Stem cells in the adult heart

- 3D culture, isolation of Side Population cells and search for a stem cell niche

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In memory of Marijan Vilić and Mladen Kajić

Who gave their young lives defending our homes

And to those who survived

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ABSTRACT

Cardiac tissue shows a poor regenerative capacity. From 2003 reports, mostly based on animal models, have showed existence of stem cells also in the heart. Using ^{14}C measurements, a slow but steady turnover of the cardiac cells was shown in humans, around 1%/year. As a source for this regeneration endogenous stem cells have been suggested.

The aim of this thesis was to identify, isolate and characterize cardiac stem cells and to find their niche. Therefore, in **Study I** a new "High Density Sphere" 3D culture system was adopted where cardiac- and progenitor biomarker levels increased over time. In **Study II** Side Population progenitors were isolated from the left human atria. In **Study III** the distribution of label retaining cells was investigated, throughout the adult rat heart and a region in the Atrio Ventricular junction (AVj) was proposed as a potential stem cell niche. To assess translatability human AVj was explored in **Study IV**. The concomitant appearance of all of the selected stem cell biomarkers in the AVj indicated that the normal human heart also harbours a potential stem cell niche which to our knowledge has not been described previously. The location of these findings in the humans coincides with the same region in rat hearts.

In conclusion we propose a new, 3D *in vitro* system for studies of cardiac cell phenotypes, identified Side Population cells and found an anatomic site, with features of a stem cell niche in rats and humans. The function of these potential niches is important to investigate in future. With these findings, we hope to contribute to better understanding of basic concepts of cardiac regeneration; an important step towards improved future therapies for patients.

Keywords: Heart, Cardiac stem cells, Stem cell niche, Atrio Ventricular junction, Side Population, 3D culture

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SAMMANFATTNING PÅ SVENSKA

Hjärt-kärl sjukdom är den vanligaste dödsorsaken i Sverige. Länge trodde man att hjärtat inte hade någon läkningsförmåga. Vid en hjärtinfarkt bildas det ärrvävnad med dålig funktion jämfört med t.ex. huden som läker ihop effektivt. Antagandet förr var att de hjärtceller man föddes med dog man med. Detta synsätt kan te sig märkligt med tanke på hur central hjärtats roll är i våra kroppar. Denna uppfattning var rådande fram till 2002-2003 då de första rapporterna från djurstudier kom som visade att man identifierat stamceller även i hjärtat. Numera är det allmänt accepterat att stamceller, d.v.s. celler som har förmåga att utvecklas till olika typer av hjärtceller och därmed ersätta skadad vävnad, förekommer i det vuxna hjärtat. Ett av de viktigaste underlagen för detta är en studie där man har använt sig av mätningar med ^{14}C och daterat humana hjärtmuskelceller. Här visade det sig att en långsam men stadig nybildning sker genom livet. Ungefär 1 % av cellerna omsätts per år.

Syftet med denna avhandling var att identifiera, odla och karakterisera stamceller från hjärtat. Vi sökte igenom hjärtats olika områden i jakt på den region där de är ansamlade, en så kallad nisch. Detta, så att vi kan förstå, aktivera och öka deras förutsättningar att förbättra hjärtats regenerationsförmåga i framtiden.

I den första studien odlades cellerna tredimensionellt i hopp om att skapa en miljö som är mer vävnadslik. Celldelning kunde aktiveras, extracellulär matrix producerades och även en höjning av stamcellernas biomarkörer över tid noterades. Vidare sökte vi efter Side Population stamceller, tidigare identifierade i benmärg. Studie II visar att dessa stamceller förekommer i hjärtans vänstra förmak. Studie III, där en råttmodell användes, leder oss till gränslandet mellan förmak och kammare, till klaffarnas fästen, där vi hittar högre antal stamceller jämfört med apex och kammare. Det framstår även som att de råttorna som sprang på löpband hade högre celldelning och att fördelningen av deras stamceller påverkats. I Studie IV utforskar vi om det området som tidigare identifierats i studie III även finns i det mänskliga hjärtat. I hjärtvävnad från organdonatorer (där hjärtat inte kunnat användas till transplantation) hittade vi, på samma plats som i råtthjärtan, ett stråk med celler som uttrycker markörer typiska för stamceller. Området

består mest av bindväv och saknar kärl. Vi hittar öar av små hjärtmuskelceller intill som förefaller dela sig. Detta fenomen ses inte i kammarvävnaden från samma hjärtan. Vi undersökte även gravt sviktande hjärtan och även där förekommer en låg andel stamceller.

Sammanfattningsvist har vi tagit fram ett nytt 3D odlingssystem för studier av de olika hjärtcellernas biologi. Vi har identifierat Side Population celler, för första gången humant. En ny stamcellsnisch påträffades vid klaffarnas infästning i råtthjärtan. Även humant kunde vi bekräfta att samma anatomiska struktur innehöll tätt packade stam celler och att detta område uppvisar nisch egenskaper. Dessa pusselbitar kan hjälpa oss att på sikt förstå regeneration i hjärtat bättre. Vidare forskning behövs för att utreda funktionalitet hos de identifierade stamcellerna och nischens kapacitet. Vår förhoppning är att genom ökad kunskap, kommer helt nya behandlings-metoder mot svår hjärtsvikt att kunna utvecklas. Detta skulle potentiellt kunna leda till förbättrad hjärtfunktion och därmed ökad livskvalitet samt minskad dödlighet i denna stora patientgrupp.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. *High density sphere culture of adult cardiac cells increases the levels of cardiac and progenitor markers and shows signs of vasculogenesis*

Vukusic K, Jonsson M, Brantsing C, Dellgren G, Jeppsson A, Lindahl A. and Asp J.
Bio Med Research International 2013; 696837

II. *Left atrium of the human adult heart contains a population of side population cells*

Sandstedt J, Jonsson M, Kajic K, Sandstedt M, Lindahl A, Dellgren G, Jeppsson A and Asp J.
Basic research in cardiology 2012 Mar; 107(2):255

III. *Physical exercise affects slow cycling cells in the rat heart and reveals a new potential niche area in the atrioventricular junction*

Vukusic K, Asp J, Henriksson HB, Brisby H, Lindahl A and Sandstedt J.
Journal of Molecular Histology 2015 Oct; 46(4-5):387-98

IV. *Atrioventricular junction of the human adult heart harbours stem cells, different stages of cardiomyocytes and shows signs of hypoxia, proliferation and migration*

Vukusic K, Jansson M, Jonsson M, Sandstedt M, Oldfors A, Jeppsson A, Dellgren G, Lindahl A and Sandstedt J.
(Manuscript)

CONTENT

ABBREVIATIONS

3D	3 dimensionally
7-AAD	7-amino-acitinomysin D
ABCG2	ATP Binding Cassette sub family G member 2
AVj	AtrioVentricular junction
BrdU	5-Bromo-2- deoxy- Uridine
BSA	Bovine Serum Albumin
CD31	=PCAM1, Platelet endothelial Cell Adhesion Molecule
cDNA	complementary DNA
CVD	Cardiovascular disease
cTnT	Cardiac Troponin T
DAPI	4,6 diamidino-2-phenylindole
DDR2	Discoidin Domaine-containing Receptor 2
DNA	deoxy-ribo Nucleic Acid
dsDNA	double stranded DNA
ECM	Extra Cellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
EMT	Epithelial to Mesenchymal Transition
ES cells	Embryonic Stem cells
FACS	Fluorescence Activated Cell Sorting
FGF	Fibroblast Growth Factor
FHF	First Heart Field
FLK-1	Fetal Liver Kinas 1
FSC	Forward Scatter
FTC	Fumitremorgin C
HDS	High Density Spheres
Hif1α	Hypoxia induced factor 1α

H3P	phosphorylated histone H3
IHC	Immunohistochemistry
iPS cells	Induced Pluripotent Stem cells
Isl1	LIM-homeodomain transcription factor
LRC	Label Retaining Cells
LV	Left Ventricle
MDR1	Multidrug resistance protein 1
MHC	Myosin Heavy Chain
MSCs	Mesenchymal Stem Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCM1	PeriCentriolar Material 1
PI	Propidium Iodide
ROI	Region Of Interest
Sca1	Stem cell antigen 1
SHF	Secondary Heart Field
SP	Side Population
SSC	Side Scatter
SSEAs	Stage Specific Embryonic Antigens
TGFβ1	Transforming Growth Factor beta-1
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand factor
Wnt	Wingles integrated
WT-1	Wilms Tumor protein
qPCR	quantitative Polymerase Chain Reaction
αSMA	α Smooth Muscle Actin

1 INTRODUCTION

Cardiovascular disease (CVD) is a major health problem worldwide and the most common cause of death, with high cost for the society [1]. Since we have an increase in life expectancy, higher prevalence of CVD is also expected in the future. The underlying causes are for example smoking, lack of physical activity, hypertension and diet.

The mortality associated with CVD has been reduced by modern treatments such as revascularization procedures. However there are no therapeutic approaches that restore the loss of cardiomyocytes, which is the underlying cause of heart failure. The only option is cardiac transplantation but the access to donor organs is strictly limited [2]. There is thus a large clinical need for development of new treatments to reduce mortality and improve quality of life for this large group of patients.

The heart was regarded as a post-mitotic organ that loses the ability to form new cardiomyocytes after birth and lacks the ability to regenerate after injury. Therefore, treatment of ischemic heart disease and heart failure was mainly focused on the protection and preservation of the cardiac muscle tissue. However, in 2002-2003, the first reports about the existence of cardiac stem cells were published [3, 4]. The existence of endogenous stem cells as well as the demonstration of turnover of existing cardiomyocytes [5, 6] have opened new possibilities. Although the field of cardiac regeneration has been growing rapidly, there is still a lack of knowledge regarding the mechanisms behind the cardiac tissue repair. In particular, there is a need for more knowledge regarding regeneration in the human heart as most studies have been carried out in animal models. The overall aim of this thesis was thus to identify, isolate and characterize cardiac stem cells in the human heart and to explore whether the adult heart contains a stem cell niche structure.

1.1 The Heart

The circulation of the blood is constantly going on in our bodies and life itself depends on the function of the heart. This muscle pump is residing in the middle of the chest, in the thoracic cavity slightly turned to the left. It has a size of a fist. It is built up of four chambers, two atria and two ventricles and between these and the outflow tract are the valves (Fig. 1).

The main function of the heart is to pump the blood through the body. The left ventricle pumps the blood through the aorta, the largest vessel, out into the systemic circulation and brings the oxygen into the body. The poorly oxygenated blood from the body is collected by veins, that carry the blood back to the right atria and then through the tricuspid valve, into the right ventricle. The right ventricle pushes the blood through the pulmonary valve and through the lungs. Oxygenated blood flows into the left atrium, the mitral valve opens and blood flows in into the left ventricle. Coronary arteries arise as direct branches of the aorta and are the ones that perfuse the myocardium. The beat frequency of the heart is normally regulated by the sinus node, which can be modulated by the sympathetic and parasympathetic nervous system. Excitation of the sinus node is spread through the atria and then through the ventricles via the atrioventricular node. The heart is enclosed within the pericardial sac [7].

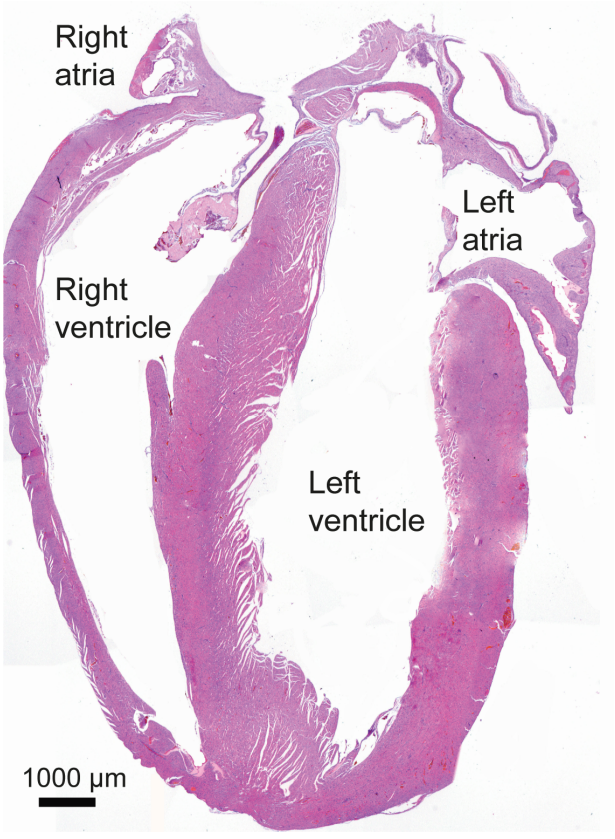

Figure 1. *Histology of one of the rat hearts from Study III. Hematoxylin Eosin staining of a cross-section displaying the four chambers of the heart*

1.1.1 Histology of the myocardium

The thickness of the cardiac wall and the diameter of the muscle fibres is depending on the workload. Therefore, the atrial walls are the thinnest, since the blood from here flows into the ventricles with minimal resistance. In contrast, the left ventricle has the highest workload and as a consequence, the thickest wall of all of the chambers of the heart.

The cardiac wall is composed of three histological layers (Fig. 2). *Epicardium* is the outer layer, where the coronary arteries are located. It is covered with mesothelial cells. *Myocardium* is the thick muscle layer responsible for the pumping action of the heart. *Endocardium* is the inner lining layer, covered by endothelial cells and in direct contact with the blood in the chambers. Cardiomyocytes appear elliptical in transverse sections with centrally placed nuclei that are irregular in shape [8].

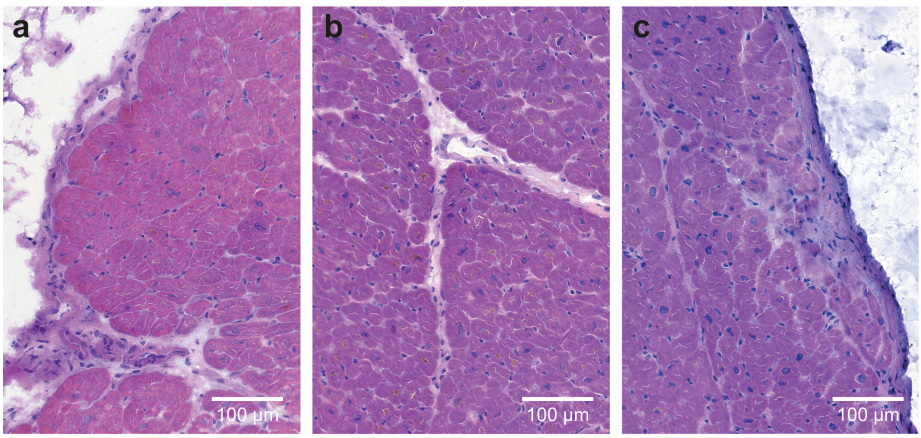

Figure 2. *Haematoxylin eosin staining showing the histology of the three main layers or the human cardiac wall: a) epicardium, b) myocardium and c)endocardium.*

Myocardium is a form of striated muscle but still different from the skeletal muscle. Actin and myosin filaments are arranged to be able to mediate contractions. The long myocardial muscles are produced by linking cardiomyocytes end to end (Fig. 3). Within the intercalated discs there are communicating gap junctions that enable the synchronization of muscle contractions. Between the fibres there is loose fibro-collagenous tissue containing small blood vessels [8].

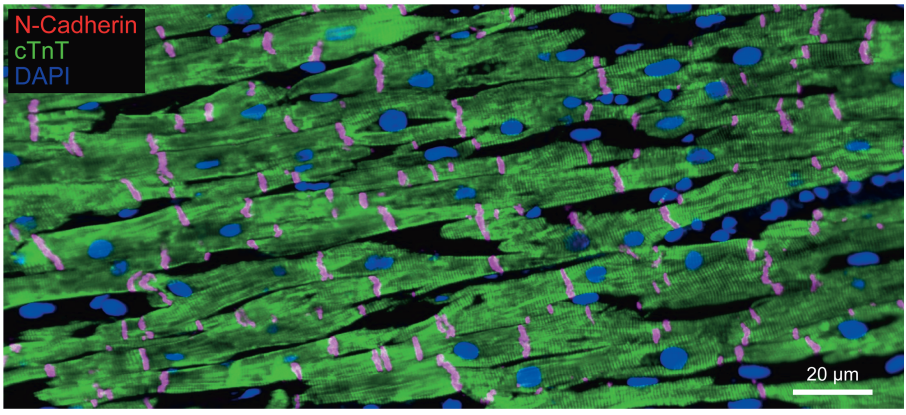

Figure 3. *Immunohistochemistry staining of a tissue section from human left ventricle, with antibodies against cTnT detecting the cytoplasm of the cardiomyocytes and n-cadherin detecting the intercalated discs. Nuclei are stained with DAPI.*

1.1.2 Histology of the atrioventricular junction (AVj) and the valves

The heart has a fibrogenous skeleton. The central fibrous body is the main component, located at the level of the valves. The valve rings are formed of extensions of the central fibrous body, surrounding the valves [8]. A fibrocollagenous skeleton anchors the chambers and the valves together. The valves are stratified into Extra Cellular Matrix (ECM) rich layers consisting of elastin, proteoglycans and collagen. The valve leaflets are lined by endothelial cells [9]. Histology of the AVj is shown in Fig. 4.

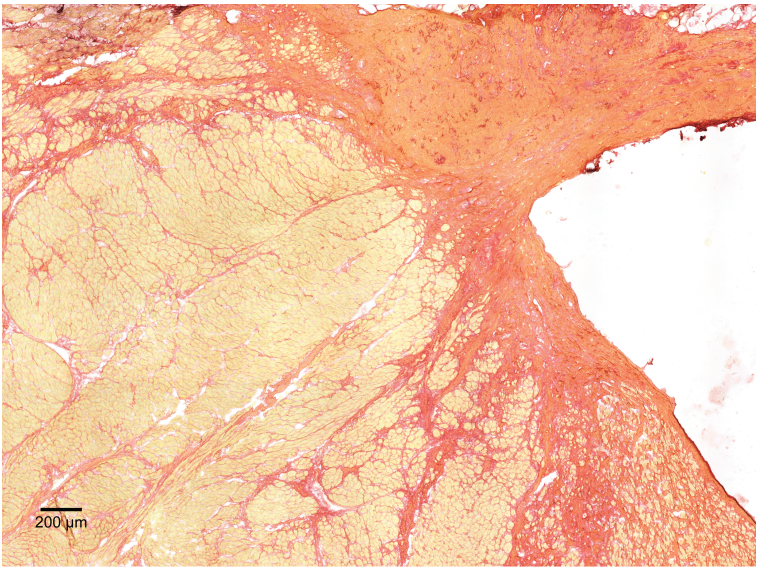

Figure 4. *Picric Sirius red staining of a tissue section from human AVj from Study IV, staining the collagen in Mitral valve and the connective tissue red.*

1.1.3 Cell phenotypes in the cardiac tissue

The most characteristic cardiac type of cell is the cardiomyocyte. However, there are also non-myocytes including fibroblasts, endothelial cells, pericytes, smooth muscle cells and resident immune cells. The proportions of the various cardiac cell phenotypes is not clear [10]. Early studies in rats concluded that myocardium consists of 70% myocytes/30% non-myocytes [11]. Others have shown 55 % myocytes /45% non-myocytes, predominantly fibroblasts, in mice [12].

In the normal human ventricular tissue 70-90% of the total volume is taken up by cardiomyocytes and increases with age [13, 14]. Only 30% of all cells are cardiomyocytes and with age this number decreases. In other words, cardiomyocytes take up most of the volume, while they are a minority within the other cell types. Lately, immunohistochemistry (IHC) and flow cytometry analysis confirmed that 31% of nuclei, in mice and human hearts, were cardiomyocytes. Among the non-myocytes, endothelial cells constitute >60%, hematopoietic-derived cells 5%-10% and fibroblasts <20%. There were no large regional differences in cardiac cellular composition [15]. Fibroblasts constitute a relatively minor population compared to earlier reports. Endothelial cells, being the majority of non-myocytes, are predicted to play a larger physiological role than previously appreciated.

Cardiomyocytes

Cardiomyocytes are the beating units of the myocardium where mitochondria take up a large portion of the cell volume. They contain myofibrils with thick and thin filaments. Polyploidization (DNA replication without nucleus division) occurs during childhood. Diploid cardiomyocytes are the most common at birth and with time polyploidization occurs. After the age of 10 no increase in polyploidization was observed [16]. The function of the polyploidization is not yet understood [17]. Furthermore, cardiomyocytes may also be multinucleated. Around 70% of the cardiomyocytes are mononucleated and the rest multinucleated, mostly binucleated. This is a result of their ability to duplicate the DNA, divide the nucleus, without undergoing the cell division [18]. The nuclei configuration is stable with age, in humans [19].

Common markers used for detection of cardiomyocytes are contractile proteins such as cardiac Troponin T (cTnT) (Fig. 5), Troponin I (cTnI) and cardiac α-actin or gap junctions proteins such as connexin 43 and n-cadherin [20] (Fig. 3). There is also a marker for human cardiomyocyte nuclei, Pericentriolar Material 1 (PCM1), (Fig. 5). PCM1 is a centrosome protein that re-localizes to the nuclear membrane, where it accumulates and forms an insoluble matrix in differentiated myocytes. PCM1-labeled nuclei were expressing the cardiac specific transcription factor NKX2.5 and surrounded by Myosin Heavy Chain (MHC) positive cytoplasm, showing the specificity of this marker. The staining pattern appeared as perinuclear [21].

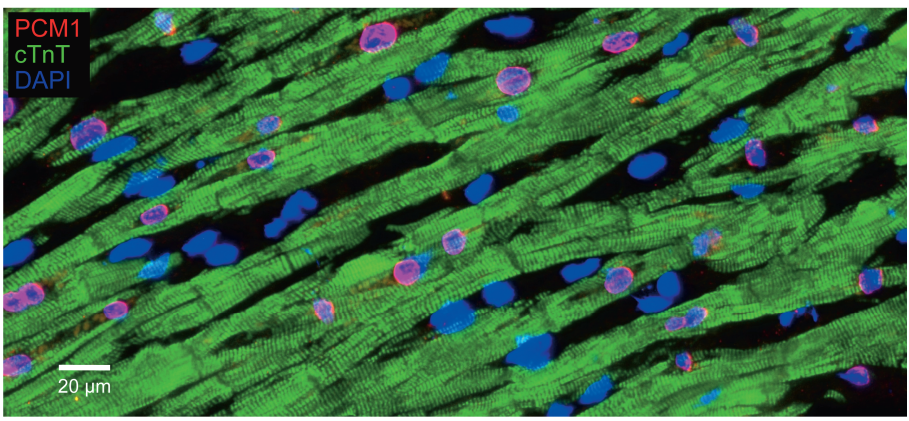

Figure 5. Immunohistochemistry with antibody against cTnT staining human cardiomyocyte cytoplasm and antibody against PCM1 detecting human cardiomyocyte nuclei, in the human left ventricular tissue.

Endothelial cells

The inner layer of the blood vessel wall is composed of flattened endothelial cells. An endothelium is also lining the cardiac lumen. The nuclei are flattened and the cytoplasm small [8]. Endothelial cells are anchored together by tight junctions to prevent diffusion. They secret factors that regulate the coagulation of the blood. Endothelial cells respond to changes in blood pressure, oxygen tension and blood flow and can regulate the contraction state of smooth muscle cells in the vessel walls. Inflammatory cells migrate into the tissue through the endothelium [7].

There are many biomarkers used for detection of endothelial cells like von Willebrand factor (vWF), involved in platelet adhesion and activation of blood coagulation [22]. Others are adhesion protein CD31 [12] (Fig. 6), Vascular Endothelial Growth Factor (VEGF) and its receptor Fetal Liver Kinase 1 (FLK1) [23].

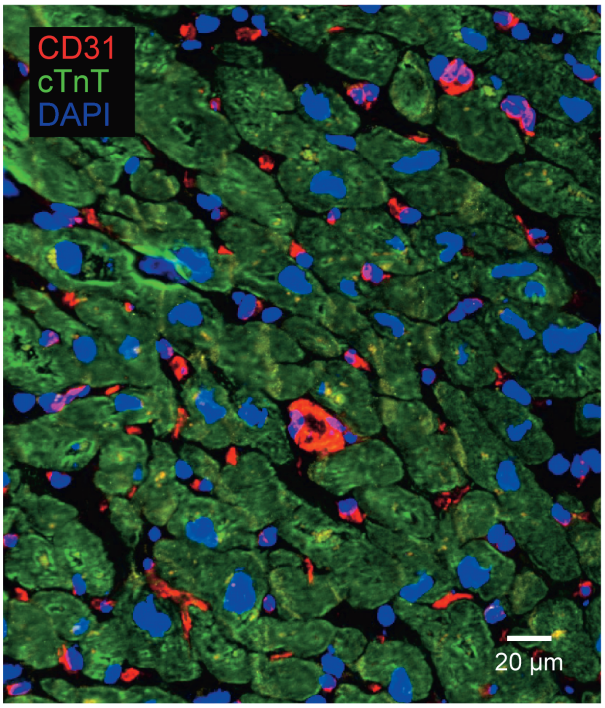

Figure 6. Immunohistochemistry with antibody against CD31 staining endothelial cells and antibody against cTnT staining cardiomyocytes in left ventricular tissue.

Cardiac Fibroblasts

The main function of cardiac fibroblasts has been regarded as production of Extra Cellular Matrix (ECM) proteins, including collagens. ECM gives the tissue its biomechanical properties and structure. Production of cytokines, growth factors and matrix metalloproteinases, needed for connective tissue homeostasis, is another function of fibroblasts. It has also been shown that fibroblasts form junctions with cardiomyocytes, playing a role in their response to mechanical stimulation [24].

As a response to hypertrophy and myocardial infarction, fibrosis occurs as a protective mechanism. Activated cardiac fibroblasts have the ability to upregulate α smooth muscle actin (αSMA) and can become myofibroblast [10, 25]. A major clinical issue, caused by long term chronic hypertension, is fibroblast accumulation resulting in excessive fibrosis [10].

There are many markers used for detection of fibroblasts. Thymocyte 1 (Thy1, CD90) and Vimentin are expressed by cardiac fibroblasts, but also by other cells [11, 24]. A more specific marker is Discoidin receptor 2 (DDR2), a member of collagen specific receptor tyrosine kinase family, which labels fibroblasts, but not endothelium, smooth muscle, or myocytes [26]. Te7 (Fig. 7) is another fibroblast-specific, connective tissue protein, that has been validated for the detection of fibroblasts in a number of tissues including human skeletal muscle [27].

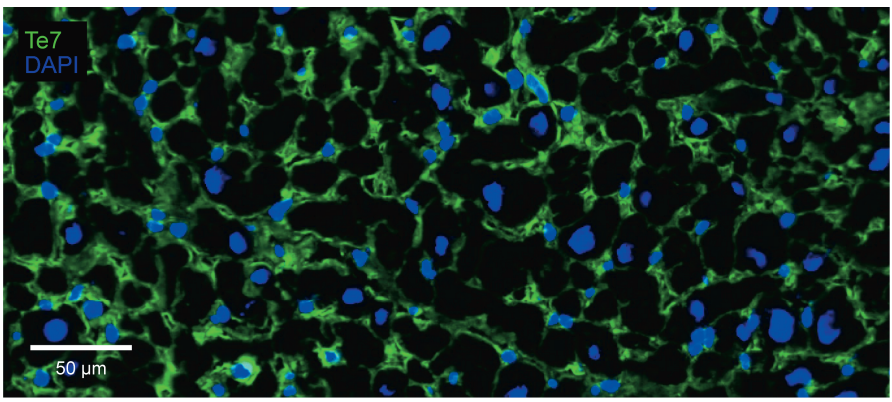

Figure 7. Immunohistochemistry with antibody against Te7, staining the network of fibroblast in the human left ventricular tissue.

Smooth muscle cells

Cardiac smooth muscle cells are the main contractile units in the blood vessel wall, involved in regulation of blood pressure. They are anchored together into functional units by basement membrane material. Their nuclei are centrally placed and elongated, the cytoplasm is spindle shaped. A common marker used for the detection of smooth muscle cells is the αSMA [12]. Although, as mentioned previously, activated cardiac fibroblast also express this marker, making the distinction difficult.

Pericytes

Pericytes are spindle shaped cells arranged around the blood vessels. They form junctions with endothelial cells as well as with each other. Pericytes are involved in regulation of angiogenesis, coagulation and blood flow in small arterioles [28]. A combination of markers is needed to distinguish them from other cells like fibroblasts [29].

1.1.4 Effects of physical exercise on the heart

Sedentary life style is a well-established risk factor for CVD. By adopting a life style with high physical activity on the other hand, the risk for coronary heart disease may be lowered by 20 – 30% [30]. Aerobic capacity testing is used as a tool in clinical practice, when diagnosing ischemic heart disease. A recent cohort study of middle-aged men, with a long follow up of 40 years, showed that low aerobic capacity was associated with increased mortality, independent of traditional risk factors. Only smoking had higher impact on mortality [31]. There may be several reasons behind the observed CVD reducing effects of physical exercise including lower resting heart rate [32, 33], better perfusion and myocardial function [32, 34]. Therefore, it is prescribed as a part of the treatment within CVD [35].

When it comes to effects of physical exercise on cardiac regeneration, it has been shown that physical exercise resulted in increased cardiomyocyte size in a rat model. Furthermore, increased numbers of small, newly formed cardiomyocytes were shown by BrdU incorporation [36]. The intensity of the training was correlated with the formation of new cardiomyocytes. Surprisingly, 7% of the cardiomyocytes were formed during only four weeks of intensive training, suggesting very high cardiomyocyte turnover. The suggested mechanism was activation of endogenous cardiac stem cells. In Paper

III in this thesis, a rat model was used to analyse the effects of moderate physical exercise on proliferation and progenitor cell distribution.

1.1.5 Aging and pathology

Cardiac aging is a process characterized by several histopathological changes including fibrosis, hypertrophic myocytes and collapse of sarcomeres. Loss of cardiomyocytes with age is compensated by changes in ECM composition leading to cardiac stiffness [37, 38]. Furthermore, hypertension and oxidative stress lead to vascular remodelling, characterized by arterial stiffness [39]. With time, these changes lead to higher prevalence of the heart failure and ischemic heart disease in elderly population.

1.2 Embryonic development of the heart

In human embryogenesis, the heart is derived from the mesodermal germ layer through a complex developmental process, controlled by signaling pathways including canonical Wnt/βcatenin, IGF, Notch, TGFβ/BMP2 and Hippo [40, 41].

During gastrulation, the primitive epiblast layer undergoes epithelial-to-mesenchymal transition (EMT) and form the primitive streak. There are some fundamental steps that take place including the formation of First and Secondary Heart Fields (FHF and SHF) and the epithelial layer; cardiac crescent. In the third embryonic week, the cardiac crescent fuses at the midline and gets together to form the heart tube that consists of two layers; endocardium and myocardium. The epicardium is derived from the proepicardium around day 21 in humans. The linear heart tube starts beating at day 22 [42]. Eventually it branches and loops, forming atria and ventricles and giving rise to the four chamber heart [41, 43]. The remodeling of the heart is complete at day 50 [44].

Expression of Isl1 is used for definition of SHF progenitors that contribute to the atria, outflow tract and the right ventricle. Expression of Tbx5, Nkx2.5, and HCN4 have been used to define FHF progenitors, contributing mainly to left ventricle development [45].

Proepicardial and epicardial stem cells undergo an Epithelial to Mesenchymal Transition (EMT) and have the ability to differentiate into cardiomyocytes, endothelial and smooth muscle cells [46, 47]. Transcription factors Tbx18 and Wilms tumor protein (WT1) were expressed and suggested as markers for the epicardial stem cells. Commonly used mesodermal cardiac lineage markers include among others: Isl1, Tbx5, NKX2.5, Gata4, SRF, and Mef2c [48].

1.2.1 Development of the AVj and the valves

The epicardium plays a significant role in the formation of the AVj. First, through the process of EMT, the subepicardial AV mesenchyme is formed. Annulus fibrosus, a fibrous sheath of tissue, is the physical barrier between atrial and ventricular working myocardium. It is formed by AV-epicardium together with the epicardium derived cells. The formation of the annulus fibrosus is a crucial step of the development of the AVj, for a functional electrical separation of atrial and ventricular tissue. Subsequently, migration of the epicardium derived cells into the valves form the leaflets [47, 49, 50].

1.3 Stem cells

Stem cells are traditionally defined by functional assays where they have to fulfil the criteria of self-renewal and multipotency [51]. Embryonic stem (ES) cells have the unlimited proliferative capacity and are *pluripotent,* meaning that they can give rise to cells from all the three germlayers; mesoderm, endoderm and ectoderm [52].

Adult stem cells are found in several different tissues. They display limited plasticity and proliferative capacity. The role of adult stem cells is to maintain the tissue homeostasis by replenishing dying cells and regenerating damaged tissue. Within the hematopoietic system, different adult stem cell populations have been characterized and a developmental hierarchy described [53, 54]. Other examples are epidermal stem cells [55], satellite cells in skeletal muscle [56] and intestinal stem cells [57].

The current theory is that differentiated cells, with a mature phenotype, cannot re-enter into a more undifferentiated stage *in vivo*. However, *in vitro,* it was first shown by Yamanaka et al. that mature fibroblasts can be re-programmed into a multipotent phenotype called induced pluripotent stem (iPS) cells. This was done by overexpressing four factors; Oct3/4, Sox2, c-Myc, and Klf4 [58]. In line with this study, reprogramming of mouse fibroblasts directly into functional cardiomyocytes was reported with combination of three early cardiac transcription factors; Gata4, Mef2c, and Tbx5 [59]. These reprogramed fibroblasts were suggested as a source of cardiomyocytes for regenerative approaches.

During development, the stem cells get more specialized through the process of differentiation and get a certain phenotype. In several tissues, reservoirs of adult stem cells, so called niches, have been described. Upon activation of the niche, stem cells differentiate into the tissue specific phenotypes of cells. In this manner, it has been reported that endogenous cardiac stem cells can give rise to cardiomyocytes, endothelial- and smooth muscle cells.

It should be noted that within the field of cardiac regeneration, the terms ”stem cells” and ”progenitor cells” are often used as equal. Progenitor cells is perhaps more reflecting the capacity of the adult cardiac endogenous populations.

1.4 Cardiac regeneration

The heart has been considered as an organ lacking regenerative capacity. The main evidence for this was the lack of mitoses within the cardiomyocytes [60] and the constant numbers of cardiomyocytes through life [61]. Hypertrophy became the accepted mechanism behind myocardium growth and tissue homeostasis. The scar tissue formation and low tissue function, upon myocardial infarction, are consequences of limited regenerative capacity, compared to other tissues.

However, in a study by Bergman et al. integration of ^{14}C into the DNA, generated during the Cold War, was used to date human cardiomyocytes. It was shown that new cardiomyocytes are formed throughout life. The rate of this turnover was however modest, with 1% turnover/year up to the age of 50, after which the rate declined [16]. Mollowa et al examined cardiomyocyte mitosis, using an antibody against phosphorylated histone H3 (H3P) and concluded that cardiomyocyte proliferation contributes to developmental heart growth in young humans [19]. Furthermore, expression of the proliferation marker Ki67 was found in human cardiomyocytes [62-64].

When BrdU incorporation was used for 6 days, in a mouse model, it was reported that 10 - 19% of all cardiomyocytes were formed during a period of 10 weeks, suggesting extremely high cardiomyocyte turnover [65]. In another BrdU retention study, also in a mouse model, BrdU staining was however not observed in cardiomyocytes at all [66]. The reason to this discrepancy is unclear.

Within the field of cardiac regeneration, there are three main hypotheses about the cell source for regeneration (Fig. 8). These are: regeneration through differentiation of a pool of endogenous cardiac stem cells, regeneration through migration and transdifferentiation of extracardiac progenitor cells and de-differentiation of pre-existing cardiomyocytes and re-entry into the cell cycle. If these three mechanisms act in parallel or if one excludes the other is not yet known.

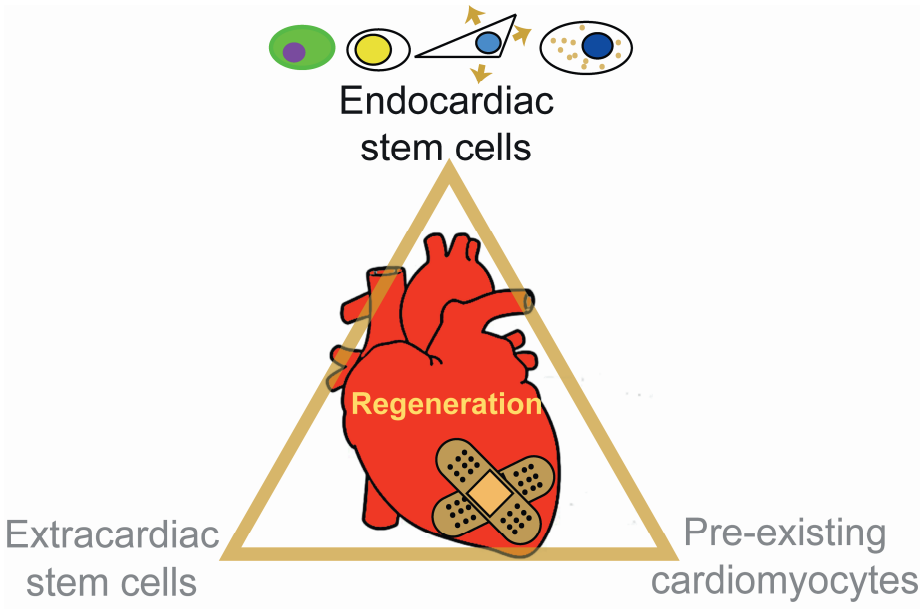

Figure 8. *Main hypotheses in the field of cardiac regeneration*

Extracardiac stem cells do not seem to be the source to regeneration since the early promising results [67, 68] could not be reproduced. The turnover of existing cardiomyocytes is demonstrated but low and decreasing with age. Whether the new cardiomyocytes are derived by division of pre-existing cardiomyocytes or by differentiation of cardiac progenitors is still not clear. Even if renewal of cardiomyocytes mainly occurs due to proliferation of cardiomyocytes [6, 19], their regenerative capacity after injury has not been fully investigated. Derivation of other cardiac cell phenotypes after injury is another important aspect. Activation of endogenous progenitor cells after injury has been shown [69-71]. Endogenous progenitors are the primary source of new cells in many other tissues. Therefore, this thesis is focused on endogenous cardiac stem cells.

1.4.1 Endogenous cardiac progenitor cells

Cardiac stem/progenitor populations are rare *in vivo*, comprising just 0.005-2% of all adult cardiac cells [72]. They have the ability to differentiate into cells within the cardiac lineage such as cardiomyocytes, endothelial- and smooth muscle cells. Existence of intracardiac stem cells was first reported in 2002-2003 [3, 4] Since then, several strategies have been used to isolate intracardiac progenitor-cells, including cell culture systems selective for stem cells, functional assays and biomarkers.

Isl1+

The transcription factor Isl1 is one of the earliest markers within cardiomyogenesis, expressed by the SHF progenitors. Knockout studies showed that mice lacking Isl1 failed to enter the looping phase and developed severe malformations [73-75].

In myocardium from neonatal rats, Isl1+ cells were observed in the outflow tract, at the junction between outflow tract and ventricular tissue. Co-staining with cTnT was interpreted as evidence of cardiac commitment. A subpopulation of the Isl1+ cells expressed the proliferation marker Ki67 [76]. The same phenomenon, Isl1+/cTnT+ cells, were found in the adult outflow tract [73]. Cardiomyogenic differentiation potential was shown in Isl1+, derived from cultures of cardiac tissue from neonatal mice [77].

Isl1 expression has been observed in human fetal tissue, in the right atrium, outflow tract, left atrial wall and appendage. The expression decreased with time [78]. Isl1+ cells have been found by immunohistochemistry (IHC) after birth in a rat model. Immature Isl1+ cells were present in the out flow tract, where they resided until adulthood [73]. Distribution of Isl1+ cells in adult mice was investigated were they were largely confined to the sinoatrial node. Few Isl1+ cells were found within the outflow tract, co-expressing the cardiomyocyte marker α-actinin. Between 1 to 18 months of age, frequency and localization of Isl1+ cells remained unchanged [79].

Side population

Side Population (SP) cells express ABC transporter proteins, and have the ability to exclude the DNA dye Hoechst 33342. It is thought that these proteins may protect stem cells by excluding certain toxic substances. The ability to exclude Hoechst 33342 is used for identification of these progenitors using flow cytometry [80]. This SP assay was originally used for identification of hematopoietic stem cells [81]. SP cells showed high potency in repopulation assays, which is a hallmark for hematopoietic stem cells. Since then, SP cells have been identified in various tissues including the limbus region of the eye [82], the aorta [83] and in lung epithelium [84].

Multidrug resistance protein 1 (MDR1) and ATP-binding cassette sub-family G member 2 (ABCG2) are two well-known ABC transporter proteins which have been linked to the SP phenotype [85, 86]. In a knock out mouse model, it was shown that ABCG2 was the protein responsible for the SP phenotype during the neonatal period. With age MDR1 expression gradually increased and was the dominant efflux protein in the cardiac SP population [87].

Cardiac SP cells, isolated from mouse, were described as Sca1- and C-kit- [4]. In contrast, another study using the same species, described the SP cells as Sca1+ and to large extend CD31+. However, it was only possible to establish culture of Sca-1+/CD31- subpopulation. Cardiomyogenic differentiation was induced by co-culture with adult cardiomyocytes [88]. Eventually, the Sca1+/CD31+ subpopulation of SP cells was shown to display functional properties of endothelial progenitor cells, forming capillary structures in vivo and in vitro [89].

Adult SP/Sca-1+CD31- cells, isolated from mice, were injected into a non-ischemic area of the infracted heart and migration was studied. SP cells migrated to the infarction zone and differentiated into cardiomyocytes and endothelial cells [90]. The migrative capacity was also demonstrated when SP cells were injected in the tail vein of adult rats with myocardial infarction. SP cells migrated all the way to the infarct zone and were shown to possess cardiomyogenic differentiation potential. Interestingly, in the control group of the non-infarcted animals, lower number of the SP cells migrated and no evidence of differentiation was reported. This indicates that paracrine communication from the infarcted tissue is necessary for activation, migration and differentiation of stem cells [91]. As for many other stem

cell populations; the frequency of SP cells is highest in the fetal heart [91, 92].

SSEAs

Stage Specific Embryonic Antigens (SSEAs), SSEA-1, 3 and 4, are glycolipids found on the cell surfaces and have commonly been used for determination of the differentiation status of embryonic stem cells [93]. In humans, SSEA-3 and 4 are markers of undifferentiated cells. With differentiation SSEA-1 is upregulated while SSEA-3 and 4 are downregulated [93, 94]. In contrast, in rodents SSEA1 is expressed by undifferentiated cells while SSEA 3 and 4 increase with differentiation.

Cardiomyogenic differentiation potential of SSEA1+ cells, isolated from rat neonatal and adult myocardium, was shown by Ott et al. [95]. The cells grew in suspension and gave rise to spontaneously beating cardiomyocytes. When co-cultured with neonatal rat cardiomyocytes, the numbers of beating cardiomyocytes increased. Cardiomyogenic differentiation *in vivo* was also shown, when injected into the infarcted myocardium.

Tissue sections of fetal and neonatal human hearts were examined for expression of SSEA-4. In both the atrial and ventricular myocardium of the fetal heart, SSEA-4+ cells were detected. The neonatal tissue showed lower expression [96]. Adult human tissue revealed SSEA-3 and 4 expression in blood vessels but also in CD31+ cells within the myocardium [97].

In our lab, SSEA-1, 3 and 4 positive cells have been isolated and characterized, from human atrial tissue. High gene expression of cardiomyocyte markers TBX5, NKX2.5 and cTnT was detected in the SSEA4+/CD34- population, isolated by flow cytometry. IHC on tissue sections showed that some SSEA4+ cells co-expressed NKX2.5 and cTnT. We concluded that the SSEA4+/CD34- population showed evidence of cardiomyogenic commitment [98].

WT1

Wilms tumour protein (WT1), is a transcription factor expressed in epicardium [46] and also by majority of the cardiac endothelial cells during murine cardiomyogenesis [99]. In adulthood only a subset of coronary endothelial cells remains positive.

In the adult mouse heart, WT1 mRNA expression was only found in the epicardium covering the atrioventricular sulcus and apex.

Furthermore, contribution of the epicardium to regeneration in response to ischemia was followed. Epicardial cells, overlaying the ischemic area, had disappeared one day after MI [100]. Upregulated expression of the WT1 was noted at the infarction site and by lineage tracing studies, these WT1+ cells were shown to regenerate the epicardial layer. The infarct area is characterized by a hypoxic environment. Hypoxia increased expression of WT1 in endothelial cells in vivo and in cultured endothelial cell line (HUVEC) in vitro [99].

Two studies showed that a small fraction of WT1+ cells could give rise to cardiomyocytes [100, 101]. Another study however, could only show differentiation into fibroblast and myofibroblast [102].

C-kit

Tyrosine kinase receptor c-kit is a well-known stem cell marker from the hematopoietic stem cell field. A population of c-kit+/ hematopoietic lineage negative cells were identified in adult rat heart in 2003 [3]. Their clonogenity and cardiomyogenic differentiation potential was shown *in vitro* and in infarction model *in vivo*. c-kit+ cells have also been isolated from myocardium of mice [103].

IHC was used on human tissue, from outflow tract, to identify c-kit+ cells. Within this population early cardiac markers MEF2c and GATA4 expression was observed. Clonogenity and differentiation potential of c-kit+ cells, isolated from right atria was also shown. Telomerase was expressed in this population indicating high proliferative capacity [104].

When searching for stem cell niches in mouse model, c-kit was chosen as the main cardiac stem cell marker. Expression was found in nests of cells, between cardiomyocytes, in the myocardium of atria, base-mid region and apex [65]. The frequency of the c-kit+ cells has been investigated in different regions of the human myocardium. Existence of c-kit+ cells was found in in all four chambers of the human heart, with highest numbers in epicardium [105] or right atrium [106, 107].

The regenerative capacity of endogenous c-kit+ cardiac cells has lately been called into question. Recent lineage tracing studies have shown minimal contribution of c-kit cells to generation of cardiomyocytes. In these studies, c-kit+ cells predominantly gave rise to new endothelial cells [108-112].

Cardiospheres

Cardiospheres is a cell culture system considered as selective for cardiac progenitor cells. Cardiospheres were originally derived by mild dissociation of mouse and human cardiac tissue. Phase bright cells were harvested and showed the ability to spontaneously form spheres that could be cultured. Spontaneous differentiation into cardiomyocytes was reported from mouse and human cardiospheres, if cocultured with adult rat cardiomyocytes [113].

Later, cardiospheres were cultured from small biopsies of adult human endomyocardial tissue [114]. These were transplanted and improved the cardiac function giving rise to cardiomyocytes and endothelial cells. This was also shown when cardiospheres were generated from early post-natal and adolescent human cardiac tissue [106]. When a direct comparison was made between cardiospheres derived from adult and neonatal human tissue, higher cardiomyogenic differentiation potential *in vitro* was reported from the neonatal origin. When transplanted into infarcted myocardium, differentiation *in vivo* was only observed in the neonatal cardiospheres and was rather low [115].

The identity and regenerative capacity of cardiospheres derived cells has also been called into questioned [116]. It was recently shown that these cells had a mesenchymal phenotype and did not improve cardiac function in a rat infarction model [117].

Sca1+

Stem cell antigen 1 (Sca1) is a cell surface protein, found on murine hematopoietic stem cells [118]. When isolated from adult mouse heart, Sca1+ cells showed expression of cardiac transcription factors but lacked expression of other cardiac lineage markers [119]. Sca1+ cells showed the ability to differentiate into cardiomyocytes, in response to 5-azacytidine treatment. Treatment with expanded Sca-1+ cells in a mouse infarction model resulted in increased repair and improvement of cardiac function [119, 120].

Importantly, there is no human equivalent of murine Sca-1. However, a method for isolation and expansion of human cardiac progenitor cells, using an antibody against the mouse Sca-1 antigen, has been developed. These cells were differentiated into beating cardiomyocytes in vitro using 5-azacytidine treatment [121].

1.4.2 Regeneration by pre-existing cardiomyocytes

During the last years a new concept of cardiac regeneration has been introduced. The impressive regenerative capacity of the zebrafish heart was first demonstrated, by complete regeneration after resection of the apex [122]. The predominant mechanism behind regeneration was later shown as de-differentiation of pre-existing cardiomyocytes and re-entry in the cell cycle [123]. It was shown that pre-existing cardiomyocytes in the wound zone de-differentiate, detach from each other, upregulated the expression of GATA4 and re-entered the cell cycle. This mechanism has in more recent studies also been shown in neonatal mice [5]. The human heart however was believed to grow by enlargement but not proliferation of cardiomyocytes. Lately, in young humans, 1-20 years, it was shown that cardiomyocyte proliferation contributes to developmental heart growth [19], indicating that this mechanism might also be present in humans. An isotope labelling study in small animals has concluded that pre-existing cardiomyocytes are the dominant source of cardiomyocyte replacement both in normal myocardium as well as after myocardial injury [6].

1.4.3 Regeneration by exogenous stem cells

Different sources of extra cardiac stem cells have been suggested to possess the cardiomyogenic differentiation potential. It was reported that bone marrow derived c-kit+ cells, injected post myocardial infarction, could regenerate much of the infarcted region by cardiomyocyte renewal [67]. The same group showed mobilization of c-kit+ cells from the bone marrow into the blood, by subcutaneous injections of cytokines, resulting in large improvement of the infarcted region [68]. Later, the new myocyte formation was explained as cell fusion of c-kit+ cells with already existing cardiomyocytes [124, 125].

Based on the initial promising studies in small animals, clinical trials using different kinds of bone marrow derived cells were initiated. Although positive effects were noted in many of the studies, more recent meta-analyses of randomized trials concluded that no statistically significant effects of treatment were observed [126, 127]. The moderate improvement in cardiac function that were observed in some studies were believed to be caused by paracrine effects rather than transdifferentiation [128].

Taken together, these reports indicate that extracardiac progenitor populations are unlikely the source of cardiac regeneration. However,

exogenous cells may contribute with paracrine signalling, reported as a mechanism behind the beneficial effects. Secretion of cytokines may have pro-angiogenic [128], anti-inflammatory [129] and anti-apoptotic effects [130].

1.5 Stem cell niches

Studies from tissues with rapid cell-turnover, such as skin, small intestine, testis and bone-marrow, have shown that stem cells are organized in histological structures called niches [131, 132]. The function of the niche is to provide a protective milieu and to keep the stem cells in a quiescent state when tissue homeostasis is in balance. When needed, the stem cells can be activated to proliferate and give rise to new daughter cells that will migrate out of the niche and differentiate into tissue specific cell phenotypes.

The concept of a stem cell niches is well studied in tissues with high cell turnover [132, 133]. The niches are well organized structures. Supporting cells are attached to the stem cells and basement membrane. Cadherins and catenins form intercellular junctions between the stem cells and the supportive cells. High amount of ECM components play a protective role and are important in paracrine communication. Another characteristic is proliferation and migration of daughter cells out of the niches. A hallmark of hematopoietic niches is low oxygen tension where the key modulators are the Hypoxia inducible factors, e.g. Hif1α [134, 135].

In the heart, only a few studies have been focused on the identification of stem cell niches. In the mouse heart, stem cell niches have been described in terms of random nests of cells between the cardiomyocytes, most frequent in the apex and atria [65, 136], or equally distributed throughout the myocardium [66]. Characteristics of stem cell niches, such as presence of extracellular matrix (ECM) proteins, including laminins and fibronectin and presence of C-kit+ cells were reported. The frequency of stem cell niches was 8-fold higher in apex and atria compared to the base-midregion of the ventricles [65].

2 AIM

The overall aim of this thesis was to identify, isolate and characterize cardiac stem/progenitor cells and to find where in the heart they reside.

2.1 Specific aims

- To investigate the suitability of a new 3D cell culture system; High Density Spheres for studies of cardiomyocyte- endothelial- and stem cell biology
- To investigate possible presence of Side Population cells in the left and right atria of the human heart
- To explore the distribution of label retaining cells throughout the adult heart in order to identify stem cell niches
- To investigate if and how physical exercise affects cell proliferation and frequency of cardiac progenitor cells
- To explore the human Atrioventricular junction, where label retaining cells were found in the previous animal model, for the expression of the stem cell biomarkers and well-known niche factors
- To compare Atrioventricular tissue from organ donors with tissue from failing hearts explanted during cardiac transplantation surgery, in order to assess the effects of heart failure on stem cell content and distribution

3 MATERIAL AND METHODS

3.1 Ethics

Studies I, II and IV were based on human cardiac tissue. Ethical permissions were obtained from the research ethics board at the University of Gothenburg and the Sahlgrenska Academy. The tissue used in Study I and II was excised as a part of routine surgical procedures and would otherwise have been discarded. Thus, there were no additional risks associated with participation in the study. A signed informative consent was collected from each patient.

In Study IV, biopsies were excised from failing human hearts removed during cardiac transplantation surgery after informed consent. Consequently, participation in the study did not result in any additional risks for the included patients. Hearts from organ donors, explanted for valve donation, were used after the harvest of the valves. All had documentation of consent from the donor, stating that their organs can be used for other medical purpose than organ donation.

Study III was based on rat tissue and was approved by the local animal ethical committee. Three animals were housed per cage with free access to food and water. They were acclimatised before the experiment. The treadmill used for the exercise group was specially designed for rodents and moderate exercise load was applied. For each rat, limb- toe- and pain control was done according to a health protocol. Other tissues from the same animals were used in two other studies [137, 138] in order to reduce the number of laboratory animals used for research at the Gothenburg University.

3.2 Patients and tissue samples

Tissue from the auricle of the right atria (Paper I and II) was collected from patients undergoing coronary artery surgery or valvular replacement at the Thoracic surgery unit, at Sahlgrenska University Hospital. From Maze surgery, a procedure for treating atrial fibrillation and flutter, both left and right atrial tissue was obtained in study II. In total, 26 patients, 32-84 years old were included. Both genders were represented.

Study IV was based on whole explanted hearts from which biopsies were obtained from the atrioventricular junction and the left ventricle.

Biopsies from two groups of research subjects were included. Through collaborations with Cell and Tissue lab and the centre for transplantations at the Sahlgrenska University hospital, cardiac tissue from organ donors was obtained. These hearts, not suitable for cardiac transplantation, were collected after the valves were harvested for future clinical use. Clinical background of the organ donors is summarized in Table 1, Study IV. A total of seven donors were included, age 19-63 years.

The other group was patients with severe heart failure, undergoing cardiac transplantation surgery. Clinical background of the included transplantation patients is summarized in Table 2, Study IV. A total of seven patients were included age 39-67 years.

3.3 Cell culture

In order to expand primary isolated cells from tissues, cell culturing methods are used. Basically, tissue biopsies are dissociated with enzymes resulting in a suspension of single cells. The most common way is to seed the isolated cells on plastic surface where they attach and start to divide. This 2-dimensional culture method is called monolayer. Cell culture medium, added in culture contains nutritional agent such as glucose, but also other factors needed for metabolic processes of the cells. When the culture reaches confluence, the cells can be detached and harvested for further passages to larger surfaces, frozen or used for analyses [139].

There are many ways to culture cells and the choice is depending on the cell type and the purpose of the experiment. For example, keratinocytes from skin grow in monolayer, bone marrow cells in suspension. A whole field of science has emerged about the materials end scaffolds where the cells can be cultured 3 dimensionally (3D) in a more tissue-like environment [140]. Furthermore, there are different cell culture media, composed to fit a certain cell type and soluble factors that can be added to change the environment and study effects.

3.3.1 Isolation of human cardiac cells

When harvested, biopsies from cardiac tissue were collected in Phosphate Buffered saline (PBS) and kept cold until dissociated. Briefly, biopsies were washed and cut into pieces. In order to digest the ECM, Collagenase type II was used in Study I and Liberase type 3/TM in Study II. Collagenase type II, a mixture of different collagenases and proteases, breaks down the ECM by dissolving collagen fibres. Liberase, a mix of a purified collagenase and the proteases termolysin. Termolysin breaks down both ECM proteins as well as protein bounds between cells, by hydrolysis of peptide bounds where hydrophobic amino acids are involved [141]. For cell sorting in Study II, an additional dissociation step was required, to obtain a single cell suspension. Another protease was used; Trypsin which works on the carboxyl side of the basic amino acids lysine and arginine. The remaining single cell suspension was filtered through a 100 μm cellstrainer to remove cardiomyocytes and remaining tissue fragments. Since only collagenase was used in Paper I, the cultured 3D constructs probably contain tissue fragments and rests of ECM.

In this thesis, papers I and II are based on primary isolated cells in order to avoid the effects of possible changes in cell phenotype due to the monolayer culture. The purpose was to stay as close as possible to tissue-like situation and in that way closest to the patients. However, this was at the cost of not being able to increase the number of cells, which limited the analytical capacity.

3.3.2 Monolayer of human cardiac cells

Culturing cardiac cells, in the conventional monolayer, in our hands often resulted in phenotypic changes. One example is SSEA4+ progenitor cells, isolated from human atria. These cells retained their expression of SSEAs while the expression of cardiac genes was lost during monolayer culture [98]. Another common issue with monolayer cultures is that rapidly dividing cells, such as fibroblasts, might take over the culture [139].

Human adult cardiomyocytes are difficult to maintain in culture and they fail to divide *in vitro* [142, 143]. Cardiomyocytes are reported to lose their sarcomeric structures the first day on plastic surface [143]. During the enzymatic digestion, the cardiomyocytes were still beating as long as they are in clusters and connected to each other. When they lose the

cell-to-cell contacts they lose their contractive capability (our unpublished observation).

3.3.3 3D culture: High density spheres (HDS)

Many different methods can be used to provide a 3D construct including cardiac tissue slices, generated simply by sectioning [144, 145] and decellularised tissue that can be repopulated by seeding of cells [146]. The approach of sphere culturing has also been used where cells migrate and self-organise into cardiospheres [113].

A more tissue-like environment can be provided in a 3D- compared to monolayer culture. More cell-to-cell interaction is beneficial for the paracrine communication. Migration of cells in a 3D manner provides possibility of organization in layers and a more sophisticated extracellular matrix modelling. In order to keep the stem cells in an undifferentiated state, a new 3D culture system has been adopted previously, by our group [147]. By including all the different cell phenotypes represented in the tissue in high density manner, we hoped to allow for paracrine communication and reorganization. Primary isolated cells were either pre-cultured in a conventional monolayer or directly put in HDS culture. These tissue-like constructs displayed increased gene expression of stem cell biomarkers over time, but also preserved cardiomyocyte morphology.

Based on this previous study, in Paper I, we wanted to further investigated the suitability of HDS for studies of cardiomyocyte-, endothelial-, and stem-cell biology. Three different culture media were used, and markers for cardiomyocytes, endothelial- cells and cardiac progenitor cells were analyzed.

Briefly, biopsies were cut, tissue digested by collagenase and the cells cultured in a propylene conical tube. By centrifugation, all the different cell types isolated from the tissue, were forced together. Within a week, these high-density pallets round up and form spheres that were floating in the culture medium. HDS were than cultured in three different culture media, chosen to fit a certain cell phenotype. The culture time was up to nine weeks. Additional factors were added in the media in order to create a milieu were biological events could be studied.

Cell Culture media

In Paper I, all the HDS were first formed in Defined medium, containing DMEM-high glucose 5 μg/mL, linoleic acid, 1% insulin-transferrin-selenium-G, 1 mg/mL human serum albumin, 10 ng/mL transforming growth factor (TGF)- β1, 10−7 M dexamethasone, 14 μg/mL ascorbic acid, 1% penicillin streptomycin. This medium was chosen in favour to the stem cell phenotype since it was successfully used for 3D cultures of mesenchymal- [148] and embryonic stem cells [149]. Serum-free medium restricts proliferation therefore most of the analyses were therefore performed in Defined media.

To the HDS from 3 patients, EGM-2 MV, a commercially available endothelial medium, was added after the first formation week.

A medium combination, successfully used for cardiomyogenic differentiation of human cardiac Sca-1+ progenitor cells [121, 150, 151], was also used and called "Cardiac medium". This medium contained 2% human serum. Differentiation was induced by treatment with 5-Azacytidine and TFG-β1. 5-Azacytidine affects methylation of DNA. It undergoes intracellular conversion into 5-aza-2´-deoxycytidine and can be incorporated into the DNA of dividing cells as a cytidine analogue. When incorporated, 5-aza-2´-deoxycytidine inhibits DNA methyltransferases which results in hypomethylation of DNA and activation of previously silenced genes [152, 153]. TGF-β1 is a multifunctional growth factor involved in phosphorylation of Smad proteins. Addition of TGF-β1 increased the differentiation efficiency of human adult cardiomyocyte progenitor cells into functional cardiomyocytes [151].

3.4 FACS

Fluorescence Activated Cell Sorting (FACS) was used in Paper II, on directly isolated cells from atrial biopsies, for characterization of SP cells. It is a powerful method that enables the analysis of several parameters simultaneously for a large number of individual cells. The principle of analysis is illustrated in Fig. 9 (next page). Briefly, the cells are suspended in a sheet fluid and pass a laser beam one by one. Forward scatter (FSC) provides information about size of the cell and side scatter (SSC) about granularity. If the cells are marked with an antibody directly conjugated with a fluorochrome they can be sorted for further analyses. Lasers emitting a certain wavelength of light passing a proper setup of mirrors and band pass filters are used to detect the fluorescence labelled cells. Sorting is carried out by giving droplets with cells a positive or negative charge. A high voltage field is applied in the path of the falling droplets and change trajectory of the charged droplets. To ensure high purity, only droplets containing one particle are allowed to be sorted. Sorting purity was 90-95%, determined by re-analysis of sorted cells.

Analysis of FACS data was done using the FACSDiva software version 6.1.1 (BD). Generally, an inclusive FSC vs SSC gate was used in order to include all populations that could be of interest. In the gating strategy, very small and very large objects were regarded as debris and aggregates of cells respectively, and excluded from the analysis. Dead cells were also excluded by staining with 7-amino- acitinomyosin D (7AAD). Isotypic controls as negative samples were used when gates were set. Generally, isotypic controls were set in the range of 0-1% false positive. When percentages of positive cells were calculated, isotypic controls were always subtracted in order to avoid a bias toward positive staining.

One general limitation with FACS analysis is the requirement of enzymatic dissociation during the isolation of cells from the tissue which can affect the epitopes for antibody binding. This can result in false negative results. However, when the aim is to identify and isolate specific cell population using cell surface antigens, this is the best method to use.

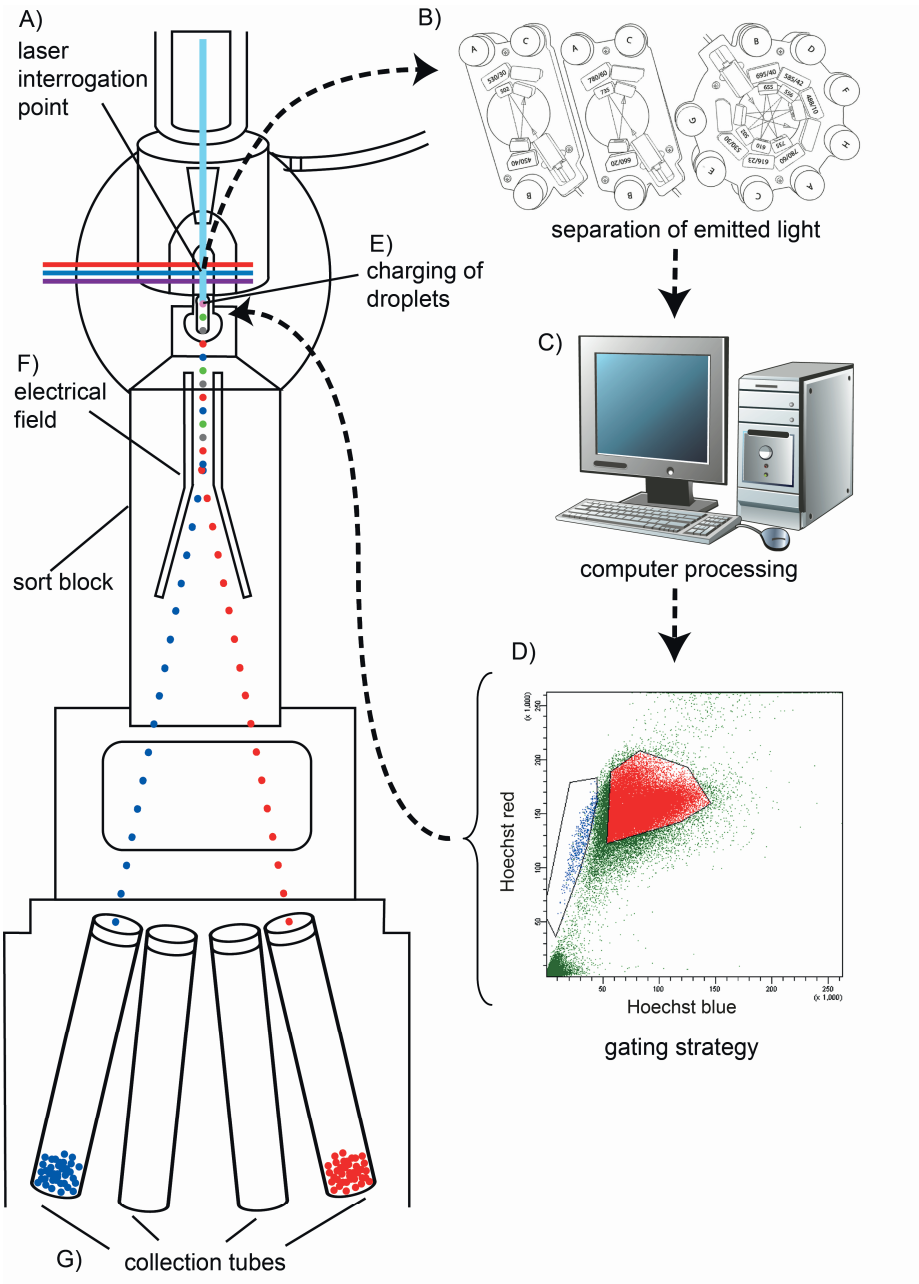
A)
laser
interrogation
point
B)
separation of emitted light
C)
computer processing
D)
Hoechst red
Hoechst blue
gating strategy
E)
charging of
droplets
F)
electrical
field
sort block
G)
collection tubes

Figure 9. *Previous page. Schematic illustration of the FACSaria system. Cells pass through the laser interrogation point in a fluid stream (A) just before the formation of droplets. Here, fluorochromes bound to the cells are excited by laser light in different wavelengths. The different laser wavelengths are separated from each other to minimize spectral bleed through. Emitted light is then separated in different wavelengths by mirrors and filters (B). Signal strength for each wavelength window is recorded and processed by a computer (C). According to the gating strategy (D) droplets with cells fulfilling the criteria for sorting are given different electrical charges (E). By applying an electrical field (F), the trajectory of the droplets are affected depending on the charge. Droplets with different charge, containing different types of cells, are collected in collecting tubes (G). (Reproduced and modified with permission from Joakim Sandstedt)*

3.4.1 Side Population assay

The definition of SP cells is based on their ability to exclude Hoechst 33342 dye. In Paper II SP cells were identified, sorted and characterized further. A single cell suspension was obtained from atrial biopsies as described in section 3.3.1. Residual erythrocytes were removed from the cell suspension by addition of lysis buffer. Cells were resuspended in staining medium, supplemented with 2% FBS at 1 million cells/ml.

Principles of the assay are outlined in Fig. 10. For the gating strategy of the SP cells as well as to elucidate which ABC transporter protein that was responsible for the SP phenotype, three different ABC transporter inhibitors were used: Fumitremorgin C (FTC), which has been described as a specific inhibitor for ABC transporter ABCG2 [154, 155]. Verapamil, which has been described as an inhibitor for another ABC transporter MDR1 [154]. Sodium azide with the addition of 2-D-Deoxyglucose which works as a general inhibitor of metabolism and should block all energy dependent transport proteins [85]. When inhibitors were used, samples were pre-incubated with these and inhibitors were then added in all subsequent steps. Next, Hoechst 33342 dye was added and cells incubated, washed and then incubated without Hoechst 33342. This two-step procedure with an additional incubation period without Hoechst 33342 differs from the original SP protocol described by Goodell et al. [81], where only one incubation period was used. It is suggested to be a way of reducing background in the SP assay [85] which was considered important when analysing directly isolated, heterogeneous cardiac cell samples. Cells were resuspended in cold FACS staining buffer and stained with 7AAD and antibodies. Cells were strictly kept on ice until FACS analysis to prevent additional Hoechst efflux in inhibitor treated samples.

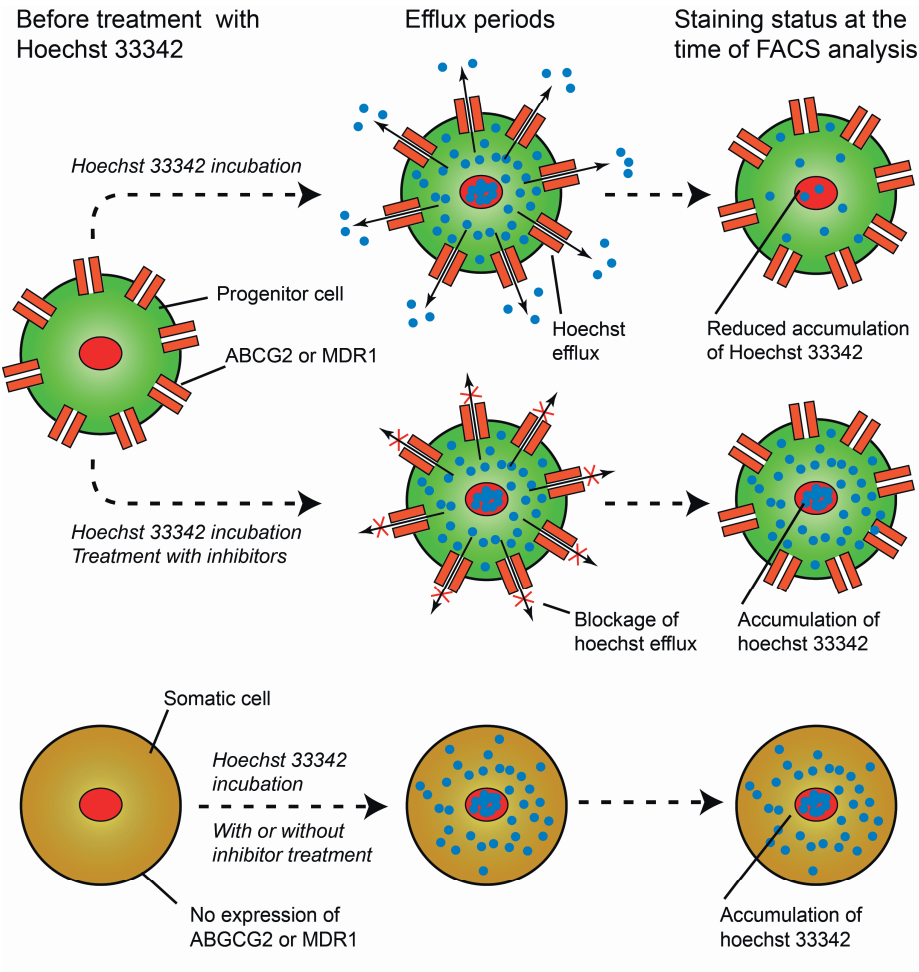

Figure 10. *Schematic illustration of the SP assay. (Reproduced with permission from Joakim Sandstedt).*

Hoechst 33342 staining was visualized using a 375nm UV laser. To identify SP cells, Hoechst blue (emission between 440–460 nm) was plotted against Hoechst red (emission above 670 nm). Hoechst emission spectra is lowered approximately 50 nm when bound to DNA [156]. Thus, Hoechst blue may mostly correspond to Hoechst 33342 bound to DNA whereas Hoechst red may correspond to Hoechst 33342 freely available in the cytoplasm. The Sodium azide and 2-D-Deoxyglucose treated samples were used for gating of the sorted SP cells since this inhibitor was most effective in blocking Hoechst 33342

efflux. As controls in the SP assay, lung cancer cell line A549 and neuroblastoma cell line SK-N-FI were used. High expression of the ABC transporter protein ABCG2 has been reported in the A549 cells [85] and ABC transporter protein MDR1 in the SK-N-FI cells [157].

The sorted cells were collected for qPCR into either RLTplus buffer including DTT or cold PBS. The RLTplus buffer has the advantage of instantly lysing the cells, protecting the RNA from degradation. When sorting larger number of cells, this was however not possible to use due to dilution of the RLTplus buffer by the sorting buffer (i.e. sheet fluid). Thus, these larger populations were sorted into cold PBS, centrifuged after sorting and snap frozen into liquid nitrogen. Samples were stored at -80°C until further analysis.

3.5 Gene analyses

RNA analysis was carried out on HDS (Paper I) and on FACS sorted cells (Paper II). The qPCR process, including cDNA synthesis, pre-amplification and real time PCR, was in principle carried out in the same way in these two studies.

3.5.1 Isolation of total RNA

HDS were first disrupted mechanically in a TissueLyser in lysis solution Qiazol. After homogenization, total RNA was extracted using RNeasy MicroKit (Qiagen) and DeoxyribonucleaseI (DNaseI). No contamination of genomic DNA was found in any of the analysed samples. From FACS sorted cells, RNA was isolated using RNeasy MicroKit or Mini Kit (Qiagen) depending on the number of cells. Removal of residual genomic DNA was done with either genomic DNA columns or enzymatic treatment. Extraction was either carried out manually or automatically in a QIAcube (Qiagen).

3.5.2 cDNA synthesis

Extracted RNA was first transcribed into complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit (Life technologies). This kit uses random hexamers primers for initiation of reverse transcription and thus all of the RNA is transcribed into cDNA. cDNA is then used for qPCR. The concentration of RNA was measured spectrophotometrically using a Nanodrop (Thermo Fischer Scientific) device. The RNA was free from DNA and protein and the purity quote was between 1,9-2,1.

Pre-amplification, 14 cycles, was used for samples with low total RNA amount using TaqMan PreAmp Master Mix (Life technologies). By this method, specific genes are amplified by PCR to allow for more genes to be analysed in the subsequent qPCR.

3.5.3 Quantitative Real Time PCR

Quantitative real-time PCR (qPCR) was used in paper I and II. qPCR offers both highly sensitive and specific quantification of mRNA expression. The qPCR analysis was carried out using commercially available TaqMan primers and probe for the genes of interest. When possible, primers spanning two different exons were chosen to eliminate the risk of detection of residual genomic DNA. For genes where genomic DNA contamination could not be excluded by primer design, qPCR was carried out on residual RNA samples that had not been reverse transcribed. If differences in cycle threshold (Ct) values (described further below) were greater than 5 between RNA samples and cDNA samples, genomic DNA contamination was considered insignificant.

PCR is based on the 5´-3´exonuclease activity of the Thermous Aquaticous (Taq) polymerase [158]. The probe containing a reporter fluorescent dye in the 5´ and a non-fluorescent quencher at 3´end is designed to anneal between two primers. As long as the probe is intact, the quencher absorbs the fluorescence from the reporter dye.

If the target of the probe is present in the sample, the probe will anneal downstream of one of the primer sites. It will then be cleaved by the 5´nuclease activity of the Taq DNA polymerase which replicates the template strand. Separation of reporter dye and the quencher increases the fluorescence of the reporter dye. Additional reporter dye molecules are cleaved with each cycle resulting in a 2-fold increase in fluorescence. The higher expression of a specific gene, the sooner a significant increase in fluorescence is detected. Compared to traditional PCR, where data is collected at the end of the reaction, in qPCR fluorescence intensity is collected continuously throughout the PCR process.

In Paper I and II, qPCR analysis was done using a 7900HT instrument (Life technologies). By setting a threshold level of fluorescence signal at the exponential phase of the curve, a Ct value was determined for each sample. The Ct value corresponds to the number of cycles needed to reach a certain fluorescence level meaning high expression of a gene will give a low Ct value.

For quantification of data, the relative comparative Ct method was used [159]. The choice of reference genes has been evaluated previously by our group [147]. CREBBP was selected as reference gene showing a stable expression in cardiac tissue. Furthermore, expression of CREBBP was compared between the FACS sorted directly isolated- and monolayer cultured cells from cardiac biopsies. Similar expression levels were noted (in house data). Values have been normalized to a calibrator sample included on each plate, used as positive control and to compensate for inter-assay variations. A water sample was included in every plate as a negative control. All samples were analysed in duplicates. The technical variability was generally observed to be very low. Gene expression data are presented in the thesis in relative units.

3.6 In vivo DNA labelling; animal model

In order to study on-going proliferation and distribution of cardiac endogenous stem cells *in vivo*, an animal model was required in Study III.

In vivo labelling techniques are well established and commonly used for detection of proliferating cells and in stem cell niche biology. 5-Bromo-2- deoxy- Uridine (BrdU) is a thymidine analogue, which is incorporated into single strand DNA during mitoses. With this method, cells undergoing mitoses during the time of administration will be labelled resulting in a traceable fraction of cells. Each time a labelled cell divides it loses half of the BrdU content. In other words, rapidly proliferating cells will dilute the BrdU fast while slow cycling cells can be detected after a chasing period. Stem cells are considered as slow cycling. The time point, at which the BrdU labelling is not detectable any more, depends on tissue type and its proliferation rate.

3.6.1 The Sprague Dawley rats

Sprague Dawley rats (Fig. 11), common animals used in medical research, were chosen for Study III. They are an outbred multipurpose breed with a lifespan around 2,5-3,5 years. Bodyweight for males is 450g and for females 250g. 20 female-, 8-10 weeks old, rats were included in the study. The animals were divided in two groups, exercise (n=9) or control (n=9). In addition, two of the animals were not given BrdU (naïve) in order to provide negative control tissue.

The animals were sacrificed by an overdose of Pentobarbital. After thoracotomy, the hearts were collected in PBS. Skin and intestine tissue were also collected from each animal as control tissue for IHC and treated in the same way.

3.6.2 Physical exercise

The rats in the exercise group were trained on a treadmill (Fig. 11), 5 days a week for 15 weeks. During the first week, the animals were acclimatized to the exercise and the intensity and duration of training was increased stepwise. Eventually, the exercise was conducted at 50 min/day and 16 m/min which is relatively moderate exercise load. Control group and naïve rats were confined to normal cage activity. No animals were excluded from the study.

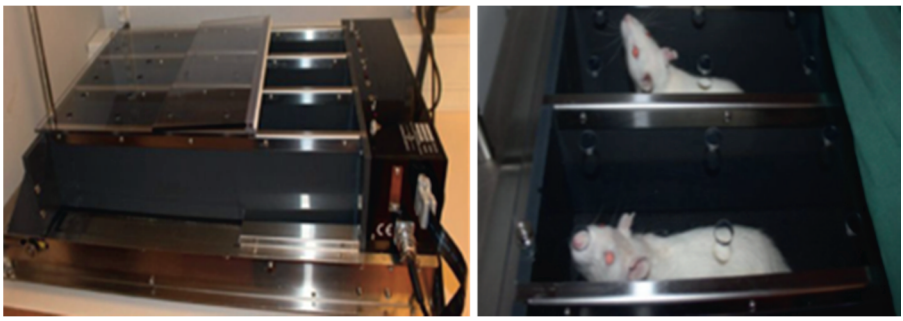

Figure 11. *Photos of the treadmill used for running exercise and Sprague Dawley rats.Modified and reproduced with permission from H. Barreto Henriksson [138]*

3.6.3 BrdU administration

The animals were given BrdU diluted in drinking water during a two weeks period. The administration began at the same time as physical exercise acclimatization. During this time, the animals were given limited amounts of water. The rats had free access to food and water during the rest of the experiment.

3.7 Histology

Microscopic structure of cells and extra cellular matrix within a tissue or a cell construct is studied by histology. The specimens need preparation and then several stainings can be used to visualize different tissue compartments.

3.7.1 Specimen preparation

Once tissues are removed from the body, they undergo a process of self-destruction or autolysis, initiated by enzymes. Many of the antigens are proteins or carbohydrates, soluble in aqueous solutions and need to be fixed in place in the tissue. The structure of the proteins needs to be preserved as well [160].

Pre-analytical treatment of HDS and rat hearts for histology included buffered 4% formaldehyde fixation and paraffin imbedding in Studies I and III. Formaldehyde is a small molecule that penetrate the tissue rapidly. Aldehydes form covalent cross-linking between proteins end-groups, stabilizing cell morphology and tissue architecture. After the fixation, rat hearts in Paper III were split in the middle, from apex to atria. The fixed tissue was than dehydrated, paraffin embedded and cut in serial sections.

Since the crosslinking fixatives act by forming chemical bonds, conformational changes of proteins can occur and also cause artifacts that might block the access to the epitope [160]. Therefore, we adopted cryofixation for the specimens in Study IV, where these risks are minimized. Human biopsies, from AVj and left ventricle from each heart, were attached onto a cork disc and embedded in a tissue mounting reagent (Tragacant), snap frozen in liquid nitrogen and preserved in -80ºC until cryosectioned (Fig. 12). Rapid freezing is preserving fine structure of cells in their native and physiologically active state.

Figure 12. *A photo of a human biopsy from AVj, attached onto a cork plate, embedded in a tissue mounting reagent and snap frozen in liquid nitrogen.*

Tissue or HDS, were sectioned by a microtome or a cryotome into 5-7 μm thin sections that were attached to glass slides. Before IHC, the frozen sections were fixed with acetone.

3.7.2 Histology stainings

Hematoxylin Eosin (HtxEo) is one of the most common histological stains. Haematoxylin is a basic dye and stains acid structures such as nuclei (ribonucleic acid) blue-violet. The acidic dye Eosin stains basic parts of the cell such as cytoplasmic proteins and extra cellular matrix components in pink [161].

Alcian Blue van Gieson (AbvG) was used for detection of ECM components in Paper I. Alcian blue is a dye that binds to negatively charged sulphated Glucose Amino Glycans. Van Gieson stains collagens in connective tissue red [162].

Picro Sirius Red was used in Paper I and IV for staining of ECM. Sirius red is an acidic hydrophilic staining that colours collagen fibres in red. Sulfonic acid groups bind with the basic groups of collagen fibres [163].

Millers Elastin was used in Paper I to visualize vascularization. Elastin fibres are coloured blue with this staining. Millers is a mixture of three aminotriarylmethanes dyes, where the hydrogen bindings of the dye complex to the elastin fibres [164].

3.8 Fluorescence IHC

Protein expression profile is often used to determine cell phenotype. Biomarkers can be detected on tissue slices using antibodies. The IHC method is based on the antibody-antigen reaction (Fig. 13). An advantage of using IHC compared to FACS is that it provides information about the location of the antigen in the tissue. An antigen has one or several epitopes, which are sites that are recognized by a specific antibody. IHC was used in studies I, III and IV.

Antibodies can be mono- or polyclonal and are produced in a variety of animal species. There are conjugated antibodies for direct detection. The most common way to visualize the immunoreaction is by use of secondary antibodies, carrying a fluorochrome that gives a signal that enables the detection. Secondary antibodies are directed to the part of the primary antibody that is from the host species.

If two primary antibodies, made in the same species, were combined it would not be possible to distinguish the signals. However, combination is possible if one of the primary antibodies is directly conjugated with a fluorochrome and incubated separately, as described in Study IV.

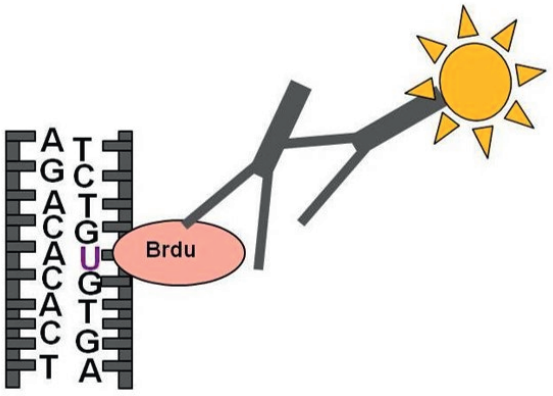

Figure 13. *Schematic illustration of the principle of IHC with a primary antibody against BrdU, which is incorporated in the DNA, detected by a secondary antibody carrying a fluorochrome. (Reproduced with permission from Helena Baretto-Henriksson)*

Monoclonal antibodies are generally more specific to the antigen as they consist of only one clone. Polyclonal antibodies on the other hand generally give higher background as they consist of multiple clones that bind to different epitopes of the chosen antigen. In order to distinguish the background from the true signal, isotypic controls can be used. Positive control tissue is also important and helps when learning the pattern of a certain biomarker expression e.g. is the marker generally expressed in the extracellular matrix, cytoplasm or in the nuclei. Negative control tissue for BrdU staining was used in Paper III, from the rats not given BrdU. When that kind of tissue is available, the isotypic controls are not as important. In most cases we did not have negative control tissue. Positive controls were used when available.

In order to exclude unspecific binding a blocking step was included where Bovine Serum Albumin (BSA) and goat serum were added.

In Paper I, paraffin sections of HDS were examined for expression of α-sarcomeric actin, VWF, cTnT, Ki67, Notch1, JAG1 and active β-catenin. After deparaffinization, the sections were rehydrated and an antigen retrieval step was performed. A blocking step with detergent and BSA followed. Sections were incubated with primary antibodies overnight in humidity chamber. After a wash step, the secondary antibody was added and sections incubated for at least 1 h. DAPI was

used as nuclear staining. In Study IV, the frozen tissue sections were fixed in –20°C acetone and stained in similar way. Three antibodies were combined in each IHC assay. Antibodies used for IHC in the Study IV are summarised in Table 3.

In Paper III a similar protocol was used for detection of cTnT, MDR1 and Sca1. However, when staining for BrdU one additional step was added to the protocol. After the antigen retrieval step, sections were incubated with 2M HCl in order to denaturate the DNA. Since DAPI is binding to double-stranded DNA, nuclear staining in combination with BrdU was not possible.

3.8.1 Image analyses

A Nikon Eclipse 90i fluorescence microscope was used in Paper I and for manual counting of BrdU cells in study III. A Nikon Eclipse Ti fluorescence microscope was used to acquire large photos in Paper III and IV, with an Andor iXon x3 camera and NIS elements software. Images of MDR1 and Sca1 in study III and all images in study IV were photographed by an Andor Zyla camera.

Large images were acquired using a motorized board and stitched together by the software. When working with large images it was necessary to acquire images in at least 3 Z-levels in order to get the nuclei in focus. Image J software was then used for image analyses. Pixel ranges displayed for each channel were set to remove most of the background. Isotypic controls were treated in the same way.

3.8.2 Quantification of IHC

Traditionally, IHC was regarded more as a qualitative method, providing the expression pattern of a biomarker. Quantitative data, needed for comparison between groups or treatments, can be provided by manual counting or by using software. Quantification of BrdU was done by both methods in Paper III.

BrdU+ cells were counted manually, using 20x objective and counting in three fields of view/region on each slide. Four independent IHCs were performed for each heart, as technical replicates and one for the skin tissue.

Intensity of the BrdU staining was of great importance and a quantification method was developed using Image J software. Comparison was made between Apex-, Left Ventricle- and AVj regions within the same rat heart. Large photos were acquired with 40x objective, ~1.8×18 mm. The following steps were performed:

- Artefacts were removed e.g. empty spaces, edges, autofluorescense
- Binary images were created
- BrdU+ cells were detected as Regions Of Interest (ROIs)
- Only ROIs within focus were selected
- Quality control step: all images manually checked
- Median intensities for each ROI were measured
- The median background intensity of the surrounding tissue for each ROI was subtracted
- A fraction of BrdU + cells (2,5%) with the highest intensity were selected as BrdU Bright
- For each region, a ratio of BrdU Bright was calculated
- The ratio was normalized to BrdU Bright/1000 BrdU+ nuclei

3.9 Statistical methods

For qPCR analyses in Paper I and II, two technical replicates were used. A mean value of these were used in subsequent statistical analyses. Two-sided Students t-test was used to calculate statistics.

For manual counting in Paper III, a mean value for each region and rat was first calculated based on four technical replicates. Three-way full factorial ANOVA analyses were used with region, time and exercise as fixed factors and animal as random block factor. For Intensity measurements, two-way full factorial ANOVA was used, with time and exercise as fixed factor and animal as random block factor. Post-Hoc analyses with SIDAC correction for mass-significance were carried out.

In most cases, log transformation was used prior to statistical analysis to obtain a more normal distribution. p-value <0,05 was considered significant. SPSS or Excel software were used for calculations. Data are generally presented as mean ± standard error of the mean (SEM).

4 SUMMARY OF THE RESULTS

4.1 Paper I

We investigated the suitability of the High Density Sphere (HDS) culture system for studies of cardiomyocyte-, endothelial- and stem cell biology. All cells isolated from right atrial biopsies were included in their relative proportion. This gave us the possibility to study the protein- and gene expression of makers for several kinds of cells and the changes with time and culture condition. Cardiac-, Endothelial- and Defined media were used in order to investigate the possibility to favour and support cardiomyocyte, endothelial- or progenitor cell phenotypes.

It was possible to establish HDS cultures from all nine patients included. Spheres were formed within a few days and in endothelial medium, the spheres gave rise to buds that were shed off as new spheres over time. In the new buds, no cardiomyocytes were seen. Histology of the HDS showed well preserved cardiomyocytes in Cardiac culture media. Both Cardiac and Endothelial media contained serum. Proliferation marker Ki67 expression was mainly located in the multilayer area on the surface of the HDS and in the buds. In endothelial medium with highest serum content, most Ki67+ cells were found. Since high cell density and serum-free media restrict proliferation, Defined medium was used for most of the phenotypic analyses.

ECM was produced over time in all three media conditions visualized by collagen staining by Picric Sirius red. Even thou not quantified, we noted an increase in collagen production in the HDS cultured in cardiac- and endothelial media where also proteoglycans were shown by AbvG staining in the periphery of the HDS.

Endothelial gene and protein expression was studied. On gene level, CD31 and FLK1 expression increased significantly over time when cultured in Defined medium. In endothelial medium the expression however decreased. In one week old HDS, analysed by IHC, VWF+ cells were found in clusters between cardiomyocytes. Over time, cavities were formed within the HDS most prominently in Defined medium. Reorganization of endothelial cells was shown as VWF+ cells lining the cavities in six weeks old HDS. The intraluminar position of endothelial cells remained also after nine weeks in culture. Vessel-like formations were found by histology studies in late HDS, regardless of choice of

culture medium. When serial sections were analysed these showed a tubular formation suggesting early vasculogenesis in HDS.

The cardiomyocyte phenotype was followed and a significant increase in expression levels of the cardiac-specific genes: *NKX2.5*, *TNNT2*, *MYH6*, and *NPPA* was noted over time in Defined medium. When cardiac medium was added no additional increase in expression of cardiac genes was noted. At one week in culture, cardiomyocytes were strongly stained for α-Sarcomeric actin. After six weeks of culture however, protein expression of cTnT and α-Sarcomeric actin had decreased.

Expression of stem cell- and early cardiac genes was analysed by qPCR in HDS cultured in Defined medium. A significant increase was shown for *C-kit, ABCG2, MDR1, ISL1, MESP1* and *MEF2c* while no significant increase was shown for *TBX5* and *GATA4*. Expression of Notch1 protein was detected throughout culture time. Specific localization of Notch1+ cells was noted in the outer multilayer area. This was formed only in Cardiac medium, where high content of proteoglycans and collagen was found.

Activity within the Wnt/β-catenin signalling was studied in early HDS cultured in Defined medium. β-catenin is reported to be required for ISL1 expression [165]. Since active form of β-catenin was detected in nuclei of many cells by IHC, we hypothesized that HDS can be used as a model for studies of interaction between Wnt and ISL1. GSK3 inhibitors dephosphorylate β-catenin and can promote expansion of ISL1 progenitors. GSK3 was added to newly formed HDS. This stimulation resulted in gene expression of ISL1 compared to control where no expression was detected. In addition, a downregulation of the Wnt inhibitor DKK1 was detected.

4.2 Paper II

We set up a functional assay for isolation of SP cells using their ability to exclude the fluorescent dye Hoechst 33342. The SP phenotype is linked to the expression of plasma bound transporter proteins, most commonly ABCG2 and MDR1. In Paper II we investigated the existence of SP cells in human atrial tissue. Characterization of SP cells, isolated by FACS, was determined by gene and protein expression analyses.

The existence of SP cells was shown in human cardiac tissue. From left atria, a small population, 0,2% of the total number of cells, was isolated. No clear population could be isolated from the right atria. Inhibitors were used: FTC selective for ABCG2, Verapamil selective for MDR1 and as a general inhibitor of metabolism, sodium azide with 2-D-Deoxyglucose. FTC did not block Hoechst 33342 efflux, Verapamil showed a partially blocking effect. The best inhibition was achieved by the Sodium azide with the addition of 2-D-Deoxyglucose. When stained with ABCG2 and MDR1 antibodies, no expression was detected within the SP.

Using FACS, SP was separated from main population and both were sorted for further characterization by gene expression analysis. ABCG2 showed very low gene expression in both populations compared to the positive control cell line A549. MDR1 showed significantly higher expression in the SP compared to main population, at the same level as the positive control cell line SK-N-FI.

Stem cell associated genes *C-KIT* and *OCT-4* were significantly higher expressed in the SP compared to main population. Expression of endothelial genes *CD31, VWF, FLK1*, and cardiac genes *TNNT2* and *NKX2.5*, showed no differences when SP was compared to main population.

Around 48% of the SP cells expressed the hematopoietic marker CD45. SP was subdivided into CD45+SP and CD45-SP. No difference was seen for expression of *ABCG2, MDR1* and *OCT4*. Endothelial genes were highly expressed in CD45-SP. There was no difference in the expression of cardiac genes that were either low or undetectable.

4.3 Paper III

DNA labelling with BrdU was used in a rat model, in order to study proliferation and distribution of slow cycling cells. Physical exercise was used and its effects on cell turnover and distribution of progenitors were investigated.

BrdU+ nuclei were detected by IHC and sections of whole rat hearts were studied. A sparse distribution of the BrdU staining was observed throughout the cardiac tissue with the exception of the Atrioventricular junction (AVj) regions. Here, densely packed BrdU+ nuclei were seen at all time points and in every section studied at the left, right and septum AVj. This anatomic site consisted of connective tissue with a fibre network continuing into the myocardium of the atria and ventricle.

Manual count of BrdU+ cells was done in apex, ventricles and AVj regions in control and exercise group and cell numbers were compared to skin tissue, used as positive control. 350 nuclei/field were counted in skin compared to half of that in cardiac tissue after BrdU administration, showing slower ongoing proliferation in cardiac tissue. Statistical analyses showed significant effects of time and region in a three way full factorial ANOVA. Over time, number of BrdU+ cells decreased as expected. AVj contained highest number of BrdU+ cells and ventricles the lowest. There was a significant difference in cell numbers when AVj was compared to apex and ventricles. We observed a minor interaction between time and exercise. In the exercise group, more cells were labelled with BrdU in the AVj region, showing higher ongoing proliferation. In apex and ventricles, similar cell numbers were shown.

BrdU content was washed out in 85% of the counted skin cells during the 13 weeks long chasing period, compared to 71% in cardiac tissue. This illustrated also slower cell turnover in cardiac tissue compared to skin. However ending up with 29% of the original number of BrdU+ nuclei, we concluded that the chasing time period was too short.

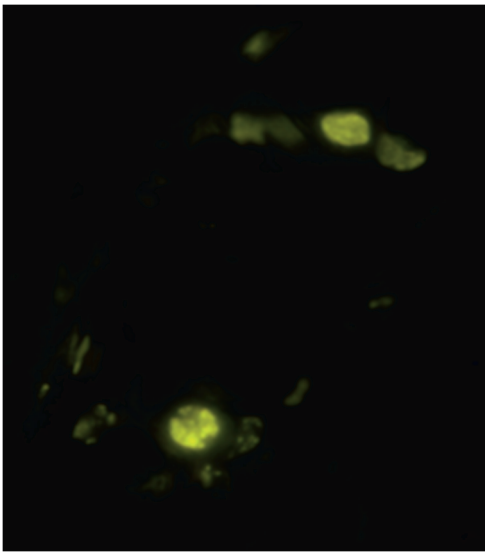

Figure 14. *Photo of BrdU positive nuclei displaying the variation in intensity of the staining after the chasing period.*

A variation in intensity (Fig. 14) of the staining was however observed after the chasing period. Therefore, we measured the intensity of BrdU staining in order to study cells with the highest intensity: BrdU-Bright (arbitrary set at 2,5 %) and consider these as stem cells. A comparison was made showing that most of the BrdU Bright cells in the exercise group were located in the suggested niche subregion in AVj with a significant difference compared to the other regions. In order to visualize the position of BrdU Bright nuclei, they were back-plotted to the photos. The results show that cells with the highest intensity were located at the border between ventricle myocardium and valves, in the suggested niche region. When different intensity levels were used is was shown that BrdU+ cells lose their intensity with distance from the suggested niche subregion.

In order to further investigate the potential niche region identified with label retention, AVj sections were stained with antibodies against cardiac progenitor markers MDR1 and Sca1. MDR1 expression was found in the proposed niche region. Sca1 was sparsely expressed in the niche region but most of the Sca1+ cells were found further down in the ventricular myocardium, between the cardiomyocytes. There was no difference in expression pattern of Sca1 between the groups while MDR1 showed stronger expression in the exercise group. However this observation was not quantified.

4.4 Study IV (Manuscript)

Since we identified a new potential cardiac stem cell niche region in AVj in a rat model we wanted to investigate if a similar niche also exist in the human adult heart. In study IV, histological stainings and IHC with antibodies against stem cell-, hypoxia-, migration- and proliferation biomarkers were used. These were combined with cardiac lineage markers in order to study co-expression. A comparison was made between AVj and Left Ventricular (LV) biopsies. Biopsies from organ donors were compared with biopsies from severely failing, explanted hearts from transplantation recipients.

Histological analyses showed that the AVj region is rich in ECM content compared to LV. In donor AVj, Picric Sirius staining showed the Mitral valve and the radiating strands of collagen fibres, in red, continuing into the myocardium of LV. Cardiomyocytes, at the boarder to LV, were small and embedded in ECM. In the AVj from failing hearts, collagen content was lower, the fine fibre network appeared as disrupted and the Mitral valve thinner. Fibrosis and hypertrophy characterized the failing LV tissue where cardiomyocyte nuclei were hollow and irregular in shape. More and larger blood vessels were noted in failing tissue.

IHC showed strong expression of cardiac progenitor marker MDR1 in a large streak in the connective tissue region of AVj, at the boarder to ventricular myocardium. In the same region, expression of CD31 was absent, showing low vascularization. In addition, strong nuclear expression of hypoxia marker HIF1α was found in many cells. MDR1 expression decreased with the distance from the valve.

Nuclear expression of the early cardiac stem cell marker ISL1 was found in clusters of cells in the connective tissue region of donor AVj, where progenitor marker WT1 was absent. WT1 was expressed from the myocardial boarder in AVj. The border zone cardiomyocytes were small with cTnT+/WT1+/ISL1+ expression profile. Elsewhere, WT1 and ISL1 did not co-stain. WT1 was otherwise expressed in cells between the cardiomyocytes and more densely at the epicardium.

Progenitor marker glycolipid SSEA4 showed similar expression pattern as MDR1. It was strongly expressed in a large streak in the connective tissue in the suggested niche region with decreasing expression with the distance from the valve. The small boarder zone cardiomyocytes were cTnT+/SSEA4+.

In blood vessels, regardless of location, nuclear HIF1α expression was seen. MDR1 expression was also noted in blood vessels and co-localized with CD31. Donor LV tissue showed very low or no expression of ISL1, WT1 or SSEA4.

Proliferation marker Ki67 expression was studied in combination with human cardiomyocyte nuclei specific marker PCM1 and cTnT. Expression of Ki67 was found in many cells in AVj where most of them were PCM1- and cTnT-, as expected. PCM1 turned out to be specific for cardiac nuclei. At the myocardium boarder in AVj, a few PCM+ nuclei were observed without the surrounding cTnT+ cytoplasm, which could be interpreted as an early stage of cardiomyocyte. Also, Ki67+/PCM1+/cTnT+ cells were found here. With increased distance from the boarder, the cTnT+ cytoplasm size increased. These findings together indicate different stages of cardiomyocyte maturity.

Migration marker Snai1 co-stained with cTnT and the intensity of the staining decreased with the distance from the myocardium boarder with no expression seen in the LV tissue. N-cadherin was expressed from the boarder to myocardium, in junctions between cardiomyocytes .

In the failing AVj, similar but weaker expression pattern was seen for HIF1α, MDR1, SSEA4, Snai1 and N-cadherin. CD31 expression revealed more and larger blood vessels in the AVj region. As for donor hearts, this expression was outside the MDR1 and SSEA4 positive streak. Opposite to the donor hearts, no expression of ISL1 of WT1 was found in the AVj. On the other hand, both were upregulated in the failing LV tissue. In LV, ISL1 was located in the large nuclei of cTnT+ cells and WT1 in nuclei between cardiomyocytes co-localizing with the fibroblast marker Te7 expression. Some PCM1+/cTnT- cells were present in the myocardium boarder also in failing heart. Ki67+ expression was absent in six out of seven patients with heart failure.

5 DISCUSSION

The purpose of this thesis was to identify, isolate and characterize adult cardiac stem cells, and to find where in the heart they reside. Using a variety of methods, the stem cells were approached in different ways. A new 3D culture system was adopted where a more tissue-like environment was provided for the isolated cells from human cardiac tissue. A functional assay was set up for SP cells that were isolated and characterized. In order to search for the cardiac stem cell niche, a rat exercise model was used where slow cycling cells were detected, using DNA labelling with BrdU. A new potential niche region in the adult rat heart was discovered. Tissue from this anatomic site was further obtained from human hearts in order to investigate if the results were translatable to the human situation. Furthermore, a comparison was made between tissue from donor and failing hearts in order to study how heart failure affects the distribution and frequency of progenitor cells. All of the *in vitro* experiments were based on human tissue.

New 3D culture system: HDS

3D culture systems are becoming more advanced lately. Models of tissues in a dish, including human brain, liver and airways have been developed, called organoids [166]. Within the cardiac regeneration field, different 3D systems have also been used including cardiospheres [113], tissue slices [145] and decellularized tissue scaffolds [146]. The goal of cardiac tissue engineering is to provide an environment allowing for paracrine communication, where cardiomyocytes, fibroblasts, endothelial cells as well as the rare stem cells, can survive and keep their functionality.

In Paper I we formed tissue-like constructs, using high cell density in a 3D manner where all the different cell phenotypes isolated from cardiac tissue were included. Based on our previous work, where expression of progenitor markers increased over time and cardiomyocytes showed well preserved morphology [147], we now wanted to investigate if we could enrich a certain cell phenotype by using different culture media. Effects on histology, proliferation, ECM production and biomarker expression were studied.

Proliferation, ECM production and vasculogenesis in HDS

To enrich the endothelial phenotype, newly formed HDS were cultured in Endothelial medium. Surprisingly, gene expression of endothelial markers *VWF, CD31* and *FLK1* decreased. That could be an effect of high proliferation that was induced in this medium, containing 5% serum. Proliferation was clearly shown by formation of buds, new small spheres that were shed of and by expression of Ki67 protein. Based on morphology, the proliferating cells were non-myocytes. However, the identity of the proliferating cells was not further investigated.

Production of ECM was studied within the HDS by stainings for collagen and proteoglycans. Larger amounts of Collagen were observed in the multilayer of cells, organised in sheets, which were formed in Endothelial and Cardiac media at the periphery of the HDS. More tissue-like ECM in this area was shown by detection of proteoglycans. ECM production was interpreted as a sign of functionality of fibroblasts, indicating that HDS is a suitable model for studies of cardiac fibroblast biology.

Cavities were formed over time within the HDS. There could be several explanations for these including cell death. However, migration and rearrangement of the cells within the HDS is another possibility for cavity formation. Interestingly, the cavities were lined by well-organized VWF+ cells. Intraluminal location is a natural position of the endothelial cells, suggesting a well preserved endothelial phenotype. Furthermore, histology revealed tubular structures that could be followed in serial sections. These structures could only be found in late HDS and were positive for elastin. Taken together, these findings point toward signs of vasculogenesis *in vitro*. Formation of new blood vessels is a crucial step in cardiac regeneration upon injury [167]. Although not fully investigated, these findings are of importance to follow up. This new *in vitro* model could potentially serve as a tool for studying the formation of new blood vessels.

Effects on cardiomyocytes

When attempts were made to establish a monolayer culture, cardiomyocytes lost their sarcomeric structures the first day on plastic surface [143]. This illustrates the difficulties with cardiomyocyte *in vitro* systems. In HDS, the morphology of cardiomyocytes was well preserved over time, up to nine weeks. However, declining expression of α Sarcomeric actin protein over time illustrate disorganization of the sarcomeres. This event has been described as a programmed cell

survival mechanism [168]. Furthermore, it has been shown that cardiomyocytes need to de-differentiate in order to be able to proliferate [169, 170]. HDS culture, with high cell density and paracrine communication, might allow for this event in a more slow and controlled way. Disorganization of the sarcomeres in HDS is interpreted as a sign of de-differentiation of Cardiomyocytes *in vitro* and might be an important step towards establishing human cardiomyocyte cell culture. To the contrary of the decrease of contractile proteins, expression of cardiac specific genes *NKX2.5*, *TNNT2*, *MYH6,* and *NPPA* increased over time in Defined medium. This was interpreted as increased transcriptional activity within the cultured cardiomyocytes with time. It could be speculated that the reason for discrepancy between protein and gene expression could be that the cardiomyocyte downregulate gene-expression at week one as a part of adaptation to the in vitro system. Then, after adaptation, gene expression is upregulated to maintain the cardiomyocyte phenotype. For future studies, it would thus be of interest to compare the gene expression with atrial tissue samples.

Increased levels of progenitor genes in HDS

Expression of cardiac stem/progenitor genes was analyzed in Defined medium. Expression of *MESP1, MEF2C, C-KIT*, *ABCG2,* and *MDR1* was significantly upregulated over time. *ISL1* expression, was detected in two out of six patients in the newly formed HDS. With culture time, its expression was upregulated in five out of six patients. Taken together, detection and increase of gene expression of early cardiac and progenitor markers in our material, where the average age of the patients was 71 years, indicates that HDSs provide a good environment also for stem cell populations.

Effects of Cardiac medium

Since the gene expression of stem cell markers significantly increased over time in Defined medium, we wanted to investigate if we could induce differentiation of progenitor cells into cardiomyocytes. Therefore, the Defined medium was changed to Cardiac medium for three additional weeks, including a pretreatment with 5-Azacytidine [121]. Since all the different phenotypes were included it was difficult to interpret the results. Did the progenitors differentiated into cardiomyocytes with the 5-azacytidine treatment in the few strongly α Sarcomeric actin positive cells, or did a few cardiomyocytes keep their expression in culture for nine weeks. In animal models, genetic lineage

tracing methods could be used to evaluate this further. This approach is unfortunately not an option when working with primary isolated human cells.

SP cells in the human left atria

A characteristic common for many stem cells is that they can exclude toxic substances. In this manner, SP cells express members of the ABC transporter proteins in their membrane and as a consequence have the ability to efflux Hoechst 33342, a fluorescent dye. In Paper II, the SP assay was set up, and for the first time in adult human cardiac tissue, the existence of these progenitor cells was reported.

Previously, SP cells have been isolated and characterised from murine hearts [88, 90, 91]. They have also been isolated from human fetal cardiac tissue, but from adults, only expression of the MDR1 [104] and ABCG2 [97, 171] has been reported. These two ABC transporter proteins have been linked to the ability of SP cells to exclude Hoechst 33342. Expression of ABC transporters has been described as age dependent. ABCG2 expression was high in neonatal mice and then shifted to MDR1 in adults [87].

Using inhibitors, Verapamil selective for MDR1 and FTC selective for ABCG2, we aimed to find the protein responsible for the transport of Hoechst 33342 dye out of the SP cells. Moreover, as a general inhibitor of energy dependent transport mechanisms, sodium azide with addition of 2-D-Deoxyglucos, was used. Hoechst 33342 efflux was partly blocked by Verapamil and the general inhibitor and was not affected by treatment of FTC. Taken together, our results indicate that MDR1 is the efflux protein responsible for the SP phenotype in the human adult heart, which is in line with what has previously been shown in the mouse [87]. When human neonatal cardiac SP cells were studied on the other hand, inhibition with FTC blocked the efflux of Hoechst 33342 linking the ABCG2 transporter to the SP phenotype in this age group [172].

In the present study, a co-expression analysis was performed on the isolated SP cells, where no protein expression of neither MDR1 nor ABCG2 could be detected. This outcome could be caused by the, necessary but somewhat aggressive, dissociation protocol that could affect the epitopes on the cell surfaces. Epitope destruction during the dissociation have been previously observed by our group [173]. However, high expression of MDR1 on mRNA level was detected by qPCR, which is in line with the results from the inhibitor analyses.

Clonogenic capacity and the self-renewal properties of cardiac SP cells have been investigated. Clones, cultured in methylcellulose and studied for over 10 months, retained the SP phenotype, expressed Sca-1 and did not undergo replicative senescence. When these clones were implanted in a mouse infarction model the hearts showed higher ejection fractions and decrease in scar tissue, 12 weeks after injury [174]. A resent review concluded that SP cells consistently prove their differentiation into the main cardiac lineages [175]. Therefore, SP cells could be promising targets for regenerative therapies.

In paper III and IV, protein expression of MDR1 was studied in the AVj region. In the rat hearts, MDR1 expression was found in the suggested niche region. The same pattern was seen in the AVj from the human donor hearts; a large streak of densely packed MDR1+ cells, with high intensity of the staining. Since the inhibitor analysis and gene expression linked MDR1 to the SP phenotype, would be of great interest to isolate SP cells from the AVj region. Compared to the other regions of the heart, AVj would be expected to show a higher frequency of these cells. MDR1 was one of few progenitor markers that was found even in AVj of failing human hearts.

Identification of a BrdU dense region in the AVj

Infrequent proliferation is another general characteristic of stem cells, used in Paper III for identification of LRC. LRC are considered as stem cells, regardless of their biomarker profile. DNA labelling techniques with BrdU have been commonly used, for the identification of stem cell niches e.g. in skin [176, 177], cartilage [178, 179] and intestine [180]. Skin tissue was used as the experimental control, since it has high proliferation rate and well-known location of LRC. Direct comparison of cell-turnover between skin and cardiac tissue was done by manual counting of BrdU+ cells. Roughly, half as many cells/field were labelled in myocardium when compared to skin, at the end of the BrdU administration, proving lower ongoing proliferation. The decrease in numbers of BrdU+ cells over the chasing period, was lower in the cardiac tissue showing also a slower cell turnover, as expected. These findings show that the BrdU experiment worked well in the rat model.

Although stem cell niches have been extensively studied in other tissues, little is known regarding the heart. In the adult rodent heart, nests of stem cells between the cardiomyocytes have been identified as stem cell niches, more frequent in apex and atria [65, 136] or equally distributed [66]. In these studies, biomarkers in combination with BrdU intensity

were used for selection of stem cells. A specific anatomic structure with high frequency of LRC in the adult heart, has to our knowledge not been described yet.

In Paper III, whole rat hearts were sectioned and the distribution of all BrdU+ cells was studied in order to analyse proliferation after the BrdU administration and to identify the slow cycling LRC at the end of the chasing period. Sparsely distribution of BrdU+ cells, throughout the myocardial tissue, was observed in line with a previous report [66]. AVj was an exception. At the mitral and tricuspid valve insertion points, the highest density of BrdU+ cells was observed. Manual counting of the BrdU+ cells showed that AVj displayed highest numbers of BrdU+ cells and ventricles the lowest, at all time points.

Ending up with roughly 30% of the BrdU+ cells left, at the end of the chasing period, we could not conclude that these represented a progenitor population, rather that the duration of the chasing period was too short. When comparing with previous studies, already after 10 weeks, numbers of BrdU+ cells decreased by 91 % in mice [65]. The same group later reported a decrease of 86% in young and by 93% in old rats after 12 weeks [136]. Even thou the same species were used, we showed a lower decrease (71%) after a longer period of 13 weeks. This discrepancy may be due to different efficiencies in the BrdU detection. The longest chasing period that has been used was 1 year in mice, where after 4 months, 97 % of the BrdU were washed out and the remaining bright cells were considered as the LRC [66].

Effects of Physical exercise

There are many beneficial effects of physical exercise. It has been shown that running results in increased neurogenesis [181] and proliferation of satellite cells in skeletal muscle [182]. Therefore, we hypothesized that physical exercise affects cell proliferation and distribution of progenitor cells, also in the adult heart. To test this hypothesis, rats given BrdU were either submitted to treadmill running or normal cage activity.

After the BrdU administration, the number of the BrdU+ cells increased in the AVj region and in skin tissue, indicating increased cell proliferation due to physical exercise. From the same animals other tissues have been studied and in line with our findings, increased proliferation rate was observed in lumbar intervertebral disc regions [138] and Achilles tendon [137]. When exercising the full organism is affected. Satellite cells in skeletal muscle proliferate and increases their

pool size during exercise stress and muscle damage [183]. Another study in adult rats, also reported increased proliferation due to exercise. Additional effects were physiologic hypertrophy, cardiac stem cell activation and new myocyte formation in the high intensity exercise group [36]. These adaptations were dependent on exercise duration and intensity. Our results show that even moderate exercise load had some impact on cell turnover.

Intensity analysis reveal a potential niche area in the AVj

As described above, a variation within the intensity of the BrdU staining was noted after the chasing period, within the specimen but also between the animals. Therefore, to identify the location of the realistic LRC we sat up and optimized intensity measurements where individual BrdU+ nuclei were analysed in large images. A small fraction of the brightest cells, arbitrary set to 2,5% of all BrdU+ cells were selected for each rat. By making a relative comparison within each rat, the inter-individual variation was eliminated. Intensity analyses showed that numbers of these BrdU Bright cells were significantly higher in the suggested niche area compared to the other areas, in the exercise group. The reason why this pattern could only be seen in the exercise group is unclear, but could be that exercise increase the proliferation rate in somatic cells. This could result in a faster wash out of BrdU labelling which gives a greater difference between slow cycling stem cells and proliferating somatic cells.

The back plotting of LRC showed a dilution of intensity within the BrdU+ cells with increased distance from the ventricular border, schematically illustrated in Fig. 15. Similar expression pattern has been described in other niche models. In the intestinal mucosa [180], a small population of LRC is located in the crypts. These LRC differentiate into the proliferating transient amplifying cells, which differentiate further into the cell phenotypes of the intestinal epithelium [57]. Similar pattern has also been shown when chondrogenic stem cell niches were identified in the zone of Ranvier in the knee joint [178] and in the anulus fibrosus of the interverterbral disc region [179].

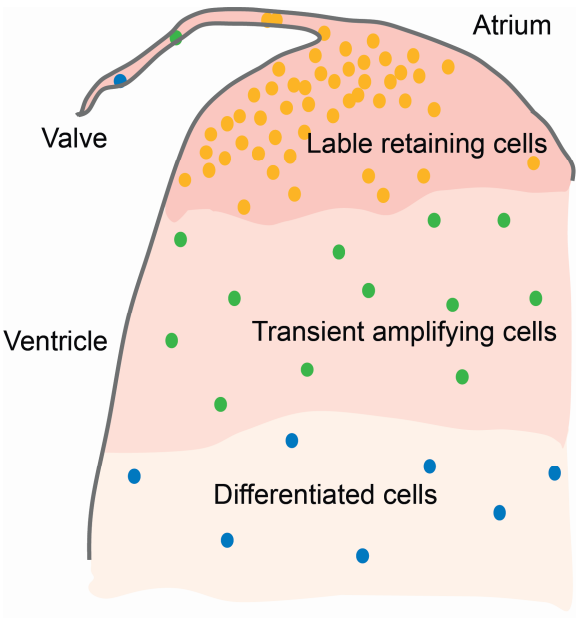

Figure 15. *Schematic drawing of the putative niche in AVj region of adult rat hearts*

This theory is further supported by the results from study IV. The intensity of the staining for the progenitor marker SSEA4 and the migration marker Snai1 showed a gradient in the myocardium of the left ventricle at AVj, decreasing with the distance from the valve.

Stem cell biomarker expression in the rat and human AVj

After the identification of LRC in a subregion in AVj, IHC was used to investigate the expression of progenitor markers in the rat heart. The detection of MDR1 and to less extent Sca-1 within the LRC dense region in the AVj of rat hearts, also point toward the existence of a stem cell niche.

Whether a similar niche also exist in the adult human heart is not known. Therefore, in Study IV, we investigated if the human AVj shows features of a stem cell niche. We studied histology and expression of stem cell- hypoxia-, proliferation- and migration- biomarkers in this region. LV tissue was used for comparison. Very little is known about the distribution of the known cardiac progenitor populations in the ventricles, probably due to difficulties to access ventricular tissue. The results showed that none of the investigated stem cell biomarkers were expressed in left ventricular tissue of the donors, except for the expression of MDR1 in some of the blood vessels.

Histology results, from donors in Study IV, show high collagen content in the AVj, displaying an ECM rich milieu. During development, annulus fibrosus of AVj and valve leaflets are derived from proepicardium [49], with the main function of electrical separation of atrial and ventricular tissue. Furthermore, importance of ECM for stem cell maintenance has been described as another important function [184]. In this ECM rich environment, Isl1, MDR1 and SSEA4 showed the strongest expression. Below this connective tissue region, at the boarder to myocardium, WT1 and Isl1 were expressed in the small border cardiomyocytes.

During early development, one of the sites were Isl1 is expressed is the outflow tract. Co-expression of cTnT/Isl1 was observed in clusters of cells, at the junction between outflow tract and ventricular tissue in neonatal [76] and adult rats [73, 185]. A subpopulation of the Isl1+ cells expressed the proliferation marker Ki67. Few Isl1+ cells in the outflow tract co-expressed the cardiomyocyte marker α-actinin in another adult mouse model. In this study Isl1 was regarded as a novel marker of the adult sinoatrial node [79]. In Study IV, we found Isl1+ cells in the connective tissue of AVj but also Isl1+/cTnT+ cells in the boarder to myocardium. Co-expression with cTnT can be interpreted as evidence of cardiac commitment in Isl1+ progenitor cells.

Small cardiomyocytes co-expressing stem cell and proliferation markers

At the suggested niche region, the boarder cardiomyocytes were small, organized in clusters and embedded in ECM. These first small clusters of cTnT+ cells were to a large extend cTnT+/Isl1+/Wt1+. In addition, the border zone cardiomyocytes were cTnT+/SSEA4+. In a previous study, where expression of SSEA-4 in human atrial tissue was investigated, we observed few SSEA4+ cells that also co-expressed NKX2.5 and cTnT [98]. Furthermore, in study IV we investigated expression of proliferation marker Ki67 in combination with cTnT and human cardiomyocyte nuclei specific marker PCM1 [21, 186]. Most of the Ki67+ cells were cTnT-/PCM1-. However, we also found expression of the Ki67 in a small fraction of PCM1+ nuclei, surrounded by cTnT cytoplasm in the AVj boarder to myocardium. A new hypothesis emerges where these results together represent properties of daughter cells that might be derived from the neighboring niche region. This hypothesis need to be investigated further, with confirmation of the co-staining by confocal microscopy as a first step.

Hypoxia in the human AVj

Hypoxia is considered as a hallmark of stem cell niches e.g. the hematopoietic niches [135]. Hif1α is one of the key factors for maintenance of the quiescent state of stem cells within niches [187].

In Study IV, nuclear expression of Hif1α was found in the connective tissue region of AVj, suggesting hypoxia. Lack of CD31 expression in the streak of Hif1α+ cells was observed.

Epicardium provides progenitor cells during development and these epicardial cells express WT1. Furthermore, WT1 is associated with the activation and reactivation of the epicardium after MI, where the embryonic expression pattern within the epicardium is reactivated. Lately, it has been shown that hypoxia induces activation of WT1 [99, 188]. Epicardium and subepicardium have been identified as hypoxic stem cell niches also in adult mice. Across the ventricle, lowest capillary density was shown in epicardium and subepicardium where 50% of the cells expressed Hif1α protein. Non-myocytes isolated from this region were clonogenic, self-renewing and showed cardiomyogenic differentiation potential [189].

Our finding of a streak of Hif1α in a region lacking CD31 expression, displays similar pattern described in the mouse epicardium [189]. We suggest therefore a new potential hypoxic stem cell niche in the connective tissue of human AVj. Furthermore, MDR1 and Hif1α antibodies were used in combination with CD31. The expression of MDR1 and Hif1α was overlapping, in the region lacking CD31, connecting MDR1 to the hypoxic region.

A new stem cell niche in the AVj?

LRC were found in the bulge regions of the hair follicles in skin of the rats in Paper III, where well known stem cell niches reside [176, 177]. Therefore, we suggest that an adult cardiac stem cell niche is present in, or nearby, the valve insertion point, in the AVj. Furthermore, human AVj displayed an extracellular matrix rich region, with high expression of four different stem cell biomarkers in the connective tissue and different stages of cardiomyocytes at the myocardium boarder. In addition, signs of hypoxia, proliferation and migration were shown, summarized in Fig. 16. Therefore, we propose a new hypoxic stem cell niche in human adult AVj, in the same region previously identified in adult rat hearts.

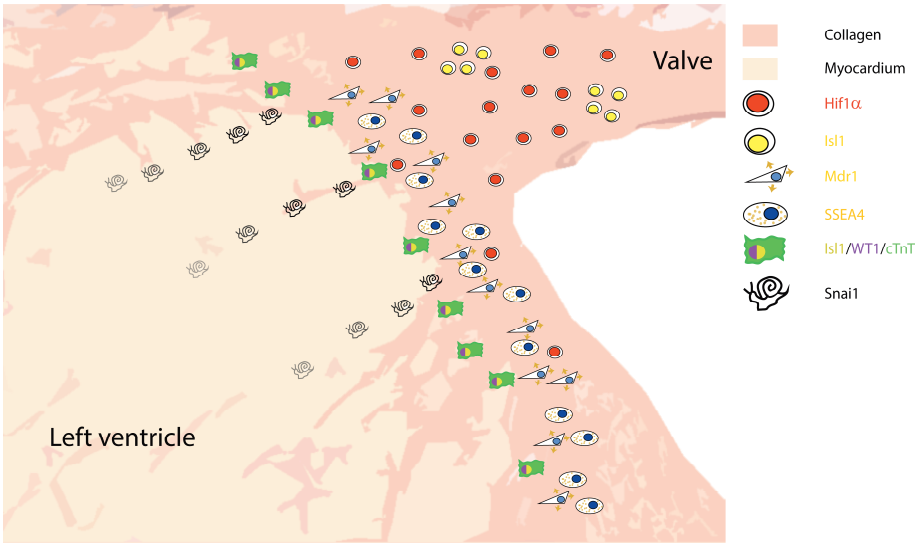

Figure 16. Summary of the expression of different markers in the AVj

AVj is a new location, not mentioned in the previous studies identifying cardiac stem cell niches [65, 66, 190]. Interestingly, increased proliferation due to physical exercise has been reported in distal parts of Achilles tendon [137] and annulus fibrosus of the intervertebral discs [138] from the same animals. Distal tendon and annulus fibrosus are described as stem cell niche regions, consisting mainly of connective tissue, which is also the case for the proposed niche region in the AVj.

I Paper III, highest numbers of BrdU+ cells were found in AVj regions, at the insertion of the mitral and tricuspid valves, also at septum. In one of these regions in human hearts, the left AVj in Study IV, features of a stem cell niche were shown in tissue from all of the seven included donors. The biopsies were harvested somewhere along the lateral side, at the insertion of the mitral valve. This points towards the theory that the niche is circle around the mitral valve, more specifically the border between annulus fibrosus and the left ventricle myocardium.

The valve rings support the base of the valves and are the centre of the fibrogenous skeleton of the heart. This fibrocollagenous skeleton anchors the chambers and the valves together [8]. The discovery of a potential stem cell niche in the centre of the collagenous skeleton of the heart opens for a new hypothesis (Fig. 17).

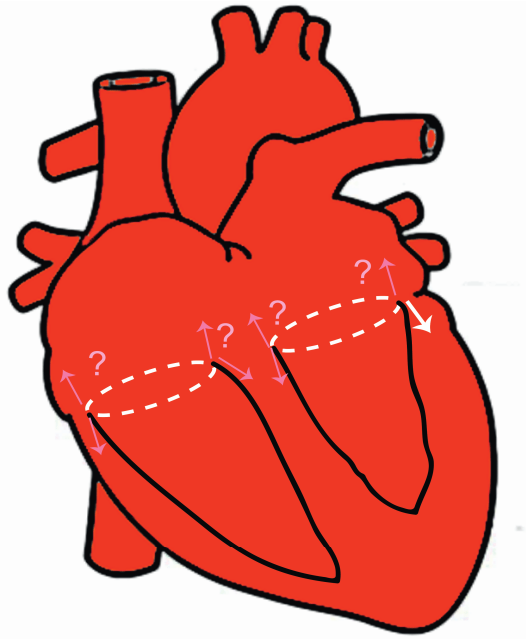

Figure 17. Illustration of a new hypothesis of the proposed adult cardiac stem cell niches

If the cardiac niches are located here the daughter cells might be derived from this site. We have shown the expression of migration marker Snai1 from the myocardium border in AVj. The intensity of the staining decreased with the distance from the putative niche. This indicates migration of cells into the myocardium. Further studies are needed to confirm this finding but also to investigate if migration occurs towards the valve or atria.

Collagenous fibres were strongly stained and well organised in this region. One could speculate that the cells might migrate along these fibres. This was not investigated in the Study IV. A combination of Snai1 and Collagen antibodies would be interesting since migration of stem cells along collagen fibres has been reported in the intervertebral disk region [191].

An ultimate stemness identifier may not exist [131]. However, BrdU Bright LRC and all of the four analyzed stem cell markers were present either coexpressing or neighboring in the same anatomic site. Isl1, MDR1 and SSEA4 showed strongest expression in the connective tissue region of AVj. From the boarder to myocardium WT1, Isl1, migration marker Snai1 and N-cadherin were expressed. Whether there is a hierarchial link between the different stem cell populations in this region remains to be investigated.

Functional aspects of this potential niche are obviously of great importance but was beyond of the scope of these two studies. In order to investigate the functionality of the identified niche region animal models with lineage tracing will be needed.

Expression of stem cell biomarkers in the failing hearts

In order to improve the treatment for heart failure, cellular mechanisms need to be investigated further. Expression of stem cell markers in severely failing hearts has not been studied to any larger extend. There is however some evidence that ischemia [97] and cardiac hypertrophy and heart failure [107] affect the frequency of progenitor cells. For example, following ischaemia, the number of progenitor cells was elevated [98]. In study IV, we investigate how severe heart failure might affect the distribution of endogenous progenitors. Two opposite hypotheses were assumed: either that niches are depleted during aging, which could be the cause of heart failure, or that the expression of stem cell markers is increased as a response to disease.

When a comparison was made between donor hearts and failing hearts from cardiac transplantation patients, the tissue from failing hearts displayed low or no expression of stem cell markers in AVj region. Lack of the early stem cell markers Isl1 and WT1 in the whole AVj points towards the depletion of stem cell niches. This is also in line with the observed lower expression of MDR1 and SSEA4 in the AVj of the failing hearts.

A comparison was further made between the AVj and LV of failing hearts. Upregulation of Isl1 and WT1 expression in left ventricular tissue of failing hearts was somewhat surprising, especially when no expression was detected in the AVj from the same hearts. When WT1 and Te7 antibodies were used in combination, WT1 expression was found in Te7+ cells. This finding was interpreted as activation of fibroblasts. In addition, many WT1+/Isl1+/cTnT+ cells were found with large nuclei. We observed this co-expression pattern in clusters of cells at the myocardium border in AVj of donors. An increase of Isl1 and WT1 expression in cardiomyocytes with large nuclei indicates activation of these transcription factors in end stage heart failure. Since the size of these WT1+/cTnT+ nuclei were large compared to the other nuclei in the same specimen it could be speculated if this could be a sign of polyploidization, as a response to disease. Indeed, increase in polyploidy of cardiomyocytes has previously been shown in pathological situations such as ischemic injury or end stage heart failure [17, 192].¨

Methodological considerations

1) Due to limited size of each biopsy it was not possible to test all the different media conditions on the cultured HDS from the same patients.

2) **Pulse-chase methods**: The stem cells are considered as quiescent in the niches. The rare events of cell division are difficult to catch if the BrdU administration is carried out during too short time period. Two weeks of BrdU administration may be too short for all slow cycling cells to pick up the label. Hypothetically, BrdU labelling could thus detect slowly proliferating cells, close to the niche rather than the true stem cells. However this method has previously been used to find LRC and niche structures. Unfortunalety, if administered for a long time BrdU treatment could cause toxicity issues.

3) **Fluorescence microscopy** used in the cardiac stem cell research field is an issue. The numbers of stem cells are low and there are many challenges. For example co-localization could be a result of low resolution and should be confirmed by confocal microscopy.

4) **DNA incorporation** does not always result in cell division. There are the issues with polyploidization, multinucleation and cell fusion. This is both related to BrdU and Ki67 results.

5) **Lack of specific markers**: biomarkers are often expressed by several different types of cells. Markers used for stem cell identification e.g.MDR1 in Paper III and IV, may also be expressed by hematopoietic cells.

6 CONCLUSIONS

In this thesis, a new 3D culture system is proposed for studies of cardiac cell biology. SP stem cells were identified in left atria for the first time in humans. In addition, a new anatomic region with features of a stem cell niche was discovered in the Atrioventricular junction of the adult rat and human hearts.

Paper I:

A novel 3D culture system, HDS, was set up for studies of primary isolated cardiac cells. High cell density in a 3D manner enables for paracrine communication between the cells, reorganization and migration. Histology showed well preserved cell morphology, including the cardiomyocytes. An increase in both cardiac- and progenitor-specific gene expression was shown over time. Endothelial cells adopted an intra-luminal position. With addition of serum, it was possible to induce proliferation and ECM production. Activities within Notch signalling pathway were shown. Moreover, it was possible to affect the Wnt signalling in HDS by addition of a GSK3 inhibitor. Taken together, these findings show the suitability of HDS as an *in vitro* model for studies of cardiac cell phenotypes in a tissue like environment.

Paper II:

Left and right atrial human adult biopsies were analysed for content of SP cells. SP cells could only be isolated from the left atrial biopsies. Using inhibitors, we suggested MDR1 as the pump protein responsible for efflux of Hoechst 33342 dye in the SP cells. Within the SP cells C-Kit and OCT4 genes were highly expressed compared to main population. Further subdivision using CD45 showed that CD45+SP cells were uncommitted to the cardiomyocyte and endothelial lineages according to gene expression. However, CD45-SP cells were expressing endothelial genes. Functionality of the isolated SP cells was not addressed.

Paper III:

Using DNA labelling in a rat model we showed that the highest amount of BrdU+ cells was seen in the AVj and lowest in ventricles at all time points. A possible niche structure was found in the AVj were the BrdU+ cells were densely packed. The number of BrdU+ cells in AVj at day 14 was affected by physical exercise, suggesting an early increase in proliferation. There was significantly higher number of BrdU Bright cells in the suggested niche region at the end of chasing period, suggesting that physical exercise affects the distribution of LRC within the heart.

Study IV:

Using histological methods, we investigated whether human AVj shows features of a stem cell niche. We found an extracellular matrix rich region with high expression of the chosen stem cell biomarkers, different stages of cardiomyocytes, signs of hypoxia, proliferation and migration. We propose a new hypoxic stem cell niche in human AVj in the same region previously identified in Paper III. Low or no expression of stem cell markers was noted in the AVj of end stage heart failure tissue. However, in the LV, expression of Isl1 and WT1 were upregulated. The function of these potential niches were not addressed and remains to be investigated.

These findings are a step towards better understanding of basic concepts of cardiac regeneration and hopefully improvement of future therapies for patients.

7 FUTURE PERSPECTIVES

Since the heart was regarded as a non-regenerative organ, treatment strategies have been focused on protection and preservation of the remaining myocardium upon injury. Today however, there are several lines of evidence supporting a continuous renewal of all types of cells in the heart throughout life. Both an intracardiac pool of stem cells as well as cardiomyocyte de-differentiation and re-enter into the cell cycle have been proposed as underlying mechanisms. This has opened a whole new research field that aim to improve cardiac function by manipulating regenerative mechanisms.

To this date, clinical trials using cell therapy for treatment of cardiac diseases have shown a good safety profile. However, recent meta-analyses of these studies indicate no clear improvements in cardiac function [126, 127]. There are many possible explanations for this outcome including the cell source, concentration of cells, delivery systems and choice of patient groups. In particular, it should be pointed out that almost all of these studies have been based on bone marrow derived cells, which are generally believed not to have the capacity to transdifferentiate into cardiomyocytes. Results from initial studies using endogenous cardiac stem cells are promising [193, 194], but larger randomized trials are needed. The use of iPS or ES derived cells may also be a viable option for cell therapy in the future, as it has been shown in a primate animal model [195], but safety of these cells has to be further evaluated.

In addition to cell therapy, development of drugs that could recruit and activate endogenous progenitor cells or stimulate cardiomyocytes to re-enter the cell cycle is another approach. Biomaterials loaded with smart molecules is another promising addition to cell therapy approach. Which route that will yield the best regenerative result obviously depends on the underlying mechanism of cardiac regeneration. One major problem in the field, however, has been the lack of *in vitro* systems for human cardiac cells that mimics the in vivo situation.

In Paper I, we present a new 3D culture system where we noted signs of de-differentiation of cardiomyocytes *in vitro* while the morphology was well preserved. Neovascularization after MI is also a crucial step. Study I provide some descriptive and preliminary, but interesting results, indicating that HDS could be a suitable *in vitro* model for studies of vasculogenesis. Taken together, HDS could thus potentially serve as

a model system for cardiac regeneration and in particular study of human cardiomyocyte biology and de-differentiation.

In Studies III and IV we discovered a new anatomic site, in AVj of adult rat and human hearts, with features of a stem cell niche. In the human donor AVj tissue, nearby the suggested niche, small cardiomyocytes occasionally expressed proliferative marker Ki67. This finding is interesting to follow up since division of pre-existing cardiomyocytes has been shown in several studies [19, 123]. Furthermore, when DNA labelling was used in a rat model, BrdU+ cardiomyocytes were mononucleated and of smaller size [36].

It would be particularly interesting to investigate the function of this proposed niche. It was found by DNA label retention in a rat model, confirmed by hypoxia- and stem cell biomarker expression. Different sizes of cardiomyocyte cytoplasm and expression of the migration marker just below indicates that we might have found a daughter cell region. If this is the case the new formation of cardiomyocytes could be understood by deeper studies of this subregion in AVj. In order to address this new hypothesis, we aim to use laser capture technology (Fig. 18) to separate the proposed niche region in the connective tissue from the proposed daughter cell region at the myocardial boarder, to enable further gene-expression and protein analyses.

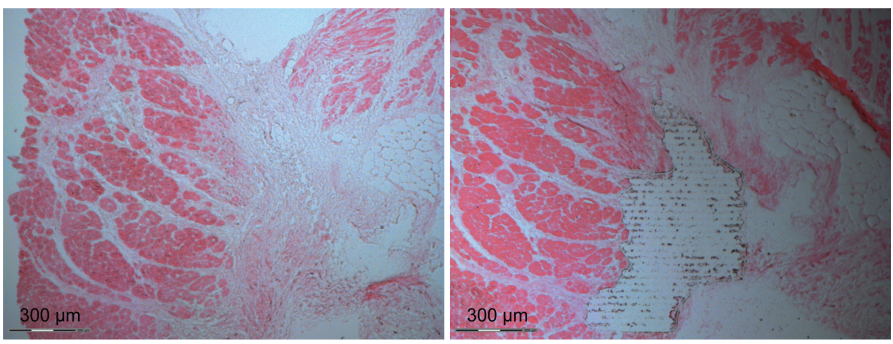

Figure 18. *Laser capture technique used for separation of the proposed niche and the proposed daughter cell region in the AVj from ongoing experiments. Hematoxylin Eosin staining, showing histology of one of the AVj sections before and after laser capture.*

In Study IV, we only focused on the AVj at the Mitral valve. It would be of great interest to evaluate if a similar structure exists also on the right side of the heart, in the AVj at the tricuspid valve. However, for

the function of the niche, animal models and lineage tracing are required.

Another important question that has not been fully investigated is how endogenous cardiac progenitor cells are distributed in the human adult heart and how cardiac disease may affect the function and distribution of these progenitors. To address this, our group is conducting a study where the previously identified progenitor populations C-kit, SSEA-4 [98, 173] and SP are isolated from the four chamber of the human adult heart. To address effects of heart failure, cells isolated from failing hearts are compared with cells from donor hearts. In the future, we aim to use the same approach on isolated cells from left and right AVj from human hearts. Furthermore, it would be interesting to further investigate the differentiation potential of isolated cells from AVj. One approach would be to culture isolated stem cells as HDS. This, since we in Study I noted an increase in stem cell markers over time in this system.

It should be emphasized that in the field of cardiac regeneration, the overwhelming majority of studies have been carried out in small animal models. These models may however not correspond to the human situation [196]. This makes the relatively unique, human cardiac sample collection, presented in this thesis of particular value. It should be pointed out that some of the donor tissue samples are from individuals without any reported cardiac disease and provide a good base for studies of normal cardiac cell biology but also negative control tissue samples when compared to diseases tissue. Tissue from younger individuals was also collected in few cases and represent another time window of cardiac tissue homeostasis for future work.

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