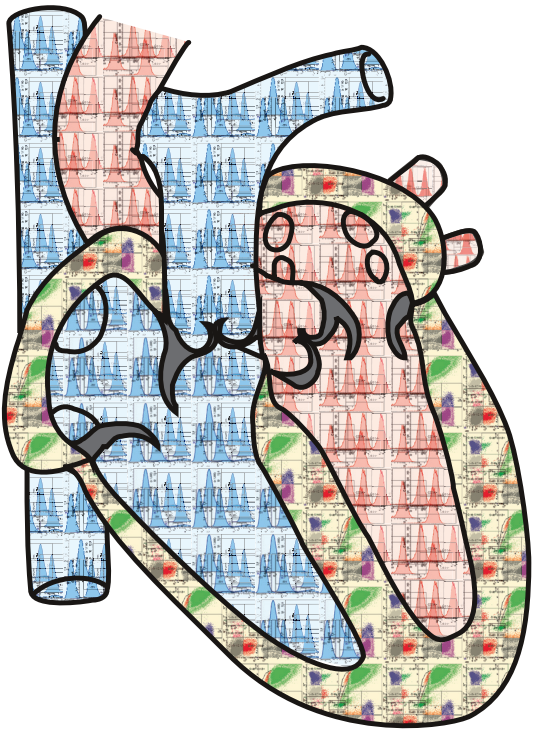
Identification and characterization of progenitor populations in the human adult heart



Joakim Sandstedt

Institute of Biomedicine
at Sahlgrenska Academy
University of Gothenburg

The Sahlgrenska Academy

UNIVERSITY OF GOTHENBURG

Identification and characterization of progenitor populations in the human adult heart

Joakim Sandstedt

Department of Clinical Chemistry and Transfusion Medicine
Institute of Biomedicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2014

Cover illustration: *The heart*

Identification and characterization of progenitor populations in the human adult heart

joakim.sandstedt@gu.se

Correspondence:
Joakim Sandstedt
Department of Clinical Chemistry and Transfusion Medicine
Institute of Biomedicine
Bruna Stråket 16
SE-413 45 Göteborg
Sweden

ISBN (E-published) 978-91-628-8896-1
ISBN (Print) 978-91-628-8892-3

Printed in Gothenburg, Sweden 2014
Ineko AB, Gothenburg

“Facts are the air of scientists.
Without them you can never fly.”

Linus Pauling

Identification and characterization of progenitor populations in the human adult heart

Joakim Sandstedt

Department of Clinical Chemistry and Transfusion Medicine, Institute of Biomedicine
Sahlgrenska Academy at University of Gothenburg,
Göteborg, Sweden

ABSTRACT

Traditionally, the heart has been regarded as a non-regenerative organ. During the last 10 years, this notion has been challenged. By ^{14}C measurements, it was calculated that at the age of 50, about 45% of all cardiomyocytes had formed after birth. An endogenous population of progenitor cells in the heart has been suggested as the source of this regeneration. Until now, most studies have however been conducted in animal models which may not fully reflect the human situation.

The overall aim of this thesis was to add to our knowledge of the identity, distribution and function of endogenous progenitor cells in the human adult heart. In **paper I**, a small population of C-kit+ cells was identified, that could be sub-divided based on expression of the hematopoietic marker CD45. The C-kit+CD45+ population was determined to be of mast cell phenotype whereas the C-kit+CD45- population expressed endothelial associated markers. Differentiation assays showed further endothelial maturation but no evidence of cardiac differentiation. In **paper II**, heterogeneity within the C-kit+CD45- population was further investigated by single cell qPCR. The results indicated that while most of the C-kit+CD45- cells were committed to the endothelial lineage, a minor portion of them could represent cardiac progenitors. In **paper III**, Side Population (SP) cells were identified in the left atrium. The SP phenotype was linked to the MDR1 protein. On gene expression level, the SP cells expressed high levels of *MDR1* as well as stem cell associated genes *C-KIT* and *OCT-4*. Furthermore, the SP could be subdivided based on expression of the hematopoietic marker CD45. The CD45- SP cells had an endothelial profile while the CD45+ SP cells were neither committed to the endothelial, nor the cardiomyogenic lineage. In **paper IV**, expression of SSEA-1, 3 and 4 was investigated. All SSEAs were expressed at variable levels. The SSEA-1+ population was determined to be of hematopoietic origin. Of the SSEA-4+ cells, some co-expressed CD34. In right atrium, the SSEA-4+CD34- population displayed a high expression of cardiomyocyte genes. By immunohistochemistry, SSEA-4+ cells were identified both within and outside the myocardium.

In conclusions, in the present thesis, three different cell populations with characteristics were isolated from human cardiac biopsy material. One C-kit+CD45- population that consisted of both endothelial and cardiac committed progenitors. SP cells where the CD45- fraction showed evidence of endothelial commitment and SSEA-4+CD34- cells that showed signs of cardiac commitment.

Keywords: Cardiac progenitor cells, heart, C-kit, Side Population, Stage Specific Embryonic Antigens, FACS
ISBN: 978-91-628-8892-3

SAMMANFATTNING PÅ SVENSKA

Hjärtat är ett av kroppens mest fascinerande organ, vars funktion är att pumpa runt blodet i kroppen. Under en genomsnittlig livstid slår det mänskliga hjärtat ungefär 3 miljarder gånger. Trots detta har hjärtat fram tills nu betraktats som ett organ utan läkningsförmåga. Detta har man bl.a. baserat på att det vid sjukdomar som drabbar hjärtat, t.ex. hjärtinfarkt, inte sker någon synbar läkning av hjärtvävnaden. Istället bildas ärrvävnad som inte har förmåga att dra sig samman då hjärtat slår.
Under de senaste 10 åren har denna bild dock kommit att nyanseras. Nya tekniker för att studera långsam cellomsättning, såsom kol-14 datering, har visat att det i det mänskliga hjärtat sker en långsam nybildning av hjärtmuskelceller under hela livet. Det finns idag också ett stort antal studier, i de flesta fall utförda i djurmodeller, som visat på förekomst av olika s.k. stam / progenitor cell populationer. Dessa har visats kunna utvecklas till både hjärtmuskelceller, som ger hjärtat förmåga att dra sig samman, och endotelceller, som bygger upp insidan av blodkärl. Dessa blodkärl är i sin tur viktiga för transport av syre och näringsämnen ut i hjärtvävnaden.

Syftet med denna avhandling var att öka vår kunskap om vilka populationer av progenitor celler som finns i det mänskliga vuxna hjärtat. Vidare ville vi studera om det finns skillnader i distribution av dessa celler mellan hjärtats högra och vänstra sida samt ta reda på vilken funktion dessa celler kan ha i hjärtat. I de två första arbetena studerades en cellpopulation som uttryckte det stamcells-associerade proteinet C-kit, men var negativ för proteinet CD45, som uttrycks av blodceller. Denna population av celler verkade i huvudsak vara inriktad mot endotelutveckling även om vi såg tecken till att en mindre del av cellerna istället var inriktad mot hjärtmuskel utveckling. Cellerna fanns till största delen i hjärtats högra förmak. I det tredje arbetet identifierades en cellpopulation baserat på förmågan att pumpa ut ett fluorescerande ämne - Hoechst 33342. Denna förmåga har man tidigare sett hos andra stamcellspopulationer i kroppen, t.ex. i benmärgen. Denna cellpopulation kunde bara identifieras i hjärtats vänstra förmak. Förmågan hos dessa celler att pumpa ut det fluorescerande ämnet kunde kopplas till proteinet MDR1.

I det fjärde arbetet studerades uttryck av s.k. stage specific embrynoic antigens (SSEAs). Dessa är socker-strukturer som finns på cellens yta. Man har tidigare kopplat ett visst mönster av SSEAs till olika utvecklingsstadier hos embryonala stamceller. I det vuxna hjärtat är dock inte så mycket känt kring dessa markörer. I vår studie kunde vi se att alla SSEAs uttrycktes i varierande utsträckning även i det mänskliga vuxna hjärtat. I en del av de celler som uttryckte SSEA-4 (ett av de SSEAs som studeras) såg vi tecken på både sen och tidig hjärtmuskelcell utveckling. Dessa celler skulle kunna representera en omogen cellpopulation med inriktning mot hjärtmuskelutveckling. Celler med denna profil kunde endast identifieras i höger förmak.

Sammanfattningsvis kan man utifrån detta avhandlingsarbete dra slutsatsen att även det mänskliga vuxna hjärtat innehåller flera potentiella progenitor populationer. Dessa kan förhoppningsvis utnyttjas i framtida utveckling av nya behandlingsmetoder för vanliga hjärtsjukdomar såsom hjärtsvikt och ischemisk hjärtsjukdom. För detta krävs dock ytterligare forskning, inte minst vad gäller funktionella aspekter hos de olika cellpopulationerna.

LIST OF PAPERS

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

I Sandstedt J, Jonsson M, Lindahl A, Jeppsson A, Asp J. C-kit+ CD45- cells found in the adult human heart represent a population of endothelial progenitor cells. *Basic Res Cardiol*. 2010 Jul;105(4):545-56

II Sandstedt J, Jonsson M, Dellgren G, Lindahl A, Jeppsson A, Asp J. Human C-kit+CD45- cardiac stem cells are heterogeneous and display both cardiac and endothelial commitment by single-cell qPCR analysis. *Biochem Biophys Res Commun*. 2014 Jan 3;443(1):234-8

III Sandstedt J, Jonsson M, Kajic K, Sandstedt M, Lindahl A, Dellgren G, Jeppsson A, Asp J. Left atrium of the human adult heart contains a population of side population cells. *Basic Res Cardiol*. 2012 Mar;107(2):255

IV Sandstedt J, Jonsson M, Dellgren G, Lindahl A, Jeppsson A, Asp J. SSEA-4+ CD34- cells in the human adult heart show molecular characteristics of a novel cardiomyocyte progenitor population. *Submitted.*

TABLE OF CONTENTS

ABBREVIATIONS

7-AAD	7-amino-acitinomysin D
ABCG2	ATP-binding cassette sub-family G member 2
ACTC1	alpha-cardiac actin
Akt	serine/threonine protein kinase
α-SMA	alpha-smooth muscle actin
BMNCs	bone marrow derived mononuclear cells
BrdU	5-bromo-2-deoxyuridine
C/EBP	also known as DNA damage-inducible transcript 3 protein
CABG	coronary artery bypass graft
CD31	also known as platelet endothelial cell adhesion molecule (PECAM-1)
CMA1	chymase
CMR	cardiac magnetic resonance
CREBBP	CREB-binding protein
cTnI	cardiac troponin I
cTnT	cardiac troponin T
CXCR4	C-X-C chemokine receptor type 4
cytokinesis	total cellular division
DAPI	4’,6-diamidino-2-phenylindole
DDR2	discoidin domain-containing receptor 2
EPCs	endothelial progenitor cells
EPDCs	epicardium derived progenitor cells
Erk	extracellular-signal-regulated kinase
ES cells	embryonic stem cells
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FLK-1	fetal liver kinase 1, also known as vascular endothelial growth factor receptor 2
FTC	Fumitremorgin C
G-CSF	granulocyte colony-stimulating factor
HGF	hepatocyte growth factor
HS	human serum
HUVECs	human umbilical vein endothelial cells
ICD	implantable cardioverter-defibrillator
IGF	insulin-like growth factor
IGF-1	insulin-like growth factor 1
IGF-2	insulin-like growth factor 2
IGF-1R	insulin-like growth factor 1 receptor
IGF-2R	insulin-like growth factor 2 receptor
IL-6	interleukin-6

IL-10	interleukin-10
iPS cells	induced pluripotent stem cells
karyokinesis	division of the cell nucleus
LIF	leukemia inhibitory factor
LVEF	left ventricular ejection fraction
MDR1	multidrug resistance protein 1
MEF2C	myocyte-specific enhancer factor 2C
MP	main population
MSC	mesenchymal stem cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PET-CT	positron emission tomography - computed tomography
PI	propidium iodide
PI3	phosphatidylinositide 3
qPCR	quantitative real time PCR
Sca-1	stem cell antigen 1, also known as Ly-6A.2/6E.1
SCF	stem cell factor
SDF-1α	Stromal cell-derived factor 1 alpha
SEM	standard error of the mean
SP	Side Population
SSEAs	stage specific embryonic antigens
SSEA-1	stage specific embryonic antigen 1
SSEA-3	stage specific embryonic antigen 3
SSEA-4	stage specific embryonic antigen 4
TGF-β1	transforming growth factor beta-1
TPSG1	tryptase
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VSELs	very small embryonic like cells
VWF	von Willebrand factor
Wt1	Wilms tumor protein

1 BACKGROUND

1.1 Normal structure and function of the heart

1.1.1 Anatomy and normal physiology of the heart

The heart works as a muscle pump, propelling the blood to all parts of the body. In the human, it is located in the thoracic cavity, in the mediastinum. It is enclosed by the pericardial sac. The mammalian heart, including that of the human, consists of four chambers, two atrias and two ventricles. The right side of the heart pumps poorly oxygenated blood to the lungs while the left side of the heart supplies the remaining body with oxygenated blood. Between the atrias and ventricles and at the outflow tracts of the ventricles are valves that prevent backflow of the blood. The cardiac tissue itself is perfused by the coronary arteries, which emanates from the ascending aorta just above the aortic valve (1). The heart rate is under normal physiological conditions governed by the sinus node, which is situated in the right atrium close to the opening of superior vena cava. The sinus node displays an electrical self excitation, the pace of which can be modulated by both the sympathetic and parasympathetic nervous system. An increase in sympathetic stimulation results in an increase in heart rate whereas an increased parasympathetic stimulation has the opposite effect. From the sinus node, excitation is spread among the rest of the atrial cardiomyocytes and then transmitted to the ventricles via the atrio-ventricular node. In the ventricles, excitation is spread partly via cardiomyocyte to cardiomyocyte transmission, partly via specialized Purkinje fibers (2).

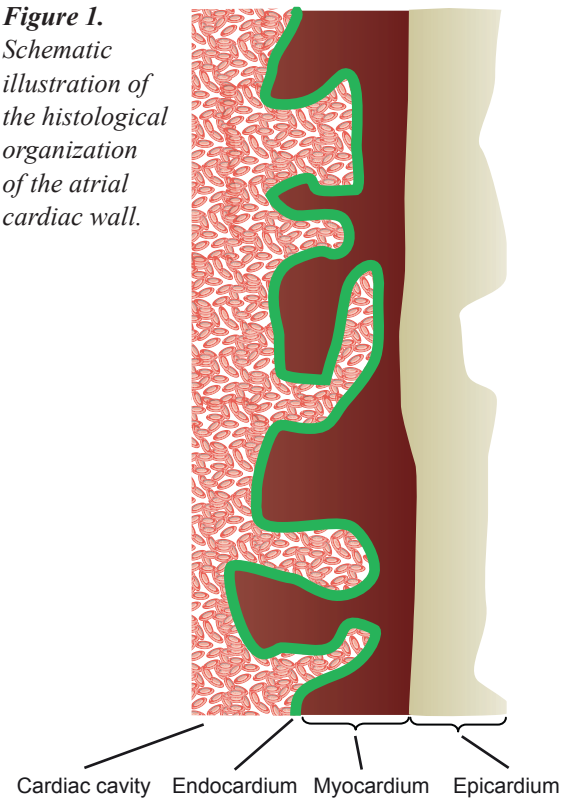


Figure 1. *Schematic illustration of the histological organization of the atrial cardiac wall.*

1.1.2 Histological organization and cellular composition of the heart

The cardiac wall is histologically organized into three layers (1, 3). Its organization is schematically illustrated in Figure 1. A corresponding histological picture of the atrial cardiac wall

is shown in Figure 2. The epicardium is the most superficial layer and constitutes the visceral layer of the pericardial sac. It mainly consists of connective tissue, where fibroblasts are the dominating type of cell. Its outermost layer is built up by mesothelial cells, which produces the pericardial fluid. This enables the heart to move in the pericardial sac in an almost frictionless manner. Parts of the epicardium also have depositions of adipose tissue (4). Furthermore, the coronary arteries are located within the epicardial layer.

The myocardium makes up the mid part of the cardiac wall. It is by far the thickest layer, particularly in the ventricles. The main volume of this layer consists of cardiomyocytes. Furthermore, branches of vessels from the coronary arteries transverse the myocardium. These are build up by endothelial cells, smooth muscle cells and pericytes.

The endocardium is the innermost layer. It is a very thin layer lining the entire inside of the heart cavity. It consists mainly of endothelial cells. Notably, the inner surface of the cardiac wall is highly irregular caused by trabeculations.

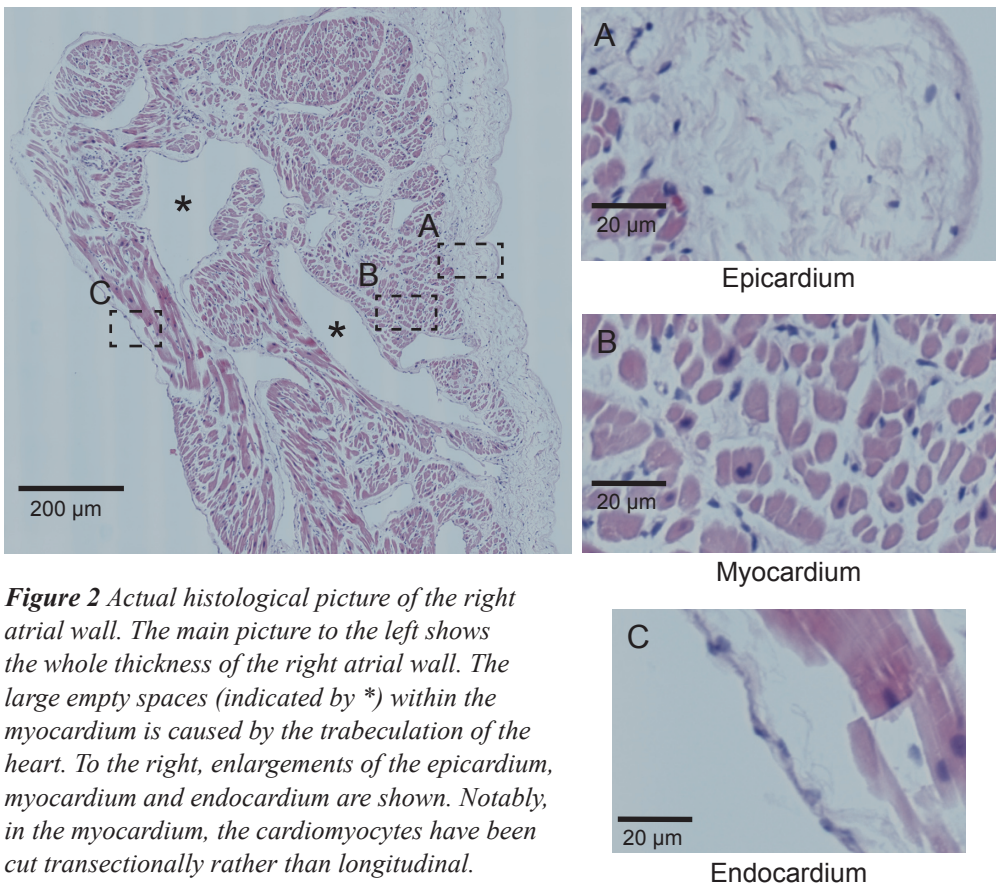


Figure 2 *Actual histological picture of the right atrial wall. The main picture to the left shows the whole thickness of the right atrial wall. The large empty spaces (indicated by *) within the myocardium is caused by the trabeculation of the heart. To the right, enlargements of the epicardium, myocardium and endocardium are shown. Notably, in the myocardium, the cardiomyocytes have been cut transectionally rather than longitudinal.*

The overall cellular composition of the heart has been determined both in the murine and human heart. In older studies, where identification of different types of cells were carried out based on morphological properties determined by electron microscopy and gradient centrifugation, 30 - 40% of the total number of cells in the rat heart were determined to be cardiomyocytes whereas 60 - 70% of the cells were other types of cells (5, 6). In a more recent study by Banerjee et al. (7), where different types of the cell were identified based on fluorescence-activated cell sorting (FACS) and immunohistochemistry, the percentage of cardiomyocytes in the adult rat heart was similarly to the results of previous studies determined to be an average 26.4%. In the adult mouse heart, the percentage of cardiomyoctes was on average 56% and thus much higher compared to the rat heart. Notably, the volume fraction of cardiomyocytes in the adult rat ventricle has been determined to be about 80% (8), thus almost inverse to the percentage of cells.

In the normal human adult heart, it has been shown by Olivetti et al. (9) that cardiomyocytes constituted between approximately 70 - 90% of the total volume of both right and left ventricle. The volume fraction of cardiomyocytes increased with increased age. The total number of cardiomyocytes, to the contrary, decreased with increased age. Vliegen et al. (10) showed similarly that on average 81% of the volume of the normal adult human heart was made up of cardiomyocytes. When cellular composition was determined on the other hand, on average only 30% of the total number of cells were made up of cardiomyocytes. Taken together, it can be concluded that while the volume of both rat and human hearts mainly are taken up by cardiomyocytes, these cells account for only a minority of the total number of cells.

1.1.3 A description of the different types of cells found within the heart

As described above, the heart is composed of several different types of cells including cardiomyocytes, fibroblasts, endothelial cells, pericytes and smooth muscle cells. In this section, a short description of the function and common markers used for identification of each type of cell will be given.

1.1.3.1 Cardiomyocytes

As described above, cardiomyocytes make up most of the volume of the heart. They are often divided into three subgroups depending on location and function. The two first groups, ventricular and atrial cardiomyocytes, have many similarities and are responsible for the contraction of the heart. In addition to these groups, there is also a heterogeneous third group of specialized cardiomyocytes that conducts the electrical impulse in a coordinated manner as well as function as impulse generators. Cardiomyocytes could generally be regarded as an intermediate between skeletal muscle cells and smooth muscle cells. As skeletal muscle cells, they have a striated pattern when observed in histological sections. This comes from the A and I bands of the sarcomeres, which are the contractile units of the cardiomyocyte. These are in its turn arranged in myofibrils. The cardiomyocytes are arranged in a syncytium, connected to each other with intercalated discs which permits the exchange of ions between cells. Furthermore, cardiomyocytes are electrophysiologically competent. During contraction of the heart, an action potential is propagated by opening of membranous voltage gated fast sodium channels and slow calcium-sodium channels. This results in influx of Ca^{2+} ions both from the sarcomplasmic reticulum and extracellular fluid which activates the contractile process of the cells. The electrophysiological resting potential is then restored by opening of potassium channels, a process called repolarization (2, 3, 11).

Compared to most other types of cells, cardiomyocytes have the ability to undergo karyokinesis without cytokinesis (i.e. duplication of DNA without cell division). This may result in multinucleation - the existence of two or more distinct cell nuclei within the same cell, as well as polyploidization - the replication of DNA in one cell nuclei without the formation of two distinct nuclei. In the human adult heart, it has been determined that most cardiomyocytes are mononucleated (74% of all cardiomyocytes). The next most common nucleus configuration is binucleation (25.5%) while trinucleation (0.4%) and tetranucleation (0.1%) are rarely seen (12). The percentages of the different nucleus configurations do not change considerably from the early postnatal time to old age in human (13). Polyploidization on the other hand has been shown to change considerably during childhood. While most cardiomyocytes are diploid at birth, most nuclei become tetraploid and a few also octaploid until the age of 10. After this, no further increase in polyploidization was observed (14). Notably, the physiological functions of both multinucleation and polyploidization in cardiomyocytes are still mostly unknown (15). The phenomena

are however very important to consider when interpreting results from many common assays used for studying cell renewal.

Commonly used markers for identification of mature cardiomyocytes are proteins of the contractile apparatus such as cardiac troponin T (cTnT), cardiac troponin I (cTnI) and cardiac α-actin as well as gap junction proteins such as connexin 43 (11, 16). For detection of early cardiomyogenic differentiation in stem and progenitor cells, transcription factors responsible for specification to the cardiomyogenic lineage such as TBX5, NKX2.5, GATA-4 and myocyte-specific enhancer factor 2C (MEF2C) (17) are often used.

1.1.3.2 Endothelial cells

Endothelial cells are found within the cardiac wall, lining blood vessels of different calibers. In addition, they make up the innermost layer of the cardiac cavity. The general functions of the endothelium involves regulation of blood coagulation, recruitment of inflammatory cells to the tissue via transendothelial migration and regulation of the contraction state of smooth muscle cells found lining all blood vessels except capillaries (3). In capillary vessels of the heart, endothelial cells are also involved in the transportation of fluid in and out of the tissue, from and into the vessel. In addition to these functions, it has also been shown that cardiac endothelial cells release factors such as nitric oxide (NO), prostaglandin I2 and endothelin that can influence cardiomyocytes and result in increased contractile force (18).

Commonly used markers for identification of endothelial cells include adhesion protein CD31 (7, 18) and fetal liver kinase 1 (FLK-1) which is a receptor for vascular endothelial growth factor (VEGF) (19). Another marker is von Willebrand factor (VWF) which is involved in initiating the coagulation of blood (20).

1.1.3.3 Smooth muscle cells

Smooth muscle cells in the heart are found in the wall of all blood vessels except capillaries. Their function is to regulate the flow of blood through contraction or relaxation. Commonly used markers for identification includes smooth muscle specific isoforms of proteins in the contractile apparatus, such as alpha-smooth muscle actin (α-SMA) (7).

1.1.3.4 Pericytes

Pericytes are found outside the endothelial layer of blood vessels of the heart. They form heterocellular junctions with endothelial cells as well as homocellular junctions with other pericytes. The physiological function of pericytes involves regulation of angiogenesis, coagulation and possibly also blood flow in small sized arteriols (21). There is no known marker specific for pericytes but rather a combination of markers must be used to distinguish them from other types of cells such as fibroblasts,

smooth muscle cells and endothelial cells (22).

1.1.3.5 Fibroblasts

Cardiac fibroblasts are found dispersed within the cardiac wall. Their functions include homeostasis of extracellular matrix, regulation of mechanical properties of the heart and possibly also electrical signaling in the heart. As an indication of the later, it has been found that fibroblasts form heterocellular gap junctions with cardiomyocytes (23). Upon increased mechanical load, cardiac fibroblasts can upregulate α-SMA, the same protein that is also expressed by smooth muscle cells. This is observed in cardiac hypertrophy as well as at sites of previous myocardial infarction (24). Fibroblasts expressing α-SMA are often called myofibroblasts.

A commonly used marker for identification of fibroblasts is vimentin, which is an intermediate filament abundantly expressed in fibroblasts. This marker is unfortunately also expressed by for example endothelial cells (23). DDR2, a collagen specific receptor tyrosine kinase, has been described as a more specific marker for cardiac fibroblasts. It has not been found in other types of cells in the heart (23, 25).

(This page intentionally left empty)

1.2 Embryological development of the heart

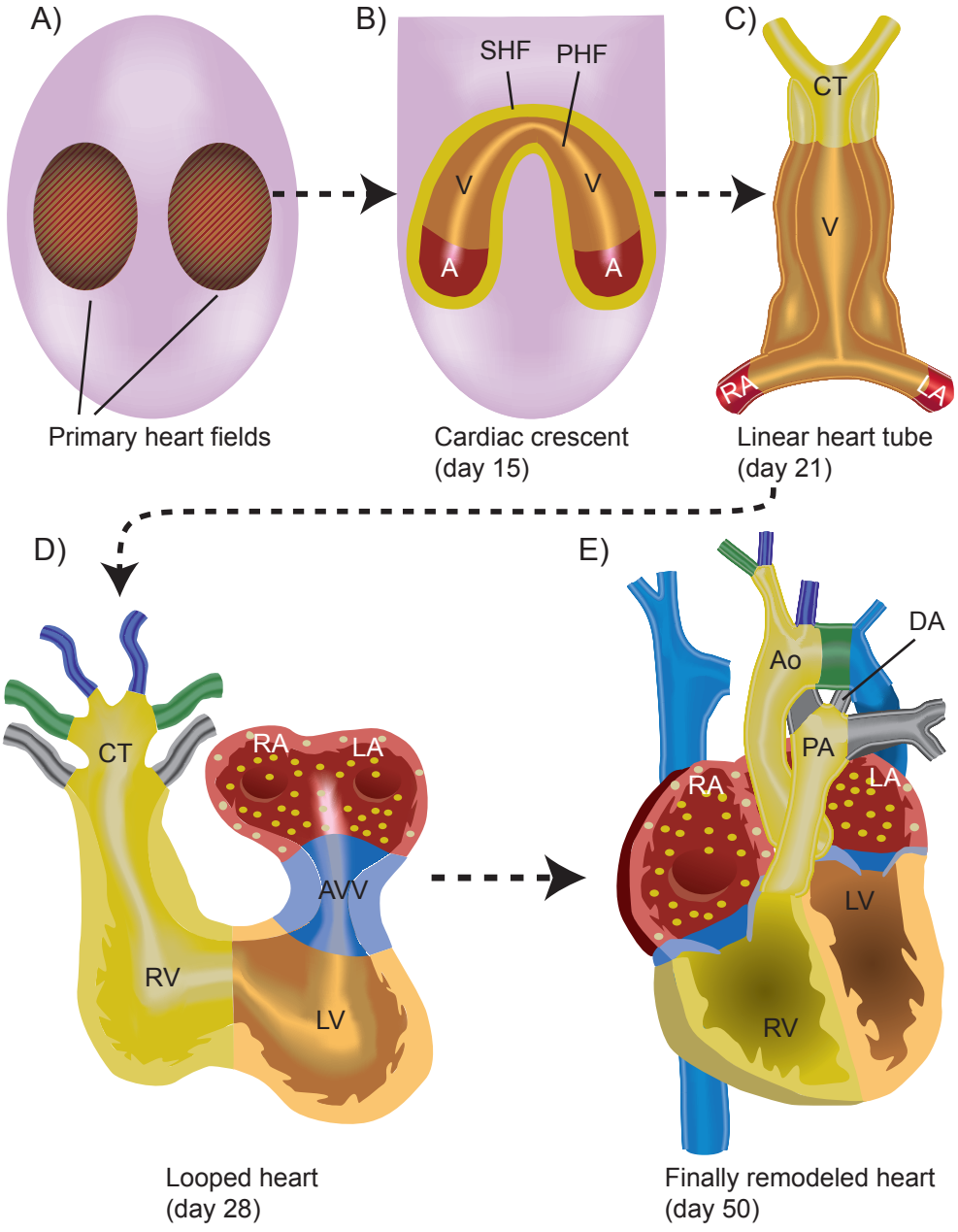


Figure 3 (overleaf). *Schematic illustration of human cardiac embryonic development. Related regions are color coded. Contribution of secondary heart field progenitors are shown according to the results by Cai et al. (35). Approximate embryonic days in human cardiac development are indicated below B - E. A) shows the two primary heart fields that arise shortly after gastrulation. B) shows the cardiac crescent where the two primary heart fields have fused in the middle. C) shows the linear heart tube. D) shows the heart after looping and E) shows the finally remodeled heart.*
SHF, secondary heart field; PHF, primary heart field; V, ventricle; A, atrium; CT, conustruncus; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; AVV, atrioventricular valve; PA, pulmonary artery; Ao, aorta; DA, ductus arteriosus.
Adapted from Srivastava (28) and Frances et al. (29)

The embryological development of the heart is a very complex process involving the development of several cell types as well as the formation of a complicated 3-D structure. It is schematically illustrated in Figure 3.

Mammalian embryonic development starts with the specification of cells into inner cell mass and trophoblasts. The later give rise to the placenta whereas the inner cell mass give rise to the embryo as well as amniotic cavity. Through gastrulation, the three germ layers, ectoderm, mesoderm and endoderm, are formed (26). The heart is developed from the mesodermal layer. During and shortly after gastrulation, a part of the mesoderm becomes specified to cardiac development and is called cardiac mesoderm. The cardiac mesoderm is bilaterally distributed on both sides of the primitive streak, which is situated in the midline of the embryo (Figure 3A). The embryonic disc then folds and the laterally separated areas of cardiac mesoderm migrate inwards, fuse in the middle and form the cardiac crescent (Figure 3B). This later develops into the cardiac tube (27-29) (Figure 3C). This process is completed at about embryonic day 7 in the mouse and day 21 in the human, shortly after which the heart starts beating (30). The heart tube then undergoes a process called looping, through which atrias and ventricles are formed (Figure 3D). In the end of this process, trabeculations of the luminal surface of the heart start to form. The function of these may be to enable the myocardium to grow in mass without a fully developed coronary circulation (31). The remodeling of the heart is complete at about embryonic day 13.5 in the mouse and day 50 in the human (28, 29) (Figure 3E). The embryonic heart remain functional throughout this extensive remodeling (32).

The mesodermal cell population that forms the heart tube is called the primary heart field (33). The ion channel HCN4 has been described as a specific marker for this population (34). In addition to this, another population of cells contributing to the cardiac development, situated anterior of the primary heart field has been identified. Called the secondary or anterior heart field, this population was originally believed to contribute to the right ventricle and outflow tract of the heart (33). Later studies

have suggested transcription factor Islet-1 as marker of the secondary heart field (35-37). In a knock out mouse model, it was shown that mice lacking Islet-1 expression developed severe cardiac malformations and failed to enter the looping phase. By genetic lineage tracing, it was estimated that most of the heart including almost all of the outflow tract and the right ventricle, approximately 2/3 of the atria and minor portions of the left ventricle were formed from secondary heart field progenitors (35) (this is graphically illustrated in Figure 3). However, in another study where a mef2c enhancer region specific for the secondary heart field was used for lineage tracing, a more limited contribution was found. It was speculated that Islet-1 might also be expressed earlier in the embryonic development and thus also give rise to some of the primary heart field in studies of lineage tracing (38). Notably, Islet-1 expression has also been found in the post-natal heart (36) and has been suggested as potential marker of adult cardiac progenitor cells (39). This will be discussed in further detail in section 1.6.3.

1.3 An introduction to stem cells

Stem cells are defined as undifferentiated cells capable of proliferation, self-renewal, production of a large number of undifferentiated progeny and regeneration of tissue (40). Stem cells are further categorized by their differentiation capabilities. Pluripotent stem cells theoretically have the ability to generate all types of cells from all three germ layers of embryonic development. Generally, only embryonic stem (ES) cells have been thought to be pluripotent. ES cells are *in vitro* expanded cells derived from the inner cell mass cells of early embryonic development (41). A few years ago, it was however also shown that adult terminally differentiated cells such as fibroblasts could be reprogrammed to a phenotype with differentiation capacity similar to that of ES cells. This was done by transfection with certain stem cell specific genes *in vitro*, resulting in overexpression of these genes. These transfected cells, called induced pluripotent stem cells (iPS cells), were shown similarly to ES cells to have the capacity to differentiate into types of cells derived from all three germ layers (42). While iPS cells display impressive properties in terms of differentiation potential and may in the future have clinical applications, it should be pointed out that they are not believed to exist *in vivo* but represents an artificial type of cell created *in vitro*.

In contrast to ES and iPS cells, stem cells in the adult have been regarded as tissue specific and multipotent – restricted in their differentiative potential to only a few types of cells.

Adult stem cells are thought to produce daughter progenitor / transient amplifying cells by asymmetric cell division. These cells are in its turn capable of fast proliferation and differentiation (43, 44). This is perhaps best characterized in the hematopoietic system, where several different stem / progenitor populations have been characterized as well as a developmental hierarchy (44, 45). Other examples of

adult stem cells are intestinal crypt stem cells renewing the intestinal epithelium (43), epidermal stem cells generating keratinocytes in the skin (46), satellite stem cells capable of repairing skeletal muscle (47) and mesenchymal stem cells (MSCs) in the bone marrow, at least capable of differentiating into bone, fat and cartilage (48). It should be observed that the terms ”stem cells” and ”progenitor cells” are not used stringently in the field of cardiac regeneration but are often used as interchangeably with each other. In this thesis, the term ”progenitor cells” will generally be used to indicate cells with uni- or multipotent differentiation capacity within the heart.

In recent years there has been a lot of debate regarding the plasticity of adult stem cells. There have been several reports indicating that some populations of adult stem cells, in particular stem cells derived from the bone marrow, may have a brooder differentiation capability than what was previously believed (49, 50). This will be discussed further in the context of cardiomyocyte regeneration from extra-cardiac stem cells. Please see section 1.5.

1.4 The concept of the heart as a regenerative organ

During most of the 20th century, the heart was predominantly viewed as a post-mitotic organ, soon after birth loosing the ability to generate new cardiomyocytes. This dogma was supported by the apparent lack of regeneration after acute myocardial infarction, as well as the inability to observe dividing cardiomyocytes in histological sections in studies conducted in the beginning of the 20th century (51). Furthermore, a rather constant number of cardiomyocytes throughout life was noted by Linzbach et al. (52) in the 1950s. This was interpreted as further proof of hypertrophy rather than hyperplasia of cardiomyocytes as the mechanism by which the heart grow and adapts to physiological stress. It has also been observed that adult cardiomyocytes fail to divide when cultured *in vitro* (53).

In the end of the 20th century this dogma started to become questioned. Studies of DNA synthesis in mammalian cardiomyocytes had shown that there might be a low degree of DNA synthesis in adult cardiomyocytes (54), possibly indicating that new cardiomyocytes are generated. Furthermore, studies of expression of the proliferation marker Ki67 (55) showed expression in a small fraction of human adult cardiomyocytes (56, 57) further strengthening this interpretation. Expression of Ki67 in cardiomyocytes was markedly upregulated after myocardial infarction (56) and tachycardia induced heart failure (57) respectively. This could be viewed as an attempt to regeneration, although inadequate.

Another indirect evidence of cardiomyocyte renewal were studies of cardiomyocyte death by apoptosis or necrosis in the failing heart (58, 59). It has been argued that the rate of cardiomyocyte death is much greater than what would be expected from the actual loss of cardiac mass. The obvious solution to this paradox that have been

suggested is the existence of a simultaneous cardiomyocyte renewal (60).

1.4.1 Direct assessment of cardiomyocyte renewal

In animal models, assessment of newly formed cells including cardiomyocytes have been conducted through administration of 5-bromo-2-deoxyuridine (BrdU). This substance works as a analogue to the DNA base thymidine and is incorporated instead of this during cell division. Thus, cell nuclei containing high levels of BrdU must have formed during the labeling period. Waring et al. (61) showed that the rat heart responded to physical exercise by both increased size of already existing cardiomyocytes but also by increase in the number of small cardiomyocytes. This group of cardiomyocytes was found to incorporate BrdU, indicating that these cells were newly formed. The extent of formation of new cardiomyocytes was correlated to training intensity. In the high intensity exercise group, about 7% of the total number of cardiomyocytes was determined to have formed during a 4 week period of training compared to below 2% in the control group. In a study by Urbanek et al. (62) of BrdU incorporation in the normal mouse heart, as high as between 10 - 19% of all cardiomyocytes (depending on which region of the heart that was assessed) were determined to have formed during a period of 10 weeks. These results indicate a surprisingly high turnover of cardiomyocytes, especially if taking into account that in the study of Urbanek et al., BrdU was only administered for the first 6 days of the study whereas in the study by Waring et al., BrdU was administered continuously during the whole study period. One reason for this discrepancy could be differences in cardiomyocyte turnover between rats and mice. However, in a study by Meinhardt et al (63), mice were administered BrdU continuously for 14 days with a subsequent chase period of 4 months (~16 weeks). After this period, BrdU retention in different types of cells were investigated by immunohistochemistry. While BrdU staining rarely was observed in endothelial cells, no incorporation of BrdU in cardiomyocytes was observed. Instead, most BrdU+ cells stained positive for progenitor marker stem cell antigen 1 (Sca-1). Although it could be argued that Meinhardt et al. used a less advanced method of microscopic analysis, it seams unlikely that such a substantial turnover of cardiomyocytes as above 10% of the total cardiomyocyte population would be missed. The reasons for the different results of cardiomyocyte turnover between the studies of BrdU incorporation thus remain unclear.

In the human heart, methods utilizing BrdU incorporation for identification of cycling cells have not been possible due to ethical considerations. However, in a study of Kajstura et al. (64), autopsy samples from the hearts of patients that had received radiosensitizer IdU as a part of cancer treatment was analyzed. IdU, similarly to BrdU, also works as a nucleotide analogue and is incorporated in newly synthesized DNA during cell replication. In this study, on average 22% of the pool of cardiomyocytes was calculated to be replaced in one year although a quite high inter-patient variation was noted. Similarly to the study by Waring et al.

(61) described above, newly formed cardiomyocytes was mostly mono-nucleated excluding the possibility of these cells being the result of karyokinesis rather than cytokinesis.

Another method of determining cardiomyocyte turnover in human, which is not dependent on a certain treatment or category of patients, is analysis of ^{14}C content in isolated cardiomyocyte nuclei. This analysis make use of the fact that during atmospheric atomic bomb tests during the 50's and beginning of the 60's, $^{14}CO2$ concentration in the atmosphere rose sharply. The level of $^{14}CO2$ is in its turn in equilibrium with ^{14}C content in food which is in equilibrium with ^{14}C content in newly synthesized DNA at that particular time (14). In the first study of human cardiomyocyte ^{14}C incorporation by Bergman et al. (14), it was shown that new cardiomyocyte are formed throughout life. The rate of turnover was however modest and negatively correlated with age. It was estimated that at the age of 50, about 45% of all cardiomyocytes had formed after birth. Furthermore, it was noted that although most cardiomyocyte nuclei were diploid at birth, most became polyploid during the first ten years of life. After this, no further increase in polyploidization was observed. These changes were compensated for in the calculations of cardiomyocyte turnover described above.

To the contrary of the study by Bergman et al., a later study by Kajstura et al. (65) found a dramatically higher turnover of cardiomyocytes both in the normal and diseased heart. It was calculated that between the age of 20 and age of 78, the cardiomyocyte compartment of the heart was turned over approximately 8 times. A similar turnover rate was estimated for endothelial cells and fibroblast. Furthermore, cardiomyocyte turnover was found to accelerate at old age rather than decrease. The reasons behind these great differences in results are unclear. When reading the study by Kajstura et al., it however seems unclear how they have come to their conclusions based on the ^{14}C data presented. The study by Bergman et al. on the other hand has a clear line of reasoning and although it has be acknowledged that the technique is very complicated, it is possible to understand how their conclusions are generated from the underlying data. Taken together, the study by Bergman et al. seems more methodologically robust. Furthermore, the results are much more reasonable in the light of the well established very limited regenerative capacity of the human heart in cardiac disease.

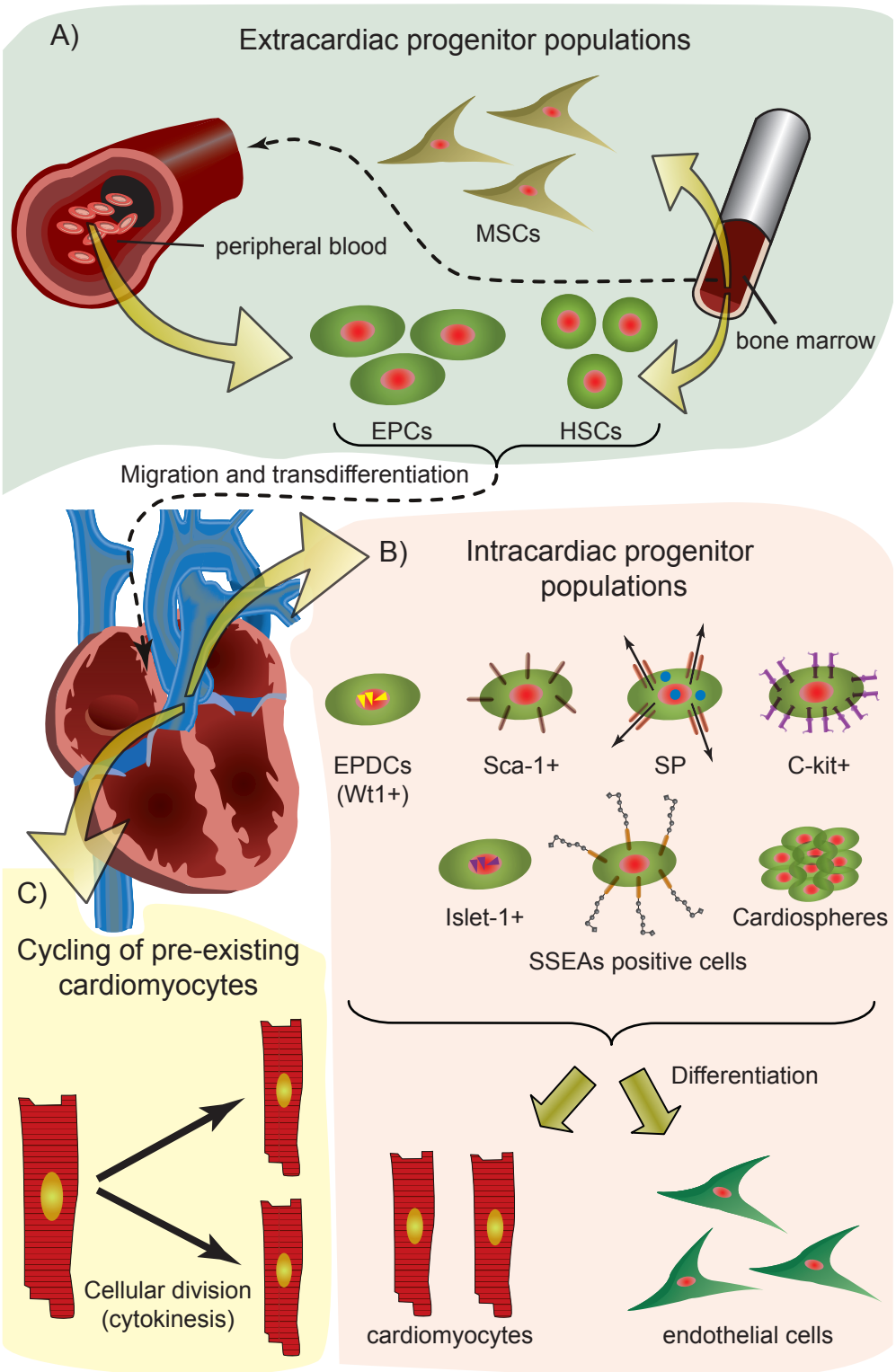

A)
Extracardiac progenitor populations
peripheral blood
MSCs
bone marrow
EPCs
HSCs
Migration and transdifferentiation
B)
Intracardiac progenitor populations
EPDCs (Wt1+)
Sca-1+
SP
C-kit+
Islet-1+
Cardiospheres
SSEAs positive cells
Differentiation
cardiomyocytes
endothelial cells
C)
Cycling of pre-existing cardiomyocytes
Cellular division (cytokinesis)

Figure 4 (overleaf). *Schematic illustration of different sources of cardiac regeneration that have been proposed in the literature. A) shows different cell populations derived from the bone marrow or peripheral blood. Both EPCs (endothelial progenitor cells) and HSCs (hematopoietic progenitor cells) have been proposed to be able to migrate to the heart and transdifferentiate into cardiomyocytes. MSCs (mesenchymal stem cells) have been used in the setting of cell therapy but are not believed to be able to migrate through the blood circulation. EPCs are believed to be at least partially derived from the bone marrow, which is indicated by the dashed arrow.*
B) shows different populations of cells identified within the cardiac tissue that have been shown to be able to differentiate into both cardiomyocytes or endothelial cells in vitro and / or in vivo. C) illustrates regeneration of cardiomyocytes through re-entry into the cell cycle of pre-existing cardiomyocytes.
EPDCs, Epicardium derived progenitor cells.

1.5 Extracardiac stem cells as the source of cardiac regeneration

Since both direct and indirect evidence points toward a regeneration of all types of cells composing the heart, the logical following question is how this regeneration takes place. Theoretically, this can be achieved by either the migration of a population of cells outside the heart via the blood circulation that in the heart differentiates into the different cell types composing the heart, or by a regenerative process confined to the heart. In this section, studies investigating contribution of external cell sources to cardiac regeneration will be discussed. These are schematically outlined in Figure 4A.

1.5.1 Hematopoietic stem cells as the source of cardiac regeneration

Just after the turn of the century, there were several reports describing the possibility of hematopoietic stem cells being able to transdifferentiate into cardiomyocytes. Orlic et al. (50) showed that mouse bone marrow derived C-kit+ lineage negative cells, when injected into the myocardium after myocardial infarction, were able to differentiate into cardiomycytes, endothelial cells and smooth muscle cells. Just 9 days after cell transplantation, on average 68% of the infarcted region was occupied by newly formed cardiomyocytes derived from the injected cells. In another study by the same group, bone marrow cells were instead mobilized by subcutaneous injections with cytokines (stem cell factor, SCF and granulocyte colony-stimulating factor, G-CSF) before and shortly after myocardial infarction. This resulted in mobilization of C-kit+ lineage negative cells into the blood, regeneration of 76% of the infarction area by functional myocardium and substantial improvements in cardiac function as measured by ultrasonic cardiography (66).
A few years after these very promising studies of differentiation capacity of hematopoietic stem cells, there were however several studies published which all failed to reproduce these initial results. In a study by Murry et al. (67), two different cardiac-restricted reporter strains of mice were used to asses differentiation capacity

of isolated hematopoietic stem cells including C-kit+ lineage negative cells. Evidence of cardiomyogenic differentiation of these hematopoietic stem cells could not be detected in a single mouse of the total of 145 mice that received cell transplantation. This, regardless of whether mice had first been subjected to myocardial infarction or not. Similarly, in a study by Balsam et al. (68) where different populations of bone marrow stem cells (including C-kit+ lineage negative cells), were isolated from GFP expressing animals and injected in the peri-infarction area, no evidence of transdifferentiation of these cells into cardiomyocytes could be observed. To the contrary, these cells adopted mature hematopoietic fates. A minor improvement of cardiac function was noted in the treatment groups, however, this was only evident after 6 weeks.

The reason behind this discrepancy between the different studies is not clear. It has however been suggested that in the original study by Orlic et al. (50), autofluorescence in the myocardium may wrongly have been interpreted as GFP positivity and as evidence of transdifferentiation of the transplanted cells (67). Notably, several studies of bone marrow transplantation assessing the contribution of marrow derived cells to the formation of new cardiomyocytes, show a minor but detectable contribution. This has been shown both in animal models (69, 70) and in studies of human female patients that have received a bone marrow transplant from a male donor (70). The mechanism behind these rare events of marrow derived cardiomyocytes have however been shown to be cell fusion with already existing mature cardiomyocytes rather than transdifferentiation (71). Cell fusion has also been shown to take place infrequently when hematopoietic cells were injected into the infarcted myocardium (72) and may thus be another possible contributing factor to the results by Orlic et al. (50). It is however far to infrequent to account for all of the massive regeneration reported in that study.

1.5.2 Other beneficial effects of hematopoietic stem cells not related to regeneration of cardiomyocytes

Although hematopoietic stem cells do not seem to be able to differentiate into cardiomyocytes to any substantial degree, studies in both animal models (68, 73-75) as well as clinical studies (76, 77) (which will be discussed in further detail in section 1.8) have observed beneficial effects of these cells when injected after myocardial infarction. Several possible mechanisms have been suggested. First of all, there are some evidence that the injected cells may transdifferentiate into endothelial cells and thus improve perfusion of the heart (78). As for cardiomyogenic differentiation, other studies have however failed to observe this (68, 75). Furthermore, long term engraftment of injected hematopoietic cells into the heart have been shown to be minimal (75, 79) and thus seems unlikely as the cause of functional improvement. Other studies have instead focused on paracrine signaling as the mechanism of action. It has been shown that bone marrow derived cells secrete cyokines that have pro-angiogenic (73, 78, 80), anti-inflammatory (74) and anti-apoptotic effects (80).

Furthermore, when supernatants of cultured bone marrow derived cells were injected into the hearts of infarcted animals as well as intraperitoneal for an additional period of time, a short term increase in cardiac function compared to control animals was observed (80).

1.5.3 Mesenchymal stem cells

MSCs, although also described in other tissues, will here refer to cells derived from the bone marrow as this is the most well described source of MSCs and thus most studies have been based on this source of cells. They were discovered during the 60's and 70's and described as a population of non-hematopoietic cells derived from the bone marrow that adhered and grew on ordinary plastic cultureware. It has been well established that they have a differentiation capability into chondrocytes, osteocytes and adipocytes (48). Although later on, several markers have been reported that can be used to prospectively isolate MSCs (81, 82), adherence to plastic is still the most common method of isolation.

In the context of cardiac regeneration, several studies have investigated MSCs as treatment after myocardial infarction. Most of these have shown a reduction in infarction size (83) or improvement of cardiac function (84-88), although notably, there is also one study of human MSCs which did not observe any positive effects of cell treatment (89). The mechanism of action of the transplanted cells is however not clear. It was first suggested that MSCs, similarly as what was proposed for hematopoietic stem cells, differentiated into cardiomyocytes and endothelial cells and by this mechanism attenuated infarction size (83). Several *in vitro* studies have also suggested that MSCs are able to differentiate into cardiomyocytes when co-cultured with cardiomyocytes (90-93). The frequency of differentiation is however only in the range of 3 - 5% of all MSCs (92, 93), although the MSC population may contain sub populations with higher cardiomyogenic differentiation capacity (93). Furthermore, it has been shown that only by co-culture with neontal cardiomyocytes, and not adult, was it possible to induce MSCs to differentiate into the cardiomyogenic lineage (90). This call the relevance of these *in vitro* differentiation data in question since only mature cardiomyocytes may be present in the setting of treatment after myocardial infarction. Notably, there is also one study which could not observe any signs of *in vitro* cardiomyogenic differentiation of human MSCs except minor effects on gene expression (94). As an additional argument against cardiomyogenic differentiation *in vivo* as a major contributing factor to improvement in cardiac function, several studies where MSCs were injected after myocardial infarction only observed a very limited long term retention of these cells in the heart (85, 88, 89).

As for hematopoietic stem cells, other mechanisms not involving transdifferentiation has been suggested for MSCs. It has been shown that human MSCs secrete a number of cytokines including VEGF, hepatocyte growth factor (HGF), leukemia inhibitory

factor (LIF) and IL-6 which may have beneficial effects such as preventing apoptosis and stimulating angiogenesis (85, 95). Consequently, MSCs have been shown to prevent apoptosis of cardiomyocytes both in direct co-culture systems (96) as well as in a system where only culture media from MSCs was evaluated (85). Treatment with MSCs after myocardial infarction have also been associated with increased capillary density (97). Finally MSCs are also well known to have immunomodulatory properties, which has been exploited in the field of hematology to prevent graft versus host disease (98). These properties may also be relevant in the context of treatment after myocardial infarction. It has for example been shown that treatment with MSCs after myocardial infarction resulted in short term increase in the levels of anti-inflammatory cyokine IL-10 gene expression relative to gene expression of other cytokines (87).

1.5.4 The role of Endothelial progenitor cells (EPCs) in cardiac regeneration

Endothelial progenitor cells (EPCs) are circulating cells in the blood with a well established capacity to differentiate into endothelial cells. It has been proposed that EPCs may be subdivided into immature EPCs that have CD133+CD34+FLK-1+ cell surface marker signature and mature EPCs that do not express CD133 but have upregulated markers of mature endothelial cells including VWF and vascular endothelial cadherin (VE-cadherin) (99). It has also been shown that there is a population of circulating CD34 negative cells positive for monocyte associated antigen CD14 which have the capacity to differentiate into endothelial cells when cultured *in vitro* (100). Whether there is a hierarchical link between this population and the CD34+ populations described above is however unclear. It should be noted that in addition to definitions of EPC identity based on cell surface markers, many studies have instead used a culture based definition where EPCs are defined as adherent growing cells from cultures of peripheral blood (101-103).

The origin of EPCs is not entirely clear. While many reviews of the EPC system suggest a model where EPCs are thought to be derived from the bone marrow (99, 104), there is also a study by Lin et al. (105) where human patients that had received gender mismatched bone marrow transplantations were examined for EPC origin. In this study, it was shown that 95% of freshly isolated EPCs were derived from the recipient. However, in EPCs that were expanded *in vitro*, a progressively higher fraction of cells were derived from the donor with increased time in culture. At day 27 in culture, approximately 83% of the cells were derived from the donor. No attempt was made to correlate these two apparently different populations to different cell surface signatures. The *in vivo* contribution of bone marrow derived EPCs to vascular homeostasis has been further illustrated by Brittmann et al. (106) In this study, autopsy material from patients that had undergone gender mismatched bone marrow transplantation were analyzed. Donor derived cells were found in the

capillary bed and co-expressed endothelial marker CD31.

Clinically, EPCs have attracted much attention as a potential marker in different settings of cardiovascular disease. For example, EPC number has been found to increase in patients with acute ischemic heart disease (101, 107) as well as in patients with heart failure due to dilated cardiomyopathy (108). When it comes to cardiac regeneration, as previously described for both hematopoietic stem cells and MSCs, several studies have indicated a positive effect after myocardial infarction of either EPC transplantation (109, 110) or increased mobilization and recruitment (111). It was initially suggested that EPCs might improve cardiac function by differentiation into cardiomyocytes. In a study by Badorff et al. (112) human EPCs were shown to differentiate into cardiomyocytes *in vitro* when co-cultured with neonatal rat cardiomyocytes. It was further shown that direct cell-cell contact was necessary to obtain differentiation. In a later study by Gruh et al. (113), the results by Badorff et al. were however not possible to reproduce. Furthermore, it was extensively shown that this discrepancy most likely was the results of methodological shortcomings in the study by Badordd et al. These involved failure of identifying autofluorescence of cardiomyocytes in differentiation analyses by FACS and incorrectly interpreting EPCs laying on top of neonatal cardiomyocytes as a sign of differentiation in immunohistochemical analyses. Furthermore, it has also been shown that EPCs rarely migrate over the endothelial barrier of blood vessels (114), which further speak against cardiomyogenic differentiation of EPCs *in vivo*. Other possible mechanisms of action of EPC treatment after myocardial infarction involve formation of new capillary vessels (111) and secretion of paracrine factors such as IGF-1 and transforming growth factor beta-1 (TGF-β1) (110). It has indeed been shown that only conditioned media from cultured EPCs, without cells, gave positive effects of cardiac function when injected intramyocardial after myocardial infarction (110, 115). In one of these studies, improvement was linked to prevention of apoptosis in cardiomyocytes, which in its turn was coupled to IGF-1 secretion (115).

1.5.5 Studies of chimerism in gender mismatched cardiac transplantation

Although both hematopoietic stem cells, MSCs and EPCs have been found to have no or very limited capacity of differentiation into the cardiomyogenic lineage, it would be theoretically possible that another extracardiac progenitor population exists that contribute to renewal of cardiac cells including cardiomyocytes under physiological conditions. To fully elucidate the contribution of extracardiac cells in a population independent way, several studies have investigated biopsy and autopsy material from patients that have undergone gender mismatched cardiac transplantations. Generally, male patients that have received female hearts have been used. All cells that have a male origin in the heart (identified by the existence of a Y-chromosome

by fluorescence in situ hybridization) are assumed to be derived from the recipient of the cardiac transplant. This technique was first demonstrated by Hruban et al. (116). In this initial study, two hearts that had been explanted in the process of re-transplantation due to accelerated arteriosclerosis were analyzed. Most of the donor derived cells within the heart were found to be infiltrating mature hematopoietic cells. No chimerism was detected in cardiomyocytes or smooth muscle cells, whereas a minor contribution of donor derived cells to the endothelial lineage was noted. In contrast to these initial results, in a study by Quaini et al. (117), a high percentage of donor derived cardiomyocytes was noted. When corrected for efficiency of Y-chromosome detection, on average 18% of the cardiomyocytes were found to be donor derived. A high degree of chimerism was also noted in endothelial cells and smooth muscle cells. Notably, at about the same period of time, two studies showing a high regenerative capacity of bone marrow derived stem cells were also published (50, 66) (described in further detail in section 1.5.1). This together generated a high interest for the contribution of extracardiac cells to cardiac regeneration. Consequently, several other studies were initiated exploring incorporation of recipient derived cells in gender mismatched cardiac transplantation. In a study by Glaser et al. (118), published shortly after the study by Quaini et al., over 6000 cardiomyocyte nuclei were examined for Y-chromosome content which would be indicative of extracardiac origin. However, not a single Y-chromosome positive nucleus could be identified. In contrast to this, a low level of chimerism in smooth muscle cells was noted. In another study by Laflamme et al. (119), which was also published shortly after the study by Quaini et al., chimeric cardiomyocytes were observed. The frequency of these, when adjusted for efficiency of Y-chromosome detection sensitivity, was however only on average 0.04% of all cardiomycoytes. It was argued that the source of this discrepancy most likely was not differences in clinical parameters of the included patients but rather technical differences. In the study by Quaini et al., confocal microscopy was used to identify chimeric cardiomycoytes whereas in the study by Laflamme, standard light microscopy was used. It was proposed that in the study by Quaini et al., infiltrating leukocyte nuclei superimposed on top of a cardiomyocyte nuclei may have incorrectly been interpreted as a chimeric cardiomyocytes. Notably, in a later study by the same group where contribution of recipient derived cells to non-cardiomyogenic lineages in the heart including endothelial cells and smooth muscle cells were investigated, a much higher degree of chimerism was noted. For endothelial cells, about 24.3% of the cells were found to be chimeric whereas the percentage of chimeric smooth muscle cells were 3.4% (120). These levels were noted to be in the same range as in the study by Quaini et al. (117). After the initial studies by Glaser et al. (118) and Laflamme et al. (119), several other groups have also reported data from studies of chimerism after gender mismatched cardiac transplantation. In these studies, levels of chimerism in cardiomyocytes range from virtually no chimeric cardiomycoytes to a maximum of 1 - 2% (121-124). Notably, although stringent criteria were used

to identify true chimeric cardiomyocytes, in one of these studies where a subset of apparent chimeric cardiomyocyte nuclei were subjected to a detailed analysis with Z-stack confocal microscopy, it was noted that most of these cells were in fact artifacts due to superimposed leukocytes (122).

Taken together, studies of gender mismatched cardiac transplantation show that while extracardiac cells make a substantial contribution to the vasculature of the heart, there seems to be very little or no contribution to the cardiomyocgenic lineage. It should finally also be noted that in most cases of cardiac transplantations, minor portions of atrial tissue of the heart of the recipient is left (119). It can thus not be excluded that the possible minor contribution of recipient cells to renewal of cardiomyocytes originates from cells that migrate out of this tissue rather than extracardiac sources.

1.6 Intracardiac progenitor cells as a source of cardiac regeneration

During the last 10 years, a number of putative progenitor populations have been identified in the adult heart (summarized in Figure 4B). Different methods have been used for identification and isolation of these populations, including culture systems regarded to be selective for stem cells, functional assays and cell surface markers. Notably, of the markers and assays that have been used, several have originally been used to identify stem cells in the hematopoietic system.

1.6.1 C-kit

C-kit is a tyrosine kinase receptor, well characterized in the hematopoietic system as a marker for hematopoietic stem cells. Upon differentiation into the different lineages of blood, these cells lose their expression of C-kit, with the exception of the mast cell lineage (125). It was one of the first markers used to identify stem cells in the adult heart. The first report of an intracardiac population of C-kit+ cells was published in 2003 by Beltrami et al. (126). In this, a population of C-kit+ cells negative for hematopoietic lineage markers was identified in the rat heart. These cells were shown to be able to differentiate into all three major cell lineages of the heart - cardiomyocytes, endothelial cells and smooth muscle cells. This was shown both *in vitro* and *in vivo*, in an infarction model. Furthermore, the cell population was shown to be clonogenic, i.e. one cell could give rise to a progeny of cells that were able to differentiate into all lineages of the heart. C-kit+ cells with similar characteristics have also successfully been isolated from cardiac tissue of other animals including mice (127, 128) and dogs (129).

1.6.1.1 C-kit as a marker for human cardiac progenitor cells

In the human heart, C-kit+ cells were first identified by immunohistochemistry in tissue samples from the outflow tract. Parts of these cells expressed markers indicative of early cardiac commitment, such as GATA-4 and MEF2 (130). C-kit+ cells isolated from human cardiac tissue samples were found to be clonogenic and to have the ability to differentiate into cardiomyocytes, endothelial cells and smooth muscle cells. They were also found to express telomerase, which is a prerequisite for long term high proliferative capacity. When human C-kit+ cells were injected in mouse or rat hearts after induction of myocardial infarction, the human cells differentiated into cardiomyocytes and formed capillary vessels (131). It should be mentioned that in paper I and II in this thesis, the isolation and characterization of human cardiac C-kit+ cells are described. To the contrary of the results described above, we observed a predominant endothelial profile of the C-kit+CD45- population. This is described in further details in the results and discussion sections of this thesis.

1.6.1.2 Location of C-kit+ progenitor cells

The distribution of C-kit+ cells has been thoroughly investigated in the mouse heart. C-kit+ cells were present as nests of cells in atria, base-mid region and apex. These nests of cells had the characteristics of stem cell niches, consisting of extracellular matrix proteins such as laminins and fibronectin. They contained both uncommitted C-kit+ cells and C-kit+ cells with signs of early lineage commitment. The frequency of stem cell niches were reported to be 8-fold higher in apex and atria compared to the base-midregion of the ventricles (62). In the human adult heart, C-kit+ cells have been described as most numerous in the left atrium and right ventricle. In contrast to the mouse heart, C-kit+ cells were significantly less common in the apex. Furthermore, most of the human C-kit+ cells were found within the epicardial layer of the heart (132). When cells were expanded as monolayer culture from different locations of the adult heart, C-kit+ cells could be isolated from cultures of all four chambers of the heart. The number of C-kit+ cells in cultures was highest from right atrium. Between other locations, there were no significant differences (133). Distribution of C-kit+ cells have also been determined in the young human heart, in patients suffering from dilated cardiomyopathy. In this study, samples from all 4 chambers of the heart were compared with each other. Right atrium had by far the highest percentage of C-kit+ cells, followed by right ventricle, left ventricle and left atrium (134).

1.6.1.3 Subpopulations of C-kit+ progenitor cells

The human cardiac C-kit+ progenitor population has been subdivided based on expression of the VEGF receptor FLK-1 and insulin-like growth factor (IGF) receptors respectively. C-kit+FLK-1+ cells were found to be present predominantly in the epicardiac layer of the heart in vicinity of coronary arteries, arterioles and

capillaries. C-kit+FLK-1- cells on the other hand were found within the myocardium. When differentiation capacity *in vitro* was investigated, C-kit+FLK-1+ cells mostly gave rise to endothelial cells and smooth muscle cells. C-kit+FLK-1- cells on the other hand mostly gave rise to cardiomyocytes. The capacity of human C-kit+FLK-1+ cells to form functional vessels was further investigated in a dog model of critical stenosis of the left anterior descending artery. C-kit+FLK-1+ cells were infused in vicinity of the stenosis and formed new collateral vessels around the stenosis area (135). C-kit+FLK-1+ cells have also been isolated from endocardial biopsies, although from this sampling location, only a minor percentage of the cultured C-kit+ population was found to co-express FLK-1 (136).

The C-kit+ population has also been subdivided based on expression of IGF 1 and 2 receptors (IGF-1R, IGF-2R). Only IGF-1R+ cells were found to respond to stimulation with IGF 1 and 2 (IGF-1, IGF-2) with increased proliferation as well as increased differentiation into cardiomyocytes. On the other hand, activation of the C-kit+IGF-2R+ population with IGF-2, especially if combined with stimulation with agiotensine II, resulted in increased apotosis. It was suggested that this may be a mechanism by which homeostasis of the intracardiac C-kit+ population is kept (137).

1.6.1.4 Effects of cardiac disease on the C-kit+ cardiac population

A few studies have investigated how disease affects the distribution, frequency and to lesser degree function of C-kit+ stem cells. In the human heart, the first study of C-kit+ cells in disease by Urbanek et al. (130) investigated frequency of C-kit+ cells in patients with aortic stenosis. Theses patients generally have increases left ventricular pressure which may induce a concentric hypertophy of the left ventricle. It was observed that in tissue samples from the outflow tract, the number of C-kit+ cells was increased 8 - 19 fold compared to control hearts. Furthermore, evidence of increased proliferation within the C-kit+ population was detected in the disease group by increased expression of Ki67 and telomerase. In another study, frequency of C-kit+ cells in end stage heart failure was investigated by flow cytometry. Compared to control hearts, failing hearts contained significantly higher number of C-kit+ cells. The cause of heart failure however did not affect the frequency of cells. The C-kit+ cells were characterized as CD45dim (138). As discussed in further detail in section 1.6.1.6, this population may however represent mast cells rather than true C-kit+ progenitor cells. In expanded cells from cardiac biopsies, from patients undergoing cardiac surgery of various reasons, frequency of C-kit+ cells were correlated to several clinical characteristics. Of these, only hypertension significantly increased the number of C-kit+ cells. Factors such as medication, smoking, left ventricular hypertension, pulmonar hypertension valvular disease and impaired left ventricular function had on the other hand no effect on the number of C-kit+ cells. This is in contrast to the report by Urbanek et al (130) where aortic

stenosis was found to dramatically increase the number of C-kit+ cells. In this study however, tissue sections were used for quantification. Since a primary monolayer culture system may increase the percentage of C-kit+ cells by several order of magnitudes by itself, the final percentage of C-kit+ cells may not accurately reflect the original percentage of C-kit+ cells. This could in its turn explain the discrepancy between the studies.

In animal models, the role of C-kit+ cardiac stem cells have recently been investigated in a beta-adrenergic overload model where administration of isoproterenol induced a diffuse subendocardial and apical cardiomyocyte death as well as heart failure. This model has similarities with the Takotsubo cardiomyopathy. Interestingly, it was shown that upon this diffuse myocardial damage, C-kit+ endogenous stem cells were activated and were able to restore the cardiac function through regeneration of cardiomyocytes (139). This is notably in contrast to damage caused by an ischemia in a myocardial infarction, where no significant regeneration takes place. It could be speculated that from an evolutionary point of view, there has been a need to protect the individual from stress induced cardiac damage whereas this has not been the case for ischemic heart disease which often occurs at high age after the reproductive period.

1.6.1.5 Functional aspects of the C-kit receptor

The C-kit receptor is activated by binding of either soluble or membrane bound SCF. This results in dimerization of the receptor and activation of its intrinsic tyrosine kinase activity. C-kit activation have been linked to activation of several downstream targets including the Ras / Erk and the PI3 / Akt pathways (140).

There are a few studies which have investigated functional aspects of C-kit and SCF in the context of myocardial regeneration. Mice with defect C-kit signaling were shown to develop heart failure at old age (12 months). This was accompanied with a decrease in the number of C-kit+ cells residing in the heart. Histologically, hypertrophy of cardiomyocytes was observed as well as lower vascular density. It was hypothesized that this cardiomyopathy state might be caused by the reduced number of resident C-kit+ cells or impairment of the function of the resident C-kit+ cells (141). In contrast to this, when mice of 8 weeks of age with defect C-kit signaling were subjected to pressure overload, defects in C-kit signaling were on the one hand found to increase the proliferative capacity of cardiomyocytes as well as the number of C-kit+ stem cells. On the other hand, differentiation of these cells into cardiomyocytes were impaired (142). This discrepancy may be explained by that different strains of mice have been used. Furthermore, effects of defect C-kit signaling may change with increased age.
Effects of SCF have been investigated in the context of myocardial infarction. Overexpression of SCF within the heart resulted in improved cardiac function and

decreased fibrosis. This was associated with increased number of C-kit+ cells within the heart as well as decreased apoptosis of cardiomyocytes (143, 144).

1.6.1.6 Cardiac mast cells also express C-kit and may inadvertently have been interpreted as progenitor cells

C-kit+ cells either directly isolated from cardiac tissue samples or isolated from primary cell cultures derived from tissue samples have mostly been described as negative for hematopoietic lineage markers (126, 131, 133). A dim expression of pan-hematopoietic marker CD45 has however also been reported in most of the C-kit+ cells directly isolated from human biopsy material (138). Although it was suggested that these C-kit+CD45dim cells actually represented cells with progenitor properties, an alternative explanation could be that they in fact represented contaminating cardiac mast cells. Mast cells are hematopoietic cells with a well established role in acute and chronic allergic reactions. They are known to reside in a number of tissues, including the heart, have a C-kit+CD45dim signature and express several specific markers intracellularly such as tryptase and chymase. The percentage of mast cells in a cell suspension of dissociated atrial tissue was determined to be in the range of 0.5 - 1.5%. Similar preparations from ventricular tissue however contained less than 0.1% mast cells (145). In tissue sections from human cardiac biopsies, co-localization of C-kit with tryptase as well as CD45 showed that 85-100% of all C-kit+ cells had in fact a mast cells identity (146, 147). This doesn't exclude the existence of a small population of non-mast cell C-kit+ progenitors, but it calls for caution when interpreting results from studies where directly isolated cells or tissues sections have been used. This is of particular importance when investigating how disease effects the C-kit+ population since mast cells also have been shown to be affected by cardiac disease. For example, the number of mast cells was shown to be correlated to the degree of fibrosis in patients with idopathic dilated cardiomyopathy (148). Furthermore, when myocardial infarction was induced in rats, mast cell number greatly increased (149). Interestingly, mast cells may also be relevant from a therapeutic point of view since infusion of mast cell granulae post myocardiac infarction have shown to have a cardioprotective effect (150). It should be pointed out that although mast cell contamination may have been a factor in studies looking at C-kit+ cells in tissue sections or directly isolated cells, mast cells generally have a limited culture capacity and are not believed to be able to differentiate into other types of cells. Thus, mast cell contamination is improbable in studies where cultured C-kit+ cells have been used and been induced to differentiate.

1.6.2 Sca-1

Sca-1 is a member of the Ly-6 protein family. It is known as a marker for bone marrow stem cells in the mouse that give rise to the thymic subset (151). In the first report about a cardiac Sca-1+ population, dissociated mouse cardiac tissue was shown to consist of approximately 14 - 17% Sca-1+ cells. These cells were negative

for hematopoietic markers as well as most endothelial markers. When cultured *in vitro*, they were shown to be able to up-regulate cardiomyocyte specific proteins upon treatment of cytosine analog 5-Azacytidine. *In vivo* differentiation into cardiomyocytes of injected Sca-1+ cells in the context of myocardial infarction were also confirmed. Notably, about half of the differentiated cells were determined to be caused by cell fusion with pre-existing cardiomyocytes (152). Further differentiation of Sca-1+ cells into fully competent beating cardiomyocyte like cells was induced by treatment with the hormone oxytocin. It should however be noted that this process was quite inefficient with only about 1% of the cells exhibiting spontaneous beating (153). In another study where differentiation capacity of mouse Sca-1+CD31- cardiac cells were assessed, a limited degree of endothelial differentiation was observed in addition to differentiation into cardiomyocytes (154).

1.6.2.1 Sca-1+ cells in the human heart

Although the Sca-1 protein has been well characterized in the mouse, currently no human homologue exists although some human gene products have shown limited sequence homology to the Ly-6 protein family (151). When expanded cells from single cell cloning of dissociated human cardiac tissue were investigated for expression of stem cell antigens, they however surprisingly showed reactivity with an antibody against the mouse Sca-1 protein. When Sca-1+ cells were instead prospectively isolated from human fetal or adult cardiac tissue, they showed similar morphology compared to the cells isolated by single cell cloning. Upon treatment with 5-Azacytidine, ascorbic acid and TGF-β1, cells differentiated into cardiomyocytes and exhibited spontaneous beating (155). Histologically, Sca-1+ cells have been identified within the atrium, the intra-atrial septum, the atrial - ventricular junction as well as scattered within the epicardial layer of the human fetal heart. The human cardiac Sca-1+ population was negative for hematopoietic markers (156).

When human fetal and adult Sca-1+ cells were compared with each other, cells derived from both stages of age were able to differentiate into cardiomyocytes as well as endothelial cells and smooth muscle cells. Fetal cells differentiated into cardiomyocytes however exhibited spontaneous beating to a greater extent compared to adult cells whereas adult cells showed a more mature electrophysiological profile. In terms of endothelial and smooth muscle differentiation, fetal Sca-1+ cells tended to generate more endothelial cells relative smooth muscle cells whereas the opposite was true for adult cells. Furthermore, fetal cells showed evidence of a broader differentiation capacity as these cells could also differentiate into adipocytes (157).

1.6.3 Islet-1

Islet-1 is a transcription factor involved in cardiac development and has been described as a marker for the secondary heart field progenitors (35-37). Its role in

this context is described in further detail in section 1.2. Its expression and function in the adult heart is however more controversial. In the early post-natal rat heart, Islet-1+ cells were observed in the outflow tract, at the junction between outflow tract and ventricular tissue as well as a few scattered cells within the right atrium. Most of these cells showed evidence of cardiac differentation by co-expression of cTnT while only a minor percentage were cycling as determined by proliferation marker Ki67. In the adult heart, scattered Islet-1+ cells were observed in the outflow tract, co-expressing cTnT (36). By using a conditional genetic marker technique, Islet-1+ cells have been isolated from primary cultured cells derived from the early post-natal mouse heart. These cells were able to differentiate into cardiomyocytes when co-cultured with neontal cardiomyocytes. No such differentiation was observed when Islet-1 negative cells were tested (39). Expression of Islet-1 has also been found in the human fetal heart. The distribution of these cells within the heart varied depending on the developmental stage (158, 159). Between 11 and 18 weeks of gestation, they were primarily found in the right atrium, outflow tract, left atrial wall and appendage. The number of Islet-1+ cells decreased with increased age of gestation (159). Since Islet-1 is an intracellular marker, this precludes it from being sorted without either intracellular staining,which at the same time kills the cells, or genetic tracing. The later is unfortunately not possible to use when working with human biopsy samples since it require either genetically modified animals or extensive *in vitro* culture of cells. As a consequence of this, differentiation potential of human Islet-1+ cells has only been determined in a model of human embryonic stem cells. In this study, Islet-1+ cells were found to be multipotent and able to differentiate into cardiomyocytes as well as endothelial and smooth muscle cells (159). It should however be noted that when Islet-1+ cells in tissue sections of human fetal hearts were co-stained for cell cycle marker Ki67, only a few Islet-1+ cells expressed this marker (158). This apparent lack of self renewal within the Islet-1+ population *in vivo* in contrast to the embryonic stem cell model could indicate that the embryonic stem cell model not fully corresponds to the *in vivo* situation.

There is currently no data available on Islet-1+ expression in the human adult heart. In 3D cultures of cells derived from the human right atrium, Islet-1 gene expression has however been detected (160, 161). Which cells that are responsible for this expression as well as its functional implications, however remain to be determined.

1.6.4 Stage specific embryonic antigens (SSEAs)

Stage specific embryonic antigens (SSEAs) were originally discovered in the late 70's. They are defined by reactivity of certain monoclonal antibodies. These have later been found to bind to different glycolipids on the cell surface (162, 163). Thus, expression of a certain SSEA does not directly correspond to expression of a certain protein but rather the expression of a certain combination of glycosyltransferases which determine the pattern of glycosylation of lipids. In embryology and ES cell

research, SSEA-1, 3 and 4 have been used to determine differentiation status of cells (164). Importantly, the expression pattern of SSEAs differs between different species. In the mouse, SSEA-1 is expressed by undifferentiated ES cells while SSEA-3 and 4 are not detectable. Upon differentiation, SSEA-1 is downregulated and SSEA-3 and 4 are upregulated. In human ES cells to the contrary, SSEA-3 and 4 are highly expressed by the undifferentiated cells while SSEA-1 is undetectable. When these cells differentiate, SSEA-1 is upregulated while SSEA-3 and 4 are downregulated (164, 165). The physiological functions of the different SSEAs are largely unknown. SSEA-1, which has been studied the most, has been implicated in the compaction phase in early embryonic development (166). Furthermore, overexpression of the glycosyltransferase FUT4, which in its turn increases expression of SSEA-1, in mouse ES cells resulted in enhanced cardiomyocyte differentiation. In human ES cells, inhibition of SSEA-3 and 4 expression have been tested and was found to neither affect pluripotency properties, nor differentiation capacity (167).

In the adult organism, expression of SSEAs is much less known compared to the expression during the embryonic period. Nevertheless, SSEA-4 expression have been described as a marker for human mesenchymal stem cells in the bone marrow (82) as well as a marker for very small embryonic like cells (VSELs) (168, 169). These are circulating cells also derived from the bone marrow which have been described as multipotent stem cells. Outside the bone marrow, SSEA-4 has been identified in periodontal ligament stem cells (170) as well as in lung cancer where it indicates poor prognosis (171).

1.6.4.1 Expression of SSEAs in the murine and human heart

Cells positive for SSEAs were first described in the rat heart by Ott et al. (172). In this study, SSEA-1+ cells were detected both in the neonatal and in the adult heart. When expanded in an *in vitro* system, SSEA-1+ cells grew in suspension and were able to differentiate into both cardiomyocytes, endothelial cells and smooth muscle cells. Differentiation into cardiomyocytes was observed spontaneously in primary cultures but could be further enhanced by co-culture with neontal rat cardiomyocytes. When injected after myocardial infarction, differentiation into these lineages were also shown *in vivo*. No expression of SSEA-4 was found within the rat heart.

In the human heart, expression of SSEA-4 has been investigated in tissue sections of fetal and neonatal hearts. While scattered SSEA-4+ cells were readily identified in both the atrial and ventricular myocardium in the fetal heart, only a minor portion of the neonatal specimens contained a SSEA-4+ population (173). In the adult heart, SSEA-3 and 4 positive cells have been identified in tissue sections in vessel structures as well as individual cells within the myocardium. These cells were found to co-express endothelial marker CD31. These cells were however not characterized further (174). In addition to this, in paper IV in this thesis, SSEA-1, 3 and 4 positive

cells were characterized and isolated from human atrial biopsy material. For further details, please see the result and discussion sections.

1.6.5 Side Population

The Side Population (SP) assay was originally developed in 1996 by Goodell et al. (175) for identification of hematopoietic stem cells. SP cells were defined as cells capable of excluding the fluorescent dye Hoechst 33342. When such cells were isolated from the bone marrow, they were found to have high potency in repopulation assays, which is a hallmark for hematopoietic stem cells. After this initial study, SP cells with progenitor properties have been identified in various tissues including the limbus area of the eye (176), the aorta (177) and in the airway epithelium (178). The ability of cells to exclude Hoechst 33342 has been attributed to the expression of membrane bound ABC transporter proteins. Of these, multidrug resistance protein 1 (MDR1) and ATP-binding cassette sub-family G member 2 (ABCG2) are the most well known. In most studies, one of these proteins have been liked to the SP phenotype. In addition to excluding Hoechst 33342, these proteins have also been shown to transport a number of drugs out of the cell, including several cytostatic drugs. They are frequently over expressed in cancer cells where they contribute to drug resistance (179, 180).

1.6.5.1 Properties of SP cells in the murine heart

In the heart, a small population of SP cells was first described in a mouse model by Hierlihy et al (181). In this initial study, these cells were described as negative for other hematopoietic stem cell markers such as Sca-1 and C-kit. In contrast to this, Pfister et al. (182) showed that most of the cardiac SP population in the mouse heart was positive for Sca-1 and could be further subdivided based on expression of endothelial marker CD31. Although most of the SP population consisted of CD31+ cells, when sorted for *in vitro* expansion, only the Sca-1+CD31- subpopulation grew in culture. This population could be induced to differentiate into functional cardiomyocytes by co-culture with adult cardiomyocytes. *In vitro* differentiation of rat cardiac SP cells into cardiomyocytes has also been accomplished by treatment with oxytocine or TSA. Furthermore, these SP cells could also be induced to differentiate into the adipogenic and osteogenic lineages (183). It should however be noted that in this study, neonatal SP cells were used, which may have a broader differentiation potential compared to cells isolated from adult animals. Furthermore, in this study, only a minor portion of the SP population was CD31+. This could potentially be due to differences between rats and mice.

Although the mouse SP Sca-1+CD31+ population was initially not studied because of culture difficulties, these problems have later been overcome. In a study of this population by Liang et al. (184), it was shown that this population had properties of endothelial progenitor cells with the ability to form capillary structures both *in vitro*

and *in vivo* after injection into the infarcted heart.

The *in vivo* differentiation potential of cardiac SP cells has been investigated in an infarction model. When SP cells isolated from neonatal rat hearts were injected in the tail vein of adult animals with myocardial infarction, they were able to home to the infarction area and differentiate into both cardiomyocytes and endothelial cells. Interestingly, when SP cells were administered to non-infarcted animals, much lower number of the cells homed to the heart and no evidence of differentiation was observed. This indicates that factors in the infarction environment is necessary both for homing of the cells as well as for inducing differentiation (183). In another study, migration of SP cells with Sca-1+CD31- cell surface marker combination derived from adult mouse hearts was investigated after injection into a non-ischemic area of the infracted heart. It was shown that also in this setting, the SP cells were able to migrate into the infarction area and although at a low frequency, were able to differentiate into cardiomyocytes and endothelial cells. Based on up regulation of Stromal cell-derived factor 1 alpha (SDF-1α) within the infarction area and *in vitro* migration studies, it was proposed that the SDF-1α / CXCR4 chemotaxis system plays a role in migration of cardiac SP cells (185).

1.6.5.2 Effects of developmental stage on frequency and ABC transporter protein profile of cardiac SP cells

In several studies, it has been observed that the frequency of SP cells is affected by developmental stage. The highest percentages of SP cells have been observed in the fetal heart (183). In the murine heart, frequency of SP cells then gradually declines with increased age during early post-natal time until adult age (183, 186, 187). In contrast, when SP frequency in adult mice was analyzed, it was found that the percentage of SP cells was higher in mice of old age (188). Interestingly, the same phenomenon has been observed for the C-kit+ population. When frequency of C-kit+ cells was measured in human biopsy material, it was found to decrease in the age span of early post-natal time to adolescence (134). When patients in the age interval between 19 to 104 years of age were studied however, there was a correlation between increased number of C-kit+ cells and increased age (189). This indicates that the limited capacity of regeneration upon injury of the old heart is not attributed to a decrease of cardiac progenitor populations.

The dynamic expression of the ABC transporter proteins mdr1 and abcg2 in the cardiac SP population has been extensively investigated in mouse models where mdr1 and abcg2 respectively were knocked out. It was shown that while abcg2 was responsible for the SP phenotype in the neonatal mouse, the significance of mdr1 expression for the SP phenotype gradually increased with increased age. At the same time, the significance of abcg2 decreased. In the adult mouse, mdr1 was determined to be the dominant efflux protein in the SP population (187).

1.6.5.3 Functional significance of ABC transporter proteins

The physiological function of the ABC transporter proteins in progenitor populations including the cardiac has to a large extent been unknown. Both mdr1 (190) and abcg2 (191) nock out mice display normal fertility, growth and phenotype in laboratory housing conditions. Interestingly, nock out of abcg2 resulted in high sensitivity to the phototoxin pheophorbide which may be present in food based on vegetables (191), indicating an evolutionary value in protecting the organism from normally occurring toxins. Although the extrusion of pheophorbide may not be directly linked to expression of ABC transporter proteins in progenitor cells, it would be plausible that the expression in these cells could have an evolutionary value in directly protecting them from various naturally occurring toxins.

Recently, a few studies have also directly investigated the role of ABC transporters in the function of cardiac SP cells. All of these have been focused on the abcg2 protein, there is currently no studies available that have investigated the functional importance of mdr1 expression for the cardiac SP population. In a study by Phister et al (187), it was shown that knock out of abcg2 resulted in impaired proliferative capacity in the cardiac SP population (isolated from adult animals) whereas overexpression resulted in increased proliferative capacity but on the other hand impaired cardiomyogenic differentiation capability. Furthermore, it was shown that lack of abcg2 expression rendered the SP cells more susceptible to oxidative stress. The protective effects of abcg2 expression on oxidative stress have also been demonstrated by overexpression of the protein in embryonic fibroblasts (192). In a recent study where the role of abcg2 for proliferation of SP cells was investigated in more detail, it was shown that lack of abcg2 expression resulted in inability of the cells to progress from G1 to S phase. Furthermore, it was shown that silencing of the abcg2 gene in SP cells resulted in a shift in cell division from primarily symmetric cell division to asymmetric cell division (193). This may explain earlier findings of impaired cardiac differentiation capability when abcg2 was overexpressed since differentiation is generally believed to be a result of asymmetric cell division (44). The role of abcg2 has finally also been investigated in the context of myocardial infarction. In this study, it was clearly observed that mice deficient of abcg2 function had a much lower survival rate compared to control mice. This was linked to impaired survival of cardiac microvascular endothelial cells. In these, lack of functional abcg2 resulted in increased oxidative stress which was caused by accumulation of protoporphyrin X (194). It should however be noted that in this study, it was not assessed whether these abcg2 expression endothelial cells was equivalent to SP cells identified by efflux of Hoechst 33342.

1.6.5.4 SP cells in the human heart

In the human heart, there are only a few studies that have investigated the occurrence of SP cells. In a study by Marah et al (195), SP cells were identified in the human

neonatal heart. In this study, no attempt was made to sort this population. Thus its properties remains to be determined. Notably, the SP population was sensitive to treatment with Fumitremorgin C (FTC) which has been described as selective for the ABCG2 transporter protein (196, 197). This indicates that ABCG2 is the dominant efflux protein also in the human neonatal cardiac SP population. In the human adult heart, the existence of a SP population has currently only been described by our group. Data from this study are presented in paper III in this thesis, for further details please see the results and discussion sections. In addition to this study, there are also two studies which have indentified ABCG2 expression in the human adult heart by immunohistochemistry (174, 198). In this context, it should however be pointed out that SP is defined by the function of excluding Hoechst 33342 rather than expression of certain ABC transporter proteins alone. Since no attempt was made to correlate the ABCG2+ cells with efflux function, those identified cells should not be regarded as SP cells.

1.6.6 Identification of stem cells based on culture system - Cardiospheres

In addition to isolation and identification of cardiac progenitor populations based on expression of cell surface markers or functional assays, several studies have instead utilized culture systems regarded as selective for progenitor cells. This was first described in 2004 by Messina et al (199). In this study, both mouse and human cardiac tissue were investigated. From cultures of mildly dissociated cardiac tissue, loosely adherent, phase bright cells could repeatedly be harvested. These cells were able to form sphere like structures (often named cardiospheres) that grew either loosely adherent or in suspension. The spheres were able to differentiate into functional cardiomyocytes either spontaneously (if derived from mouse hearts) or through co-culture with mature cardiomyocytes (if derived from human hearts). This technique was later on further optimized, and was shown to also be able to generate cardiospeheres from small endomyocardial biopsies of the human adult heart (200). In this study, the regenerative potential of these cells was also investigated *in vivo* in an infarction model. It was shown that cardiospheres improved cardiac function and was superior to fibroblasts as well as vehicle (phosphate buffered saline, PBS) injection. By immunostaining, differentiation of the cardiospheres into cardiomyocytes and endothelial cells was observed. Cardiospheres have also successfully been generated from the early post-natal and adolescent human heart. Although these inefficiently differentiated into cardiomyocytes *in vitro*, they similarly to the cardiospheres generated from adult hearts improved cardiac function *in vivo* in an infarction model and showed evidence of cardiomyogenic differentiation in this context (134).

In the studies described above, no direct comparison was done between cardiospheres derived from adult and neontal human hearts. This was investigated in further detail

by Simpson et al (201). It was found that although cardiospheres could successfully be generated from both neonatal and adult hearts, cardiospheres dervied from neonatal hearts showed higher efficiency in cardiac differentiation *in vitro*. Notably, the differentiation efficiency for both neonatal and adult derived cardiospheres was quite low with only a few percent of the cells differentiating into cardiomyocytes. In contrast to previous results (200), when cardiospheres in the study by Simpson et al. were injected *in vivo* in an infarction model, only those cardiospheres derived from neonatal hearts improved cardiac function. Furthermore, *in vivo* differentiation into cardiomyocytes was only observed in neonatal cardiospheres.

1.6.6.1 Criticism of the cardiosphere system

Several studies have noted a considerable heterogeneity within cardiospheres, with cells staining positive for both stem cell markers like C-kit and Islet-1 (200, 201) as well as markers for fibroblasts (202, 203), smooth muscle cells (202), endothelial cells (200, 202) cardiomyocytes (201, 202) and mesenchymal cells (134, 200, 202). Furthermore, the cellular composition varies considerably between different studies. For example, while some studies have found that either no or just a few percent of the cells in cardiospheres express C-kit (202-204), others have found levels up to about 20 percent (200). Furthermore, it seems like the percentage of C-kit+ cells is higher if cardiospheres were generated from neontal hearts compared to adolescent or adult hearts (134, 201). Although it has been argued that the mix of different cells in a cardiosphere may function as a 3D niche for the cardiac stem cells (201), the heterogeneity of the system makes it hard to correlate which cells *in vivo* that actually form the cardiospheres. More importantly, as there is no separation of different cell types when cells are detached from the primary outgrowth of biopsies to form cardiospheres, these could potentially be contaminated by mature types of cells. These could then be detected in differentiation assays and interpreted as a result of a differentiation process. This hypothesis was thoroughly investigated by Andersen et al. (205) in the murine heart. In this study, cardiospheres were found to partly consist of contaminating explant tissue. If cells used for cardiosphere generation were filtered, expression of cardiomyogenic proteins as well as spontaneous beating were abolished. To the contrary to a previous study which suggested a clonal origin of cardiospheres (199), cardiospheres were found to be formed in a process of cellular aggregation. Furthermore, when cardiospheres derived from filtered cells were co-cultured with rat cardiomyocytes, no signs of cardiac differentiation was observed. In this study, those phase bright cells referred to in many studies as migrating out from cultured explants were characterized as contaminating CD45+ hematopoietic cells. These also made up a part of the final cardiospheres. In this regard, it should be pointed out that other studies have not detected CD45 expression in cardiospheres (200, 203). Additionally, in a study on cardiospheres generated from biopsy material from patients that had undergone cardiac transplantation, it was concluded that cardiospheres were not derived from extracardiac sources such as contaminating

blood cells (206). The reasons for this discrepancy is unclear.

1.6.6.2 Effects of cardiospheres unrelated to differentiation

Although the cardiosphere system, as described above, has been criticized for being heterogeneous and possibly be a result of contaminating mature tissue fragments, several studies have found beneficial effects of transplantation of cardiospheres after myocardial infarction (134, 200, 207, 208). Although differentiation of cardiospheres into functional cardiac tissue may contribute to these effects, it has also been suggested that paracrine signaling may play a role. It has been shown that cardiospheres secrete several cytokines including VEGF, HGF and IGFs, both *in vitro* and when transplanted *in vivo* after myocardial infarction. *In vitro*, paracrine signalling from cardiospheres was shown mediate both anti apoptotic effects on cardiomyocytes and stimulate capillary formation of endothelial cells (209). Furthermore, diluted conditioned media from cardiosphere cultures has been shown to increase contractility of cardiomyocytes *in vitro* and prevented cardiomyocyte contractile dysfunction associated with angiotensine II treatment (204). These effects, if also present *in vivo*, could potentially enhance the contractility of the remaining cardiomyocytes after a myocardial infarction.

1.6.7 Epicardium derived progenitor cells

Epicardium derived progenitor cells have been described as a population of cells derived from the proepicardium and epicardium during embryonic development. These cells undergo an epithelial to mesenchymal transition and can differentiate into cardiomyocytes, endothelial and smooth muscle cells (210, 211). Fate mapping have suggested transcription factors Tbx18 (211) and Wilms tumor protein (Wt1) (210) respectively as markers for these embryonic progenitor populations. The developmental potential of these cells was found to be determined by the embryonic developmental stage. Only Tbx18+ cells in the early embryo were able to form cardiomyocytes whereas Tbx18+ cells later in embryonic development were restricted to endothelial and smooth muscle cell development (211).

In the adult heart, expression of Wt1 mRNA expression was restricted to the epicardium covering atrioventricular sulcus and apex. No expression was found in myocardial or endocardial layers of the heart (212). Upon induction of myocardial infarction, a transient upregulation of Wt1 expression in the epicardium bordering the infarction zone was noted (212-214). By lineage tracing, these cells were found to regenerate the epicardial layer in the infarction area. Whereas one study also found a small population of Wt1 derived cardiomyocytes after infarction (212), others could not detect this in animals with myocardial infarction that had not been subjected to treatment (214). Several studies have investigated the possibility of augmenting the activation of epicardium derived progenitors after myocardial infarction by treatment of thymosin beta 4. This is monomer binding peptide involved in normal

heart development where it affects growth of the heart, vasculogenesis and epicardial development (215). Treatment with thymosin beta 4 after myocardial infarction has been shown to improve cardiac function (216) and resulted in upregulation of Wt1+ cells in the epicardium (214, 217). Whether the beneficial effects of tymosin beta 4 treatment on cardiac function are attributed to activation of a Wt1+ progenitor population is however unclear. While two studies showed differentiation into cardiomyocytes of Wt1+ cells (214, 217), others could only detect fibroblast and myofibroblast / smooth muscle cell differentiation in the Wt1 progeny (215). This is in agreement with the results of Huang et al. (213) which showed that inhibition of C/EBP, the transcription factor responsible for upregulation of Wt1 after myocardial infarction, resulted in decreased myocardial fibrosis and improved cardiac function.

1.6.8 Other putative progenitor populations in the adult heart.

In addition to the different cardiac progenitor populations described earlier in this sections, there are also a few additional populations that have not yet received as much attention. These will be briefly reviewed in this section.

1.6.8.1 Telocytes

By Hinescu et al. (218), a population of interstitial cells with similarities to Cajal cells of the intestine was identified in the human atrium. These cells were identified based on ultrastructural properties with electron microscopy and described as cells with long very thin processes with a small cell body. In later studies, these cells have been named “Telocytes” (219). These cells have been found to form heterocellular junctions with other types of cells including cardiomyocytes (220). By immunohistochemistry, Telocytes have been characterized as CD34+SB100+C-kit+ (219). Thus it seems unclear whether this population is distinct from the population of C-kit+ cardiac progenitor cells described by other groups (126, 139), since these cells have not been characterized ultrastructurally.

1.6.8.2 Nestin+ progenitorcells

A population of cells positive for the neural progenitor marker Nestin was first detected after myocardial infarction, only in the infarction area. These cells have been associated with neural and oligodendrocyte differentiation (221) as well as angiogenesis in the infarction area (222).

1.6.8.3 Aldehyde dehydrogenase bright cells

Aldehyde dehydrogenase is a cytosolic enzyme involved in oxidation of aldehydes. It is highly expressed in a number of different stem cell populations and have been shown to mediate resistance to certain cytostatic drugs in hematopoietic cells. A method for isolation of cells expressing high levels of aldehyde dehydrogenase was first developed in the hematopoietic field. These cells were characterized as

hematopoietic stem cells (223). Recently, a population of cells expressing high levels of aldehyde dehydrogenase has also been found in the human heart. These cells were distinct from the previously described C-kit+ population but expressed Islet-1. When sorted and co-cultured with neonatal rat cardiomyocytes, the cells expressing high levels of aldehyde dehydrogenase differentiated into functional cardiomyocytes. The cardiomyogenic differentiation was found to be superior of that of cardiosphere derived cells (224).

1.7 Division of pre-existing cardiomyocytes as a mechanism of cardiac regeneration

In previous sections, the possibilities of a contribution of either extra or intracardiac stem / progenitor cells to cardiac regeneration have been discussed. Principally however, there is also a third mechanism by which new cardiomyocytes may be formed. This is by de-differentiation and re-entry into the cell cycle of already terminally differentiated cardiomyocytes (outlined in Figure 4C). This may in some respects seem more intuitive since it is well established that DNA synthesis in cardiomyocytes may occur postnatally (14, 54). Although this mostly results in either multinucleation or popyploizidation, it would seem plausible that this in some cases also results in a complete cellular division.

1.7.1 Cardiac regeneration by de-differentiation of pre-existing cardiomyocytes in the zebrafish

The teleost fish zebrafish is well known for its much greater regenerative capacity compared to mammals. It has been shown that the zebrafish is able to regenerate structures such as fins, optic nerve and spinal cord upon injury (225). In 2002, it was shown by Poss et al. (226) that the adult Zebrafish also had a remarkable ability to regenerate cardiac tissue. When approximately 20% of the apical region of the ventricle was surgically removed, this tissue was fully regenerated after just 60 days. This ability has later been shown to be present during the whole life of the zebrafish, and was not attenuated by old age (227). In contrast to the mammalian heart, minimal amount of scarring was observed in the study by Poss et al. Based on the BrdU incorporation pattern in cardiomyocytes after injury, it was proposed that the regeneration was accomplished by re-entry into the cell cycle of already existing cardiomyocytes. Most later studies using transgenic zebrafish models to trace the origin of newly formed cardiomyocytes upon injury confirm this hypothesis (228, 229). Interestingly, it was noted by electronic microscopy that this process involved ultrastructural changes of these pre-existing cardiomyocytes including disorganization of the sarcomeric structure (228). It should however also be noted that the literature is not entirely clear as there is also one study of genetical tracing that indicate a contribution of epicardial undifferentiated progenitors to the regenerative process (230).

In human, damage to the heart is infrequently caused by direct trauma / amputation of tissue but rather by ischemia. This was mimicked in a zebrafish model of hypoxia / reoxygenation by Parente et al. (231). In this study, it was shown that this treatment similar to the mammalian heart, caused oxidative stress, an inflammatory response and myocardial death by apoptosis and necrosis. In contrast to the mammalian heart however, this was followed by proliferation of cardiomyocytes without any detectable fibrosis. This was accompanied by recovery of the cardiac function. Interestingly, proliferation of cardiomyocytes in the zebrafish has also been observed in a model of long term exercise (232). On the other hand, no signs of hypertrophy was observed which would have been the principal response in the mammalian heart (233). It thus seems that the principal response of the zebrafish heart, not only in the setting of traumatic amputation but also in other contexts, is that of proliferation of cardiomyocytes.

1.7.2 Evidence of cardiac differentiation by re-entry into the cell cycle of pre-existing cardiomyocytes in the mammalian heart

Although it is obvious that certain animals such as the zebrafish posses a great capacity for cardiac regeneration, this is obviously not the case for mammals including human. However, in a study by Porello et al. (234), it was shown that this is not true for the neonatal heart. When a part of the left ventricular apex was surgically resected in day 1 old mice, a complete regeneration was observed within 21 days. By genetical lineage tracing as well as histological observations, it was concluded that this regeneration was due to de-differentiation and re-entry into the cell cycle of pre-existing cardiomyocytes. Notably, the time window of regneration was narrow since a similar surgical resection in 7 days old mice did not result in any regeneration but rather the formation of scare tissue. A complete regeneration in the neontal heart has also been observed in a sheep model where the injury was caused by ischemia rather than trauma. While no cardiac regeneration was observed in adult sheep, neonatal sheep displayed evidence of cardiomyocyte renewal determined by BrdU incorporation as well as no deterioration in cardiac function (235).

In another study by Senyo et al. (236), the origin and rate of the formation of new cardiomyocytes was measured by a combination of genetical lineage tracing and multiple-isotope imaging mass spectrometry in mice. By this approach, it was determined that a slow physiological regeneration of cardiomyocytes takes place throughout life although the rate of regeneration declines with increased age. Furthermore, it was determined that physiological regeneration was caused by replication of pre-existing cardiomyocytes rather than differentiation of progenitor cells. Furthermore, it was shown that the rate of regeneration increased after myocardial infarction. In this context, it would also be possible with a contribution from a progenitor population to the observed cardiomyocyte renewal.

In the human heart, few modern studies have investigated cardiomyocyte renewal by cellular division of pre-existing cardiomyocytes. In a study by Mollova et al. (13), it was however shown by an image analysis based strategy that mitotic cardiomyocytes existed throughout life although it decreased with increased age. Direct evidence of cytokinesis in cardiomyocytes was observed in samples from patients <20 years of age but was undetectable in patients of older age. Furthermore, it was noted that in age range of 1 - 20 years of age, the heart grow both by increased size of existing cardiomyocytes as well as increased number of cardiomyocytes (3.4 fold increase).

1.8 Clinical studies

During the past decade, a number of clinical studies have been initiated to test safety as well as efficacy of transplantation of various adult cell populations to treat ischemic heart disease (both acute and stable) as well as heart failure. Most of the initial studies were small non-randomized safety studies with relatively short time of follow up (237). However, as will be discussed later in this section, there are now also data available from randomized trials with longer follow up times. This section aims to give a short summary of clinical results from the most well studied cell populations.

1.8.1 Skeletal myoblasts

The first type of cell to be tested clinically in cardiac disease was the skeletal myoblast. In the first case study by Menasche et al. (238), one patient who suffered from advanced heart failure due to ischemic heart disease was given intramyocardial injections with expanded autologous skeletal myoblast during coronary artery bypass graft (CABG) surgery. After this, several studies have been conducted, most of which have been small and carried out without randomization or even control groups (239). Notably in one of the few studies where randomization was employed and a proper control group included, primary endpoints of increase in global left ventricular function was not reached. For those patients receiving the highest dose of cells, a reversion of adverse left ventricular remodeling was however noted (240). From a safety point, skeletal myoblasts have raised concerns of increased risk of serious arrhythmias including sustained ventricular tachycardia and ventricular fibrillation. As a consequence of this, in more recent trials, patients have as a precaution received implantable cardioverter-defibrillators (ICDs) (239). The mechanism of action for transplanted myoblasts is not clear. It has been suggested that theses cells may form functional electrophysiological junctions with cardiomyocytes (241). However, in animal studies, long term engraftment has been poor (242) which argues against this mechanism. Paracrine effects of the injected cells may be another way by which skeletal myoblasts can have positive effects on the cardiac function (243).

1.8.2 Bone marrow derived cells

A large number of clinical studies have investigated beneficial treatment effects of bone marrow derived cells in cardiac disease. Different populations of cells have been used. Although most studies have used relatively heterogeneous populations of bone marrow derived mononuclear cells (BMNCs) (77), studies of purified populations of CD34+ (244, 245) or CD133+ (246, 247) cells as well as cultured MSCs (248, 249) have also been reported. Most studies have used autologous cells, but for MSCs there are also studies on allogeneic cells (249, 250). In these studies, no adverse effects of signs of rejection were noted. One explanation to this could be the immunomodulatory properties of MSCs that previously have been described in the context of bone marrow transplantation (98).

It should also be noted that different routes of administration have been tried. While some of the studies on bone marrow derived cells have used direct injection of cells into the myocardium from either the epicardial side during CABG surgery (251), or from the endocardial side via a catheter based delivery system (252), most studies have used intracoronary infusion of cells (77). Notably, the retention of bone marrow mononuclear cells has been determined to be very low regardless of mode of delivery. In a study in pig, it was shown that by direct intramyocardial injection, which produced the highest retention, only 11% of the cells were retained in the heart. For the intracoronary route, only about 3% of the cells ended up in the heart (253). A similar degree of short term retention for the intracoronary route was also demonstrated in human where radiolabeled cells were traced non-invasively. Notably, retention was markedly better for purified CD34+ bone marrow derived cells (in the range of 14 - 39%) which indicate better homing of this population of cells (254).

Most studies using bone marrow derived cells have focused on treatment after acute myocardial infarction, although there are also studies that have investigated treatment effects in chronic ischemia as well as heart failure (255). In a recent Cochrane meta-analysis by Clifford et al. (77), results from all randomized trials investigating effects of bone marrow derived cells on treatment of acute myocardial infarction were summarized. In this study, 33 trials with a total of 1765 participants were included. A high degree of heterogeneity between the included trials of both clinical and statistical nature was observed. No statistically significant effects of treatment were observed for either mortality or morbidity. In contrast to this, a moderate and statistically significant effect on cardiac function measured by left ventricular ejection fraction (LVEF) was observed both short term (< 12 months) and long term (12 - 61 months). Furthermore no difference in adverse events between treatment and controls were noted, except for trials where G-CSF had been administered to mobilize bone marrow derived progenitor cells to the peripheral blood for harvest. These events were described as related to G-CSF administration and resolved when

G-CSF administration was discontinued.

For other indications than acute myocardial infraction, considerable fewer trials have been carried out and no meta-analyses exists. For refractory angina pectoris, catheter based infusion of CD34+ mobilized bone marrow cells has been tried. It was shown that treatment compared to placebo reduced both symptoms of angina pectoris as well as the consumption of nitroglycerine medication (244). In a similar study where instead the total mononuclear fraction from bone marrow aspirates was used for cell transplantation, symptomatic improvements were also reported. Furthermore, a minor but significant improvement in LVEF was noted (256). For heart failure, there have also been few studies conducted, and most of them have been small and non randomized. In studies where BMNCs have been administered by intramyocardial injection during CABG surgery, no or very small positive effects have been observed except in one study where purified CD133+ cells were used (257). In this study by Stamm et. al. (247), a moderate increase LVEF in the cell treatment group compared to CABG only was noted at 6 months follow up. Furthermore, it was shown by positron emission tomography - computed tomography (PET-CT) that cell treatment increased perfusion of the heart. Finally, there is one study by Assmis et al. (258) where BMNCs were compared with circulation derived mononuclear cells in a cross-over design of 75 patients suffering from heart failure. Both cell populations were administered intracoronary. At 3 months follow up, patients that had received BMNCs showed a moderate improvement in LVEF compared to control. No improvement was observed in the group of patients that had received circulating mononuclear cells. Similar results were noted in the crossover part of the study.

1.8.3 Cardiac derived stem cells

There are today two clinical studies that have investigated treatment effects of *in vitro* expanded cardiac derived stem cells. Both are phase 1 trials and thus of limited size with the primary objective to establish safety of the cell transplantation procedure. In the SCIPIO trial (259), patients with LVEF < 40% that had experienced a previous myocardial infarction and were planned for CABG surgery were included. The study was designed as partly open labeled, partly randomized. During CABG, atrial biopsies were harvested and from these, cells were expanded from which C-kit+ cells in its turn were isolated. Patients that still 3-4 months after CABG surgery had a LVEF < 40% received autologous C-kit+ cells via intracoronary infusion. In the treatment group, a progressive increase in LVEF was noted with time. At 4 months, the absolute increase in LVEF was an average 8.2 LVEF percent points whereas at 12 months it had increased 12.3 percent points. In contrast, no increase in LVEF was noted in the control group. A part of the patients that had received cell treatment was subjected to detailed analysis of infarction size by cardiac magnetic resonance (CMR) imaging (260). In this, it was shown that at 12 months follow up point, infarction size was reduced by approximately 30 or 40%, depending on which

method of calculation that was used. Concurrently, viable LV mass increased by an average 21.5% at 12 months of follow up. From a safety standpoint, no adverse events was noted in the treatment group.

In the CADUCEUS trial (261), patients were included that recently (< 4 weeks) had experienced a myocardial infarction that had been successfully reperfused and that had a moderate left ventricular dysfunction (LVEF 25 - 45%). The trial was randomized but open label. In the treatment group, endomyocardial biopsies were sampled and from these, cardiosphere derived cells were expanded (see section 1.6.6 for more information regarding cardiospheres). These cells were then delivered intracoronary at the site of previous occlusion. From a safety point of view, seven adverse events were noted in the treatment group. One of these, a non-Q wave myocardial infarction, was determined to be possibly related to the cell treatment procedure. No increase in arrhythmia incidence was noted in the treatment group.

From an efficiency point of view, a reduction in scare size was noted by CMR analysis at both 6 months and 12 months of follow up. At 12 months, patients in the treatment group had an absolute decrease in scare size of 12.3%. To the contrary of this, in the control group, only a small decrease of 2.2% was noted. When viable myocardial mass was measured, a reciprocal increase was noted in the treatment group. In contrast to these results, no differences in LVEF between treatment group and controls were observed neither at 6 months follow up, nor at 12 months (262).

Although both the SCIPIO study and CADUCEUS study overall show promising results, it should be emphasized that both studies have been designed to primarily evaluate safety, are limited in size and open label. Furthermore, controls were not been given a placebo treatment. Thus, to establish treatment effects of expanded cardiac derived stem cells, further studies with proper sizes, randomization and ideally placebo treatment will be needed.

2 AIMS OF THE THESIS

The overall aim of this thesis was to add to our knowledge of endogenous progenitor cells in the human adult heart.

The specific aims were:

- To study the identity and differentiation properties of human cardiac C-kit+ cells, including the development of a suitable culture system and the comparison of directly isolated and cultured C-kit+ cells (addressed in paper I).

- To study heterogeneity within the C-kit+CD45- progenitor population in regard of endothelial, cardiac and stem cell properties on single cell level by using single-cell quantitative real time PCR (single cell qPCR) technique (addressed in paper II).

- To elucidate whether also the human heart contains a population of SP cells and to further characterize these cells in regard of SP phenotype determinant and lineage commitment (addressed in paper III).

- To investigate the expression of SSEAs within the human adult heart and to explore whether these markers may be used to identify cardiac progenitor cells (addressed in paper IV).

- To investigate differences in distribution of C-kit+ cells, SP cells, and cells expressing SSEAs between right and left atrium (addressed in paper II, III and IV).

3 METHODOLOGICAL CONSIDERATIONS

For a detailed description of methods used in this thesis, please see the material and methods sections of each individual paper included in the thesis. The aim of this section is to provide a general discussion of the methods used.

3.1 Ethical considerations

All of the studies have been based on human biopsy samples. Only tissue that was removed as a part of the routine surgical procedure and otherwise would have been discarded was used. Thus, there were no adverse effects associated with participation in the studies. An ethical approval was obtained from the local ethical committee at the University of Gothenburg. In addition, an informed consent was obtained from each individual patient.

3.2 Sources of human cardiac biopsy material

Biopsy material was obtained from the right atrium, from patients undergoing either CABG or valvular replacement surgery at the thoracic surgery unit of Sahlgrenska University Hospital. This material was used in all four papers. From patients undergoing Maze surgery, a surgical procedure to treat atrial fibrillation (263), it was possible to obtain material from both left and right atrium of the heart. Biopsy material from this group of patients was also used in all four papers. It has unfortunately not been possible to obtain tissue from hearts without any cardiac disease. The patients included ranged from 32 to 84 years of age. Patients from both genders were included.

3.3 Isolation of cells

Cardiac biopsies (Figure 5A) were collected in cold PBS during surgery and directly transported to the cell culture lab. Biopsies were washed with additional PBS to remove residual blood. Then, remaining epicardial adipose tissue was removed and biopsies were weighted. Biopsies from patients undergoing Maze surgery were generally much larger (weights in most cases over 1 g) compared to biopsies from patients undergoing surgery of other causes (weights in most cases under 1 g). Tissue material were cut into small pieces (Figure 5B) and dissociated with Liberase type 3 / TM (Roche, Basel, Switzerland) at 37 °C, 4.5h. This dissociation product consists of combination of two highly purified collagenases and a protease, thermolysin. Collagenases work by breaking down collagen fibers of the extracellular matrix. Thermolysin on the other hand break down proteins of the extracellular matrix as well as proteins that attach cells to each other. This is done by hydrolysis of peptide bounds involving hydrophobic amino acids (264). By this initial dissociation, extracellular matrix was removed to a high degree but we observed that cell-

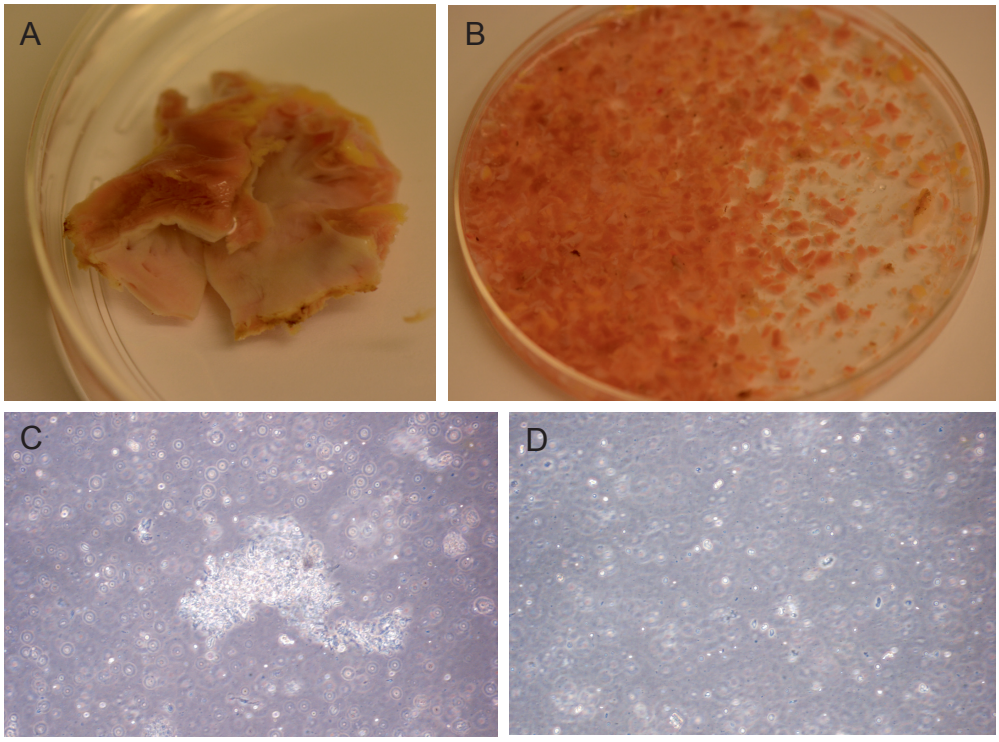


***Figure 5**. A - B shows a representative left atrial biopsy before (A) and after (B) mechanical mincing of the tissue. C - D show an enzymatically dissociated biopsy before (C) and after (D) treatment with trypsin-EDTA. By this treatment, cellular aggregates still remaining after collagenase treatment are broken into single cells.*

cell attachment still remained. To obtain a better single cell suspension to enable subsequent FACS analysis, the dissociated tissue was also incubated with 0.05% Trypsin-EDTA (Life technologies, Carlsbad, CA, USA) for about 10 minutes (Figure 5C - D). Trypsin is also a protease but to the contrary of thermolysin, it breaks down peptide bounds on the carboxyl side of the basic amino acids lysine and arginine. Thus, this proteolytic enzyme is complementary to thermolysin. By this additional incubation, a sufficient single cell suspension was obtained. This cell suspension was filtered through a 100 µm cell strainer to remove residual tissue fragments as well as most cardiomyocytes. The remaining cell suspension was either seeded to monolayer culture (paper I, IV), stained for FACS analysis (paper I, III, IV) or underwent and epitope regeneration treatment prior FACS analysis (paper I, II, IV). Cells that would be subjected to direct FACS analysis were also treated with an erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) to remove residual erythrocytes.

3.4 Epitope regeneration treatment

In paper I, II and IV, an epitope regeneration treatment step was used on directly isolated cells from tissue samples, to enhance detection of cell surface antigens in subsequent FACS analysis. Cells were incubated in suspension in DMEM:F12 (Life Technologies) supplemented with 5% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) and 1 mM EDTA (Sigma) with mild agitation for 7 - 10 hours. The rational for this step is that during dissociation, not only proteins of the extracellular matrix and cell - cell attachment proteins are broken down but also cell surface antigens that are of interest to detect in FACS analysis. By allowing the cells to recover in suspension, they have time to at least partially replace cell surface antigens by endocytic recycling pathways (265). After the epitope regeneration treatment, cell suspension was briefly incubated with an erythrocyte lysis buffer (described in the previous section) to remove residual erythrocytes, then subjected to FACS staining.

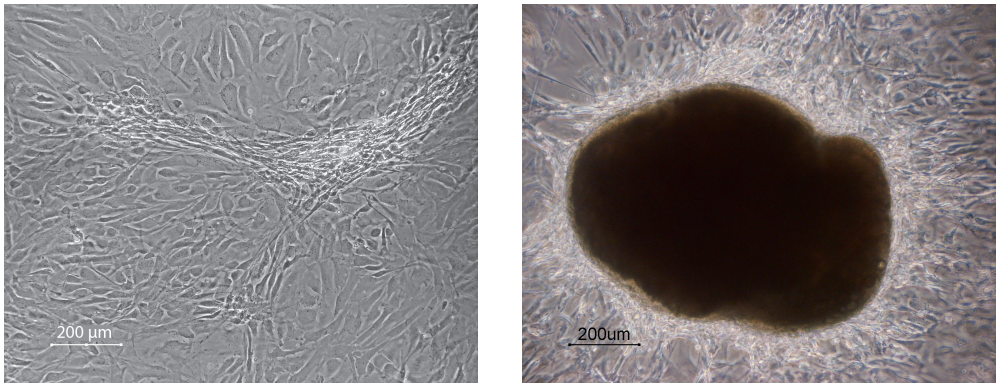


Figure 6. *Representative pictures of cultured cardiac cells prior dissociation for FACS analysis.*

3.5 Culture of cardiac derived cells

For culture of cells from cardiac biopsy material, both an explant culture system and a monolayer culture system were assessed in paper I. An explant culture system has previously been used both in the generation of cardiospheres (199, 200) and for isolation of C-kit+ cells (131). An explant culture system was established by cutting biopsies in small pieces and then seeding these into Primaria® flasks (BD, Franklin Lakes, NJ, USA) with no prior treatment with dissociative enzymes. Explants were cultured in a basic growth media consisting of DMEM:F12 supplemented with 10% human serum (HS), penicillin/streptomycin (PEST, PAA Laboratories, Pasching, Austria) and L-glutamine (Life technologies). Explants were observed to attach to the bottom of the culture vessels and cells started to migrate out of them. Explants were cultured for 19 - 24 days without passages, then subjected to FACS analysis.

At this time, a layer of fibroblast like cells around the explants had been established (shown in Figure 6). Notably, this culture time was about the same as what was used in the first study of cardiospheres by Messina et al. (199).

A monolayer culture system was used for expansion of dissociated cardiac cells in paper I and IV. Cells were seeded at about 1 mg of tissue/cm2 in the same basic growth media used for explant cultures. A weight to surface area measurement of seeding density was used since this was deemed to have less variability compared to counting of cells. Cells were grown without passages until confluence (11 - 15 days), then subjected to FACS analysis. Typical cell morphology at the time of FACS analysis is shown in Figure 6.

3.6 Culture of cell lines

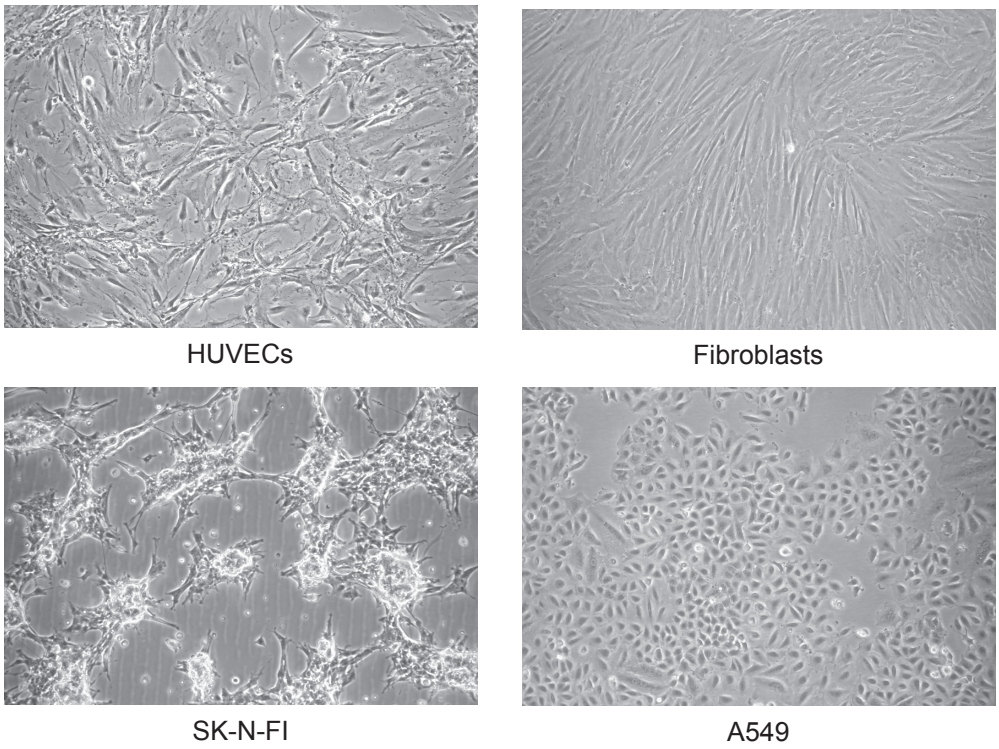


Figure 7. *Representative pictures of the morphology of the cell lines used in paper I and III.*

In paper I, human umbilical vein endothelial cells (HUVECs) and human fibroblasts (LGC Standards AB, Borås, Sweden) were used as control cell lines. Both cell lines were cultured as monolayer and passaged at 70-80% of confluence, according to manufacturer's description.
In paper III, lung cancer cell line A549 and neuroblastoma cell line SK-N-FI (both obtained from LGC standards) were used as control cell lines in the SP assay. The

A549 cell line is known to highly express the ABC transporter protein ABCG2 (266) whereas the SK-N-FI cell line expresses ABC transporter protein MDR1 (180). Both cell lines were cultured according to manufacturer's description. While A549 cells grew as a monolayer, SK-N-FI cells were observed to form 3 dimensional structures. Typical morphology of the different cell lines are shown in Figure 7.

3.7 Methods of inducing differentiation

In paper I, *in vitro* differentiation into the endothelial and cardiomyogenic lineages of sorted C-kit+CD45- cells was assessed.

3.7.1 Endothelial differentation

For endothelial differentiation, sorted cells were seeded at 8 - 10,000 cells / cm2 into collagen type 1 (BD) coated primaria® plates. Differentiation was induced by culturing the cells in endothelial growth media EGM-2 MV for 10 days. This media includes several growth factors important for endothelial development, including VEGF. It has previously been shown to be able to induce endothelial differentiation in ES cells (267). Endothelial differentiation was assessed by FACS analysis, qPCR and by cell morphology.

3.7.2 Cardiomyogenic differentation

For cardiomyogenic differentiation in paper I, sorted cells were seeded at 30,000 cells/cm^2 into gelatin coated 48 well plates. Differentiation was induced by treatment with 5-Azacytidine and TFG-β1 according to a protocol which previously successfully have induced cardiomyogenic differentiation of human cardiac Sca-1+ progenitor cells (268). 5-Azacytidine is often described as a de-methylating agent of DNA, but should perhaps more stringently be described as an inhibitor of methylation of DNA. It undergoes intracellular conversion into 5-aza-2´-deoxycytidine which is incorporated into the DNA of replicating cells as an analogue to the nucleoside cytidine. When incorporated, 5-aza-2´-deoxycytidine inhibits DNA methyltransferases which results in hypomethylation of DNA and activation of previously silenced genes (269, 270). Cardiac differentation was assessed by qPCR and visual inspection of cells for beating colonies.

3.8 FACS analysis

FACS analysis and sorting of cells have been used in all four papers. FACS technology enables the analysis of several parameters simultaneously for a large number (> 10^6) of individual cells. These parameters include basic cellular properties such as relative size (forward scatter, FSC) and granularity (side scatter, SSC) as well as fluorescence intensities in a number of different wavelength bands. Depending on the instrument configuration, several different wavelengths of excitation may also be used simultaneously with minimal interference between each other. If the

instrument is equipped with a cell sorting unit, cells of interest may also be sorted for further analysis. In all FACS analyses, a FACSaria II instrument (BD) was used. Cells were either obtained from directly dissociated tissue (all four papers) or from dissociated monolayer cultured cells (paper I, IV). Cells were resuspended in FACS staining buffer which consisted of PBS supplemented with 5% FBS, 1% BSA and 2 mM EDTA. FBS and BSA were included to reduce unspecific binding of antibodies whereas EDTA was included to minimize aggregation of cells. To remove any residual aggregates of cells or mature cardiomyocytes, cells were poured through a 40 μm cell strainer prior FACS analysis.

In paper I, II and IV, cells were incubated with antibodies directly conjugated with fluorochromes directed to cell surface antigens for 30 minutes, 4 C°. The usage of directly conjugated antibodies makes it possible to analyze several different antigens simultaneously using antibodies produced in the same species but conjugated to different fluorochromes.
In addition, 7-amino-acitinomysin D (7-AAD) was included as a dead cell discriminator. Live cells are normally non-permeable to 7-AAD and are thus not stained. In dead cells, the cell membrane is damaged and 7-AAD is able to transverse the cell membrane and bind to DNA in the nucleus (271). Dead cells are important to exclude since they have high autofluorescence and bind antibodies unspecific. Furthermore, dead cells may affect results of subsequent analyses on sorted cells. Compared to propidium iodide (PI) which also is a DNA binding dye often used for dead cell discrimination, 7-AAD has the advantage of less spectral overlap with PE and is not excited by UV light which may pose a problem when analyzing cells for SP. Furthermore, 7-AAD seems to be more specific for dead cells whereas PI stain a larger portion of live cells (in house, unpublished data). In paper III and IV, cells were also stained for SP. For details on this staining procedure, please see the section 3.9.

Analysis of FACS data was carried out using the FACSDiva software version 6.1.1 (BD). Generally, an inclusive FSC vs SSC gate was used to not miss any populations that could be of interest. Events with very high FSC values were however excluded as these probably represent small aggregates of cells. 7-AAD+ cells were also excluded in the gating strategy. Gates for the different parameters were set using isotypic controls as negative samples. 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. FACS data are generally presented in the papers by representative plots from one experiment with statistics reflecting mean ± standard error of the mean (SEM) of all experiments carried out.

3.8.1 FACS sorting

In all papers, FACS sorting was used to isolate progenitor populations for further culture or analysis by qPCR. The principles of FACS sorting using the FACSaria instrument are outlined in Figure 8. Sorting is carried out by giving droplets with cells that fulfill criteria for sorting 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 is allowed to be sorted. Furthermore, for single cell sorting, a special sorting protocol which optimizes trajectory of the sorted droplets at the expense of sorting efficiency was employed. Sorting purity was determined to be 90 - 95% pure by re-analysis of sorted cells.

In paper I, cells were sorted for further culture into polypropylene FACS tubes containing cold PBS supplemented with 20% FBS and PEST. In paper I, III and IV, cells were sorted for qPCR into either RLTplus buffer including DTT (Qiagen, Hilden, Germany) or cold PBS. The RLTplus buffer has the advantage of instantly lysing the cells and it then protects 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.

In paper II, cells were single cells sorted for further qPCR analysis directly into 96 well plates with lysis buffer and DNAse type 1 (both from Life technologies). Thus, cells were instantly lysed and RNA was then protected by RNase inhibitors included in the lysis buffer. According to manufacturer's description, "stop solution" was added to the wells before storage at -80 °C.

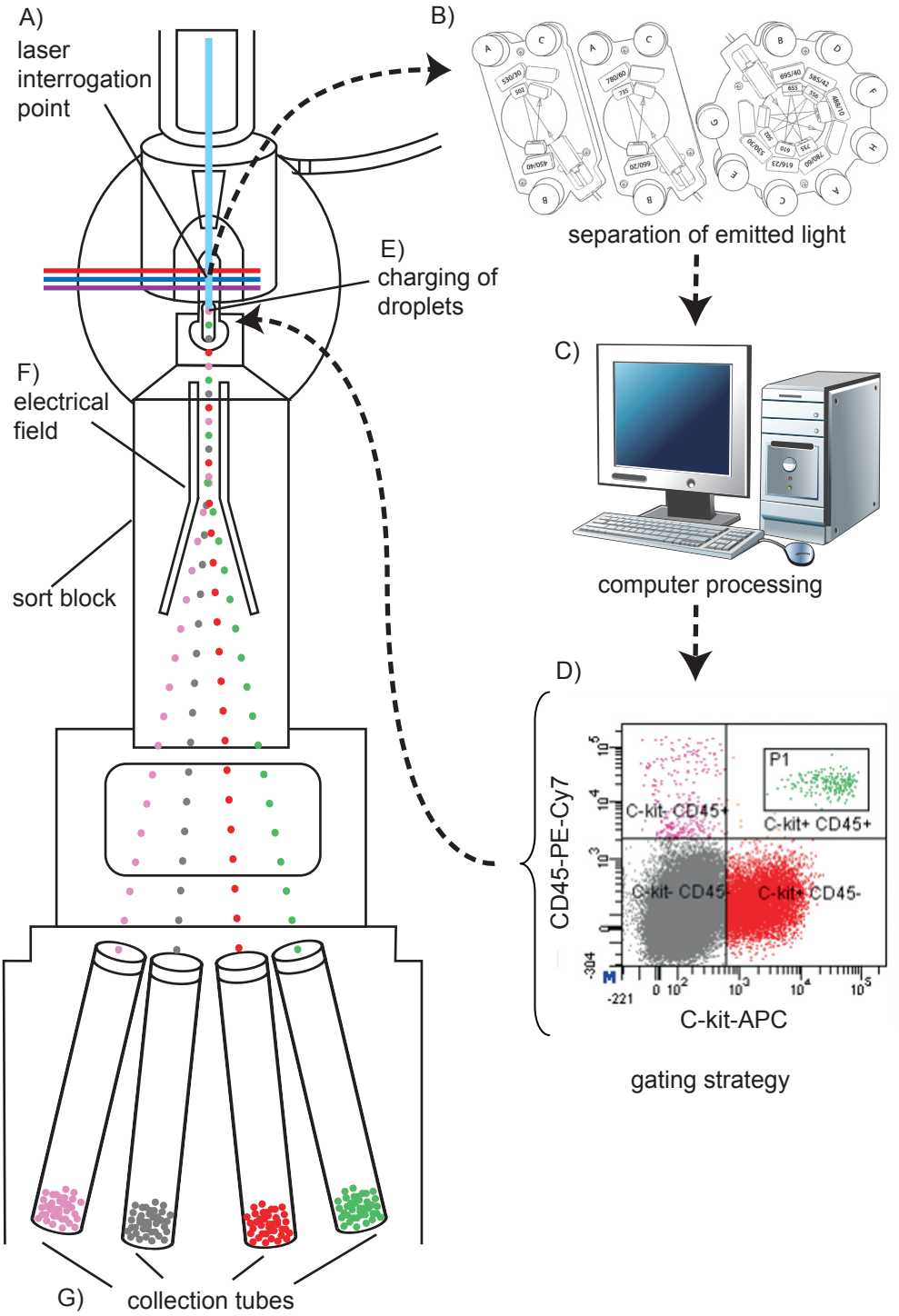

A)
laser
interrogation
point
B)
separation of emitted light
C)
computer processing
D)
CD45-PE-Cy7
C-kit-APC
P1
C-kit- CD45+
C-kit+ CD45+
C-kit- CD45-
C-kit+ CD45-
gating strategy
E)
charging of
droplets
F)
electrical
field
sort block
G)
collection tubes

Figure 8 (overleaf). *Schematic illustration of the FACSaria system. Cells pass through the laser interrogation point in a stream of fluid (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 different collecting tubes (G). It is also possible to sort cells into plates, but then only one population of cells can be sorted.*
Modified from the FACSaria instrument manual.

3.9 Side Population assay

In the SP assay used in paper III and IV, cells with the ability to exclude Hoechst 33342 were identified. Principles of the staining are outlined in Figure 9. Cells were resuspended in staining medium (DMEM hepes modified medium (Life technologies) supplemented with 2% FBS) at 1×10^6 cells / ml. To be able to gate the SP population as well to elucidated which ABC transporter protein that was responsible for the SP phenotype, ABC transporter inhibitors Fumitremorgin C (FTC), Verapamil and sodium azide with the addition of 2-D-Deoxyglucose (all obtained from Sigma) were included. FTC has been described as a specific inhibitor for ABC transporter ABCG2 (196, 197) whereas Verapamil has been described as an inhibitor to ABC transporter MDR1 (197). Sodium azide with the addition of 2-D-Deoxyglucose works as a general inhibitor of metabolism and should block all energy dependent transport proteins (266). When inhibitors were used, samples were pre-incubated with these for 20 minutes and inhibitors were then added in all subsequent steps. Next, Hoechst 33342 (Life technologies) was added at a concentration of 5 µg/ml. Cells were incubated for 45 minutes at 37 °C, then washed and incubated for additional 45 minutes 37 °C 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. (175), where only one incubation period was used. It has however been described in the literature as a way of reducing background in the SP assay (266) which is of particular importance when analyzing an heterogeneous sample of cells such as that of directly isolated cells from a cardiac biopsy. After the second incubation period, cells were washed and resuspended in cold FACS staining buffer and stained for dead cells and cell surface antibodies as described in section 3.8. Cells were strictly kept at 4 °C / on ice until FACS analysis to prevent additional Hoechst efflux in inhibitor treated samples.

On the FACSaria II instrument, Hoechst 33342 staining was visualized using a 375 nm near UV laser. To indentify SP cells, Hoechst blue (emission between 440 - 460

nm) was plotted against Hoechst red (emission above 670 nm). Hoechst emission spectra has been shown to be blue shifted approximately 50 nm when bound to DNA (272). 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 SP population was generally gated based on the 2-D-Deoxyglucose treated samples since this inhibitor was most effective in blocking Hoechst 33342 efflux.

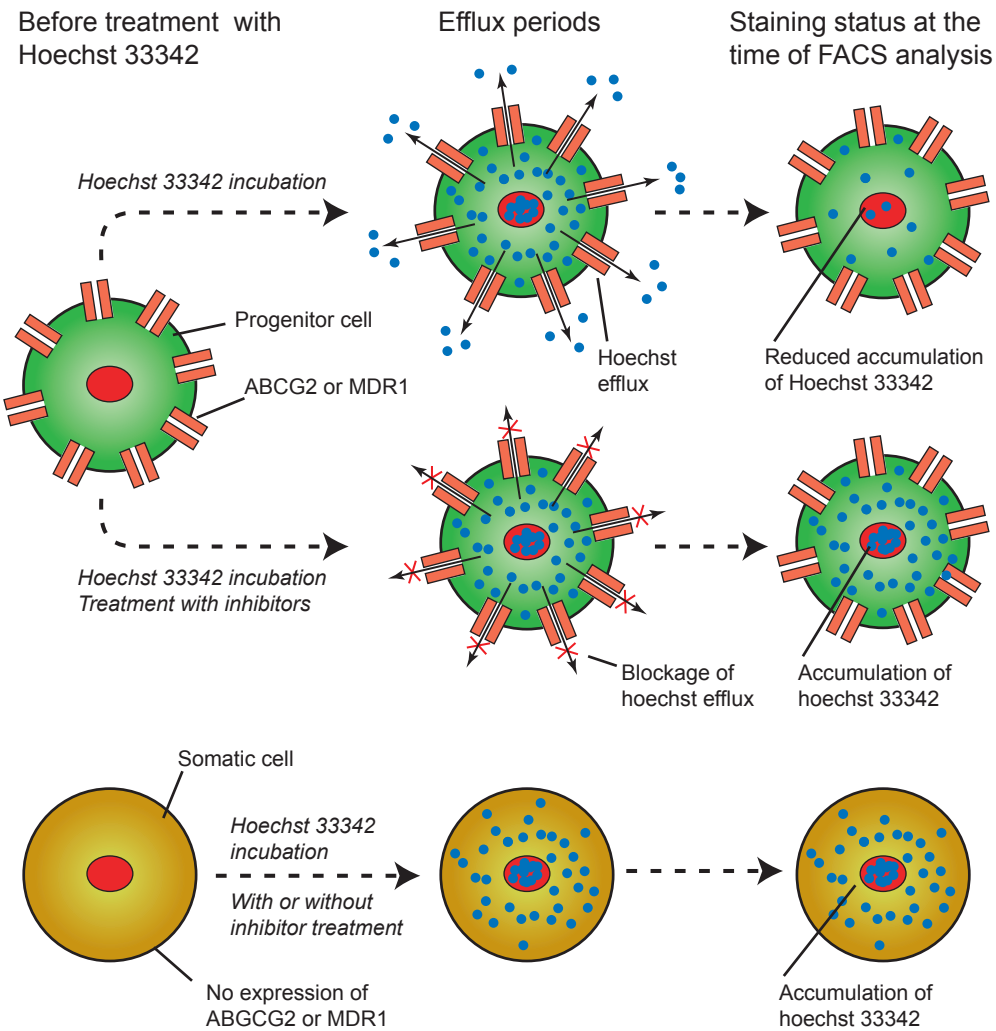


Figure 9. *Schematic illustration of the SP assay. In the upper part of the figure, staining of a SP progenitor cell is shown while in the lower part, staining of a non SP somatic cell is illustrated. For further details, please see the section 3.9 in the main text.*

3.10 Immunohistochemistry and image analysis

Compared to FACS analysis, immunohistochemistry has the advantage of providing information of location of a protein expression in a tissue. Furthermore, there is no need to use dissociative enzymes which may adversely affect detection of cell surface antigens. On the other hand, immunohistochemistry does generally not provide any statistical information or intensity measurements of individual cells. It may thus be hard to reliably identify populations of cells that are small in number.

In paper IV, immunohistochemistry was used to study the location of SSEA-4 expression in tissue slides. Biopsies from right and left atrium were imbedded in TissueTek OCT (Histolab, Gothenburg, Sweden) and frozen on dry ice. From these, 7 µm frozen tissue sections were prepared (Histo-center, Gothenburg, Sweden). Although frozen sections generally have less good morphology compared to sections from formalin fixed and paraffin embedded tissue, they generally provide better compatibility with staining with antibodies. In the case of SSEA-4 staining, both frozen and formalin fixed, paraffin embedded sections were tested but only in frozen sections could a positive staining be detected (data not shown).

Sections were fixed with -20 °C acetone for 10 minutes, washed with PBS and incubated for 30 minutes with blocking solution consisting of PBS supplemented with 1% BSA (Sigma) and 5% goat serum (Life technologies). Serum and BSA were used to reduce unspecific staining of antibodies. Primary antibodies were then added and incubated in a moisture chamber over night at 4 °C. Primary antibodies used were mouse anti SSEA-4 (eBioscience, San Diego, CA, USA) and rabbit monoclonal anti CD34 (Abcam, Cambridge, UK). Corresponding isotypic controls were used to evaluate background staining.
Sections were then washed and incubated with secondary antibodies conjugated to fluorchromes (alexa 488 and alexa 546 dyes) for 1 hour at room temperature in a moisture chamber. To enable triple staining with cTnT, a mouse anti cTnT antibody (Thermo Scientific, Waltham, MA, USA) were labeled with alexa 647 using the Zenon kit (Life technologies). This kit works by creating an immune complex between the primary cTnT antibody and a secondary fluorochrome conjugated antibody before staining the tissue. Surplus secondary antibody is removed by adding additional mouse IgG of the same isotype as the primary antibody. This prevents remaining secondary antibody from staining antibodies that are already present in the tissue sections. The labeled cTnT antibody and a corresponding isotypic control were added after washing of tissue sections, for 45 minutes in a moisture chamber, at room temperature. The tissue sections were then washed and fixed with Histofix (Histolab) for 15 minutes to prevent slow dissociation of immun-complexes between primary and secondary antibodies which could result in undesired cross labeling of the cTnT and SSEA-4 antibodies. After additional washing, sections were mounted using Prolong gold antifade with 4’,6-diamidino-2-phenylindole (DAPI)

(Life technologies). The DAPI dye binds to double stranded DNA and was used to visualize cell nuclei.

Staining was visualized using a Nikon EclipseTi inverted fluorescence microscopy (Nikon, Tokyo, Japan). Images were acquired using and Andor iXon X3 camera and NIS elements 4.12 software (Nikon). Large images (approximately 2 × 2 mm^2) were acquired using a motorized board and the "stich images" option in the software. The ability to create high resolution large images is important since only a small percentage of the total number of cells in the tissue were SSEA-4+. To further enable accurate identification of SSEA-4+ cells, images were scanned in at least 3 levels in Z-axis.

Aquired images were exported to ImageJ v. 1.47h, Fijii distribution. This is an open source software for biological image analysis (273) which enables detailed analyses and quantification of images. In order to distinguish background staining from positive staining, displayed pixel ranges for each fluorochorme channel were set so that the corresponding isotypic control was essentially black. The upper limits of the displayed pixel ranges were adjusted so that the whole dynamic range of intensities was shown without saturated pixels. Areas with artifacts related to for example folding of the section were excluded from further analysis. SSEA-4+ cells were identified manually and counted. A clear staining morphology around a nucleus was required for positive identification. Customized macros were developed for determining co-localization between SSEA-4 and CD34, to determine the total number of nuclei in an image and to determine the number of SSEA-4+ cells outside and inside myocardium as defined by cTnT staining. An approximate percentage of SSEA-4+ cells was calculated by assuming that one DAPI nucleus corresponds to one cell.

3.11 RNA analysis by qPCR

RNA analysis of FACS sorted cells by qPCR was used in paper I, III and IV. In addition, RNA analysis was carried out on cultured cells in differentiation experiments in paper I. In paper II, single cell qPCR was conducted. The qPCR process including cDNA synthesis, preamplification and real time PCR was in principle carried out in the same way as described in this section. However, RNA isolation as well as data analysis were carried out differently and will be described further in section 3.11.1.

Total RNA was extracted with RNeasy Micro or Mini Kit (Qiagen) depending on the number of cells. Removal of residual genomic DNA from samples was done with either genomic DNA columns or DNaseI treatment (Qiagen). Extraction was either carried out manually or automatically in a QIAcube (Qiagen) according to manufacturer's description.

Extracted RNA was first transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Life technologies). This kit uses random hexameres primers for initiation of reverse transcription and thus all of the RNA is transcribed into cDNA. Samples with low cell number and correspondingly low total RNA quantity as measured by a Nanodrop instrument were pre-amplified using TaqMan PreAmp Master Mix (Life technologies). By this method, specific genes are amplified by PCR in an unbiased manner to allow for more genes to be analyzed in the subsequent qPCR. 14 cycles of amplification was used. The pre-amplification step has been validated in house by comparing with identical samples that had not been pre-amplified. Relative gene expression levels were similar regardless of whether pre-amplification had been used or not.

qPCR offers both highly sensitive and specific quantification of mRNA expression. Compared to traditional PCR, where data is collected at the end of the reaction, in qPCR, data is collected continuously throughout the PCR process. The qPCR analysis was carried out using commercially available TaqMan primers and probes 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.

The principle behind TaqMan qPCR analysis was first described by Holland et al (274) and is outlined in Figure 10. It is based on an oligonucleotide probe containing a reporter fluorescent dye and a quencher. When the probe is intact the reported dye and quencher are in close proximity to each other and fluorescence is quenched by fluorescence resonance energy transfer (FRET). 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. This results in separation of reporter dye and the quencher, which increases the fluorescence of the reporter dye. For each cycle, fluorescence will increase approximately 2 fold. Fluorescence intensity is measured continuously during the qPCR analysis. In all papers included in this thesis, 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 quantification of data, the relative comparative Ct method was used (275). CREBBP was selected as reference gene as this has been shown have a stable expression in cardiac tissue in previous work by our group (160). Furthermore, we have compared expression of CREBBP in FACS sorted directly isolated and

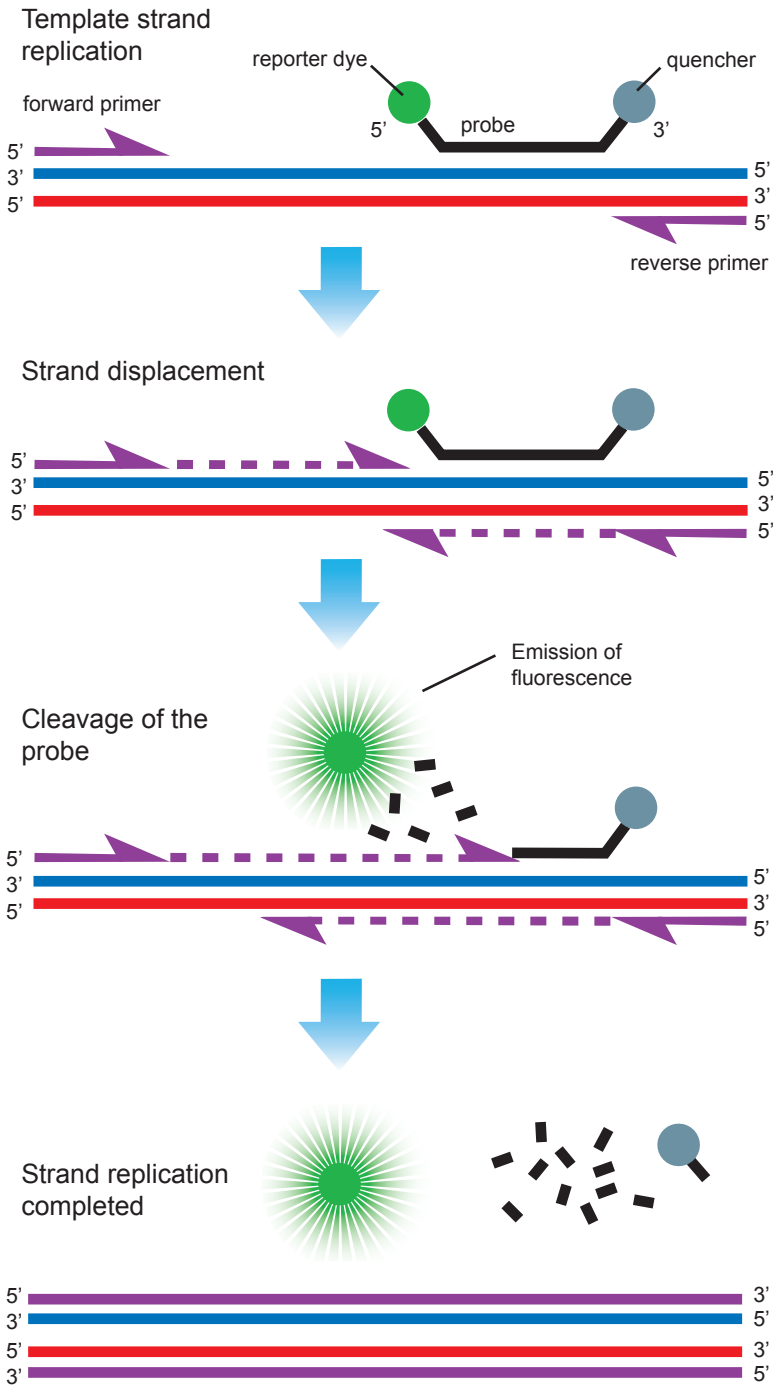


***Figure 10**. Schematic illustration of the qPCR assay showing template strand replication, strand displacement and cleavage of the probe which results in emission of fluorescence. For further details, please see section 3.11 in the main text. Modified from www.lifetechnologies.com.*

monolayer cultured cells from cardiac biopsies and obtained similar expression levels (in house data). Furthermore, values have been normalized to a calibrator sample included on each plate to be able to compare data between different plates. All samples were analyzed in duplicates. The technical variability was generally observed to be very low. Gene expression data are presented in the thesis in relative units.

3.11.1 Single cell qPCR analysis

Single cell qPCR was carried out in paper II. As described in section 3.8.1, cells were sorted as single cells into a lysis solution. Lysis of cells, cDNA synthesis, preamplification and gene expression analysis was carried out using the TaqMan® PreAmp Cells-to-CT™ Kit (Life technologies) with minor modifications as described in detail in the supplemental material and methods section to paper II. cDNA synthesis, pre-amplification and real time PCR were carried out in principle as described in the previous section (3.11). When it comes to data analysis, the relative comparative Ct method can not be used for single cells. This is due to that while a reference gene is stably expressed in large group of cells, this is not the case on single cell level (276). On the other hand, cells had been sorted with high precision as one cell per well. Thus, data was instead expressed as raw Ct values / cell. This method precluded from the usage of a calibrator sample for normalization. However, such a sample was included in each plate and showed minimal variation between plates. 2-dimensional cluster analysis was carried out using GenEx v. 5 software (MultiD Analyses AB, Gothenburg, Sweden) to find subgroups of cells. Ward's algorithm with eucledian distance was used for both gene and cell clustering.

3.12 Statistical analyses

For statistical analysis, depending on nature of the analyzed data, two-sided Student's t-test for either group wise or pair wise comparison was carried out. A $p<0.05$ was considered statistical significant. Statistical calculations were carried out using either SPSS v. 20 (IBM, New York, NY, USA) or Excel v. 2003 (Microsoft, Redmond, WA, USA). The t-test assumes normal distribution of data. To obtain a better normal distribution of gene expression data, these were log transformed before calculation.

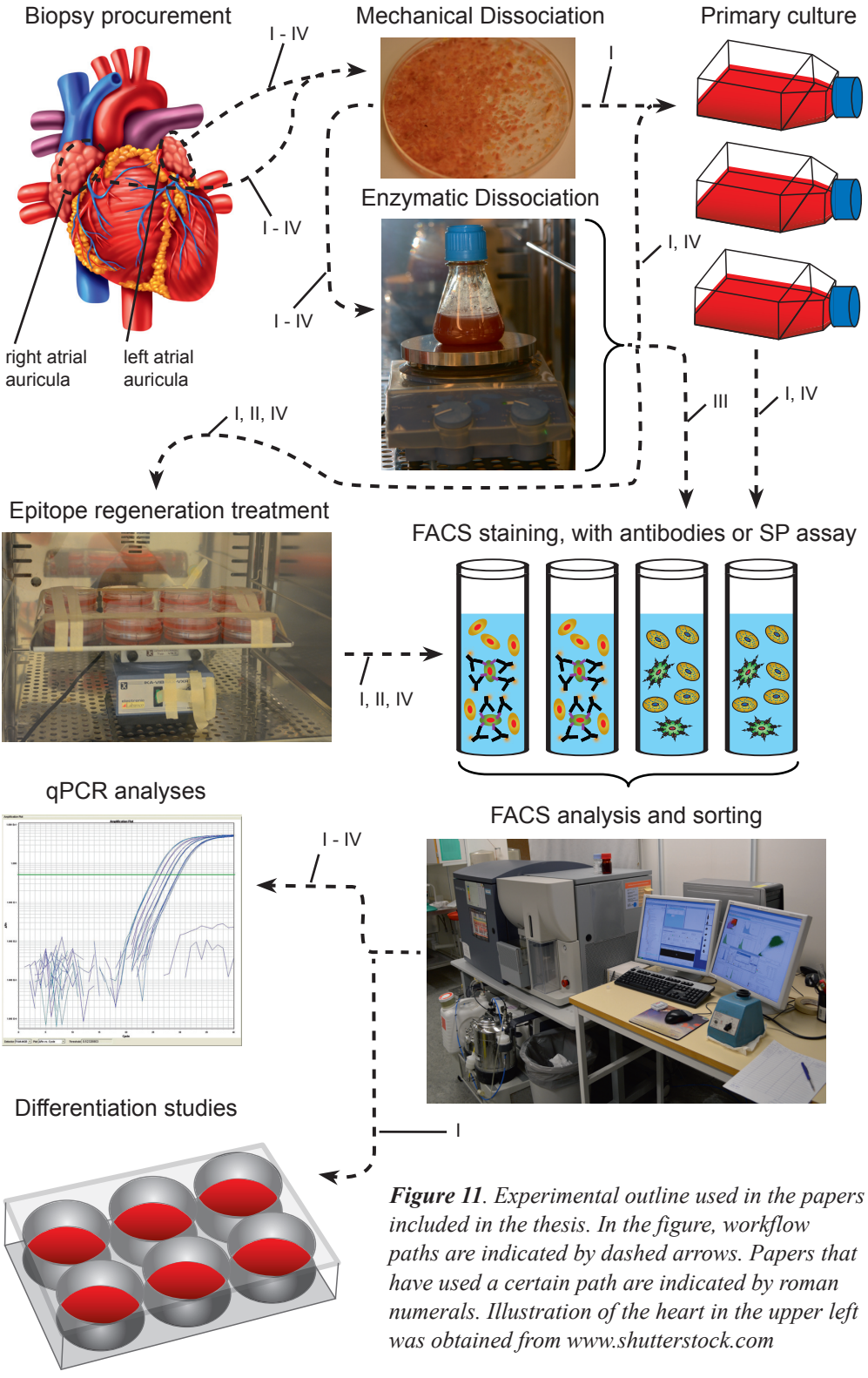


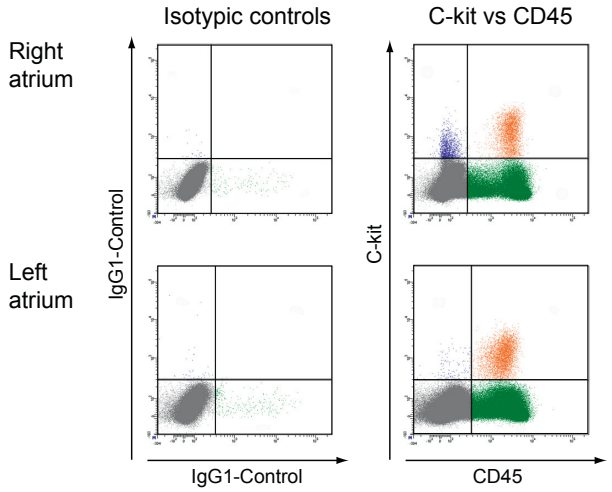
***Figure 11**. Experimental outline used in the papers included in the thesis. In the figure, workflow paths are indicated by dashed arrows. Papers that have used a certain path are indicated by roman numerals. Illustration of the heart in the upper left was obtained from www.shutterstock.com*

4 SUMMARY OF RESULTS

An overview of the study designs of the included papers are given in Figure 11. Here follows a summary of the most important results. For further details, please see the individual papers.

4.1 C-kit+ cells are present in both directly isolated cells and cultured cells from human atrial tissue and could be subdivided based on hematopoietic marker CD45 (Papers I and II)

Cardiac atrial biopsies from left and right atrium were dissociated and cardiomyocytes were depleted by filtration. There was a clear population of C-kit+ cells in both right and left atrium (Figure 12). Most of these cells co-expressed hematopoietic marker



C-kit- CD45-	89	± 2	%
C-kit+ CD45-	*0.078	± 0.018	%
C-kit+ CD45+	*0.42	± 0.09	%
C-kit- CD45+	7.7	± 0.6	%

n = 7

C-kit- CD45-	87	± 3	%
C-kit+ CD45-	*0.010	± 0.004	%
C-kit+ CD45+	*0.86	± 0.12	%
C-kit- CD45+	9.5	± 3.6	%

n = 6

Figure 12. *FACS analysis of C-kit and CD45 staining in cells from right and left atrial biopsies. Plots to the left show gating strategy for one representative experiment while statistic tables to the right show mean value for all analyzed biopsies ± SEM. Numbers (n) of biopsies analyzed are indicated in the figure. * denotes a significant difference (P < 0.05) in expression between right and left atrium.*

CD45 while in the right atrium, there was a small but clearly identifiable population of C-kit+CD45- cells. This population was on the other hand barely detectable in cells derived from left atrial biopsies. By FACS analysis, the directly isolated C-kit+CD45- population was determined to co-express EPC marker CD34 and as well as CD31 (Figure 13). The C-kit+CD45+ population on the other hand was negative for these markers but instead expressed CD33. This is a cell surface maker known to be expressed by mast cells. Notably, by FACS analysis, no expression of progenitor

markers Sca-1, CD133, MDR1 and ABCG2 were found in either directly isolated, or monolayer cultured cells.

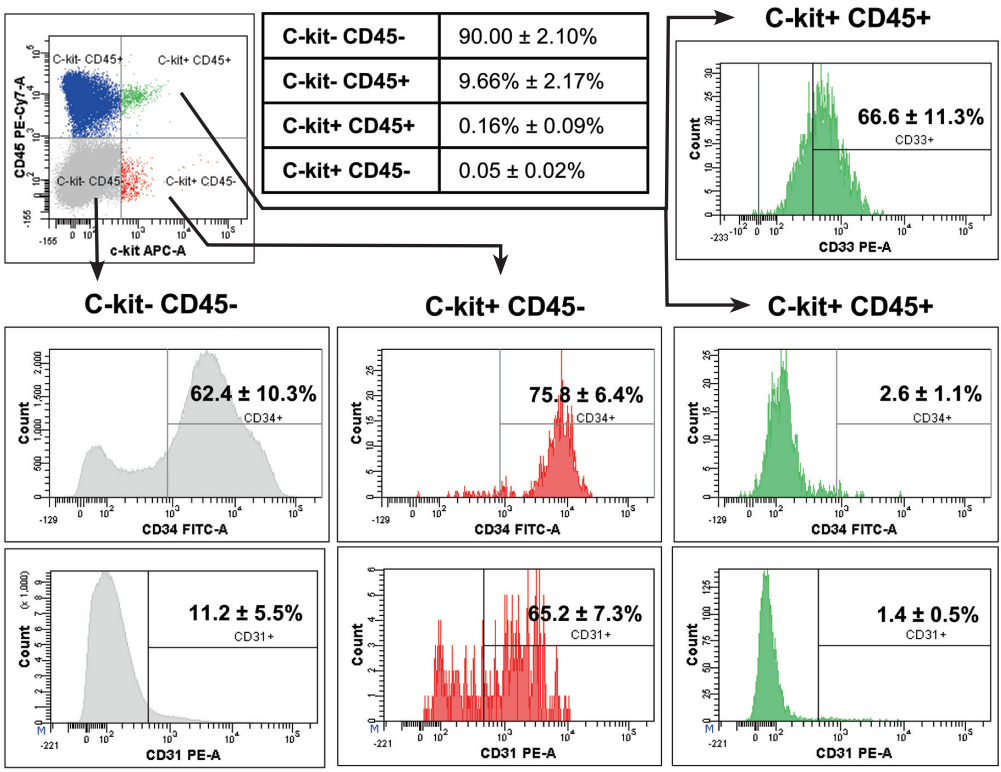


Figure 13. *Cell surface antigen expression of directly isolated cells. In the upper left part of the figure, C-kit plotted against CD45. Co-expression of CD34 and CD31 are shown for the different cell populations as indicated by the arrows. For C-kit+CD45+ cells, expression of mast cell antigen CD33 is shown as well.*

As the number of C-kit+CD45- cells that could be isolated from directly dissociated tissue was rather low, a culture system was developed to expand cells from cardiac biopsies prior sorting. Initially, an explant culture system was compared to a monolayer culture system. Although both these culture systems retained a population of C-kit+CD45+ cells that stained positive for mast cell associated markers CD33 and CD69, the monolayer culture system was found to greatly increase the number of C-kit+CD45- cells and was thus used in further experiments. Monolayer cultured C-kit+CD45- cells similar to directly isolated cells co-expressed EPC markers CD34, CD31 and chemokine receptor CXCR4 to almost 100% (Figure 14). They dimly expressed FLK-1. By gene expression analysis, the C-kit+CD45- cells were shown to highly express *C-KIT*. Both directly isolated and monolayer cultured C-kit+CD45- cells expressed endothelial genes (*CD31*, *VWF*, *FLK1*) significantly

more than C-kit-CD45- cells. In addition, C-kit+CD45- cells expressed higher levels of cell fate determiner *NOTCH1*. In analysis of whole population gene expression (paper I), cardiac specific markers were either undetectable or expressed in very low levels. No difference was observed between C-kit+ and C-kit- cells. C-kit+CD45+ cells were observed to express of mast cell specific genes *TPSG1* (tryptase) and *CMA1* (chymase).

C-kit- CD45-	95.8 ± 0.6%
C-kit- CD45+	0.11 ± 0.03%
C-kit+ CD45+, P1	0.03 ± 0.01%
C-kit+ CD45-	4.0 ± 0.6%

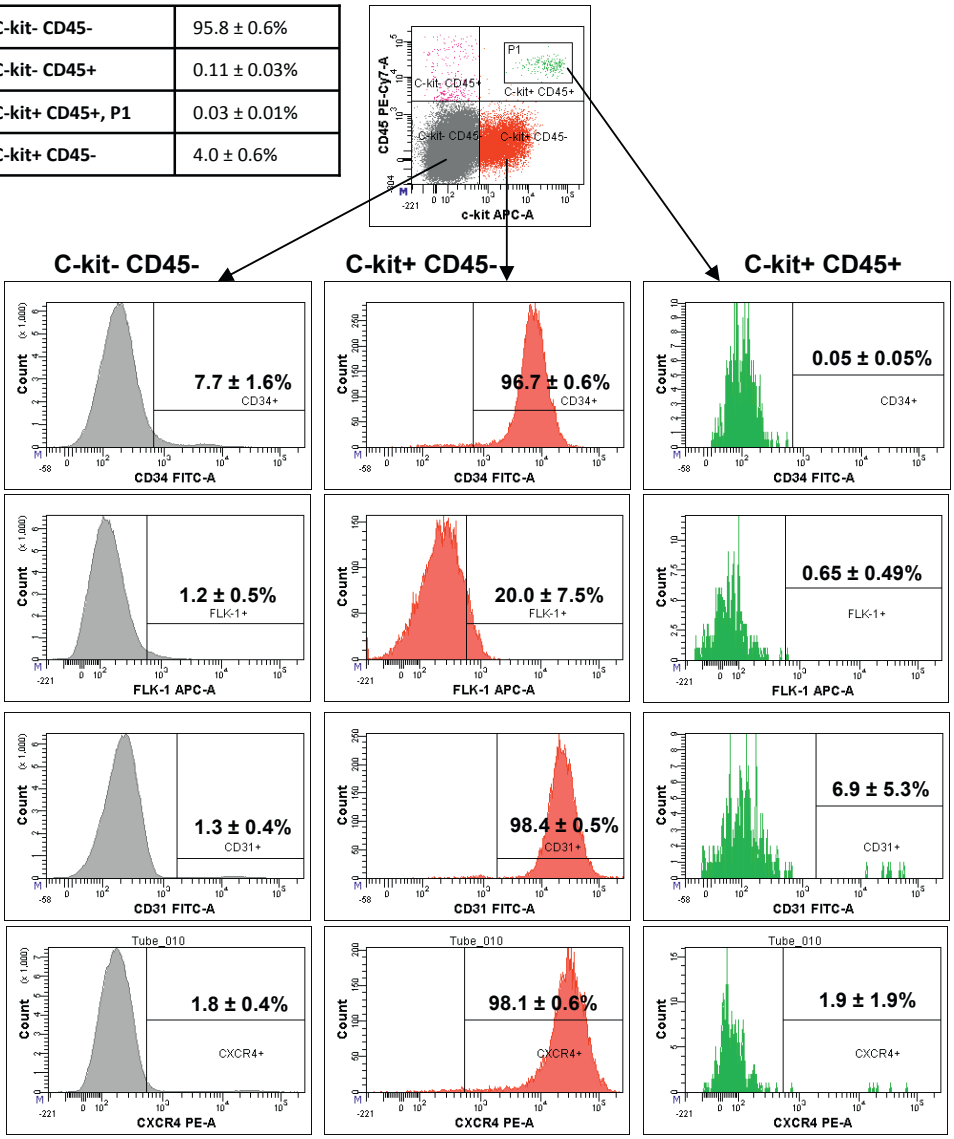


Figure 14. *Cell surface antigen expression of monolayer cultured cells. C-kit plotted against CD45. A smaller gate (P1) is drawn around the clearly positive CD45+ C-kit+ cells. Co-expression for EPC markers are shown for the different cell populations as indicated by the arrows.*

4.2 Differentiation capacity of monolayer cultured C-kit+CD45- cells (Paper I)

Differentiation capacity into the endothelial lineage was evaluated by culturing sorted C-kit+CD45- cells in a media containing endothelial growth factors on collagen type I coated culture vessels. In this media, cells were observed to assume an endothelial morphology (Figure 15 A, thin arrow). By FACS analysis, EPC marker CD34 as well as C-kit were downregulated which could be interpreted as further differentiation into the endothelial lineage. By qPCR analysis, C-kit+CD45- cells expressed similar levels of C-kit as the endothelial control cell line HUVEC whereas expression of endothelial genes were similar or higher compared to the HUVEC cell line.

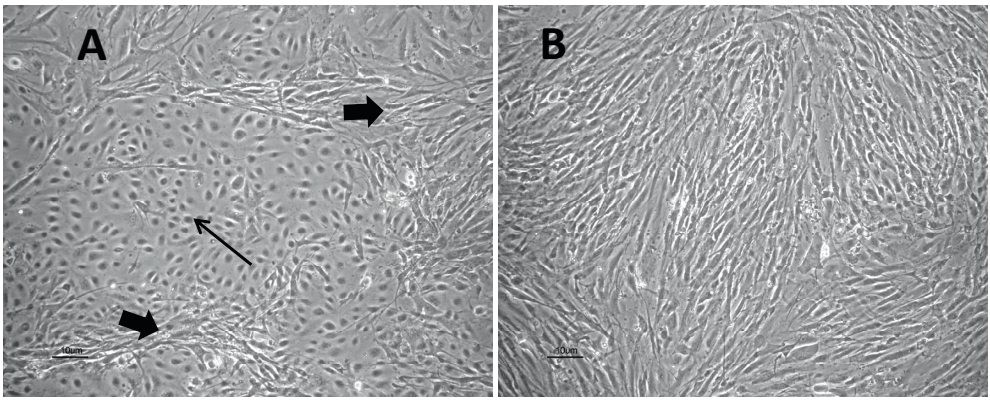


Figure 15. *(A) C-kit+ cells grown in media optimized for microvascular endothelial cells. Two distinct cell morphologies appeared one fibroblast like (thick arrows) and one with a rounded appearance growing in clusters (thin arrow).*
(B) C-kit- cells grown in media optimized for microvascular endothelial cells.

Cardiac differentiation was assessed by a combination of 5-Azacytidine and TGF-β1 treatment. However, no signs of cardiac differentiation was observed by either visual inspection for beating cells or by qPCR analysis for cardiac specific genes.

4.3 Investigation of heterogeneity within the C-kit+CD45- population in regard of cardiac and endothelial commitment by single cell qPCR (Paper II)

Although the C-kit+CD45- population both when directly isolated from tissue biopsies and when isolated from cultured cells primarily showed an endothelial profile, it could not be excluded that a small subpopulation of cardiac committed progenitors was also present. To elucidate this further, C-kit+CD45- cells from right atrial biopsies were FACS sorted as single cells for qPCR analysis. Most of the cells with verified *C-KIT+CD45-* gene expression profile were found to express *VWF*

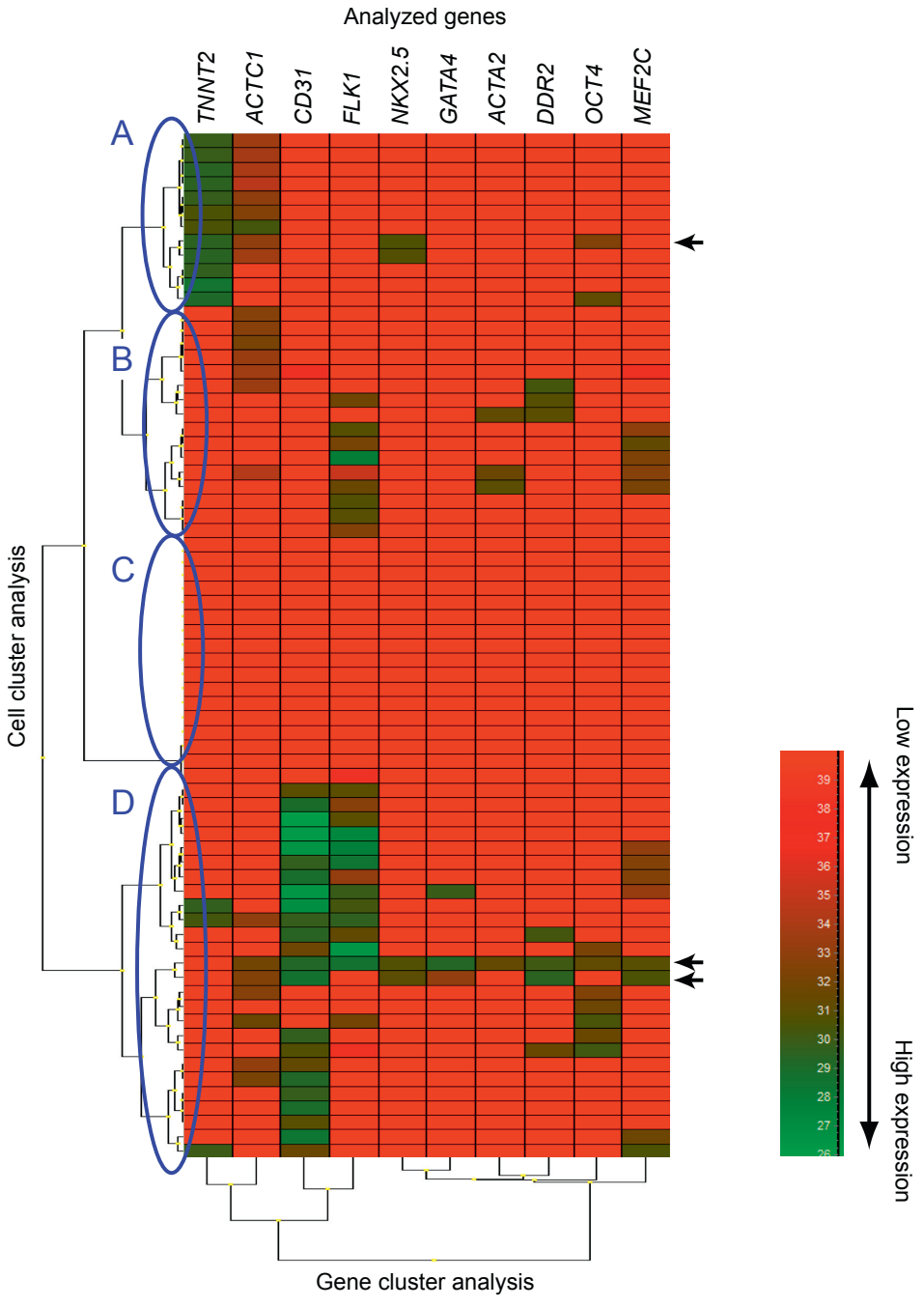


Figure 16. *Cell and gene cluster analysis of single-cell sorted C-kit+CD45- cells with either CKIT+ VWF- or NKX2.5+ gene expression. Cluster analysis was carried out according to Wards algorithm, Euclidean distance. Clustering of genes is shown on the X-axis whereas clustering of cells is shown on the Y-axis. Arrows denotes NKX2.5+ cells also positive for VWF.*

indicative of late endothelial differentiation. This is in line with our previous results presented in paper I. However, a minor portion of the cell (19%) were found to be negative for *VWF* expression. In addition, a few cells (1.1%) expressed cardiac transcription factor *NKX2.5*. These cells were further analyzed for additional genes indicative of cardiac, endothelial, smooth muscle cells, fibroblast and stem cell identity. By 2-dimensional cluster analysis, four groups of cells were identified (Figure 16). One of these expressed late cardiac genes but no endothelial markers while another expressed endothelial genes with a few cells also expressing one or two cardiac genes. Of the two remaining groups, one was negative for all analyzed genes (but as mentioned above, positive for *C-KIT*) and the other consisted of two subpopulations where one expressed *FLK-1* and the other expressed cardiac gene alpha-cardiac actin (*ACTC1)*. The gene cluster analysis showed, as expected, a clustering of endothelial and cardiac genes.

4.4 Identification of Side Population cells in the human adult heart (Paper III)

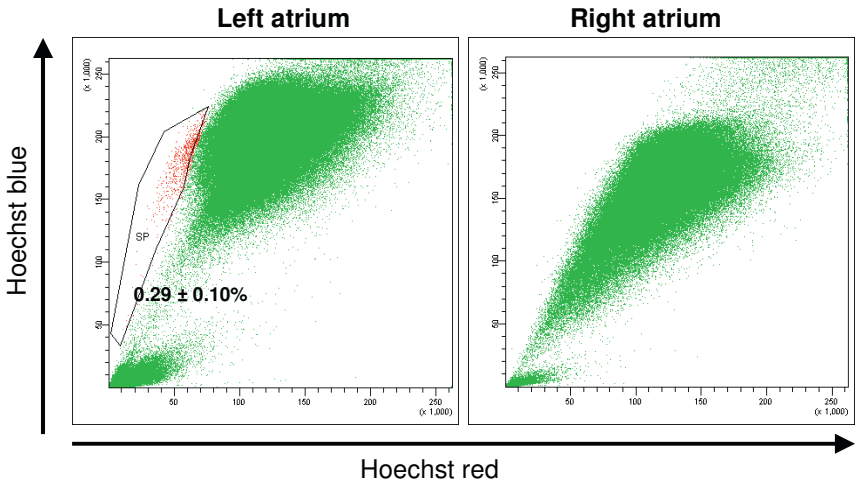


Figure 17. *SP analysis by FACS. Comparison between cells derived from the left and the right atrium. A gate is drawn around the SP population in the left*

In parallel with the identification and characterization of cardiac C-kit+ cells, an assay for identification of cells based on the ability to efflux Hoechst 33342 was developed. Right and left atrial biopsies were dissociated and stained with this assay for identification of SP cells. In cells derived from right atrial biopsies, no clearly distinguishable SP population could be identified. On the other hand, in cells derived from left atrial biopsies, a SP population was observed (Figure 17). Cells from left atrium were further analyzed by known inhibitors of Hoechst 33342 efflux. It was observed that while ABCG2 inhibitor FTC did not affect the SP population, the SP

population was partially decreased by treatment with MDR1 inhibitor Verapamil. The SP was almost completely abolished by treatment with a combination of sodium azide and 2-D-Deoxyglucose, which work as a general inhibitor of metabolism (Figure 18A). Control cell lines known to highly express ABCG2 and MDR1 respectively were also included. In both of these cell lines, Hoechst 33342 efflux was attenuated by treatment with sodium azide and 2-D-Deoxyglucose. As expected, the SP in the MDR1 control cell line was only affected by Verapamil treatment while the ABCG2 control cell line was only affected by FTC treatment (Figure 18B - C) By co-expression analysis, SP cells were found to express hematopoietic marker CD45 to a variable degree.

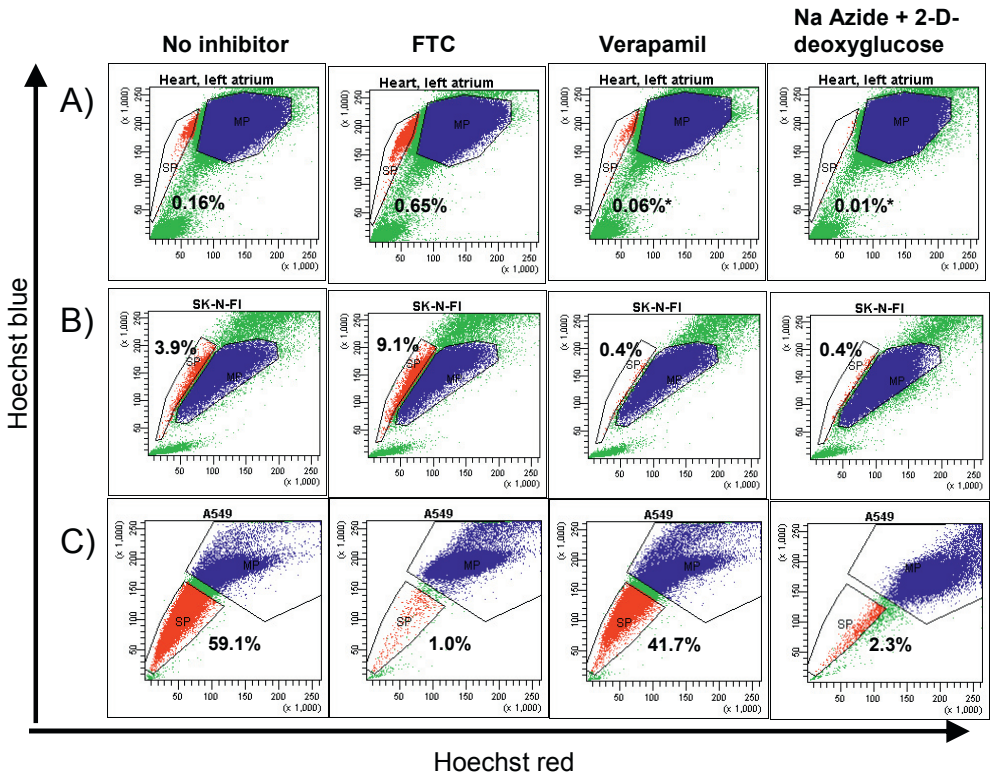


Figure 18. *Inhibitor analysis of SP by FACS. FTC, Verapamil and sodium azide with addition of 2-D-Deoxyglucose were compared with samples with no added inhibitor. Gates for SP cells were set to get a minimum of positive events in the inhibitor-treated samples. Percentages for one representative experiment are shown. A) Cardiac cells derived from the left atrium. * denotes a significant difference ($p < 0.05$) between inhibitor and untreated sample. Statistical calculations were performed using the paired Student's t-test. B) SK-N-FI cell line expressing MDR1. C) A549 cell line expressing ABCG2.*

SP and other cells except SP (named main population, MP cells) were sorted for qPCR analysis. In line with inhibitor results, a high *MDR1* gene expression was observed in the sorted SP cells. Furthermore, expression of stem cell associated genes *C-KIT* and *OCT4* were significantly higher expressed in SP cells compared to MP cells. When SP cells were subdivided based on CD45 expression and both these subpopulation were sorted, it was found that the SP CD45- population expressed higher levels of endothelial genes compared to SP CD45+ cells and MP cells. The expression of cardiac genes on the other hand was low or undetectable in both MP and SP cells, regardless of CD45 expression status. Expression of *C-KIT* was noted to be mostly confined to the CD45+ subpopulation.

4.5 Expression of SSEAs in directly isolated and monolayer cultured cells derived from human adult heart biopsies (Paper IV)

To characterize expression of SSEAs in the human adult heart, biopsies from left and right atrium from patients undergoing Maze surgery as well as biopsies from right atrium from patients undergoing surgery of other causes were obtained, dissociated and analyzed by FACS. Both right and left atrial biopsies had a clear expression of SSEA-4 and SSEA-3. Expression of SSEA-1 on the other hand was generally low (Figure 19A). These SSEA-1+ cells stained dimly for CD45 and thus probably consisted of contaminating hematopoietic cells. SSEA-4 expressing cells were also detected in biopsies from patients not undergoing Maze surgery, although the percentage was lower compared to biopsies from patients undergoing Maze.

When cells derived from cardiac biopsies were monolayer cultured, expression of SSEA-4 and SSEA-3 could still be identified. Furthermore, expression of SSEA-1 was markedly upregulated.

4.6 Subdivision of the SSEA-4+ cardiac population based on CD34 expression reveals a potential cardiomyocyte progenitor population (Paper IV)

In analyses of co-expression, it was noted that the SSEA-4+ cardiac population could be subdivided based on expression of EPC and hematopoietic progenitor marker CD34 (Figure 19B). When the SSEA-4+CD34- population was FACS sorted for qPCR from right atrial biopsies, it was found to have a high expression of both early and late genes indicative of the cardiomyogenic lineage (Figure 20). This pattern was not observed when SSEA-4+CD34- cells from left atrium were analyzed. Expression of endothelial genes was low in the SSEA-4+CD34- population regardless of location, but high in the SSEA-4-CD34+ population. When SSEA-4+CD34- cells were isolated after a primary monolayer culture, expression of cardiac genes was

lost in the right atrial derived cells.

Expression of SSEA-4 was also investigated in tissue sections by immunohistochemistry. Expression could be identified both within and outside the myocardium as defined by cTnT staining. Similarly to analysis of directly isolated cells by FACS, most SSEA-4+ cells were found to be CD34+.

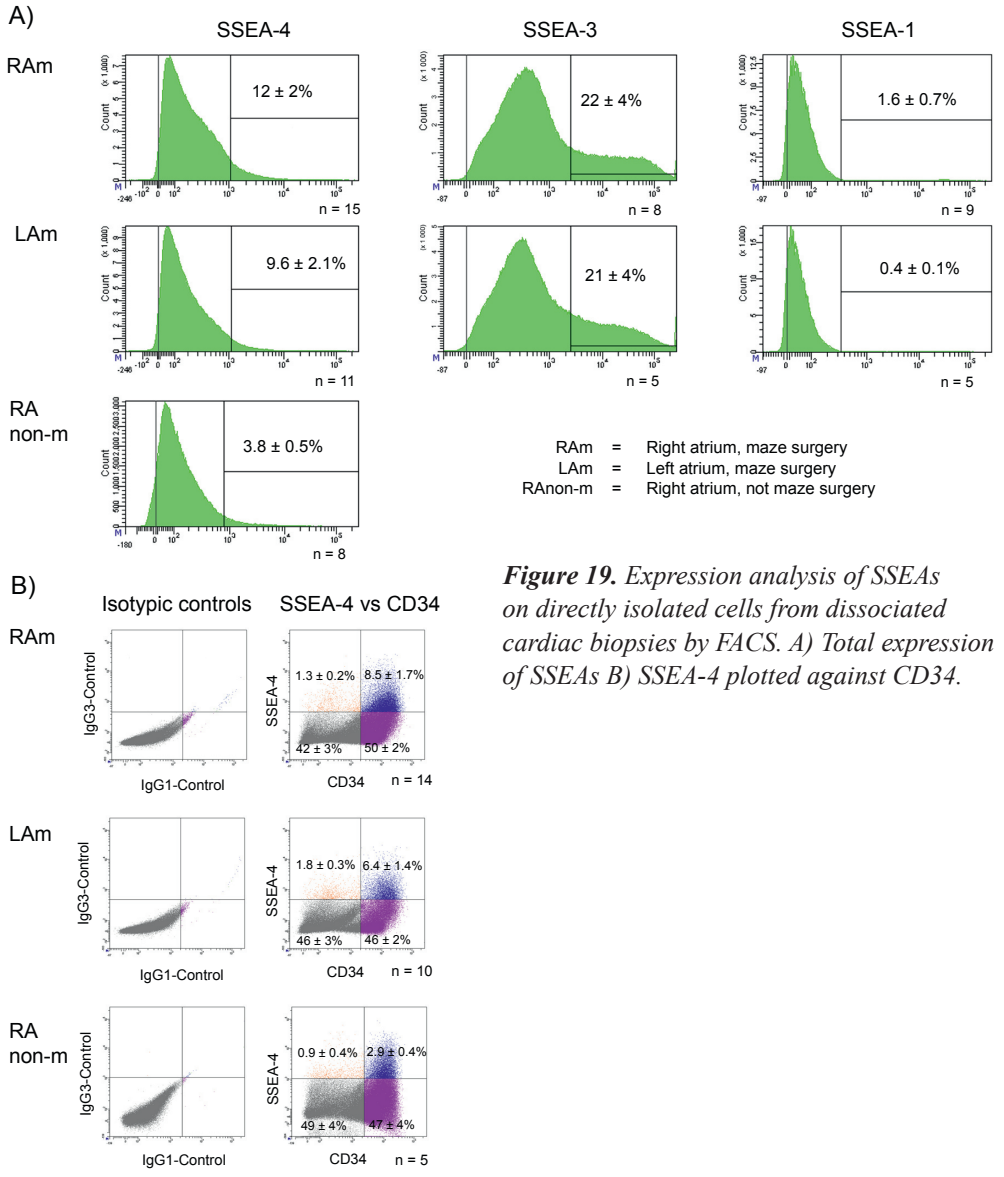


Figure 19. *Expression analysis of SSEAs on directly isolated cells from dissociated cardiac biopsies by FACS. A) Total expression of SSEAs B) SSEA-4 plotted against CD34.*

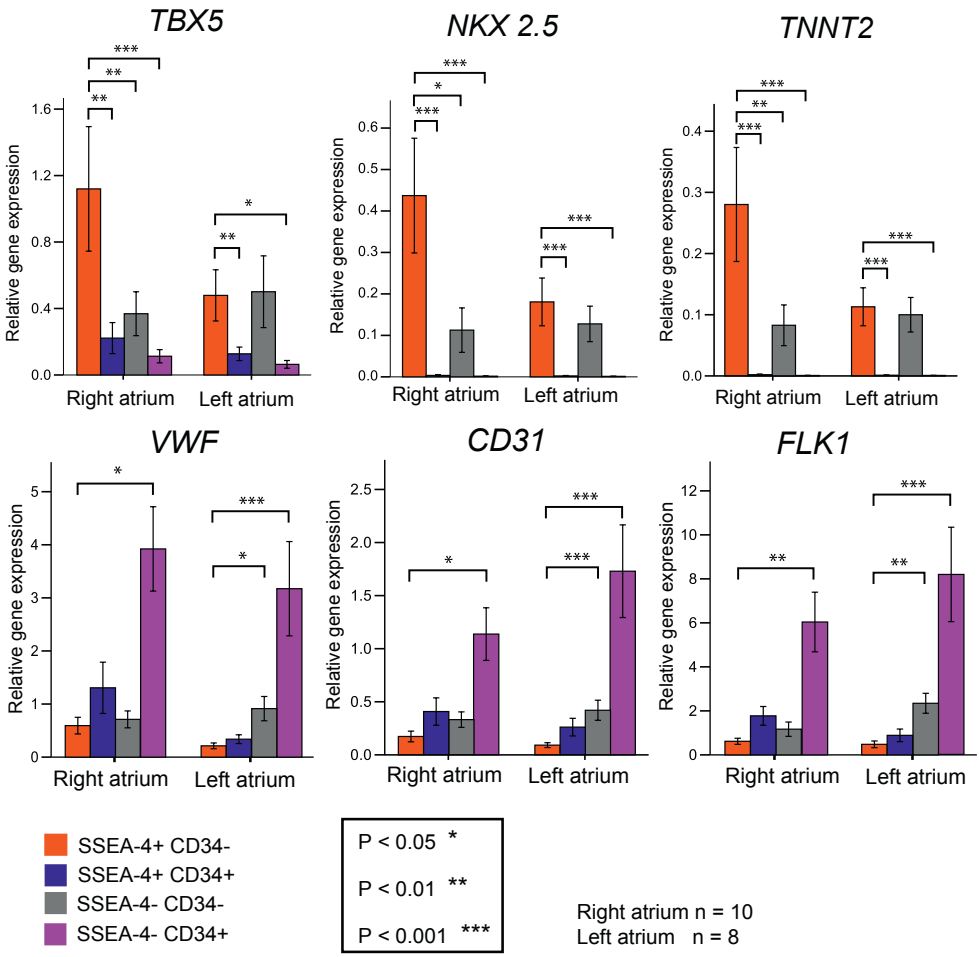


Figure 20. *Cardiac and endothelial gene expression analysis of sorted cells from dissociated cardiac biopsies when SSEA-4 was plotted against CD34. Mean relative values for the different populations ± SEM are shown. Statistical calculations, where the SSEA-4+CD34- population was compared to the other populations, were performed using Student's t-test, pair wise comparison.*

4.7 Relationship between SSEA-4+ cells, C-kit+ and SP cardiac progenitor cells (Paper IV)

By FACS analysis, the C-kit+CD45- population derived from right atrial biopsies was found to be enriched in SSEA-4 expression both when directly isolated from tissue and after monolayer culture. These cells were predominantly CD34+. Notably, as the C-kit+CD45- population identified in directly isolated cells was much smaller compared to the SSEA-4+ population, only a minor portion of the SSEA-4+ cells were C-kit+.

As described above, cardiac SP cells could only be identified in directly isolated cells from left atrial biopsies. When these were co-stained for SSEA-4 expression, no expression was found in the SP cells.

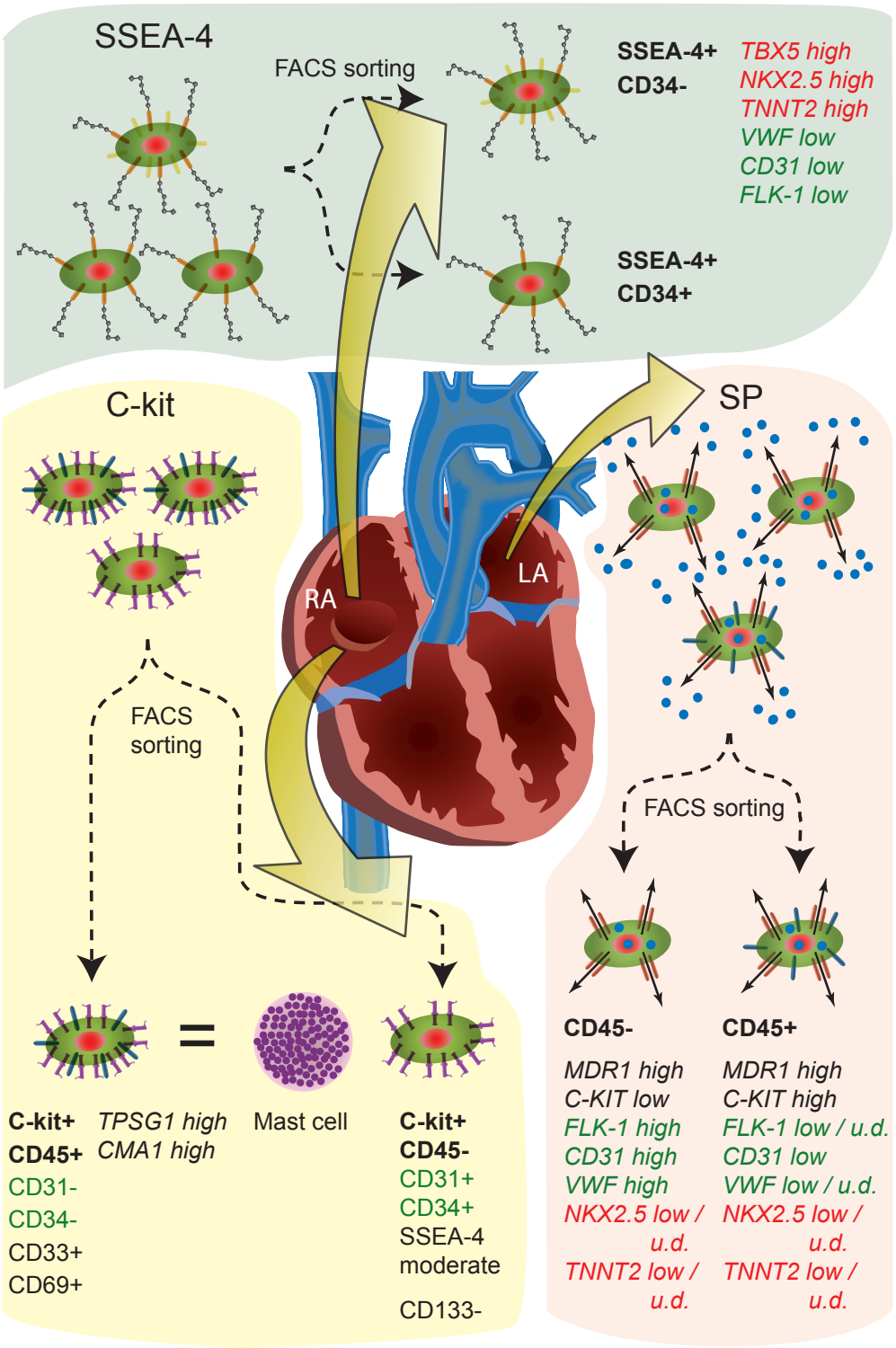

SSEA-4
FACS sorting
SSEA-4+
CD34-
TBX5 high
NKX2.5 high
TNNT2 high
VWF low
CD31 low
FLK-1 low
SSEA-4+
CD34+
C-kit
SP
RA
LA
FACS sorting
FACS sorting
=
C-kit+
CD45+
CD31-
CD34-
CD33+
CD69+
TPSG1 high
CMA1 high
Mast cell
C-kit+
CD45-
CD31+
CD34+
SSEA-4
moderate
CD133-
CD45-
MDR1 high
C-KIT low
FLK-1 high
CD31 high
VWF high
NKX2.5 low / u.d.
TNNT2 low / u.d.
CD45+
MDR1 high
C-KIT high
FLK-1 low / u.d.
CD31 low
VWF low / u.d.
NKX2.5 low / u.d.
TNNT2 low / u.d.

***Figure 21 (overleaf)**. Summary of the major molecular characteristics and distribution of progenitor cells in the human heart based on the results in paper I - IV. Characterization on protein level is indicated by roman typeface whereas characterization on gene levels are indicated by italic typeface. Markers that were used for isolation of the cell populations are indicated by bold typeface. Furthermore, markers of endothelial differentiation are written in green whereas markers of cardiac differentiation are written in red. RA, right atrium; LA, left atrium.*
In the upper part of the figure, the subdivision of SSEA-4+ cells based on co-expression of CD34 is shown. Although SSEA-4+CD34- cells could be identified in both right and left atrium, only cells derived from the right atrium showed evidence of cardiomyogenic commitment. In the lower left of the figure, subdivision of C-kit+ cells based on hematopoietic marker CD45 is shown. In paper II, differentiation status of the C-kit+CD45- population was further investigated on single cell level. For a summary of these results, please se Figure 22. In the lower right part of the figure, the subdivision of cardiac SP cells based on the hematopoietic marker CD45 is shown.

5 DISCUSSION

The aim of this thesis has been to identify, isolate and further characterize cell populations from human cardiac tissue with stem / progenitor properties. Based on previously published work, mostly carried out in animal models, we have focused on the cardiac C-kit+CD45- cells, SP cells and cells expressing SSEAs. Since the percentage of progenitor cells in the cardiac tissue is very low, FACS analysis and sorting has been the main method of identification and isolation of cells of interest. A summary of the results in regard of molecular characteristics and location of these cell populations are found in Figure 21.

5.1 The cardiac C-kit+ population predominantly consists of CD45+ mast cells

In paper I, a population of C-kit+ cells was identified in directly isolated cells from human atrial tissue. C-kit is a well known marker for stem cells in the hematopoietic system (125). In previous studies, cardiac C-kit+ cells have been isolated both from small (126-128) and large (129) animals as well as from the human (131). These cells have been described as clonogenic and multipotent with the ability to differentiate into cardiomyocytes, endothelial cells and smooth muscle cells. In paper I, we observed that most of the C-kit+ cells were also CD45+. This was even more pronounced in paper II, where an epitope regeneration treatment was used. This method enhances the detection sensitivity of cell surface antigens including C-kit. The C-kit+CD45+ cells were found to co-expression cell surface antigens CD33 and CD69 to a high degree and on gene expression level expressed *TPSG1* and *CMA1*. All these markers have previously been associated to mast cells (145, 277) which strongly suggest that the C-kit+CD45+ cells identified in paper I and II have

a mast cell identity.

In addition to the C-kit+CD45+ population, a much smaller population C-kit+CD45- cells of less then 0.1% of the total cell population was identified. This is in contrast to a study by Bearzi et al. (131), which found a much higher percentage of about 1% of C-kit+CD45- cells in similar human cardiac tissue samples. The reason behind this discrepancy is unclear. Surprisingly, in the study by Bearzi et al., virtually no C-kit+ cells co-expressed CD45. On the other hand, when the density of mast cells were determined in dissociated human atrial tissue, about 0.5 - 1.5% of the total cell suspension was determined to have a mast cell phenotype (145). Furthermore, when identity of C-kit+ cells in the human atrium was investigated by co-expression analysis for a panel of mast cell markers, the majority of all C-kit+ cells (85 - 100%) was determined to have a mast cell identity (146). This is also in agreement with a study by Kubo et al. (138) who observed a dim expression of CD45 in most C-kit+ cells isolated. Taken toghether, it thus seems like the study by Bearzi et al. underestimates the number of C-kit+CD45+ mast cells and that these are included in the C-kit+CD45- population. This could potentially be a result of usage of different antibodies to CD45 binding to different epitopes of the protein with different susceptibility to dissociation associated destruction. In the study by Kubo et al. (138), differentiation of C-kit+CD45+ cells into cardiomyocytes was shown. Since it is highly unlikely that mast cells would be able to transdifferentiate in this manner, this could be potentially be explained by a small non-mast cell subpopulation present within the C-kit+CD45+ population. Another possible explanation to the results Kubo et al. is contamination by other cells with cardiomyogenic differentiation properties since sorting purity was reported to be not more than 38%.

5.2 Within the human cardiac C-kit+ population, there is also a small population of CD45- cells with predominant endothelial commitment

In both paper I and paper II, a minor portion of the C-kit+ cells directly isolated from atrial biopsies were found to be negative for CD45. These cells to a large extent co-expressed late/mature EPC markers CD34 and CD31 but were negative for early/immature EPC marker CD133. Endothelial commitment within the directly isolated C-kit+CD45- population was also shown by qPCR analysis on groups of cells in paper I.

In contrast to these results, cardiac C-kit+ cells isolated from the rat heart was originally described by Beltrami et al. (126) as mostly consisting of uncommitted stem cells that were clonogenic and multipotent. The C-kit+ cells were negative for endothelial, fibroblast and smooth muscle markers with a minor portion (about 7 - 10%) of the cells expressing transcription factors indicative of early cardiomyogenic

commitment. These results are in line with an immunohistochemical study where C-kit+ cells were characterized as either uncommitted or committed to the cardiomyogenic lineage in patients suffering from hypertrophic cardiomyopathy (130). In another study by Urbanek et al. (278), where cardiac biopsies from patients suffering from ischemic heart failure were examined, both uncommitted C-kit+ cells as well as C-kit+ cells committed to the endothelial, smooth muscle cell and cardiomyogenic lineages were detected. In the study by Bearzi et al (131), C-kit+ cells were isolated from human hearts and were similarly to C-kit+ cells isolated from the murine heart described as clonogenic, multipotent and mostly negative for markers indicative of endothelial or cardiac differentiation.

In later studies, the VEGF receptor FLK-1 has also been suggested as marker for further subdivision of the C-kit+ population into cells with either predominant endothelial (C-kit+FLK-1+ cells) or cardiac (C-kit+FLK-1- cells) developmental potential (135, 136). Notably, in a study of primary cultured cells from human cardiac biopsies, only a minor portion (about 3%) of the C-kit+ cells were described as FLK-1+ (136). Taken together, these previous studies suggest that most C-kit+ cells are either uncommitted or committed to the cardiomyogenic lineage *in vivo*. This is summarized in Figure 22, upper part.

To further elucidate the differentiation state of human cardiac tissue derived cells as close to the *in vivo* situation as possible, a single cell qPCR approach was used in paper II. Although this technique does not allow for detection of protein expression but only gene expression, it has the advantage of being both highly sensitive and specific. In contrast, identification by immunohistochemistry is always more or less subjective and observer dependent. Indeed, over-interpretation of immunofluorescence has for example been suggested as one possible reason to the initially reported very high cardiac differentiation potential of hematopoietic stem cells (50, 66) that in later studies was not reproducible (67, 68). Furthermore, with single cell qPCR, a large number of different genes may be analyzed for the same cell whereas immunohistochemistry only allows for simultaneous analysis of a rather limited number of markers.

In paper II, in contrast to previous studies described above, but in line with the results in paper I (discussed above), most cells with confirmed *C-KIT+CD45-* gene expression showed evidence of endothelial commitment expressing either late endothelial marker *VWF* or *FLK-1*. A high degree of overlap between the *VWF+* and *FLK-1+* population was noted. Very few cells on the other hand expressed *NKX2.5*, a transcription factor expressed in different stages of developing as well as mature cardiomyocytes (17, 279). Furthermore, its expression has been described in a part of the C-kit+ population in several previous studies (126, 134, 139) where it has been interpreted as a sign of early cardiomyogenic specification. The major findings

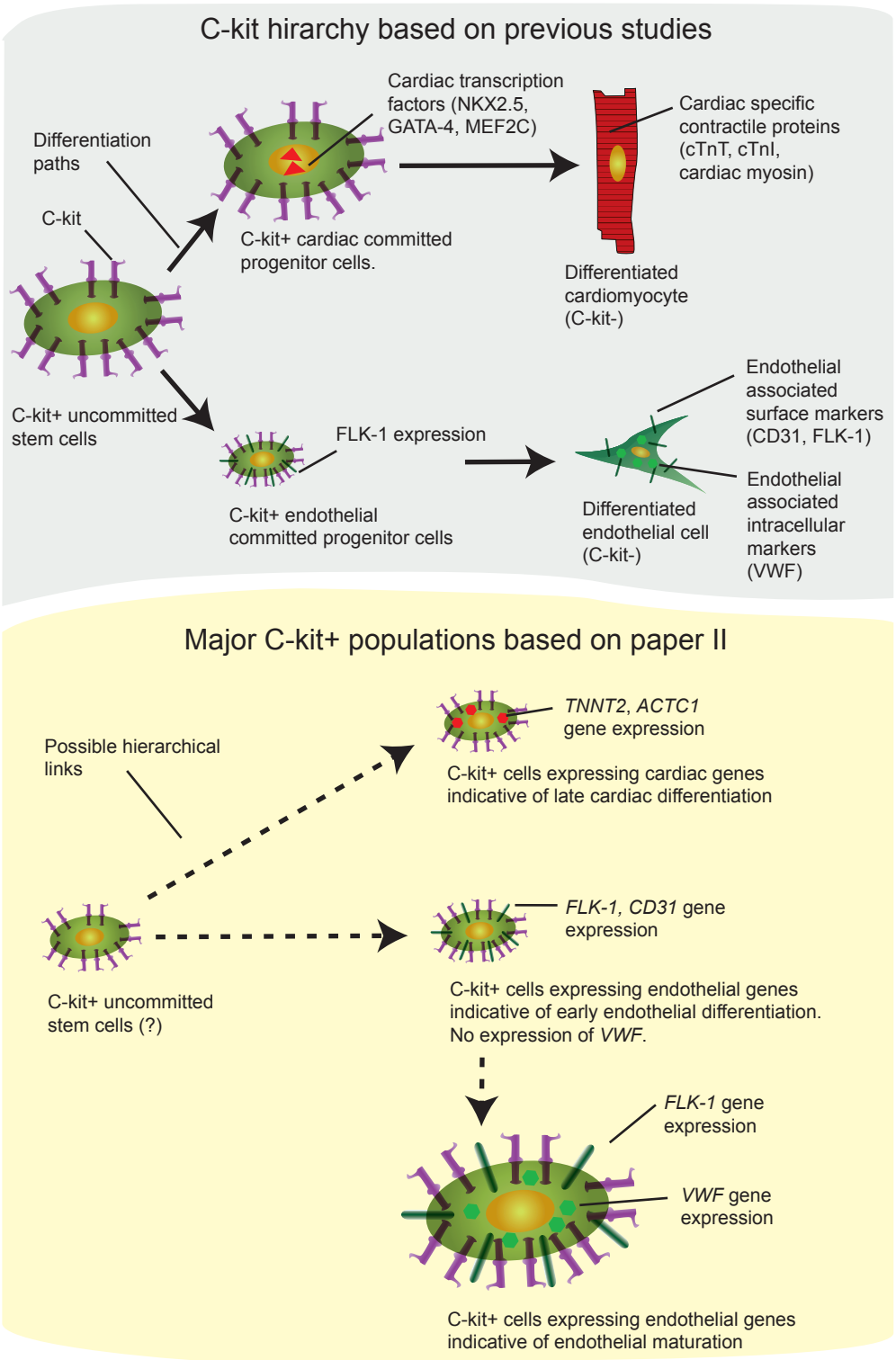

C-kit hirarchy based on previous studies
Differentiation paths
C-kit
C-kit+ uncommitted stem cells
Cardiac transcription factors (NKX2.5, GATA-4, MEF2C)
C-kit+ cardiac committed progenitor cells.
Cardiac specific contractile proteins (cTnT, cTnI, cardiac myosin)
Differentiated cardiomyocyte (C-kit-)
FLK-1 expression
C-kit+ endothelial committed progenitor cells
Endothelial associated surface markers (CD31, FLK-1)
Endothelial associated intracellular markers (VWF)
Differentiated endothelial cell (C-kit-)
Major C-kit+ populations based on paper II
Possible hierarchical links
C-kit+ uncommitted stem cells (?)
TNNT2, ACTC1 gene expression
C-kit+ cells expressing cardiac genes indicative of late cardiac differentiation
FLK-1, CD31 gene expression
C-kit+ cells expressing endothelial genes indicative of early endothelial differentiation. No expression of VWF.
FLK-1 gene expression
VWF gene expression
C-kit+ cells expressing endothelial genes indicative of endothelial maturation

Figure 22 (overleaf). *Schematic illustration of the hierarchy of C-kit+ cardiac progenitor cells. Sizes of the C-kit+ cells indicate an approximate relative fraction of the total C-kit+ population that is made up of that particular sub-population.*
In the upper part of the figure, the C-kit hierarchy based on previous studies (126, 130, 131, 135, 136). Proposed differentiation paths are indicated by arrows. Notably, most of the C-kit+ cells are either uncommitted stem cells or cardiac committed progenitor cells.
In the lower part of the figure, major sub-populations of C-kit+CD45- cells based on the single cell qPCR analysis in paper II are shown. Possible hierarchical links are indicated by dashed arrows. Most of the C-kit+CD45- cells expressed markers indicative of endothelial maturation.

in paper II are summarized in Figure 22, lower part.

The reason behind this difference in commitment of the C-kit+ population between our study and previous studies is not clear. One potential reason could be different disease background of the included patients. In order to obtain large volumes of tissue for isolation of a sufficient number of cells for single cell qPCR, biopsy material from patients undergoing Maze surgery was used. This is a surgical procedure to treat atrial fibrillation or flutter (263). Consequently, although we have not had access to the medical history of the patients, it can be assumed that these patients suffer from either constant or paroxysmal atrial fibrillation or flutter. These conditions have been linked to structural changes within the atria including fibrosis (280). Whether this may affect the C-kit+ population is no known. In patients with ischemic heart failure, it was noted that the number of both endothelial and cardiac committed C-kit+ precursor cells were increased compared to control hearts (278). In the study by Bearzi et al. (131), where human C-kit+ cells were isolated, no data are available on either medical history, cause of surgery or if the biopsies were obtained from atrial or ventricular tissue. In an animal model of isoproterolol induced cardiomyopathy, a clear increase in cardiomyogenic commitment within the C-kit+ population was noted (139). Taken together, no of these previous studies have identified a disease dependent endothelial commitment of the C-kit+ population. It thus seems unlikely that patients suffering from atrial fibrillation or flutter which are not associated with ischemia but caused by improper impulse conduction should have a fundamentally different commitment profile of the C-kit+ population. Furthermore, it should be noted that in paper I, a high expression of endothelial genes was noted in the total C-kit+CD45- population. Importantly, biopsies used for this analysis were derived from patients undergoing other types of surgery than Maze (not disclosed in the published paper). This further argues against that discrepancies between paper II and previous studies in regard of commitment of the C-kit+ population are due to inclusion of different groups of patients.

It should also be noted that there are a few studies of genetic lineage tracing of cardiac C-kit+ cells that similarly to us have found a predominant endothelial commitment

in this population. In a study by Jesty et al. (281), where differentiation potential of C-kit+ cells were investigated both in the neonatal and adult mouse heart, only C-kit+ cells isolated from neonatal mice could differentiate into cardiomyocytes. Adult cardic C-kit+ cells were however still able to contribute to an angiogenic response in the context of myocardial infarction. In another study by Tallini et al. (282), expression of C-kit after myocardial infarction was associated with fibrous and vascular repair, but not cardiomyogenic differentation in the adult mouse.

5.3 Single cell qPCR reveals subgroups of cells within the VWF negative C-kit+CD45- population

In paper II *VWF*- and *NKX2.5*+ cells of the *C-KIT+CD45*- population were analyzed for a wider panel of genes and subjected to 2 dimensional cluster analysis. This revealed four different groups of cells. The cardiac group was observed to express genes coding for structural proteins indicating late cardiac development. Surprisingly, very few cells expressed early transcription factors *NKX2.5*, *GATA-4* or *MEF2C*. One potential explanation to this could be methodological reasons. While single qPCR is sensitive and specific, it could potentially fail to detect genes with very low copy number. Thus, if structural genes are expressed more abundantly than transcription factors, these would be detected while expression of transcription factors would be underestimated. Another explanation could be re-expression of C-kit in already mature cardiomyocytes. Although mature cardiomyocytes may express transcription factors such as *NKX2.5*, expression decreases with increased age and was only noted in a fraction of all cardiomyocytes in the human adolescent heart (22). In the study by Tallini et al. (282), expression of C-kit was noted in already terminally differentiated cardiomyocytes. The classification of cardiomyocytes as terminally differentiated was based on mature cTnT staining and a lack of mitotic figures or pHH3 staining indicative of cell cycle activity. On the other hand, in a study on *in vitro* expanded rat cardiomyocytes, de-differentiation of these cells was noted which was accompanied by expression of C-kit (283). While it is highly improbable that the cells included in the single cell qPCR study would represent mature cardiomyocytes due to multiple filtration steps prior FACS analysis, it can not be ruled out that they represent de-differentiated cardiomyocytes with smaller cell size that potentially could pass through the filtering process. In a study by Mollova et al. (13), proliferating cardiomyocytes were indeed observed also *in vivo*, in the human heart. Although no detailed analysis of size of these cycling cardiomyocytes was carried out, based on the presented images they seem to be about 100 µm of size. This would argue that these cells would have been excluded during the filtering process prior FACS analysis. However, notably, as newly formed cardiomyocytes derived from cellular division logically would be only half the size of the parent cell, these could theoretically in some cases pass through the smallest filter of 40 µm. It should also be noted that it is still unknown whether cycling cardiomyocytes *in vivo* upregulate C-kit in any part of this process.

In paper II, there was also one group of cells that did not express *VWF* but instead showed expression of other endothelial genes (*CD31, FLK-1*). In both the maturation of EPCs and the differentiation of human ES cells into endothelial cells, expression of endothelial markers such as CD31 and FLK-1 have been shown to precede the expression of VWF (284, 285). Thus, the group of *VWF*- cells with expression of other endothelial genes could be regarded as a more undifferentiated endothelial progenitor population compared to the group of *VWF*+ cells. Within the group of *VWF*- cells with expression of other endothelial genes, a few cells also expressed cardiac genes which could be interpreted as cells with bi-potent differentiation capacity. Interestingly, there was also one group of cells expressing no other genes than *C-KIT*. It could be hypothesized that this group consists of cells not committed to any lineage of differentiation. Although no expression of stem cell associated marker *OCT-4* was identified, this has also been noted previously in for example Sca-1+ progenitor cells in the mouse (286) and human fetal heart (156). Thus, it may not be a prerequisite in cardiac progenitor populations.

5.4 Expanded C-kit+CD45- cells showed endothelial differentiation potential but could not be induced to differentiate into the cardiomyogenic lineage

In paper I, C-kit+CD45- cells isolated after monolayer culture expressed markers indicative of endothelial commitment both on protein level by FACS and on mRNA level by qPCR analysis. When these cells were cultured in an endothelial growth media, expression of CD34 and C-kit were downregulated which could be interpreted as further endothelial maturation. As a further evidence of endothelial maturation of the C-kit+CD45- population, it was noted in a pilot experiment that these cells could form capillary structures *in vitro* (unpublished data).

Cardiomyogenic differentiation was also tested in paper I, on isolated C-kit+CD45- cells, with a protocol based on 5-Azacytidine treatment together with TGF-ß1. This protocol has previously been used to induce differentiation of Sca-1+ progenitors into cardiomyocytes (268). Notably, 5-Azacytidine treatment has also been used to induce cardiomyogenic differentiation of C-kit+ cells (133). In paper I however, no evidence of cardiomyogenic differentiation was observed. In addition to 5-Azacytidine treatment, other agents previously shown to induce cardiomyogenic differentiation including dexamethasone (126) and oxytocine (153) were tested in pilot experiments during the work with paper I. No sign of cardiomyogenic differentiation was however noted (data not shown).

C-kit+CD45- cells have previously been described as multipotent with the ability to differentiate *in vitro* into both endothelial, smooth muscle cells and cardiomyocytes.

This has been shown in small animals by Beltrami et al. (126), large animals by Linke et al. (129) and in human by Bearzi et al. (131), D'Amario et al. (136) and Itzhaki-Alfia et al. (133). In all of these studies except that of Itzhaki-Alfia et al., dexamethasone appears to have been used as a general inducer of differentiation into all three lineages. In the study by Itzhaki-Alfia et al. on the other hand, 5-Azacytidine was used to selectively promote differentiation into the cardiomyogenic lineage. The reason behind this discrepancy in differentiation potential between paper I and the studies listed above is not clear. Possible explanations however include different methods of expansion and isolation of C-kit+ cells, different cellular composition, different biopsy locations and contamination of other types of cells. Each of these topics will be discussed below.

In previous studies, different methods of expansion of C-kit+ cells have been used. In the study by Bearzi et al., C-kit+ cells were either isolated from directly dissociated tissue or from explant cultures. On the other hand, in the study by D'Amario et al., C-kit+ cells were isolated from monolayer cultured cells similarly to the method used in paper I. Thus, it does not seem like the method of expansion affects the differentiation capacity of the C-kit+ cells.

When it comes to cellular composition of the isolated C-kit+ population, it is notable that in the study by D'Amario et al., only a minor fraction of the C-kit+ cells (about 3%) were observed to express FLK-1. These predominantly were able to differentiate into endothelial cells and smooth muscle cells but also cardiomyocytes upon addition of dexamethasone. The vast majority of FLK-1 negative cells on the other hand preferentially adopted cardiomyogenic fate with a minor portion differentiating into endothelial cells and smooth muscle cells. Although no subdivision of C-kit+ cells based on FLK-1 was carried out in paper I, this does not seem to be a likely explanation why no cardiomyogenic differentiation was observed since also in this study, the majority of the C-kit+CD45- cells (about 80%) were FLK-1 negative as determined by FACS. Furthermore, in the study by D'Amario et al. (136), C-kit+ cells did not express other endothelial markers such as CD31 prior dexamethasone treatment. This was regardless of FLK-1 expression status. In paper I, on the other hand, nearly 100% of the C-kit+CD45- cells co-expressed CD31 as well as other markers associated with the endothelial lineage when isolated from primary monolayer cultured cells. Importantly, in paper I, a similar high expression of endothelial markers in the C-kit+CD45- population was also observed in directly isolated cells. Thus, it can not be regarded as an effect of the *in vitro* culture system.

Another possible explanation to the difference in differentiation capacity of the C-kit+ population between paper I and the study by D'Amario et al. could be different biopsy locations. In the study by D'Amario et al., endomyocardial biopsies were used whereas in paper I, whole wall atrial biopsies were used. In the study by Bearzi

et al. (131) no data were available on either patient characteristics or location origin of the biopsies. Thus, it is not possible to tell whether different biopsy locations may be an explanation to different differentiation capacity compared to paper I.

In the study by Itzhaki-Alfia et al. (133), monolayer expanded human cardiac cells were analyzed for C-kit expression and used for *in vitro* differentiation. *In vitro* differentiation into cardiomyocytes was confirmed by positive staining for cTnT. Notably, in this study, cells in passage 3 were used whereas in paper I primary cultured cells were used. Furthermore, as judged by the C-kit FACS data presented, background staining seems rather high and thus the cells sorted as C-kit+ may have been contaminated by cells false positive for this antigen. Furthermore, about 60% of all monolayer cultured cells showed evidence of cardiomyogenic differentiation based on GATA-4 and cardiac alpha actin positive staining. Thus, it is possible that the cTnT+ cells observed after 5-Azacytidine treatment were the result of contaminating cells already positive for cardiac markers but negative for C-kit.

5.5 Characterization of SP cells in the human adult heart

SP was originally described by Godell et al. (175) as a population of highly potent hematopoietic stem cell population within the bone marrow. Thereafter, SP cells have been described in a number of tissue specific stem / progenitor cells (176-178). In the heart, SP cells have been characterized as progenitor cells in the murine system (182, 183, 185). In the human, SP cells have previously only been identified in fetal cardiac tissue. Although expression of both MDR1 (130) and ABCG2 (174, 198) have been shown in the human adult heart, no functional assay of Hoechst 33342 efflux had been carried out. This was done in paper III, where for the first time, it was shown that also the human adult heart contains a population of SP cells. These cells could only be identified in left atrium which will be further discussed in the section 5.8.

In previous studies of SP stem / progenitor cells, the ability of these cells to exclude Hoechst 33342 has been linked to expression of MDR1 and / or ABCG2 which are both ABC transporter proteins. In mouse cardiac SP cells, a dynamic expression profile of these transporter proteins was found depending on age. While the SP phenotype was predominantly determined by ABCG2 expression in early post-natal mice, this was shifted towards MDR1 dependence with increased age. In paper III, ABC transporter dependence of the human adult cardiac SP population was extensively investigated by inhibitor analysis, FACS and qPCR. In the inhibitor analysis, Verapamil was used as an inhibitor to MDR1 while FTC was used as an inhibitor for ABCG2. In addition, sodium azide together with 2-D-Deoxyglucose was used as a general inhibitor to energy dependent transport systems. The ability of cardiac cells to efflux Hoechst 33342 was attenuated by Verapamil and sodium azide and 2-D-Deoxyglucose treatment respectively while it was not affected by FTC

treatment. This points toward MDR1 as the predominant efflux protein in human adult cardiac SP cells similar to adult mouse cardiac SP cells. Notably, in a previous study of human neonatal cardiac tissue, the SP population was in contrast to our study attenuated by treatment with FTC. No other inhibitors were tried (195). This indicates that also in the human heart, there is a shift from ABCG2 to MDR1 as the dominant efflux protein of the SP with increased age. In paper III, a high expression of MDR1 in the cardiac SP was shown on mRNA level by qPCR analysis. In accordance with the inhibitor analysis, no difference in expression of ABCG2 was noted between the SP cells and all other cells. On the other hand, surprisingly, no expression of either ABCG2 or MDR1 was observed on protein level by FACS co-expression analysis. Although it can not be fully excluded that the Hoechst 33342 efflux in human SP cells is caused by another ABC transporter protein previously not described in cardiac SP cells, a more likely explanation to this difference between gene and protein expression data would be destruction of epitopes during tissue dissociation. This has also been observed as a problem in paper I and II. Notably, in paper III, no epitope regeneration treatment was used as this was not necessary for the SP assay. Although the epitope regeneration treatment used in paper I and II can enhance detection of cell surface antigens, it unfortunately also results in loss of cells. As the SP assay in itself also results in loss of cells due to aggregation, cell yield was prioritized over the potential benefits of using an epitope regeneration treatment step for protein identification.

It should also be noted that although the results in paper III clearly indicates MDR1 as the major determiner of the human adult SP phenotype, as mentioned above, ABCG2+ cells have previously been identified in the human adult heart by immunohistochemistry in a study by Emmert et al. (174) and Wohlschlaeger et al (198). The reason why these cells apparently do not have a SP phenotype is unclear. Although most focus on regulation of Hoechst 33342 efflux have been on differential expression of different efflux proteins as well as the effects of inhibitors to these proteins, there may also be other regulatory mechanisms. In study by Zhou et al. (287) it was observed that ABCG2 was located both in the cytoplams and bound to the cell membrane in a tumor cell line. By overexpression of CD147, a transmembrane glycoprotein, localization was shifted to the cell surface. Furthermore, CD147 expression resulted in homodimerization of ABCG2 proteins. Together, this resulted in an enhanced efflux activity of ABCG2 without increased ABCG2 mRNA expression. In another study by Takada et al. (288), activation of the PI3K-Akt signaling pathway was linked to internalization of ABCG2 in LLC-PK1 cells stably overexpressing ABCG2. Interestingly, in the study on cardiac ABCG2 positive cells by Wohlschlaeger et al (198), ABCG2 staining was noted to be cytoplasmatic. This also seems to be the case in the study by Emmert et al. (174) judged by the immunohistochemical pictures presented. It could thus be hypothesized that ABCG2 expression in the adult heart has a predominant cytoplastmic localization and

thus do not contribute to the SP. This underlines the importance of distinguishing between SP which is only defined by the functional assay of Hoechst 33342 efflux, and expression of individual ABC transporter proteins.

In paper III, a variable co-expression of the pan hematopoietic marker CD45 was found in the cardiac SP cells, indicating partial hematopoietic origin. This has previously been observed in the rat (183) but not in the mouse heart (182, 289). CD45+ SP cells were found to express low levels of endothelial genes and tended to have a higher expression of *C-KIT* compared to CD45- SP cells. CD45+ SP cells with a moderate expression of C-kit but negative for CD34 have previously been described in human peripheral blood by Preffer et al. (290). *In vitro,* these cells gave rise to lymphoid and dendritic progeny in an assay of hematopoietic differentiation. Although it could be argued that the CD45+ cardiac SP cells merely represent contaminating peripheral blood, this would not explain why these cells could only be detected in left atrial biopsies. As shown in paper II, the percentage of contaminating CD45+ hematopoietic cells is about the same in both left and right atrial biopsies, indicating a similar degree of peripheral blood contamination regardless of biopsy location. Thus, it seems like the CD45+ SP in human left atrium represent a resident population of cells. Whether these cells adopt hematopoietic fates or possibly contribute to renewal of cardiac cells remains to be determined. Although contribution of hematopoietic cells to regeneration in the heart is highly controversial (as described in further detail in the background section), differentiation of CD45+ SP cells into non-hematopoietic progeny has for example been observed in such cells derived from human liver tissue (291).

The CD45- human cardiac SP cells were on the other hand found to express high levels of endothelial genes. Previous studies of murine cardiac SP cells have showed that these could be subdivided based on expression of endothelial marker CD31. While the CD31- SP cells were able to differentiate into the cardiomyogenic lineage (182), the CD31+ SP cells displayed properties of endothelial progenitor cells (184). Notably, the majority of all SP cells were CD31+ (182). Our qPCR data of sorted CD45- SP cells supports a predominant endothelial commitment of this population also in the human. On the other hand, no evidence of early commitment to the cardiomyogenic lineage was observed. This does however not exclude the possibility of non endothelial progenitor population within the SP CD45- population. Notably, the total SP population had a higher expression of stem cell associated gene *OCT-4* compared to all other cells. This could indicate that within the total SP population, there is s small subpopulation of uncommitted progenitors with a high *OCT-4* expression. Further studies are needed to elucidate possible sub-populations within the human cardiac SP population, potentially with the aid of single cell qPCR analysis.

It should also be acknowledged that while repeatedly tried, it was not possible to establish a reproducible *in vitro* culture system of directly isolated SP cells. Possible explanations to this include general problems with a long dissociation and staining period which may render the cells functionally impaired. Furthermore, Hoechst 33342 staining has been observed to have toxic side effects on the cells (292, 293). Future studies have to be carried out in order to optimize these conditions further. In this regard, it should however be noted that it may not be possible to substantially lower the concentration of Hoechst 33342 since the fluorescence properties of this dye in the "blue" and "red" spectra may depend on a certain concentration (272). As an alternative approach it might be possible to exchange Hoechst 33342 for another non-toxic substrate of MDR1 efflux such as rhodamine 123 (292).

5.6 SSEAs are expressed in the human adult heart where a subpopulation of SSEA-4+CD34- cells may represent a cardiomyogenic progenitor population

SSEAs were originally described as markers for different stages in embryonic development and differentiation of embryonic stem cells. In more recent years, there have also been some reports regarding expression of SSEAs in adult stem cells (82, 168-171). In the murine heart, SSEA-1+ cells have previously been described as a population with progenitor properties (172). In the human fetal, neonatal (173) and recently also adult (174) heart, SSEA-4+ cells have been identified in tissue sections by immunohistochemistry. However, these cells were not quantified or characterized further. In paper IV, the existence of SSEA-1, 3 and 4 positive cells in both left and right atrium of the human heart is shown by FACS analysis. The SSEA-1+ population also dimly expressed pan hematopoietic marker CD45. As this antigen is also known to be expressed by certain granulocytic populations (166, 294), these cells probably represent contaminating hematopoietic cells.

In co-expression analyses with EPC and hematopoietic progenitor marker CD34, it was noted that the SSEA-4+ population could be subdivided based on expression of this antigen. While the majority of SSEA-4+ cells co-expressed CD34, there was also a smaller subpopulation of SSEA-4+CD34- cells. When this population was isolated from directly isolated cells derived from right atrium, it showed a very high expression of both early and late genes associated with cardiomyogenic development. Expression of endothelial genes was on the other hand generally low. Taken together, these results indicate that the SSEA-4+CD34- population may harbor cells specified to the cardiomyogenic lineage. This would be in line with previous results from the murine heart where cardiomyogenic differentiation potential was shown for SSEA-1+ cells (172). In this context, it should be reminded that SSEA-1 in the murine system has the same expression pattern as SSEA-3 and 4 in the human system (164, 165). In the adult human heart, SSEA-3 and 4 positive cells

were recently described by Emmert et al. (174) as present in vessels and as single cells co-staining for endothelial marker CD31 by immunohistochemistry. In paper IV, to the contrary, qPCR analysis of several endothelial genes including CD31 showed generally low expression in SSEA-4+ cells with a tendency toward higher expression in the SSEA-4+CD34+ population compared to the SSEA-4+CD34- population. One possible explanation to the discrepancy between our results and the results by Emmert et al. could be that different locations of the heart were used for SSEA-4 profiling. Furthermore, our results does not exclude the possibility of some cells within the SSEA-4+ population being committed to endothelial lineage as these cells were not entirely negative for endothelial gene expression. In particular, the SSEA-4+CD34+ population as described above, tended to have a higher expression of endothelial genes compared to the SSEA-4+CD34- population which potentially could be explained by some of the cells in the SSEA-4+CD34+ population being committed to the endothelial lineage. Future studies by for example single cell qPCR are needed to elucidate this potential heterogeneity further.

Although *in vitro* culture of directly isolated SSEA-4+CD34- cells was tried, it unfortunately proved to be difficult to set up such culture system in a reproducible manner. As discussed in the previous section (5.5), this may be due to a combination of a long dissociation and staining procedure which rendered the cells functionally impaired. To circumvent the problems with culturing of directly isolated FACS sorted cells and to potentially increase the number of SSEA-4+CD34- cells available for sorting, a mixed monolayer culture system similar to that used in paper I was tried. Also in monolayer cultured cells, all SSEAs could be identified. SSEA-1 was markedly upregulated while SSEA-4 was a bit donwnregulated. The SSEA-4+ population could still be subdivided based on CD34 expression. However, in contrast to directly isolated cells, the expression of genes indicative of cardiomyogenic differentiation was lost in the SSEA-4+CD34- population. In paper I, it is shown that monolayer culture of the cardiomyocyte depleted fraction of cardiac cells may profoundly alter cellular composition and increase the percentage of C-kit+ cells. It is thus not unreasonable that this system also may alter the differentiation path of the SSEA-4+CD34- cells or favor the growth of a subpopulation of non-cardiomyogenic progenitors within this population. It could be speculated that either cell-cell contact or paracrine signaling from mature cardiomyocytes may be necessary for cardiomyogenic specification of the SSEA-4+CD34- population. This is in line with previous studies showing induction of cardiomyogenic differentiation of stem cells by co-culture with cardiomyocytes (91, 295, 296).

5.7 Relationship between different stem / progenitor populations in the adult heart

As described in the background section, many putative cardiac progenitor populations have been described during the last decade. These include C-kit+ (126-129, 131), Sca-1+ (152, 153, 155), Islet-1+ (35-37, 39) and SSEAs+ (172, 173) cells as well as cells identified based on functional assays such as SP (181-183) and Aldehyde bright (224). Furthermore, there is also a population of epicardium derived progenitor cells (210, 211) which have been associated with Wt-1 expression (210, 212-214). Whether these populations overlap each other or are hierarchically linked have however not been fully elucidated, especially in the human.

5.7.1 Relationship between C-kit+ cells and other progenitor populations

In paper I, C-kit+ cells were identified in human atrial biopsies. In this study, both directly isolated and monolayer cultured cells were also stained for ABCG2, MDR1, Sca-1 and CD133 but were found to be completely negative for these antigens. In contrast to these results, in a study by Urbanek et al. (278), C-kit, MDR1 and Sca-1 positive cells were all identified in the human heart by immuohistochemistry. The majority of these cells (about 60%) expressed all three of these antigens. A similar degree of co-expression was observed by the same group in the dog (129) and the mouse heart (127). When cells expressing either one, two or all three of these antigens were isolated from dog heart and compared to each other, all cell populations had similar cloning efficiency and gave rise to approximately the same fraction of cardiomyocytes, endothelial cells and smooth muscle cells. However, it was noted that cells only expressing C-kit gave rise to a higher total number of differentiated cells (129). Other studies of mouse cardiac Sca-1+ cells did however not detect any co-expression of C-kit within this population (152, 286). Furthermore, in a study by Dey et al. (297) where C-kit+, Sca-1+CD31-CD45- and SP cells respectively were isolated from mouse hearts and analyzed by micro-array, the C-kit+ population was found to be the most distinct and least correlated with the other two progenitor populations. The total Sca-1+ cardiac population was also noted to be about 3 times as large as the C-kit+ population. Both these results strongly argue against a large overlap between these two populations.

It should be acknowledged that as others have identified Sca-1+ cells in the human heart by immunohistochemistry (155, 278), it can not be excluded that the lack of Sca-1 expression in paper I may be a result of dissociation associated epitope destruction. As it is not known which human protein that is detected by antibodies with reactivity to the mouse Sca-1 protein, it was unfortunately not possible to investigate Sca-1 expression on gene level in sorted cells.

As mentioned above, a high overlap between C-kit and MDR1 was observed in the human heart by Urbanek et al. (278). This is in contrast to paper I where no expression of MDR1 was found on either protein or gene expression level in directly isolated C-kit+CD45- cells. MDR1 expression has previously been linked to the SP phenotype in the adult mouse heart (187) and was also found to be the major determinant of the human adult SP population in paper III. Previous studies of SP cells, isolated from the adult murine heart have shown no or very little co-expression of C-kit (152, 182, 184, 289). Although one of these studies stated dissociation associated epitope destruction as a potential reason of no C-kit expression (182), taken together, these studies argues against a significant overlap between MDR1 and C-kit. Furthermore, in the human heart, the C-kit+CD45- progenitor population was preferentially located to the right atrium (paper II) whereas the SP was only clearly distinguishable in the left atrium (paper III). On the other hand, in paper III, the SP was found to express C-kit significantly higher compared to all other cells. This expression tended to be confined to the CD45+ SP population. In line with these results, in a pilot experiment where SP cells were co-stained for C-kit, a few C-kit+ SP cells were noted that had a dim expression of CD45 (unpublished data). Although as discussed in section 5.1, most C-kit+CD45+ cells represent mast cells, it can not be excluded that a minor portion of these cells consists of hematopoietic derived progenitors. Importantly, however, these cells can not represent the C-kit+MDR1+ cells found in the study by Urbanek et al. (278) as those cells were described as of non-hematopoietic origin. Notably, in a study by Kubo et al. (138), C-kit+ cells with a dim expression of CD45 was sorted from human hearts and were found to be able to differentiate into cardiomyocytes in a co-culture system. Although this could be due to contamination of other cells as the sorting purity was noted to be rather low, another explanation would be the existence of a progenitor sub population within the C-kit+CD45+ population that otherwise predominantly consists of mast cells. As SP is indicative of stem / progenitor properties in many different tissues, it could be hypothesized that this subpopulation consists of C-kit+CD45+ SP cells. Further studies are however needed to elucidate this possible relationship.

For the other ABC transporter protein, ABCG2, which has been linked to SP phenotype predominantly in the early post natal heart (187), we did similarly as for MRD1 not find any expression on either protein or gene expression level in the directly isolated C-kit+CD45- population (paper I). This is in accordance with a previous immunohistochemical study of human cardiac tissue where no co-expression between ABCG2+ cells and C-kit+ cells were observed, although these cells were sometimes found in close proximity to each others (174).

In the embryonic heart, Islet-1 has been identified as a marker for progenitor cells of the secondary heart field (35-37). Islet-1+ cells have been identified in human fetal heart (298), but there are no data on Islet-1+ cells in the human

adult heart. Interestingly, these cells were characterized as largely C-kit+ by immunohistochemistry. In this context, it should be noted that during the work on paper II, Islet-1 was analyzed in all *C-KIT+VWF-* or *NKX2.5+* cells. No expression of this gene was however found in any of these cells (data not shown). This indicates that in contrast to the human fetal heart, C-kit+ cells in the adult heart are negative for Islet-1 expression.

5.7.2 Relationship between SSEA-4 and other progenitor populations

In paper IV, expression of SSEA-1, 3 and 4 was investigated in the human adult heart. For SSEA-4, a more detailed co-expression analysis was carried out with SP and C-kit since the SSEA-4+CD34- population showed signs of being a cardiomyogenic progenitor population based on qPCR analysis of sorted cells. When SP cells were co-stained SSEA-4, no co-expression was found. This indicate that the SSEA-4+ population was distinct from the SP population. On the other hand, when directly isolated cells from right atrium were co-stained for C-kit and SSEA-4, the C-kit+CD45- progenitor population was found to be enriched for SSEA-4 expression. This was even more apparent in monolayer cultured cells. Importantly, however, the total SSEA-4+ population was approximately two orders of magnitude larger than the C-kit+CD45- population in directly isolated cells, i.e. the overwhelming majority of SSEA-4+ cells were not C-kit+. Furthermore, as almost all of the C-kit+CD45- cells were CD34+, the C-kit+SSEA-4+ cells were distinct from the SSEA-4+CD34- putative cardiomyogenic progenitor population. Few previous studies have investigated expression of SSEAs in the heart. In a study of primary cultured SSEA-1+ cells derived from rat heart, no co-expression of C-kit or Sca-1 was found (172). SSEA-4+ cells have been identified in human fetal (173) and adult heart (174). In these studies however, no co-expression analysis was carried out. In a study by Hou et al (299) on sheep heart, SSEA-4+ cells were identified in tissue sections as well as in clonally expanded cells. Although some of the expanded clones expressed both moderate levels of C-kit and SSEA-4 by FACS and qPCR analysis, no direct co-expression analysis by FACS or single cell analysis by qPCR were carried out. Co-expression analysis was neither carried out in tissue sections. Thus, data presented in paper IV represent the first direct comparison between SSEA-4 and C-kit and SP cells respectively.

5.8 Distribution of progenitor populations in the adult heart

In paper II, III and IV, distribution of C-kit+, SP and SSEAs were compared between right and left atrium. These both tissue locations were possible to obtain from patients undergoing Maze surgery. The C-kit+CD45+ population that in paper I was characterized as predominantly consisting of mast cells, was readily identified in both right and left atrium in paper II with approximately twice as many cells in the left compared to the right atrium.

The number of cardiac mast cells have previously been shown to be affected by disease such as idopatic dilated cardiomyopathy (148) and myocardial infarction (149). This was also noted in an animal model of pressure overload induced susceptibility to atrial fibrillation. Interestingly, in this study, it was shown that attenuation of mast cell function by treatment with cromolyn strongly reduced the incidence of atrial fibrillation after atrial burst stimulation. This was in its turn linked to reduced fibrosis (300). Although the mechanism behind atrial fibrillation is not entirely clear, it has more recently been suggested that it is sustained through a non-random electrophysiological activity emanating from the left atrium (301). Based on this, it could be hypothesized that the increased number of mast cells in left atrium is associated with maintenance of atrial fibrillation in this group of patients. To further elucidate this, a comparison with biopsy material from both left and right atrium of patients not suffering from atrial fibrillation would obviously be needed.

In paper II, The C-kit+CD45- progenitor population was only clearly identifiable in right atrium and only barley detectable in left atrium. In paper III on the other hand, SP cells could only be clearly identified in the left atrium. The reason behind this observed difference in distribution between C-kit+CD45- and SP progenitor cells is not clear. Few previous studies have investigated the atrial distribution of these cell populations. In a study by Urbanek et al. (62), mouse cardiac C-kit+ cells were identified as nests of cells present throughout the heart. The frequency was approximately 8 fold higher in apex and atria compared to the base - mid region of the ventricles. In a immunohistochemical study by Castaldo et al. (132), distribution of C-kit+ cells in left and right atria was investigated in human normal and failing heart. To the contrary of the results in paper II, the number of C-kit+ cells were slightly higher in the left compared to the right atrium. One possible explanation to this discrepancy could be inadvertent interpretation of mast cells as C-kit+ progenitor cells in the study by Castaldo et al. Although it is stated that hematopoietic cells were excluded in the material and method section in the study, no direct co-expression analysis between C-kit and hematopoietic markers seems to have been carried out. In another study of the early post natal and adolescent human heart by Mishra et al. (134), by far the highest percentage of C-kit+ cells was found within the right atrium (5%) while the lowest percentage was found within the left atrium (0.3%). These results comply well with the results in paper II. Although percentages of C-kit+ cells were much higher in the study by Mishra et al. compared to paper II, this could be attributed to the much younger age of the included patients. Indeed, it is shown by Mishra et al. that the number of C-kit+ cells clearly decline up to the oldest included patients of 13 years of age. Based on our results and as well as the results by Mishra et al., it could be speculated that the human right atrium might serve as a pool for human cardiac C-kit+CD45- progenitor cells. In line with this theory, it has been possible to induce migration of C-kit+ cells from the atrioventricular groove area of the mouse heart to an area within the ventricle by injections HGF and IGF-1 (127).

In contrast to the C-kit+ population, there are currently no other studies that have investigated the distribution of SP cells within the heart. One possible reason to the differential distribution of SP cells and C-kit+ progenitor cells could be different embryonic origin. However, as both right and left atrium have the same developmental source with approximately 2/3 of the cells originating from the secondary heart field with the remaining 1/3 of the cells from the primary heart field (35), this does not seem to be a likely explanation. Similarly to C-kit+ cells, SP cells have been found to be able to migrate through the cardiac tissue. This ability was linked to the CXCR4 / SDF-1a system (185). Interestingly, in paper I, C-kit+ cells were also observed to highly express CXCR4. In a study by Liang Tang et al. (302), CXCR4 expression in C-kit+ cells was further augmented by hypoxic conditions. Similarly to SP cells, recruitment of C-kit+ cells to an infarction area was linked to the CXCR4 / SDF-1a system. Based on these results, it could be speculated that while C-kit+CD45- cells in the human adult heart serve as progenitors for the right atrium and potentially also the right ventricle, SP cells has a similar function in the left atrium and potentially left ventricle. Further studies are needed to elucidate this in further detail.

In paper IV, a population of SSEA-4+CD34- cells was identified in both right and left atrium. Although equally distributed, only cells isolated from right atrium were committed to the cardiomyogenic lineage. The reason for this difference in commitment between the localizations is unclear.

It should be acknowledged that in the included papers, only atrial biopsy samples have been included. Although it would have been ideal to also have ventricular tissue for comparison, this has not been possible to obtain. From patients undergoing routine cardiac surgery such as CABG or valvular replacement surgery, only needle biopsies would be possible from ventricular locations. These are unfortunately to small for cell identification and isolation by FACS. To address this issue, we have initiated a study on explanted hearts from patients undergoing cardiac transplantation. This will hopefully in the future add to our knowledge of tissue distribution of cardiac progenitors also in the ventricles.

5.9 General limitations associated with conducting research on human cardiac material

It should be acknowledged that in both paper III and IV, no *in vitro* differentiation data are presented. Furthermore, in paper I, it would have been desirable to include more assays to assess endothelial and cardiac differentiation such as detailed analysis of capillary forming ability, uptake of acetylated LDL and intracellular flow cytometry to identify cardiomyogenic differentiation combined with qPCR analysis of sorted cells for verification. These analyses would ideally have been conducted

both before and after induction of differentiation. A general limitation when working with human biopsy material is however both the availability and the amount of tissue material that is possible to obtain. Furthermore, the cell populations of interest only represent small fractions of the total number of cells. Taken together, this greatly limits the number of assays and conditions that can be included. On the other hand, results from human cardiac material compared to animal models can be considered to be much more relevant from a clinical perspective (303, 304). It should also be acknowledged that in papers I - III, no comparison is carried out between different groups of patients. In paper IV on the other hand, results from patients undergoing Maze surgery and patients undergoing surgery of other causes were accounted for separately. Unfortunately, it has not been possible to obtain control samples of cardiac tissue from patients without any cardiac disease. Notably, in studies by other groups, autopsy material has often been used to obtain normal control heart tissue (130, 132, 174). This source of tissue unfortunately only allows for analysis by immunohistochemistry and is not possible to use for analysis and isolation of live cells by FACS.

It should also be noted that the ethical permit used in paper I - IV has not allowed for storage of patient data other than cause of surgery and age. This have precluded us from carrying out post-hoc analyses of different groups of patients. In this context, it should be mentioned that we have currently initiated a study on explanted cardiac tissue from patients undergoing transplantation surgery, where patient data will be recorded. As comparison, we plan to use tissue samples from explanted donor hearts not suitable for transplantation that are used for extraction of aortic valves for later valvular replacement surgery.

6 CONCLUSIONS

In this thesis, three different progenitor cell populations have been identified and isolated from the human adult heart. These have been found to be largely distinct from each other.

In **paper I**, we showed that C-kit+ cells could be isolated from the human adult heart. These cells could be subdivided based on the hematopoietic marker CD45. The C-kit+CD45+ cells were determined to have a mast cell identity whereas the C-kit+CD45- cells had a predominant endothelial commitment. C-kit+CD45- cells could also be successfully isolated from monolayer expanded cells. These cells had a similar gene and protein expression when compared with directly isolated cells. Although no cardiac differentiation could be induced, these cells showed evidence of further endothelial maturation when cultured in an endothelial differentiation media.

In **paper II**, heterogeneity within the C-kit+CD45- cardiac population was investigated by single cell qPCR. While most of the cells were found to have an endothelial gene expression profile, a minor portion of the cells showed evidence of cardiomyogenic lineage commitment. Furthermore, some of the cells did not express any markers of lineage commitment. These cells could represent uncommitted progenitor cells.

In **paper III**, SP cells were identified in the human adult heart. Inhibitor analyses as well as gene expression analyses pointed toward MDR1 as the major determinant of the SP phenotype. The cardiac SP was partly positive for the hematopoietic marker CD45. While the SP CD45- population showed evidence of endothelial lineage commitment, expression of cardiac genes were either low or undetectable in SP cells regardless of CD45 expression status.

In **paper IV**, expression of SSEA-1, 3 and 4 were shown in cells derived from the human adult heart. SSEA-1+ cells when isolated directly from the heart, were determined to be of hematopoietic origin. SSEA-4+ cells could be further subdivided based on CD34 expression status. SSEA-4+CD34- cells directly isolated from the right atrium were found to be committed to the cardiomyogenic lineage based on gene expression analysis.

Location of progenitor cells were investigated in **paper II, III and IV**. C-kit+CD45- cells were predominantly confined to the right atrium. SP cells on the other hand, could only be identified in the left atrium. SSEAs were expressed in both right and left atrium, but only SSEA-4+CD34- cells derived from the right atrium showed evidence of cardiomyogenic lineage commitment.

In conclusions, this thesis adds to our knowledge of the existence, identity and distribution of cardiac progenitor populations in the human adult heart. It both provides new information regarding previously described C-kit and SP cell populations as well as suggests SSEA-4+CD34- cells to represent a novel progenitor cell population with cardiomyogenic commitment.

7 FUTURE PERSPECTIVES

Cardiovascular disease, together with cancer, is the leading cause of death in the western world. For major cardiac diseases such as ischemic heart disease and heart failure, the treatment strategy until now has been to protect and preserve remaining myocardial tissue. As the heart has been regarded as a non-regenerative organ, loosing its ability to form new cardiomyocytes shortly after birth, little effort has been put into developing treatment strategies of regenerating myocardial tissue. The knowledge of a limited regenerative capacity in the human adult heart however, open new possibilities. These include cell therapy with expanded cardiac or extracardiac derived progenitor cells, development of drugs that stimulate endogenous progenitor cells or development of drugs that stimulate cardiomyocytes to re-enter the cell cycle and start to proliferate. Which path that should be chosen however, depends on the underlying mechanism of cardiac regeneration.

Until now, three different mechanisms of cardiac regeneration have been proposed. These are regeneration through differentiation of a pool of endogenous cardiac progenitor cells, regeneration through migration and transdifferentiation of extracardiac derived progenitor cells and re-entry into the cell cycle and proliferation of pre-existing cardiomyocytes. In the literature, there is a lot of conflicting data regarding the properties and developmental potential of different cell populations. Furthermore, as has been shown in this thesis for the C-kit+ population, there is often a low degree of reproducibility between the results of different groups. Importantly, this is not unique for the field of cardiac regeneration but has been pointed out as a growing general problem in the field of biomedical basic research (305, 306). Thus, there is a need for more replicating studies in the future, ideally by groups independent from each other, to firmly establish or reject the potential of different cell populations in the context of cardiac regeneration. In particular, there is a need for more studies on human biopsy material as there may be differences between species as well as differences between different groups of patients.

Although not complete, the previously published literature when taken together, indicate that extracardiac progenitor populations are unlikely to be the source of cardiac regeneration. Notably however, such cells may in the future have a therapeutic role as they seem to have other beneficial effects when administered after myocardial infarction. Further studies are however needed to optimize factors such as type of cell, route of administration and timing.

When it comes to endogenous cardiac progenitor cells versus cycling of pre-existing cardiomyocytes as the source of cardiac regeneration, studies in favor of both mechanisms exist and it is theoretically possible that these co-exist. Further studies comparing different progenitor populations with each other are however needed to

elucidate which endogenous progenitor populations that have the greatest potential to regenerate myocardial tissue. Furthermore, it remains largely unknown whether these different populations are hierarchically linked to each other. Indeed, at least seven different cell populations in the heart that have been described as multipotent progenitors with the ability to differentiate into cardiomyocytes and endothelial cells. Taken into consideration the rather limited regenerative capacity of the heart, it seems unlikely that the heart would harbor such large number of different progenitor populations that are unrelated to each other. In animal models, hierarchal links could be further elucidated by genetic lineage tracing. In the human, this method is however unfortunately not possible. Instead, it might be possible to use a single cell analysis approach, which was employed in this thesis to investigate heterogeneity in the C-kit+CD45- population. If two cell populations are hierarchically linked, it would be reasonable to assume that the transition is continuous and that it thus would be possible to identify individual cells that are on different stages of transitioning from one of the cell populations to the other.

In this thesis, it has been demonstrated that different progenitor populations are distributed unevenly between right and left atrium. The functional implications of this distribution however remain to be determined. Furthermore, little is known about the distribution of different progenitor populations in the right and left ventricle and whether this may be affected by cardiac diseases.

In this thesis, data are presented for three different and largely distinct progenitor populations in the human heart. Of these, C-kit+CD45- and SSEA-4+CD34- cells would theoretically be possible to isolate and expand for cell therapy purposes whereas identification of SP cells currently require staining with hoechst 33342 which makes it unsuitable for clinical applications. The discovery of a population of SP cells within the human adult heart is however of potential clinical value as it may be possible to stimulate this populations of cells by for example local injections with cytokines. This has been shown in animal models, but before initiation of clinical studies, further investigations regarding the differentiation potential and function of human SP cells are needed. In this regard, it will also be necessary to optimize the staining procedure used to identify SP cells in order to improve viability after sorting of cells. This may either be carried by titrating the concentration of hoechst 33342 or by adopting another staining that is effluxed by MDR1.

There is currently one published clinical study where C-kit+ cells were used (259, 260). In that study, cells were expanded in a way similar to the monolayer culture system presented in this thesis. Although clinical improvement was noted in that study, the results in this thesis indicate that this expansion system may not be optimal as it seems to favor the expansion of endothelial committed progenitor cells over a smaller subpopulation of cardiac committed progenitors. Further studies are thus

needed to investigate whether culture conditions may be optimized to instead favor the expansion of the cardiac committed C-kit+ subpopulation. Conditions that may be of importance include coating of culture vessels, media composition and oxygen tension. In this regard, factorial design of experiments may be a possible approach to systematically evaluate several culture condition factors simultaneously. This methodology has previously successfully been used to optimize culture conditions for other types of cells, such as chondrocytes (307).

When it comes to the population of SSEA-4+CD34- cells, this has previously not been described in the literature. In this thesis, it has only been possible to do an initial characterization by FACS and qPCR. As this population on group level shows evidence of cardiomyogenic commitment, in contrast to both C-kit+ and SP cells, it may be superior to both these populations of cells in terms of cardiomyogenic differentiation potential. Further studies are however needed to establish a reproducible culture system for these cells, that retain the cardiomyogenic commitment profile. Furthermore, it would be of interest to carry out a single cell qPCR analysis of these cells to determine whether all cells show evidence of cardiomyogenic commitment or if this is confined to a sub population of the cells.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to everyone that has contributed to this thesis. In particular, I would like to thank:

Anders Lindahl, my main supervisor. For giving me the opportunity to work with this project and in particular for giving me much freedom to pursue my own ideas as well as for your always encouraging and positive attitude.

Julia Asp, co-supervisor. For being a great discussion partner during design of studies and in the process of manuscript writing as well as sharing with me your in depth knowledge of gene expression analysis. You have shown me the importance of not just planning and carrying out experiments but also to compile it in a way that makes it interesting for others to read.

Göran Dellgren, co-supervisor. For your always positive and encouraging attitude and for being a discussion partner regarding clinical aspects as well as giving me support with writing ethical applications.

Anders Jeppsson (co-author), for supporting this project from the start and always having a positive attitude.

Marianne Jonsson (co-author), for your great knowledge and thoroughness in all aspects of gene expression analysis.

Mikael Sandstedt, my brother and co-author. We have had many fruitful discussions during this work. Furthermore, when time is late and the cells are many, I can always count on your support.

Kristina Vukusic (co-author). For always being supportive as well as a great discussion partner. I look forward to continue to unravel the mysteries of cardiac regeneration with you!

Håkan Berggren, for supporting this project from the start and bringing valuable suggestions.

Linda Thimour-Bergström, Erika Backlund, Christine Roman-Emanuel and Åsa Israelsson for always being helpful and for coordinating procurement of cardiac biopsies in an excellent way. All surgeons at the department of thoracic surgery, Sahlgrenska University hospital, for procurement of biopsy material. Without you, this thesis would not have been possible!

All former and present members of the molecular cell biology group at Clinical Chemistry and the cell and tissue laboratory. In particular, I would like to thank Camilla Brantsing for friendship and valuable ideas and suggestions. Marion Walser for carrying out some of the practical work with trying to induce cardiac differentiation of cells. Ruth Wickelgren for also being a part of the cardiac group and bringing valuable suggestions. Lars Enochsson and Camilla Karlsson for a great collaboration with work on HL-1 cells (although not included in this thesis) and for the experience of the longest per review session ever. Johan Stenberg, Vivien Lettry, Eva Schöldebrant, Cecilia Granéli, Emilia Svala and Anna Torfve for friendship and interesting research discussions regarding cartilage, bone but also the heart. Narmin Bigdeli for showing me embryonic stem cells and for being a great travel companion in Boston during conference. Stina Simonsen and Cecilia Boreström for valuable discussions regarding iPS cells. Catherine Concaro, Marie Leander, Josefine van der Lee, Margret Hagman and Pia Andersson for friedship and for showing interest in this work. Hanna Stenhamre for friendship and earlier collaboration.

I also would like to thank Katarina Junevik for all support and great discussions regarding FACS sorting and analysis. Petra Bergström, Anna Staffas and Mette Diswall, for interesting discussions in other research areas. Ola Hammarsten for inspiration and interesting discussions regarding cardiomyocytes. Ulla Strandberg for administrative support. Mattias Johansson, for being the only one working at the lab at the same times as me when I started my work at clinical chemistry. You have been a great friend and we have had many interesting discussions about science as well as life. I hope you will come back to science.

My parents Gun and Bengt Sandstedt. For your love, unconditional support and encouragement. I would in particular like to thank my father for truly being a source of inspiration and for all discussions we have had regarding various aspects of the vast field of cardiology.

My sister, Maria Sandstedt. You will be a great scientist in the future, I can tell!

Gabriella, for all of your love and patience.

REFERENCES

1. Moore KL, Dalley AF. Clinically oriented anatomy. 4 ed: Lippincott Williams and Wilkins; 1999.
2. Guyton AC, Hall JE. Textbook of Medical Physiology. 10 ed: W. B. Saunders Company. An Imprint of Elsvier Science; 2000.
3. Kierszenbaum AL. Histology and Cell Biology An introduction to Pathology: Mosby Inc., An affilate of Elsvier Science; 2002.
4. Sacks HS, Fain JN. Human epicardial adipose tissue: a review. Am Heart J. 2007 Jun;153(6):907-17.
5. Nag AC. Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution. Cytobios. 1980;28(109):41-61.
6. Zak R. Development and proliferative capacity of cardiac muscle cells. Circ Res. 1974 Aug;35(2):suppl II:17-26.
7. Banerjee I, Fuseler JW, Price RL, Borg TK, Baudino TA. Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. Am J Physiol Heart Circ Physiol. 2007 Sep;293(3):H1883-91.
8. Dammrich J, Pfeifer U. Cardiac hypertrophy in rats after supravalvular aortic constriction. I. Size and number of cardiomyocytes, endothelial and interstitial cells. Virchows Archiv B, Cell pathology including molecular pathology. 1983;43(3):265-86.
9. Olivetti G, Melissari M, Capasso JM, Anversa P. Cardiomyopathy of the aging human heart. Myocyte loss and reactive cellular hypertrophy. Circ Res. 1991 Jun;68(6):1560-8.
10. Vliegen HW, van der Laarse A, Cornelisse CJ, Eulderink F. Myocardial changes in pressure overload-induced left ventricular hypertrophy. A study on tissue composition, polyploidization and multinucleation. Eur Heart J. 1991 Apr;12(4):488-94.
11. Severs NJ. The cardiac muscle cell. Bioessays. 2000 Feb;22(2):188-99.
12. Olivetti G, Cigola E, Maestri R, Corradi D, Lagrasta C, Gambert SR, Anversa P. Aging, cardiac hypertrophy and ischemic cardiomyopathy do not affect the proportion of mononucleated and multinucleated myocytes in the human heart. J Mol Cell Cardiol. 1996 Jul;28(7):1463-77.
13. Mollova M, Bersell K, Walsh S, Savla J, Das LT, Park SY, Silberstein LE, Dos Remedios CG, Graham D, Colan S, Kuhn B. Cardiomyocyte proliferation contributes to heart growth in young humans. Proc Natl Acad Sci U S A. 2013 Jan 22;110(4):1446-51.
14. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J. Evidence for cardiomyocyte renewal in humans. Science. 2009 Apr 3;324(5923):98-102.
15. Liu Z, Yue S, Chen X, Kubin T, Braun T. Regulation of cardiomyocyte polyploidy and multinucleation by CyclinG1. Circ Res. 2010 May 14;106(9):1498-506.

16. Xu C, Police S, Hassanipour M, Gold JD. Cardiac bodies: a novel culture method for enrichment of cardiomyocytes derived from human embryonic stem cells. Stem Cells Dev. 2006 Oct;15(5):631-9.

17. Olson EN. Gene regulatory networks in the evolution and development of the heart. Science. 2006 Sep 29;313(5795):1922-7.

18. Brutsaert DL, Fransen P, Andries LJ, De Keulenaer GW, Sys SU. Cardiac endothelium and myocardial function. Cardiovasc Res. 1998 May;38(2):281-90.

19. Shibuya M. Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. J Biochem Mol Biol. 2006 Sep 30;39(5):469-78.

20. Furlan M. Von Willebrand factor: molecular size and functional activity. Ann Hematol. 1996 Jun;72(6):341-8.

21. Nees S, Weiss DR, Juchem G. Focus on cardiac pericytes. Pflugers Archiv : European journal of physiology. 2013 Jun;465(6):779-87.

22. Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell. 2011 Aug 16;21(2):193-215.

23. Camelliti P, Borg TK, Kohl P. Structural and functional characterisation of cardiac fibroblasts. Cardiovasc Res. 2005 Jan 1;65(1):40-51.

24. Wang J, Chen H, Seth A, McCulloch CA. Mechanical force regulation of myofibroblast differentiation in cardiac fibroblasts. Am J Physiol Heart Circ Physiol. 2003 Nov;285(5):H1871-81.

25. Goldsmith EC, Hoffman A, Morales MO, Potts JD, Price RL, McFadden A, Rice M, Borg TK. Organization of fibroblasts in the heart. Dev Dyn. 2004 Aug;230(4):787-94.

26. Gilbert SF. Developmental biology. 2003.

27. Moorman AF, Christoffels VM. Cardiac chamber formation: development, genes, and evolution. Physiol Rev. 2003 Oct;83(4):1223-67.

28. Srivastava D. Genetic regulation of cardiogenesis and congenital heart disease. Annual review of pathology. 2006;1:199-213.

29. High FA, Epstein JA. The multifaceted role of Notch in cardiac development and disease. Nat Rev Genet. 2008 Jan;9(1):49-61.

30. Sissman NJ. Developmental landmarks in cardiac morphogenesis: comparative chronology. The American journal of cardiology. 1970 Feb;25(2):141-8.

31. Sedmera D, Pexieder T, Vuillemin M, Thompson RP, Anderson RH. Developmental patterning of the myocardium. Anat Rec. 2000 Apr 1;258(4):319-37.

32. Brand T. Heart development: molecular insights into cardiac specification and early morphogenesis. Dev Biol. 2003 Jun 1;258(1):1-19.

33. Kelly RG, Buckingham ME. The anterior heart-forming field: voyage to the arterial pole of the heart. Trends in genetics : TIG. 2002 Apr;18(4):210-6.

34. Spater D, Abramczuk MK, Buac K, Zangi L, Stachel MW, Clarke J, Sahara M, Ludwig A, Chien KR. A HCN4+ cardiomyogenic progenitor derived from the first heart field and human pluripotent stem cells. Nat Cell Biol. 2013 Sep;15(9):1098-106.

35. Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell. 2003 Dec;5(6):877-89.

36. Genead R, Danielsson C, Andersson AB, Corbascio M, Franco-Cereceda A, Sylven C, Grinnemo KH. Islet-1 cells are cardiac progenitors present during the entire lifespan: from the embryonic stage to adulthood. Stem Cells Dev. 2010 Oct;19(10):1601-15.

37. Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, Qyang Y, Bu L, Sasaki M, Martin-Puig S, Sun Y, Evans SM, Laugwitz KL, Chien KR. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell. 2006 Dec 15;127(6):1151-65.

38. Verzi MP, McCulley DJ, De Val S, Dodou E, Black BL. The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within the secondary/anterior heart field. Dev Biol. 2005 Nov 1;287(1):134-45.

39. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature. 2005 Feb 10;433(7026):647-53.

40. Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? Cell. 2001 Jun 29;105(7):829-41.

41. Gepstein L. Derivation and potential applications of human embryonic stem cells. Circ Res. 2002 Nov 15;91(10):866-76.

42. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006 Aug 25;126(4):663-76.

43. Rizk P, Barker N. Gut stem cells in tissue renewal and disease: methods, markers, and myths. Wiley interdisciplinary reviews Systems biology and medicine. 2012 Sep-Oct;4(5):475-96.

44. Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol. 2006 Feb;6(2):93-106.

45. Pelayo R, Welner R, Perry SS, Huang J, Baba Y, Yokota T, Kincade PW. Lymphoid progenitors and primary routes to becoming cells of the immune system. Curr Opin Immunol. 2005 Apr;17(2):100-7.

46. Stern MM, Bickenbach JR. Epidermal stem cells are resistant to cellular aging. Aging Cell. 2007 Aug;6(4):439-52.

47. Kuang S, Kuroda K, Le Grand F, Rudnicki MA. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell. 2007 Jun 1;129(5):999-1010.

48. Vaananen HK. Mesenchymal stem cells. Ann Med. 2005;37(7):469-79.

49. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002 Jul 4;418(6893):41-9.

50. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. Nature. 2001 Apr 5;410(6829):701-5.

51. Karsner HT, Saphir O, Todd TW. The state of the cardiac muscle in hypertrophy and atrophy. Am J Pathol. 1925 (1):351-71.

52. Linzbach AJ. [The muscle fiber constant and the law of growth of the human ventricles]. Virchows Archiv : an international journal of pathology. 1950;318(5):575-618.

53. Piper HM, Jacobson SL, Schwartz P. Determinants of cardiomyocyte development in long-term primary culture. J Mol Cell Cardiol. 1988 Sep;20(9):825-35.

54. Soonpaa MH, Field LJ. Survey of studies examining mammalian cardiomyocyte DNA synthesis. Circ Res. 1998 Jul 13;83(1):15-26.

55. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol. 2000 Mar;182(3):311-22.

56. Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA, Anversa P. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med. 2001 Jun 7;344(23):1750-7.

57. Leri A, Barlucchi L, Limana F, Deptala A, Darzynkiewicz Z, Hintze TH, Kajstura J, Nadal-Ginard B, Anversa P. Telomerase expression and activity are coupled with myocyte proliferation and preservation of telomeric length in the failing heart. Proc Natl Acad Sci U S A. 2001 Jul 17;98(15):8626-31.

58. Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, Nitahara JA, Quaini E, Di Loreto C, Beltrami CA, Krajewski S, Reed JC, Anversa P. Apoptosis in the failing human heart. N Engl J Med. 1997 Apr 17;336(16):1131-41.

59. Guerra S, Leri A, Wang X, Finato N, Di Loreto C, Beltrami CA, Kajstura J, Anversa P. Myocyte death in the failing human heart is gender dependent. Circ Res. 1999 Oct 29;85(9):856-66.

60. Nadal-Ginard B, Kajstura J, Anversa P, Leri A. A matter of life and death: cardiac myocyte apoptosis and regeneration. J Clin Invest. 2003 May;111(10):1457-9.

61. Waring CD, Vicinanza C, Papalamprou A, Smith AJ, Purushothaman S, Goldspink DF, Nadal-Ginard B, Torella D, Ellison GM. The adult heart responds to increased workload with physiologic hypertrophy, cardiac stem cell activation, and new myocyte formation. Eur Heart J. 2012 Oct 25.

62. Urbanek K, Cesselli D, Rota M, Nascimbene A, De Angelis A, Hosoda T, Bearzi C, Boni A, Bolli R, Kajstura J, Anversa P, Leri A. Stem cell niches in the adult mouse heart. Proc Natl Acad Sci U S A. 2006 Jun 13;103(24):9226-31.

63. Meinhardt A, Spicher A, Roehrich ME, Glauche I, Vogt P, Vassalli G. Immunohistochemical and flow cytometric analysis of long-term label-retaining cells in the adult heart. Stem Cells Dev. 2011 Feb;20(2):211-22.

64. Kajstura J, Urbanek K, Perl S, Hosoda T, Zheng H, Ogorek B, Ferreira-Martins J, Goichberg P, Rondon-Clavo C, Sanada F, D'Amario D, Rota M, Del Monte F, Orlic D, Tisdale J, Leri A, Anversa P. Cardiomyogenesis in the adult human heart. Circ Res. 2010 Jul 23;107(2):305-15.

65. Kajstura J, Rota M, Cappetta D, Ogorek B, Arranto C, Bai Y, Ferreira-Martins J, Signore S, Sanada F, Matsuda A, Kostyla J, Caballero MV, Fiorini C, D'Alessandro DA, Michler RE, Del Monte F, Hosoda T, Perrella MA, Leri A, Buchholz BA, Loscalzo J, Anversa P. Cardiomyogenesis in the aging and failing human heart. Circulation. 2012 Oct 9;126(15):1869-81.

66. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci U S A. 2001 Aug 28;98(18):10344-9.

67. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature. 2004 Apr 8;428(6983):664-8.

68. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature. 2004 Apr 8;428(6983):668-73.

69. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, Entman ML, Michael LH, Hirschi KK, Goodell MA. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest. 2001 Jun;107(11):1395-402.

70. Deb A, Wang S, Skelding KA, Miller D, Simper D, Caplice NM. Bone marrow-derived cardiomyocytes are present in adult human heart: A study of gender-mismatched bone marrow transplantation patients. Circulation. 2003 Mar 11;107(9):1247-9.

71. Ishikawa F, Shimazu H, Shultz LD, Fukata M, Nakamura R, Lyons B, Shimoda K, Shimoda S, Kanemaru T, Nakamura K, Ito H, Kaji Y, Perry AC, Harada M. Purified human hematopoietic stem cells contribute to the generation of cardiomyocytes through cell fusion. Faseb J. 2006 May;20(7):950-2.

72. Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, Taneera J, Fleischmann BK, Jacobsen SE. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med. 2004 May;10(5):494-501.

73. Uemura R, Xu M, Ahmad N, Ashraf M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res. 2006 Jun 9;98(11):1414-21.

74. Burchfield JS, Iwasaki M, Koyanagi M, Urbich C, Rosenthal N, Zeiher AM, Dimmeler S. Interleukin-10 from transplanted bone marrow mononuclear cells contributes to cardiac protection after myocardial infarction. Circ Res. 2008 Jul 18;103(2):203-11.

75. Limbourg FP, Ringes-Lichtenberg S, Schaefer A, Jacoby C, Mehraein Y, Jager MD, Limbourg A, Fuchs M, Klein G, Ballmaier M, Schlitt HJ, Schrader J, Hilfiker-Kleiner D, Drexler H. Haematopoietic stem cells improve cardiac function after infarction without permanent cardiac engraftment. Eur J Heart Fail. 2005 Aug;7(5):722-9.

76. Zhang C, Sun A, Zhang S, Yao K, Wu C, Fu M, Wang K, Zou Y, Ge J. Efficacy and safety of intracoronary autologous bone marrow-derived cell transplantation in patients with acute myocardial infarction: insights from randomized controlled trials with 12 or more months follow-up. Clin Cardiol. 2010 Jun;33(6):353-60.

77. Clifford DM, Fisher SA, Brunskill SJ, Doree C, Mathur A, Watt S, Martin-Rendon E. Stem cell treatment for acute myocardial infarction. The Cochrane database of systematic reviews. 2012;2:CD006536.

78. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, Masaki H, Mori Y, Iba O, Tateishi E, Kosaki A, Shintani S, Murohara T, Imaizumi T, Iwasaka T. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation. 2001 Aug 28;104(9):1046-52.

79. Templin C, Kotlarz D, Faulhaber J, Schnabel S, Grote K, Salguero G, Luchtefeld M, Hiller KH, Jakob P, Naim HY, Schieffer B, Hilfiker-Kleiner D, Landmesser U, Limbourg FP, Drexler H. Ex vivo expanded hematopoietic progenitor cells improve cardiac function after myocardial infarction: role of beta-catenin transduction and cell dose. J Mol Cell Cardiol. 2008 Sep;45(3):394-403.

80. Takahashi M, Li TS, Suzuki R, Kobayashi T, Ito H, Ikeda Y, Matsuzaki M, Hamano K. Cytokines produced by bone marrow cells can contribute to functional improvement of the infarcted heart by protecting cardiomyocytes from ischemic injury. Am J Physiol Heart Circ Physiol. 2006 Aug;291(2):H886-93.

81. Stewart K, Monk P, Walsh S, Jefferiss CM, Letchford J, Beresford JN. STRO-1, HOP-26 (CD63), CD49a and SB-10 (CD166) as markers of primitive human marrow stromal cells and their more differentiated progeny: a comparative investigation in vitro. Cell Tissue Res. 2003 Sep;313(3):281-90.

82. Gang EJ, Bosnakovski D, Figueiredo CA, Visser JW, Perlingeiro RC. SSEA-4 identifies mesenchymal stem cells from bone marrow. Blood. 2007 Feb 15;109(4):1743-51.

83. Kudo M, Wang Y, Wani MA, Xu M, Ayub A, Ashraf M. Implantation of bone marrow stem cells reduces the infarction and fibrosis in ischemic mouse heart. J Mol Cell Cardiol. 2003 Sep;35(9):1113-9.

84. Jiang W, Ma A, Wang T, Han K, Liu Y, Zhang Y, Zhao X, Dong A, Du Y, Huang X, Wang J, Lei X, Zheng X. Intravenous transplantation of mesenchymal stem cells improves cardiac performance after acute myocardial ischemia in female rats. Transpl Int. 2006 Jul;19(7):570-80.

85. Iso Y, Spees JL, Serrano C, Bakondi B, Pochampally R, Song YH, Sobel BE, Delafontaine P, Prockop DJ. Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. Biochem Biophys Res Commun. 2007 Mar 16;354(3):700-6.

86. Boomsma RA, Swaminathan PD, Geenen DL. Intravenously injected mesenchymal stem cells home to viable myocardium after coronary occlusion and preserve systolic function without altering infarct size. Int J Cardiol. 2006 Dec 20.

87. Chen G, Nayan M, Duong M, Asenjo JF, Ge Y, Chiu RC, Shum-Tim D. Marrow stromal cells for cell-based therapy: the role of antiinflammatory cytokines in cellular cardiomyoplasty. Ann Thorac Surg. 2010 Jul;90(1):190-7.

88. Hashemi SM, Ghods S, Kolodgie FD, Parcham-Azad K, Keane M, Hamamdzic D, Young R, Rippy MK, Virmani R, Litt H, Wilensky RL. A placebo controlled, dose-ranging, safety study of allogenic mesenchymal stem cells injected by endomyocardial delivery after an acute myocardial infarction. Eur Heart J. 2008 Jan;29(2):251-9.

89. Grinnemo KH, Mansson-Broberg A, Leblanc K, Corbascio M, Wardell E, Siddiqui AJ, Hao X, Sylven C, Dellgren G. Human mesenchymal stem cells do not differentiate into cardiomyocytes in a cardiac ischemic xenomodel. Ann Med. 2006;38(2):144-53.

90. Yoon J, Shim WJ, Ro YM, Lim DS. Transdifferentiation of mesenchymal stem cells into cardiomyocytes by direct cell-to-cell contact with neonatal cardiomyocyte but not adult cardiomyocytes. Ann Hematol. 2005 Oct;84(11):715-21.

91. Wang T, Xu Z, Jiang W, Ma A. Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. Int J Cardiol. 2006 Apr 28;109(1):74-81.

92. He XQ, Chen MS, Li SH, Liu SM, Zhong Y, McDonald Kinkaid HY, Lu WY, Weisel RD, Li RK. Co-culture with cardiomyocytes enhanced the myogenic conversion of mesenchymal stromal cells in a dose-dependent manner. Mol Cell Biochem. 2010 Jun;339(1-2):89-98.

93. Ishimine H, Yamakawa N, Sasao M, Tadokoro M, Kami D, Komazaki S, Tokuhara M, Takada H, Ito Y, Kuno S, Yoshimura K, Umezawa A, Ohgushi H, Asashima M, Kurisaki A. N-Cadherin is a prospective cell surface marker of human mesenchymal stem cells that have high ability for cardiomyocyte differentiation. Biochem Biophys Res Commun. 2013 Sep 6;438(4):753-9.

94. Koninckx R, Hensen K, Daniels A, Moreels M, Lambrichts I, Jongen H, Clijsters C, Mees U, Steels P, Hendrikx M, Rummens JL. Human bone marrow stem cells co-cultured with neonatal rat cardiomyocytes display limited cardiomyogenic plasticity. Cytotherapy. 2009;11(6):778-92.

95. Markel TA, Wang Y, Herrmann JL, Crisostomo PR, Wang M, Novotny NM, Herring CM, Tan J, Lahm T, Meldrum DR. VEGF Is Critical for Stem Cell Mediated Cardioprotection and a Crucial Paracrine Factor for Defining the Age Threshold in Adult and Neonatal Stem Cell Function. Am J Physiol Heart Circ Physiol. 2008 Oct 10.

96. Xu M, Uemura R, Dai Y, Wang Y, Pasha Z, Ashraf M. In vitro and in vivo effects of bone marrow stem cells on cardiac structure and function. J Mol Cell Cardiol. 2007 Feb;42(2):441-8.

97. Tang YL, Zhao Q, Qin X, Shen L, Cheng L, Ge J, Phillips MI. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann Thorac Surg. 2005 Jul;80(1):229-36; discussion 36-7.

98. Kim EJ, Kim N, Cho SG. The potential use of mesenchymal stem cells in hematopoietic stem cell transplantation. Exp Mol Med. 2013;45:e2.

99. Hristov M, Erl W, Weber PC. Endothelial progenitor cells: mobilization, differentiation, and homing. Arterioscler Thromb Vasc Biol. 2003 Jul 1;23(7):1185-9.

100. Harraz M, Jiao C, Hanlon HD, Hartley RS, Schatteman GC. CD34- blood-derived human endothelial cell progenitors. Stem Cells. 2001;19(4):304-12.

101. George J, Goldstein E, Abashidze S, Deutsch V, Shmilovich H, Finkelstein A, Herz I, Miller H, Keren G. Circulating endothelial progenitor cells in patients with unstable angina: association with systemic inflammation. Eur Heart J. 2004 Jun;25(12):1003-8.

102. Werner N, Wassmann S, Ahlers P, Schiegl T, Kosiol S, Link A, Walenta K, Nickenig G. Endothelial progenitor cells correlate with endothelial function in patients with coronary artery disease. Basic Res Cardiol. 2007 Nov;102(6):565-71.

103. Kunz GA, Liang G, Cuculi F, Gregg D, Vata KC, Shaw LK, Goldschmidt-Clermont PJ, Dong C, Taylor DA, Peterson ED. Circulating endothelial progenitor cells predict coronary artery disease severity. Am Heart J. 2006 Jul;152(1):190-5.

104. Smadja DM, Cornet A, Emmerich J, Aiach M, Gaussem P. Endothelial progenitor cells: characterization, in vitro expansion, and prospects for autologous cell therapy. Cell Biol Toxicol. 2007 Jul;23(4):223-39.

105. Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest. 2000 Jan;105(1):71-7.

106. Bittmann I, Hentrich M, Bise K, Kolb HJ, Lohrs U. Endothelial cells but not epithelial cells or cardiomyocytes are partially replaced by donor cells after allogeneic bone marrow and stem cell transplantation. J Hematother Stem Cell Res. 2003 Aug;12(4):359-66.

107. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, Imaizumi T. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation. 2001 Jun 12;103(23):2776-9.

108. Roura S, Planas F, Prat-Vidal C, Leta R, Soler-Botija C, Carreras F, Llach A, Hove-Madsen L, Pons Llado G, Farre J, Cinca J, Bayes-Genis A. Idiopathic dilated cardiomyopathy exhibits defective vascularization and vessel formation. Eur J Heart Fail. 2007 Oct;9(10):995-1002.

109. Schuh A, Liehn EA, Sasse A, Hristov M, Sobota R, Kelm M, Merx MW, Weber C. Transplantation of endothelial progenitor cells improves neovascularization and left ventricular function after myocardial infarction in a rat model. Basic Res Cardiol. 2008 Jan;103(1):69-77.

110. Doyle B, Sorajja P, Hynes B, Kumar AH, Araoz PA, Stalboerger PG, Miller D, Reed C, Schmeckpeper J, Wang S, Liu C, Terzic A, Kruger D, Riederer S, Caplice NM. Progenitor cell therapy in a porcine acute myocardial infarction model induces cardiac hypertrophy, mediated by paracrine secretion of cardiotrophic factors including TGFbeta1. Stem Cells Dev. 2008 Oct;17(5):941-51.

111. Jujo K, Hamada H, Iwakura A, Thorne T, Sekiguchi H, Clarke T, Ito A, Misener S, Tanaka T, Klyachko E, Kobayashi K, Tongers J, Roncalli J, Tsurumi Y, Hagiwara N, Losordo DW. CXCR4 blockade augments bone marrow progenitor cell recruitment to the neovasculature and reduces mortality after myocardial infarction. Proc Natl Acad Sci U S A. 2010 Jun 15;107(24):11008-13.

112. Badorff C, Brandes RP, Popp R, Rupp S, Urbich C, Aicher A, Fleming I, Busse R, Zeiher AM, Dimmeler S. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation. 2003 Feb 25;107(7):1024-32.

113. Gruh I, Beilner J, Blomer U, Schmiedl A, Schmidt-Richter I, Kruse ML, Haverich A, Martin U. No evidence of transdifferentiation of human endothelial progenitor cells into cardiomyocytes after coculture with neonatal rat cardiomyocytes. Circulation. 2006 Mar 14;113(10):1326-34.

114. Funcke F, Hoyer H, Brenig F, Steingen C, Ladage D, Muller-Ehmsen J, Schmidt A, Brixius K, Bloch W. Characterisation of the interaction between circulating and in vitro cultivated endothelial progenitor cells and the endothelial barrier. Eur J Cell Biol. 2007 Oct 16.

115. Hynes B, Kumar AH, O'Sullivan J, Klein Buneker C, Leblond AL, Weiss S, Schmeckpeper J, Martin K, Caplice NM. Potent endothelial progenitor cell-conditioned media-related anti-apoptotic, cardiotrophic, and pro-angiogenic effects post-myocardial infarction are mediated by insulin-like growth factor-1. Eur Heart J. 2013 Mar;34(10):782-9.

116. Hruban RH, Long PP, Perlman EJ, Hutchins GM, Baumgartner WA, Baughman KL, Griffin CA. Fluorescence in situ hybridization for the Y-chromosome can be used to detect cells of recipient origin in allografted hearts following cardiac transplantation. Am J Pathol. 1993 Apr;142(4):975-80.

117. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Chimerism of the transplanted heart. N Engl J Med. 2002 Jan 3;346(1):5-15.

118. Glaser R, Lu MM, Narula N, Epstein JA. Smooth muscle cells, but not myocytes, of host origin in transplanted human hearts. Circulation. 2002 Jul 2;106(1):17-9.

119. Laflamme MA, Myerson D, Saffitz JE, Murry CE. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. Circ Res. 2002 Apr 5;90(6):634-40.

120. Minami E, Laflamme MA, Saffitz JE, Murry CE. Extracardiac progenitor cells repopulate most major cell types in the transplanted human heart. Circulation. 2005 Nov 8;112(19):2951-8.

121. Muller P, Pfeiffer P, Koglin J, Schafers HJ, Seeland U, Janzen I, Urbschat S, Bohm M. Cardiomyocytes of noncardiac origin in myocardial biopsies of human transplanted hearts. Circulation. 2002 Jul 2;106(1):31-5.

122. Hocht-Zeisberg E, Kahnert H, Guan K, Wulf G, Hemmerlein B, Schlott T, Tenderich G, Korfer R, Raute-Kreinsen U, Hasenfuss G. Cellular repopulation of myocardial infarction in patients with sex-mismatched heart transplantation. Eur Heart J. 2004 May;25(9):749-58.

123. Fogt F, Beyser KH, Poremba C, Zimmerman RL, Ruschoff J. Evaluation of host stem cell-derived cardiac myocytes in consecutive biopsies in long-term cardiac transplant patients. J Heart Lung Transplant. 2003 Dec;22(12):1314-7.

124. Bayes-Genis A, Salido M, Sole Ristol F, Puig M, Brossa V, Camprecios M, Corominas JM, Marinoso ML, Baro T, Vela MC, Serrano S, Padro JM, Bayes de Luna A, Cinca J. Host cell-derived cardiomyocytes in sex-mismatch cardiac allografts. Cardiovasc Res. 2002 Dec;56(3):404-10.

125. Katayama N, Shih JP, Nishikawa S, Kina T, Clark SC, Ogawa M. Stage-specific expression of c-kit protein by murine hematopoietic progenitors. Blood. 1993 Oct 15;82(8):2353-60.

126. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell. 2003 Sep 19;114(6):763-76.

127. Urbanek K, Rota M, Cascapera S, Bearzi C, Nascimbene A, De Angelis A, Hosoda T, Chimenti S, Baker M, Limana F, Nurzynska D, Torella D, Rotatori F, Rastaldo R, Musso E, Quaini F, Leri A, Kajstura J, Anversa P. Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. Circ Res. 2005 Sep 30;97(7):663-73.

128. Hosoda T, D'Amario D, Cabral-Da-Silva MC, Zheng H, Padin-Iruegas ME, Ogorek B, Ferreira-Martins J, Yasuzawa-Amano S, Amano K, Ide-Iwata N, Cheng W, Rota M, Urbanek K, Kajstura J, Anversa P, Leri A. Clonality of mouse and human cardiomyogenesis in vivo. Proc Natl Acad Sci U S A. 2009 Oct 6;106(40):17169-74.

129. Linke A, Muller P, Nurzynska D, Casarsa C, Torella D, Nascimbene A, Castaldo C, Cascapera S, Bohm M, Quaini F, Urbanek K, Leri A, Hintze TH, Kajstura J, Anversa P. Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. Proc Natl Acad Sci U S A. 2005 Jun 21;102(25):8966-71.

130. Urbanek K, Quaini F, Tasca G, Torella D, Castaldo C, Nadal-Ginard B, Leri A, Kajstura J, Quaini E, Anversa P. Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc Natl Acad Sci U S A. 2003 Sep 2;100(18):10440-5.

131. Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, Lecapitaine N, Cascapera S, Beltrami AP, D'Alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P. Human cardiac stem cells. Proc Natl Acad Sci U S A. 2007 Aug 28;104(35):14068-73.

132. Castaldo C, Di Meglio F, Nurzynska D, Romano G, Maiello C, Bancone C, Muller P, Bohm M, Cotrufo M, Montagnani S. CD117-positive cells in adult human heart are localized in the subepicardium, and their activation is associated with laminin-1 and alpha6 integrin expression. Stem Cells. 2008 Jul;26(7):1723-31.

133. Itzhaki-Alfia A, Leor J, Raanani E, Sternik L, Spiegelstein D, Netser S, Holbova R, Pevsner-Fischer M, Lavee J, Barbash IM. Patient characteristics and cell source determine the number of isolated human cardiac progenitor cells. Circulation. 2009 Dec 22;120(25):2559-66.

134. Mishra R, Vijayan K, Colletti EJ, Harrington DA, Matthiesen TS, Simpson D, Goh SK, Walker BL, Almeida-Porada G, Wang D, Backer CL, Dudley SC, Jr., Wold LE, Kaushal S. Characterization and Functionality of Cardiac Progenitor Cells in Congenital Heart Patients. Circulation. 2011 Jan 17.

135. Bearzi C, Leri A, Lo Monaco F, Rota M, Gonzalez A, Hosoda T, Pepe M, Qanud K, Ojaimi C, Bardelli S, D'Amario D, D'Alessandro DA, Michler RE, Dimmeler S, Zeiher AM, Urbanek K, Hintze TH, Kajstura J, Anversa P. Identification of a coronary vascular progenitor cell in the human heart. Proc Natl Acad Sci U S A. 2009 Sep 15;106(37):15885-90.

136. D'Amario D, Fiorini C, Campbell PM, Goichberg P, Sanada F, Zheng H, Hosoda T, Rota M, Connell JM, Gallegos RP, Welt FG, Givertz MM, Mitchell RN, Leri A, Kajstura J, Pfeffer MA, Anversa P. Functionally competent cardiac stem cells can be isolated from endomyocardial biopsies of patients with advanced cardiomyopathies. Circ Res. 2011 Apr 1;108(7):857-61.

137. D'Amario D, Cabral-Da-Silva MC, Zheng H, Fiorini C, Goichberg P, Steadman E, Ferreira-Martins J, Sanada F, Piccoli M, Cappetta D, D'Alessandro DA, Michler RE, Hosoda T, Anastasia L, Rota M, Leri A, Anversa P, Kajstura J. Insulin-like growth factor-1 receptor identifies a pool of human cardiac stem cells with superior therapeutic potential for myocardial regeneration. Circ Res. 2011 Jun 10;108(12):1467-81.

138. Kubo H, Jaleel N, Kumarapeli A, Berretta RM, Bratinov G, Shan X, Wang H, Houser SR, Margulies KB. Increased cardiac myocyte progenitors in failing human hearts. Circulation. 2008 Aug 5;118(6):649-57.

139. Ellison GM, Vicinanza C, Smith AJ, Aquila I, Leone A, Waring CD, Henning BJ, Stirparo GG, Papait R, Scarfo M, Agosti V, Viglietto G, Condorelli G, Indolfi C, Ottolenghi S, Torella D, Nadal-Ginard B. Adult c-kit(pos) Cardiac Stem Cells Are Necessary and Sufficient for Functional Cardiac Regeneration and Repair. Cell. 2013 Aug 15;154(4):827-42.

140. Ronnstrand L. Signal transduction via the stem cell factor receptor/c-Kit. Cell Mol Life Sci. 2004 Oct;61(19-20):2535-48.

141. Ye L, Zhang EY, Xiong Q, Astle CM, Zhang P, Li Q, From AH, Harrison DE, Zhang JJ. Aging Kit mutant mice develop cardiomyopathy. PLoS One. 2012;7(3):e33407.

142. Li M, Naqvi N, Yahiro E, Liu K, Powell PC, Bradley WE, Martin DI, Graham RM, Dell'Italia LJ, Husain A. c-kit is required for cardiomyocyte terminal differentiation. Circ Res. 2008 Mar 28;102(6):677-85.

143. Yaniz-Galende E, Chen J, Chemaly E, Liang L, Hulot JS, McCollum L, Arias T, Fuster V, Zsebo KM, Hajjar RJ. Stem Cell Factor Gene Transfer Promotes Cardiac Repair After Myocardial Infarction via In Situ Recruitment and Expansion of c-kit+ Cells. Circ Res. 2012 Nov 9;111(11):1434-45.

144. Xiang FL, Lu X, Liu Y, Feng Q. Cardiomyocyte-specific overexpression of human stem cell factor protects against myocardial ischemia and reperfusion injury. Int J Cardiol. 2013 May 13.

145. Sperr WR, Bankl HC, Mundigler G, Klappacher G, Grossschmidt K, Agis H, Simon P, Laufer P, Imhof M, Radaszkiewicz T, Glogar D, Lechner K, Valent P. The human cardiac mast cell: localization, isolation, phenotype, and functional characterization. Blood. 1994 Dec 1;84(11):3876-84.

146. Zhou Y, Pan P, Yao L, Su M, He P, Niu N, McNutt MA, Gu J. CD117-positive cells of the heart: progenitor cells or mast cells? J Histochem Cytochem. 2010 Apr;58(4):309-16.

147. Pouly J, Bruneval P, Mandet C, Proksch S, Peyrard S, Amrein C, Bousseaux V, Guillemain R, Deloche A, Fabiani JN, Menasche P. Cardiac stem cells in the real world. J Thorac Cardiovasc Surg. 2008 Mar;135(3):673-8.

148. Batlle M, Perez-Villa F, Lazaro A, Garcia-Pras E, Ramirez J, Ortiz J, Orus J, Roque M, Heras M, Roig E. Correlation between mast cell density and myocardial fibrosis in congestive heart failure patients. Transplant Proc. 2007 Sep;39(7):2347-9.

149. Engels W, Reiters PH, Daemen MJ, Smits JF, van der Vusse GJ. Transmural changes in mast cell density in rat heart after infarct induction in vivo. J Pathol. 1995 Dec;177(4):423-9.

150. Kwon JS, Kim YS, Cho AS, Cho HH, Kim JS, Hong MH, Jeong SY, Jeong MH, Cho JG, Park JC, Kang JC, Ahn Y. The novel role of mast cells in the microenvironment of acute myocardial infarction. J Mol Cell Cardiol. 2011 May;50(5):814-25.

151. Bamezai A. Mouse Ly-6 proteins and their extended family: markers of cell differentiation and regulators of cell signaling. Arch Immunol Ther Exp (Warsz). 2004 Jul-Aug;52(4):255-66.

152. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A. 2003 Oct 14;100(21):12313-8.

153. Matsuura K, Nagai T, Nishigaki N, Oyama T, Nishi J, Wada H, Sano M, Toko H, Akazawa H, Sato T, Nakaya H, Kasanuki H, Komuro I. Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J Biol Chem. 2004 Mar 19;279(12):11384-91.

154. Wang X, Hu Q, Nakamura Y, Lee J, Zhang G, From AH, Zhang J. The role of the sca-1+/CD31- cardiac progenitor cell population in postinfarction left ventricular remodeling. Stem Cells. 2006 Jul;24(7):1779-88.

155. Goumans MJ, de Boer TP, Smits AM, van Laake LW, van Vliet P, Metz CH, Korfage TH, Kats KP, Hochstenbach R, Pasterkamp G, Verhaar MC, van der Heyden MA, de Kleijn D, Mummery CL, van Veen TA, Sluijter JP, Doevendans PA. TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. Stem Cell Res. 2008 Nov;1(2):138-49.

156. van Vliet P, Roccio M, Smits AM, van Oorschot AA, Metz CH, van Veen TA, Sluijter JP, Doevendans PA, Goumans MJ. Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. Neth Heart J. 2008 May;16(5):163-9.

157. van Vliet P, Smits AM, de Boer TP, Korfage TH, Metz CH, Roccio M, van der Heyden MA, van Veen TA, Sluijter JP, Doevendans PA, Goumans MJ. Foetal and adult cardiomyocyte progenitor cells have different developmental potential. J Cell Mol Med. 2010 Apr;14(4):861-70.

158. Genead R, Danielsson C, Wardell E, Kjaeldgaard A, Westgren M, Sundstrom E, Franco-Cereceda A, Sylven C, Grinnemo KH. Early first trimester human embryonic cardiac Islet-1 progenitor cells and cardiomyocytes: Immunohistochemical and electrophysiological characterization. Stem Cell Res. 2009 Nov 5.

159. Bu L, Jiang X, Martin-Puig S, Caron L, Zhu S, Shao Y, Roberts DJ, Huang PL, Domian IJ, Chien KR. Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature. 2009 Jul 2;460(7251):113-7.

160. Jonsson M, Henriksson HB, Hagman M, Kajic K, Lindahl A, Jeppsson A, Berggren H, Asp J. Novel 3D culture system with similarities to the human heart for studies of the cardiac stem cell niche. Regen Med. 2010 Sep;5(5):725-36.

161. Vukusic K, Jonsson M, Brantsing C, Dellgren G, Jeppsson A, Lindahl A, Asp J. High Density Sphere Culture of Adult Cardiac Cells Increases the Levels of Cardiac and Progenitor Markers and Shows Signs of Vasculogenesis. BioMed Research International. 2013;2013(Article ID 696837):11 Pages.

162. Kannagi R, Cochran NA, Ishigami F, Hakomori S, Andrews PW, Knowles BB, Solter D. Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. The EMBO journal. 1983;2(12):2355-61.

163. Kannagi R, Levery SB, Ishigami F, Hakomori S, Shevinsky LH, Knowles BB, Solter D. New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to a developmentally regulated antigen, stage-specific embryonic antigen 3. J Biol Chem. 1983 Jul 25;258(14):8934-42.

164. Fenderson BA, De Miguel MP, Pyle AD, Donovan PJ. Staining embryonic stem cells using monoclonal antibodies to stage-specific embryonic antigens. Methods Mol Biol. 2006;325:207-24.

165. Draper JS, Pigott C, Thomson JA, Andrews PW. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J Anat. 2002 Mar;200(Pt 3):249-58.

166. Muramatsu T, Muramatsu H. Carbohydrate antigens expressed on stem cells and early embryonic cells. Glycoconj J. 2004;21(1-2):41-5.

167. Brimble SN, Sherrer ES, Uhl EW, Wang E, Kelly S, Merrill AH, Jr., Robins AJ, Schulz TC. The cell surface glycosphingolipids SSEA-3 and SSEA-4 are not essential for human ESC pluripotency. Stem Cells. 2007 Jan;25(1):54-62.

168. Wojakowski W, Tendera M, Kucia M, Zuba-Surma E, Paczkowska E, Ciosek J, Halasa M, Krol M, Kazmierski M, Buszman P, Ochala A, Ratajczak J, Machalinski B, Ratajczak MZ. Mobilization of bone marrow-derived Oct-4+ SSEA-4+ very small embryonic-like stem cells in patients with acute myocardial infarction. J Am Coll Cardiol. 2009 Jan 6;53(1):1-9.

169. Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J, Ratajczak MZ. A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia. 2006 May;20(5):857-69.

170. Fukushima H, Kawanabe N, Murata S, Ishihara Y, Yanagita T, Balam TA, Yamashiro T. SSEA-4 is a marker of human deciduous periodontal ligament stem cells. Journal of dental research. 2012 Oct;91(10):955-60.

171. Gottschling S, Jensen K, Warth A, Herth FJ, Thomas M, Schnabel PA, Herpel E. Stage-specific embryonic antigen-4 is expressed in basaloid lung cancer and associated with poor prognosis. The European respiratory journal. 2013 Mar;41(3):656-63.

172. Ott HC, Matthiesen TS, Brechtken J, Grindle S, Goh SK, Nelson W, Taylor DA. The adult human heart as a source for stem cells: repair strategies with embryonic-like progenitor cells. Nat Clin Pract Cardiovasc Med. 2007 Feb;4 Suppl 1:S27-39.

173. Amir G, Ma X, Reddy VM, Hanley FL, Reinhartz O, Ramamoorthy C, Riemer RK. Dynamics of human myocardial progenitor cell populations in the neonatal period. Ann Thorac Surg. 2008 Oct;86(4):1311-9.

174. Emmert MY, Emmert LS, Martens A, Ismail I, Schmidt-Richter I, Gawol A, Seifert B, Haverich A, Martin U, Gruh I. Higher frequencies of BCRP+ cardiac resident cells in ischaemic human myocardium. Eur Heart J. 2013 Sep;34(36):2830-8.

175. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med. 1996 Apr 1;183(4):1797-806.

176. Park KS, Lim CH, Min BM, Lee JL, Chung HY, Joo CK, Park CW, Son Y. The side population cells in the rabbit limbus sensitively increased in response to the central cornea wounding. Invest Ophthalmol Vis Sci. 2006 Mar;47(3):892-900.

177. Sainz J, Al Haj Zen A, Caligiuri G, Demerens C, Urbain D, Lemitre M, Lafont A. Isolation of "side population" progenitor cells from healthy arteries of adult mice. Arterioscler Thromb Vasc Biol. 2006 Feb;26(2):281-6.

178. Giangreco A, Shen H, Reynolds SD, Stripp BR. Molecular phenotype of airway side population cells. Am J Physiol Lung Cell Mol Physiol. 2004 Apr;286(4):L624-30.

179. Doyle LA, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene. 2003 Oct 20;22(47):7340-58.

180. Helson L, Helson C, Malik S, Ainsworth S, Mangiardi J. A saturation threshold for taxol cytotoxicity in human glial and neuroblastoma cells. Anticancer Drugs. 1993 Aug;4(4):487-90.

181. Hierlihy AM, Seale P, Lobe CG, Rudnicki MA, Megeney LA. The post-natal heart contains a myocardial stem cell population. FEBS Lett. 2002 Oct 23;530(1-3):239-43.

182. Pfister O, Mouquet F, Jain M, Summer R, Helmes M, Fine A, Colucci WS, Liao R. CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. Circ Res. 2005 Jul 8;97(1):52-61.

183. Oyama T, Nagai T, Wada H, Naito AT, Matsuura K, Iwanaga K, Takahashi T, Goto M, Mikami Y, Yasuda N, Akazawa H, Uezumi A, Takeda S, Komuro I. Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo. J Cell Biol. 2007 Jan 29;176(3):329-41.

184. Liang SX, Khachigian LM, Ahmadi Z, Yang M, Liu S, Chong BH. In vitro and in vivo proliferation, differentiation and migration of cardiac endothelial progenitor cells (SCA1+/CD31+ side-population cells). Journal of thrombosis and haemostasis : JTH. 2011 Aug;9(8):1628-37.

185. Liang SX, Tan TY, Gaudry L, Chong B. Differentiation and migration of Sca1+/CD31- cardiac side population cells in a murine myocardial ischemic model. Int J Cardiol. 2010 Jan 7;138(1):40-9.

186. Tomita Y, Matsumura K, Wakamatsu Y, Matsuzaki Y, Shibuya I, Kawaguchi H, Ieda M, Kanakubo S, Shimazaki T, Ogawa S, Osumi N, Okano H, Fukuda K. Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. J Cell Biol. 2005 Sep 26;170(7):1135-46.

187. Pfister O, Oikonomopoulos A, Sereti KI, Sohn RL, Cullen D, Fine GC, Mouquet F, Westerman K, Liao R. Role of the ATP-binding cassette transporter Abcg2 in the phenotype and function of cardiac side population cells. Circ Res. 2008 Oct 10;103(8):825-35.

188. Mulligan JD, Schmuck EG, Ertel RL, Brellenthin AG, Bauwens JD, Saupe KW. Caloric restriction does not alter effects of aging in cardiac side population cells. Age (Dordr). 2010 Oct 5.

189. Kajstura J, Gurusamy N, Ogorek B, Goichberg P, Clavo-Rondon C, Hosoda T, D'Amario D, Bardelli S, Beltrami AP, Cesselli D, Bussani R, del Monte F, Quaini F, Rota M, Beltrami CA, Buchholz BA, Leri A, Anversa P. Myocyte turnover in the aging human heart. Circ Res. 2010 Nov 26;107(11):1374-86.

190. Schinkel AH, Mayer U, Wagenaar E, Mol CA, van Deemter L, Smit JJ, van der Valk MA, Voordouw AC, Spits H, van Tellingen O, Zijlmans JM, Fibbe WE, Borst P. Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. Proc Natl Acad Sci U S A. 1997 Apr 15;94(8):4028-33.

191. Jonker JW, Buitelaar M, Wagenaar E, Van Der Valk MA, Scheffer GL, Scheper RJ, Plosch T, Kuipers F, Elferink RP, Rosing H, Beijnen JH, Schinkel AH. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl Acad Sci U S A. 2002 Nov 26;99(24):15649-54.

192. Martin CM, Ferdous A, Gallardo T, Humphries C, Sadek H, Caprioli A, Garcia JA, Szweda LI, Garry MG, Garry DJ. Hypoxia-Inducible Factor-2{alpha} Transactivates Abcg2 and Promotes Cytoprotection in Cardiac Side Population Cells. Circ Res. 2008 May 9;102(9):1075-81.

193. Sereti KI, Oikonomopoulos A, Unno K, Cao X, Qiu Y, Liao R. ATP-binding cassette G-subfamily transporter 2 regulates cell cycle progression and asymmetric division in mouse cardiac side population progenitor cells. Circ Res. 2013 Jan 4;112(1):27-34.

194. Higashikuni Y, Sainz J, Nakamura K, Takaoka M, Enomoto S, Iwata H, Sahara M, Tanaka K, Koibuchi N, Ito S, Kusuhara H, Sugiyama Y, Hirata Y, Nagai R, Sata M. The ATP-binding cassette transporter BCRP1/ABCG2 plays a pivotal role in cardiac repair after myocardial infarction via modulation of microvascular endothelial cell survival and function. Arterioscler Thromb Vasc Biol. 2010 Nov;30(11):2128-35.

195. Alfakir M, Dawe N, Eyre R, Tyson-Capper A, Britton K, Robson SC, Meeson AP. The temporal and spatial expression patterns of ABCG2 in the developing human heart. Int J Cardiol. 2010 Nov 24.

196. Shen H, Lee FY, Gan J. Ixabepilone, a novel microtubule-targeting agent for breast cancer, is a substrate for P-glycoprotein (P-gp/MDR1/ABCB1) but not breast cancer resistance protein (BCRP/ABCG2). The Journal of pharmacology and experimental therapeutics. 2011 May;337(2):423-32.

197. Henrich CJ, Bokesch HR, Dean M, Bates SE, Robey RW, Goncharova EI, Wilson JA, McMahon JB. A High-Throughput Cell-Based Assay for Inhibitors of ABCG2 Activity. J Biomol Screen. 2006 Feb 20.

198. Wohlschlaeger J, Levkau B, Takeda A, Takeda N, Stypmann J, Schmid C, Milting H, Schmid KW, Baba HA. Increase of ABCG2/BCRP+ side population stem cells in myocardium after ventricular unloading. J Heart Lung Transplant. 2012 Mar;31(3):318-24.

199. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res. 2004 Oct 29;95(9):911-21.

200. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marban E. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation. 2007 Feb 20;115(7):896-908.

201. Simpson DL, Mishra R, Sharma S, Goh SK, Deshmukh S, Kaushal S. A strong regenerative ability of cardiac stem cells derived from neonatal hearts. Circulation. 2012 Sep 11;126(11 Suppl 1):S46-53.

202. Chan HH, Meher Homji Z, Gomes RS, Sweeney D, Thomas GN, Tan JJ, Zhang H, Perbellini F, Stuckey DJ, Watt SM, Taggart D, Clarke K, Martin-Rendon E, Carr CA. Human cardiosphere-derived cells from patients with chronic ischaemic heart disease can be routinely expanded from atrial but not epicardial ventricular biopsies. J Cardiovasc Transl Res. 2012 Oct;5(5):678-87.

203. Carr CA, Stuckey DJ, Tan JJ, Tan SC, Gomes RS, Camelliti P, Messina E, Giacomello A, Ellison GM, Clarke K. Cardiosphere-derived cells improve function in the infarcted rat heart for at least 16 weeks--an MRI study. PLoS One. 2011;6(10):e25669.

204. Maxeiner H, Krehbiehl N, Muller A, Woitasky N, Akinturk H, Muller M, Weigand MA, Abdallah Y, Kasseckert S, Schreckenberg R, Schluter KD, Wenzel S. New insights into paracrine mechanisms of human cardiac progenitor cells. Eur J Heart Fail. 2010 Jul;12(7):730-7.

205. Andersen DC, Andersen P, Schneider M, Jensen HB, Sheikh SP. Murine "cardiospheres" are not a source of stem cells with cardiomyogenic potential. Stem Cells. 2009 Jul;27(7):1571-81.

206. White AJ, Smith RR, Matsushita S, Chakravarty T, Czer LS, Burton K, Schwarz ER, Davis DR, Wang Q, Reinsmoen NL, Forrester JS, Marban E, Makkar R. Intrinsic cardiac origin of human cardiosphere-derived cells. Eur Heart J. 2013 Jan;34(1):68-75.

207. Li TS, Cheng K, Lee ST, Matsushita S, Davis D, Malliaras K, Zhang Y, Matsushita N, Smith RR, Marban E. Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell-matrix interactions, rationalizing their enhanced functional potency for myocardial repair. Stem Cells. 2010 Nov;28(11):2088-98.

208. Tseliou E, Pollan S, Malliaras K, Terrovitis J, Sun B, Galang G, Marban L, Luthringer D, Marban E. Allogeneic cardiospheres safely boost cardiac function and attenuate adverse remodeling after myocardial infarction in immunologically mismatched rat strains. J Am Coll Cardiol. 2013 Mar 12;61(10):1108-19.

209. Chimenti I, Smith RR, Li TS, Gerstenblith G, Messina E, Giacomello A, Marban E. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. Circ Res. 2010 Mar 19;106(5):971-80.

210. Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, von Gise A, Ikeda S, Chien KR, Pu WT. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature. 2008 Jul 3;454(7200):109-13.

211. Cai CL, Martin JC, Sun Y, Cui L, Wang L, Ouyang K, Yang L, Bu L, Liang X, Zhang X, Stallcup WB, Denton CP, McCulloch A, Chen J, Evans SM. A myocardial lineage derives from Tbx18 epicardial cells. Nature. 2008 Jul 3;454(7200):104-8.

212. van Wijk B, Gunst QD, Moorman AF, van den Hoff MJ. Cardiac regeneration from activated epicardium. PLoS One. 2012;7(9):e44692.

213. Huang GN, Thatcher JE, McAnally J, Kong Y, Qi X, Tan W, DiMaio JM, Amatruda JF, Gerard RD, Hill JA, Bassel-Duby R, Olson EN. C/EBP transcription factors mediate epicardial activation during heart development and injury. Science. 2012 Dec 21;338(6114):1599-603.

214. Smart N, Bollini S, Dube KN, Vieira JM, Zhou B, Davidson S, Yellon D, Riegler J, Price AN, Lythgoe MF, Pu WT, Riley PR. De novo cardiomyocytes from within the activated adult heart after injury. Nature. 2011 Jun 30;474(7353):640-4.

215. Zhou B, Honor LB, Ma Q, Oh JH, Lin RZ, Melero-Martin JM, von Gise A, Zhou P, Hu T, He L, Wu KH, Zhang H, Zhang Y, Pu WT. Thymosin beta 4 treatment after myocardial infarction does not reprogram epicardial cells into cardiomyocytes. J Mol Cell Cardiol. 2012 Jan;52(1):43-7.

216. Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. Nature. 2004 Nov 25;432(7016):466-72.

217. Chen S, Shimoda M, Chen J, Grayburn PA. Stimulation of adult resident cardiac progenitor cells by durable myocardial expression of thymosin beta 4 with ultrasound-targeted microbubble delivery. Gene therapy. 2012 Nov 15.

218. Hinescu ME, Popescu LM. Interstitial Cajal-like cells (ICLC) in human atrial myocardium. J Cell Mol Med. 2005 Oct-Dec;9(4):972-5.

219. Popescu LM, Manole CG, Gherghiceanu M, Ardelean A, Nicolescu MI, Hinescu ME, Kostin S. Telocytes in human epicardium. J Cell Mol Med. 2010 Aug;14(8):2085-93.

220. Gherghiceanu M, Popescu LM. Cardiac telocytes - their junctions and functional implications. Cell Tissue Res. 2012 May;348(2):265-79.

221. Drapeau J, El-Helou V, Clement R, Bel-Hadj S, Gosselin H, Trudeau LE, Villeneuve L, Calderone A. Nestin-expressing neural stem cells identified in the scar following myocardial infarction. J Cell Physiol. 2005 Jul;204(1):51-62.

222. El-Helou V, Beguin PC, Assimakopoulos J, Clement R, Gosselin H, Brugada R, Aumont A, Biernaskie J, Villeneuve L, Leung TK, Fernandes KJ, Calderone A. The rat heart contains a neural stem cell population; Role in sympathetic sprouting and angiogenesis. J Mol Cell Cardiol. 2008 Nov;45(5):694-702.

223. Storms RW, Trujillo AP, Springer JB, Shah L, Colvin OM, Ludeman SM, Smith C. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci U S A. 1999 Aug 3;96(16):9118-23.

224. Koninckx R, Daniels A, Windmolders S, Mees U, Macianskiene R, Mubagwa K, Steels P, Jamaer L, Dubois J, Robic B, Hendrikx M, Rummens JL, Hensen K. The cardiac atrial appendage stem cell: a new and promising candidate for myocardial repair. Cardiovasc Res. 2013 Mar 1;97(3):413-23.

225. Poss KD, Keating MT, Nechiporuk A. Tales of regeneration in zebrafish. Dev Dyn. 2003 Feb;226(2):202-10.

226. Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. Science. 2002 Dec 13;298(5601):2188-90.

227. Itou J, Kawakami H, Burgoyne T, Kawakami Y. Life-long preservation of the regenerative capacity in the fin and heart in zebrafish. Biology open. 2012 Aug 15;1(8):739-46.

228. Jopling C, Sleep E, Raya M, Marti M, Raya A, Belmonte JC. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. Nature. 2010 Mar 25;464(7288):606-9.

229. Gupta V, Poss KD. Clonally dominant cardiomyocytes direct heart morphogenesis. Nature. 2012 Apr 26;484(7395):479-84.

230. Lepilina A, Coon AN, Kikuchi K, Holdway JE, Roberts RW, Burns CG, Poss KD. A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. Cell. 2006 Nov 3;127(3):607-19.

231. Parente V, Balasso S, Pompilio G, Verduci L, Colombo GI, Milano G, Guerrini U, Squadroni L, Cotelli F, Pozzoli O, Capogrossi MC. Hypoxia/reoxygenation cardiac injury and regeneration in zebrafish adult heart. PLoS One. 2013;8(1):e53748.

232. Jean MJ, Deverteuil P, Lopez NH, Tapia JD, Schoffstall B. Adult zebrafish hearts efficiently compensate for excessive forced overload cardiac stress with hyperplastic cardiomegaly. BioResearch open access. 2012 Apr;1(2):88-91.

233. Kemi OJ, Hoydal MA, Macquaide N, Haram PM, Koch LG, Britton SL, Ellingsen O, Smith GL, Wisloff U. The effect of exercise training on transverse tubules in normal, remodeled, and reverse remodeled hearts. J Cell Physiol. 2011 Sep;226(9):2235-43.

234. Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA. Transient regenerative potential of the neonatal mouse heart. Science. 2011 Feb 25;331(6020):1078-80.

235. Herdrich BJ, Danzer E, Davey MG, Allukian M, Englefield V, Gorman JH, 3rd, Gorman RC, Liechty KW. Regenerative healing following foetal myocardial infarction. Eur J Cardiothorac Surg. 2010 Dec;38(6):691-8.

236. Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Guerquin-Kern JL, Lechene CP, Lee RT. Mammalian heart renewal by pre-existing cardiomyocytes. Nature. 2013 Jan 17;493(7432):433-6.

237. Zhang SN, Sun AJ, Ge JB, Yao K, Huang ZY, Wang KQ, Zou YZ. Intracoronary autologous bone marrow stem cells transfer for patients with acute myocardial infarction: A meta-analysis of randomised controlled trials. Int J Cardiol. 2008 Jul 19.

238. Menasche P, Hagege AA, Scorsin M, Pouzet B, Desnos M, Duboc D, Schwartz K, Vilquin JT, Marolleau JP. Myoblast transplantation for heart failure. Lancet. 2001 Jan 27;357(9252):279-80.

239. Seidel M, Borczynska A, Rozwadowska N, Kurpisz M. Cell-based therapy for heart failure: skeletal myoblasts. Cell Transplant. 2009;18(7):695-707.

240. Menasche P, Alfieri O, Janssens S, McKenna W, Reichenspurner H, Trinquart L, Vilquin JT, Marolleau JP, Seymour B, Larghero J, Lake S, Chatellier G, Solomon S, Desnos M, Hagege AA. The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. Circulation. 2008 Mar 4;117(9):1189-200.

241. Pedrotty DM, Klinger RY, Badie N, Hinds S, Kardashian A, Bursac N. Structural coupling of cardiomyocytes and noncardiomyocytes: quantitative comparisons using a novel micropatterned cell pair assay. Am J Physiol Heart Circ Physiol. 2008 Jul;295(1):H390-400.

242. van der Bogt KE, Sheikh AY, Schrepfer S, Hoyt G, Cao F, Ransohoff KJ, Swijnenburg RJ, Pearl J, Lee A, Fischbein M, Contag CH, Robbins RC, Wu JC. Comparison of different adult stem cell types for treatment of myocardial ischemia. Circulation. 2008 Sep 30;118(14 Suppl):S121-9.

243. Kan CD, Lee HL, Yang YJ. Cell transplantation for myocardial injury: a preliminary comparative study. Cytotherapy. 2010 Sep;12(5):692-700.

244. Losordo DW, Schatz RA, White CJ, Udelson JE, Veereshwarayya V, Durgin M, Poh KK, Weinstein R, Kearney M, Chaudhry M, Burg A, Eaton L, Heyd L, Thorne T, Shturman L, Hoffmeister P, Story K, Zak V, Dowling D, Traverse JH, Olson RE, Flanagan J, Sodano D, Murayama T, Kawamoto A, Kusano KF, Wollins J, Welt F, Shah P, Soukas P, Asahara T, Henry TD. Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial. Circulation. 2007 Jun 26;115(25):3165-72.

245. Tendera M, Wojakowski W, Ruzyllo W, Chojnowska L, Kepka C, Tracz W, Musialek P, Piwowarska W, Nessler J, Buszman P, Grajek S, Breborowicz P, Majka M, Ratajczak MZ. Intracoronary infusion of bone marrow-derived selected CD34+CXCR4+ cells and non-selected mononuclear cells in patients with acute STEMI and reduced left ventricular ejection fraction: results of randomized, multicentre Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction (REGENT) Trial. Eur Heart J. 2009 Jun;30(11):1313-21.

246. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schumichen C, Nienaber CA, Freund M, Steinhoff G. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet. 2003 Jan 4;361(9351):45-6.

247. Stamm C, Kleine HD, Choi YH, Dunkelmann S, Lauffs JA, Lorenzen B, David A, Liebold A, Nienaber C, Zurakowski D, Freund M, Steinhoff G. Intramyocardial delivery of CD133+ bone marrow cells and coronary artery bypass grafting for chronic ischemic heart disease: safety and efficacy studies. J Thorac Cardiovasc Surg. 2007 Mar;133(3):717-25.

248. Katritsis DG, Sotiropoulou PA, Karvouni E, Karabinos I, Korovesis S, Perez SA, Voridis EM, Papamichail M. Transcoronary transplantation of autologous mesenchymal stem cells and endothelial progenitors into infarcted human myocardium. Catheter Cardiovasc Interv. 2005 Jul;65(3):321-9.

249. Hare JM, Fishman JE, Gerstenblith G, Difede Velazquez DL, Zambrano JP, Suncion VY, Tracy M, Ghersin E, Johnston PV, Brinker JA, Breton E, Davis-Sproul J, Schulman IH, Byrnes J, Mendizabal AM, Lowery MH, Rouy D, Altman P, Wong Po Foo C, Ruiz P, Amador A, Da Silva J, McNiece IK, Heldman AW. Comparison of Allogeneic vs Autologous Bone Marrow-Derived Mesenchymal Stem Cells Delivered by Transendocardial Injection in Patients With Ischemic Cardiomyopathy: The POSEIDON Randomized Trial. JAMA : the journal of the American Medical Association. 2012 Nov 6:1-11.

250. Hare JM, Traverse JH, Henry TD, Dib N, Strumpf RK, Schulman SP, Gerstenblith G, DeMaria AN, Denktas AE, Gammon RS, Hermiller JB, Jr., Reisman MA, Schaer GL, Sherman W. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am Coll Cardiol. 2009 Dec 8;54(24):2277-86.

251. Zhao Q, Sun Y, Xia L, Chen A, Wang Z. Randomized study of mononuclear bone marrow cell transplantation in patients with coronary surgery. Ann Thorac Surg. 2008 Dec;86(6):1833-40.

252. de la Fuente LM, Stertzer SH, Argentieri J, Penaloza E, Miano J, Koziner B, Bilos C, Altman PA. Transendocardial autologous bone marrow in chronic myocardial infarction using a helical needle catheter: 1-year follow-up in an open-label, nonrandomized, single-center pilot study (the TABMMI study). Am Heart J. 2007 Jul;154(1):79 e1-7.

253. Hou D, Youssef EA, Brinton TJ, Zhang P, Rogers P, Price ET, Yeung AC, Johnstone BH, Yock PG, March KL. Radiolabeled cell distribution after intramyocardial, intracoronary, and interstitial retrograde coronary venous delivery: implications for current clinical trials. Circulation. 2005 Aug 30;112(9 Suppl):I150-6.

254. Hofmann M, Wollert KC, Meyer GP, Menke A, Arseniev L, Hertenstein B, Ganser A, Knapp WH, Drexler H. Monitoring of bone marrow cell homing into the infarcted human myocardium. Circulation. 2005 May 3;111(17):2198-202.

255. Menasche P. Cardiac cell therapy: lessons from clinical trials. J Mol Cell Cardiol. 2011 Feb;50(2):258-65.

256. van Ramshorst J, Bax JJ, Beeres SL, Dibbets-Schneider P, Roes SD, Stokkel MP, de Roos A, Fibbe WE, Zwaginga JJ, Boersma E, Schalij MJ, Atsma DE. Intramyocardial bone marrow cell injection for chronic myocardial ischemia: a randomized controlled trial. JAMA : the journal of the American Medical Association. 2009 May 20;301(19):1997-2004.

257. Menasche P. Cell-based therapy for heart disease: a clinically oriented perspective. Mol Ther. 2009 May;17(5):758-66.

258. Assmus B, Honold J, Schachinger V, Britten MB, Fischer-Rasokat U, Lehmann R, Teupe C, Pistorius K, Martin H, Abolmaali ND, Tonn T, Dimmeler S, Zeiher AM. Transcoronary transplantation of progenitor cells after myocardial infarction. N Engl J Med. 2006 Sep 21;355(12):1222-32.

259. Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, Beache GM, Wagner SG, Leri A, Hosoda T, Sanada F, Elmore JB, Goichberg P, Cappetta D, Solankhi NK, Fahsah I, Rokosh DG, Slaughter MS, Kajstura J, Anversa P. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet. 2011 Nov 26;378(9806):1847-57.

260. Chugh AR, Beache GM, Loughran JH, Mewton N, Elmore JB, Kajstura J, Pappas P, Tatooles A, Stoddard MF, Lima JA, Slaughter MS, Anversa P, Bolli R. Administration of cardiac stem cells in patients with ischemic cardiomyopathy: the SCIPIO trial: surgical aspects and interim analysis of myocardial function and viability by magnetic resonance. Circulation. 2012 Sep 11;126(11 Suppl 1):S54-64.

261. Makkar RR, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, Czer LS, Marban L, Mendizabal A, Johnston PV, Russell SD, Schuleri KH, Lardo AC, Gerstenblith G, Marban E. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet. 2012 Mar 10;379(9819):895-904.

262. Malliaras K, Makkar RR, Smith RR, Cheng K, Wu E, Bonow RO, Marban L, Mendizabal A, Cingolani E, Johnston PV, Gerstenblith G, Schuleri KH, Lardo AC, Marban E. Intracoronary cardiosphere-derived cells after myocardial infarction: evidence for therapeutic regeneration in the final 1-year results of the CADUCEUS trial. J Am Coll Cardiol. 2013 Sep 2.

263. Cox JL, Boineau JP, Schuessler RB, Jaquiss RD, Lappas DG. Modification of the maze procedure for atrial flutter and atrial fibrillation. I. Rationale and surgical results. J Thorac Cardiovasc Surg. 1995 Aug;110(2):473-84.

264. Kamo M, Inouye K, Nagata K, Tanokura M. Preliminary X-ray crystallographic analysis of thermolysin in the presence of 4 M NaCl. Acta crystallographica Section D, Biological crystallography. 2005 Jun;61(Pt 6):710-2.

265. Maxfield FR, McGraw TE. Endocytic recycling. Nat Rev Mol Cell Biol. 2004 Feb;5(2):121-32.

266. Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood. 2002 Jan 15;99(2):507-12.

267. Kaufman DS, Lewis RL, Hanson ET, Auerbach R, Plendl J, Thomson JA. Functional endothelial cells derived from rhesus monkey embryonic stem cells. Blood. 2004 Feb 15;103(4):1325-32.

268. Smits AM, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology. Nat Protoc. 2009;4(2):232-43.

269. Patra SK, Bettuzzi S. Epigenetic DNA-(cytosine-5-carbon) modifications: 5-aza-2'-deoxycytidine and DNA-demethylation. Biochemistry Biokhimiia. 2009 Jun;74(6):613-9.

270. Issa JP, Kantarjian H. Azacitidine. Nature reviews Drug discovery. 2005 May;Suppl: S6-7.

271. Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV. Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry. 1992;13(2):204-8.

272. Petersen TW, Ibrahim SF, Diercks AH, van den Engh G. Chromatic shifts in the fluorescence emitted by murine thymocytes stained with Hoechst 33342. Cytometry A. 2004 Aug;60(2):173-81.

273. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012 Jul;9(7):676-82.

274. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A. 1991 Aug 15;88(16):7276-80.

275. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.

276. Stahlberg A, Kubista M, Aman P. Single-cell gene-expression profiling and its potential diagnostic applications. Expert review of molecular diagnostics. 2011 Sep;11(7):735-40.

277. Hauswirth AW, Florian S, Schernthaner GH, Krauth MT, Sonneck K, Sperr WR, Valent P. Expression of cell surface antigens on mast cells: mast cell phenotyping. Methods Mol Biol. 2006;315:77-90.

278. Urbanek K, Torella D, Sheikh F, De Angelis A, Nurzynska D, Silvestri F, Beltrami CA, Bussani R, Beltrami AP, Quaini F, Bolli R, Leri A, Kajstura J, Anversa P. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci U S A. 2005 Jun 14;102(24):8692-7.

279. Kasahara H, Ueyama T, Wakimoto H, Liu MK, Maguire CT, Converso KL, Kang PM, Manning WJ, Lawitts J, Paul DL, Berul CI, Izumo S. Nkx2.5 homeoprotein regulates expression of gap junction protein connexin 43 and sarcomere organization in postnatal cardiomyocytes. J Mol Cell Cardiol. 2003 Mar;35(3):243-56.

280. Everett THt, Olgin JE. Atrial fibrosis and the mechanisms of atrial fibrillation. Heart Rhythm. 2007 Mar;4(3 Suppl):S24-7.

281. Jesty SA, Steffey MA, Lee FK, Breitbach M, Hesse M, Reining S, Lee JC, Doran RM, Nikitin AY, Fleischmann BK, Kotlikoff MI. c-kit+ precursors support postinfarction myogenesis in the neonatal, but not adult, heart. Proc Natl Acad Sci U S A. 2012 Aug 14;109(33):13380-5.

282. Tallini YN, Greene KS, Craven M, Spealman A, Breitbach M, Smith J, Fisher PJ, Steffey M, Hesse M, Doran RM, Woods A, Singh B, Yen A, Fleischmann BK, Kotlikoff MI. c-kit expression identifies cardiovascular precursors in the neonatal heart. Proc Natl Acad Sci U S A. 2009 Feb 10;106(6):1808-13.

283. Zhang Y, Li TS, Lee ST, Wawrowsky KA, Cheng K, Galang G, Malliaras K, Abraham MR, Wang C, Marban E. Dedifferentiation and proliferation of mammalian cardiomyocytes. PLoS One. 2010;5(9):e12559.

284. Guo S, Cheng Y, Ma Y, Yang X. Endothelial progenitor cells derived from CD34+ cells form cooperative vascular networks. Cell Physiol Biochem. 2010;26(4-5):679-88.

285. Sun X, Cheng L, Duan H, Lin G, Lu G. Characterization and comparison of embryonic stem cell-derived KDR+ cells with endothelial cells. Microvasc Res. 2012 Sep;84(2):149-54.

286. Ryzhov S, Goldstein AE, Novitskiy SV, Blackburn MR, Biaggioni I, Feoktistov I. Role of A2B adenosine receptors in regulation of paracrine functions of stem cell antigen 1-positive cardiac stromal cells. The Journal of pharmacology and experimental therapeutics. 2012 Jun;341(3):764-74.

287. Zhou S, Liao L, Chen C, Zeng W, Liu S, Su J, Zhao S, Chen M, Kuang Y, Chen X, Li J. CD147 mediates chemoresistance in breast cancer via ABCG2 by affecting its cellular localization and dimerization. Cancer letters. 2013 Sep 1;337(2):285-92.

288. Takada T, Suzuki H, Gotoh Y, Sugiyama Y. Regulation of the cell surface expression of human BCRP/ABCG2 by the phosphorylation state of Akt in polarized cells. Drug metabolism and disposition: the biological fate of chemicals. 2005 Jul;33(7):905-9.

289. Yamahara K, Fukushima S, Coppen SR, Felkin LE, Varela-Carver A, Barton PJ, Yacoub MH, Suzuki K. Heterogeneic nature of adult cardiac side population cells. Biochem Biophys Res Commun. 2008 Jul 11;371(4):615-20.

290. Preffer FI, Dombkowski D, Sykes M, Scadden D, Yang YG. Lineage-negative side-population (SP) cells with restricted hematopoietic capacity circulate in normal human adult blood: immunophenotypic and functional characterization. Stem Cells. 2002;20(5):417-27.

291. Hussain SZ, Strom SC, Kirby MR, Burns S, Langemeijer S, Ueda T, Hsieh M, Tisdale JF. Side population cells derived from adult human liver generate hepatocyte-like cells in vitro. Dig Dis Sci. 2005 Oct;50(10):1755-63.

292. Liu WH, Qian NS, Li R, Dou KF. Replacing Hoechst33342 with rhodamine123 in isolation of cancer stem-like cells from the MHCC97 cell line. Toxicol In Vitro. 2010 Mar;24(2):538-45.

293. Fried J, Doblin J, Takamoto S, Perez A, Hansen H, Clarkson B. Effects of Hoechst 33342 on survival and growth of two tumor cell lines and on hematopoietically normal bone marrow cells. Cytometry. 1982 Jul;3(1):42-7.

294. Mozley PD, Stubbs JB, Dresel SH, Barraclough ED, Smith T, Patel M, Finkelman C, Sparks RB, Thakur ML, Mathur A, Alavi A. Radiation dosimetry of a 99mTc-labeled IgM murine antibody to CD15 antigens on human granulocytes. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 1999 Apr;40(4):625-30.

295. Miyamoto S, Kawaguchi N, Ellison GM, Matsuoka R, Shin'oka T, Kurosawa H. Characterization of Long-Term Cultured c-kitpos Cardiac Stem Cells (CSCs) Derived from Adult Rat Hearts. Stem Cells Dev. 2009 Jul 6.

296. Arminan A, Gandia C, Bartual M, Garcia-Verdugo JM, Lledo E, Mirabet V, Llop M, Barea J, Montero JA, Sepulveda P. Cardiac differentiation is driven by NKX2.5 and GATA4 nuclear translocation in tissue-specific mesenchymal stem cells. Stem Cells Dev. 2009 Jul-Aug;18(6):907-18.

297. Dey D, Han L, Bauer M, Sanada F, Oikonomopoulos A, Hosoda T, Unno K, De Almeida P, Leri A, Wu JC. Dissecting the molecular relationship among various cardiogenic progenitor cells. Circ Res. 2013 Apr 26;112(9):1253-62.

298. Serradifalco C, Catanese P, Rizzuto L, Cappello F, Puleio R, Barresi V, Nunnari CM, Zummo G, Felice VD. Embryonic and foetal Islet-1 positive cells in human hearts are also positive to c-Kit. European journal of histochemistry : EJH. 2011;55(4):e41.

299. Hou X, Appleby N, Fuentes T, Longo LD, Bailey LL, Hasaniya N, Kearns-Jonker M. Isolation, Characterization, and Spatial Distribution of Cardiac Progenitor Cells in the Sheep Heart. Journal of clinical & experimental cardiology. 2012 Oct 11;S6.

300. Liao CH, Akazawa H, Tamagawa M, Ito K, Yasuda N, Kudo Y, Yamamoto R, Ozasa Y, Fujimoto M, Wang P, Nakauchi H, Nakaya H, Komuro I. Cardiac mast cells cause atrial fibrillation through PDGF-A-mediated fibrosis in pressure-overloaded mouse hearts. J Clin Invest. 2010 Jan;120(1):242-53.

301. Jalife J. Experimental and clinical AF mechanisms: bridging the divide. J Interv Card Electrophysiol. 2003 Oct;9(2):85-92.

302. Tang YL, Zhu W, Cheng M, Chen L, Zhang J, Sun T, Kishore R, Phillips MI, Losordo DW, Qin G. Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression. Circ Res. 2009 May 22;104(10):1209-16.

303. Dogterom P, Zbinden G, Reznik GK. Cardiotoxicity of vasodilators and positive inotropic/vasodilating drugs in dogs: an overview. Critical reviews in toxicology. 1992;22(3-4):203-41.

304. Milani-Nejad N, Janssen PM. Small and large animal models in cardiac contraction research: Advantages and disadvantages. Pharmacol Ther. 2013 Oct 15.

305. Problems with scientific research. How science goes wrong. The Economist. 2013 Oct 19th.

306. Hawkes N. Most laboratory cancer studies cannot be replicated, study shows. BMJ (Clinical research ed). 2012;344:e2555.

307. Enochson L, Brittberg M, Lindahl A. Optimization of a chondrogenic medium through the use of factorial design of experiments. BioResearch open access. 2012 Dec;1(6):306-13.