TRANSCRIPTIONAL PROFILE OF C-KIT POSITIVE CARDIAC STEM-PROGENITOR CELLS (c-kit^{pos} eCSCs) ISOLATED FROM THE FOUR CHAMBERS OF THE ADULT HUMAN HEART

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DEDICATION

To my wife Georgina, and my two little daughters, Martha and Maria. I apologise to them as during this period of working on this project they never saw me at home.

ACKNOWLEDGEMENTS

This work will not be successfully completed if a number of very important people would not participate to help me. Family, educational supervisors, cardiac surgery consultants, patients, mentors and laboratory researches colleagues were near to me throughout this difficult period of this scientific exercise. I am grateful to them for life and I hope that this little piece of work will give to the scientific community a little help to understand cardiac stem cell biology and mechanisms of repair the damaged myocardium, so patients with cardiac diseases will not suffer any more.

ABSTRACT

Introduction: Our findings and those of others show that the adult myocardium, including human, harbours a population of resident (endogenous) cardiac stem cells (eCSCs). They express the stem cell factor receptor c-kit, are distributed throughout the myocardium, are clonogenic, self-renewing and multi-potent, in that they differentiate into the 3 main cardiac lineages; cardiomyocytes, smooth muscle and endothelial cells *in vitro* and *in vivo*. The objective of this study is to determine whether c-kit^{pos} eCSCs isolated from the different cardiac chambers have a distinct transcriptional profile depending on the chamber of origin.

Methods: Pieces of myocardium have been obtained from all the 4 chambers of the adult human heart. All patients were fully consented before undergone open heart surgery. They were suffered of various cardiac pathologies such as ischemic heart disease, aortic, mitral and tricuspid valve insufficiency or stenosis, and various aortic pathologies. Ethical approval for these procedures has been given by NREC (08/H1306/91).c-kit^{pos} eCSCs were isolated by enzymatic digestion and purified by Magnetic Activated Cell Sorting (MACS) from samples taken from the right and left atria (RA, LA), right and left ventricle (RV, LV) of the adult human heart. mRNA was isolated using Qiagen® mRNA kit, and reverse transcribed using first strand cDNA synthesis with random hexamers. qRT-PCR was performed using SYBR Green on a MyIQ thermocycler Bio-Rad® of specific genes representative of the primary and secondary heart field, and the developmental program of their chamber of origin.

Results: c-kit^{pos} eCSCs isolated from 15 human samples (5LA, 1RV, 4LV, 5RA) were processed. c-kit^{pos} eCSCs are distributed throughout the human myocardium and in all 4 chambers of the heart. Transmitted light microscopic observations of c-kit^{pos} eCSCs revealed that the c-kit^{pos} cells from the human biopsies were generally small and rounded, consistent with a stable c-kit^{pos} eCSCs phenotype, regardless of the chamber of origin. The eCSCs c-kit^{pos} cells could be cultured under hypoxic conditions between 7 and 12 days to attain full

confluency. Expression of transcripts for c-kit, Foxh1, Hand1, Hand2, Pitx2, Tbx5, Tbx20, Hrt1, Hrt2, Fgf8, Fgf10, and Isl1 were found at differential levels in c-kit^{pos} CSCs isolated from the four cardiac chambers.

Conclusion: This study is the first to show that c-kit^{pos} eCSCs derived from human adult cardiac samples, do not appear to have a 'chamber-specific' transcript footprint, and are therefore potentially interchangeable between cardiac chambers, raising the potential of their therapeutic application.

Key words: human c-kit^{pos} cardiac stem cells, left atria, right atria, left ventricle, right ventricle, transcriptional profile, primary heart field, secondary heart field.

LIST OF CONTENTS……...Page

A. LIST OF TABLES.....Page

B. LIST OF FIGURES……...Page

ABBREVIATIONS

C-kit: or tyrosine-protein kinase Kit or CD117 is a receptor tyrosine kinase protein that in humans is encoded by the kit gene.

eCSCs: endogenous cardiac stem cells

MACS: Magnetic-activated cell sorting

RV: Right ventricle

LV: Left ventricle

RA: Right atria

LA: Left atria

m-RNA: messenger RNA

qRT-PCR: quantitative reverse transcriptase Polymerase chain reaction

c-DNA: complementary DNA

SYBR: Syber green

EU: European Union

UK: United Kingdom

NICE: National Institute of Clinical Excellence

PCI: Percutaneous Coronary Intervention

MI: Myocardial Infarction

CRT: Cardiac Resynchronization Therapy

ESCs: embryonic stem cells

iPS: induced pluripotent stem cells

BMDCs: bone marrow derived cells

MSCs: mesenchymal stem cells

EPCs: endothelial progenitor stem cells

HSC: haematopoietic stem cells

EB: embryonic bodies

Oct3/4: octamer binding transcription factor 3/4

Sox2 or SRY: (sex determining region Y)-box 2

C-Myc: Myc (c-Myc) is a regulator gene that codes for a transcription factor. The protein encoded by this gene is a multifunctional, nuclear phosphor-protein that plays a role in cell cycle progression, apoptosis and cellular transformation.

KLF-4: Kruppel-like factor 4 (KLF4) is a member of the KLF family of transcription factors and regulates proliferation, differentiation, apoptosis and somatic reprogramming.

MAGIC TRIAL: The **M**yoblast **A**utologous **G**rafting in **I**schemic **C**ardiomyopathy Trial

BM: bone marrow

Lin$^-$: Lineage negative

Sca-1$^+$: stem cell antigen 1^+

Thy1.1: Thymosite antigen 1.1 or Cluster of Differentiation 90.

VEGF-R2: Vascular endothelial growth factor receptor 2

MHC-I: Major Histocompatibility complex I

MHC II: Major Histocompatibility complex II

RCT: Randomised control trial

AMI: acute Myocardial Infarction

ATP: adenosine triphosphate

BrdU: 5-bromo-2'-deoxyuridine

α-sarc: alpha sarcomeric actin

CSC: cardiac stem cell

vW-FVIII: von Willebrand factor VIII

CPCs: Cardiac Progenitor Cells

SP: side population cells

BMP2: Bone morphogenetic protein 2

BMP4: Bone morphogenetic protein 4

MDR1: P-glycoprotein 1 (permeability glycoprotein, abbreviated as P-gp or Pgp) also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1) or cluster of differentiation 243 (CD243) is an important protein of the cell membrane that pumps many foreign substances out of cells.

KDR: Kinase insert domain receptor (KDR, a type III receptor tyrosine kinase) also known as vascular endothelial growth factor receptor 2 (VEGFR-2) is a VEGF receptor.

ALCADIA: AutoLogous Human CArdiac-Derived Stem Cell To Treat Ischemic cArdiomyopathy.

TICAP: Intracoronary Autologous Cardiac Progenitor Cell Transfer in Patients with Hypoplastic Left Heart Syndrome

CADUCEOUS: CArdiosphere Derived aUtologous stem CElls to reverse ventricUlar dySfunction.

SCIPIO: Stem Cell In Patients with Ischemic cardiomyopathy

ALLSTAR: Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration

PERSEOUS: Cardiac Progenitor Cell Infusion to Treat Univentricular Heart Disease

MRI: Magnetic Resonance Imaging

Nkx-2.5: homeobox protein

Tbx5: T-box transcription factor

Tbx20: T-box transcription factor

bHLH: basic helix-loop-helix transcription factor

Hand1: Heart and neural crest derivatives expressed protein-1

Isl-1: Insulin gene enhancer protein-1

Hand2: Heart and neural crest derivatives expressed protein-2

FoxH1: Forkhead box protein H1

Fgf8: Fibroblast growth factor 8

Fgf10: Fibroblast growth factor 10

HGF: Hepatocyte growth Factor

IGF: Insulin Growth Factor

LHCH: Liverpool Heart & Chest Hospital

ANOVA: Analysis of variance

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Ct: cycle threshold

Tm: Melting temperature

CDS: Coding DNA sequence

RPM: rounds per minute

RLT: Buffer RLT is a lysis buffer for lysing cells and tissues prior to RNA isolation and simultaneous RNA/DNA/Protein isolation

RW1: Buffer RW1 is for washing membrane-bound RNA when following RNeasy

PBS: Phosphate buffered saline

DMEM: Dulbecco's modified Eagle's medium

ABs: Antibiotics

NAAA: N-acetylaspartate aminoacid

FBS: fetal bovine serum

b FGF: Fibroblast Growth Factor Basic

h EPO: human erythropoietin

DPBS: Dulbecco's Phosphate-Buffered Saline

EDTA: Ethylenediaminetetraacetic acid

1. INTRODUCTION

1.1 Impact of Cardiovascular Disease.

1.1.1 Cardiovascular disease and mortality.

In the 21st century when most illnesses potentially can be cured, cardiovascular disease still remains one of the most serious pathologies that kill thousands of people worldwide. Each year cardiovascular disease causes over 4 million deaths in Europe, approximately 47% of all deaths and is equally prevalent in males and females. Overall cardiovascular disease is estimated to cost the EU economy ~ €196 billion per year (1). Of this total cost around 54% is due to patient formal health care costs, 24% due to productivity losses and 22% due to the informal care of people with cardiovascular disease (2).

In the UK as in Europe, cardiovascular disease is the most prevalent pathology with chronic heart failure the most common cause of death and commonly associated with significant poor prognosis: 30–40% of patients diagnosed with chronic heart failure die within a year, but thereafter the mortality rate is 10% per year (3,4). Recent statistics in the UK identified approximately 900,000 people suffer from heart failure and every year 25,000 new cases recorded (5). Many of these patients have suffered from one or more myocardial infarctions in the past that have triggered myocardial damage and failure over time. Cardiac pathologies that could gradually damage the myocardium and progress to chronic heart failure include: hypertension, valve disease and cardiomyopathy.

There are a number of risk factors that can trigger coronary artery disease and consequently ischemic, chronic heart failure: family history, smoking, increased alcohol consumption, diabetes, obesity and high cholesterol together with loss of physical activity and adoption of a sedentary lifestyle (6). All of these factors can result in damage of the coronary vasculature, placing the cardiac muscle in danger of a myocardial infarction with progressive and gradual cardiac failure.

Both the incidence and prevalence of chronic heart failure increase with age, with the average age at first diagnosis being 76 years in 2006 (7). The prevalence of chronic heart failure is expected to rise in the future as a result of an ageing population, improved survival of people with ischaemic heart disease and more effective treatments for heart failure (8). There is also evidence of a trend for improved prognosis, with 6-month mortality rate decreased from 26% in 1995 to 14% in 2005 (9). More than £500 million is spent annually on the treatment of chronic heart failure, which is ~ 1.4% of the annual health care budget (10). The overall rate of chronic heart failure hospitalisation per 10,000 people did not change significantly from 2000 to 2010 (35.5 compared with 32.8), but the trends were different between those under and over age 65 showing that chronic heart failure is increasingly prevalent in younger individuals (11).

1.1.2 Mechanisms of cardiomyocyte failure.

Improved medical and surgical management in the past 30 years has produced a decline in the death rate due to cardiovascular disease. However, damage to the heart often leads to negative cardiac remodelling (12). Left ventricular remodelling can progress to biventricular heart failure and accelerated ventricular remodelling is a probable contributor to the increased mortality observed after myocardial infarction in hypertensive patients.

A number of studies have shown that the failing human myocardium undergo significant changes that can lead to a steady and progressing loss of its contractile function. This can be seen in a molecular level and as well in a neuro-hormonal regulation level. There is in fact a decreased expression of α-myosin heavy chain gene with increased expression of β-myosin heavy chain (13, 14, 15).Simultaneously there is progressive loss of myofilaments in cardiomyocytes, alterations in cytoskeletal proteins, alterations in excitation contraction coupling and desensitization of β-adrenergic signalling (16). Some of these changes it could be presumed that they are mechanisms of adaptation and could be beneficial in terms of

protecting cardiomyocytes against the potential deleterious consequences of excessive neuro-hormonal activation, however when these changes are seen in a molecular and then functional level they could lead to a defect in cardiomyocyte contractile function, as well as decreased and loss of responsiveness to normal adrenergic control mechanisms, both of which are characteristics of failing human myocardium.

Thus, the contractile dysfunction that develops within cardiomyocytes during the process of LV remodelling is likely to involve a number of genes, including those that regulate calcium handling, sarcomere turnover, α-adrenergic signalling and the cytoskeleton, all of which may interact in an exceedingly complex manner within the cardiac myocytes to produce contractile dysfunction (17). The cellular basis of this cardiac deterioration is a decrease in the number of viable cardiomyocytes, secondary to either acute ischemic injury or chronic cell apoptosis/necrosis, which is not balanced by the adaptive hypertrophy of the remaining cardiomyocytes. To overcome the myocardial damage medical and surgical therapies have been implemented to reduce the loss of the healthy cardiomyocytes and improve cardiac function and patient symptoms with better quality of life and better outcomes; unfortunately the success on curing chronic heart failure is still debatable.

1.1.3. Current treatment of cardiovascular disease.

The use of triple medical therapy (ace-inhibitors, diuretics and β-blockers) in patients with chronic heart failure has improved the outcomes and delayed the progression of the disease. However, due to other multiple comorbidities, e.g. diabetes, obesity, hypertension despite maximal medical therapy and recently cardiac resynchronization therapy or CRT (18), the failing heart also affects other vital organs, including the kidneys and liver. More than 800 medical clinical trials have been implemented, to determine the best medical treatment to cure and improve the quality of life of patients who are suffering with heart failure (www.clinical trials.gov). Surgical therapy cannot offer a better advantage to medical therapy

and in most of patient cases the long term solution is assist devices as a destination therapy or as a bridge to transplantation (19, 20). However the lack of donors and the long-term side effects of the anti-rejection medications together with the enormous cost of the assist devices, questions the future validity of this mode of intervention. Furthermore, current therapy cannot reverse the harmful remodelling and loss of myocytes that accompany injury. In light of the limited efficacy and poor side effect profile of current treatment options, alternative long-term therapeutic strategies are needed which focus more on the cardiac repair, regeneration and remodelling processes. In theory if therapies could be developed to reverse or repair the myocardial damage, they could ameliorate an enormous public health burden, with an immediate effect on the quality of life and productivity of these patients.

Over the last 20 years stem cell biologists and cardiovascular basic researchers have made interesting observations regarding revolutionary cell based therapies and their potential as viable treatment options for various cardiovascular conditions, including: acute myocardial infarction and chronic heart failure (21, 22). A number of clinical studies have been implemented to deliver stem cells, including: cells from the bone marrow, skeletal muscle myoblasts, adipose tissue-derived cells and more recently the use of endogenous resident cardiac stem cells (eCSCs), to repair the damaged myocardium. All have shown potential for myocardial repair and regeneration in patients with ischemic heart disease and chronic heart failure (23, 24).

1.2. Cell-based therapy for the treatment of cardiovascular disease.

Cell-based therapy is a relatively new approach to treat myocardial damage and is aimed at replacing diseased and lost myocardium with new healthy myocytes and vasculature. There are a number of ways that cell transplantation therapy could repair/regenerate damaged myocardial tissue and improve cardiac function, such as firstly direct trans-differentiation of

transplanted cells to new myocytes and vasculature (25), secondarily as paracrine effects of the transplanted cells on cardiomyocytes and vascular cells in recipient tissue (26), and thirdly as direct effects of growth factors on the eCSC population within the recipient tissue stimulating their differentiation into new cardiac cells (27). A number of different stem-progenitor cells have been studied in the last decade as a potential source of new cardiomyocytes to ameliorate the injured myocardium, compensate for the loss of ventricular mass and contractility and eventually restore cardiac function (28). These include embryonic stem cells (ESCs), induced pluripotent stem cells (iPS cells), skeletal myoblasts, bone marrow derived cells (BMDCs) including mesenchymal stem cells (MSCs), endothelial progenitor stem cells (EPCs) and haematopoietic stem cells (HSCs), and recently endogenous cardiac stem-progenitor cells (eCSCs). Moreover, as opposed to cell transplantation, a new concept involves the activation of the resident eCSCs by growth factors (27, 29) and could be the future of regeneration of the damaged myocardium (30).

1.2.1. Embryonic stem cells.

Embryonic Stem Cells (ESCs) are derived from the inner mass of the blastocyst (31) and they can grow *in vitro* and be propagated indefinitely in their undifferentiated state while retaining a normal karyotype (32). They can maintain their property of multi-lineage commitment and therefore can differentiate into all cell types present in an organism (33, 34). Embryonic stem cells from a variety of animal species including human, which can differentiate into cardiomyocytes and other cell types present in the heart (35, 36). *In vitro* experiments have mostly relied on spontaneous differentiation of ESCs in embryonic bodies (EBs) to form contracting outgrowths containing cardiomyocytes (37). A range of different cardiomyocyte cell types can be obtained from ESCs including: nodal-like, atrial-like and ventricular-like cells (38). Indeed, implantation of beating EBs has been shown to reverse atrio-ventricular block in pigs (39). Techniques are evolving to enrich and purify these different populations of

cardiomyocytes, together with the impact of certain cytokines or growth factors on cardiac lineage differentiation of ESCs (40). Animal studies *in vivo* suggest that ESC-derived committed cardiac cells have the capacity to significantly improve myocardial function and structure after MI, through generation of new cardiomyocytes in the infarcted area (41). Furthermore, ESC-derived endothelial cells positively stimulate angiogenesis and improve myocardial contractility in mice following Myocardial Infarction (42), which may facilitate recovery or reduce damage.

Although the research on ESCs is growing, there are some serious concerns regarding their safety, use and their effectiveness in the treatment of human diseases, with significant ethical and regulatory concerns regarding their retrieval and usage for the purpose of treating patients with chronic diseases. In addition to these issues, there is a risk of developing malignancies, intra and extra-cardiac teratomas or other tumours associated with the transplantation of ESCs (43, 44). Furthermore, following allogeneic application, ESCs will trigger an immune response in the recipient (45, 46), requiring the need for immunosuppression, if the grafted cells are to be sustained. Finally, it remains elusive as to whether human ESCs efficiently engraft and electromechanically integrate into ischemic myocardium (47). As a result of these limitations, potentially hindering the transition of ESC based therapy into clinical translation (48), viable alternatives must be sought. Indeed, scientists have invested extensive research into identifying and characterising alternative models. The discovery of Takahashi and Yamanaka of the induced pluripotent stem cells (iPs) cells and the use of them has opened new opportunities for stem cell research (49), offering a number of alternative routes for furthering clinical applications without compromising health or individual beliefs (50).

1.2.2. Induced pluripotent stem cells (iPS cells).

The discovery by Takahashi & Yamanaka (49) that embryonic and mature mouse fibroblasts can be induced to become pluripotent stem cells by retroviral transduction of four transcription factors Oct3/4, Sox2, c-Myc and KLF-4 has revolutionised regenerative biology. The creation of iPS cells from human fibroblasts (51) heightened clinical appeal and led to rapid implementation of these cells as a source of cardiomyocytes (52, 53). These clonogenic, multipotent and autologous iPS cells can be created from a skin biopsy, hair follicle cells or blood (54). They allow potential disease modelling, drug discovery, as well as the generation of potentially large numbers of autologous cardiomyocytes, which could be used for transplantation, without the risk of rejection. Like the ESCs, iPS cells can form various types of cardiomyocytes including atrial, ventricular and nodal cells (55). However, as with ESCs, these cells also retain the potential to form tumours, posing a future risk, which needs to be verified and surmounted. Alternative methods to create iPS cells avoiding the use of viral vectors (i.e. small molecules) are currently being investigated to overcome this important limitation (56). Another important issue concerning the cardiogenesis of iPS cells is their long term stability and integration into the myocardium, as many iPS-derived cells are incompletely differentiated and remain immature in phenotype compared to their mature cell counterparts (57, 58). Therefore, while promising, these cells are currently not viable for clinical use in the regeneration of damaged myocardium.

1.2.3. Skeletal myoblasts.

Skeletal myoblasts have been used and transplanted into the infarcted heart with the goal to restore cardiac function (59). Two large, well conducted, phase I-II studies carried out by the MAGIC TRIAL showed that there was attenuation in LV remodelling, which was cell number dependant. Unfortunately this was not accompanied by functional cardiac

improvements (60). The failure of the myoblasts to improve cardiac function in humans has been attributed to their inability to differentiate into cardiac myocytes and the *in situ* development of dysfunctional electrical coupling with the resident cardiomyocytes (61). One of the major concerns raised by the early-phase studies of myoblast transplantation was that it could increase the risk of ventricular arrhythmias, a complication attributed to the slowing of conduction velocity by the electrically isolated islet-like clusters of myoblasts and the subsequent occurrence of re-entry cardiac arrhythmias. Results from the double blinded randomised MAGIC trial showed that at the 6-month study point, the proportions of patients who had experienced arrhythmias did not differ significantly between the pooled treatment groups and the placebo group. Nevertheless, the actual number of arrhythmias was two times greater in each of the myoblast treated groups than in the placebo group, which clearly calls attention to a pro-arrhythmic risk of myoblast implantation. Indeed, there are on-going concerns, despite these early results that skeletal myoblast therapy can precipitate cardiac arrhythmias making them a non-viable cell model (62).

1.2.4. Bone marrow derived cells (BMDCs).

On the search for potential transplantable stem cells, experimental and clinical studies have tested BMDCs for a range of therapeutic strategies including their mobilization to sites of cardiac injury. Three meta-analyses (23,24,63) evaluating data from approximately 18 trials concluded that BMDC-based therapies contribute to modest improvements in cardiac function by reducing infarct size of injury, preserving LV dimensions and increasing ejection fraction by 2-3% within 6-12 months from transplantation. The mechanisms underlying the modest benefits of BMDC therapy remain poorly understood, although enhanced angiogenesis and reduction in myocyte apoptosis through a paracrine effect of the BMDCs, (64), differentiation of BMDCs into cardiac cells (65) and activation of the resident eCSCs (66) have all been suggested and are discussed further below.

1.2.5 Haematopoietic stem cells (HSCs).

A hematopoietic stem cell is a cell isolated from the blood or bone marrow (BM) that can renew itself, can differentiate to a variety of lineage specific specialized cells, can mobilize out of the bone marrow into circulating blood and can undergo programmed cell death. Identifying and characterizing properties of HSCs began with studies in mice, which laid the groundwork for human studies (67). Approximately 1/10,000 to 1/15,000 bone marrow cells are believed to be stem cells. In the blood stream the proportion falls to 1 in 100,000 blood cells. New treatments using HSCs include graft-versus-tumour therapy for currently incurable cancers, autologous transplants for autoimmune diseases and gene therapy and tissue repair for a host of other problems. The adequate production of blood cells is maintained by a set of immature HSCs located in the bone marrow after birth. HSC are able to reconstitute the hematopoietic system in disease-related bone marrow failure and bone marrow aplasia (68).

In current treatments, HSCs can be mobilized from the bone marrow into the peripheral blood using hematopoietic cytokines, allowing a convenient harvest of these cells for clinical transplantation. Moreover, reports suggest that HSCs of mesenchymal lineage can trans-differentiate into a wide variety of phenotypes, including cardiomyocytes (25). Studies by Orlic (69) reported extensive regeneration in sites of myocardial infarcts, after direct injection of an adult BM population enriched for hematopoietic stem cells (Lin^-, $c\text{-}kit^+$) into the ischemic heart. In another study by the same group, similar effects were observed following the mobilization of BM cells into the peripheral circulation by growth factors (70). These promising results have prompted several clinical trials (71, 72, 73, 74). However, the underlying concept is currently being challenged by studies in which several populations of HSCs (c-kit-enriched, Lin^- $c\text{-}kit^+$, Lin^- $c\text{-}kit^+$ $Sca\text{-}1^+$, and Lin^- $Thy1.1^{low}$ $c\text{-}kit^+$ $Sca\text{-}1^+$ BM cells of either a-MHC–nLAC or h-Act–EGFP transgenic donor mice) did not readily acquire a cardiac phenotype but rather adopted traditional hematopoietic fates after direct

transplantation into ischemic myocardium (75, 76). Despite the techniques and cells available, the knowledge that researchers have in terms of stem cell transplantation is still in development, therefore we await further studies in order to realise the full promise of HSC-based therapy, in for example, myocardial regeneration.

1.2.6 Endothelial progenitor cells (EPCs).

EPCs can be isolated from peripheral blood as circulating BMDCs, based on the expression of HSC surface markers such as CD34, CD133 and vascular endothelial growth factor receptor 2 (VEGF-R2). The main mechanism of action of these cells is the formation of endothelial cells and new vessels in the infarcted myocardium (77) and their ability to produce endothelium has been demonstrated *in vitro* (78). In rats With Acute Myocardial Infarction, intravenous injection of EPCs can stimulate development of collateral vessels from pre-existing vessels as well as *de novo* capillary formation (79).

This group reported that there was decreased apoptosis of myocytes in the borderline zone, reduced fibrosis and scar formation resulting in prevention of LV remodelling and improvement in myocardial function. In patients with old MI and chronic, total coronary artery occlusion, intracoronary infusion of EPCs after recanalization of the occluded artery improved myocardial perfusion, reduced infarct area and ameliorated myocardial function (80). However, a clinical trial using EPCs was terminated prematurely due to potential adverse reaction of increased restenosis (81). There is little evidence for their *in vivo* trans-differentiation into new cardiac myocytes (65).

1.2.7 Mesenchymal stem cells (MSCs).

Mesenchymal stem cells (MSCs) can be isolated from a variety of tissues such as adipose, umbilical cord, umbilical cord blood and bone marrow. They lack hematopoietic lineage markers such as CD14, CD34 and CD45 but they express stromal surface markers such as

Stro1, CD105, CD90 and CD71. They have reduced expression of levels of MHC class I and lack class MHC II so they potentially provide prototypic immune-privileged cell based therapies and have been tested in phase I double blind randomised clinical trials as an allogeneic graft (82). These cells have a multifactorial action on cardiac regeneration with trans-differentiation capacities (83), and effects mediated by secreted factors and cytokines that evoke a therapeutic response (84). Recent studies reported that the MSCs can facilitate cardiac regeneration through mechanisms that involve differentiation and paracrine stimulation of innate repair pathways (85). Particularly MSCs seem to have a unique capacity to activate the endogenous c-kit^{pos} cardiac stem cells (eCSCs) and establish the correct cardiopoietic milieu that can support or even guide the eCSCs to regenerate a myocardial scar (86, 87) demonstrated that allogeneic MSCs transplanted into the infarcted mouse myocardium survived for up to 6 months. At 2 weeks, the transplanted MSCs did not express cardiomyocyte specific markers (e.g. α-actinin, myosin heavy chain, phospholamban, and tropomyosin), when assessed by confocal microscopy. However at 3 and 6 months expression of α-actinin, myosin heavy chain, phospholamban, and tropomyosin were evident. MSCs improved global LV function at 4 weeks, at a time when the transplanted MSCs did not express a full complement of cardiomyocyte markers and ultimately differentiation was incomplete, with myofibril organization being immature. In addition, all of hearts at 6 months contained MSCs that expressed von Willebrand factor, which indicates a role for MSCs in angiogenesis. Although by 6 months post transplantation, the transplanted MSCs did express markers of adult mature cardiomyocytes, the improvement in LV function was no longer present; suggesting that the improvement in LV function at 4 weeks was due to a paracrine effect of these cells rather than their differentiation into new cardiomyocytes. Furthermore, not all the studies provide evidence of myocardial regeneration following MSC transplantation (87, 88) again suggesting further research is required.

The MSCs are promising cells for myocardial regeneration; the degree to which they differentiate does not explain in full their cardiac reparative properties (83). The most likely mechanism is that they stimulate regeneration of cells already present in the myocardium that are in a quiescent state, triggering mobilization of the eCSCs to repair the myocardial damage. This synergistic effect may enhance the potential of the endogenous cardiac stem cells in the process of regeneration, which could consolidate a link between MSC/eCSCs and the myocardial regeneration process (89, 90).

1.2.8 Endogenous cardiac stem-progenitor cells (eCSCs).

The discovery of populations of resident endogenous cardiac stem-progenitor cells including those in the adult human heart (Table 1.1) has revealed great potential in the field of myocardial regeneration and future clinical application for the treatment of the cardiac failure (91). In the last decade, cardiac stem cell research has made significant steps and challenged the dogma of the adult heart as a post-mitotic organ. Cells in the post-natal murine heart with a phenotype of 'stem-like' cells were first reported in 2002. These cells expressed the ATP binding cassette transporter Abcg2, extruded Hoechst dye (92), represented 1% of total cardiac cells and differentiated into cardiac myocytes *in vitro*. Subsequently two groups Anversa & Nadal-Ginard (93) reported endogenous c-kit positive (c-kit^{pos}) cardiac stem cells (eCSCs) resident in the adult mammalian heart. More recently, endogenous cardiac stem-progenitor cells have been shown to play a significant role in cardiac cell homeostasis and myocardial response to injury (94) and are viewed as integral in myocardial regeneration, representing probably the best candidate stem cells for long term reconstitution of the damaged myocardium either by direct cell transplantation or by stimulation of their regenerative capacities to produce new myocytes and vessels *in situ* by growth factors (95).

The existence of cardiac-derived stem-progenitor cells has been confirmed by several independent groups (95, 96, 97, 98, 99,100,101) and although a variety of markers have been

proposed to identify eCSCs in different species, it still remains to be determined whether these markers identify different populations of eCSCs or, more likely, different developmental and/or physiological stages of the same cell type (see Table 1.1) (102). The different cardiac stem-progenitor cells identified to date are described in detail in the next paragraph.

1.3. Tissue specific endogenous cardiac stem-progenitor cells.

1.3.1. c-kit positive (c-kit^{pos}) CD45 negative (CD45^{neg}) eCSCs.

One population of resident cardiac stem-progenitor cells can be identified through expression of the stem cell factor receptor, c-kit (c-kit^{pos}), in addition to Sca-1 and MDR-1 (ABCG2). They are negative for markers of the blood cell lineage, CD31, CD34 and CD45. They have capacities of self-renewal, clonogenicity and multipotency, with significant myocardial regenerative capacity *in vivo* (97). They are capable of generating the 3 major cell types of the myocardium; cardiomyocytes, smooth muscle and endothelial vascular cells (see Figure 1.2). When BrdU or EGFP tagged c-kit^{pos} eCSCs were injected into the border zone of an experimentally produced infarction, a band of regenerating myocardium composed of tagged cells were observed in the infarcted zone. The labeled cells were either small cardiomyocytes expressing sarcomeric proteins or vascular cells forming new vascular structures (97).

Since their discovery in the rodent heart, these endogenous c-kit^{pos} eCSCs have been identified and characterized in other species, such as the dog (103), pig (27),sheep(104) and human (89,95,100,105). In the human, the density of eCSCs corresponds to 1 c-kit^{pos} eCSC per ~1000 cardiomyocytes or ~50,000 eCSCs per gram of tissue (106). The c-kit^{pos} eCSCs are the only cardiac progenitor cells proven to fulfil the requirements to be *bona fide* stem cells, being clonogenic, self-renewing and multipotent with significant cardiac regenerative potential *in vivo*. Not all these characteristics have been proven for all the other cardiac cell types described in Table 1.1 as shown below.

Table 1.1 Cardiac stem-progenitor cells and their characteristics identified in the mammalian heart.

AUTHORS	PHENOTYPE	CLONOGENIC	MULTIPOTENT	IN VIVO CARDIAC REGENERATIVE POTENTIAL
Beltrami et al (2003)	c-kit^{+}, Lin^{-} , CD34^{-}CD45^{-}	Yes	Yes, gave rise to cardiomyocytes, endothelial and smooth muscle cells *in vitro* and *in vivo*	Yes, injected green fluorescent protein (GFP$^{+)}$, c-kit^{+} cells into the infarcted myocardium and gave rise to a band of regenerating myocardium, including new cardiomyocytes and microvasculature.
Oh et al (2003)	Sca1^{+} c-kit^{-}CD31^{+},Lin^{-} CD34^{-} CD45^{-},	Not cloned	Myocyte differentiation in vivo and in vitro	Yes, ischemia reperfusion injury - 1.5% myocyte differentiation in infarct border zone
Matsuura et al (2004)	Sca1^{+} c-kit^{+}, CD34^{+}, CD45^{+},	Not cloned	Myocyte (beating), adipogenic osteogenic differentiation, *in vitro*	Not determined
Martin et al (2004)	SP, Sca1^{+} Abcg2^{+} c-kit$^{+/low}$ CD31^{-} CD34$^{+/low}$ CD45$^{-/low}$	Not cloned	Myocytes differentiation (α-actin^{+}) *in vitro*	Not determined
Pfister et al(2005)	SP, Sca1^{+} Abcg2^{+} c-kit$^{+/low}$ CD31^{-} CD34$^{+/low}$ CD45$^{-/low}$ CD44$^{low/}$ Nkx2.5^{+}Gata-4^{+}, SMA^{+}Desmin^{+} Tie2^{+}	Not cloned, Colony forming units	Myocyte differentiation with sarcomeric organization and spontaneous contractions at low frequency in vitro	Not determined
Tomita et al (2005)	SP Sca1^{+}, c-kit^{+} CD45^{-}, CD34^{+} CD44^{+} Flk-1^{+}, Nestin^{+}, Musashi^{+}, Mdr1^{+}	cardiosphere formation	Differentiation of cardiosphere derived cells into neurons, glia of CNS and PNS lineage (in vitro and in vivo) and beating cardiomyocytes (in vitro)	Not determined

Chong et al. (2011)	$CD44^+$, $CD90^+$, CD29+, $SCA1^+/PDGFR\alpha^+/CD31^-$ $CD105^+$,Gata5CRE+,Mesp1CRE +,$Oct4^+$,$cMyc^+$	Not cloned	Myocyte differentiation only in vitro	Not determined
Messina et al (2004) Smith et al(2007)	c-kit^+, $Sca1^+$, $CD34^+$, KDR/$Flk-1^+$, $CD31^+$	cardiosphere formation	Endothelial and beating myocytes differentiation, *in vitro* and *in vivo*	Yes, gave rise to new myocytes and capillaries in infarcted myocardium
Smart et al. (2011)	Wt1+,GFP+,YFP+,Sca1+,	Not cloned	Transdifferentiation to cardiomyocytes with no fusion	Yes gives cardiomyocytes and capillaries in infarcted myocardium
Langwitz et al (2005) Morretti et al (2006) Zhou et al (2008)	$Isl-1^+$, $Sca-1^-$,$c-kit^-$,$Flk1^+$, $Nkx2.5^+$ Wt1,GATA-4, $Nkx-2.5^+$,$Isl1^+$	Cloned on cardiac mesodermal layer	Yes	Not determined Not determined

The c-kit^{pos} eCSCs reside throughout the myocardium and have a high density in the atria and LV apex (107). c-kit^{pos} eCSCs can be found in a minimum of three different states: *i. Quiescent undifferentiated cells; ii. Activated cycling undifferentiated cells; and iii. Cycling cells that already express transcription factors characteristic of one cardiac cell lineage.* The first two cellular development states identify cells that can give rise to the three cardiac cell types, whereas cells expressing lineage-specific transcription factors probably correspond to the so-called *transit cells* in other systems and are already committed to a specific cell lineage (Figure 1.1).

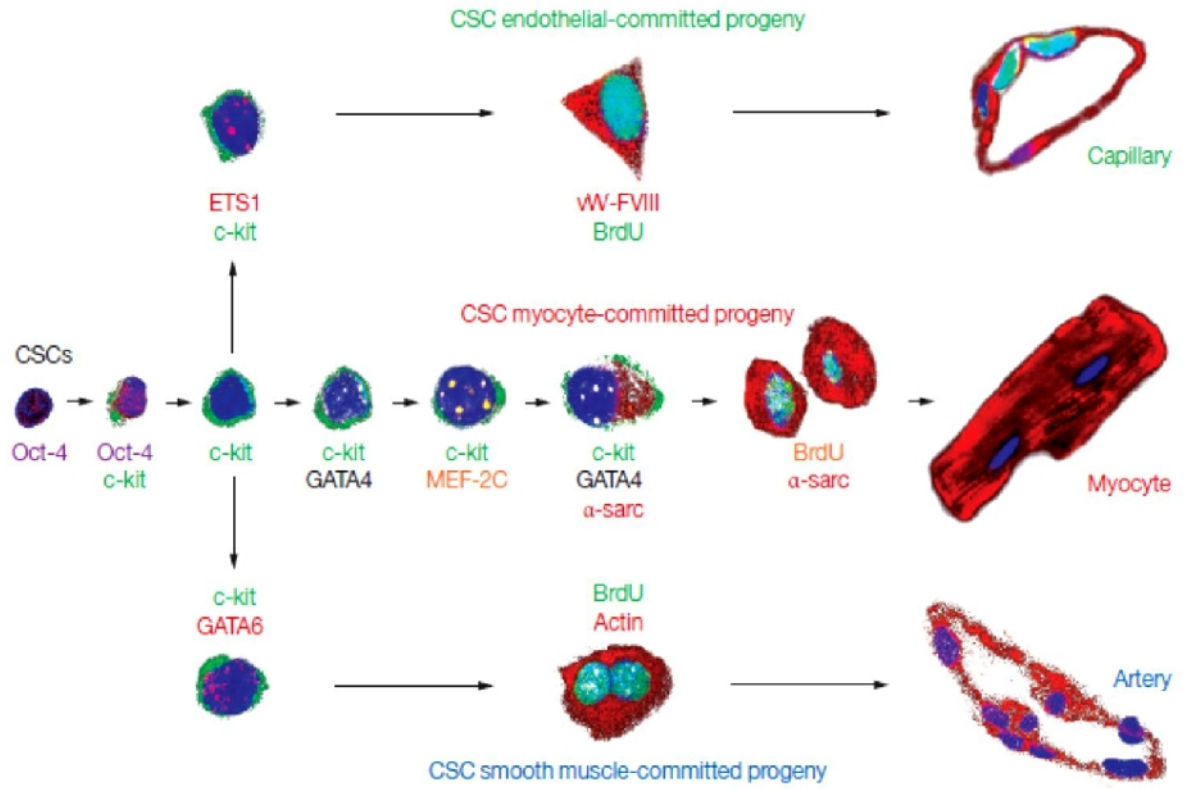


Figure 1.1. Schematic representation of the transitional sequence of cardiac stem-progenitor cell committed progeny. Quiescent, primitive, undifferentiated eCSCs express Oct-4 (red fluorescence), are activated, and start expressing c-kit (green fluorescence). In response to stress, these cells multiply and lose expression of Oct-4. The resulting c-kit^{pos} eCSCs are still uncommitted to one specific cardiac cell lineage. After further expansion and differentiation, the cells induce expression of transcription factors specific to one cardiac lineage (GATA4, ETS1, or GATA6) and differentiate into one of the three cardiac cell types: cardiomyocytes, endothelial, and smooth muscle cells, respectively. These newly formed cardiac cells can undergo a few rounds of replication before becoming terminally differentiated. Abbreviations: α-sarc: alpha sarcomeric actin; CSC: cardiac stem cell; vW-FVIII: von Willebrand factor VIII. (Adapted from GM Ellison *et al* 2006).

The c-kit^{pos} eCSCs can be isolated and amplified *in vitro* so regenerative protocols of cell transplantation could be employed to treat damaged myocardium. There are two methods of isolating these cells; the first technique involves the enzymatic digestion and mechanical dissociation of cardiac tissue yielding the small cell population from which eCSCs can be purified with antibody selection procedures. The second technique as been described by Smits for c-kit and Sca-1 cells, involves the explant culture of pieces of myocardial biopsy from which cells migrate from the explant and eCSCs can be purified from these explant-

derived cells using antibody selection procedures similar as the techniques used for the Sca-1 derived cells (108).

1.3.2 Sca-1^{pos} and Side Population (SP) cardiac stem-progenitor cells.

Stem cell antigen (Sca-1) is a member of the Ly-6 family and an important surface marker for somatic cells and especially hematopoietic stem cells (109). This stem cell antigen has been used as a marker in the adult heart when isolate progenitor cardiac stem cells in conjunction with SP cells and other stem cell antigens like c-kit,CD45,CD34 (101). A number of heterogeneous subpopulation of Sca1$^+$ Cardiac Progenitor Cells (CPCs) has been described based on the different subset of stem cell markers co-expressed with Sca1.These Sca1$^+$ CD31$^+$ cardiac progenitors were identified in the mouse adult myocardium (96) as small interstitial cells c-kit$^-$, CD31$^+$, Lin$^-$, CD34$^-$, and CD45$^-$.

In the above study it has been found that the Sca1$^+$ CD31$^+$ progenitors, express initially cardiogenic transcription factors as Gata 4, MEF2C, TEF-1 and when stimulated in vitro with 5-azacytidine they can acquire a phenotype of cardiomyocytes and can express structural cardiac genes. When stimulate in vitro the cardiac Sca1$^+$,c-kit$^+$,CD45$^+$,and CD34$^+$ CPCs with oxytocin it could result in the generation of beating cardiomyocytes, and in these conditions Sca1$^+$ CD45$^-$ could differentiate towards osteogenic and adipogenic lineages showing the multipotency of these CPCs (101). When these cells examined in vivo in the murine model the transplanted Sca-1$^+$, CD31$^-$ CPCs had beneficial effect on the cardiac remodelling following myocardial infarction and seems that they promote regeneration via paracrine effects to the local myocardial damaged tissue (110).

Although other studies have since reported their presence in the human myocardium (111); the homology/phenotype of the cells initially identified in the mouse has not yet been confirmed in any other species. Sca-1^{pos} have significant overlap and co-expression of transcription factors that other cardiac stem-progenitor cells expressed like the c-kit^{pos}

eCSCs, SP cells etc. (see Table 1.1), and although they show potential as a source of cells for cardiac regeneration more studies required for further characterisation.

Side population (SP) stem progenitor cells were first identified in the murine bone marrow by their ability to extrude Hoechst dye 33342 (112). This property is attributable to expression of ATP binding cassette transporters ABCG2 and MDR1.The existence of SP in the heart was first reported in the post-natal murine myocardium where resident verapamil-sensitive SP cells with stem cell activity and cadiomyocyte differentiation potential were detected by Hierlihy (92). These cardiac SP cells are highly positive for Sca-1 and low expression of c-kit, CD34 and CD45. They are negative for CD31 indicating that these SP cells differ from the Sca-1^{pos} cells described by Oh in (96). They cells were capable of differentiation into a cardiomyocyte phenotype after co-culturing with primary cardiomyocytes (92) or as recently reported (113) in order to induce differentiation into cardiomyocytes, these cells were cultured with various growth factors, such as BMP2, BMP4, and Oxytocin, or on the feeder layers of the mesenchymal cells. Only treatment with Oxytocin was able to induce Cardiac SP cells into beating cardiomyocytes. In addition Pfister (98) demonstrated that among cardiac SP cells the greatest potential for cardiomyocyte differentiation is restricted to cells negative for CD31 but positive for Sca-1.

These cells showed a potent in vivo activity as their transplantation of Sca-1^{+} $CD31^{-}$ cells into the acutely infarcted mouse heart lead to myocardial regeneration and significant improvement in cardiac function (110).In addition the cardiac $Sca1^{+}/CD31^{-}$ SP in a different study showed to migrate into the damaged myocardium and to differentiate into cardiomyocytes or endothelial cells upon modulation of the SDF-1a/CXCR4 system (114). These cells are heterogeneous in nature have distinct subpopulations identified by expression of VE-cadherin, CD31,CD34,Sca1 and consisting of vascular endothelial cells, smooth muscle cells and mesenchymal progenitors including cardiomyocyte precursors (115).

However despite the apparent cardiovascular potential there is not absolute proof that cardiac SP cells are a bona fide stem cell population defined by clonogenic and self-renewal properties has not yet been provided.

1.3.3. Cardiosphere-derived cells.

Cardiospheres are clusters of heterogeneous cells that are exclusively heart derived (116). The heterogeneity of the cardiospheres results in a microenvironment that mimics the stem cell niche with peripheral supporting cell populations (117). Primary cardiospheres are derived from endomyocardial biopsies that are partially digested enzymatically then plated on fibronectin coated dishes. After several days in culture small phase bright cells migrate out from the explant and these cells form cardiospheres in suspension (118). Cardiosphere cells are positive for c-kit, Nkx2.5, desmin connexin 43, KDR, and Isl-1 (116). Cardiospheres can be mechanically disaggregated and plated as monolayers, where they are termed cardiosphere-derived cells (CDCs), and when exposed to EGF can reform secondary cardiospheres. Cardiospheres secrete a number of factors like VEGF, HGF and IGF-I, giving them a paracrine role in enhancing regeneration (118). However, there are safety concerns associated with the administration of the cardiospheres via intracoronary infusion and the potential for adverse events as coronary microembolism. Therefore, the CDCs are viewed as a favourable alternative (119).

*1.3.4. Epicardial derived progenitor cells (*EPDCs*).*

Recently epicardial-derived progenitor cells (EPDCs) have been suggested as a population of cells with possible cardiogenic potential. Adult mouse EPDCs induced to express Wilm's tumor 1 (Wt is a key embryonic epicardial gene) through pre-treatment (priming) with thymosin β4 (Tβ4), an actin monomer binding protein, can result in neovascularization and *de novo* cardiogenesis, after myocardial infarction. $Wt1^{pos}$ EPDCs are positive for Sca-1

(~80%) and co-express early cardiac progenitor markers Isl-1, Nkx2.5 and Gata-4, but were negative for c-kit (120).

Human-derived EPDCs differentiate into endothelial and smooth muscle cells but not cardiomyocytes (121). However, they have been shown to improve cardiac function after transplantation following an infarct through paracrine protection of the surrounding tissue (122). These results identify EPDCs as candidate cardiac stem-progenitors in the adult heart and lay the foundation for future efforts to harness the cardiogenic potential of these progenitors for cardiac regeneration and repair.

1.3.5. Isl-1^{pos} cardiac progenitor cells.

The heart is formed from cardiogenic progenitors expressing the transcription factors Nkx2-5 and Isl-1(114). These multi-potent progenitors give rise to the major lineages of the mature heart; cardiomyocytes, smooth muscle and endothelial cells (129). Isl-1 cardiac progenitor cells are found in the second (anterior) heart field during development and play a crucial role in creation of the right ventricle, atria and outflow tract during the embryogenesis of the cardiac chambers (130). These progenitor cells could be optimal cells for myocardial regeneration, however the presence of Isl-1^{pos} cardiac progenitors is correlated with age, where the embryonic heart has abundant levels of Isl-1^{pos} cells, but these reduce in neonatal, and are almost absent in adult heart (131).

1.4 Clinical potential of cardiac stem-progenitor cells.

Endogenous, cardiac stem-progenitor cells have already been used in phase I clinical trials (SCIPIO, ALCADIA, TICAP and ALLSTAR) in which they have been shown to contribute to myocardial regeneration. These studies are based on the concept that the eCSCs can trigger regeneration of the damaged myocardium that they belong to. They have a number of advantages in comparison to other cells such as BMSC, MSC, ESC etc. in that they have a

relationship with the cardiac milieu and the fact that their regenerative potential can be triggered *in situ* via paracrine signals from the damaged myocardium (29). In the trials described below, the feasibility and safety of the use of these cells is described.

1.4.1 Autologous cell transplantation.

Patients with post-infarction LV dysfunction (ejection fraction [EF] ≤40%) before coronary artery bypass grafting were enrolled into the SCIPIO TRIAL. One million autologous c-kit^{pos} CSCs were administered by intracoronary infusion ~113 days after surgery. 16 patients were assigned to the treatment group and seven to the control group; no CSC-related adverse effects were reported. In 14 CSC-treated patients who were analysed, LVEF increased from 30.3% before CSC infusion, to 38.5% at 4 months after infusion. Importantly, the effects of CSCs were even more pronounced at 1 year in eight patients with LVEF increasing by 12.3 ejection fraction units. In contrast on the 7 control patients group during the corresponding time interval LVEF didn't change 30.1% at 4 months after CABG and 30.2% at 8 months respectively. A reduction in infarct size was also noted using the semi-quantitative infarct score index which was confirmed the echocardiographic analysis. Cardiac MRI showed a reduction in mean infarct weight from 32.6g before infusion of the CSCs to 24.8g (24% reduction) in 4 months and 22.8g (30% reduction) at 6 months respectively. By contrast in the control group there were no significant change in the regional wall motion score index at 4 months after baseline either in the infarcted LV segments (1.99vs 1.91) at baseline with p=0.144 or in all LV segments combined (1.89 vs 1.88) at baseline p=0.846 (128).

The CADUCEUS TRIAL similarly used intracoronary administered CDCs for heart regeneration after myocardial infarction. Patients assigned to receive CDCs received autologous CDCs derived from endomyocardial biopsy specimens, which were infused into the infarct-related artery 1.5-3 months after myocardial infarction. Four patients (24%) in the CDCs group had serious adverse events compared with one control (13%; p=1.00).

Compared with controls at 6 months, MRI analysis of patients treated with CDCs showed reductions in scar mass, increases in viable heart mass, regional contractility and regional systolic wall thickness. However, changes in end-diastolic volume, end-systolic volume and LVEF were not different between the two groups at 6 months (120).

The ALCADIA trial was designed to test the efficacy of transplanting both CSCs from endomyocardial biopsies and hydrogel infused with FGF. Injected cells expanded for this trial expressed CD105, CD90, CD29, Nanog, Oct 4 and Gata4 and were negative for CD45. Data of seven patients after combination therapy at 6 months reported an improvement in LVEF from 26% to 35.8% by 3D echocardiography, and 22.6% to 34.7% by MRI. Findings also showed a decrease in infarct volume by 23% to 19.7% and a concomitant decrease in wall motion score from 17.2 to 6.6. Recently these authors presented their results after one year in the AHA meeting in Los Angeles USA, and concluded that transplantation of human cardiac derived stem cell with controlled release of bFGF is safe and feasible to ischemic cardiomyopathy patients. They suggested that it is a novel biotherapy and it may have a potential to restore the injured heart to functional repair with reconstruction of post ischemic environment (129).

In the TICAP trial, transcoronary infusion of cardiac progenitor cardiosphere derived cells (CDCs) in patients with a single ventricle was designed to treat hypoplastic left heart syndrome in patients less than 6 years old. Phase I consisted of a safety and feasibility trial in which fourteen patients received 0.3 million cells/kg of autologous cardiac progenitor cells via intracoronary delivery one month after cardiac surgery. Patients were monitored for 3 months to 1 year after cell transplantation TICAP trial (130). The results of the Phase I trial have been released and no complications have been reported, including cardiac death, myocardial ischemia, pro-arrhythmia, hospitalization for heart failure, or tumor formation, within 24 months of CDC infusion. Echocardiography showed that improvement of right

ventricular ejection fraction (RVEF) was greater in the CDC-treated group (+5.3±3.2%) than in controls (+0.1±3.4%, P=0.01) at 3 months after CDC infusion. This cardiac function enhancement was manifested even 1 year and 2 years after CDC infusion (+7.8±4.9% vs. +2.2±3.1% at 1 year, P=0.03; +8.8±3.7% vs. +3.4±6.4% at 2 years, P=0.04). In addition, RVEF measured by cardiac MRI was also markedly improved in CDC-treated patients from 36.1±7.5% at baseline to 42.7±8.7% at 1 year (P=0.04) and to 42.4±7.7% at 2 years (P=0.047). The Phase I study concludes that intracoronary infusion of autologous CDCs is feasible and safe to treat children with Hypoplastic Left Heart Syndrome (HLHS). This novel therapeutic strategy may impact on cardiac function, clinical symptoms of heart failure and somatic growth in long-term recovery. The Phase II of the same trail which has been called PERSEUS is now underway and will investigate the efficacy of cell transplantation as the primary endpoint. Cardiac ejection fraction will be assessed by echocardiography and ventriculography and cardiac MRI will be conducted before and after treatment between the control (non-transplanted) and the transplanted treated patients. This phase II trial is due to conclude in March 2018.

The ALLSTAR (allogeneic heart stems cells to achieve myocardial regeneration) trial is designed to test safety of allogeneic CDCs. This study is currently recruiting participants with LVEF<45% and expects enrolment of 274 patients. 25 million cardiosphere derived cells (CAP-1002) will be delivered with intracoronary infusion in these patients which will be compared with a control group .The CDCs will be transplanted <3 months or 3-12 months after myocardial infarction to assess the ideal time frame for cell delivery. This is the first trial to assess allogeneic use of cardiac stem-progenitor cells in humans ALLSTAR TRIAL (131).The study is still undergoing and still recruiting participants with completion date the December 2015. Until now no interim results have been published or presented in the literature.

1.4.2 Autologous regeneration without cell transplantation.

Transplantation of autologous stem cells is a long and expensive procedure still in its relative infancy and is not available to all patients who require regenerative therapy for their damaged myocardium. Given that the eCSCs express receptors for transduction pathways relating to various growth factors i.e. Insulin like growth factor I (IGF-I), hepatocyte growth factor (HGF), a new concept has emerged to stimulate endogenous repair and regeneration mechanisms *in vivo*, without the need for cell transplantation. This concept has already been demonstrated in rodent trials, proving to be very effective in producing myocardial regeneration after MI in mice (132). To prove this concept in larger mammals, a recent study was conducted by Ellison (27), firstly identified c-kit^{pos} eCSCs in the adult pig myocardium, which possess stem cell properties i.e. clonogenicity, multipotency and self-renewal. The porcine c-kit^{pos} eCSCs expressed receptors for IGF-I and HGF and responded to stimulation with IGF-I and HGF via downstream effectors *in vitro*, increasing c-kit^{pos} eCSC proliferation, migration and cardiomyogenic differentiation.

Subsequently, IGF-I and HGF (2 to 8µg IGF-I and 0.5 to 2µg HGF), were administered via intracoronary injection, just below the site of left anterior descendent occlusion, 30 minutes after Acute Myocardial Infarction (AMI) during coronary reperfusion. IGF-I/HGF in a dose-dependent manner improved cardiomyocyte survival and reduced fibrosis with cardiomyocyte reactive hypertrophy. Furthermore, this growth factor cocktail triggered a regenerative response from the c-kit^{pos} eCSCs, without the need for cell transplantation (27). Histological changes were correlated with a reduced infarct size and an improved ventricular segmental contractility and ejection fraction at the 2 month follow-up assessed by cardiac MRI.

1.5 c-kit^{pos} eCSCs within the four cardiac chambers.

1.5.1 The c-kit^{pos} CD45^{neg} eCSC niche.

The c-kit^{pos} eCSCs reside in potential stem cell niches, which provide a stable microenvironment designed to preserve the survival and replication potential of stem cells (133). In cardiac niches, c-kit^{pos} eCSCs are regulated by the surrounding cellular and non-cellular constituents. The niches are located more in areas of less stress like the atria and LV apex, meaning exposed to moderate and minimal mechanical forces in the anatomic components of the heart (134). In contrary the base and mid-region of the heart have high stress areas due to the organization of the myocyte bundles and the level of the mechanical forces. CSC niches have been identified by Urbanek (132) and their role has been assumed to be related as a reserve cells to support regeneration of the damaged tissues. The general trend of the niches is to reserve a local milieu of quiescent eCSCs cells that can be activated when needed in the myocardium and they can be mobilised and stimulate regeneration after production of paracrine stimulation of the local endogenous cells or even distant cells in different cardiac chambers. The c-kit^{pos} eCSCs can undergo either symmetric (one eCSC gives rise to two eCSCs) or asymmetric (one eCSC gives rise to one eCSC and one committed cell) division as shown in vivo in the mouse model by Cesselli (134), are reportedly dynamic in their function and can be mobilized to areas of injury soon after infarction (135).

Data suggest that the atrial appendages in humans are rich in cardiac progenitors (136). Studies by Itzhaki (137) identified a number of cell niches in the right atrium a chamber rich of eCSCs positive of c-kit and Isl-1. Completing the recent evidence by the group of Arsalan is been reported that the left atrium contained viable numbers of eCSCs too (105). Studies by Castaldo (138) identified the presence of CD117 positive cells localized in the sub-epicardium, and their activation was associated with Laminin-1 and alpha-6 integrin

expression. Interestingly it has been demonstrated that cardiac progenitor cells exist in the right atria of the sheep (104).

To date very few studies have interrogated the presence of eCSCs in the human myocardium and no studies have investigated their profile in terms of their chamber of origin nor correlated them with the primary and secondary heart field. This will be one objective of this thesis and will be explored in the next paragraphs.

1.5.2 Embryological heart development.

In mammals the development and morphogenesis of the cardiac chambers is under the control of chamber-specific genetic programs. In the adult, these differences in gene expression result in cardiac muscle (particularly between atria & ventricles) with different biochemical, contractile and functional properties (139). In animal hearts (mouse and chick) at the embryological level, a number of transcripts have been identified and mark the evolution and genotypic mapping of each chamber accordingly to the evolution stage. Primarily the *ballooning theory* of the embryological origin of the heart gave a new insight into heart chamber development and the genes that are expressed throughout the process of the development of each chamber see Table 1.2 (140,141).

Table 1.2 General and specific transcription factors from each chamber depending on the stage of cardiac embryogenesis according to the *ballooning theory* (Adapted from Moorman et al 2003).

Chamber of Origin	Early	Medium	Late
Atrial			
RIGHT ATRIUM	**GATA 4,5,6** ***Retinoic acid* (Raldh2)** **Tbx-20**	**ANF , TBX-5, MLC-2a**	**CX40, CX43, Hrt1**
LEFT ATRIUM	**Tbx-20** ***Retinoic acid***	**TBX-5** **Hesr1, MLC-2a**	**CX40, CX43** **Pitx-2, Hrt1**

Ventricular			
RIGHT VENTRICLE	**SERCA-2, MLC-2v, Isl-1**	**Hand-2, GIN-2**	**Hand-2, GIN-2**
	Hrt2	**Mlclv-nlacZ-24**	**CX43**
LEFT VENTRICLE	**SERCA-2, MLC-2v**	**Hand-1** **TBX-5**	**Hand-1, IRX4** **TBX-5**
	Hrt2	**Mlc3f-nlacZ-2**	**CX43**

1.5.3 The primary and secondary heart field.

The recent theory of the primary and secondary heart fields (142,143,144) challenged the embryological origin of the 4 cardiac chambers and their topographical genotypic location. It is important to point out that the identification of transcripts for the origin of each chamber and heart field have been made in mouse but not in human see Figure 1.2.

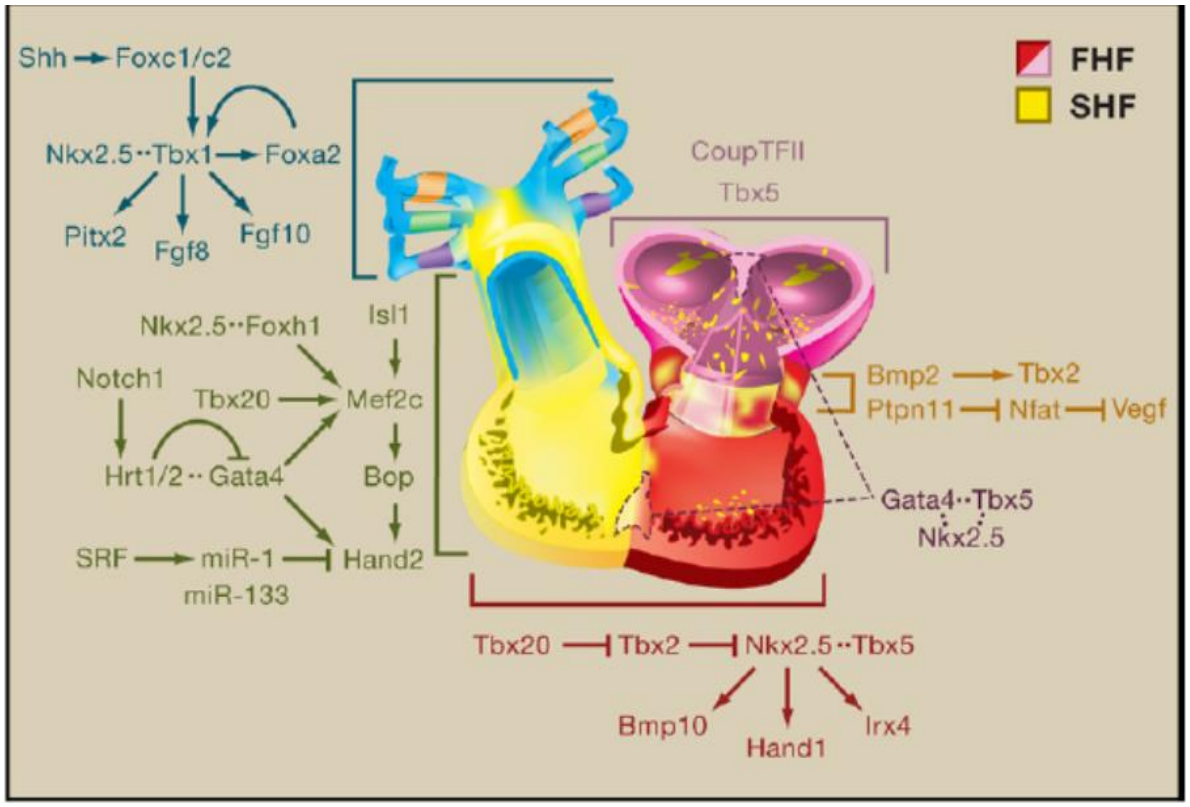


Figure 1.2 Transcriptors expressed according to primary and secondary heart fields (Adapted from Srivastava *et al* 2009).

This theory suggested that later in embryogenesis and in adult life there is a consistent phenotypic pattern and function of all the cardiac chambers. A mutation in these genes can give an abnormal phenotype of the cardiac chamber and in consequence abnormal function of the cardiac muscle or the valve apparatus. As mentioned earlier these recent studies have demonstrated that the cardiogenic mesoderm maintains two multi-potent progenitor cell populations known as first heart field (FHF) and secondary heart field (SHF) which give rise to various cardiac structures. The FHF originates in the anterior splanchnic mesoderm and forms the cardiac crescent from which cells migrate medially to form the linear heart tube. Ultimately the FHF contributes to left ventricle (LV) (127,144,145), while the SHF which originates in the pharyngeal mesoderm medial to the cardiac crescent and lies anterior and dorsal to the linear heart tube contributes to the right ventricle (RV) and outflow tract and both atrial tissues, accounting for two thirds of all the cells in the heart (146). While not all genetic markers unique to the FHF and SHF have been identified, some transcription factors and signalling molecules are known to characterise each population. Both heart fields are marked by the homeobox protein Nkx-2.5, but the FHF progenitors are distinguished by the expression of the T-box transcription factors such as Tbx5 and Tbx20 and the basic helix-loop-helix (bHLH) transcription factor, and Heart and neural crest derivatives expressed protein-1 (Hand1). The SHF progenitors contribute to the formation of all the chambers except the LV (which is formed by the FHF) and are distinguished by Insulin gene enhancer protein-1 (Isl-1), Heart and neural crest derivatives expressed protein-2 (Hand2), Tbx1, Forkhead box protein H1 (FoxH1), Fibroblast growth factor 8 (Fgf8), and Fibroblast growth factor 10 (Fgf10),(142) (see Table 1.3). Although a retrospective clonal analysis in the mouse embryo suggested the FHF and SHF progenitors originate from a common precursor (147), further investigations must be pursued in order to determine whether the FHF and SHF progenitors stem from a single precursor or perhaps a subset of precursors (148).

Table 1.3 Transcripts from primary & secondary heart fields. Mutants of these genes are responsible for congenital cardiac pathologies as shown on the left of the table. OFT: outflow tract, INF: inflow tract, LV: left ventricle, RV: right ventricle, A: atria (Adapted from Buckingham et al, 2005).

Transcript	Crescent expression	Second heart field expression	Cardiac tube expression	Mutant phenotype in the cardiac tube
Nkx-2-5	yes	yes	yes	Single atrial and ventricular compartment Loss of ventricular tissue, no Hand 1 expression
Hand1	yes	no	LV (high in outer curvature)	LV Disrupted
TBX-5	yes	no	LV ,A, INF	Sino-atrial defects; Hypoplastic LV
Fgf10	no	yes	no	No early phenotype detected
Fgf8	no	yes	no	OFT Defects
TBX1	yes	yes	no	OFT Defects
Isl1	no	yes	OFT, RV, LV, A, INF	Single atrial and ventricular compartments; no OFT; Hand 1 and TBX-5 expression which indicates that LV identity is intact; atria at the venous pole abnormal
Foxh1	no	yes	no	OFT reduced or absent; RV doesn't develop
Mef2c	yes	yes	yes	OFT reduced; RV doesn't develop(HAND2 down regulated) INF abnormalities
Hand2	yes	yes	RV	RV Abnormalities
TBX-20	yes	yes	OFT,RV,LV	Chambers do not develop, no Hand 1 expression; hypoplastic RV; OFT disrupted

The classification of these two cardiac heart fields simplifies the embryological origin of each chamber and suggests that transcripts that are specific for each chamber could be used as markers of recognition for the eCSCs that reside in each chamber to identify their origin.

1.5.4 Do c-kit^{pos} eCSCs reflect their chamber of origin?

As mentioned previously, c-kit^{pos} eCSCs cells reside in all chambers of the myocardium and are most prevalently in the LA, RA and LV apex, and less prevalent in areas of higher stress such as the mid-base region of the LV (107). Two recent studies have identified in the murine (149) and human (105) heart that the maximum number of c-kit^{pos} eCSCs reside in the LA. However, no studies have explored whether their transcriptional profile is related to their embryological origin, being reflective of the chamber where they reside in. Therefore, are c-kit^{pos} eCSCs chamber specific, expressing a transcriptional profile reflective of the chamber in which they reside and were isolated from, or do c-kit^{pos} eCSCs express a generic, global transcriptional profile, encompassing transcripts reflective of all cardiac chambers? The answer to this question is critical when it comes to whether c-kit^{pos} eCSCs can be used for regenerative therapies. Indeed, this information is of a significant biological and clinical significance because if the c-kit^{pos} eCSCs are remnants of the chamber anlagen and reflect their chamber of origin, it is likely that they would also generate the corresponding tissue (i.e. right atria eCSCs give raise to right atria cardiomyocytes). Therefore cell transplantation therapy could run the risk of producing inappropriate myocardium, giving rise to arrhythmias or regional wall abnormalities. It is the LV myocardium which is damaged in Myocardial Infarction (MI) and needs to be replaced using stem cell therapy. If on the other hand, the c-kit^{pos} eCSCs are indistinguishable and interchangeable throughout the myocardium, then their source for cell therapy becomes simpler and the right atrial appendix which is the easily accessible and obtainable becomes the source of choice. With this information in hand the most optimal myocardial regeneration strategies, either through direct cell transplantation or *in situ* activation of c-kit^{pos} eCSCs post MI, can be applied to treat cardiac failure and regenerate the damaged myocardium.

1.6 Objectives & hypotheses.

The first objective of this thesis is to isolate c-kit^{pos} eCSCs from all 4 cardiac chambers of the human heart and compare the number of c-kit^{pos} eCSCs isolated between chambers. Due to the high prevalence of c-kit^{pos} eCSCs in the atria and LV apex and low number in the RV and LV mid-base region reported in the literature, it is hypothesized that more c-kit^{pos} eCSCs will be successfully isolated from the left and right atria and fewer obtained from the LV base and RV samples. The LV samples, as shown in previous studies, have a different distribution of cardiac stem cells throughout the myocardium with higher number on the LV apex and atria and fewer at the base of the heart. In this study the size of the LV and RV samples were small as a consequence of these areas of harvesting being vulnerable areas of bleeding and thus running the risk of compromising the patient safety.

The second objective of this thesis is to characterize the transcriptional profile of c-kit^{pos} eCSCs isolated from all four chambers using quantitative real time polymerase chain reaction (qRT-PCR) to assess expression of genes specific to the first and second heart field and to determine whether the transcriptional profile of these cells is related to their chamber of origin. It is hypothesized that eCSCs isolated form the 4 chambers of the human heart would have a universal transcriptional profile rather than a profile that reflects their chamber of origin. This would give the added advantage of being able to isolate c-kit^{pos} eCSCs from any of the four cardiac chambers to produce cardiomyocytes that exhibit specific characteristics of the chamber that they are required to regenerate. This will increase their viability for future cell therapy in patients with cardiac disease.

2. MATERIALS & EXPERIMENTAL METHODS

2.1 Cardiac sample/biopsy collection.

Between August 2011 and April 2012, small pieces of myocardium (0.189 ±0.22g) were obtained from all 4 chambers of the adult human heart of fully consented patients (male & female, >18 years and < 90 years, n=5 for each cardiac chamber) undergoing cardiac surgery at the Liverpool Heart & Chest Hospital (LHCH), Liverpool, United Kingdom. Ethical approval for these procedures was given by NREC (08/H1306/91; PI, Dr. G.M Ellison) and local R&D LHCH ethical approval. Upon arrival at the laboratory the sample was weighed and photographed before proceeding immediately to cell isolation (Figure 2.1).

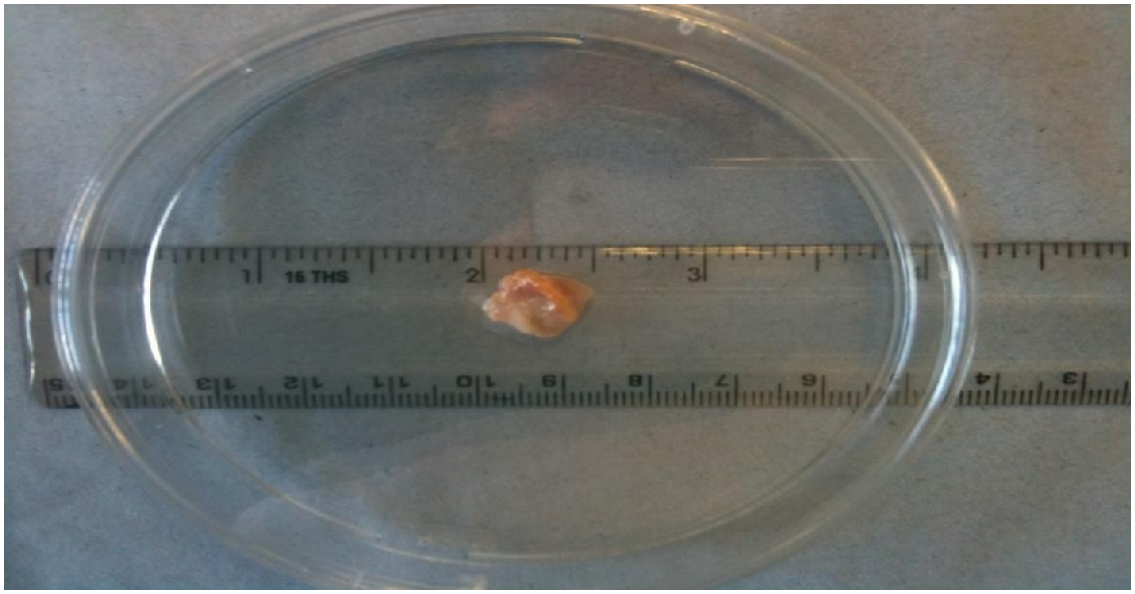


Figure 2.1 Example sample before enzymatic isolation of cells.

2.2 Small cell isolation by enzymatic digestion.

Cardiac small cells were isolated from human myocardial samples by enzymatic digestion. The sample was cut with sterile scissors into very small pieces (~1-2mm) inside a small,

25ml conical flask containing 15mls of Collagenase type 2 Worthington® 1mg/ml in DMEM (Figure 2.2).

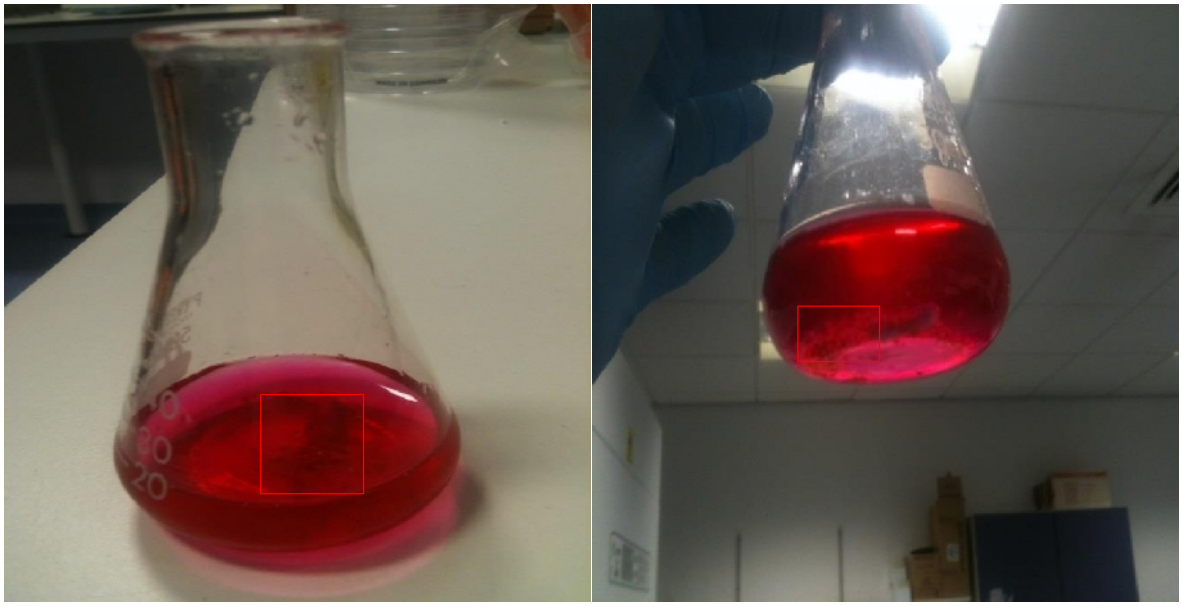


Figure 2.2 Photographs showing a myocardial sample in a conical flask before cutting (left) and after sufficient cutting (right); notice the small size (less than 1-2mm) of the pieces after cutting as shown inside the red box for been noticeable.

A small magnetic stir bar was placed into the conical flask, which was transferred to a 37°C oven on a stirrer plate set at 250 RPM for 5 min, to further dissociate the tissue. The supernatant was transferred to a 15ml falcon tube and centrifuged at 300G for 5 min. After centrifugation, the pellet was re-suspended in 10ml of 10% foetal bovine serum (FBS) in DMEM and left on ice. A further 15ml of 1mg/ml Collagenase type 2 Worthington® in DMEM was added to the conical flask containing the sample sediment and returned to the 37°C oven to continue mixing. This process was continued every 5 min for a further 10-15 times or until complete digestion of the tissue. When the digestion process was completed all the re-suspended cell pellets were combined and passed through a 40 micron filter into a 50ml falcon tube and centrifuged for 7 min at 400G. The pooled pellet was re-suspended in 3ml of DMEM and the number of viable small cells were determined using a haemocytometer and trypan blue stain. To perform cell counts, 10µl of undiluted cell

suspension was mixed with 10µl of Trypan blue to identify dead/non-viable cells and loaded onto the haemocytometer. Clear bright cells were counted in four of the 1mm² squares and the average taken to determine the number of viable cells in 100µl (0.1mm³). This value was multiplied by 2 to account for the 50% dilution with Trypan blue and then multiplied by 10000 to determine the number of viable cells per cm³ or per ml (150). The total number of cells isolated were calculated before (cardiac small cells) and after MACS separation (c-kit^{pos} cardiac stem-progenitor cells). The number of cells per gram of tissue was calculated for each cardiac sample and used to determine average number of cells per gram of tissue for each cardiac chamber.

2.3 c-kit^{pos} cardiac stem-progenitor cell isolation.

The c-kit^{pos} endogenous cardiac stem/progenitor cells (eCSCs) were purified from the small cell population by magnetic activated microimmunobead sorting (MACS; Miltenyi®), according to the manufacturers protocol (Figure 2.3). The cell suspension was centrifuged at 300G for 10 min, supernatant removed and the cell pellet re-suspended in 300µl of incubation media (DPBS 492mls, Bovine Serum Albumin 2.5gr, Fungizone 500µl, Gentamycin 500µl, Pen/Strep 5mls, and EDTA 2mls). After adding 100µl of FcR Blocking reagent (MACS, Miltenyi® Biotec) and 100µl CD117 microbeads (MACS, Miltenyi® Biotec), the suspension was mixed for 15 min at 4°C. After washing the cells by adding 1ml of incubation media, centrifugation of the cells at 300G for 10 min was performed.

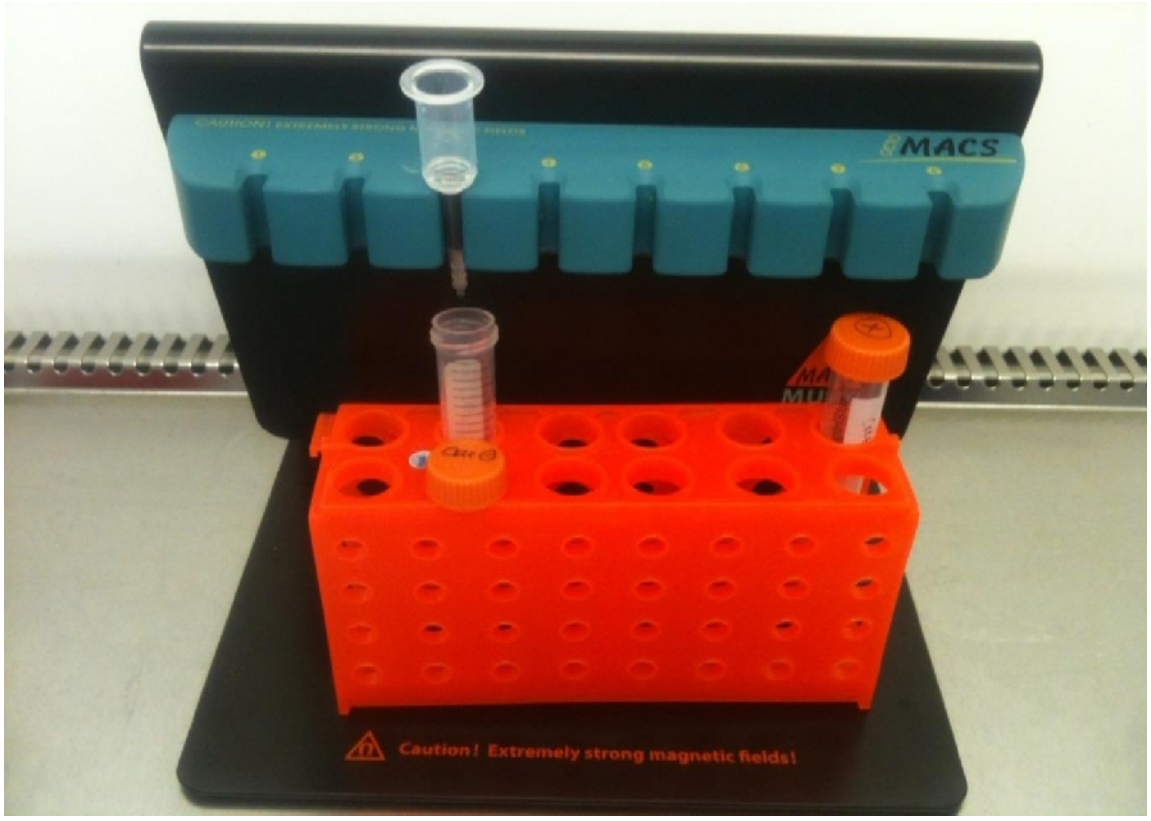


Figure 2.3 Photographic representation of MACS technology (MACS; Miltenyi®) of c-kit^{pos} CSCs from the cardiac small cells. Magnetically labelled (c-kit^{pos}) cells are retained in the column by the magnet while unlabelled (c-kit^{neg}) cells pass through into the collection tube of 15mls on the left of the picture.

The magnetic MS column was rinsed with 500µl incubation media, before passing the cell suspension through the column. The unlabelled cell fraction (c-kit^{neg}) was collected in the flow through and discarded. Then the column was washed three times with 500µl of incubation media. The column was removed from the magnet and 1ml of incubation buffer was added to the column and was immediately flushed with the plunger into a fresh collection tube and the magnetically labelled (c-kit^{pos}) cells were collected (Figure 2.4).

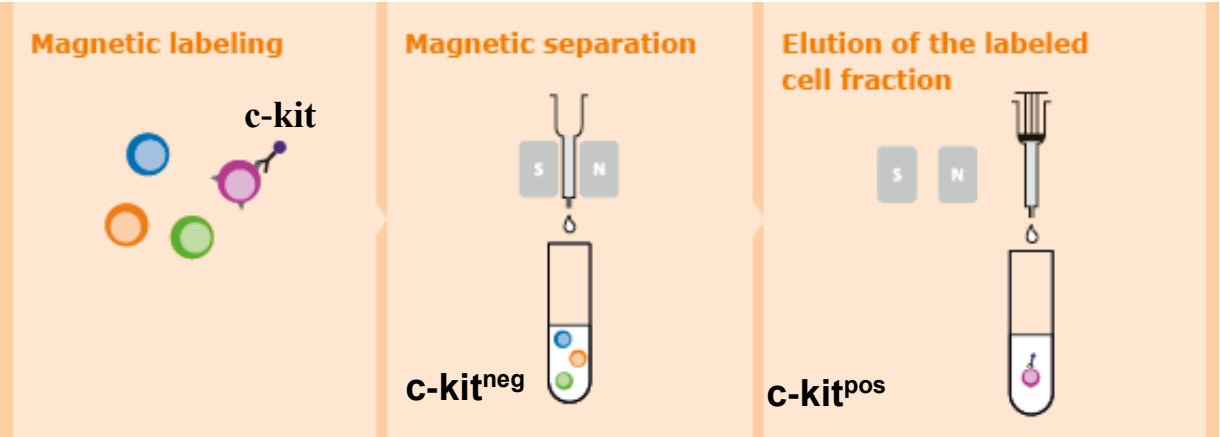


Figure 2.4 Diagrammatic representation of the isolation of c-kit^{pos} eCSCs (Adapted from www.miltenybiotec.com).

The c-kit^{pos} population was centrifuged for 3 min at 1300 rpm, the supernatant removed and the pellet re-suspended in growth media (see below for recipe) and plated for cell culture.

2.4 Cell Culture.

Human c-kit^{pos} eCSCs were plated onto gelatin coated 12-well plates (BD) in human eCSC growth media [DMEM/F12K (Gibco) supplemented with 10% FBS-ESQ, 10 ng/ml bFGF and 10 ng/ml LIF, 1% NAAA, 1/% Glutathione, 0.005U/ml hEPO (sigma) and antibiotics (1% Pen/strep, 0.1 % Fungizone & 0.1% Gentamycin (10mg/ml liquid Invitrogen). Cells were cultured at 37°C on 2% O_2 and 5 % CO_2 for up to 2-3 weeks. Media was changed after 5 days, and then every 2-3 days thereafter. 70% of the media was removed and replaced with fresh media to enable cells to condition their media through the secretion of growth factors and cytokines that benefit their own growth and proliferation. When ~1 x 10^6 cells had been propagated (usually after 2 weeks), cells were trypsinised and lysed for total RNA isolation. The population of c-kit^{pos} CD45^{-} cells were collected after 2 weeks of culture. The c-kit^{pos} CD45^{+} cells, such as mast cells, which do not grow in adherent culture conditions they have been discarded.

Upon reaching full confluency the cells were harvested as follows: media was aspirated and the adherent cell monolayer washed with 5mls of phosphate buffered saline (PBS+Antibiotics) at room temperature to remove residual protein or dead, detached cells. PBS+ABs was removed and replaced with 2mls of trypsin. Trypsinization was performed at 37°C for ~5 min. Cell detachment was monitored using transmitted light microscopy (Olympus®, CKX 41 Japan) until approximately 90% of cells had detached, at which point the trypsin was neutralised by addition of 6 mls of serum-rich passaging media [10% fetal bovine serum (FBS) in DMEM]. The trypsin/cell suspension was transferred to a 15ml falcon tube and centrifuged at 1300g for 3 min at 25°C to pellet the cells. The resulting supernatant was aspirated and the remaining cell pellet lysed in 350µl of RLT buffer* with the addition of 10µl 1M β-mercaptoethanol (Sigma-Aldrich) for 5 min at room temperature before being transferred to a QIA shredder column (Qiagen®) and centrifuged at 12000RPM for 2 min. The resulting lysate was stored at -80°C until further analyses.

*RLT is a lysis buffer which contains a high concentration of guanidine isothiocycanate, which supports the binding of RNA to the silica membrane for lysing cells and tissues prior to RNA isolation and simultaneous RNA/DNA/Protein isolation from (Qiagen)®.

2.5 RNA isolation.

At all stages gloves were worn, and filtered, DNase-RNase free pipette tips (Rainin) were used. All equipment was cleaned thoroughly with ethanol before and throughout the procedure. Total RNA was isolated from cell lysates using an RNeasy mini-kit (Qiagen®), according to the manufacturer's protocol. The lysate was removed from the -80°C freezer and placed in a 37°C water bath to thaw. When fully thawed, 700µl (or equal part volume to lysate) of 70% molecular grade ethanol was added and mixed well using a micropipette. This sample solution was then transferred to an RNeasy mini spin column (pink) and centrifuged for 15sec at 10000RPM. The flow through was discarded and the column was then rinsed with 350µl of buffer RWI and centrifuged for 15sec at 10000RPM. The flow through was

discarded and 10µl of DNase (Qiagen) mixed with 70µl of buffer RDD was added to the membrane of the column and left to stand at room temperature for 15 min. This step allowed the removal of residual contaminating DNA from the sample. The column was rinsed with 350µl of buffer RWI and centrifuged for 15sec at 10000RPM. After discarding the flow through, 500µl of buffer RPE was added to the column and it was centrifuged for another 15 sec at 10000RPM. The flow through was discarded and a further 500µl of buffer RPE was added and the column was centrifuged for 2 min at 10000RPM. Subsequently, the RNeasy spin column was placed into a new 2ml collection tube and centrifuged at full speed at 20000 RPM for 1 min. This last step was performed to eliminate any possible carryover of buffer RPE, or residual flow-through. The RNeasy spin column was next placed in a new 1.5ml collection tube and 30µl of RNase free water was applied directly to the column membrane. After centrifugation for 1 min at 8000RPM, the total RNA was eluted into the water and collected into the 1.5ml tube. The tube containing the RNA sample was kept on ice until spectrophotometric analysis with the Nanodrop to determine RNA quantity and quality.

2.6 Nanodrop spectrophotometric analysis.

The quantity and purity of the total RNA was determined using a Nanodrop (LabMode Washington Delaware USA) spectrophotometer. 2µl of the sample was analysed for the purity (260/230 and 260/280 ratios) and quantity (ng/µl) of the RNA. The 260/280 ratio shows ethanol or protein contamination of the sample while the 260/230 ratio shows DNA or other contamination (Figure 2.5). A ratio < 2.0 is generally accepted as *pure* for DNA, but a ratio of 2 is generally accepted as pure for RNA (151). If the ratio is lower in either case it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280nm. After quantity and purity Nanodrop assessment, total RNA samples were stored at -80°C until processed for cDNA synthesis.

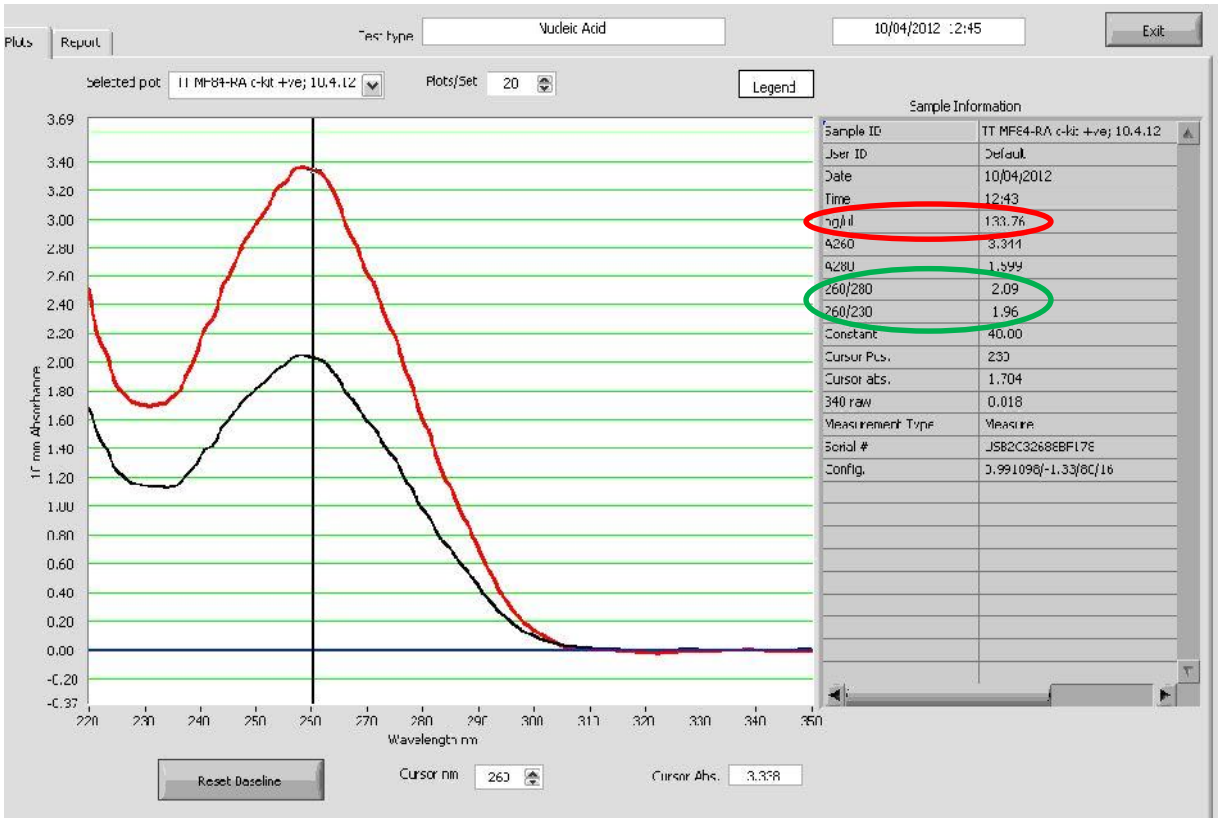


Figure 2.5 Nanodrop quantification of RNA quantity and purity. In red circle is the RNA concentration of the sample, this is used to calculate the volume of the sample needed for 1µg of RNA required for the c-DNA synthesis. In green circle shown the purity ratios of 260/280 and 260/230 respectively.

2.7 Reverse transcription or cDNA synthesis.

Reverse transcription (RT) was performed using a Taqman reverse transcription kit (Applied Biosystems) ® on a Biorad I-cycler. For each sample, 1µg of total RNA was required for the RT reaction, and the volume of RNA sample needed for 1µg was calculated using the RNA concentration determined with the Nanodrop. Each sample was made up to a total volume of 38.5µl by the addition of RNase free H_2O. To each sample, 61.5µl of RT master mix was added to make a total volume of 100µl for each RT reaction. The volumes and concentrations of the components of the RT master mix are listed in Table 2.1.

Table 2.1 Table showing components of reverse transcriptase reaction mixture; (adapted from User guide Applied Biosystems Life Biotechnologies, Chapter 3 Reverse Transcription).

REVERSE TRANSCRIPTASE REACTION MIX		
Component	**Volume/Tube(µl)**	**Final concentration**
RNase free water	Variable*	
10xRT Buffer	10	1x
25mM MgCl2	22	5.5mM
deoxyNTPs Mixture(2.5mM)	20	500µM per d NTP
Random Hexamers[1] (50µM)	5	2.5µM
RNase Inhibitor (20U/L)	2	0.4U/µl
Multiscribe Reverse Transcriptase (50U/µl)	2.5	1.35U/µl
Total	61.5**	
*the volume of RNase free water (µl) will be 38.5-RNA sample volume in 100 µL reaction		
[1]Random Hexamers, oligo d (T) 16 or sequence specific reverse primers can be used for primers of c-DNA synthesis.		
** if changing the reaction volume make sure the final proportions are consistent with the recommended values above		

Reverse Transcription was performed on a Biorad I cycler as follows:

a. First step is the hexamer incubation period of 10 minutes at 25°C

b. Second step is to perform reverse transcriptase for 30 min at 48°C

c. Last step of 5 min at 95°C to stop the reverse transcriptase reaction.

The cDNA synthesised from the RT reaction was stored at -80°C for qPCR analysis.

2.8 Primer design.

Firstly, coding strand cDNA sequences of genes of interest were identified using the genome search platform (www.ncbi.nlm.nih.gov). The Coding DNA sequence (CDS) was copied into the primer design program Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast), which performs a basic local alignment search of human genome sequence databases to find regions of local similarity within sequences. Once a successful BLAST had been performed a

primer search was carried out allowing the selection of regions of the coding strand sequence suitable for amplification. Primers were designed to range between 20-25bp along with a guanine –cytosine (GC) content ranging between 40-60%, and an annealing temperature of 60.0 ± 3°C. In addition, all primers were designed to span exon-exon junctions to ensure that they do not associate with genomic DNA. All primers (Table 2.2) were designed in-house and were purchased from Sigma Aldrich.

Table 2.2 List of qPCR primers used in the study.

SPECIES/GENE	SEQUENCE	cDNA product size (bp)	Tm	NCBI Ref Seq
human HRT-1-F	CGAGGTGGAGAAGGAGAGTG	177	60	NM 012258.3
human HRT-1-R	CTGGGTACCAGCCTTCTCAG		59.9	
human HRT-2-F	TGGGGAGCGAGAACAATTAC	181	60.1	NM 012259.2
human HRT-2-R	TCAAAAGCAGTTGGCACAAG		60	
human Hand1-F	ACATCGCCTACCTGATGGAC	138	60	NM 004821.2
human Hand1-R	GAAAACCTTCGTGCTGCTG		59.6	
human Hand2-F	ACATCGCCTACCTCATGGAC	162	59.9	NM 021973.2
human Hand2-R	TGGTTTTCTTGTCGTTGCTG		59.9	
human Tbx-20-F	GCGGCTCTAAGGAGAAGGAG	165	60.6	NM 001077653.2
human Tbx-20-R	TGCACAGAGAGGAGGAGGAC		60.5	
human Tbx-5-F	TCAAGCTCACCAACAACCAC	150	59.7	NM 000192.3
human Tbx-5-R	CAGGAAAGACGTGAGTGCAG		59.6	
human PITX-2-F	CTGAGTGCTTGCCAGTATGC	137	59.6	NM 001204399.1
human PITX-2-R	CTTTAGTGCCCACGACCTTC		59.7	
human FGF-10-F	GAGATGTCCGCTGGAGAAG	194	59.9	NM 004465.1
human FGF-10-R	TTTCCCCTTCTTGTTCATGG		59.7	
human FGF-8-F	GTAGGGCACCCAAAACTCAA	117	59.9	NM 001206389.1
human FGF-8-R	AACAGCAAAAACCCAACAGC		60	
human ISL-1-F	AAACAGGAGCTCCAGCAAAA	157	59.9	NM 0022022
human ISL-1-R	AGCTACAGGACAGGCCAAGA		59.7	
human FOXH-1-F	AGCCCCCTAAGAGGAGGAAG	134	60	NM 003923.2
human FOXH-1-R	GACCTGACGGATGATCTGGG		59.9	

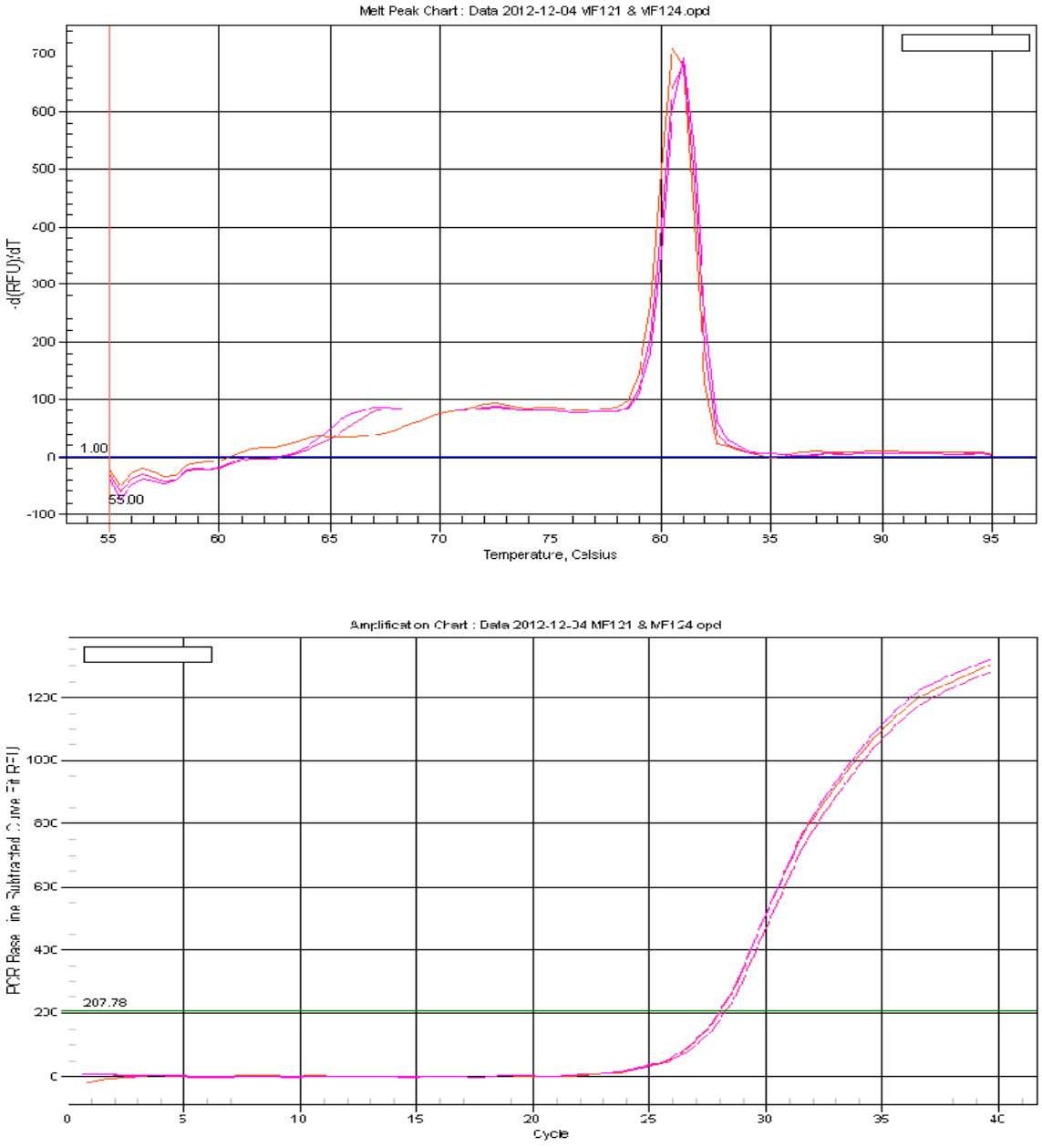
2.9 Quantitative Polymerase Chain Reaction (qRT-PCR).

Quantitative real time polymerase chain reaction (qPCR) allows amplification and simultaneous quantification of double stranded DNA. In this study a fluorescent dye (SYBR green) which binds double stranded DNA was used to assess PCR reaction kinetics. For each gene of interest, a mixture of 10µl of SYBR green, 1µl forward primer, 1µl reverse primer, 7µl of RNase free H_2O and 1µl of cDNA was prepared in triplicate for each sample in a 96 well plate on ice**.** Once setup and plate preparation was complete the plate was sealed with a film and centrifuged at 1300rpm for 20 sec at full speed to ensure that all the reagents had collected at the bottom of the wells of the PCR plate.

Plates were transferred to a Biorad® MyIQ thermocycler and qPCR was conducted as follows: samples were denatured for 5 min at 95°C followed by 40 cycles of amplification at 95°C for 15 seconds, 30 seconds at an annealing temperature of 60°C and 30 seconds at 72°C. Following this, melt curve analysis was performed of 81 cycles on 55-95°C for 30 seconds each.

Once amplification had been performed melt curve analysis of the product was undertaken where plates were subject to an increase in temperature of 1°C per 30 seconds, starting at 55°C and terminating at 95°C. The melt curve analysis will identify different reaction products and non-specific products. On completing the amplification reaction a melt curve is generated. When the double stranded DNA in the reaction denatures the fluorescence decreases intensity. As the melting temperature of double stranded DNA varies depending upon its base composition, this analysis indicates the identity of the amplified products when compared against the melting temperature of an amplified product for a specific primer (Figures 2.6a, b). The negative first derivative of the change in fluorescence is plotted as a function of temperature (see Figures 2.6a, b). A characteristic peak at the amplicons melting

temperature (Tm, the temperature at which 50% of the base pairs of a DNA duplex are separated) distinguishes it from other products such as primer-dimers, which melt at different temperatures. In the example of the Figures 2.6a, b the Tm of this specific product which successfully analysed during qPCR was 82°C.



Figures 2.6 (a, b) Example of a melting curve analysis (a) and melting peak chart (b) in one of the reactions. Notice that for this specific product the Tm was at 82°C.

Ct values were calculated by the MyIQ when fluorescence from SYBR green binding to double stranded DNA entered its exponential phase during amplification. All reactions were performed in triplicate. Amplicons were stored at 4°C.

2.10 Quantitative PCR normalisation Algorithm.

For each gene or transcript relative expression was calculated and normalized to expression of the two housekeeping genes GAPDH and beta-Actin using the following equation used as our standard reference in our laboratory (152):

Copy number of target = 2500*1.93^ (HKi - target Ct)

HKi = GAPDH Ct - (Median GAPDH Ct - Median Actin Ct)

2.11 Statistical Analysis.

Due to the variability of the speciments taken form each patient in order to analyse efficiently all the genes expressed from each specimen and from each chamber we used the sequent statistical methodology: after correcting for multiple testing and analyse the data on the linear model,we checked for normality of the distribution of the the data using the Shapiro-Wilk significance value which needed to be (greater than 0.05), and then we used the one-way ANOVA with Tukey post hoc tests to locate significant differences. Significance was set at $p<0.05$. Multiple data are shown as Mean ± SD.

3. RESULTS

3.1 Isolation of c-kit^{pos} eCSCs and their quantity in the four cardiac chambers.

Cardiac tissue was obtained from consenting patients and the weight of each sample was recorded. Endogenous c-kit^{pos} cardiac stem cells (eCSCs) were then isolated following enzymatic digestion of the tissue and magnetic-activated cell sorting (MACS). The number of ckit^{pos} cells in each chamber was recorded after their isolation, and the number per gram of tissue was calculated. These results are shown in Table 3.1.

The samples obtained from the RA showed the greatest weight being 0.46±0.36g. However, ANOVA analysis revealed no statistical significance between samples for weight (Table 3.1). The highest numbers of c-kit^{pos} eCSCs were also isolated from the RA samples (4860±2130)/cm^3 which is in line with the RA samples being bigger and from the LV samples which were more muscular (4626±2945)/cm^3. However, ANOVA analysis between the LA, RA and LV revealed that there were no significant differences between chambers for the number of ckit^{pos} cells isolated. When the number of c-kit^{pos} eCSCs per gram of tissue was calculated, the LV showed the greatest number of c-kit^{pos} eCSCs/gram of tissue, however similarly to the previous analysis, there were no significant differences detected between chambers after the ANOVA analysis (see Table 3.1).

Table 3.1 Sample weight, number of c-kit^{pose} eCSCs isolated and number of c-kit^{pos} eCSCs per gram of tissue, from all 4 human cardiac chambers accordingly to sex, age and pathology of the patients.(AS: aortic stenosis, MS: mitral stenosis, MR: mitral regurgitation, TR: tricuspid regurgitation, 3VD: three vessel disease,4VD: four vessel disease).

Samples	Weight (gr)	No of c-kit^{pos} isolated/cm^3	No of c-kit^{pos} cells/gr of tissue	Sex	Pathology	Age	p-value
LA1	0.123	2700	21951	M	MS	77	
LA2	0.147	4275	29082	M	MR	68	
LA3	0.086	3600	41860	M	MS/TR	73	
LA4	0.086	4400	51163	M	3VD/MS	80	
LA5	0.114	3300	28947	F	AS/MR	78	
mean±SD	**0.11±0.03**	**3655±704**	**34601±11720**			**75.2±5**	**NS**
RA1	1	6400	6400	M	3VD	77	
RA2	0.277	1800	6498	M	AS/3VD	64	
RA3	0.147	5000	34014	F	AS	90	
RA4	0.227	3900	17181	M	4VD	75	
RA5	0.638	7200	11285	F	3VD	74	
mean±SD	**0.46±0.36**	**4860±2130**	**15076±11470**			**76±14**	**NS**
LV1	0.025	3000	120000	M	AS	82	
LV2	0.243	9600	39506	M	MR	72	
LV3	0.068	3600	52941	M	MS/TR	72	
LV4	0.186	4800	25806	F	MS	68	
mean±SD	**0.18±0.13**	**4626±2945**	**49945±42097**			**73.5±8**	**NS**
RV1	0.033	2475	75000	M	MS/TR	74	

3.2. Cell morphology of c-kit^{pos} eCSCs isolated from LA, RA, LV, and RV chambers.

Transmitted light microscopic observations of c-kit^{pos} eCSCs in culture revealed that most of the isolated c-kit^{pos} cells were generally small and rounded, consistent with a c-kit^{pos} eCSC phenotype.

All the cells from most of the LA samples showed a uniformly pattern of growth in culture without any evidence of change of their phenotype during their culture period. The culture period for the c-kit^{pos} eCSCs isolated from the LA was 16.2±2.6 days, before they reached full confluence and were lysed for RNA isolation (Figure 3.1).

c-kit^{pos} eCSCs isolated from the RA were also generally small and rounded with the exception of RA5, which appeared more spindle shaped as shown in Figure 3.2. Similar to LA, the c-kit^{pos} eCSCs isolated from the RA cells had a mean growth time in culture of 15.4±3.6 days. With the exception of RA5 that was in culture for 21 days before reaching full confluency.

Similarly, c-kit^{pos} eCSCs isolated from the LV samples were also generally small and rounded, with the exception of LV3 which appeared more flattened, generally indicative of a more differentiated or precursor-like phenotype (Figure 3.3). The mean time of growth of c-kit^{pos} eCSCs from the LV samples was 10.9±4.3 days. With the exception of the LV1 sample which reached full confluency after 19 days.

c-kit^{pos} eCSCs isolated from the single RV sample were not as small and rounded as from other samples, and appeared more spindle-shaped (Figure 3.3). Cells from the RV1 sample reached full confluency in 8 days.

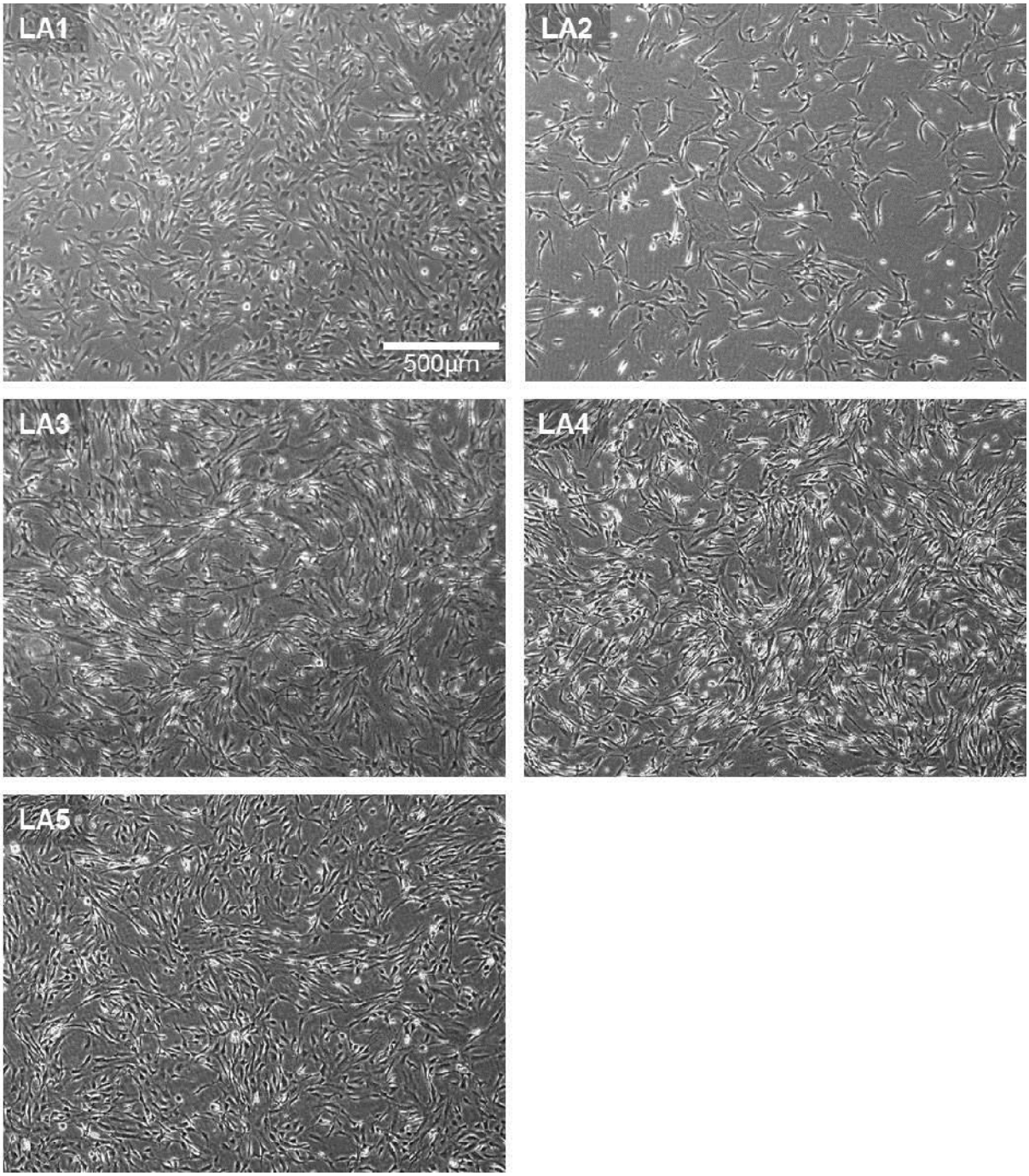


Figure 3.1 Phase contrast microscopic images of c-kit^{pos} eCSCs in culture isolated from each LA sample, taken at (16.2±2.6) days *in vitro*.

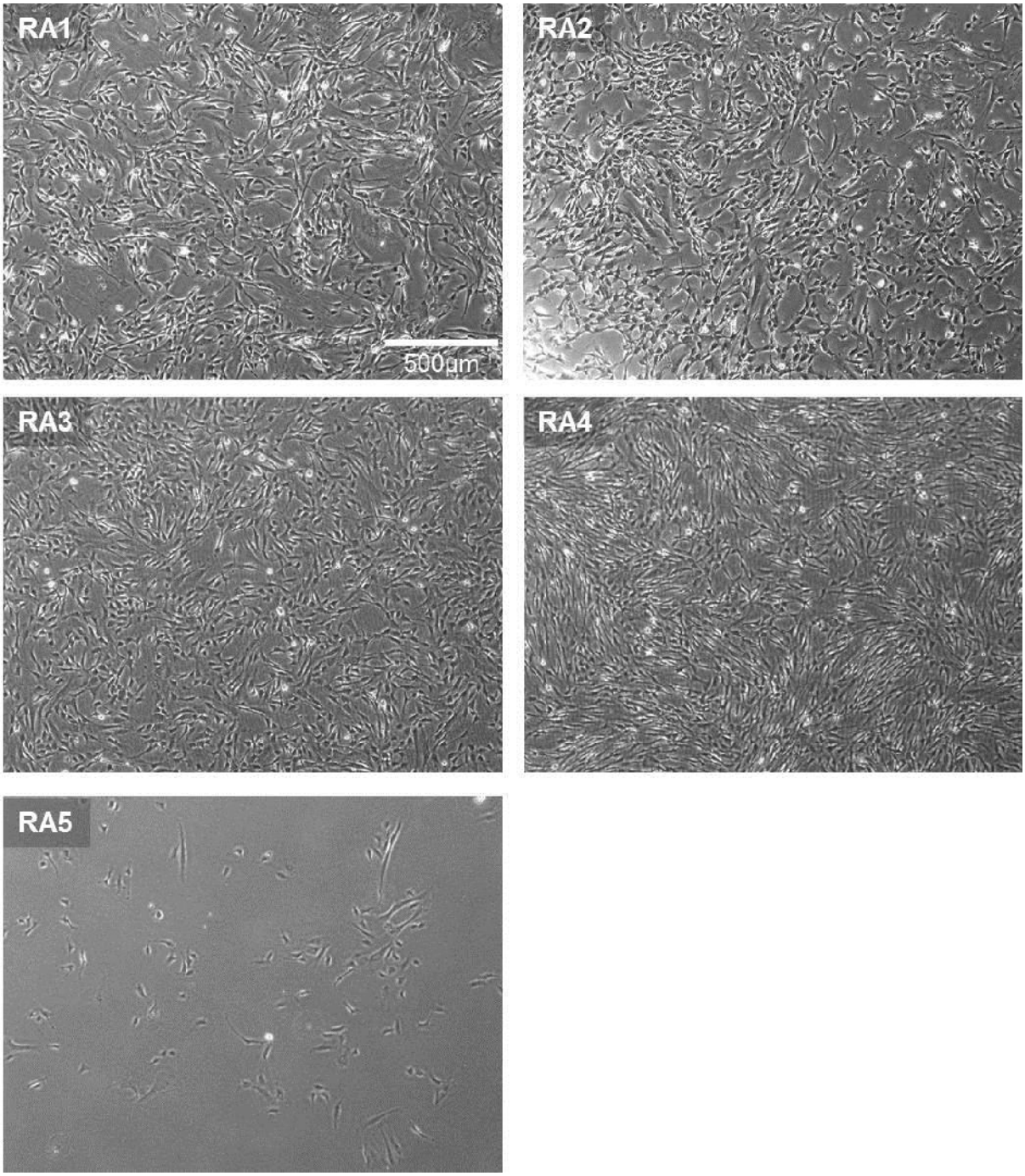


Figure 3.2 Phase contrast microscopic images of c-kit^{pos} eCSCs in culture isolated from each RA sample, taken at (15.4±3.6) days *in vitro*.

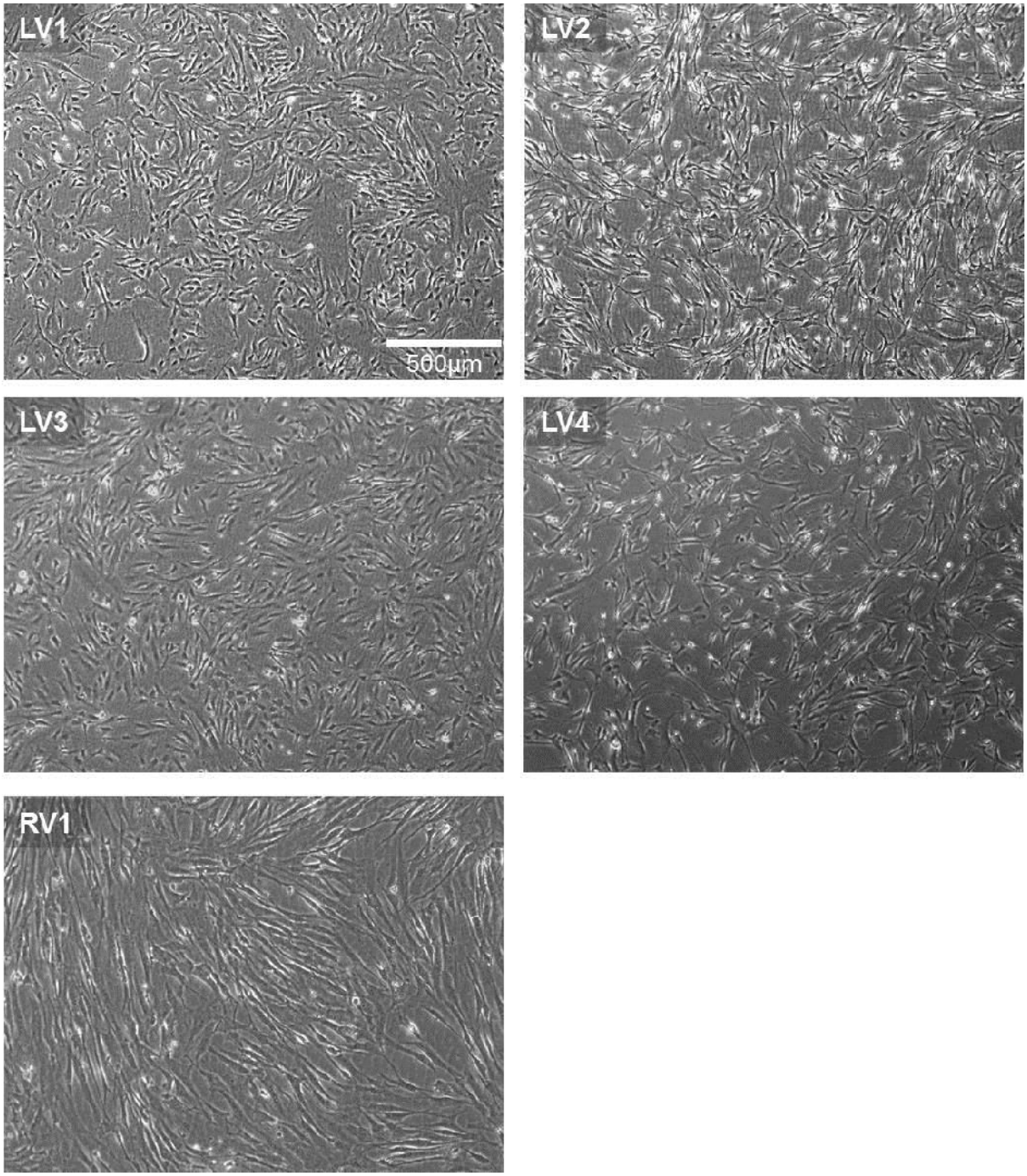


Figure 3.3 Phase contrast microscopic images of c-kit^{pos} eCSCs in culture isolated from each LV sample at (10.9±4.3) days, and RV1 sample, taken at 8 days *in vitro*.

3.3. RNA isolation and purity for human c-kit^{pos} eCSCs isolated from the 4 cardiac chambers.

In order to determine the mRNA expression levels of each c-kit^{pos} eCSCs sample, it was first necessary to isolate total RNA of sufficient quantity and quality. Table 3.2 shows the concentration and purity ratios for each c-kit^{pos} eCSCs sample from each of the four chambers. In terms of RNA quantity, c-kit^{pos} eCSCs isolated from the samples of all chambers showed a concentration >200ng/µl, except LA1 which was 40ng/µl (Table 3.2). Through utilising the 260/280 and 260/230 ratios, the purity of each sample was determined. The 260/280 ratio was consistent with an excellent level of *pure* RNA (>2), devoid of phenol or protein contamination for all samples (Table 3.2). The 260/230 ratio, which is reflective of genomic contamination, was not as consistent, with 5 samples having a 260/230 ratio of <1.7 (Table 3.2).

Table 3.2 Nanodrop spectrophotometric analysis for quantity and quality of the RNA isolation for c-kit^{pos} eCSCs isolated from each cardiac chamber. (*denotes genomic DNA contamination).

Sample	RNA quantity (ng/µl)	(260/280)	(260/230)
LA1	**40.03**	**2.01**	**0.78***
LA2	**270.9**	**2.05**	**2.14**
LA3	**565.7**	**2.05**	**2.18**
LA4	**520.7**	**2.09**	**0.68***
LA5	**429.5**	**2.07**	**2.08**
mean±SD	**365.3±213.9**	**2.05±0.02**	**1.57±0.77**
RA1	**453.2**	**2.07**	**1.93**
RA2	**508.8**	**2.08**	**1.03***
RA3	**448.9**	**2.07**	**1.74**
RA4	**1345.9**	**2.1**	**1.31***
RA5	**789.5**	**2.08**	**2.03**

mean±SD	**709.2±382.5**	**2.08±0.01**	**1.60±0.42**
LV1	**643.7**	**2.05**	**1.79**
LV2	**248.4**	**2.08**	**0.32***
LV3	**881.5**	**2.07**	**1.98**
LV4	**416.1**	**2.05**	**2.15**
mean±SD	**547.4±275.4**	**2.06±0.01**	**1.56±0.83**
RV1	**1042.5**	**2.08**	**2.16**

3.4. qRT-PCR analysis.

Following growth, mRNA isolation and PCR technologies key genes of interest can be monitored. By plotting fluorescence accumulation against cycle number an amplification plot representing the accumulation of product can be generated. Example plots for all analysed genes are presented in Figures 3.4 and 3.5. Data also reveal the CT value for each gene of interest, which is representative of expression levels – higher CTs being indicative of lower expression. Having determined the gene expression levels it was important to assess the quality of the reaction. From the PCR-derived melting curves it is possible to ascertain that genes including: c-kit, Tbx5, Tbx20, Isl-1, Hand-2, FGF-10, HRT-1 and HRT-2 together with the house keeping genes GAPDH and beta-actin showed a normal range of melting curves and no evidence of primer-dimer formation (Figures 3.6 and 3.7).By contrast, genes including: the Foxh-1, FGF-8, Hand-1 and Pitx2 show evidence of abnormal shape of the melting curves with evidence of probable mispriming and/or some primer-dimer formations.

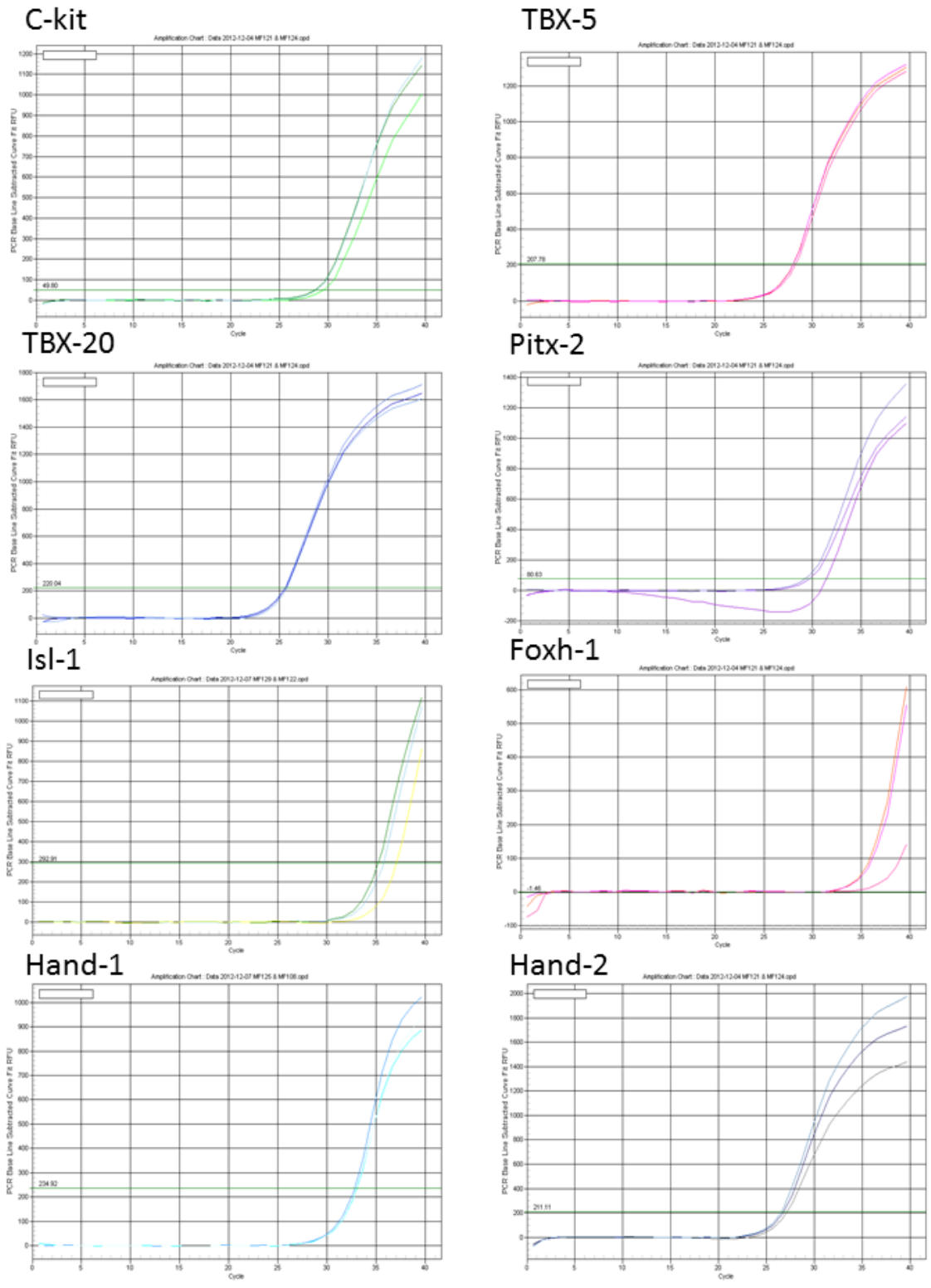


Figure 3.4 Typical amplification curves of c-kit, Tbx-5, Tbx-20, Pitx-2, Isl-1, Foxh-1, Hand-1 and Hand-2. Cycle number is shown along the X axis and arbitrary fluorescence units are shown on the Y axis.

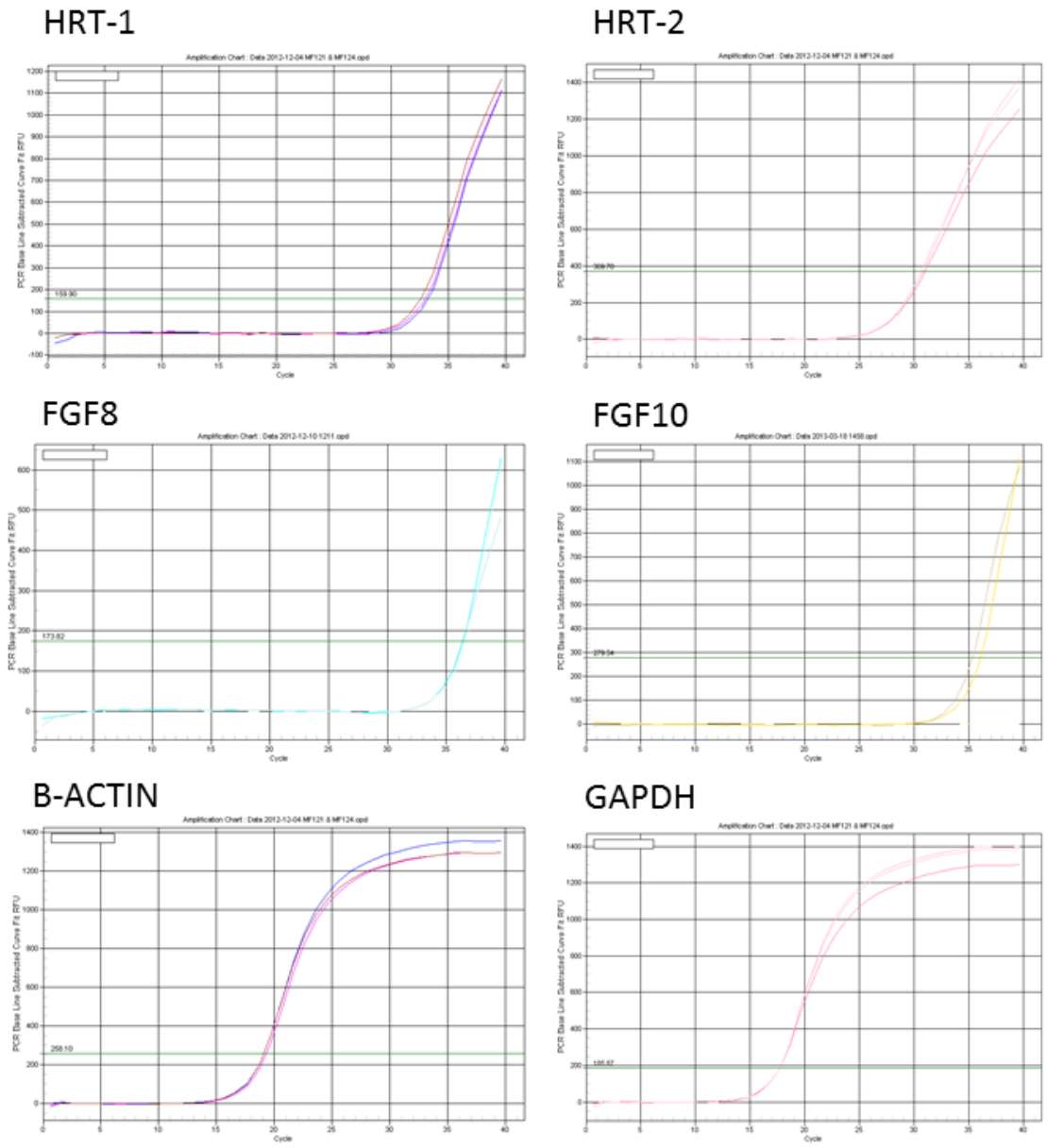


Figure 3.5 Typical amplification curves of for HRT-1, HRT-2, FGF8, FGF10, and housekeeping genes β-Actin and GAPDH. Cycle number is shown along the X axis and arbitrary fluorescence units are shown on the Y axis.

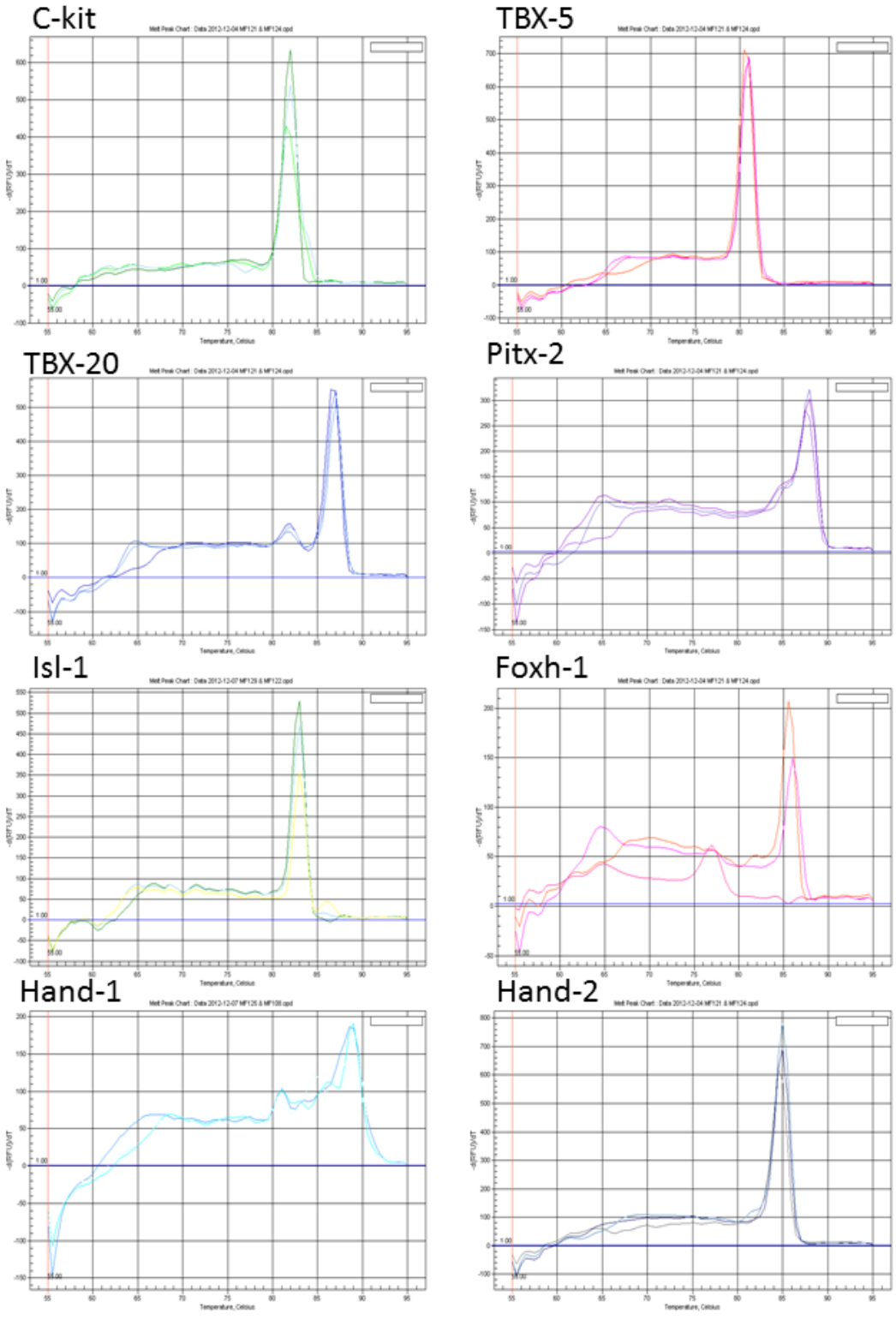


Figure 3.6 Typical melting curves for c-kit, Tbx5, Tbx20, Pitx-2, Isl-1, Foxh-1, Hand-1, and Hand-2. The X axis shows the change of the relative fluorescence units with time d (RFU)/d T, and the Y axis the Temperature with the peak melting temperature Tm.

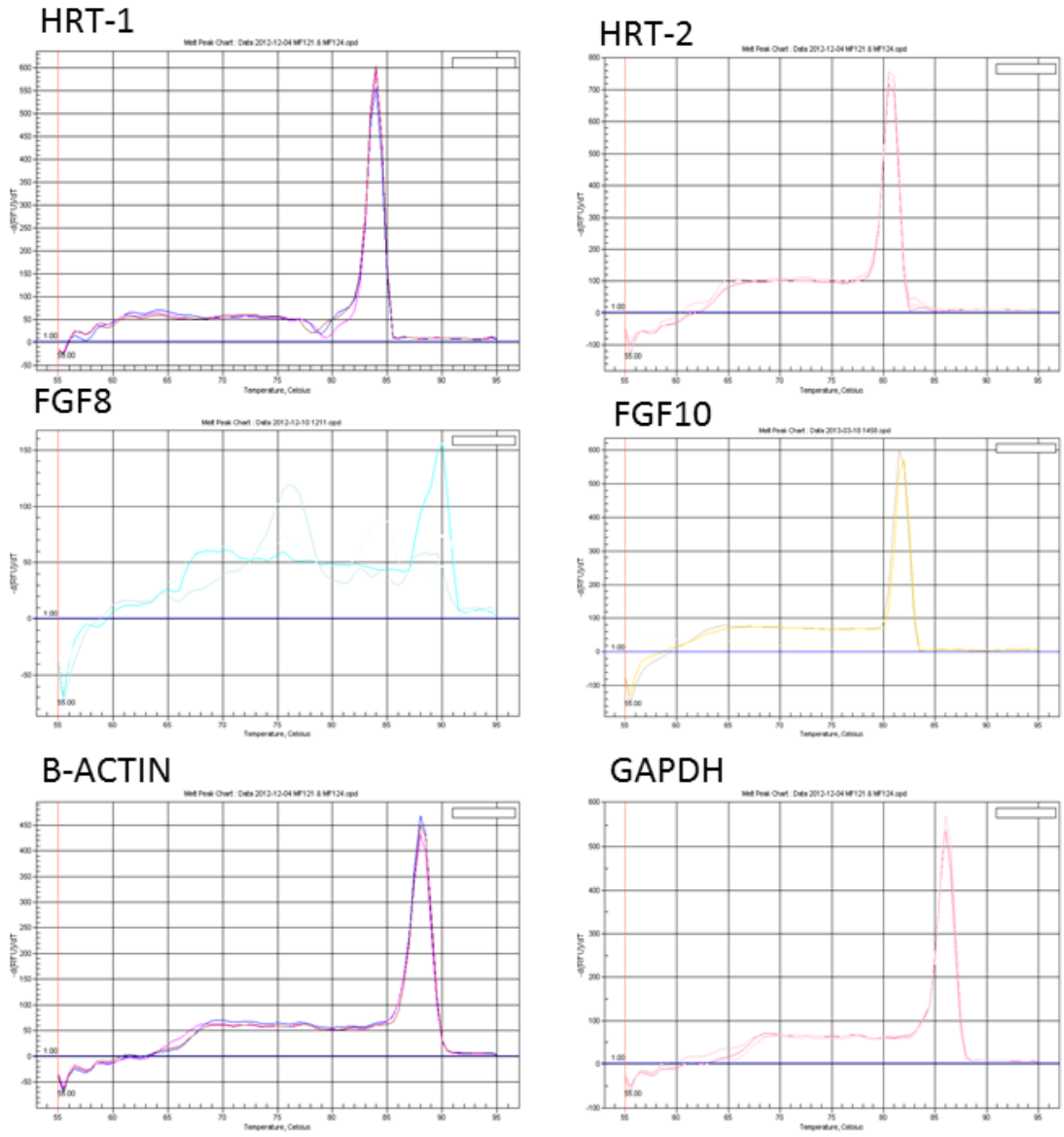


Figure 3.7 Typical melting curves for HRT-1, HRT-2, FGF8, FGF10, and housekeeping genes B-Actin and GAPDH. The X axis shows the change of the relative fluorescence units with time d (RFU)/d T, and the Y axis the Temperature with the peak melting temperature Tm.

3.5. c-kit mRNA expression levels of c-kit^{pos} eCSCs isolated from all four cardiac chambers.

All of the samples harvested from the 4 different cardiac chambers were analysed for c-kit mRNA expression using qRT-PCR. All samples showed expression of c-kit but at varying levels (Figure 3.8). LA, RA, and LV samples were found to express c-kit with a mean relative

expression level of (1.52±1.58), (6.04±10.48), and (2.56±4.55) respectively. LA samples were the most consistent for c-kit expression, with eCSCs expressing relatively similar amounts. The sole RV sample expressed the lowest level of c-kit (0.05).

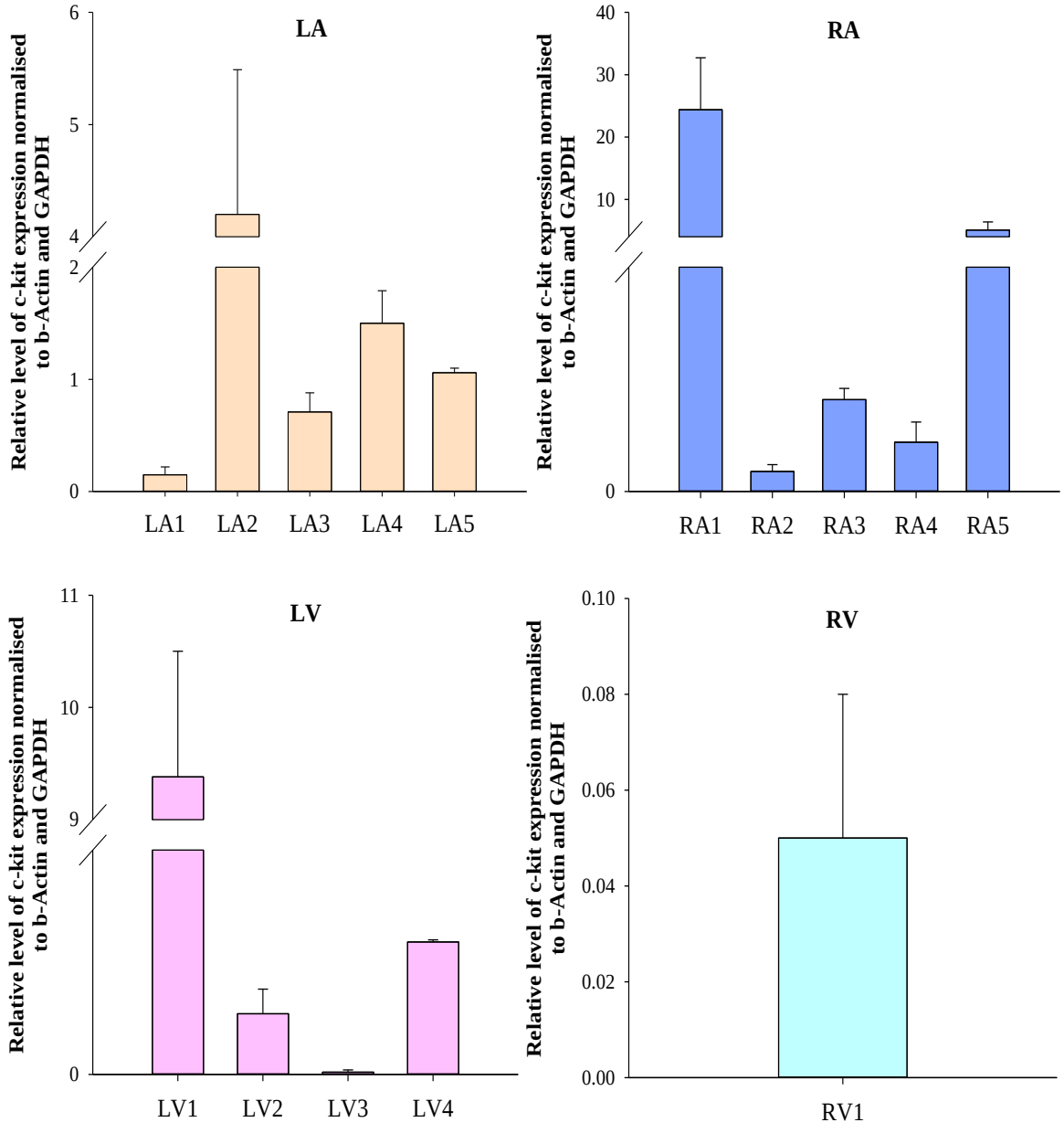
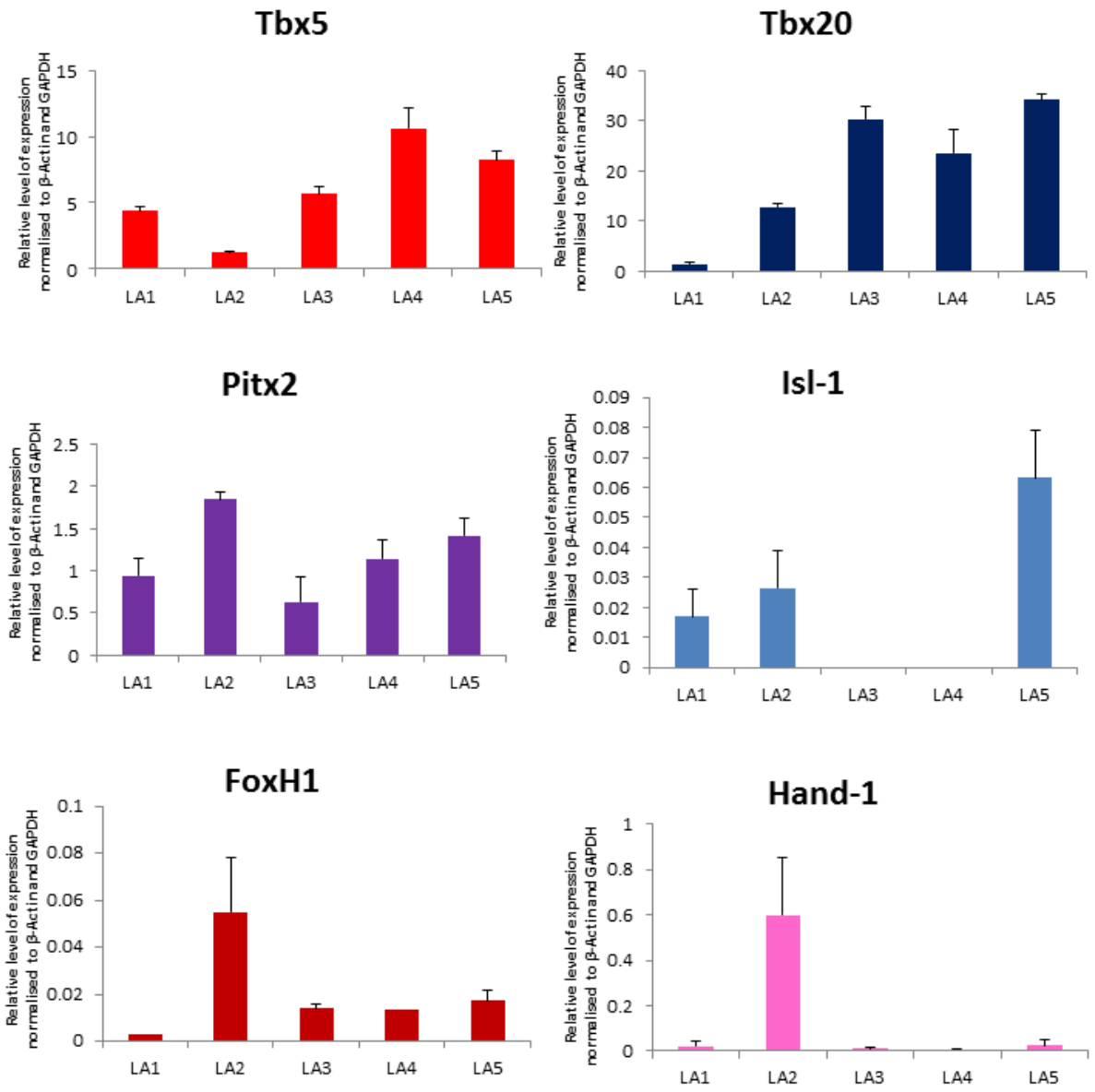


Figure 3.8 qRT-PCR analysis for c-kit expression of c-kit^{pos} eCSCs harvested from the 4 different cardiac chambers: Bars represent c-kit relative expression levels; error bars represent the standard deviation of the mean. (LA: left atria, RA: right atria, LV: left ventricle and RV: right ventricle), p value showed no significance difference between chambers.

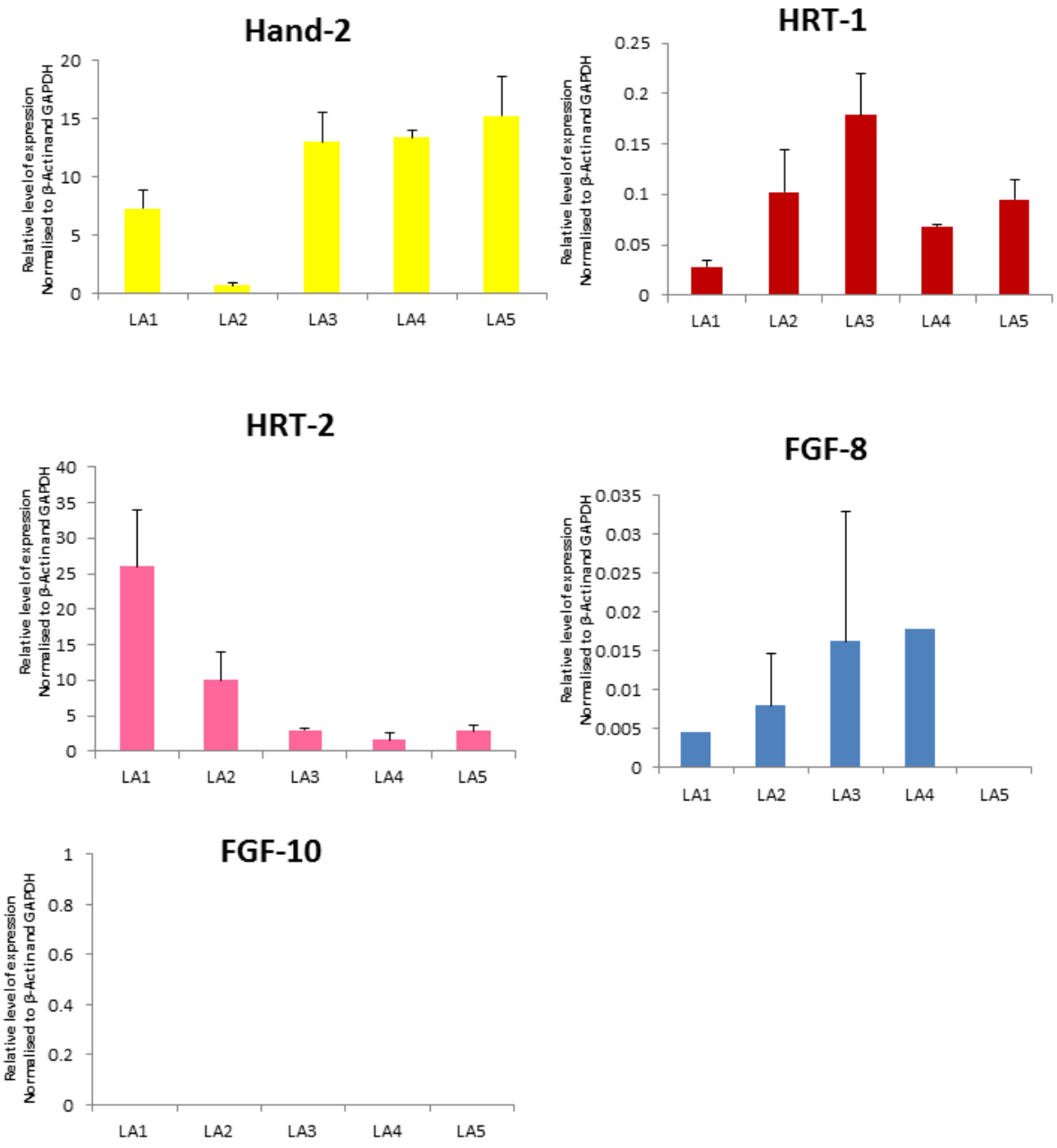
3.6 Transcriptional profile of c-kit^{pos} eCSCs isolated from all the 4 cardiac chambers.

3.6.1 Left atrium.

qRT-PCR analysis of the LA samples revealed relative expression of >1 for Tbx5, Tbx20, Pitx2, Hand-2, and HRT-2 in all 5 c-kit^{pos} eCSCs samples (Figures 3.9 and 3.10). HRT-1 showed expression across all 5 c-kit^{pos} eCSCs samples, however this was low being <0.25 relative expression (Figure 3.10). Hand-1 showed expression, albeit low, in only one sample (LA2; ~0.6 relative expression). The other samples were negligible for Hand-1 being <0.05 relative expression (Figure 3.9). Isl-1, Foxh-1, FGF-8 and FGF-10 all had no or negligible relative expression levels, being <0.08, in all 5 c-kit^{pos} eCSCs samples (Figures 3.9 and 3.10). These relative expression levels corresponded with the Ct values (See Table 3.3).



Figures 3.9 qRT-PCR analysis of the c-kit^{pos} eCSCs isolated from LA. Bars represent mRNA relative expression levels; error bars represent the standard deviation of the mean. All samples were carried out in triplicate, p=NS.



Figures 3.10 qRT-PCR analysis of the c-kit^{pos} eCSCs isolated from LA. Bars represent mRNA relative expression levels; error bars represent the standard deviation of the mean. All samples were carried out in triplicate, p=NS.

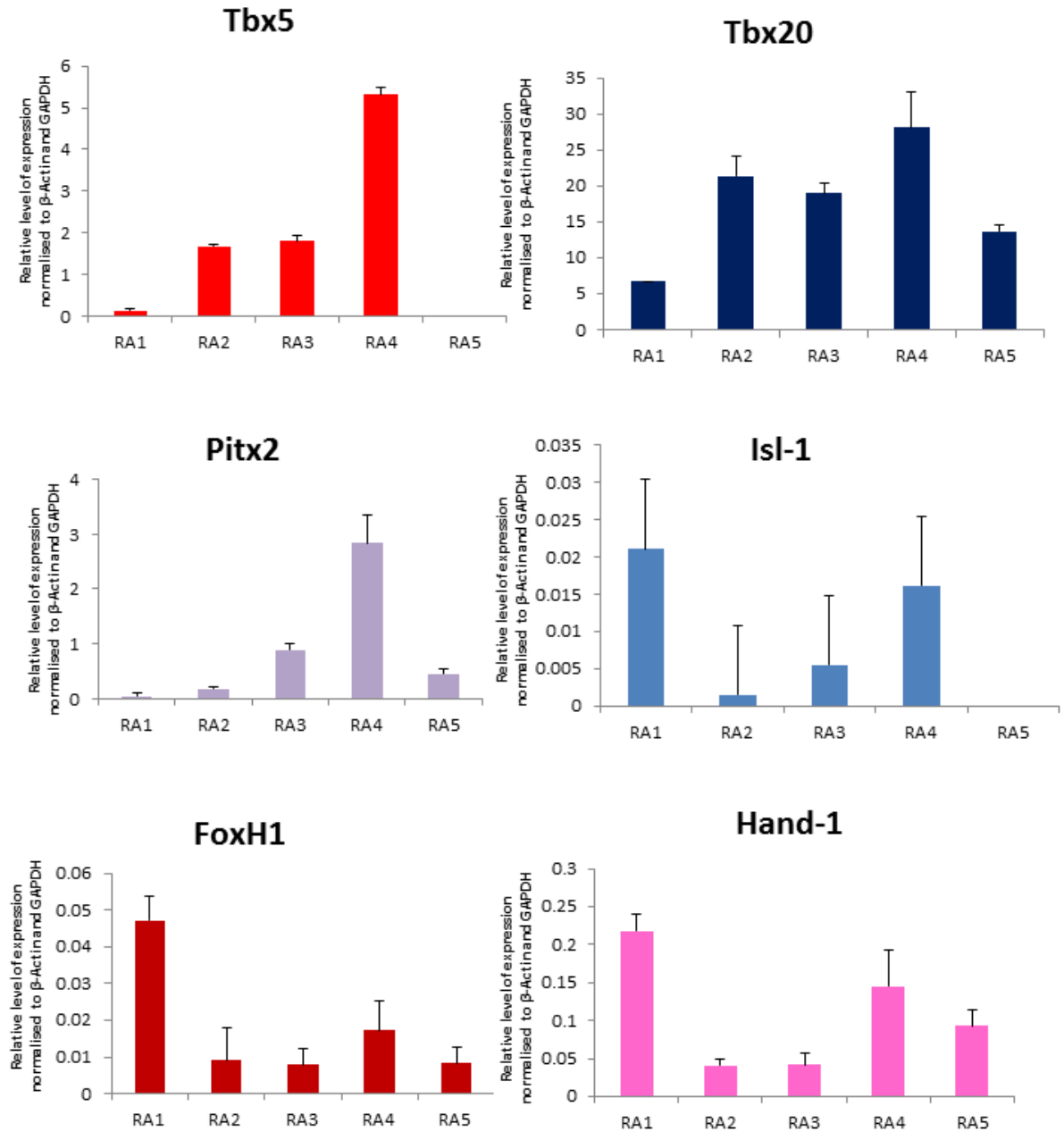
Table 3.3 Ct values of the LA samples for all the genes of the study (mean±SD). No values indicate no expression of the transcripts.

SAMPLES	LA1	LA2	LA3	LA4	LA5
c-kit	32.55 ±0.94	25.78 ±0.04	30.53 ±0.4	29.29 ±0.31	29.99 ±0.06
Tbx5	27.21 ±0.13	27.6 ±0.15	27.34 ±0.15	26.3 ±0.22	26.87 ±0.12
Tbx20	29.05 ±0.57	24.05 ±0.08	24.8 ±0.13	25.11 ±0.32	24.71 ±0.05
Pitx2	29.57 ±0.33	26.99 ±0.08	30.83 ±0.87	29.7 ±0.29	29.56 ±0.21
Isl-1	35.79 ±0.81	33.6 ±0.02			34.3 ±0.38
FoxH1	38.33	32.44 ±0.75	36.47 ±0.22	36.46	36.31 ±0.46
Hand-1	35.76 ±1.92	28.78 ±0.6	37.12 ±0.86	37.28 ±0.81	36.15 ±1.52
Hand-2	26.46 ±0.32	28.54 ±0.39	26.1 ±0.3	25.94 ±0.06	25.96 ±0.36
HRT-1	34.93 ±0.42	31.49 ±0.74	32.62 ±0.33	33.97 ±0.04	33.67 ±0.29
HRT-2	24.54 ±0.45	24.49 ±0.67	28.29 ±0.1	29.45 ±1.27	28.52 ±0.4
FGF-8	37.67	35.85 ±1.87	36.83 ±1.99	36.02	
FGF-10					
β-Actin	22.46 ±0.35	16.02 ±0.08	18.08 ±0.12	17.99 ±0.48	18.18 ±0.08
GAPDH	19.79 ±0.06	17.03 ±0.6	17.13 ±0.01	17.69 ±0.45	17.45 ±0.05

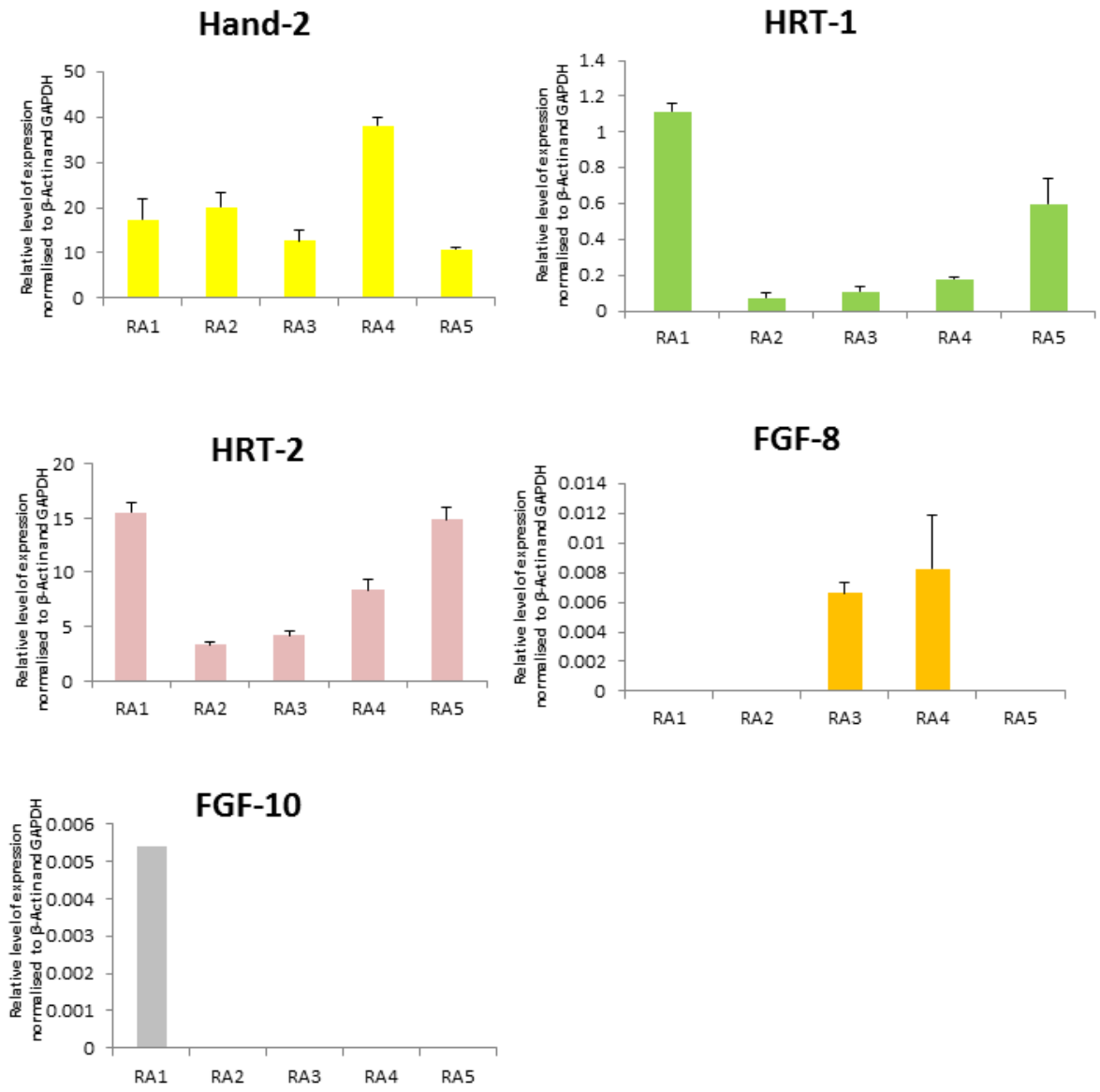
3.6.2 Right atrium.

qRT-PCR analysis of the RA samples revealed relative expression of >1 for Tbx20, Hand-2, and HRT-2 in all 5 c-kit^{pos} eCSCs samples (Figures 3.11 and 3.12). 3 c-kit^{pos} eCSCs samples (RA2, 3 and 4) showed a relative expression level of >1 for Tbx5, however the other 2 samples (RA1 and 5), showed negligible or no relative expression (Figure 3.11). Similarly for Pitx2, RA4 had a relative expression of ~3; however the other c-kit^{pos} eCSCs RA samples

were all ≤1 (Figure 3.11). Hand-1 relative expression was <0.25 for all 5 c-kit^{pos} eCSCs samples. HRT-1 showed relative expression >1 for RA1, ~0.6 for RA5, yet <0.2 for RA2, 3 and 4 (Figure 3.12). Similar to LA, Isl-1, Foxh-1, FGF-8 and FGF-10 all had no or negligible relative expression levels, being <0.05, in all 5 c-kit^{pos} eCSCs samples (Figures 3.11 and 3.12). These relative expression levels corresponded with the Ct values (see Table 3.4).



Figures 3.11 qRT-PCR analysis of the c-kit^{pos} eCSCs isolated from RA. Bars represent mRNA relative expression levels; error bars represent the standard deviation of the mean. All samples were carried out in triplicate, with p=NS.



Figures 3.12 qRT-PCR analysis of the c-kit^{pos} eCSCs isolated from RA. Bars represent mRNA relative expression levels; error bars represent the standard deviation of the mean. All samples were carried out in triplicate, with p=NS.

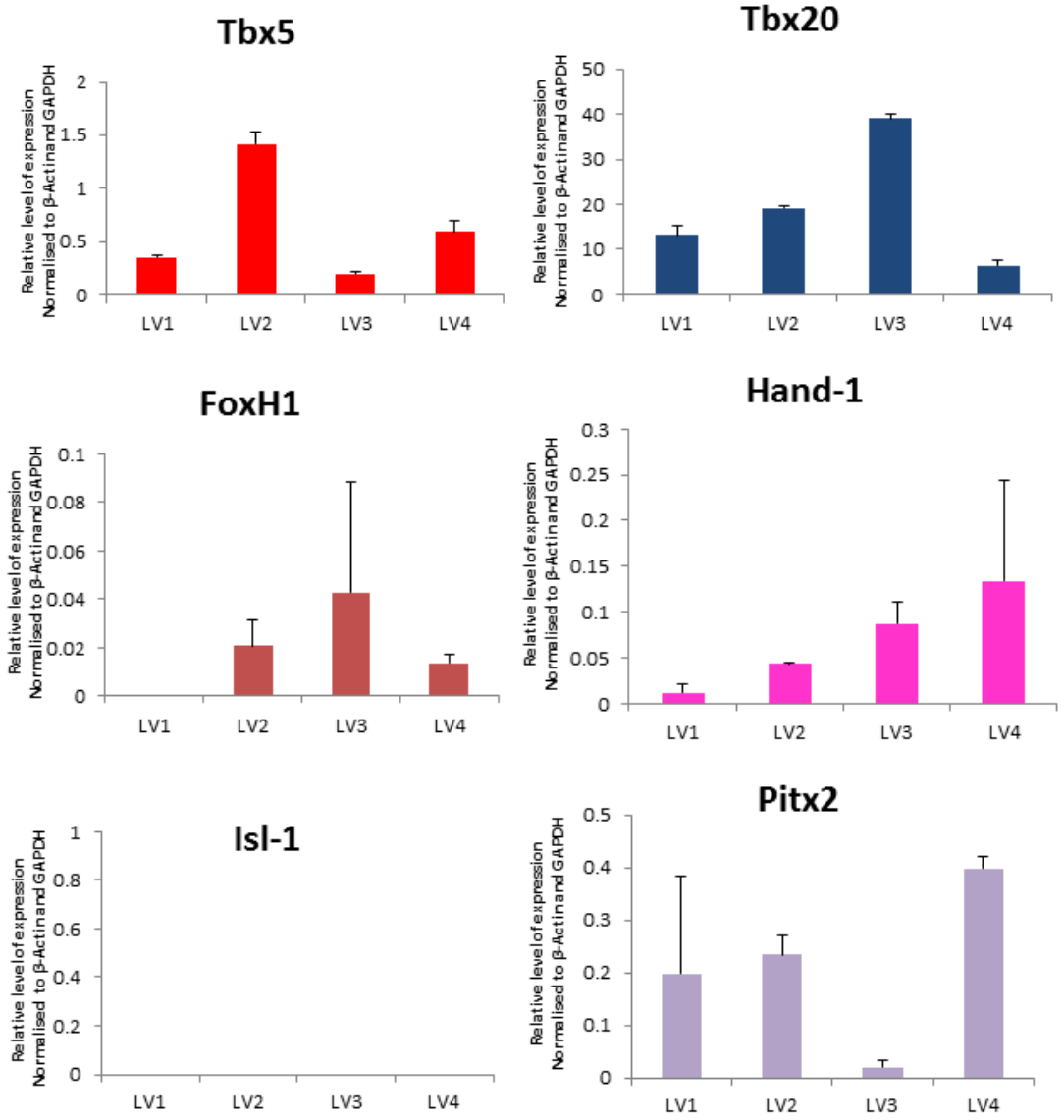
Table 3.4 Ct values of the RA samples for all the genes of the study (mean±SD).No values indicate no expression of the transcripts.

SAMPLES	RA1	RA2	RA3	RA4	RA5
c-kit	25.25 ±0.55	31.52 ±0.54	30.34 ±0.19	32.12 ±0.66	26.55 ±0.42
Tbx5	33.07 ±0.31	27.07 ±0.05	28.08 ±0.11	27.18 ±0.05	35.89 ±0.04
Tbx20	27.15 ±0.02	23.2 ±0.18	24.5 ±0.11	24.66 ±0.28	25 ±0.08
Pitx2	34.98 ±1.42	30.42 ±0.19	29.17 ±0.19	28.15 ±0.27	30.2 ±0.3
Isl-1	35.92	37.84 ±0.58	36.89	35.99	
FoxH1	34.7 ±0.22	35.46 ±1.41	36.56 ±1.12	36.03 ±0.8	36.46 ±0.98
Hand-1	32.36 ±0.15	32.72 ±0.31	33.86 ±0.58	32.71 ±0.54	32.62 ±0.35
Hand-2	25.75 ±0.44	23.31 ±0.24	25.12 ±0.27	24.18 ±0.08	25.38 ±0.09
HRT-1	29.88 ±0.66	31.85 ±0.48	32.36 ±0.33	32.35 ±0.07	29.79 ±0.37
HRT-2	25.88 ±0.09	26.04 ±0.09	26.82 ±0.16	26.5 ±0.18	24.88 ±0.12
FGF-8			36.61 ±0.17	37.12 ±0.7	
FGF-10	37.98				
β-Actin	18.14 ±0.35	15.95 ±0.1	17.08 ±0.09	17.82 ±0.16	17.08 ±0.33
GAPDH	18.05 ±0.3	16.33 ±0.35	17.72 ±0.05	17.34 ±0.24	17.09 ±0.26

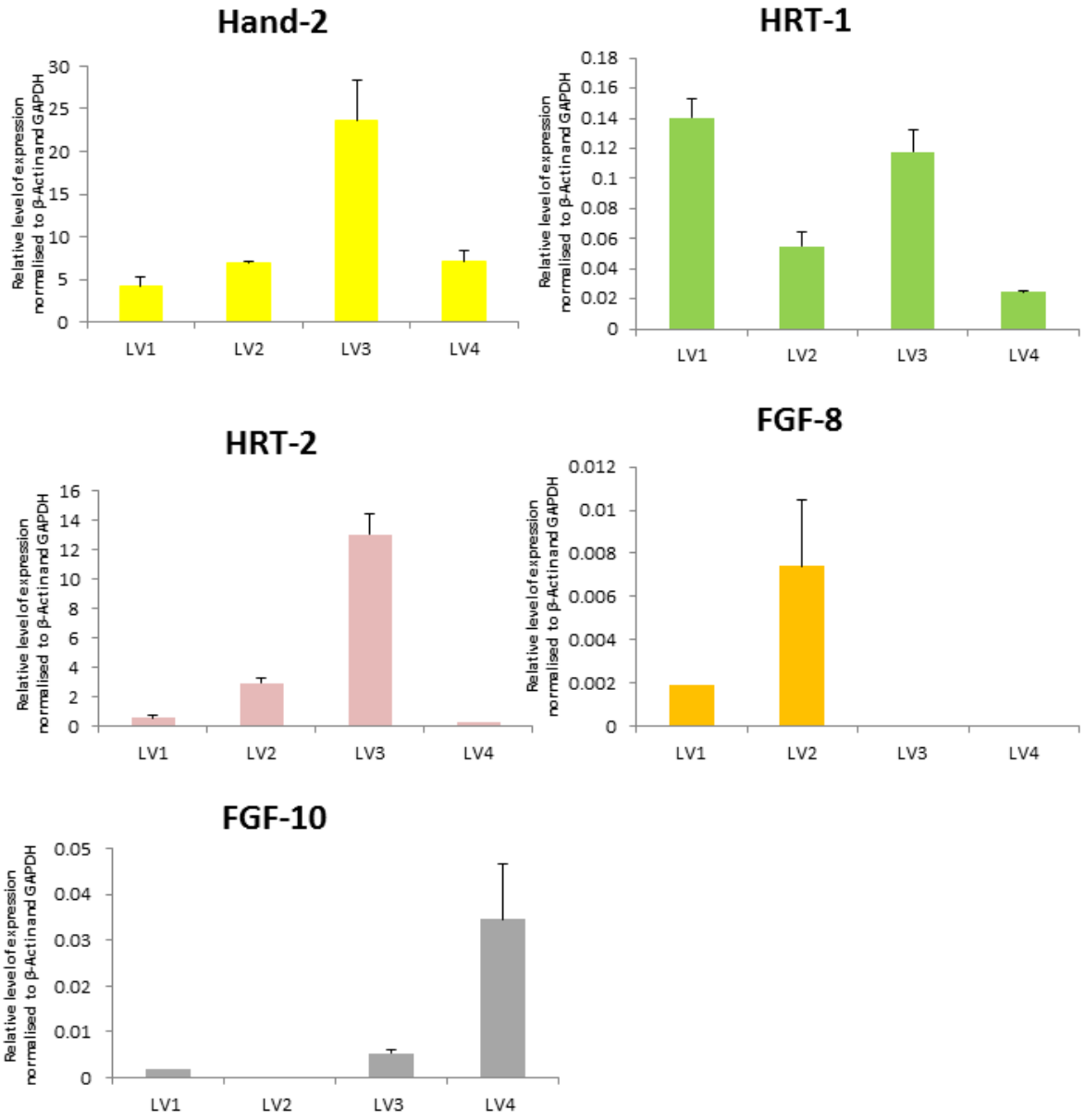
3.6.3 Left ventricle.

qRT-PCR analysis of the LV samples revealed relative expression of >1 for Tbx20, Hand-2, and HRT-2 in all 4 c-kit^{pos} eCSCs samples (Figures 3.13 and 3.14). 1 c-kit^{pos} eCSCs LV sample (LV2) showed a relative expression level of >1 for Tbx5, however the other 3 samples (LV1, 3 and 4), showed a lower relative expression of <0.7 (Figure 3.13). Pitx2 had a low expression level of <0.4 across all 4 c-kit^{pos} eCSCs LV samples (Figure 3.13). Hand-1

relative expression was <0.25 for all 4 c-kit^{pos} eCSCs LV samples. HRT-1 relative expression was <0.15 for all 4 c-kit^{pos} eCSCs LV samples (Figure 3.14). Similar to LA and RA, Isl-1, Foxh-1, FGF-8 and FGF-10 all had no or negligible relative expression levels, being <0.08, in all 4 c-kit^{pos} eCSCs LV samples (Figures 3.13 and 3.14). These relative expression levels corresponded with the Ct values (see Table 3.5).



Figures 3.13 qRT-PCR analysis of the c-kit^{pos} eCSCs isolated from LV. Bars represents mRNA relative expression levels; error bars represent the standard deviation of the mean. All samples were carried out in triplicate, with p=NS.

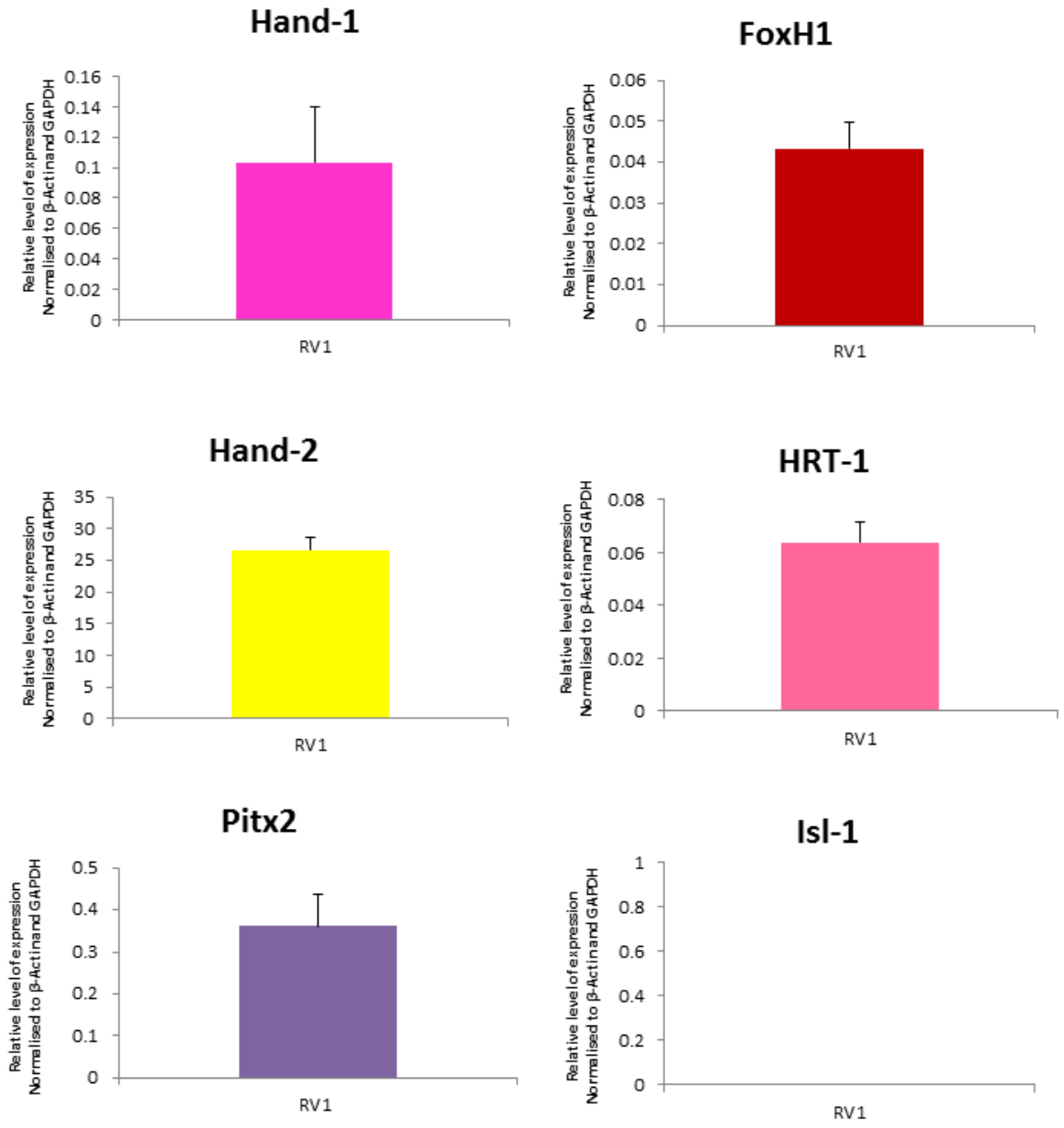


Figures 3.14 qRT-PCR analysis of the c-kit^{pos} eCSCs isolated from LV. Bars represents mRNA relative expression levels; error bars represent the standard deviation of the mean. All samples were carried out in triplicate, with p =NS.

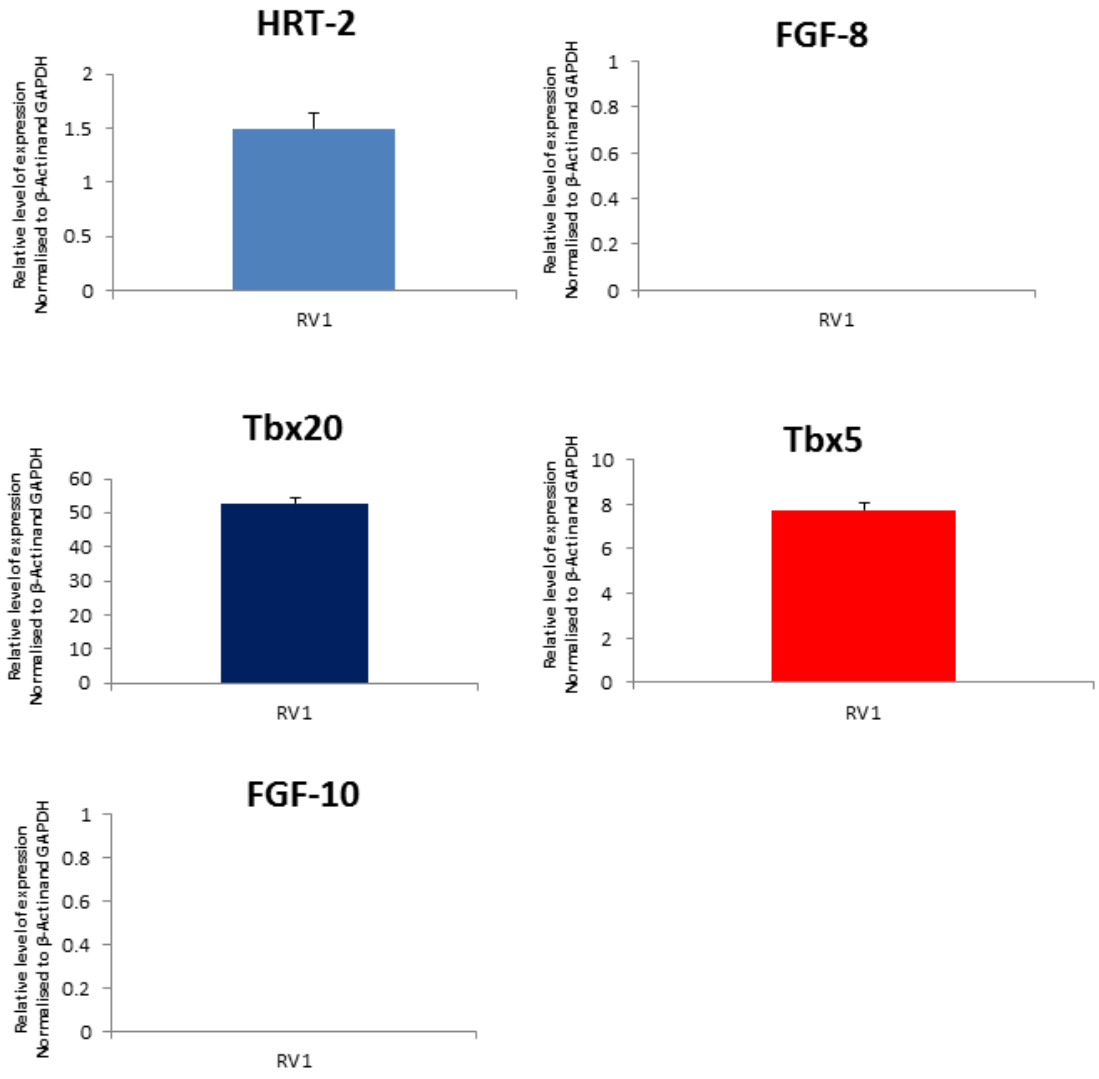
3.6.4 Right ventricle

After harvesting and isolating 5 RV samples, only the RV1 sample successfully yielded cells which could be grown in culture and fully analysed. This sample revealed relative expression of >1 for Tbx5, Tbx20, Hand-2, and HRT-2 (Figures 3.15 and 3.16). Pitx2 had a low expression level of <0.4 (Figure 3.15). Hand-1 relative expression was <0.15 (Figure 3.15).

HRT-1 relative expression was negligible being <0.07 (Figure 3.15). Similar to LA, RA and LV, Isl-1, Foxh-1, FGF-8 and FGF-10 all had no or negligible relative expression levels, being <0.05, in all 4 c-kit^{pos} eCSCs LV samples (Figure 3.15 and 3.16). These relative expression levels corresponded with the Ct values (see Table 3.5).



Figures 3.15 qRT-PCR analysis of the c-kit^{pos} eCSCs isolated from RV. Bars represent mRNA relative expression levels; error bars represent the standard deviation of the mean. All samples were carried out in triplicate with p=NS.



Figures 3.16 qRT-PCR analysis of the c-kit^{pos} eCSCs isolated from RV. Bars represent mRNA relative expression levels; error bars represent the standard deviation of the mean. All samples were carried out in triplicate with p=NS.

Table 3.5 Ct values of the LV and the RV samples for all the genes of the study (mean±SD).No values indicate no expression of the transcripts.

SAMPLES	LV1	LV2	LV3	LV4	RV1
c-kit	25.58 ±0.19	32.37 ±0.59	36.41 ±1.03	31.15 ±0.04	34.37 ±0.95
Tbx5	30.54 ±0.07	29.74 ±0.12	32.01 ±0.23	31.14 ±0.26	26.64 ±0.07
Tbx20	25.08 ±0.27	25.77 ±0.04	23.97 ±0.05	27.55 ±0.28	23.72 ±0.05
Pitx2	31.88 ±1.37	32.49 ±0.23	35.85 ±1.26	31.74 ±0.09	31.32 ±0.32
Isl-1	36.45				36
FoxH1		36.27 ±0.81	34.99 ±1.75	36.91 ±0.45	34.53 ±0.22
Hand-1	36.11 ±1.54	35.03 ±0.06	33.29 ±0.43	33.78 ±1.36	33.26 ±0.51
Hand-2	26.85 ±0.42	27.31 ±0.03	24.75 ±0.32	27.38 ±0.26	24.76 ±0.12
HRT-1	31.97 ±0.14	34.7 ±0.28	32.8 ±0.18	35.98 ±0.05	33.94 ±0.19
HRT-2	29.86 ±0.48	28.64 ±0.15	25.65 ±0.16	32.21 ±0.28	29.14 ±0.15
FGF-8	38.51	37.8 ±0.65			
FGF-10	38.49		37.5 ±0.16	35.51 ±0.55	
β-Actin	17.08 ±0.11	18.37 ±0.21	17.64 ±0.17	18.44 ±0.66	17.85 ±0.17
GAPDH	18.04 ±0.32	17.99 ±0.09	17.48 ±0.16	18.38 ±0.18	17.42 ±0.19

3.7 Cumulative qRT-PCR analysis between all 4 cardiac chambers.

In order to compare the expression of our genes of interest across the four cardiac chambers, the individual sample data described above were cumulated (Figures. 3.17 and 3.18). qRT-PCR analyses across LA, RA and LV cardiac chambers revealed a significant ($p<0.05$) increased relative expression of Tbx5 in the LA, compared to LV (Figure 3.17). There were no significant differences in relative expression between chambers for the other transcripts

(Figures 3.17 and 3.18). Data reveals that TBX20, TBX5, HAND2 and HRT2 show elevated relative expression levels, whereas the relative level of expression of all others is low. When compared to basal Ct values of these genes TBX20, TBX5, HAND2 and HRT2 with all the other genes, it is obvious that they have the lowest Ct values which confirm their higher expression during qPCR (see Table 3.6).

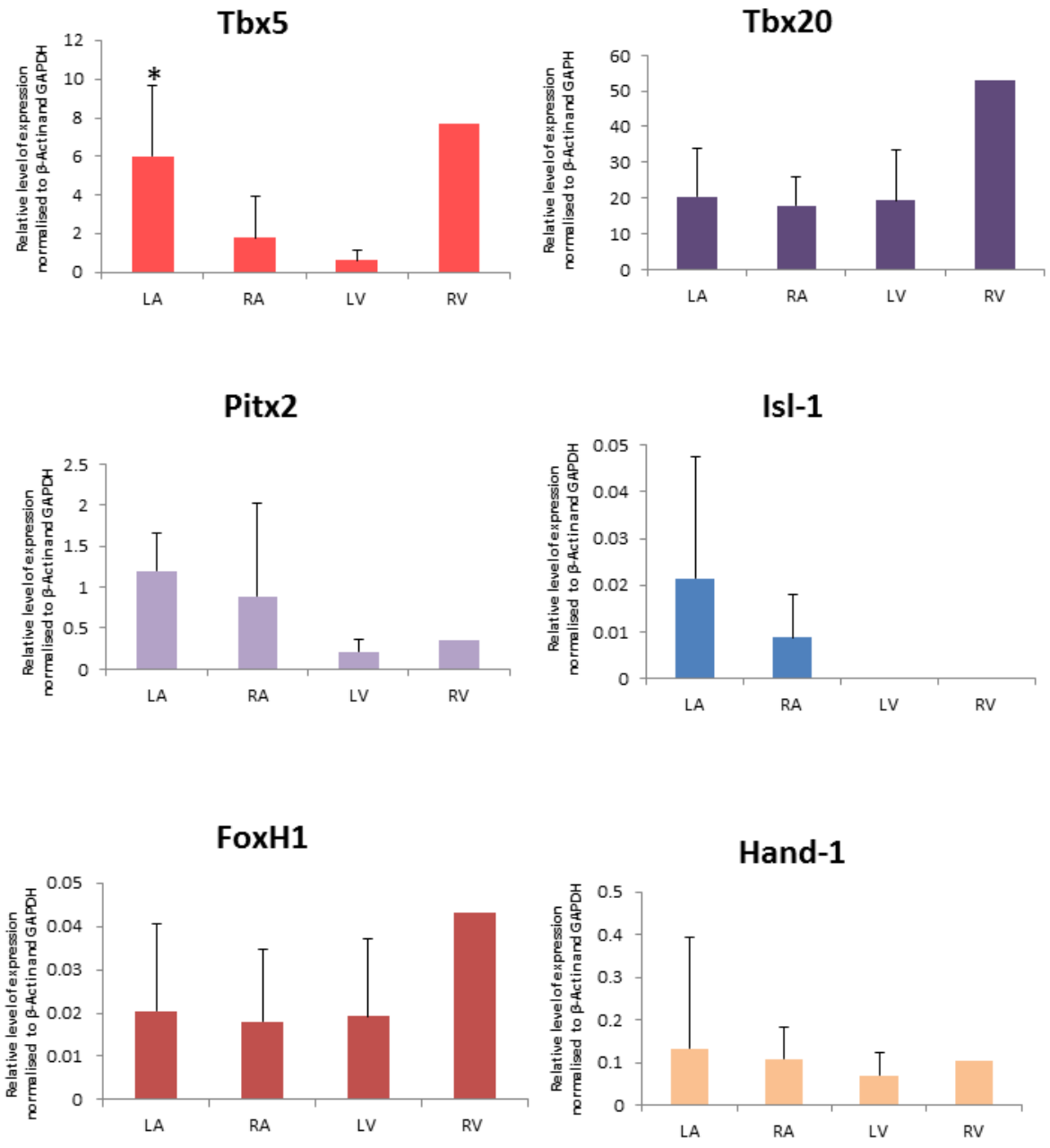


Figure 3.17 cumulative qRT-PCR analyses for all 4 cardiac chambers. Bars represent mRNA relative expression levels; error bars represent the standard deviation of the mean. n=5, 5, 4 and 1 for LA, RA, LV and RV, respectively.*$p<0.05$ vs. LV.

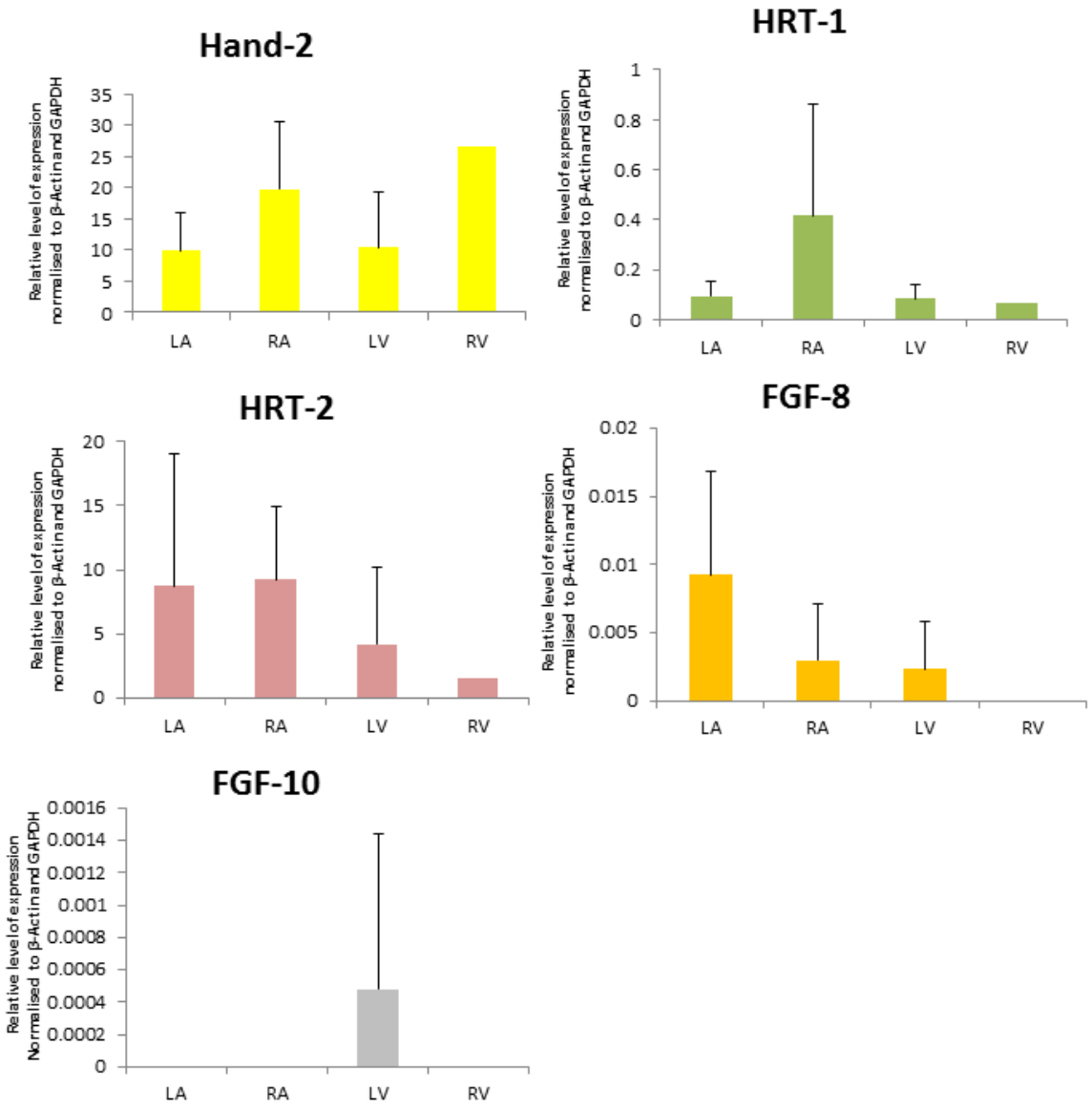


Figure 3.18 cumulative qRT-PCR analyses for all 4 cardiac chambers. Bars represent mRNA relative expression levels; error bars represent the standard deviation of the mean. n=5, 5, 4 and 1 for LA, RA, LV and RV, respectively with p=NS.

Table 3.6. Ct values of the most expressed genes TBX5, TBX20, HRT2 and HAND2 from all the cardiac chambers.

LA	**Tbx5**	27.21±0.13	27.6 ±0.15	27.34 ±0.15	26.3 ±0.22	26.87 ±0.12
	Tbx20	29.05 ±0.57	24.05 ±0.08	24.8 ±0.13	25.11±0.32	24.71 ±0.05
	Hand-2	26.46 ±0.32	28.54 ±0.39	26.1 ±0.3	25.94±0.06	25.96 ±0.36
	HRT-2	24.54 ±0.45	24.49 ±0.67	28.29 ±0.1	29.45±1.27	28.52 ±0.4
RA	**Tbx5**	33.07 ±0.31	27.07 ±0.05	28.08 ±0.11	27.18±0.05	35.89 ±0.04
	Tbx20	27.15 ±0.02	23.2 ±0.18	24.5 ±0.11	24.66±0.28	25 ±0.08
	Hand-2	25.75 ±0.44	23.31 ±0.24	25.12 ±0.27	24.18±0.08	25.38 ±0.09
	HRT-2	25.88 ±0.09	26.04 ±0.09	26.82 ±0.16	26.5 ±0.18	24.88 ±0.12
LV/RV	**Tbx5**	30.54 ±0.07	29.74 ±0.12	32.01 ±0.23	31.14±0.26	26.64 ±0.07
	Tbx20	25.08 ±0.27	25.77 ±0.04	23.97 ±0.05	27.55±0.28	23.72 ±0.05
	Hand-2	26.85 ±0.42	27.31 ±0.03	24.75 ±0.32	27.38±0.26	24.76 ±0.12
	HRT-2	29.86 ±0.48	28.64 ±0.15	25.65 ±0.16	32.21±0.28	29.14 ±0.15

3.8 Statistical analysis results of the data.

Due to the vast variability of the samples from different chambers and from different patients with a number of co-factors and as the number of genes was analysed from each chamber a complex statistical analysis was performed in order to avoid errors. Firstly the data has been analysed for multiple testing and this proved to be that the data had no linearity.Secondarly using the Shapiro-Wilk test to look for normalisation of the data we calculated the significance value of each gene which needed to be greater than 0.05.We find out that most of the genes were normaly distributed except two the Hand -1 and HRT-1 gene (see table 3.7 below). Finally when we look for significant differences using the ANOVA with Tukey post

hoc tests and setting the significance at p<0.05 we identified only in the TBX-5 gene (as it is been shown in figure 3.17) after cumulative qRT-PCR analyses for all 4 cardiac chambers,there was significance difference between the RV,RA,LA versus the LV chamber with a p value of less than 0.05 (p<0.05) .In all the other analysis of the genes during PCR the data didn't showed any statistical significance between the genes from all the samples taken from the 4 cardiac chambers.

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Tbx5	.200	7	.200*	.939	7	.631
Pitx2	.146	7	.200*	.968	7	.886
Tbx20	.164	7	.200*	.958	7	.803
Isl-1	.261	7	.161	.838	7	.096
FoxH1	.324	7	.026	.822	7	.067
Hand-1	.376	7	.003	.655	7	.001
Hand-2	.250	7	.200*	.930	7	.554
HRT-1	.417	7	.001	.561	7	.000
HRT-2	.303	7	.051	.813	7	.055
FGF-8	.267	7	.140	.903	7	.352

Table 3.7. Tests of normality of the distribution of the data.Please note that the data in green are nortmally distributed with a value of greater than 0.05 and the data in red are not with values more than 0.05.

Key Points

- The number of c-kit^{pos} eCSCs and number of c-kit^{pos} eCSCs per gram of tissue isolated from all the 4 human cardiac chambers seems to be consistent, with minor variances (not statistical significant) accordingly to the weight and size of the samples.
- Transmitted light microscopic observations of c-kit^{pos} eCSCs in culture from all the four cardiac chambers revealed that most of the isolated c-kit^{pos} cells from the human biopsies were generally small and rounded, consistent with a stable c-kit^{pos} eCSCs phenotype despite the chamber of origin.
- eCSCs c-kit^{pos} can grow in cell culture in hypoxic conditions (2-5% O_2) between 7±5.5 days where they reach full confluency.
- All samples showed expression of the stem cell factor receptor c-kit but at varying levels. LA samples were the most consistent for c-kit expression, with eCSCs expressing relatively similar amounts of this gene.
- Transcriptional analysis using qRT-PCR revealed that all the 4 cardiac chambers host c-kit^{pos} eCSCs which express genes from the primary and secondary heart field with variances.Cells expressing much more consistenly genes from the secondary heart field like TBX-5,TBX-20,HAND-2,HRT-2 and minor expression of the PITX-2 gene again from the secondary heart field.
- Minimal or no expression was revealed for Hrt-1, Isl-1, Foxh1, Fgf-8 and Fgf-10 in all 4 chambers. This has been confirmed with the higher or absent Ct values of these genes.

4. DISCUSSION

The key objectives of this study were primarily to isolate c-kit^{pos} eCSCs from all the four cardiac chambers and compare their numbers isolated between chambers. A secondary objective was to investigate, using qRT-PCR whether these c-kit^{pos} eCSCs isolated from the four chambers of the human myocardium, could express uniformly all the genes of the primary and secondary heart fields.

It was hypothesized that c-kit^{pos} eCSCs would be obtained with greater ease from the right and left atria, and fewer number of eCSCs would be obtained from the left ventricle and right ventricle respectively. It was further hypothesized that the eCSCs when isolated from the four different cardiac chambers and analyzed by qRT-PCR for their transcriptional profile of genes specific to the primary and secondary heart field and chamber of origin, they could have the same genetic profile and could be ‘’generic’’ and not ‘’chamber specific’’.

The major finding of this study was that the eCSCs isolated from the 4 chambers of the human myocardium are c-kit^{pos} and they reside in all the cardiac chambers RA, LA, LV and RV with no significant differences between these chambers. When qRT-PCR used to investigate their transcriptional profile, revealed that they express genes from both the primary and secondary heart fields, suggesting they are not *chamber specific* but *generic* regardless of their location of origin.

4.1 The number of eCSCs isolated varied across the cardiac chambers.

All the samples harvested from the 4 cardiac chambers showed variability in their weight and number of eCSCs per gram of tissue after enzymatic isolation and MACS procedure.This was an inevitable variable which was introduced by the discretion of the surgeon who was harvesting the sample and from the operator who was isolating the eCSCs in the laboratory. Sometimes despite asking for the biggest sample in order to achieve more cells at the end of

the enzymatic digestion [an optimal size between 5mgr-1gr as has been recorded by our group (106) and by others from previous studies (105,128,136,153), due to safety to avoid bleeding a small size samples were given for analysis by the surgeon who was performing the operation. So when the samples were arriving in the laboratory in order to proceed with the enzymatic digestion and finally MACS procedure, a number of times the expected amounts of cells that were isolated in comparison to the weight of the samples were not satisfactory. Because of these limitations and in order to achieve consistent and meaningful results, more than 30 samples were processed and eCSCs processed to isolation by the same operator to ensure a level of consistency and optimization of the learning curve. The *trial and error* period, was enough to achieve sufficient practice of the procedures of isolation and optimize the protocols in order to isolate and process safely the samples. From all these 30 cardiac biopsies only 15 samples were analyzed to completion from all the 4 human cardiac chambers and the results have been recorded and analyzed for this thesis.

Some samples were small in weight but had a good amount of cells isolated per gram of tissue (see for example in Table 3.1 that LV1 sample with a weight of 0.025gr and a number of c-kit^{pos} cells per gram of tissue of 120000; similarly LV3 sample with weight of 0.068gr and number of c-kit^{pos} cells per gram of tissue of 52941 and RV1 sample with 0.033gr and number of c-kit^{pos} cells per gram of tissue of 75000); Most of these samples were more muscular in comparison of the LA and RA samples as they have been harvested from the endocardial layers of the LV and RV during mitral or tricuspid valve repair/replacements respectively. Some had a higher weight but after enzymatic digestion of the tissue and MACS procedure no cells were isolated (data not shown as not cells isolated so the samples have been discarded and not been included in the data of the analysis of this thesis).

Finally some were big in size with a good yield of cells per gram of tissue after isolation as for example the LV2 sample with 0.243gr of sample weight and 39506 c-kit^{pos} cells per gram

of tissue; in contrary with the RA1 sample who had 1gr of weight but only 6400 of c-kit^{pos} cells per gram of tissue. The difference in the variability of the weight could be attributed to the presence of epicardial fat and fibrous tissues depending of the area of harvesting; samples harvested from the RA appendix had more epicardial fat in comparison of the other samples from the other cardiac chambers. LA samples had more fibrous tissues in comparison of the LV or RV samples. The ventricular samples as they were harvested from the endocardial layers of the LV and RV chambers, they were more muscular with less fat but less volume in size. Based on our previous experience protocols of harvesting and processing human cardiac samples (106) and with the Smitts protocol publication (108) who tried to give an insight of the in vitro model of the cardiomyocyte progenitor cells and standardize their methods of differentiation in vitro, the above variability of the harvested samples and process during enzymatic isolation deemed to be acceptable according the methodology and aims of this study.

In addition there is no evidence yet of how the Cardio Pulmonary Bypass (CPB) can affect the samples and in consequence the number of the eCSCs in response to the CPB inflammation-induced process. All the samples from RA were harvested before initiation of Cardio-pulmonary Bypass and were 2-3cm in size as shown in Figure 2.1 in the methods section; the same techniques of harvesting were reported (128) in the SCIPIO trial. It was reported that harvesting myocardial samples from the right atrial tissues was easier, especially for the RA appendix as it provided the safest access during venous cannulation of the RA/IVC, prior to initiation of cardiopulmonary bypass. The LA samples instead were harvested after the initiation of the CPB and cross clamp of the aorta with cooling the heart to 34 ^{o}C. As the LA is the most posterior chamber of the heart and its appendix is in an awkward anatomical position, with the potential to bleed all these five LA samples harvested for this study were taken from the inside of the LA during mitral valve procedures

when access is viable from the Waterstones groove (154). The LA samples harvested in the human study by Arsalan (105) used similar methodology.

Similarly the LV and RV samples were obtained after initiation of the CPB and cross clamp the aorta with temperature of 34 ºC in order to have a stable and safe area of harvested tissue. Three out of 4 samples were harvested from the inside of the LV in the area of the muscular septum below the aortic valve during aortic valve procedures and/or aortic valve root procedures. Only one sample which was the smallest one in size was harvested from the outside of the deep epicardial layer of the LV and no samples were harvested from the LV apex in this study as other studies recorded in the literature (106,107). From the five samples that were harvested from the RV throughout the period of the study only one was fully processed and analyzed as the other RV samples after enzymatic isolation yielded no cells after the MACS procedure. Due to the difficulty of harvesting samples from the ventricular epicardial layer of the RV wall with risk of bleeding -especially with patients with pulmonary hypertension- surgeons were reluctant to harvest these samples from the outside epicardial RV layer.

To our knowledge except of our study (in humans) there are only three other studies in which human samples were tempted and harvested as surgical biopsies from the RV. One study by Itzhaki-Alfia (137) obtained RV samples in order to identify c-kit^{pos} cells and correlate their number to the original pathology in living patients. Another study by Castaldo (138) the RV samples were obtained from normal adult hearts derived from patients who had died for reasons other than cardiovascular disease in order to identify positivity for CD117 and to assess whether it localized in the sub-epicardium and whether their activation was associated with Laminin-1 and a6 Integrin expression**.** Recently a study performed by Matuszczak (155) has analyzed the distribution of the c-kit^{pos} eCSCs from myocardial tissues biopsied from all the cardiac chambers including the RV from explanted hearts obtained from recipients during

cardiac transplantation. It is been found after FACS analysis that there is equal distribution of eCSCs c-kit^{pos} thorough the heart including the RV chamber.

It is of interest to notify that in this study all these samples were from adult patients who were suffering from ischemic cardiac disease (3VD, 4VD) amenable to coronary artery bypass grafting (CABG) or a valve pathology (mitral aortic or tricuspid stenosis or regurgitation) amenable to surgical repair or replacement of these valves. As we can see on the Table 3.1 the mean age of all the patients were about 75 years old with prevalence of males in comparison to females and with not any significant difference of age between patients from the different harvested samples from different cardiac chambers: (75.2±5 for LA samples, 76±14 for RA samples and 73.5±8 for LV samples with 74 on the RA sample patient group).This can be in accordance with the recent report of Ellison (156) where the age is not a limiting factor of the presence or absence of the eCSCs, however in accordance with the study of Tallini (157) fetal and young mammalian hearts they have a better number of eCSCs as Matuszczak confirmed from human pediatric cardiac biopsies who underwent transplantation (155). The number of cells could be important when the regeneration process is triggered but the quality of the cells and their response to the physiological cardiac adaptation to the stress is definetly crucial. The combination of cardiomyocyte hypertrophy and hyperplasia (cardiomyocytes and capillaries) plays an important role on the viability of these cells during the process of re-adaptation after exercise stress (158). On the other hand all these patients were on simvastatin treatment and several studies suggested that (3-hydroxy-3-methylglutaryl coenzyme A) HMG-CoA reductase inhibitors benefit the patient with an ischemic and non-ischemic cardiomyopathy (159). This mechanism has been poorly understood if the statins protect against myocyte hypertrophy and cell death together with the stimulation of new myocytes from the eCSCs pool. As mentioned earlier despite been adult patients with cardiac pathologies the number of the eCSCs was not different between the

patients but the quality of these cells who could depend on the endogenous growth factors/ statins could give to the cells a better response and adaptation to stress despite the less number in comparison of the pediatric samples as has been recorded (155).

4.2. Morphologically all the eCSCs showed similar pattern of growth in culture.

Transmitted light microscopic observations of c-kit^{pos} eCSCs in culture revealed that most of the isolated c-kit^{pos} cells were generally small and rounded, consistent with a c-kit^{pos} eCSC phenotype (106). The eCSCs were cultured in hypoxic conditions for 14±7 days and after reaching full confluency they were tripsinized and stored to -80 ºC for further RNA analysis. This techniques of culture has been adopted from our previous work in our laboratory that have been optimised based on the same protocols that other researchers have reported (111), (108). All cells, isolated from each chamber, had a similar morphological aspect in culture. The phenotypes of the sample cells were consistent with the c-kit^{pos} eCSCs, reported in our previous studies (106). Interestingly, the single RV sample showed very rapid progress in culture. The c-kit^{pos} eCSCs were not as small and rounded as other samples, appearing more spindle-shaped and reaching confluency in 8 days.

These morphological and growth differences may be due to a number of factors that affect cell growth in culture, including the age of patients, comorbidities, gender and underlying pathology (137). Pouly (160) recorded that the location of the harvested samples can be a factor on the type of eCSCs and Parker (161) argued that the time in culture can underpin alterations in the phenotype of cells. The age and pathology of the patients could play a role in the quantity and quality of the cells but although the cloning efficiency was inversely age-related, single-cell derived eCSC clones obtained from young and old hearts were indistinguishable by their gene expression and differentiation potential, strongly suggesting that eCSC aging is a stochastic process as has been recorded from a recent study done by

Ellison (156). Interestingly, while failing hearts have increased numbers of c-kit^{pos} progenitors, age has been correlated with a decrease in the number of functional progenitors. Disease and gender affect the distribution of cells; females tend to have a greater number of c-kit^{pos} cardiac progenitors (137). Medical history, including the use of β-blockers, statins, smoking, atrial fibrillation, and prior myocardial infarction, can also influence the frequency of c-kit^{pos} progenitors in the heart (162). Failing hearts have an approximate fourfold increase in c-kit^{pos} progenitor cells, and it has been shown that approximately 80% of these cells co-express CD45 which are mast cells (163). All patients recruited to this project were sick patients who underwent open heart surgery and suffered of ischemic cardiomyopathy, aortic or mitral valve disease and tricuspid valve pathology. As previously mentioned, techniques and patient related characteristics may play a significant role in the growth and progress of the cells in culture. However, in this study the morphology and average number of cultured eCSCs was generally consistent.

4.3 m-RNA purity & c-DNA synthesis.

In our study samples harvested from all the chambers showed a normal ratio level of the 260/280 which showed good level of DNA purity of the samples with the mean value for the LA chambers of 2.05±0.02, for the RA chambers of 2.08±0.01, for the LV of 2.06±0.01 and for the unique RV of 2.08 respectively. This confirms good operator technique and purity of the samples. However there were samples as LA1, LA4, RA2, RA4, LV2 that had low 260/230 ratio and this could suggest presence of contaminants or low RNA content as the samples were small in size. As mentioned earlier in previous paragraphs, the learning curve of these specialist and delicate techniques of the operator might have a contribution on these m-RNA purity results in additional of the small samples sizes who some of them had low number of c-kit^{pos} eCSCs; (see Table 3.1 and Table 3.2 in the Results Section). It is obvious that LA1 sample has a low weight 0.123gr with an RNA quantity of 40.03ng/μl, where the

sample LA4 had been low weight 0.086gr with an RNA quantity of 520.7ng/μl but with a low ratio of 260/230 suggesting genomic contamination and operator technique. Similarly with RA2, RA4, LV2 despite a weight of 0.277g, 0.227g, and 0.243g respectively the 260/230 ratio of 1.03,1.31 and 0.32 respectively, suggesting poor operator technique.

4.4 qRT-PCR and c-kit expression.

As has been recorded in the results section c-kit^{pos} eCSCs were distributed throughout the 4 cardiac chambers between LA, RA, LV, and RV. All of the samples showed expression of c-kit but at varying levels LA, RA, and LV samples were found to express c-kit with a mean relative expression level of (1.52±1.58), (6.04±10.48), and (2.56±4.55) respectively. LA samples were the most consistent for c-kit expression, with eCSCs expressing relatively similar amounts across samples. The RA samples showed variability in their ckit^{pos} eCSCs, and three of the four LV samples showed good amount of c-kit^{pos} expression. The sole RV sample expressed the lowest level of c-kit (0.05).When examining closely the number of c-kit^{pos} eCSCs distributed at the 4 cardiac chambers (see Table 3.1 at the results section) there were differences of c-kit^{pos} accordingly to the different anatomical locations with distribution in the LA with mean±SD of 34601±11720, for the RA 15076±11470 and for the LV 49945±42097 respectively. The sole sample of the RV had 75000 cells per gram of tissue. These numbers are in accordance with previous studies of our group were the amount of cells were about 25000 to 50000 cells per gram of tissue using the same techniques of enzymatic isolation (106).

Regarding the variability of the c-kit^{pos} eCSCs between chambers this study agreed with the studies of Arsalan (105) regarding the superiority of the LA to contain a higher amount of eCSCs c-kit^{pos} cells per gr of tissue in comparison with the other cardiac chambers. Arsalan used the same harvesting techniques and similar enzymatic isolation of the eCSCs however

he analyzed the c-kit^{pos} expression using FACS analysis not qRT-PCR. The presence or not of the c-kit^{pos} cells can be confirmed or excluded with either analytical technique but because the purpose of our study was to look for c-kit^{pos} gene expression of the eCSCs of the primary and secondary heart fields, the qRT–PCR was primarily used in order to have meaningful and accurate results for each gene expression. In addition Arsalan has not studied the RV but reached conclusions with RA, LA and LV samples only.

Recent studies (155) analyzed the distribution of the c-kit^{pos} eCSCs from the myocardial tissues and in cell cultures derived from explanted hearts obtained from recipients during transplantation. It has been found equal distribution of eCSCs c-kit^{pos} throughout the myocardial chambers and the RV, while FACS analysis was performed. In our study we tempted to analyze RV samples also, despite the fact that only one sample -of the 5 RVs that were processed- has been fully and completely analyzed. The patient that this sample has been harvested was a 74 years old male who suffered of mitral valve stenosis and tricuspid valve regurgitation. He was generally fit and well with cycling of weekly basis 10km all his life. As he recently had more shortness of breath due to his progressing pathology he underwent an echocardiographic imaging that relieved the diagnosis. It could be speculated as reported in the literature (156) that despite of this patient age and because of his fitness and general health status his myocardial mass was well refurnished of eCSCs who were distributed in his RV in order to encourage reverse remodeling and regeneration despite his underlying valve pathology (158).

In the two studies where RV samples were harvested from living and cadaveric samples (137,138), the presence of eCSCs were c-kit^{pos} confirmed, but their number were minimal in comparison to the other cardiac chambers. Our results (albeit of only one sample) agreed with these findings as fewer eCSCs were isolated from the RV and of 5 samples harvested,

only one yielded able to be cultured cells, suggesting that eCSCs may be reduced in number in the RV.

The RA chambers in our study showed variability in the number of the c-kit^{pos} eCSCs but still agreed with previous studies (106,128,137), supporting the idea that the RA provides and accessible deposit of myocardial progenitors that can be easily mobilized in case of myocardial injury in order to maintain myocardial integrity. The right atrium, a region of low mechanical stress, with the highest CD117-positive cell and laminin content as supported by the study of Castaldo (138), may constitute the privileged site for the primitive cells, which survive during pathological conditions involving the ventricles and migrate across the sub-epicardial space or myocardium to the regions of damage and regeneration.

Another relevant observation that can affect the number of the c-kit^{pos} cells in the already sick hearts of the patients of our study is the inverse relationship between cardiac pathology and presence of c-kit^{pos} cells. Most of the patients were sick with abnormal LV ejection function and early stage heart failure. Previous reports also correlated increased numbers of CPCs in humans with significant aortic stenosis, acute and chronic ischemia, and other cardiomyopathies (163,164,165,166). Together, these findings support the notion that the endogenous reparative system is stimulated in the injured heart in different level and intensity (167). A recent report has suggested that c-kit^{pos} cells have increased proliferative capacity when cultured with extracellular matrix obtained from a failing heart (138). Thus, local signals that can stimulate eCSCs in response to injury could explain the mobilization of these progenitor cells from the rich atria to the damaged ventricles and support myocardial regeneration using paracrine messages.

4.5 Variable expression of genes from both the cardiac fields from the eCSCs of the four cardiac chambers.

In order to understand better the genetical profile of the eCSCs and their gene expression in this study, a correlation with the primary and secondary heart fields has been implemented. A heart field it defines as a discrete embryonic region in which cells that have myocardial potential are located (168) .The theory of heart fields gives an insight of the process of cardiogenesis and a better understanding of the congenital pathologies. The inter-relation dynamics of these genes between the heart fields is enormous and poorly understood however during cardiac embryogenesis some genes are expressed and some switch off according to the time and production of the transcription factors to activate key developmental pathways.

Hand-1 is the Heart and neural crest derivatives expressed protein 1. In humans is encoded by the Hand-1 gene (169,170). The protein encoded by this gene belongs to the basic helix-loop-helix family of transcription factors (171). The Hand-1 gene expressed form the LV (as equally the Nkx-2.5 and TBX-5 genes) is expressed with the myocardium of the primary heart field and plays an essential but poorly understood role in cardiac morphogenesis. Hand factors function in the formation primarily of the left ventricle, right ventricle, aortic arch arteries, epicardium, and endocardium implicating them as mediators of congenital heart disease. After analyzing our 15 samples using the qRT-PCR we identified that the Hand-1 gene is miniamlly expressed in all of the eCSCs from all the chambers with less expression on the LV but uniform and very low expression on the RV and better expression on the LA samples and RA sample. Despite the Hand-1 been a marker of the Primary Heart Field (PHF), when this marker was investigated in the eCSCs in this study it was found in minor quantities according to the size and location of the sample in all the cardiac chambers (see figure 3.17 in the results section). Unfortunately due to the design of this study we haven't investigated the gene expression of the Hand-1 gene in the cardiac chambers samples before

the extraction of the eCSCs for comparison. However in accordance to the study of McFadden (172) the cardiac samples when only analyzed for expression of these genes they do flag Hand-1 and Hand-2 genes during cardiac embryogenesis in the mouse model.

The secondary heart field describes a population of cardiac progenitor cells identified in the pharyngeal mesoderm that gives rise to a major part of the amniotic heart (173). These multipotent progenitor cells, termed the second heart field (SHF), contribute progressively to the poles of the elongating heart tube during looping morphogenesis, giving rise to myocardium, smooth muscle, and endothelial cells. The secondary heart field divides on its anterior part which is programmed to make outflow tract and RV and its posterior part where the atrial myocardium it gets formatted. A number of transcripts have been identified who are marking the evolution and formation of these cardiac structures. These genes are listed in the Table 4.1 below with the cardiac and vascular structures related.

In our study after analyzing the 15 cardiac samples from all the cardiac chambers the eCSCs they express TBX5, TBX20, and HAND-2 & HRT-2 (with low levels of PITX-2) genes universally in the myocardium (see figures 3.17 and 3.18 in the results section), which suggests the potential of these genes to be still presence in adult heart and support differentiation of these potent cells when the myocardium needed in case of myocardial ischemic insults and loss of myocardial function.

Table 4.1.Genes expressed by the Secondary Heart Field and associated structures that are involved on their expression. (*Noticed that TBX-5 is not expressed in the SHF but it plays a role on suppressing genes of the PHF/SHF during cardiac embryogenesis).

GENE	STRUCTURE
FGF-8	outflow tract
FGF-10	aorta & pulmonary trunk

TBX-1	RV, outflow tract
ISL-1	outflow tract, RV, atria
FOXH-1	RV
MEF2C	from cardiac crescent to SHF,RV
HAND-2	RV
TBX-20	RV, LV
**TBX-5*	*LV, atria, inflow tract, in PHF potentiates LV, in SHF suppress other structures*

In this study as the TBX genes are typical marker genes for the cardiac mesoderm, all the eCSCs isolated form all the cardiac chambers showed persistently good expression of the TBX5 and TBX20 genes. All their melting curves from all the samples had optimal Ct values and excellent curve morphology (see figure 3.6 and table 3.6 in the results section). The fact that these genes showed a very good expression in all the eCSCs form all the chambers confirms the importance of these genes on the cardiac chamber formation (174). In the adult myocardium the eCSCs who were isolated still retain the memory of these genes in all the cardiac chambers. This comes in accordance with the recent studies by Mauritz (175) in murine samples, which showed that when iPS cells can differentiate in cardiac myocytes they still express the TBX genes together with all the other genes of the mesodermal cardiac lineage.

The importance of the HRT1 and HRT2 genes in the cardiogenesis is crucial and already a gene defect can produce a serious malformation as has been reported by the team of Srivastava (176). In this study it has been identified that all the eCSCs that have been isolated from the 4 cardiac chambers showed consistency of expression only of the HRT2 gene (see

figure 3.18 in the results section). This consistency has been confirmed when has been correlated to the Ct values of these genes in the melting curves during qRT-PCR recorded in the figure 3.7 in the results section.Unlike the HRT-2, the HRT-1 gene didn't show any significance expression thoroughout the 4 cardiac chambers in all the samples taken.We can speculate that the HRT-1 gene who belongs on the primary hart field switches off after the embryogenesis after the LV chamber is been formatted so the secondary heart field has genes who can be still on even in the adult eCSCs genes.

Similarly in genes with high embryological importance and multipotency like the ISL-1, in this study it was found in minimal or negligible expression and only in the atrial and not in the LV or RV chambers. This confirms previous studies of the importance of the atria as depositors of progenitor cells instead of the ventricles (149). The presence of the ISL1 genes early in the cardiogenesis and the fact that these genes are expressed not only in the embryo but also in the adult (177) and especially with cells of the conductive system (178) suggests that the eCSCs that express this gene in our study is a more atrial in nature gene and not a ventricular one. Atria have been already identified as the areas of eCSCs deposits from previous studies of Kajstura (179) and as has been mentioned earlier in the work of Castaldo (138) these deposits of the eCSCs can be mobilized to the areas needed when a myocardial injury happens. It seems that these deposits of the eCSCs found in our study which only express ISL1 in the atria can confirm the above hypothesis of the group of Castaldo.

Genes as the Pitx2 member of the RIEG/PITX homeobox family, recently in the studies of Kirchhof (180) showed the importance of this gene which demonstrate that PITX2c is expressed in the adult human and mouse left atrium and that reduced expression of this isoform in mice causes shortening of atrial action potential duration and susceptibility to induced AF. In our study this gene was expressed minimally in eCSCs isolated throughout

the myocardial chambers but with better expression in the LA and even less expression to the other chambers (see figure 3.17 in the results section).

The Foxh1 is a forkhead-DNA binding transcription factor in the TGF-β Smad pathway. In our study the expression of this gene, which belongs to the secondary anterior heart field, was most of the samples from all the cardiac chambers, in minor concentrations (see figure 3.17 in the results section). Identification of this gene in the adult cardiac samples of the eCSCs shows how these cells can still retain a memory of the cardiac embryogenesis.

Genes of the fibroblast growth family as FGF8, FGF10, have been minimally or not expressed in our study; (see figures 3.17 and 3.18 in the results section). FGF-8 gene was minimal in LA RA, LV and no expression at all was evident in the RV. It appears that the RV does not retain expression these genes in the eCSC progenitors and this is probably due to the dynamics of the gene expression and down regulation during the cardiac embryogenesis of the secondary heart field (180). The Ct values of the above findings correlate well with the expression or minimal expression of this gene in the qRT PCR results in these studies (see tables 3.3-3.6 in the results section). Similarly the FGF-10 gene in our study showed minimal expression in RA and LV (see figures 3.12 & 3.14 in the results section) and no expression at all in LA and RV. Previous studies by Itzaki-Alfia *(*137) supported that the RA is the richest chamber of endogenous progenitors and as such the RA in this study confirms minimal but some expression of this gene. The fact that we couldn't identify expression in the other chambers could be explained simply by the fact that the eCSCs have no expression or that the primers have failed, although the latter is unlikely given the stringent primer design protocol.

Another explanation of the absence or minimal expression of these important mesoderm genes could be that during completion of the cardiac embryogenesis they quit their expression as other more powerful genes as TBX and HAND family genes keep suppress

their expression in order to keep the identity of the chamber of origin but have a memory to the other chambers in endogenous progenitor cells as the eCSCs and reactivate them in case the myocardium needs them. The age of the patient could be another reason of these eCSCs not to express the above genes. We can speculate that Wnt/β-catenin signaling playing an upstream role in promoting proliferation of the SHF in vivo, in addition to promoting expansion and maintaining multipotency of ISL-1 positive ESC derived cardiac progenitor cells (182,183). Furthermore studies by Ellison (156) provide evidence for a role of the Wnt/β-catenin and Bmi-1 pathways on eCSC senescence. When eCSCs isolated from old hearts were grown in Wnt3A conditioned media or transfected with a lentivirus driving Bmi-1 expression, the *aged/senescent* impairment in proliferation was corrected.

When considering these facts it appears that the original hypothesis for this study has been proven where the eCSCs express most of the genes from the primary and secondary heart field with half of them expressed in a relevant level and a number of them in minor or negligible quantities in the adult human myocardium. With this result they showed a *global and generic* genetical profile throughout the myocardium despite minor variances and non-expression of some genes from the SHF which probably suggests that these genes are not presented (or switched off by other genes), in the adult myocardial eCSCs.

5. CLINICAL RELEVANCE OF THE STUDY

In this thesis it was hypothesized that eCSCs isolated from the 4 chambers of the human heart would have a universal transcriptional profile rather than a profile that reflects their chamber of origin. This would give the added advantage of being able to isolate c-kit^{pos} eCSCs from any of the four cardiac chambers to produce cardiomyocytes that exhibit specific characteristics of the chamber that they are required to regenerate. This will increase their viability for future cell therapy in patients with cardiac failure.

In order to replace the damaged myocardium without compromising the local cardiac milleau and create any kind of disturbances like arrhytmias, regional wall abnormalities, or any regional non viability and fibrosis the cells that needed to be supplied or stimulated via growth factors needed to be the same in their genetical and functional characterictics.

In order to do so as this thesis suggested and confirmed other previous studies, harvesting the eCSCs from the right atrial chamber is the most easy and less harmfull way. Genes expressed from the atria are more likely to be the same form all the other chambers with minor variances. Cells can be easily produced in a cardiac stem cell laboratory and used afterwards to be introduced in the human damaged myocardium or together with growth factors in order to trigger regeneration. This will give better myocardial contractility and clinical improvement of the patients exercise tolerance with better quality of life.

6. LIMITATIONS OF THE STUDY

Every study in basic science research has limitations which can be attributed to the design of the study its material and methods and its analysis of the results. In this study we appreciate that some limitations exist.

Firstly the operator of this project is a senior trainee in heart surgery with major interest in cardiovascular research and translation medicine and as such it was his first time to work and get used on basic science laboratory procedures, so a learning curve time has been passed before he achieved optimal handling in the instruments and be familiar with the laboratory techniques of the micro-cosmos of the cardiac stem cell biology. For that reason more than 30 samples were analyzed and only 15 were finally achieved complete results in order to successfully complete this project. Some samples despite using the same technique of harvesting and analysis they have not progressed so other factors could be affected their process. These can be listed as *a. Patient and sample characteristics prior the analysis b.*

Laboratory bench work and growth of the cells during the analysis c. Optimization of the fine techniques of analysis as RNA extraction, Nanodrop purity analysis and finally qRT-PCR process. In all these 3 points as mentioned before in the paragraph it was a period of training and familiarization of the techniques and change of the philosophy of been a researcher and not a cardiac surgeon!

Secondarily and together with the same idea of the previous limitation it is evident in this thesis that only one sample of the RV reached a full analysis. A number of 5 samples that have been analyzed from RV chamber after being harvested from human myocardium have not been grown in culture despite using the same techniques for more than 2 weeks. This can be a limitation but even a finding on its own as the RV is probably hosting less numbers of eCSCs due to her low pressure system with less chances of require regeneration so niches of eCSCs are locating in chambers with high turnover and chance of myocardial injury like LV. On the other hand as the RA and the LA appendices have embryological remnants of cell progenitors so the heart keeps niches of eCSCs for further need in case of myocardial injury. The unique sample that has been successfully shown isolated eCSCs cells and completed all the analysis of the RV was from the endocardial layer of the RV near to the tricuspid valve and not the epicardial layer. This was a surgeon's choice in order to eliminate the risks of bleeding during harvesting and avoid patient mortality and morbidity. The participating surgeon had been asked to give a minimum of a 2-3 cm size sample from the cardiac chamber location; however this was on his discretion after taking in consideration the size of the heart and the complexity of the operation. Thus the size of the harvested sample was a limiting factor of the amount of cells that can be isolated. The RV sample was lower in weight, but still sufficient to give a number of cells after isolation. All the other RV samples which were used have been small and muscular but after the enzymatic isolation and cell

culture no cells were identified and probably were lost during the various procedures of the isolation.

Thirdly the size of the samples was not the only limiting factor of the number of cells that been isolated as small samples showed a good amount of eCSCs and bigger samples showed fewer eCSCs. Samples like the RA1 which weighed ~ 1 gram, at the end of the isolation and MACS procedure yielded a minimal number of cells and lower than those published in our previous studies of our group by Torella (106) who suggested a number of about 45,000 eCSCs per gram of tissue; specifically of this sample RA1 the sample harvested was a RA appendix which was having not only muscular layers but a significant layer of epicardial fat which was involving most of the area of the RA appendix. So the ideal weight has not been precisely calculated after eliminating the fat tissues or other fibrous tissues even after meticulous and precise sample mincing prior to enzymatic isolation. Due to this limitation despite the careful and meticulous enzymatic isolation and cell culture, the cells from some samples were still less from the cells reported in the literature. For this reason a small sample with fewer cells after MACS procedure when platted and put on in cell culture grow well in a period of 2-3 weeks in culture with CSCs growth medium. Probably for future work when we redesign of the protocol of harvesting the samples, instead of taking in consideration the real weight, it will be more accurate the ideal weight of the sample and correlate with the number of cells presented during the enzymatic isolation and MACS procedure.

Lastly using the qRT-PCR we tried to give a better and real time analysis of the expression of the genes from the primary and secondary heart field. As mentioned earlier in the discussion some of the genes had minimal or negligible expression. This can be explained with poor design of primers however for the FGF genes that showed poor expression and abnormal melting curves there were only 3 isoforms for humans and only the best of these have been

used in order to achieve results. It could be argued that redesign again and redo the same analysis could show different results or even confirm these results.

7. CONCLUSIONS

Based on our data it would appear that isolated eCSCs are indistinguishable and interchangeable throughout the myocardium. These c-kit^{pos} CD45^{neg} e-CSCs are distributed throughout the myocardium in all 4 chambers of the human heart. They don't appear to have a *chamber-specific* transcript footprint, and are therefore interchangeable between cardiac chambers. When isolated from all the 4 human cardiac chambers, they appear to have a universal genetic profile despite their chamber of origin; with expression of most of the transcripts from the secondary heart field and neglibile express from the PHF. Their source for cell therapy becomes simpler and the atrial appendix which is the most approachable and safe structure to harvest becomes the source of choice. Having this information, optimal myocardial regeneration strategies either through direct cell transplantation or *in situ* activation of these eCSCs post myocardial infarction or other myocardial insult could be applied to treat cardiac failure and regenerate the damaged myocardium with not having the risk of regional arrhythmias or change of the function of the cardiac chambers. So we can safely conclude that the myocardium harbours generic and not chamber specific eCSCs, and this it will determine future cell replacement therapy in patients.

8. FUTURE DIRECTIONS

With the results and new findings of this thesis we tried to prove the hypothesis that the human myocardium harbours eCSCs which are universal and can be potentially interchangeable between cardiac chambers. In order to explore more this hypothesis the next step of this project has a specific aim which is describing on the next paragraph and it will be taking place in the next year in our laboratory. The following step will have the objective to

use these cells in an in vivo model and then in clinical practice in order to cure ischemic heart disease and chronic heart failure.

The next steps specific aim is taking in consideration the same idea where we will try to characterize and compare the growth, multi-potential and differentiation properties of human eCSCs isolated from each of the four chambers of the heart. Pieces of myocardium will be obtained as before from all 4 chambers of the adult human heart of patients (male, >18 years, n=10 for each cardiac chamber) undergoing cardiac surgery or transplant surgery. Ethical approval for these procedures will be given by NREC (08/H1306/91). Cardiac cells will be isolated using enzymatic dissociation of the human myocardial tissue. eCSCs will be isolated from the mixed population of small cardiac cells by immune-sorting for CD45 negative and then c-kit using magnetic microbeads (Miltenyi)® . The degree of enrichment of the sorted cells obtained by magnetic activated cell sorting will be determined by FACS and Cytospin immunocytochemistry. An aliquot of c-kit^{pos} eCSCs will be characterised for expression of genes for 'stemness' i.e. Oct3/4, Bmi-1, Rex1, TERT, by quantitative RT-PCR (qRT-PCR) and other stem cell markers, i.e. CD133, Flk1 by FACS.

The rest of the eCSCs will be plated on gelatin-coated dishes in DMEM/F12 (Gibco®) media supplemented with 10% serum, 10ng/ml bFGF and 10 ng/ml LIF (Growth Medium). To optimize the expansion of eCSCs we will define a media conditioned with small molecules (e.g. a cocktail of TGF-b inhibitor, PDK-1 activator, and ERK1/2 inhibitor) without the need for adding animal serum. Then we will assess and compare the growth, multi-potency and differentiation potential of eCSCs from different cardiac chambers using in vitro assays of clonogenicity, cardiosphere formation, self-renewal, cell proliferation and multipotency. For clonogenicity, eCSCs will be serially diluted and deposited into one cell/well in 96-well plates, for the generation of single-cell clones. The clonal efficiency of eCSCs isolated from each chamber will be assessed for 10 x 96 well plates and expressed as a percentage. For cell

proliferation, eCSCs and clones will be measured over 24 hours using the BrdU incorporation assay (Roche®). For self-renewal, eCSCs will be grown past the population doubling limit of somatic cells (>40), and sub-cloned at every 10th passage and assessed for maintenance of clonogenicity and primitive *stemness* genes by qRT-PCR. For cardiosphere formation, eCSC clones will be grown and expanded and then placed into bacterial coated dishes with cardiosphere-generation media (DMEM/Hams F12, 10ng/ml LIF, Insulin-Transferrin-Selenium, B27 and N2 supplements).

The number of cardiospheres generated will be assessed and the ability of eCSCs from each chamber to generate primary, secondary and tertiary cardiospheres evaluated. To determine eCSC multi-potency, the progeny of a single clone generated cardiosphere expresses biochemical markers of cardiomyocytes (Nkx2.5, GATA-4, MEF2C, α-sarcomeric actin, α-cardiac actinin, troponin I, troponin T, cardiac myosin heavy chain, connexin 43), smooth muscle (GATA-6, α-smooth muscle actin) and endothelial (Ets1, CD31, von Willebrand factor) cells. The stained cells will be analysed and counted by confocal microscopy (Zeiss LSM-510). eCSCs myogenic differentiation potential will be assessed utilising a unique cardiosphere myogenic beating assay. The type of cardiac muscle cells produced from eCSCs from each chamber will be assessed for their mechanical and electrical properties. Also, they will be assessed for the expression of chamber-specific atrial and ventricular myosin light chain (MLC) by immune-staining and qRT-PCR (i.e. MLC-2a, which is atrial specific, and MLC2v, which is the ventricular specific. In a further step small animal myocardial infarction regeneration assay in vivo will determine the regenerative potential of the c-kit^{pos} eCSCs isolated form the atria and ventricles using animal myocardial infarction regeneration assay in vivo in the nude rat heart.

In this stage in the in vivo model we will determine whether the pattern of gene and protein expression (by qRT-PCR, immunocytochemistry cardiosphere myogenic beating assay in

vitro and calcium transients) of new cardiomyocytes derived from the eCSCs both in vitro and in vivo recapitulates ontogeny or is common for all the eCSCs irrespective of the chamber of origin.

These studies have a fundamental importance on designing protocols for myocardial regeneration, and it will make these protocols standardized for the treatment of myocardial infarction and chronic heart failure. In the short term it will be expected that the results of these studies will be used to devise a protocol to undertake a clinical trial utilising eCSCs for myocardial regenerative therapy. This could have a major impact on the future treatment of ischemic heart disease and chronic heart failure.

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