to renew in all stages of postnatal and adult life, and that heart disease has the potential to increase it. The non-myocyte cell populations are more dynamic, both with respect to changes in cell number and baseline renewal. The findings presented in this thesis suggest that it is both reasonable and realistic to work towards improving regenerative heart therapies focusing on enhancing the endogenous regeneration. The results presented here would not have been possible without the consequences of the above ground detonations of nuclear weapons during the Cold War; it has truly given us a unique opportunity to study human tissues retrospectively. The multidisciplinary aspect of the ^{14}C birth dating project is also expected to result in improvements in fields other than regenerative heart medicine. It is now already possible to measure smaller carbon samples using accelerator mass spectrometry, which benefit therapeutic microdosing experiments with ^{14}C labeled carbon compounds. Moreover, our developments and extensions of the mathematical model for cell turnover could be useful for describing other populations that change dynamics in time, such as populations of animals and humans.

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