The Distribution and Characteristics of Endogenous Cardiac Stem Cells in the Adult Human Heart

This Thesis For The Degree Of Doctor Of Philosophy

Imperial College London

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By

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Declaration of Originality

I, Sajiram Sarvananthan declare that this work is my own and that all else is appropriately referenced.

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ABSTRACT

The human myocardium harbours resident multi-potent cardiac progenitor cells (CPCs). We investigated the distribution, properties, differentiation potential and effect of LV function on CPCs in all chambers of the human heart.

Biopsies from all chambers of the heart from the same patient with good (EF>45; n=5) and impaired LV function (EF<45; n=5) was analysed for c-kitpos and MDR-1pos CPCs. CPCs were isolated using MACS from ten patients (Good and Impaired LV, n=5/group) and was characterised.

CPCs were identified in all chambers of the heart in both groups. The RA from good LV group had significantly ($p<0.05$) less c-kitpos (6± 0CPCs/mm2) and MDR-1pos CPCs (5± 1 CPCs/mm2). In the impaired LV group, the LV (38± 2 CPCs /mm2) had significantly more c-kitpos CPCs. Overall, the impaired LV group had significantly ($p<0.05$) more c-kitpos (32± 1CPCs /mm2) and MDR-1pos (47± 1 CPCs /mm2). Irrespective of LV function both c-kitpos and MDR-1pos CPCs were significantly higher ($p<0.05$) in ventricle than atria.

CPCs from the LV (80±2%) are significantly ($p<0.05$) more proliferative than RV (64±4%) and RA (64±6%) in good and impaired LV group, respectively. Regardless of LV function the atria and ventricle showed no difference in proliferation. Cardiosphereogenesis was significantly ($p<0.05$) higher in the good LV group. Irrespective of the LV function, cardiosphereogenesis, α-sarcomeric actin and calponin expression were significantly increased ($p<0.05$) in the LV chamber. In impaired LV group, the LV showed significant ($p<0.05$) expression for Nkx2.5. Overall, the cardiomyogenic and calponin expression were significantly ($p<0.05$) increased in impaired LV patients. The vWF expression was significantly ($p<0.05$) increased in LA and the atria of the good LV group.

In conclusion, there is a variation in the distribution, stem cell properties and differentiation potential of CPCs across all 4 chambers of the human heart. These variations are also affected by the LV function.

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ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ALDH	Aldehyde Dehydrogenase Activity
AR	Arrhythmogenicity
ASC	Adult Stem Cell
AV	AtrioVentricular
AVR	Aortic Valve Replacement
BIVAD	Bi ventricular Assist Device
BMDC	Bone Marrow Derived Cell
BMMNC	Bone Marrow Derived Mononuclear Cells
BMT	Bone Marrow Transplant
CABG	Coronary Artery Bypass Surgery
CASC	Cardiac Atrial Appendage Stem Cells
CARDIA	The Coronary Artery Risk Development in Young Adults Study
cCFU	Cardiac-derived Colony Forming Unit
CHD	Congenital Heart Disease
CDC	Cardiosphere Derived Cell
CM	Cardiomyocytes
CO	Cardiac Output
CPC	Cardiac Progenitor Cell
CSC	Cardiac Stem Cell

cSP Cardiac Side Population

DB Double-blinded

DMSO Dimethyl Sulfoxide

eCSC Endogenous Cardiac Stem cell (c-kit^{+})

EF Ejection Fraction

EPC Endothelial Progenitor Cells

EPDC Epicardial Derived Cell

ESC Embryonic Stem Cell

ESCQ-FBS Embryonic Stem Cell Qualified – Fetal Bovine Serum

FACS Flow Assisted Cell Sorting

FBS Fetal Bovine Serum

FHF First Heart Field

FC Flow Cytometry

GFP Green Fluorescent Protein

HF Heart Failure

HFpEF Heart Failure with Preserved Ejection Fraction

HFrEF Heart Failure with Reduced ejection fraction

hESC Human Embryonic Stem cell

HSC Hematopoietic Stem Cell

ICC Immunocytochemistry

IHC Immunohistochemistry

IC Intracoronary

IF	Immunofluorescence
iPSC	Induced Pluripotent stem cell
LA	Left Atrium
LAA	Left atrial appendage
LV	Left Ventricle
LVAD	Left Ventricular Assist Device
LVEDV	Left Ventricular End diastolic Volume
LIF	Leukemia Inhibitory Factor
MAB	Mesoangioblast
MACS	Magnetic Activated Cell Sorting (Miltenyi)
MI	Myocardial Infarction
MiRNAs	microRNAs
MSCs	Mesenchymal Stem Cells
MVR	Mitral Valve Repair/Replacement
NYHA	New York Heart Association
P	Passage
PC	Placebo-controlled
PBS	Phosphate Buffered Saline
PD	Population Doubling
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RA	Right Atrium
RAA	Right atrial appendage

RV	Right Ventricle
RT	Room Temperature
SB	Single-blinded
SC	Satellite cell
SEC	Suspension Explant Culture
SHF	Second Heart Field
SM	Skeletal myoblasts
SP	Side Population
TEN	Trans-endocardial
TEP	Transepicardial
TF	Transcription Factors

1. INTRODUCTION

1.1 The Physiology of the Human Heart

1.1.1 The Development and Anatomy of the heart

In humans, the heart is the first organ to form, and it begins to beat at around day 22-23 following fertilisation; furthermore, by the 4th week, the circulation of the blood is established (Figure1.1). The formation of this truly remarkable organ is due to four characteristic pools of progenitor cells; the cardiac crescent (First Heart Field; FHF), the Second Heart Field (SHF), the proepicardial organ and the cardiac neural crest. These progenitors differentiate into cardiomyocytes, endothelial cells, vascular smooth muscle cells, fibroblasts, and the conduction system to form a heart. This differentiation process is tightly controlled by complex signalling cascades which are securely orchestrated by various genes. The ability to switch these genes on and off ultimately allows normal cardiac development to occur. This orchestration is achieved by transcription factors (TF), growth factors and microRNAs (miRNAs) (Roche P et al. 2013).

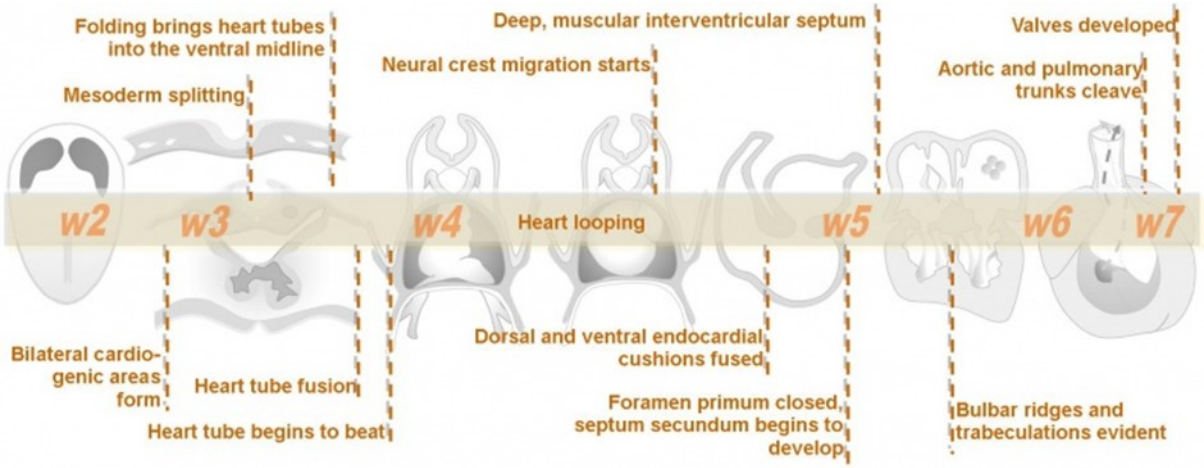


Figure 1. 1 Time line illustrating the development of the heart. (Adapted from https://embryology.med.unsw.edu.au/embryology/index.php/Intermediate-Heart_Valves).

The onset of cardiogenesis begins in the embryonic disc, within the mesodermal layer of the trilaminar region, known as the cardiogenic plate. It is located cranially and laterally to the neural plate. The right and left endocardial heart tubes, containing the progenitor cells eventually fuse (cranio-caudally) to form a single primordial tube. This process occurs at the ventral midline following lateral folding of the embryo and is complete by day 21. The cardiac crescent is made up of early cardiovascular progenitors comprising of characteristic epithelial cells and expresses specific cardiac transcription factors (Harvey RP. 2002). The progenitors within the endoderm, through various regulatory mechanisms, direct cardiac specification. Wnt is one of the earliest markers associated with cardiac specification within the mesodermal progenitors. However, endogenous Wnt antagonist such as Crescent and Dickkopf-1 plays a crucial role in regulating this (Pandur P. et al. 2002; Kwon C et al. 2007).

The primordial tube is divided into several regions, such as truncus arteriosus, bulbus cordis (forms right ventricle), primordial ventricle (forms left ventricle), primordial atrium (will give rise to common atrium) and sinus venosus. The developing heart has distinctive layers (Figure 1.2). The splanchnic mesoderm gives rise to the myocardial layer, the endothelium within the tube gives rise to the endocardial layer, and the mesothelial cells from sinus venosus contribute to the formation of the epicardium.

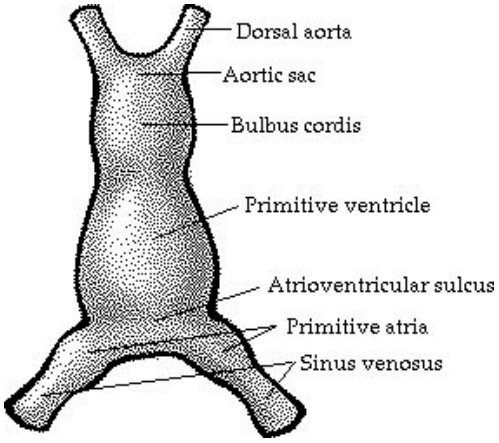


Figure 1. 2 Different regions of the heart tube. (Adapted from http://www.meddean.luc.edu/lumen/meded/grossanatomy/thorax0/heartdev/main_fra.html)

During the fourth week, the process of cardiac looping occurs and is completed by the fifth week (Figure 1.3). This looping allows the straight tube to form a complex structure that begins to look like an adult heart. The primitive right and left atria fuse to create common atrium and around day 28 the septum primum forms and contribute to the partitioning of atria.

The superior and inferior vena cava becomes incorporated into the RA as the sinuatrial orifice moves to the right side, leaving the left sinus horn to form the coronary sinus. The pulmonary veins are formed in the back wall of the ventricle. During early ventricular development, minor trabeculations are evident throughout the primordial ventricle; which enlarge as the ventricles grow. The primordial muscular interventricular ridge contributes to the formation of the septum. The partitioning of the truncus arteriosus and the conus cordis into the aorta and pulmonary trunk constitute the final changes. The truncus swelling and the conus swelling from the walls of the outflow tract contribute to the septum that achieves this partitioning.

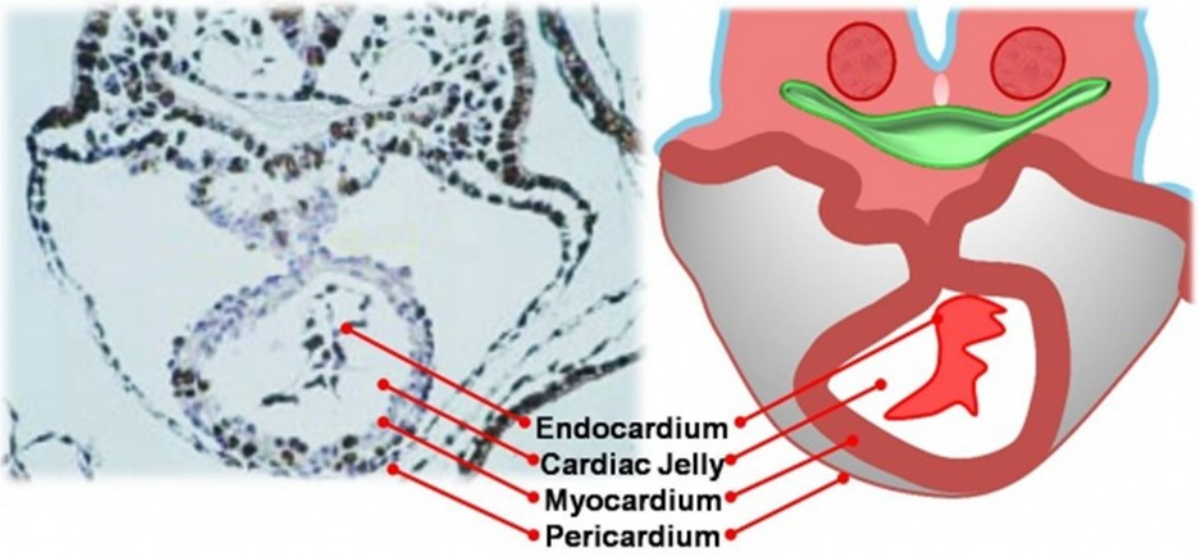


Figure 1. 3 Crossection of the heart tube showing the ventricular section. (Adapted from https://embryology.med.unsw.edu.au/embryology/index.php/Intermediate_-_Heart_Valves)

Altogether there are four valves within the heart, 2 AV valves composed of leaflets and chordae. The aortic and pulmonary (semilunar) valves are located in the aorta and the pulmonary trunk. The development of the AV valves usually occurs around 5 to 8 weeks following gestation. The AV valve has two leaflets (bicuspid/Mitral) and attached via fibrous chords (chordae tendineae) to the papillary muscle. The bulbar ridge and subendocardial tissue contribute to the formation of the semilunar valve.

The above intricate process of heart formation relies heavily on conserved cardiogenic TF. The key TFs such as Nkx2.5, MEF2C, Hand1, Hand2, Tbx5 and GATA4 are implicated in the early cardiogenesis. The understanding of the influence of Nkx2.5 comes from fruit fly and mice (Bodmer R et al. 1993; Lyons I et al. 1995; Tanaka M et al. 1999). This TF plays a crucial role in early cardiogenesis; in establishing cardiac lineages within the primary (first) heart field and contribute to the formation of the cardiac crescent. Nkx2.5 null mice have been shown to have a deficiency in cardiac looping and reduced ventricular markers. Mutations that alter the ability of Nkx2.5 to bind to DNA has been associated with an autosomal dominant form of CHD (atrial septal defects with atrioventricular conduction defects) within humans (Reamon-Buettner SM et al. 2010). Subsequent studies to date have associated over 40 Nkx2.5 related mutations that contribute CHD in humans.

Myocyte enhancer-binding factor 2C (MEF2C) gene is required for muscle formation and expressed in the mesodermal layer before the development of the heart tube (Nguyen HT et al. 1994). The MEF2C null mouse show impaired cardiac looping, RV development and reduced expression of cardiomyocyte-specific genes (Edmondson DG et al. 1994; Lin Q et al. 1997). In humans, MEF2C mutation may lead to non-syndromic congenital heart defects (Kodo K et al. 2012).

MEF2C indued Hand 1/2 expression plays a supportive role in ventricular development. The development of right and left ventricular myocardium relies on the expression of helix-loop-helix transcription factors, eHAND/Hand1 and dHAND/Hand2. The role of both Hand1/2 is less clear within humans. The Hand2 mutation causes outflow tract stenosis within humans, and mutation of Hand1 is associated with ventricular septal defect (VSD) amongst Chinese patients. (Holler KL et al. 2010; Shen L et al. 2010).

Evidence from human and mouse models show that the functional loss of one allele of the TF Tbx5 is associated with atrial and ventricular septal defects seen in Holt-Oram syndrome (Basason CT et al. 1997). Tbx5 is expressed throughout the cardiac crescent during early development and contributes to the formation of the linear heart tube, cardiac looping, chamber specification, septation and cardiomyocyte differentiation (Bruneau BG et al. 2001; Hiroi Y et al. 2001). During cardiac looping, Tbx5 expression is limited to the atria and left ventricle, marking the boundary for the formation of the ventricular septum (Bruneau BG et al. 1999). Tbx5-null mice show stunted cardiac development due to impaired looping and hypoplasia of the left ventricle (Bruneau BG et al. 2001).

The other crucial TF in cardiogenesis is GATA4 (Liang Q et al. 2002; Zhou P et al. 2012; van Berlo JH et al. 2010); which is expressed during development of endoderm (Watt AJ et al. 2004; Zeisberg EM et al. 2005) and in the adult CM. GATA4 also plays an essential role in cardiac differentiation and morphogenesis (Kuo CT et al. 1997). GATA4 deficient mice have major cardiac defects resulting in incompatibility with life (Kuo CT et al. 1997). GATA 4 also has a direct influence on other TF such as Mef2C, Hand2 and GATA6, which contribute to cardiovascular progenitor differentiation. The complex generated from GATA4 association with Tbx5 and Nkx2-5 triggers signalling cascades that contribute to cardiac development (Bruneau BG et al. 2001, Belaguli NS et al. 2000). Heterozygous GATA4 mutations in human cause familial septal defects, right ventricular hypoplasia and cardiomyopathy (Rajagopal SK et al. 2007).

During development, the proepicardial organ is generated at the sinus venosus and contributes to the formation of the epicardium. The protrusion of cells from the proepicardial organ, through the extracellular matrix, results in the formation of the epicardium. This process is mediated by the bone morphogenetic protein (BMP) signalling (Nahirney PC et al. 2003; Ishii Y et al. 2010) and has been shown to differentiate into coronary vascular smooth muscle cells and endothelial cells. Finally, the outer epicardial layer is composed of epithelial cells is the last to form

1.1.2 The Four chambers of the heart

Characteristically the adult heart is made of four chambers; right atrium, left atrium, right ventricle and left ventricle. Although the thickness of the wall across the chambers varies, the underlying structural organisation remains the same. The myocardium is sandwiched by the inner endocardial layer and an outer epicardial layer (Figure 1.5).

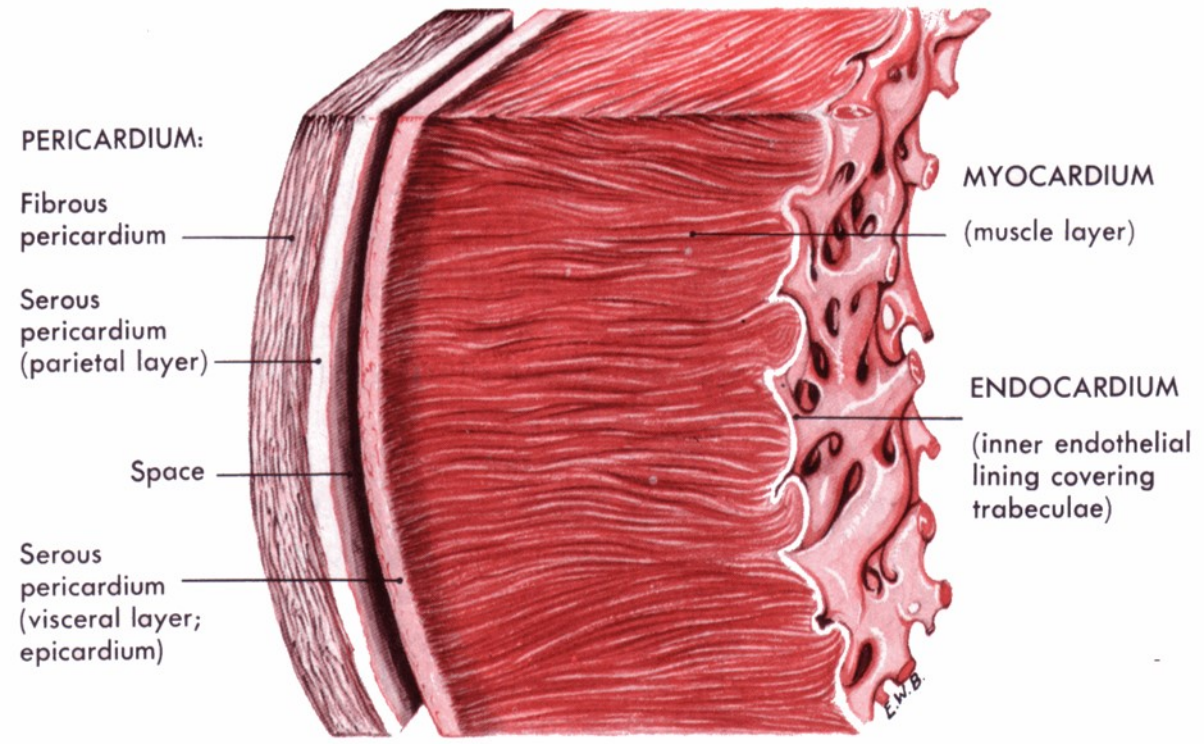


Figure 1. 4 Cross-section of the heart outlining the layers

1.1.2.1 The right atria

The right atrium is a thin-walled muscular compartment which consists of the appendage, the venous part, the vestibule and the septum. The arrangement of the pectinate muscle within the RA is characteristic. The muscle arises from the terminal crest and inserts into the inferior-lateral wall of the atrium. The appendage wall is entirely composed of the pectinate muscles, and typically it overlaps and branches but never encroaches near the orifice of the tricuspid valve.

The myofibres within the atria are circumferentially orientated around the fossa and the peripheral fibres spread towards the origin of the terminal crest. Also, the fibres that run anteriorly combine with fibres that arise from the apex of Koch's triangle and the eustachian ridge. The fibres from the posterior part merge into obliquely organised fibres that covers the epicardial surface of the venous sinus (Wang K et al. 1995; Ho SY et al. 2002).

1.1.2.2 The left atria

The left atria have several layers of fibres that are aligned differently with regional differences in the thickness of the myocardium. However, the walls are uniformly smooth textured (Wang K et al. 1995). Due to the absence of the terminal crest, the pectinate muscle in the LA is not as organised compared to the RA. The prominent interatrial bundle is located on the epicardial side and continues in parallel with circularly arranged fibres. These fibres originate from the anterior/antero-superior margin of the atrial septum and arch sideways to merge with the interatrial bundle before dividing to cover the appendage circumferentially. These fibres then remerge to generate a broad-band in the inferior wall which subsequently joins the septal raphe.

Deep to the circular fibres, is a layer of oblique fibres originating from the anterosuperior septal raphe. These fibres move to the top to become longitudinal with branches that circumvent the pulmonary veins. However, the septopulmonary bundle in the posterior wall divides into two oblique fascicles and merge with the superficial circular bundle.

1.1.2.3 The right ventricle

The right ventricle (RV) is typically crescentic in shape and composed of predominantly transverse fibres Buckberg G et al. 2014). These transverse fibres when contracts draw the tricuspid annulus toward the RV apex. The interventricular septum is composed of oblique helical fibres that cross each other at 60° angles.

1.1.2.4 The left ventricle

The left ventricle consists of three layers as per the longitudinal alignment of the myocardial strands. These layers include subepicardial (superficial), middle, and subendocardial (inner) (Figure 1.5). The orientation of these fibres are different in each layer, but they are interconnected with the superficial layer extending from one ventricle to another. The more superficial strands pass obliquely over the interventricular groove, across the obtuse margin and run towards the apex of the LV.

The superficial layer of the ventricle contributes towards 25% of the wall thickness (Sanchez-Quintana D et al. 1999; Ho S.Y et al. 2009). The spiral convolution of the myocardial strands contributes to the formation of the subendocardial layer, which makes accounts for 20% of the wall thickness (Sanchez-Quintana D et al. 1999; Ho S.Y et al. 2009).

The middle layer of the left ventricle makes up 53–59% of the ventricular wall thickness; thickest at the base and thins out toward the apex of the LV. The strands within this layer are more circumferentially organised and run parallel to the mitral orifice. The fibres within the subendocardial layer run longitudinally and insert into the aortic, mitral valves and the membranous septum. Eventually, they fuse with trabeculations and merge with the papillary muscles

The orientation of the fibres determines the biventricular function. The more transverse fibres in the RV contribute to more circumferential strain which contracts the narrow crescentic cavity. This force accounts for about 20-30% of the EF. Moreover, in the LV, the helical fibres induce longitudinal strain, while the oblique spirals thicken and coil. This action produces >60% of the EF (Ho S.Y et al. 2009).

1.1.3 The heart as a self-renewing organ

For a long time, it was believed that the adult mammalian heart was a post-mitotic organ with no intrinsic capacity to regenerate after myocardial injury. Traditionally cardiomyocytes have been categorised as terminally differentiated cells that adapt to increased work and compensate for the disease through hypertrophy rather than proliferation (Soonpaa et al. 1998). However, compelling evidence accumulated over the last decade has refuted the above notion and suggests that the heart has regenerative potential (Kajstura J et al. 1998; Nadal-Ginard et al. 2003; Beltrami et al. 2003; Bergmann O et al. 2009; Ellison et al. 2013). Indeed, cells undergoing mitosis and cytokinesis with subsequent generation of cardiomyocytes (1%) have been demonstrated in human hearts under normal and pathological conditions (Ahuja et al. 2007; Senyo SE et al. 2013; Scalise et al. 2019).

It was in 1998; the mitosis within adult cardiac myocytes was first described (Kajstura J et al. 1998). The study reported a rate of 14 myocytes per million amongst healthy adult hearts, with a subsequent 10-fold rise in mitotic cells in patients with dilated cardiomyopathy and severe IHD. Through integrating carbon-14 (14C) into DNA, Bergmann O et al. (2009) studied the cardiomyocyte turnover in the normal human heart. The study reported cardiomyocyte DNA synthesis to be an ongoing process throughout life with a turnover of 0.2-2% of cardiomyocyte annually. However, this rate was shown to decrease with age. In another study using 15N mass spectrometry in a mouse model, a similar proportion of cardiomyocyte turnover was observed with a reported reduction with age. (Senyo SE et al. 2013). Despite the generation of cardiomyocytes, confusion remains as to the source from which they arise (Bergmann O et al. 2009. However; Senyo SE et al. (2013), was able to demonstrate new cardiomyocyte generation from pre-existing cardiomyocytes.

Alongside this, there is evidence that a primitive population of stem cells may contribute to the formation of new cardiomyocytes in the adult heart. Hsieh et al. (2007) used a pulse of 4-OH-tamoxifen followed by induction of green fluorescent protein (GFP- achieving 82.7% expression), in a double-transgenic mice model to track the fate of adult cardiomyocytes. They created the double transgenic mouse (Mer-CreMer-ZEG) by crossbreeding mice that were cardiomyocyte-specific MerCreMer and ZEG. They demonstrated that the percentage of GFP+

cardiomyocytes remained unchanged during normal ageing up to one year. Therefore, indicating that stem or precursor cells did not contribute to the formation of new cardiomyocytes at a significant rate in the normal ageing heart. Following an injury to the myocardium (myocardial infarction or pressure overload), there was a 15% reduction in GFP^{pos} cardiomyocytes in areas associated with the injury; hence suggesting that after injury, stem cells contributed to the replacement of cardiomyocytes. Several studies (Beltrami et al. 2003; Bearzi et al. 2007; Ellison et al. 2013; Uchida S et al. 2013) confirm that the adult myocardium, including the human, harbours tissue-specific bona fide endogenous cardiac progenitor cells (CPCs) with true intrinsic regenerative capacity and distributed throughout the heart.

The above evidence highlight the ability of the heart to self-renew and regulate itself during the onset of cardiovascular disease, ageing and the subsequent development of heart failure. The recognition and application of the CPCs will help clinical translation.

1.2 Resident cardiac stem/progenitor cells in the myocardium

Stem/progenitor cells were first discovered in the adult mammalian heart in 2003 (Beltrami et al. 2003). A cell which can clone, self-renew and be multipotent is classified as a stem cell (Wagers AJ et al. 2004). The cardiac stem/progenitor cells (CPCs) are positive for various stem cell surface markers such as c-kit (c-kit^{pos}); Sca-1, Islet-1 and CDCs or can be isolated as side-population cells (SP) (Figure 1.5). Several independent groups have confirmed the existence of CPCs, (Ellison et al. 2013b), and although a variety of markers have been proposed to identify CPCs in different species; (Figure 1.6) (Torella et al. 2006, Oh et al. 2003; Matsuura et al. 2004; Messina et al. 2004; Smith et al. 2007; Laugwitz et al. 2005; Moretti et al. 2006; Kattman et al. 2006; Wu et al. 2006; Bearzi et al. 2007; Chong et al. 2011), it remains to be determined whether this plethora of primary and secondary markers used to identify the CPCs population to date may represent a sole CPCs at various stages of its development and physiological state. (Ellison et al. 2010; Smith AJ et al. 2014) (Figure 1.5).

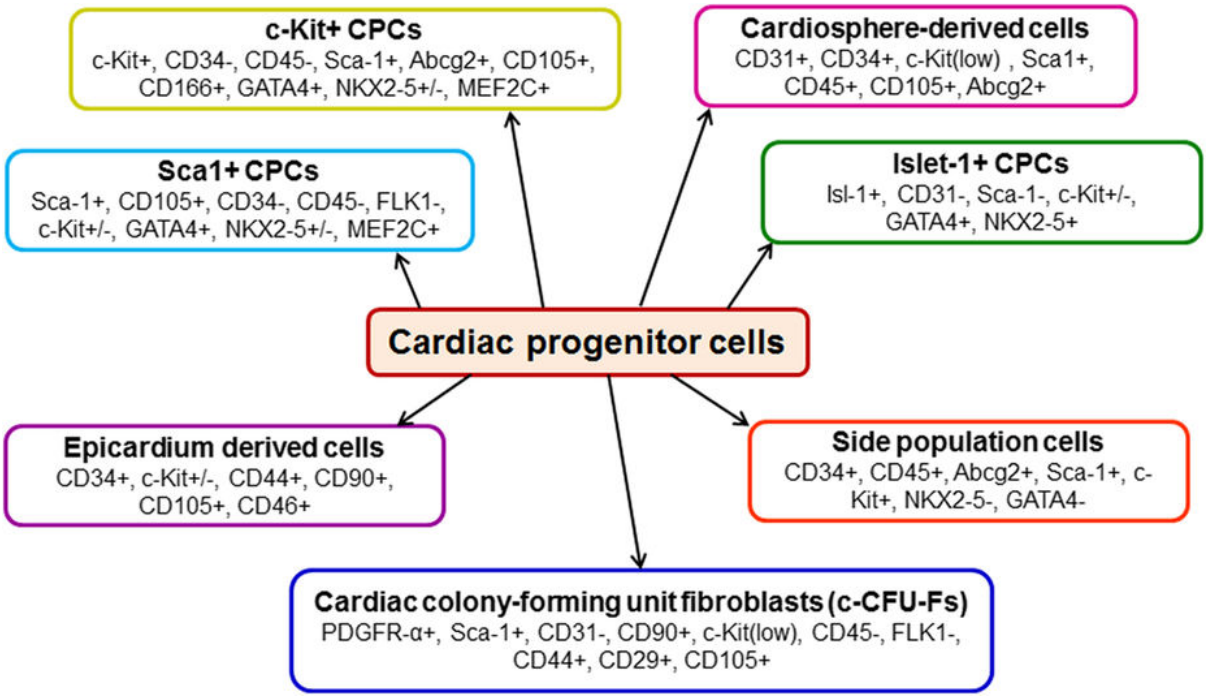


Figure 1. 5 Endogenous cardiac progenitor cell populations identified and characterised thus far (adapted from Tyl Le& JJH Chong 2016)

1.2.1. c-kit positive CSCs

So far, as many as seven different phenotypic CPC populations have been described; primarily based on a single antigen have been identified in the adult heart, including humans. The identified population of c-kit^{pos} CPCs are characterised to be CD34^{neg}, CD45^{neg}, Sca-1^{pos}, MDR-1^{pos}, CD105^{pos}, CD166^{pos}, GATA4^{pos}, NKX2-5$^{pos/neg\ or\ low}$, and MEF2C^{pos} (Chong JJ et al. 2016; Scalise et al. 2019)

c-kit is a proto-oncogene which encodes a transmembrane tyrosine kinase receptor. Also known as CD117, it binds to its ligand, stem cell factor, and triggers biological effects in the regulation of cell differentiation and proliferation in various systems. (Wehrle-Haller et al. 2003; Chi et al. 2010; Kasamatsu et al. 2008). Adult transgenic mice that were heterogeneous for c-kit receptor mutation showed alterations in cardiac anatomy and function together with defects in haematopoiesis, skin pigmentation and immune response (Reith et al. 1990; Cable et al. 1995; Theoharides et al. 1993). c-kit positive cells are relatively small (~ 5μm diameter)

and express markers for cardiomyocyte lineage (e.g. GATA-4, Nkx2.5 and MEF2), whereas blood cell lineage (CD45), skeletal muscle and neural markers were not detected. These cells can be propagated over long-term culture passages and maintained in a stable undifferentiated, self-renewing state without signs of abnormal karyotype (Miyamoto S et al. 2010; Vicinanza et al. 2017). They have also been shown to be multipotent and can regenerate cardiomyocytes and vasculature when injected into the infarcted heart in vivo (Beltrami et al. 2003). Indeed, injecting c-kit^{POS} CPCs into the infarcted myocardium in animals showed these cells targeted the border zone of the injured myocardium and differentiated into cardiomyocytes (Dawn et al. 2005; Oh et al. 2003; Wang et al. 2006).

Therefore, c-kit- positivity and hematopoietic-lineage-markers negativity has been the hallmarks for identifying CPCs from and within cardiac tissue (Bolli et al. 2011; Arsalan et al. 2012).

The evidence from rodent models (Sarvanakumar et al. 2013) suggests that the distribution of CPCs is higher in the atrium than ventricle and implies that the atria may be the origin of CPCs (Sarvanakumar et al. 2013; Arsalan M et al. 2012). The analysis from several studies shows CPCs to exist in a very small percentage. Beltrami AP et al. (2003), showed there to be 1 per 10000 myocytes, but other studies have reported a range from 1 CPCs per 20000-30000 CMs (Torella et al. 2007; Mishra R et al. 2011). Recently, Vicinanza et al. (2017); documented that only 1–2% of total c-kit^{pos} myocardial cells are the ‘truly’ clonogenic, multipotent CPCs.

In mouse models, stem cell niches that harbour long-term BrdU-retaining cells were identified within the myocardial interstitium (Urbanek et al. 2006). These cardiac niches contain CPCs and lineage-committed cells, which are committed to supporting cells represented by myocytes and fibroblasts (Urbanek et al. 2006). Furthermore, it was shown that in the mouse heart, the number of niches was higher for the atrial myocardium than at the base–mid-region of the left ventricle (Urbanek et al. 2006). They reported CPCs in the atria to be 0.08% of the atrial CMs vs 0.05% of the ventricular CMs. However, these niches are yet to be identified and characterised in the human myocardium.

Cardiac progenitor cells play a crucial role in normal cardiac homeostasis and response to 'wear-and-tear' injury (Nadal-Ginard B et al. 2014). A study by Ellison et al. (2013), using various rodent models of diffuse myocardial injury showed that c-kit^{pos} CPCs have potential to restore cardiac function through the regeneration of lost cardiomyocytes. However, this regeneration and functional recovery were not observed if the CPCs were ablated; but with the progeny of one c-kit^{pos} CPC, the regenerative process was restored. With their data, they were able to conclude that c-kit^{pos} CPCs are necessary and essential for regeneration and repair of the myocardium.

A recently published article (Van Berlo et al. 2014) argues that endogenous c-kit positive cells generated cardiomyocytes at a functionally insignificant level within the heart. The authors used mice with Cre/lox knock-in within the c-kit locus. In one line they introduced Cre-IRES-nGFP and tamoxifen based mER-Cre-mER in the other line. Thus, introducing the CRE in the kit gene at the ATG start codon of exon 1. However, this mouse model is sub-optimal (Torella et al. (unpublished data)) and doesn't sufficiently label the c-kit positive CPCs. The cells express c-kit at low to moderate levels but the CRE readily labels the CD45^{+}mast cells and CD31^{+} endothelial (progenitor) cells, which express c-kit at high levels (Vicinanza et al. 2017). Additionally, the study did not show that the labelled lineage-traced c-kit cells were stem/progenitor cells with properties and characteristics similar to those properties and characteristics of CPCs described by others (Bearzi et al. 2007; Ellison et al. 2013; Smith et al. 2014; Vicinanza C et al. 2017).

A study by Bearzi et al. 2007 isolated and expanded the c-kit positive CPCs from human myocardium. Human c-kit positive, lineage negative cells were shown to be clonogenic and differentiated into cardiomyocytes, smooth muscle cells and endothelial cells. In addition to this, when these cells were injected intramyocardially into the infarcted myocardium of immunodeficient rats and mice, they contributed to the formation of myocytes and neovasculature. The newly formed human myocardium was shown to integrate into rodent myocardium without any structural deformity while contributing to the improvement of the overall performance of the infarcted heart. Further chromosomal analysis alongside Cre-Lox experiments

demonstrated that the newly formed cardiomyocytes were due to the injected human CPCs; hence excluding the theory, this may be due to cell fusion (Bearzi et al. 2007).

Further studies in the human myocardium, showed a high yield of c-kit positive cells within the human atria compared to other chambers. In this study (Itzhaki-Alfia A et al. 2009), biopsies were obtained from 94 patients and put into explant culture. c-kit^{pos} cells were isolated using fluorescence-activated cell sorting (FACS). Although these were cells were not lineage negative sorted the study report higher number of c-kit^{pos} cells derived from atria compared to other chambers; right atrium (24±2.5%), left atrium (7.3±3.5%), right ventricle (4.1±1.6%), and left ventricle (9.7±3%; P=0.001). Indeed, a group of German scientists (Arsalan et al. .2012) assessed this by taking small tissue biopsies (0.36± 0.09 gram) from the left ventricle, the appendages of the right atrium and left atrium of 20 adult patients undergoing cardiac surgery. The number of c-kit positive cells isolated from these three regions was measured using flow cytometry. The study showed the prevalence of c-kit positive cells in both atria (4.90 ± 1.29%) was about five times greater than in the left ventricle (0.62 ± 0.14%, P = 0.035). This finding implies that the regenerative capacity of the atria is likely to be higher than that of the left ventricle.

In a recent publication, atrial samples obtained from patients who underwent LVAD implantation was used to extract CPCs (c-kit^{pos}), endothelial progenitor cells (EPC) and mesenchymal stem cells (MSC) using enzymatic digestion, explant culture technique and MACS sorting (Monsanto et al. 2017). The MAC sorting was used to isolate c-kit positive CPCs and endothelial progenitor cells (EPCs; CD31^{pos}). Subsequently, CD90 and CD105 were used to isolate c-kit^{neg} MSCs. The authors showed that from single atrial biopsy, three characteristically different progenitor cells could be isolated (Monsanto et al. 2017).

Recent clinical trial utilising eCPCs, (SCIPIO) has shown improvements in cardiac function in ischemic heart failure patients (Bolli et al. 2011). Cardiac Stem Cell Infusion in Patients with Ischemic Cardiomyopathy (SCIPIO) is a phase one randomized trial of autologous CSCs as a therapy for ischemic HF. In this trial the investigators isolated c-kit+ CPCs from the right atrial appendage obtained from 16 patients with HF induced by ischemia, undergoing coronary artery bypass surgery and propagated them ex vivo (2 million cells per patient). Subsequently, after five

months following cardiac surgery, 500,000 to 1 million CPCs were injected via intracoronary into infarcted areas, and the control was not given any treatment. Four months after the injection they showed a significant improvement in LVEF (from 30.3% at baseline to 38.5%) when compared to the control group (30·1%) and at one year follow up, there was an increase in LVEF by 12.3 EF units compared to baseline and showed a significant reduction in infarct size at 4 months and 12 months (by 7·8 g (24%) and 9·8 g (30%) respectively).

1.2.2. Sca-1 positive and side population CSCs

While c-kit has been used as a marker for cardiac progenitor cell, some scientists based the isolation of cardiac progenitor cells on another stem cell marker, stem cell antigen (Sca-1). Sca-1 is involved in cell signalling, and cell adhesion in mice (Patterson et al. 2000) and Sca-1^+ cardiac resident cells were first described by Oh et al. (2003). Characteristically these cells are 2-3μm, small, rounded and have a high nucleus to cytoplasm ratio (Samal R et al. 2012). The Sca1^+ CPCs express cardiac-specific factors such as Gata-4 and MEF2C early on; however, only a minority of them have self-renewing and multi-potent capabilities (Matsuura K et al. 2004; Chong JJ et al. 2011; Noseda et al. 2015).

The cardiac resident Sca-1 positive cells lack haematopoietic stem cell markers (CD45 and CD34). Still, unlike c-kit positive cells, they do not spontaneously differentiate in vitro unless stimulated with chemicals (i.e. 5-azacytidine) or after being co-cultured with cardiomyocytes (Wang et al. 2006). When Sca-1^+ CPCs were treated with 5-azacytidine, they expressed α-sarcomeric actinin, cardiac troponin, Nkx2.5 and α-MHC (Oh et al. 2003). Moreover, spontaneous contractions of these cells were induced with the addition of oxycontin (Matsuura K et al. 2004). Sca-1^+ CPCs have also been shown to differentiate into smooth muscle and endothelial lineages (Iwakura et al. 2011; Noseda et al. 2015). In murine heart Sca-$1^{+,}$ CPCs were shown to contribute to the generation of new cardiomyocytes, including after injury to the heart (Uchida S et al. 2013).

The use of Sca-1 to identify and isolate CPCs is promising; however, Sca-1 does not share the homology with other species, including human. Hence, restricting any

potential translational research to enhance human myocardial regenerative therapies. It has been shown that c-kit^{pos} CPCs also express Sca-1 and comparable numbers of c-kit^{pos}CD45^{neg}CD31^{neg} and Sca-1^{pos}CD31^{neg} cell populations have been reported (Smith et al. 2014) along with self-renewing, clonogenicity and differentiation potential in vitro and in vivo (Vicinanza et al. 2017). Hence it would appear that they are of the same cell population, and their expression of the surface markers vary according to their physiological/differentiation state.

Cardiac side population (SP) cells are closely related to Sca-1 cells and are identified in their ability to release Hoechst 33342 using Abcg2 transporter. It has been shown that around 80-90% of the SP cells are Sca-1$^{+,}$ and only 1% of Sca-1^{+} are SP cells (Unno K et al. 2012).

Despite Sca-1's lack of human homology, van Vliet et al. (2008) showed a rat anti-mouse Sca-1 antibody was able to bind a homogenous population of cells isolated from adult human atrial biopsies. This population, named as human cardiomyocyte progenitor cells, had the ability to self-expand, they express early myogenic transcription factors such as GATA-4, Mef2c, Isl-1, and Nkx-2.5 and differentiate into mature cardiomyocytes upon stimulation with 5-azacytidine in-vitro (Smits AM et al. 2009). It has also been suggested that Sca-1 $^{+}$ 'very small embryonic-like stem cells' (VSELs) in mice are the same population that express CD133 in humans (Zuba-Surma EK et al. 2011).

1.2.3. Cardiosphere-derived cells (CDCs)

Cardiosphere derived cells (CDCs) have the potential to regenerate myocardium (Messina et al. 2004; Smith R et al. 2007). CDCs form multicellular clusters (20–150 μm cellular spheres) from clonally derived cells in suspension and can contribute to multiple lineages (Messina E et al. 2004). The assessment of functional outcome following CDCs transplantation into the murine model post MI show better ischemic tissue protection with the enhanced remodelling with reported functional benefits (Carr CA. et al. 2011; Li at al. 2012). Also, upon stimulation with 5'-azacytidine and TGFβ1 in vitro, CDCs were shown to have the capabilities to differentiate into endothelial cells (Perbellini, F et al. 2015). A recent study by Gallet et al. (2016)

assessed the potential of allogeneic rat CDCs to decrease the effects of HFpEF amongst hypertensive rats. Hypertension was induced to dhal fed rats with a high salt diet for 7 weeks, and treatment was randomised to CDCs vs control (saline) group. Rats treated with CDCs normalised LV relaxation and LV diastolic pressures (17±10mmHg vs 9±4 mmHg in control, P=0.015 and 8±3mmHg in CDC, P=0.002).

CDCs have in fact been isolated from various species including humans by several groups (Messina E et al. 2004; Smith RR et al. 2007; Davis DR et al. 2009; Mishra R et al. 2011). Messina E et al. (2004) showed CDCs derived from humans myocardium to express c-kit. Furthermore, it has been shown that CDCs can be isolated from all four chambers of the human heart (Mishra R et al. 2011). In addition, the CDCs derived from humans are clonogenic and can self-renew (Messina E et al. 2004; Bearzi et al. 2007). Several clinical trials have evaluated the potential of CDCs, including ALCADIA and TICAP. In the ALCADIA trial, the CDCs were expanded from endomyocardial biopsies obtained during CABG. The investigators used a hybrid therapy with scaffolds. The cultured CDCs were injected intracoronary. The trial showed a 12.1% increase in EF at 6 months, with a 3.3 % reduction in infarct size. The TICAP trial (Ishigami S et al. 2015) enrolled 7 patients, and CDCs were obtained from the RA of patients. The CDCs were expanded and injected via intracoronary, one month following surgery. They were able to show an increase in EF from 46.9% to 54.0% (P = 0.0004) compared to control, 46.7% to 48.7%. These studies demonstrate that autologous CDCs are safe and could be effective in treating dysfunctional myocardium.

1.2.4 ADLH high cells

In 2013 Koninckx et al. described progenitor cells isolated from human atrial appendages based on high aldehyde dehydrogenase activity (ALDH). ALDH is an enzyme that metabolises the aldehydes related to their carboxylic acids and aids resistance to alkylating agents. These progenitor cells were called cardiac atrial appendage stem cells (CASC) and were isolated from right atrial appendages from patients undergoing cardiac surgery using the enzymatic technique. These cells are ALDH+CD34+CD45- and negative for c-kit. Around 30% of these cells are positive for platelet-derived growth receptor alpha (PDGFαr), and surprisingly all expressed Isl-1. The frequency of CASCs within the RAA of the human heart is low; 0.9 ± 0.8% total heart cell population. Characteristically these cells were mononuclear with broad cytoplasm with little vacuoles and demonstrated morphological differences to c-kit^{pos} CPCs. A single cell sort experiment showed a clonogenicity rate of 16% and they expressed pluripotency-associated genes. When subjected to medium enriched with growth factors stimulating HSC cell growth; CASCs did not grow. Also, compared to BM derived ALDH+ cells, the CASC cells failed to differentiate into either adipogenic or osteogenic lineages when incubated in identical culture settings. These properties suggest that CASCs are different to that of BM derived ALDH cells. When CASCs were co-cultured with primary cultures of neonatal rat cardiomyocytes, they showed a superior cardiac differentiation, compared to that of CDC-derived cells. Furthermore, the electrophysiological properties of the differentiated cells derived from CASCs were comparable to mature cardiomyocytes and were capable of generating action potentials (Koninckx R et al. 2012). Intramyocardial injection of these cells showed successful engraftment, preserved LV function, improved cell viability and cardiac differentiation in a minipig MI model.

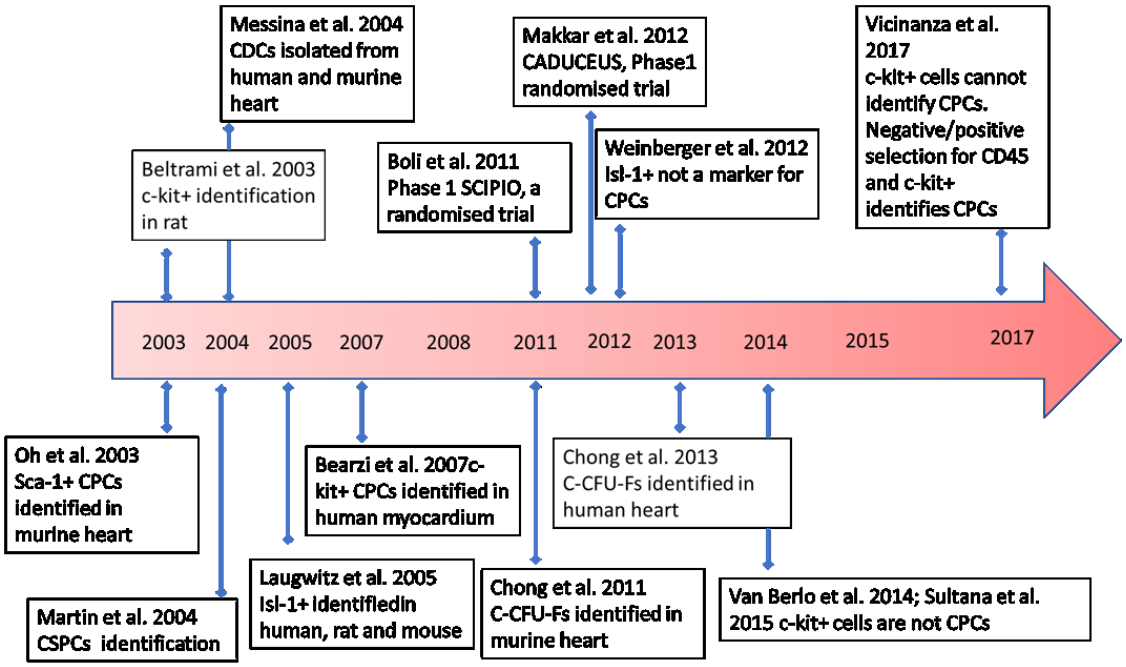


Figure 1. 6 Timeline of landmark events in cardiac CPC history.

The current evidence to date clearly shows that human myocardium harbours endogenous cardiac progenitor cells (Table 1.1). These cells are multipotent, clonogenic and capable of self-renewal in both vitro and in vivo; therefore, ensuring myocardial regeneration remains a real possibility. However, it is important to isolate and characterise these cells in accordance with specific phenotypes that have been described, including negative for markers of blood lineage such as CD34, CD45 and CD31.

1.3 Isolation of CSCs from human myocardial samples

Mainly two isolation methodologies have been employed to extract CPCs from both human and murine hearts (Beltrami AP et al. 2003; Messina E et al. 2004, Cesselli D et al. 2011). These include both enzymatic digestion and explant techniques; following which cells are isolated based on transcriptional or surface markers. The weight of the sample received contributes to the choice of either enzymatic digestion and explant methodology (Table 1.1).

1.3.1. Enzymatic digestion

In enzymatic digestion, the tissues are exposed to various enzymes such as trypsin and collagenase to breakdown and release the small cells deep within the myocardial tissue. The dissociated cells are then purified to isolate CPCs. The enzymatic digestion of myocardial tissue and the isolation of CPCs using magnetic-activated cell sorting (MACS) or Flow cytometry activated cell sorting (FACS) based on specific markers; led to the identification of various progenitor cells including that of c-kit^{pos} CPCs (Bearzi et al. 2007; Beltrami AP et al. 2003).

This process was dependant on the sample size; any samples of less than 30 mg were not suitable for FACS analysis (Bearzi C et al. 2007). Hence many studies have used enzymatic approach for isolation of CPCs from human myocardial samples; mainly obtained from explanted hearts or atrial appendages.

Retrograde perfusion of the heart containing enzymes has been successfully employed to isolate CPCs from whole rodent hearts (Torella D et al. 2004; Ellison GM et al. 2007; Smith A et al. 2014). In this technique, the aorta is cannulated, and the retrograde perfusion allows removal of blood, perfuse and penetrate enzymes thorough the entire tissue to yield effective digestion. Hence, the retrograde perfusion methodology allows optimal enzymatic digestion across the whole heart, allowing maximum dissociation of CPCs from deep within the myocardium. Since the entire heart is required for retrograde perfusion technique; it is impractical to use it to isolate human CPCs. Another drawback is over-digestion which has been shown to damage surface markers (Levesque JP et al. 2003; Mishra R et al. 2011) and can

cause internalisation of the cell surface receptors, like c-kit, leading to lower yield (Smith A et al. 2014).

Table 1.1 Types of CPC isolated using both enzymatic digestion and explant techniques and their phenotypes

0	Species	Site of Biospy	Weight of sample	Culture Technique		Phenotype	Frequency	Clonogenic	Multipotent	Tested in in vivo regenerative assay
				Enzymatic	Explant					
Beltrami et al.(2007)	Patients undergoing cardiac surgery	na	~20-100 mg	Yes-Collagenase	Yes	Lin-neg CD45neg CD34neg c-kitpos	1.1 ± 1.0% of the entire cell population and 1.6 ± 1.4% from explant derived cells	Yes.	Gave rise to 3 different cardiac lineages(myocytes., endothelial, smooth muscle),	Yes., injected cKitPOScells gave rise to myocyles., coronary arterioles and capillaries.
Arsalan et al. (2012)	Human- adult	LA/RAA/LAA	0.36 ± 0.09 g.	collagenase IV and trypsin	no	c-kitpos and lineageneg	(RA 4.80 ± 1.76% versus LA 4.99 ± 1.69% of isolated MNCs	na	na	na
Torella et al. (2006)	Human- adult	All four chambers	na	no	yes	Lin-neg CD45neg CD34neg/ CD 31neg c-kitpos	na	yes	Gave rise to 3 different cardiac lineages(myocytes., endothelial, smooth muscle), -	na
Messina et al. (2004)	Transgenic mice. 1-month to 80 year old humans.	atria and ventricle	na	no	yes-partial digestion for 5min x3 with 0.2% trypsin (Invitrogen) and 0.1% collagenase IV	CS, CD34, CD31, c-kit,	~10% of cardiac cells	Yes., Cardiosphere formation.	Endothelial and myocyte differentiation in vitro and in vivo.	Yes., gave rise to new functional myocardium in infarcted myocardium.
Steele A et al 2012	Human- adult	RAA	0.21-10.9g	no	suspension explant culture (SEC)	c-kit	na	Yes	Gave rise to 3 different cardiac lineages	na
Smith R et al 2007	Human- adult	right ventricular septal wall	21.0±1.9 mg	no	yes-partial digestion and explanted on fibronectin coated plates	CD105, CD90, CD34, c-kit,	na	Yes., Cardiosphere formation.	Gave rise to 3 different cardiac lineages	Yes., gave rise to new myocardium in infarcted myocardium.
Smits AM et al 2009	Human- adult/Foetal	na	na	Yes-Collagenase		c-kit,Sca1 (like),CD105,CD31,	na	yes	Differentiate into CM	
vanBuyn J et al 2007	Human-adult	epicardium derived from RAA	na		Yes	CD44,CD90,CD105,	na	na	Yes	na
Limana et al 2007	Human-adult	epicardium	na	Yes-Collagenase		c-kit, MDR-1,CD34,GATA-4,CD44,CD90,CD105	na			Yes., gave rise to new myocardium and vascular cells
Monsanto MM et al 2017	Human-adult	na	na	Yes-Collagenase		c-kit, CD90,CD105, CD133	na	yes	na	na
Cesselli D et al 2011	Human-adult	atrial	3-6g	Yes	Yes	c-kitpos and lineageneg, OCT3/4, mNOG, KLF-4, SOX2	na	yes	yes	na
van Vliet P et al 2008	Human- adult/Foetal	Atrial	na	Yes-Collagenase		CD105, Sca-1pos.,	na	yes.,	Differentiate into CM	na

1.3.2 Explant culture

The explant culture technique is an alternative approach to extract CPCs. In this methodology, fragments of myocardial tissue are explanted in culture media, and the cells which migrate out of the tissue are expanded and sorted for CPCs by surface marker expression.

The first isolation of adult human CPCs from the atria and the ventricle using explant culture technique was successfully reported in 2004 by Messina et al. Tissues were initially minced and subjected to brief enzymatic digestion (0.2% trypsin and 0.1% collagenase IV) before cultured as explants in complete explant medium. The cells isolated by FACS were c-kit^{pos} and were clonogenic, expressed stem and endothelial progenitor cell markers. Cells grown as outgrowth cells from the tissue explants were able to form cardiospheres in suspension culture.

This technique was refined to improve the efficiency by Smith R et al. (2007), during which partial enzymatic digestion was introduced prior to explanting on culture dishes coated with fibronectin. The biopsies were obtained percutaneously from the right ventricular septal wall. The biopsies from 70 patients had an average weight of 21.0±1.9 mg. The CDCs derived from the isolated cells differentiated into cardiac lineages and expressed antigenic properties of stem cells. When CDCs were injected into the border zone of the infarcted area in the SCID mouse, they were able to engraft and migrate into the infarcted zones; with the percentage of viable myocardium being more significant in CDC group, compared to the fibroblast-treated group (Smith R et al. 2007).

Bearzi et al. (2007) explored the conditions needed to isolate and expand human c-kit^{pos} CPCs from myocardial samples. The group compared both enzymatic and explant culture techniques and the isolated cells were MACS sorted for c-kit and further characterised. The weight of the samples from 88 patients undergoing cardiac surgery varied from ~20-100 mg; which essentially determined the isolation process as samples less than 30mg was deemed insufficient for FACS analysis. The enzymatic digestion used enzymes, such as collagenase for dissociation; while in the explant culture, minced myocardium was seeded onto uncoated Petri dishes with media. The c-kit^{pos} cells from the enzymatic digestion was 1.1 ± 1.0% of the entire

cell population and 1.6 ± 1.4% from explant-derived cells. At P0 the lineage negative (Lin−) c-kit^{pos} cells obtained from enzymatically dissociated cells was 41 ± 14% of small cells, and GATA4-positive cells (early committed) was 59 ± 14%; the corresponding values were 52 ± 12% and 48 ± 12% for explant derived cell technique. They showed that cells isolated using both techniques maintained a stable phenotype and not reach growth arrest. When subjected to serum culture, human CPCs derived from both techniques, attached rapidly with sustained growth to P8 and did not show any signs of growth arrest during this period.

In addition to explant culture and enzymatic digestion, suspension explant culture (SEC) has also been introduced as a new culture methodology (Steele A et al. 2012). This paper assessed the SEC technique to isolate CPCs from atrial appendages of paediatric patients with end-stage HF. Overall, 25 patients were evaluated, with a mean weight of the sample being 1.19g (0.21-10.9g). The atrial appendage sample from humans was suspended in a high volume of media without exposing them to enzymes. This suspension of biopsies reduced the fibroblast outgrowth that occurs as a result of contact with a substrate (Steele A et al. 2012). Cells isolated were shown to be c-kit^{pos} and showed differentiation capabilities to cardiac lineages through the expression of flk-1^{positive}, smooth muscle actin^{positive}, troponin-I^{positive}, and myosin light chain^{positive} markers.

Various groups have successfully isolated CPCs using either method as described above. Each technique has its own advantages and disadvantages; however, the choice of methodology is largely dictated by the sample size, with a smaller sample not being suitable for enzymatic digestions.

1.4 Heart Failure and its treatment

The European Society of Cardiology (ESC) defines heart failure (HF) as a clinical syndrome with typical symptoms, associated with clinical signs due to structural and/or functional cardiac abnormalities that impair the ability of the ventricle to fill or eject blood, therefore reducing cardiac output.

1.4.1 Global incidence of heart failure

The prevalence of heart failure (HF) is rising due to ageing populations and improved survival following advanced therapeutic availabilities. However, the mortality rate amongst the HF patients, remain woefully high (Ziaeian B et al. 2016). This epidemic problem contributes to significant morbidity, mortality and contributes to climbing healthcare expenditure. It is estimated that there are about 38 million people with HF worldwide and 1-2% of the total healthcare expenditure is attributed to HF in Europe and North America (Ziaeian B et al. 2016). According to the British Heart Foundation, about 1-2 million people are living with HF in the UK alone. The incidence of the HF, along with the prevalence, has been shown to increase with age (Bleumink GS et al. 2004, Lloyd-Jones DM et al. 2002). The lifetime risk of developing HF amongst both men and women at the age of 40 was estimated to be one in five by the Framingham study. In addition, this study also found one in nine men and one in six women were at risk of developing HF over their lifetime without myocardial infarction (MI) being the aetiology of their HF. A large prospective cohort study involving over twenty thousand men examined the relationship between modifiable lifestyle factors and the lifetime risk of heart failure. This study showed the lifetime risk of developing HF was around 13.8% at the age of 40 and demonstrated that maintaining a healthy lifestyle is associated with a lower lifetime risk of HF development (Djoussé L et al. 2009).

HF can occur in patients with either preserved or a reduced left ventricular ejection fraction (HFpEF or HFrEF, respectively). The PREVEND cohort study assessed the incidence between HFpEF and HFrEF over an eleven and a half year period. During this time, 4.5% were diagnosed with HF, of which 34% had HFpEF, and 66% had HFrEF (Brouwers FP et al. 2013). In addition to this, HFpEF is more common in the young black population. The CARDIA study prospectively examined the incidence of

HF between blacks and whites of both sexes (18 to 30 years of age) over 20 years. This study concluded that black people were significantly at higher risk of developing HF before the age of 50 when compared to the white population (Bibbins-Domingo K et al. 2009).

Overall survival following the diagnosis of HF remains poor. Once HF is diagnosed, the survival rates are 50% and 10% at 5 and 10 years, respectively (Ziaeian B et al. 2016). In addition, HFpEF has been shown to confer better prognosis compared to HFrEF cohorts (8-9 % vs 19% respectively; Bursi F et al. 2006). Another meta-analysis of patients with HF found an overall 30% reduction in mortality amongst patients with preserved LV compared to reduced LV function (Berry C et al. 2012).

1.4.2 Pathophysiology of heart failure

Cardiac output (CO) is the amount of blood pumped by the heart over a given period. This is affected by heart rate (HR) and stroke volume (SV (the amount of blood ejected by the ventricle per heartbeat, which is approximately 1 cc/kg)) and is usually 4–8 L/min (Mohrman DE et al. 2010). Furthermore, the competency of the valves, ventricular contraction and wall integrity also contribute to CO. In turn, the contractility, preload and afterload of the heart affect the SV. These important and complex relationships primarily contribute to maintaining good synchronicity and cardiac function. Therefore, the failure of the heart to contract effectively (Systolic dysfunction) and abnormal relaxation (diastolic dysfunction) contributes to the development of HF. Although it is easier to consider the term systolic and diastolic dysfunction to understand the underlying pathophysiology of HF, the two processes are very closely related. The diastolic heart failure is also denoted as heart failure with preserved ejection fraction (HFpEF), and the systolic heart failure signified as heart failure with reduced ejection fraction (HFrEF). There are distinctive features such as epidemiologic factors, morphologic differences of the LV, as well as differences in the cardiomyocytes and extracellular matrix that defines both HFrEF and HFpEF in its entirety (Quiñones MA et al. 2006; Aurigemma GP et al. 2006; Drazner MH et al. 2011).

1.4.2.1 Normal LV function

In HFpEF, the LVEF is typically normal and usually characterised by abnormal diastolic function. Often in this group, the LV undergoes hypertrophy with concentric remodelling (Zile MR et al. 2001; Baicu CF et al. 2005) (Figure 1.7). An injury to the myocardium, such as MI can convert HFpEF to HFrEF; otherwise, such translation is uncommon (Desai RV et al. 2011 and Hwang SJ et al. 2014).

The diastolic dysfunction describes the mechanical abnormalities of the ventricle, resulting in impaired relaxation and filling in diastole. This occurs irrespective of the EF and may or may not contribute to symptoms. Importantly it has been shown that diastolic dysfunction is a risk factor for developing HFpEF (Borlaug BA et al. 2013).

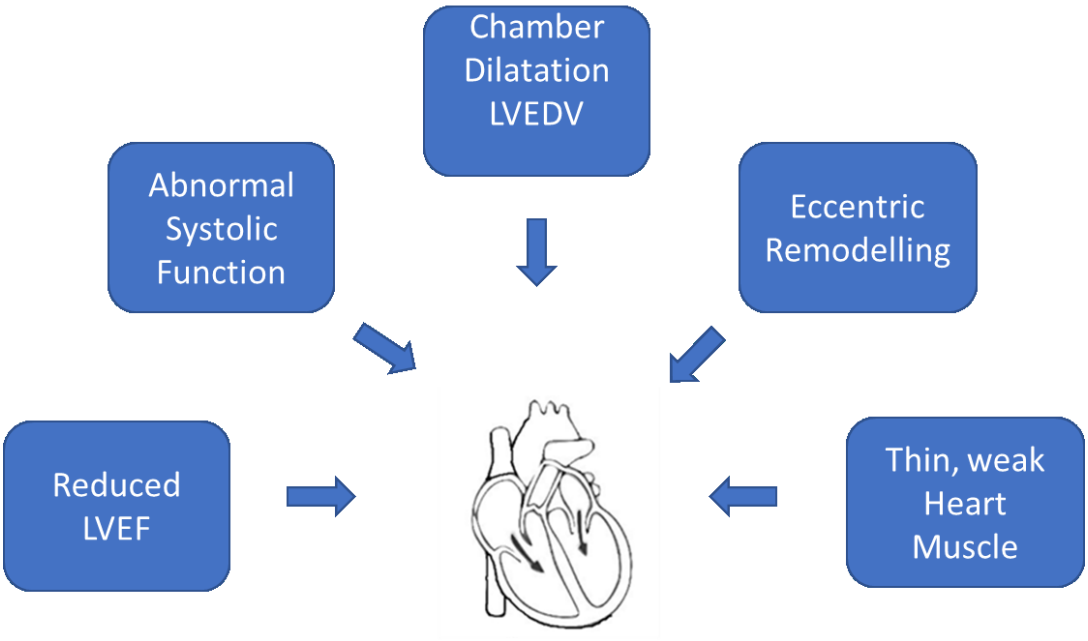
Patient's heart with HFpEF undergoes considerable structural remodelling affecting the cardiomyocytes and the extracellular matrix; therefore contributing to the overall different morphological features of both the LV and LA. Thus, altering the functionality of the heart and contributing to the symptoms experienced by the patient. In this group, the LV undergoes concentric remodelling associated with hypertrophy, and characteristically the end-diastolic volume is normal with an increased relative wall thickness with an associated increase in LV mass. (Quiñones MA et al. 2006; Aurigemma GP et al. 2006; Drazner MH et al. 2011; Lam CS et al. 2007 and Melenovsky V et al. 2007).

Microscopically, the cardiomyocytes typically have a large diameter with minimal change in length, hence the increased wall thickness but little changes to chamber volume. Also, increased collagen deposition and fibrillation in the extracellular matrix component were seen (Aurigemma GP et al. 2006; Zile MR et al. 2015). The interstitial fibrosis is also a recognised a feature but many patients do not characteristically have these (Mohammed SF et al. 2015).

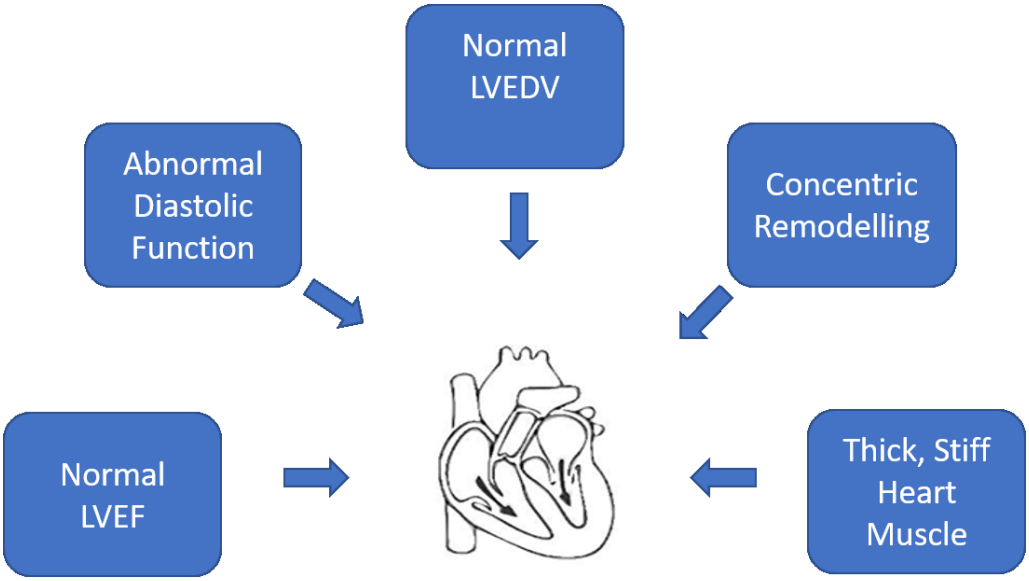
1.4.2.2 Impaired LV function

In the HFrEF (Figure 1.7) group, the EF is impaired with dilatation of cardiac chamber, increased end-diastolic volume and eccentric remodelling (Yancy CW et al. 2013 and Ponikowski P et al. 2016). However, there is little change in wall thickness but can be explained microscopically. In HFrEF the cardiomyocytes are

elongated with no changes to the diameter. Hence, while there are no changes to wall thickness, but there is an increase in LV volume (Quiñones MA et al. 2006; Aurigemma GP et al. 2006; Lam CS et al. 2007 and Melenovsky V et al. 2007). At the early stages, there is a breakdown and disruption of the collagen. During the later stages, the fibrotic changes along with subsequent scaring, contribute to the decreased collagen deposition (Aurigemma GP et al. 2006 and van Heerebeek L et al. 2006).



HF with reduced EF (HFrEF)



HF with preserved EF (HFpEF)

Figure 1. 7 The characteristics of HFpEF and HFrEF

1.4.3 Current treatment of heart failure

At present, the management of HF is mainly focused on symptomatic relief and limiting disease progression via pharmacology and limited surgical therapies. These strategies do not lead to repair or restoration of healthy cardiac tissue and function (Deedwania and Carbajal et al. 2011). For those with terminal HF, the only effective treatment option is cardiac replacement therapy in the form of heart transplantation, mechanical circulatory support and cell transplantation therapy (Akhmedov et al. 2012). Figure 1.8 shows the overall management of HF through stages. Due to the destructive nature of the disease, considerable efforts have been placed on research from all fronts to combat this. Before the 80s, in the non-pharmacological era, the treatment was mostly palliative; with the emphasis placed on bed rest and fluid restriction. However, the first pharmacological therapy was introduced in the mid-80s in the form of digitalis and diuretics. Also, the first Vasodilator Heart Failure Trial (V-Heft) (Cohn et al. 1986) was commenced. This trial showed a reduction in mortality when patients were treated with a combination of vasodilators such as hydralazine and isosorbide dinitrate; as compared with either placebo or prazosin (anti-hypertensive).

In the 90s, neurohormonal therapeutic agents such as Angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, and spironolactone were trialled and introduced to the existing pharmacological strategies. The CONSENSUS (Swedberg K et al. 1987) and SOLVD-Treatment (Yusuf S et al. 1991) trials reported an overall reduction in mortality (up to 40%) with the use of ACE such as enalapril. The SOLVD-Prevention trial (Yusuf S et al. 1991) showed that the use of Enalapril in asymptomatic patients with impaired EF reduced hospitalisation related to HF.

Beta-blockers are now a valuable addition to a cocktail of various pharmacological drugs, but at the beginning, the therapeutic effectiveness of the beta-blocker was questioned amongst patients with reduced ejection fraction and the side effects that arise from adrenergic blockade (Chana A et al. 2014). However, the U.S. Carvedilol Heart Failure Study (Packer M et al. 1996), and COPERNICUS trial (Packer M

2001), both showed conclusive evidence that use of carvedilol contributed to a reduction in mortality.

Mineralocorticoid-receptor antagonist (MRA) such as spironolactone and eplerenone are essential drugs used in the management of heart failure. The evidence from both RALES trial (Pitt B et al. 1999) and the EMPHASIS-HF trial (Zannad F et al. 2011), which assessed the efficacy of spironolactone and eplerenone respectively; showed an overall reduction in mortality of 30% when used in combination with an ACE inhibitor and a loop diuretic.

In 200, In addition to this multi-cocktail drug therapy, cardiac devices were introduced and evaluated as an adjunct to the current treatment of HF. Several types of devices such as left ventricular assist device (LVAD), the implantable cardioverter-defibrillator (ICD) and biventricular pacemakers, CRT; have been used, and several trials showed a reduction in mortality. The LVAD/BIVADs are mainly used as a bridge to transplant and in few patients used as destination therapy (REMATCH (Rose EA et al. 2001), COMPANION (Bristow MR et al. 2016), RAFT (Tang ASL et al. 2010). In some patients, despite the current treatment modalities, heart transplant remains the only option.

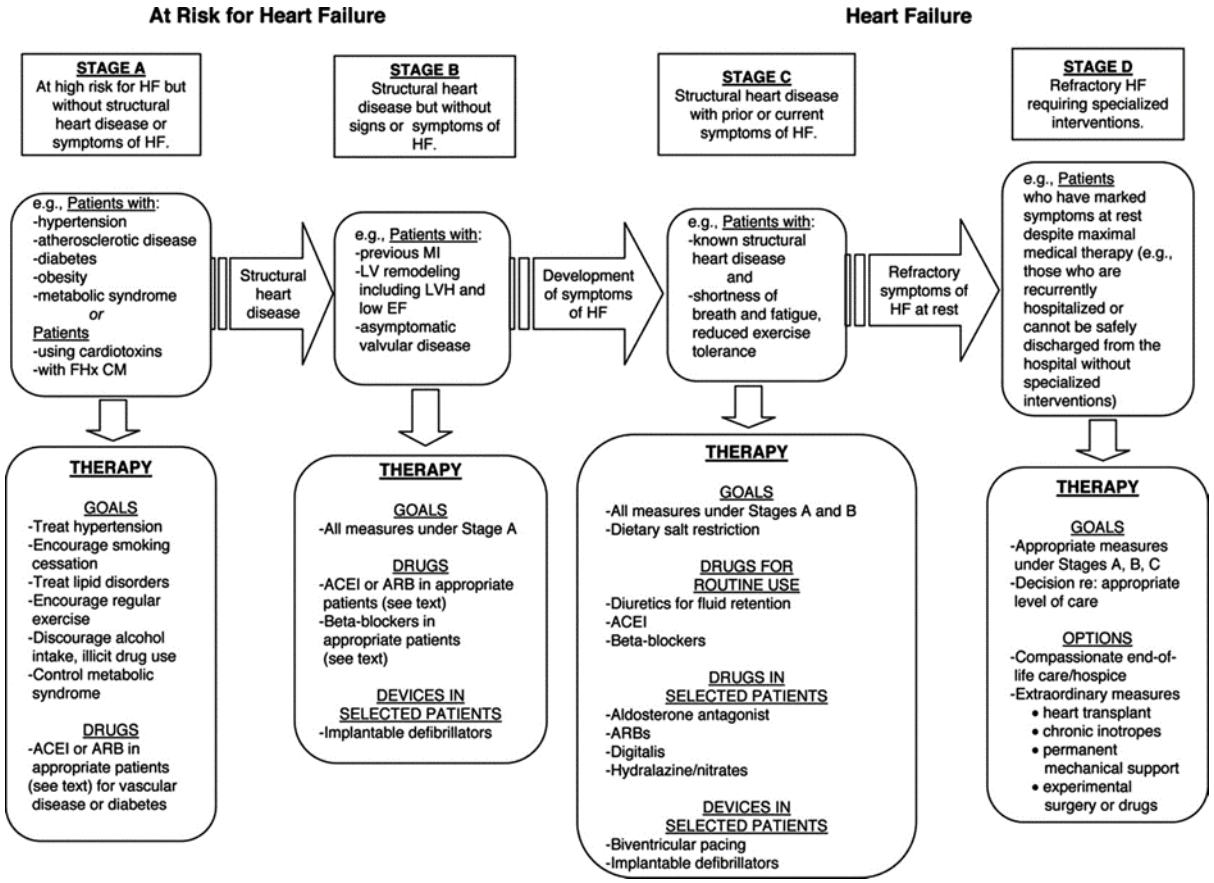


Figure 1. 8 Overall management of HF through stages. Adapted from NICE guidelines

1.4.4 Cellular therapies for the treatment of heart failure

Cell transplantation therapy is a way to prevent or treat human disease by administration of cells that have been selected, multiplied and pharmacologically altered outside the body (Preston et al. 2003; Mason and Dunnill et al. 2008). Once successful, cell transplantation therapy has the potential to be administered early post-cardiac injury to the myocardium to stimulate sufficient myocardial regeneration and prevent the onset of end-stage heart failure.

The idea that resident cardiac stem cells would be the best cell type to repair a myocardial infarction is logical. However, numerous studies focused on identifying suitable non-cardiac sources of cells for cardiac repair because of the long-held

belief that the mammalian heart is an organ with no intrinsic capacity to regenerate after myocardial injury. There have been many promises and challenges of using non-cardiac cells for transplantation as a potential therapy for heart diseases (Hare and Chaparro et al. 2008; Pallante and Edelberg et al. 2006). Indeed, several studies have investigated various cell types; such as skeletal myoblasts, bone marrow mononuclear cells, bone marrow-derived hematopoietic stem cells, mesenchymal stem cells and cardiac progenitor cells as potential cell types to treat patients post-MI and those with chronic HF (Sanganalmath SK 2013, Leong YY et al. 2017). Figure 1.9 shows the progression of cell therapy trials for HF to date.

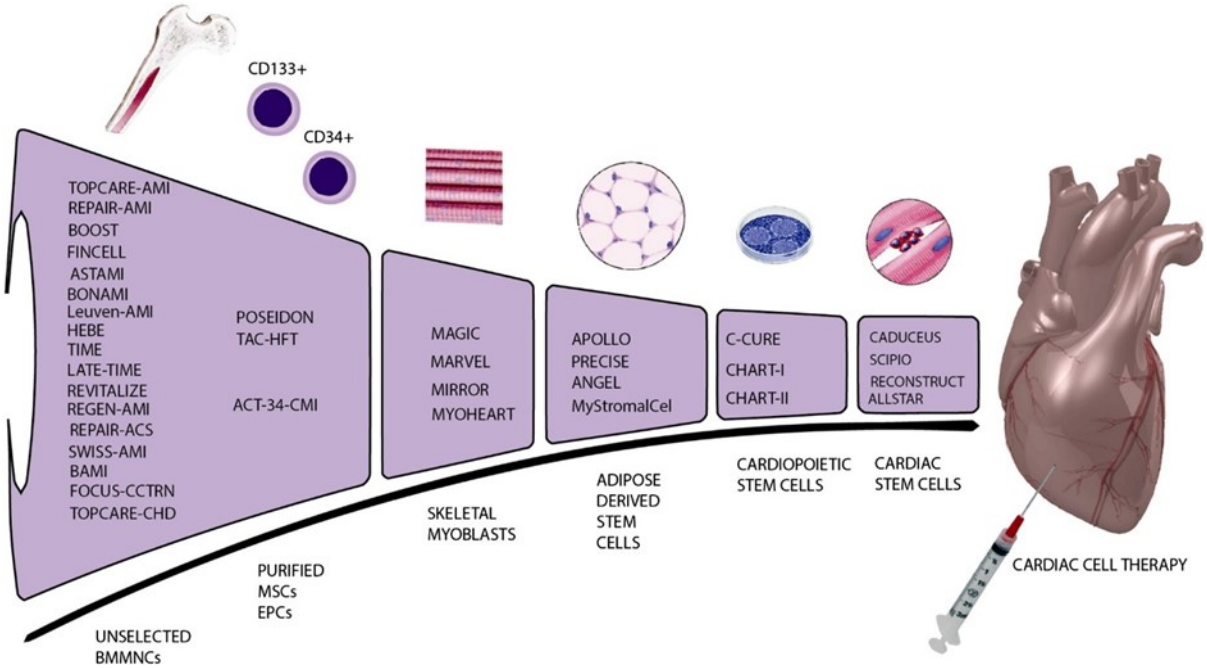


Figure 1. 9 Progression of cellular-based therapy to date. Adapted from Hastings et al. 2015.

1.4.4.1 Skeletal Myoblasts

The use of skeletal myoblasts in cardiac regeneration was explored almost 20 years ago in animal models post-infarction (Chiu R.C et al. 1995; Yoon P.D et al. 995 and Ghostine et al. 2002). In 2003, Menasche et al. performed the first human myoblast transplantation in patients with severe HF due to ischemia. In this study, about 870 million cells were injected into the scar region at the time of coronary artery bypass grafting (CABG). Despite showing functional improvements, the majority of the patients developed ventricular tachycardia. The subsequent multicentre clinical study (Magic Trial) had skeletal myoblasts injected (400 or 800 million; n=33 and n=34, respectively) in and around the scar site; in 97 patients. The skeletal myoblasts were harvested from the patient's skeletal muscle, purified and expanded before injection. All patients were fitted with a defibrillator. The trial showed an increased uptake on PET scan with an improvement of left ventricular ejection fraction (LVEF) on echocardiogram; however, the trial was terminated due to malignant ventricular arrhythmias (Menasche P et al. 2008).

1.4.4.2 Bone marrow-derived cells (BMDCs)

Most clinical trials have used bone marrow-derived mononuclear cells (BMMNCs). These trials have shown BMMNCs to be safe, but their efficacy has been less than convincing. In a mouse model, when lineage negative c-kit^{pos} (Lin-c-kit^{POS}) cells obtained from the bone marrow were injected into the peri-infarcted myocardium; they were shown to improve contractility, reduce infarct size and form new cardiomyocytes. This study reported a 70% reduction in infarct size and showed new cardiomyocytes formation in excess of 50%. Also, the left ventricular (LV) end-diastolic pressure (LVEDP) was shown to be 36 % lower in Lin-c-kit^{POS} group (Orlic.D. et al. 2001). However, a further study by Murry et al. (2004) showed that Lin^{neg}c-kit^{POS} BMDC's were not capable of transdifferentiating into cardiomyocytes.

The results from the BMDC clinical trials (Table1.2) where BMDC have been transplanted into HF patients have been varied. The trials were randomized, placebo-controlled and often multi-centred. The REPAIR-AMI (Schächinger v. et al. 2006) trial was the first randomised, double-blinded study to recruit more than 200

patients and give an intracoronary infusion of autologous BMMNCs or placebo. This trial demonstrated a significant increase in LVEF compared to the placebo group (5.5% vs 3 %; p>0.01) at six months. As with BOOST trial (Meyer G.P et al. 2006 et al.), this trial also showed more significant benefit in patients with LVEF <50%. However, the five years follow up from these studies has not been that conclusive; this is primarily due to variety of study design, patient selection and differences in the number of cells, phenotype of cells and mode of cell delivery amongst studies. In addition, the BMMNC is primarily made up of early committed cells, and only a fraction of these are hematopoietic stem cells (2%), EPCs (4%), and MSCs (0.01%) (Dimmeler S et al. 2009). This heterogeneity of cell type, number and the subsequent viability may also affect the therapeutic potential of the BMMNCS (Nguyen P et al. 2016). The postulated mechanism of their action is that of a 'paracrine' effect on the recipient's myocardial cells (Ellison et al. 2012). The paracrine effect refers to a mechanism whereby cardiac repair and regeneration is stimulated from the factors released from transplanted cells. These factors act in a paracrine fashion to induce neovascularisation, protect cells, improve survival and orchestrate regeneration via activation of endogenous cardiac stem/progenitor cells.

Moreover, clinical trials such as The Late TIME randomised trial (Traverse et al. 2011) failed to demonstrate improvement in cardiac function or symptoms in patients post-myocardial infarction following intracoronary delivery of autologous BMCs. In this trial, 150 million cells were infused and compared against the placebo group. Between the two groups, there was no significant change in LVEF at six months when compared to baseline (48.7% to 49.2% vs 45.3% to 48.8%). However, a recent systematic review and meta-analysis (Fisher et al. 2014) evaluated the clinical safety and efficacy of autologous adult bone marrow-derived stem cells (BMSC) as a potential therapy for chronic IHD and heart failure. This analysis highlighted evidence that BMSC treatment improves LVEF but showed only a small clinical benefit in long term performance status and mortality; concluding cell therapy to be safe. Importantly this analysis suggested that cell therapy is more beneficial to patients with IHD and HF than those with acute MI (Fisher et al. 2014).

Table 1. 2 BMDC based therapy in acute myocardial infarction (Adapted from Nguyen P et al 2016).

Trial	Year	Pathology	Design of the Trial	No of Pt's	Types Cells Used	Cell Harvest Site	Mode of Delivery	Follow up Period months	LVEF
TOPCARE-AMI	2002	Acute MI s/p PCI	non-randomized, matched control	31	autologous BMMNCs/PBSCs	BM iliac crest	IC	4	Improved from 51.6 ± 9.6% to 60.1 ± 8.6%
BOOST	2004	STEMI s/p PCI	randomized, open-label, single-center	60	autologous BMMNCs	BM iliac crest	IC	18	No change
LEUVEN-AMI	2006	STEMI s/p PCI, LV dysfunction	Randomized, DB, PC	67	autologous BMMNCs	BM iliac crest	IC	4	No change

Trial	Year	Pathology	Design of the Trial	No of Pt's	Types Cells Used	Cell Harvest Site	Mode of Delivery	Follow up Period months	LVEF
REPAIR-AMI	2006	Acute MI s/p PCI, LVEF< 45%	Randomized, DB, PC	204	autologous BMMNCs	BM iliac crest	IC	4	Increase in EF by 2.5% above baseline
ASTAMI	2006	Ant STEMI	randomized, open-label, single-center	97	autologous BMMNCs	BM iliac crest	IC	6	No change
FINCELL	2008	STEMI s/p thrombolysis + PCI	Randomized, DB, PC	80	autologous BMMNCs	BM iliac crest	IC	6	Increase in EF by 5% above baseline
BONAMI	2011	STEMI s/p PCI, LVEF <45%	Randomized, DB, PC	101	autologous BMMNCs	BM iliac crest	IC	3	No change

Trial	Year	Pathology	Design of the Trial	No of Pt's	Types Cells Used	Cell Harvest Site	Mode of Delivery	Follow up Period months	LVEF
LATE-TIME	2011	Acute MI s/p PCI, LVEF< 45%	Randomized, DB, PC	87	autologous BMMNCs	BM iliac crest	IC	6	No change
TIME	2012	anterior STEMI s/p PCI, LV dysfunction	Randomized, DB, PC	120	autologous BMMNCs	BM iliac crest	IC	12	No change
SWISS-AMI	2013	Acute MI s/p PCI	randomized, 3-arm trial, DB, PC	192	autologous BMMNCs	BM iliac crest	IC	12	No change

Trial	Year	Pathology	Design of the Trial	No of Pt's	Types Cells Used	Cell Harvest Site	Mode of Delivery	Follow up Period months	LVEF
TECAM	2015	STEMI s/p PCI	randomized, open-label, SB,PC	120	autologous BMMNCs, G-CSFs, both	Autologous	IC	12	No change

MI: myocardial infarction, SB: single-blinded, DB: double-blinded, PC: placebo-controlled, BM: bone marrow, BMMNCs: bone marrow derived mononuclear cells, MSCs: mesenchymal stem cells, ADSCs: adipose-derived stem cells, CDCs: cardiosphere-derived cells, IC: intracoronary, TEN: trans-endocardial, SM: skeletal myoblasts, TEP: transepicardial, AR: arrhythmogenicity

1.4.4.3 Mesenchymal Stem Cells (MSCs) and Endothelial Progenitor Cells (EPCs)

Both Mesenchymal Stem Cells (MSCs) and Endothelial Progenitor Cells (EPCs) are derived from the purification of bone marrow aspirate.

The human mesenchymal stem cells (hMSCs) and BMNCs populations were compared in the TAC-HFT trial for HF. This randomised controlled trial studied 65 patients with HF and compared hMSCs vs placebo; BMNCs vs placebo for a year. The study found these cells to be safe and showed some evidence towards reverse remodelling (a reduction in infarct size (-18.9%; 95% CI, -30.4 to -7.4, P = .004)) within the hMSCs group but not in BMNCs or placebo group. In addition to bone marrow, adipose tissue has also been used as a source for MSCs. The adipose tissue-derived MSCs have been implicated in several clinical trials (Table 1.2), including the APOLLO trial (Houtgraaf HJ et al. 2012). This randomised control trial assessed the relevance of adipose tissue-derived MSCs in patients with ST-elevation MI. In this study, 17 million MSCs were infused via the coronaries. At six months, they showed MSCs to be safe in a clinical setting and showed an improvement in cardiac function, perfusion and around 50% reduction in scar size (Houtgraaf HJ et al. 2012).

The role of EPCs was assessed by several trials (Table 1.3); including ACT-34-CMI trial (Losordo DW et al. 2011). In this randomised, double-blinded study; patients were given 1 of 2 doses (1×10^5 or 5×10^5 cells/kg of EPCs) of EPCs. A significant reduction in the frequency of angina and increased exercise tolerance was observed in the low dose group, compared to the placebo at both six and twelve-month intervals.

Table 1.3 Cellular based therapy in Chronic Myocardial Infarction (Adapted from Nguyen P et al 2016).

Trial	Year	Pathology	Design of the Trial	No of Pt's	Types Cells Used	Cell Harvest Site	Mode of Delivery	Follow up Period months	LVEF
Losordo et al.	2007	intractable angina, ischemia	Randomized, DB, PC	24	peripheral CD34+ after G-CSF × 5d	peripheral blood	TE	6	n/a
ACT34-CMI	2011	refractory angina, ischemia	Randomized, DB, PC	167	peripheral CD34+ after G-CSF × 5d	peripheral blood	TE	3/6	n/a

MI: myocardial infarction, SB: single-blinded, DB: double-blinded, PC: placebo-controlled, BM: bone marrow, BMMNCs: bone marrow-derived mononuclear cells, MSCs: mesenchymal stem cells, ADSCs: adipose-derived stem cells, CDCs: cardiosphere-derived cells, IC: intracoronary, TEN: trans-endocardial, SM: skeletal myoblasts, TEP: transepicardial, AR: arrhythmogenicity

1.4.4.4 Cardiac Stem/Progenitor Cells (CPCs or CSCs)

More recently, cardiac-derived stem/progenitor cells (CSCs or CPCs) have been used in clinical trials. SCIPIO a Phase I/II clinical trial (Bolli R et al. 2011) isolated c-kit+ CPCs from the right atrial appendage obtained from 16 patients with HF induced by ischemia, undergoing coronary artery bypass surgery and propagated them ex vivo (2 million cells per patient). Three to five months following surgery, 500,000 to 1 million CPCs were injected via intracoronary into infarcted areas, and the control was not given any treatment. The initial results after four months showed a significant improvement in LVEF (from 30.3% at baseline to 38.5%) when compared to the control group (30·1% [at four months after CABG). In addition, at one year there was an increase in LVEF by 12.3 EF units compared to baseline. The study also demonstrated a significant reduction in infarct size at four months and 12 months (by 7·8 g (24%) and 9·8 g (30%) respectively).

In addition to this a further phase I/II clinical trial, the CADUCEUS trial recruited 25 patients between 2-4 weeks following MI with LVEF between 25% to 45%. Patients had intracoronary stents to treat the blocked arteries. Patient's assigned to cardiosphere-derived cells (CDC) received autologous CDCs (12.5-25 million cells) generated from endomyocardial biopsy, 1.5-3 months post-infarct and cells were infused into coronary artery related to the infarct area. Over the 12 months, there was a significant reduction in scar size in CDC treated group (−11.1 ± 4.6%, $p < 0.001$ within-group, $p = 0.004$ between groups) and the infarcted hypokinetic zones showed recovery and improvement. However, the trial failed to show a significant change in LVEF in CDC-treated patients (5.4 ± 10.6%) vs control patients (5.8 ± 3.3%), an improvement NYHA functional class or quality of life. Of note, cardiosphere-derived cells are a mixed, heterogeneous population of cells and only ~1% has the characteristics of being defined as cardiac progenitor cells (Makkar RR et al. 2012) (Table1.5).

Table 1. 4 Cellular based therapy using MSCs and EPCs (Adapted from Nguyen P et al 2016).

Trial	Year	Pathology	Design of the Trial	No of Pt's	Types Cells Used	Cell Harvest Site	Mode of Delivery	Follow up Period months	LVEF
POSEIDON	2012	ICM, LVEF <40	randomized, NO placebo controlled	30	Autologous and Allogenic hMSCs[*]	EMBX	TEN	30d/12	No change
C-CURE	2013	ICM with LVEF <40%, NYHA> =2	randomized, DB, PC	36	Autologous Cardiopoietic hMSCs[*]	BM iliac crest	TEN	6	Increase in EF by 7% above baseline
PRECISE	2014	ICM with LVEF <45%, NYHA> =2	randomized, DB, PC	27	Autologous ADRCs	liposuction	TEN	36	No change
TAC-HFT	2014	ICM, LVEF <50%	randomized, DB, PC	59	Autologous BMMNC and MSCs	BM iliac crest	TEN	1	No change

Table 1.5 Cellular based therapy using cardiac progenitor cells (Adapted from

Nguyen P et al. 2016).

Trial	Year	Pathology	Design of the Trial	No of Pt's	Types Cells Used	Cell Harvest Site	Mode of Delivery	Follow up Period months	LVEF
CADUCEUS	2012	AMI s/p PCI LVEF <45%	randomized	25	Autologous CDCs	Endomyocardial	IC	6	No change

The vast amount of clinical studies has concentrated on BMDCs as the source for cell-based therapy. Still, it is becoming more evident from current literature that regeneration of functional myocardium requires a high level of coordination, cellular interaction between various cell types and extracellular matrix. Co-administration and transplantation of more than one cell have the potential to enhance myocardial regeneration than the use of single-cell line (Leong YY et al. 2017).

The promise of cell therapy remains a real possibility. The ongoing quest to find the best cell type and the necessary conditions to stimulate sufficient myocardial regeneration and prevent the onset of end-stage heart failure remains a significant challenge.

1.5 Alterations to CPC activity and potency in the aged and diseased human heart

The process of ageing imposes a physiological challenge to many organs, including the heart leading to the pathogenesis of HF (Dutta et al. 2012; North & Sinclair, 2012). The effects attributed to ageing is evident at both macroscopic and microscopic level. These changes alter the homeostatic function and response to injury within the heart (Oh J et al. 2014), therefore, increasing the risk of developing cardiovascular diseases (Lakatta EG. et al. 2003; North & Sinclair, 2012). The cellular changes that follow the above process contribute to the alterations of cardiac physiology leading to early diastolic filling, reduced ventricular filling with impaired cardiac output and subsequent development of HF (Lakatta EG. et al. 2003). Such significant physiological changes lead to myocardial structural changes, including atrial dilatations and ventricular hypertrophy. Vascular changes, such as arterial thickening and stiffness, also occur with ageing (Kusumbe AP. et al. 2016). These changes alter the endothelial structure leading to dysfunction and cause an abnormal response to vascular injury.

Alterations to CPC activity and potency are associated with ageing (Figure 1.10A), cardiovascular diseases (such as ischemic injury, cardiac hypertrophy and heart failure), metabolic disorders and both genetic and environmental factors (Chimenti C

et al. 2003; Dimmeler et al. 2008; Beltrami et al. 2011; Cheung T.H et al. 2013). Although the number of CPCs is increased in response to the above conditions; its potency remains questionable. This decline in CPC function is due to DNA damage, telomere shortening and external factors; which ultimately leads to impaired cardiac homeostasis (Cesselli D et al. 2017; Lewis-McDougall F et al. 2019; Cianflone E et al. 2019). The above intrinsic and extrinsic components alter cellular activities and induce senescence. The interaction of both mechanisms works synergistically and is orchestrated through a paracrine fashion (Beltrami AP et al. 2011). Figure 1.10B.

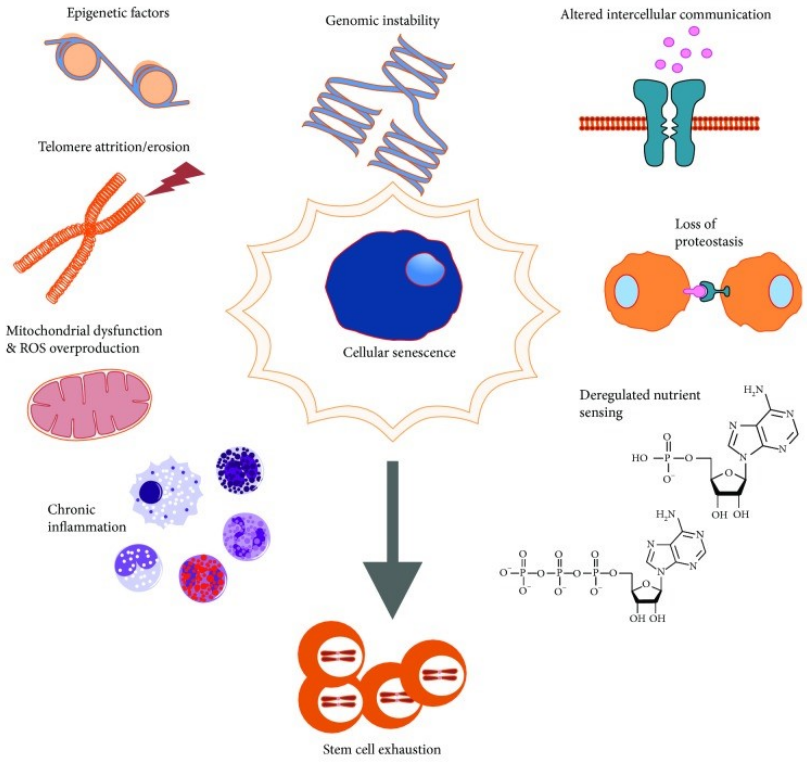


Figure 1. 10A Mechanisms associated with stem/progenitor cells senescence (Cianflone E et al. 2019)

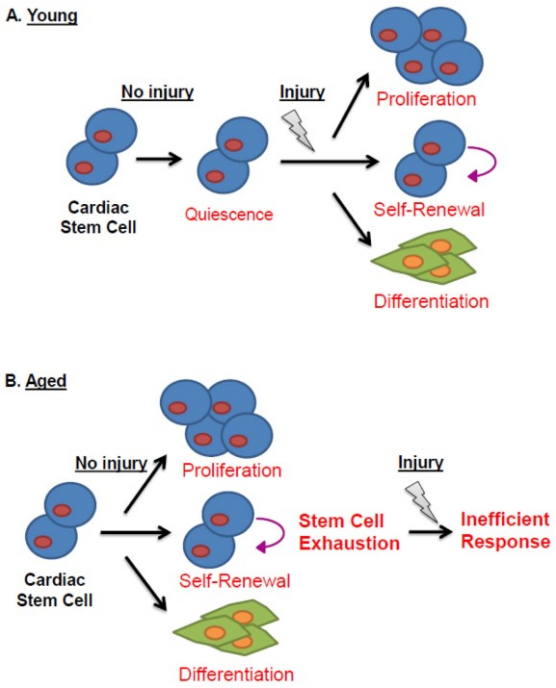


Figure 1.10B Characteristic features between young and aged cardiac stem/progenitor cells (adapted from Hariharan and Susman 2016).

Telomerase activity and length have been shown as key components in regulating stem cells (Sahin E et al. 2010; Cianflone et al. 2019). Telomerase is a reverse transcriptase enzyme that elongates the telomere, using its RNA molecules as templates (Blackburn EH et al. 2000). The truncation of telomeres beyond a critical length induces cells into senescence, therefore causing growth arrest at G1 phase (Cheung T.H et al. 2013). Once activated, this process of senescence appears to be a vicious cycle (Figure 1.11). The subsequent build-up of senescent and dysfunctional cardiomyocytes and CPCs leads to a decline in functional capacity to maintain cardiac homeostasis. The CPCs typically display DNA damage, shortened telomeres with reduced activity, high expression of cyclin-dependent kinase inhibitors (CDKIs), $p16^{INK4a}$ and p21Cip1 (Sharpless & DePinho, 2007; Chimenti et al. 2003; Torella et al. 2004; Urbanek et al. 2005; Kajstura et al. 2010; Cesselli et al. 2011; Baker et al. 2011). In a study (Cesselli et al. 2011); which evaluated the impact of ageing and CHF on CPCs, showed telomere shortening, reduction in CPCs telomerase activity, increased frequency of telomere-induced dysfunction foci within CPCs with higher expression of $p16^{INK4A}$ and p21CIP1. This study assessed 18 control patients and 23 explanted CHF hearts. The disease was shown to impair the equilibrium between the cardiomyocytes pool and non-senescent CPCS, therefore enhancing myopathy. The capacity of the CPC to clone, proliferate and migrate was altered along with gene expression. The changes to the gene expression were associated with activation of the senescence-associated secretory phenotype (SASP), such as IL-6 and IGFBP7.

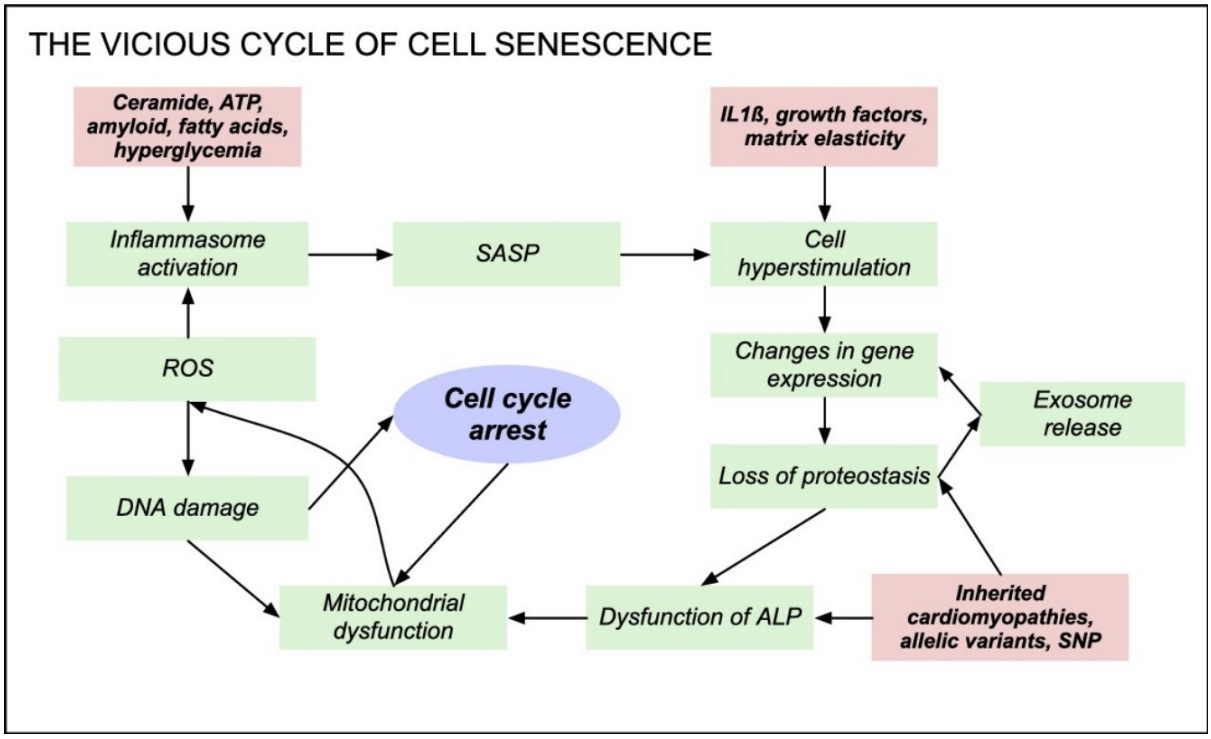


Figure 1. 11 The cascade of intrinsic events that leads to the vicious senescence cycle (adapted from Cesselli D 2017).

The properties and numbers of CPCs isolated have been compared between different age groups and in diseased hearts. Generally, the neonatal human heart tends to have more c-kit^{pos} CPCs, and this number declines by a factor of three between the ages 2-13 (Mishra R et al. 2011). Also, the ability of the cells to proliferate is highest amongst cells isolated from foetal cardiac tissues (Cesselli D et al. 2011) and declined with increasing age. Both neonatal and young CPCs showed similar ability in differentiating into all three lineages (Mishra R et al. 2011). Furthermore, the effects of nucleostemin (NS) have been evaluated in myocardial ageing (Hariharan N et al. 2015). This study compared the CPCs isolated from the human foetal heart (FhCPC), adult human diseased heart (AhCPC), as well as from young (YCPC) and old mice (OCPC) for features of senescence and NS expression. They used a knock out NS heterozygous mouse model (NS+/-) to assess the contribution of NS. The expression of NS was low, with a reduction in proliferation and telomere length in the AhCPC group compared to the FhCPC group. The features of CPCs from AhCPC and OCPC were comparable and of similar characteristics to NS silenced CPCs. These cells were typically flat, multinucleated, slower S-phase, reduced expression of stemness markers with up-regulated p53 and p16. The resultant senescent CPCs following NS silencing was mediate partly by p53. The NS induction stabilised the c-Myc pathway via Pim-1 kinase.

A study by Mohsin et al. (2013) investigated hCPC senescence characteristics through ex vivo modification of Pim-1. The biopsies were obtained from patients undergoing LVAD implantation, and c-kit^{pos} hCPCs were isolated. hCPCs with a green fluorescent protein (hCPC-eGFP) and hCPC-Pim1 (transduced with a lentivirus for eGFP and Pim-1). They showed that hCPCs isolated from multiple patients with heart failure had characteristic differences in growth rate, telomere length and senescence expression. Genetically modified Pim-1 boosted the hCPCs with advanced biological age, increasing their ability to proliferate, increase telomere length and reduce senescence capabilities.

Both animal and human studies have shown CPCs to increase by at least two-fold amongst aged and diseased heart. However, about 70 % of CPCs in aged and diseased hearts is deemed senescent and positive for p16^{Ink4a}, (Torella D et al. 2004; Beltrami AP et al. 2011; Cianflone et al. 2019). Work from our lab has shown,

irrespective of age, gender or disease the number of CPCs isolated from human myocardial sample to be comparable. However, the isolated CPCs expressed increased markers for senescence (p16, Senescence-Associated-β-gal) with an associated reduction in multipotent and proliferative markers (Lewis et al. 2016). These aged-senescent CPCs were characteristically incompetent with reduced clonal efficiency and differentiation capacity. When the CPCs isolated for aged hearts were grown in Wnt3A enriched media or transfected with lentivirus inducing Bmi-1 expression, the senescent-induced proliferative impairment in CPCs was corrected. Regardless of this, clonally derived CPCs from a single CPCs of young or old hearts, showed similar differentiation capabilities and gene expression. These findings demonstrate that CPC ageing is a stochastic process (Ellison GM. et al. 2012; Cianflone E et al. 2019).

CPCs with a normal functional capacity which expresses telomerase, cycling protein, Ki67 along with reduced senescent markers have been identified within the ageing heart (Urbenek et al. 2003; Dawn et al. 2005). These cycling competent CPCs can migrate to the damaged area of the myocardium and induce regeneration and cardiac function (Gonzalez et al. 2008 Lewis-McDougall F et al. 2019; Cianflone E et al. .2019).

The evidence accumulated thus far shows a complex interaction of various factors outlined above at the cellular level, which alter the CPC activity directly and indirectly; therefore, altering their potency. This complex interaction is seen in both aged and diseased human hearts. In this situation, the increase in CPCs that is observed is not sufficient to replace the loss of cardiomyocytes. It is possible through an understanding of the complex interaction of factors described above; novel strategies may be explored to reduce, stop or reverses cellular senescence and ageing.

1.6 Aims & Hypotheses

Our lab group has specified the CPC population to be Sca-1pos/c-kitpos/CD31^{neg}/CD45^{neg}/Tryptase^{neg} (Ellison et al., 2013; Vicinanza et al., 2017) distinguishing them from cardiac c-kit^{pos} CD31^{pos} and CD45^{pos}/Tryptase^{pos} (endothelial and mast cells respectively). Such a distinction should be established to characterise the CPCs appropriately.

The aim is to obtain a better understanding of the chamber-specific location, distribution, incidence and biology of the human CPCs as defined by our lab group, to design better protocols for the regeneration of functional contractile mass in human post-myocardial infarction, either by autologous human CPC transplantation and/or through the activation of these regenerative cells in situ.

The first specific aim is to identify, quantify and characterise eCSCs within human myocardial samples taken from all four cardiac chambers. It is hypothesised that there will be more CPCs in the atria, compared to ventricles.

The second specific aim is to assess whether human CPCs isolated from the different cardiac chambers of patients with normal LV function behave in the same way, exhibiting similar growth and multipotency in vitro compared to human CPCs isolated from patients with the disease. It is hypothesised that human CPCs isolated from patients with the disease have decreased proliferative capacity, clonogenicity, and multipotency potential compared to human CPCs isolated from patients with normal LV function. In addition, there will be no differences in growth and multipotency of the human CPCs isolated from the four cardiac chambers.

2. METHODS

2.1 Patients

This study was ethically approved by Imperial College NHS trust with NEC approval (13/LO/0457). Patients suitable for the study were identified and gave full informed consent for all four-chamber (RV, LV, RA and LA) biopsies to be obtained. Patient age, gender and demographics were also recorded (Table 2.1).

Biopsies were obtained from four chambers (Figure 2.1) during an elective cardiac surgical procedure in patients with normal (EF>50; n=5) and impaired left ventricular (LV) function (EF<49; n=5). Samples were collected for immunohistochemical analysis and cardiac stem/ progenitor cell isolation.

Table 2. 1 Patient demographics of whom the biopsies were taken from:

Age	Gender	Operation	Biopsy Site	LV Function	EF	RV Function	CCS	NYHA	Arrthymia	Hypertension (on ACEi/ARB)	Hypercholesterolemia (On Statins)	Family History	IDDM	NIDDM
63	M	CABG	RA,LA,RV, LV	Good	55-60	Good	3	3	No	Yes	Yes	Yes	No	Yes
41	M	CABG	RA,LA,RV, LV	Good	60	Good	2	2	No	Yes	Yes	Yes	Yes	No
63	M	CABG	RA,LA,RV, LV	Good	55-60	Good	3	3	No	Yes	Yes	Yes	No	Yes
56	F	CABG	RA,LA,RV, LV	Good	55-65	Good	2	2	No	Yes	Yes	No	Yes	No
59	M	CABG	RA,LA,RV, LV	Good	55-65	Good	1	2	No	Yes	Yes	Yes	No	No
77	M	MVR/AVR/LAA	RA,LA,RV, LV	Impaired	40-45	Good	3	3	Yes	Yes	No	No	No	Yes
79	M	CABG	RA,LA,RV, LV	Impaired	45-50	Good	0	3	No	Yes	Yes	Yes	No	No
74	M	CABG	RA,LA,RV, LV	Impaired	45-50	Good	2	2	No	Yes	Yes	Yes	No	No
64	M	CABG	RA,LA,RV, LV	Impaired	40	Good	2	2	No	No	Yes	No	Yes	No
61	M	CABG	RA,LA,RV, LV	Impaired	45	Good	2	2	No	Yes	Yes	Yes	No	Yes

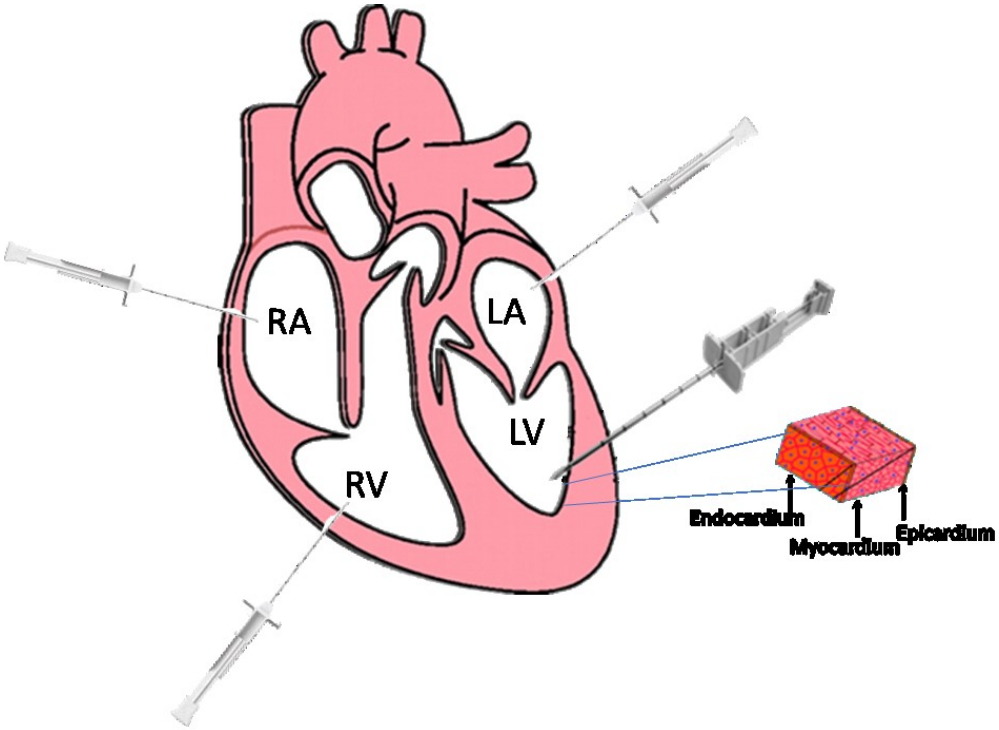


Figure 2. 1A Myocardial Biopsy sampling. Using a Tru-Cut needle, a full-thickness biopsy was taken from all four chambers of the human heart. Each biopsy consisted of all three layers; Endocardium, Myocardium and Epicardium.

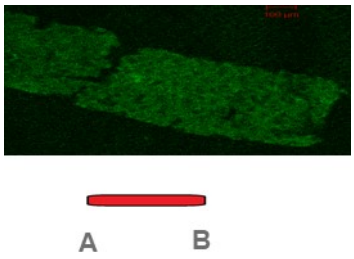


Figure 2. 2~~1~~B Each biopsy consisted of all three layers; Endocardium (A), Myocardium and Epicardium (B).

2.2 Tissue collection for immunohistochemical analysis

Samples were fixed in formalin for 24 hours and then processed in a tissue processor (Leica, TO 1020) on the following cycle:

1. Formalin – 1 hour
2. Formalin – 1 hour
3. Alcohol 70% – 1 hour 30 minutes
4. Alcohol 80% – 1 hour 30 minutes
5. Alcohol 96% – 1 hour 30 minutes
6. Alcohol 100% – 1 hour
7. Alcohol 100% – 1 hour
8. Alcohol 100% – 1 hour
9. Xylene – 1 hour 30 minutes
10. Xylene – 1 hour 30 minutes
11. Paraffin – 2 hours
12. Paraffin – 2 hours

Paraffin wax blocks containing the samples were obtained using an embedding station (Leica, EG 1160A). A microtome was used to cut 5μm tissue sections (Leica RM 2235) mounted on to poly-lysine coated microscope slides (Sigma-Aldrich, cat. no. P0425-72). Microtome sectioning was across the myocardium in the longitudinal plane, with epicardium to endocardium right to left.

2.3 Immunohistochemistry on cardiac chamber cross-sections

Immunohistochemistry (IHC) is a great tool to visualise and assess cellular architecture using microscopy. IHC allows recognition of antigens in cells of tissues by exploiting the principle of antibodies to antigens binding specificity using primary (target antigen) and secondary (fluorochrome) antibodies (Figure 2.2).

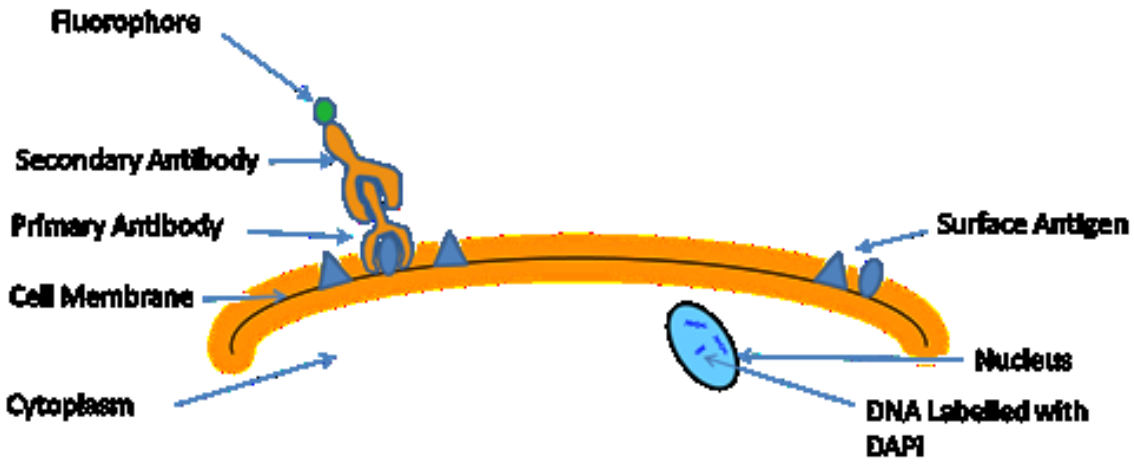


Figure 2. ~~3~~2 Schematic to show antibody binding and immunofluorescence on cell/tissue proteins.

To prepare for this process, deparaffinisation and rehydration of the sections were achieved by placing slides in the oven at 70°C for 30 minutes to melt the wax, followed by further washes in the following:

1. Xylene – 2 x 5 minutes
2. 96% ethanol – 1 x 4 minutes, 1 x 3 minutes
3. 90% ethanol – 3 minutes
4. 80% ethanol – 3 minutes
5. H_20 – 4 minutes

Warm citric acid buffer (0.35g/L citric acid monohydrate, Sigma, 2.4g/L Citric acid trisodium, Sigma; pH6), heated at full power for 10 minutes in a microwave was used to achieve antigen retrieval.

Slides were allowed to cool to room temperature (RT) for 30-45 minutes then washed with distilled water for 1 minute, followed by further multiple washes in PBS (Sigma 5 x 2-minute washes).

ImmEdge™ Pen (Vector) was used to create a hydrophobic barrier around each tissue section before blocking with 10% donkey serum (Sigma) in PBS for 30 minutes at RT in a humidified chamber. After discarding the

donkey serum, the primary antibody was applied, followed by the secondary antibody. In between each antibody incubation and after the secondary antibody incubation, 5 x 2 minutes washes with PBS were performed. Table 2.2 lists the primary and secondary antibodies used, the dilution and incubation times.

DNA binding, 4, 6-diamidino-2-phenylindole (DAPI) dye (1:1000, Sigma) was applied for 15 minutes at RT, followed by multiple PBS washes (6 x 2 minutes) to counterstain the nuclei before mounting in Vectashield™ mounting medium (Vector laboratories).

Table 2. 2 Antibodies used on paraffin embedded tissue sections.

Primary Antibody	Antigen	Raised In	Location	Incubation period	Temperature	Dilution	Secondary antibody	Incubation period	Temperature	Dilution
c-kit DAKO (A4502)	CD117, tyrosine kinase receptor	Rabbit	Surface receptor	Overnight	4°C	1/50	Donkey anti-rabbit 488 Alexa (Stratech)	One hour	37°C	1/100
MDR 1 Biorbyt (Orb18237)	Human ABCB1 / P Glycoprotein	Mouse	Surface receptor	One hour	37°C	1/50	Donkey anti-mouse 594 (Stratech)	One hour	37°C	1/100
Tryptase Abcam (ab194854)	Mast cells	goat	Cell Membrane	One hour	37°C	1/50	goat anti-mouse 594 (Invitrogen)	One hour	37°C	1/100
α-sarc Sigma-Aldrich (A2127)	Sarcomeric actin	mouse	cytoplasm	One hour	37°C	1/50	Dylight 594 Donkey anti-Mouse IgM(Stratech)	One hour	37°C	1/100

2.4 Immunohistochemistry Quantification

Cardiac sections from all four chambers were stained to identify cardiac progenitor cells and mast cells as phenotyped by our lab group, with the following characteristics:

c-kit positive, MDR-1 positive, Tryptase negative (c-kit^{pos}/MDR-1^{pos}/Tryptase^{neg})- cardiac progenitor cells (CPCs)

c-kit positive, MDR-1 positive, Tryptase positive (c-kit^{pos}/MDR1^{pos}/Tryp^{pos})- mast cells.

All quantification was conducted at x 60 magnification on a confocal microscope (Zeiss, LSM 710).

c-kit^{pos}/MDR-1^{pos} and Tryptase^{pos}, mast cells and c-kit^{pos}/MDR-1^{pos} and Tryptase ^{neg} CPCs cells were identified and counted across the entire section. The area of the cardiac section was measured using ImageJ. The number of cells was expressed per area (mm^2).

2.5 Human Cardiac small Cell Isolation

2.5.1 Enzymatic Digestion Technique

For larger samples (>100 mg) enzymatic digestion technique was used to dissociate the tissue. First, samples were transferred into a bacteriological petri dish and cleared of fat/connective, before being weighed. Following washes with PBS (2-3 times), 3ml DMEM (Invitrogen) was added and the sample minced with a surgical blade into 0.5-1mm3 fragments. The minced tissue was incubated at 37oC in a conical flask containing 10ml of dissociation media (Table 2.3) and agitated using a magnetic stirrer for 5 minutes. After the 5-minute dissociation, the minced tissue solution was gently pipetted with a transfer pipette to dissociate the tissue further. The solution was filtered through a 100µm cell strainer into a 50ml centrifuge tube. The tissue fragments collected on the strainer were transferred back

into the conical flask with fresh 5ml of dissociation media for another cycle of digestion. The strainer was washed through with an equal amount of quenching medium (Table 2.3) into the 50ml tube, which was left on ice until the whole process was complete. The cycles of dissociation were repeated (7-9 times) until all the tissue fragments had disappeared and/or became pale in colour. All 50ml tubes are centrifuged at 400g, brake 7, for 7 minutes at room temperature. The supernatant was then discarded, and the pellet was mixed with incubation media.

To remove the cellular debris, the incubation media with the pellet was suspended with 8 ml of Optiprep/DMEM mixture (with a density of 1.09 g/ml) and centrifuged at 800g for 15 min at room temperature. The top layer was discarded, and the pellet was resuspended in incubation media and proceeded to CPC isolation (Figure 2.3).

Table 2. 3 Media composition used for CPC isolation

Media	Formulations
Quenching Medium	DMEM (Invitrogen, 31885-023), FBS (Life Technologies), Fungizone (Invitrogen, 15290-018) (1% (vol/vol) , Gentamicin (Sigma-Aldrich, G1397) (1% (vol/vol), 1 % Penicillin/Streptomycin (Invitrogen, 15140-122)
Incubation Medium	DPBS (-Ca, -Mg) (Invitrogen, cat. no. 14190-136), BSA (5mg/ml), EDTA (2mM), Fungizone (Invitrogen, 15290-018) (0.1% (vol/vol), Gentamicin (Sigma-Aldrich, G1397)(0.1% (vol/vol) , 1 % Penicillin/Streptomycin (Invitrogen, 15140-122) (1% (vol/vol)
Dissociation Media	Collagenase II (Worthington), DMEM (Invitrogen, 31885-023) final concentration of 250 U/ml
Human CSC Growth Media	DMEM-F12-HAMS (Sigma-Aldrich D8437) , Neurobasal medium (500 ml) (Invitrogen, 10888-022), ITS (0.1% (vol/vol)) (Invitrogen 51500-056), Glutamax (Sigma-Aldrich, G7029), Fungizone (Invitrogen, 15290-018)(0.1% (vol/vol)), Gentamicin (Sigma-Aldrich, G1397)(0.1% (vol/vol)), Penicillin/Streptomycin (1% (vol/vol)) (Invitrogen, 15140-122) ESQ-FBS (Invitrogen, 10439-024), B27 supplement (2% (vol/vol)) (Invitrogen, 17504-044), N2 supplement (1% (vol/vol)) (Invitrogen, 17502-048), Human EGF (20 ng/ml) (Peprotech), Human (10 ng/ml) bFGF (Peprotech) , Human LIF (10 ng/ml) (Millipore, cat. no. LIF2010).
Differentiation Media	α-MEM with FCS (final concentration of 2% (vol/vol)) (Invitrogen), dexamethasone (1 μM), penicillin-streptomycin (1% (vol/vol)(Invitrogen, 15140-122)), gentamicin (0.1% (vol/vol)) (Invitrogen, 15140-122) and Fungizone (0.1% (vol/vol)(Invitrogen, 15140-122).

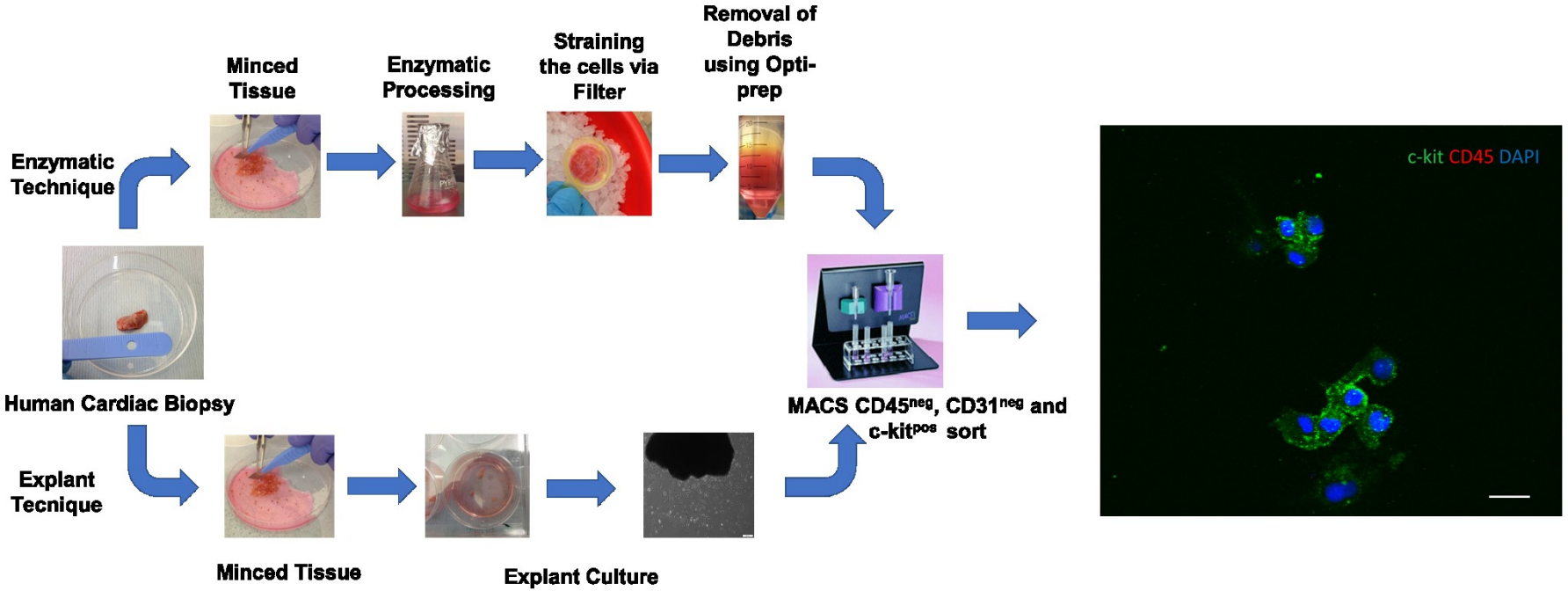


Figure 2. ~~43~~ Isolation of CD45^{-ve}, CD31^{-ve} and c-kit^{+ve} (green) CPCs using enzymatic and explant techniques. Stained by IHC for c-kit (green), CD45 (red) and DAPI (blue).

2.5.2 Explant culture technique

Biopsies from all four chambers of the human myocardium were transferred into a bacteriological petri dish and washed with PBS, before being minced with a surgical blade into 0.5-1mm^3 fragments. Fragments were plated on fibronectin (0.01units/ml of Hanks salt solution) coated six-well plates with 2ml of growth media (Table 2.2). Cultures were incubated at 37°C hypoxic incubator (Humidified 37 °C, 5% CO2 sterile incubator (HealForce HF90)) until there was a confluent layer of adherent cells outgrowing from each tissue fragment. Then the cells were trypsinised and re-plated in a T25 flask to reach confluence before CPC isolation (Figure 2.3).

2.5.3 CPC Isolation following either Enzymatic digestion or Explant culture of samples

The small cell suspension was spun at 400g for 10 minutes, and the pellet obtained was then resuspended in the appropriate amount of incubation media (Table 2.2). The standard protocol (Miltenyi) was used to sort for CPCs using Magnetic Activated Cell Sorting (MACS)(Figure 2.3). To deplete CD45^{pos} cells, the cardiac cell pellet was resuspended in 80µl incubation media before adding 20µl of CD45 microbeads (Miltenyi, 120-000-250). This was then incubated at 4°C for 15 minutes on a rocker. The cell suspension was washed with 1ml of incubation media and centrifuged at 300g for 5 minutes at 4 °C. After aspiration of the media, the labelled pellet was resuspended in 500µl of incubation media. At this stage the MS magnetic sorting column was set up with a 30µm pre-separation filter fitted above the column and the MS column primed with 500µl of incubation media. Then the labelled cell suspension was run through the column. Then two washes with 500µl of incubation media was performed to rinse through all the unbound cells. The unlabelled CD45^{negative} cell fraction was collected in a 15ml falcon tube, and the positively-labelled cells remained within the MS column was discarded. To obtain c-kit^{pos} cells, the collected unlabelled CD45^{negative} cell suspension was centrifuged at 300g for 5

minutes at 4 °C to obtain a cell pellet. The pellet was then resuspended in 80µl of incubation media and 20µl human CD117 microbeads (Miltenyi, 130-091-332). This was then incubated at 4°C for 15 min on rocker. The cell suspension was washed with 1ml of incubation media and centrifuged at 300g for 5 minutes at 4 °C. After aspiration of the media, the labelled pellet was resuspended in 500µl of incubation media. The MS magnetic sorting column was set up again with a 30µm pre-separation filter fitted above the column primed with 500µl of incubation media. Then the labelled cell suspension was run through the column. Then two washes with 500µl of incubation media was performed to rinse through all the unbound cells. This time the unlabelled c-kit^{neg} cell fraction was collected and discarded. To retrieve the c-kit^{pos} labelled cells within the MS column, the column was removed from the magnetic stand carefully and placed into a 10ml falcon tube. 500µl incubation media was used to plunge the column twice and the c-kit^{positive} labelled cells were obtained. The c-kit^{positive} CD45^{negative} fraction was then centrifuged at 300g for 5 mins at 4°C. Following aspiration of the media, the pellet was resuspended in growth media and plated on T25 coated flask with human growth media for proliferation of CPCs.

2.6 Cell culture

2.6.1 Purification of Human Cardiac Stem / Progenitor Cells

Isolated c-kit^{pos} and CD45^{neg} cells were plated on a non-coated flask for 20 minutes with growth media and incubated at 37°C in 5% CO2. This step was employed to remove contaminating fibroblasts which stick to the plastic within 20 minutes.

After 20 minutes, the supernatant from the flask, containing the non-adhered cells was collected and plated on to CELLstart™ CTS™ I (Gibco) coated flasks in growth media (Table 2.2). The cells were incubated at 37°C in 5% CO2 and passaged when reached ~80% confluence; approximately after 6-8 days. Cells were characterised for the properties of stem/progenitor cells (see below).

2.7 Immunocytochemistry of c-kit^{pos} and CD45^{neg}

Using a Cytospin 4 centrifuge and Shandon EZ double cytofunnels (Thermo Scientific), 200ul of 50,000 cells/ml were spun onto poly-L-sine coated slides (Thermo Scientific) and fixed using Shandon cellfix (Thermo Scientific).

The slides were immersed in 95% ethanol for 15 minutes to remove the fixative followed by 5 x 2 minutes washes with PBS. The slides were then permeabilised using 0.1% triton-x for 10 minutes at RT to stain for nuclear proteins; followed by 5 x 2 minutes washes with 0.1% Tween PBS. After blocking with 10% donkey serum in 0.1% Tween PBS for 30 minutes at RT, the primary antibody was applied. Then slides were washed for 5 x 2 minutes in Tween PBS. Following which the secondary antibody was applied, followed by 5 x 2 minutes in Tween PBS washes. Table 2.4 contains a list of antibodies used. DAPI was used to counterstain the nuclei for 15 minutes at RT. A final wash with PBS for 6 x 2 minutes was carried out before mounting with Vectashield™ (Vector laboratories).

The number of positive cells/nuclei was quantified as a percentage of total nuclei at x 40 magnification using a confocal microscope.

Table 2. 4 Antibodies used in immunocytochemistry of cytospin slides.

Primary Antibody	Host	Dilution	Incubation	Secondary Antibody	Dilution	Incubation
c-kit DAKO (A4502)	Rabbit	1/50	Overnight 4°C	Alexa Fluor 488 Donkey anti-Rabbit (Stratech)	1/100	1 hour at 37°
CD31 Santa Cruz (sc-1505)	Goat	1/50	1 hour at 37°	Dylight 488 Donkey anti-Goat (Stratech)	1/100	1 hour at 37°
DDR2	Goat	1/50	1 hour at 37°	Dylight 488 Donkey anti-Goat (Stratech)	1/100	1 hour at 37°
CD34 Santa Cruz	Goat	1/50	1 hour at 37°	Dylight 488 Donkey anti-Goat (Stratech)	1/100	1 hour at 37°
CD45 Santa Cruz	Rat	1/50	1 hour at 37°	Alexa Fluor 594 Donkey anti-Rat (Stratech)	1/100	1 hour at 37°

2.8 Clonogenicity

Clonogenicity assay was used to determine the clonogenic potential of the c-kit^{pos} and CD45^{neg} CPCs at P4. This was achieved through serial dilution, and a single cell was deposited into a well of a 96-well coated plate (n=3). The number of wells which formed colonies from a single cell was quantified and expressed as a percentage of the total number of wells that had a single cell at the beginning. The fastest growing and the best looking colonies were picked and expanded further through cell culture.

2.8.1 Cardiosphere formation

Fifty thousand clonally derived c-kit^{pos,} and CD45^{neg} CPCs at P5 were placed on 100mm^2 bacteriological dishes with normal growth media (10ml) without human LIF for six days. Three bacteriological dishes were set up for each cardiac chamber. The average number of cardiospheres were quantified from 10 fields of view and expressed as the number of cardiospheres /mm^2.

2.8.2 Differentiation

CM, smooth muscle and endothelial differentiation were evaluated by staining for α-actinin sarcomeric, Nkx-2.5, calponin and vWF, respectively. These markers have been previously validated by our lab group.

The nuclei were counterstained with 4, 6diamidino-2-phenylindole (DAPI). Cells were assessed using a confocal microscope. At day 5, clonally derived cardiospheres were transferred to 12 well plates containing non-coated coverslips with base differentiation media (Table 2.3). At day 10 they were fixed with 4% (vol/vol) formaldehyde (in PBS) for 20 min on ice. The fixative was then removed and washed 5 x 2 minutes washes with PBS. This was then stored at 4 °C for up to 4 days. The slides were then washed 5 x 2 minutes with 0.1% Tween PBS and blocked with 10% donkey serum in 0.1% Tween PBS for 30 minutes at RT. The primary

antibody was applied, and after each antibody, incubation slides were washed for 5 x 2 minutes in Tween PBS. Then secondary antibody was applied and after each antibody incubation slides were washed for 5 x 2 minutes in Tween PBS. Table 2.5 contains a list of antibodies used. DAPI was used to counterstain the nuclei and washed with PBS for 6 x 2 minutes before mounting with Vectashield™ (Vector laboratories).

Table 2. 5 Antibodies used in differentiation assay.

Primary Antibody	Host	Dilution	Incubation	Secondary Antibody	Dilution	Incubation
α sarcomeric actin Sigma-Aldrich (A2127)	Mouse	1/50	Overnight 4°C	Alexa Fluor 594 Donkey anti-Mouse (Stratech)	1/100	1 hour at 37°
Nkx2.5 R&D	Goat	1/20	Overnight 4°C	Dylight 488 Donkey anti-Goat (Stratech)	1/100	1 hour at 37°
Calponin	Goat	1/50	Overnight 4°C	Dylight 488 Donkey anti-Goat (Stratech)	1/100	1 hour at 37°
vWF DAKO (A0082)	Rabbit	1/50	Overnight 4°C	Dylight 488 Donkey anti-Rabbit (Stratech)	1/100	1 hour at 37°

2.9 Statistical Analysis

Data represented as Mean ±SEM. Independent T-test was used to determine the statistical significance between 2 groups, and One-way ANOVA was used for more than two groups. Significance was reported when $p<0.05$.

RESULTS

3. CPC location and distribution within all four chambers of the human heart.

3.1 Introduction

The existence of progenitor cells of cardiac origin – CPCs - has been confirmed by numerous animal studies (Oh et al. 2003 Bearzi et al. 2007; Chong et al. 2011; Ellison et al. 2013). However, the use of myocardial samples to isolate and characterise these unique and rare cells from the human heart has been limited. The original identification of human CPCs raised the possibility of the human heart to regenerate and form new functional cardiomyocytes in ageing and following myocardial injury (Quaini et al. 2002; Bearzi et al. 2007; Bergmann et al. 2009). Various CPC markers including that of c-kit^{pos}/MDR-1^{pos} CPCs have been extensively characterised and shown to comply with all the properties of a 'stem cell;' (Bearzi et al. 2007; Ellison et al. 2013). However, their importance to cardiac regeneration remains unclear and continually questioned (Zaruba et al. 2010; van Berlo & Molkentin, 2014).

Pouly et al. (2008) showed c-kit^{pos} cells to be rare (1/mm^2 atrial tissue and 2.7/mm^2 RV tissue) following a study which involved analysing endomyocardial biopsies taken from the right ventricle (RV) and right atrial appendages of patients six years post-heart transplantation. However, these c-kit^{pos} cells were cardiac mast cells as they expressed CD45 and tryptase and were negative for Nxk2.5 and CD105. Ellison et al. 2011, showed that cardiac mast cells represent ~80% of the total number of c-kit^{pos} cells within the pig atria. Moreover, Vicinanza et al. (2017) showed that 90% of the c-kit^{pos} cells in the mouse and rat heart are CD45^{pos} and CD31^{pos}, identifying them as haematopoietic (mast cell) and endothelial origin, respectively.

c-kit positive CPCs have been characterised, as mentioned previously. It has been shown that c-kit positive CPCs are also positive for Sca-1 (60 ± 10% of c-kit+ eCSCs are also Sca-1 positive) and MDR-1 (ABCG2) (Leong Y et al. 2017). Sca-1 cells have been shown play a crucial role but have been shown to lack human homology (Vicinanza et al. 2017). However, human MDR-1 positive CPCs are less well

studied, therefore understanding this marker, expressed in SP cells, will help evaluate cardiac CPC characteristics further. Therefore, the identification of c-kit CPCs alone would not be representative due to the heterogeneity of the phenotype (Scalise M et al.2019). Hence our study also included the study of MDR-1 positive cells.

Despite these efforts, the distribution and characterisation of c-kit^{pos} cells and thus CPCs across all four chambers of the human heart has not been determined, nor has there been a comparison of their number between patients with normal (>50% EF) and impaired (<49% EF) LV function.

This study first identified the abundance of c-kit^{pos} and MDR-1^{pos} cells across all four chambers of the human heart from good and impaired LV patients. Second, the abundance of c-kit^{pos} or MDR1^{pos} mast cells (tryptase^{pos}) were determined. Third, the abundance of c-kit^{pos} or MDR1^{pos} CPCs (tryptase^{neg}) were determined. Fourth, a comparison of c-kit^{pos} cells, MDR1^{pos} cells, c-kit^{pos} CPCs, MDR1^{pos} CPCs, c-kit^{pos} mast cells and MDR1^{pos} mast cells between patients with good and impaired LV function was made.

3.2 Results

3.2.1 Cardiac biopsy from all four chambers of the human heart from good and impaired LV patients.

Cardiac tissue samples were obtained from patients undergoing cardiac surgery, as described in the method section. The biopsies were taken using Tru-Cut needle to achieve two full-thickness biopsies from the marked (using a sterile marker to identify epicardial surface) area of each chamber. One of the samples was used for histological analysis, and the other was used for cell isolation. Furthermore, all the samples were obtained after establishing cardiopulmonary bypass.

The patient demographic analysis is represented on table 3.1. There was a significant statistical difference in age between good and impaired LV patients. Furthermore, regardless of LV function, there was no statistical difference observed

between NYHA class 2/3 and between NIDDM and IDDM within my patient population.

The samples were processed for histological analysis, as outlined in the Methods Chapter. Microtome sectioning was done across all layers of the myocardium in the longitudinal plane, with epicardium (right side) to the endocardium (left side). Immunohistochemistry (IHC) identified cells expressing c-kit, MDR1 and tryptase. Whole sections of all four chambers (RV, LV, RA and LA) from good (n=5) and impaired LV (n=5) patients were analysed. c-kit^{pos} cells, MDR1^{pos} cells, CPCs (c-kit^{pos} or MDR1^{pos} and Tryptase^{neg}) and mast (c-kit^{pos} or MDR1^{pos} and Tryptase^{pos}) cells were quantified and expressed as the number of cells per mm^2 (cross-sectional area).

Table 3. 1 Patient Demographics. (LV-left ventricular; AF-Atrial Fibrillation; ACEi- Angiotensin-converting-enzyme inhibitors; ARB- Angiotensin II receptor blockers; IDDM- Insulin-dependent diabetes mellitus; NIDDM-Non-Insulin-dependent diabetes mellitus; NS -not significant).

	All	Good LV Function	Impaired LV Funtion	p- Value
Total	10	5	5	
Age, mean (SD)	63.7±11	56.4±9	71±8	<0.05 (G vs Imp LV)
Gender M/F	9/1	4/1	5	
CCS (1/2/3/4)	2/5/3/0	1/2/2/0	1/3/1/0	
NYHA 2/3	6/4	3/2	3/2	NS
Arrthymia (AF)	1	0	1	
Hypertension (ACEi/ARB)	9	5	4	
Hypercholesterolemia (Statins)	9	5	4	
Family History (Y/N)	7	4	3	
IDDM	3	2	1	NS
NIDDM	4	2	2	NS
β Blockers	10	5	5	
Smokers	2	1	1	
Ex-smokers	5	3	2	

3.2.2 Quantification of c-kit^{pos} cells in all four chambers of the human heart from good and impaired LV patients.

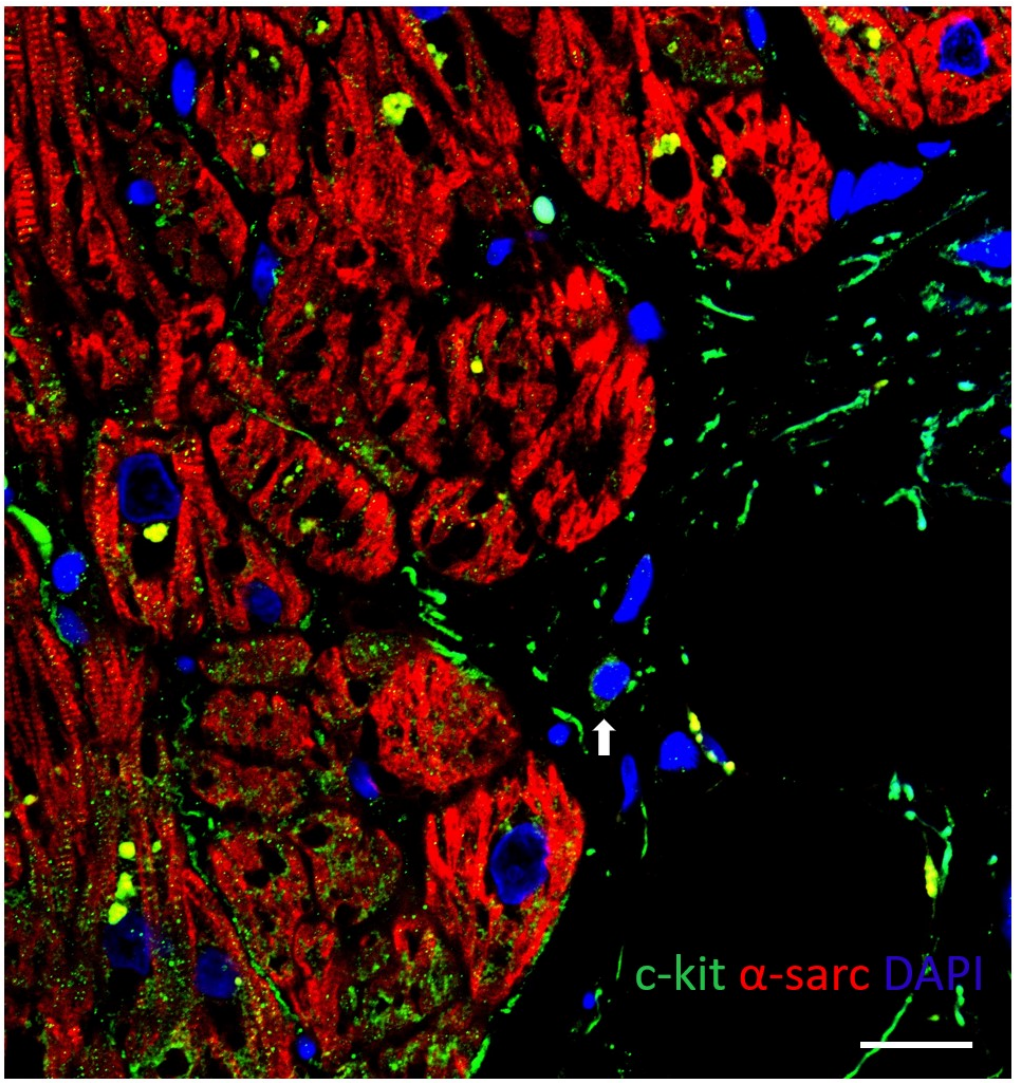


Figure 3. 2 Identification of c-kit^{pos} Cells. Representative confocal image showing a c-kit^{pos} (green) cell indicated by the white arrow within the myocardial/ endocardial region. Cardiomyocytes are stained in red (α-sarcomeric actin). Nuclei are stained in blue by DAPI.. Scale = 20μm

In good LV (Figure 3.2A) patients, the total number of c-kit^{pos} cells were significantly ($p<0.05$) lower in RA (20±1/mm^2) compared to LA (44±4/mm^2), RV (66±6/mm^2) and LV (69±3/mm^2 (Figure 3.2A). In addition, a significantly ($p<0.05$) greater number of c-kit^{pos} cells were present in the RV (66±/mm^2) and LV (69±3/mm^2) when compared to LA (44±4/mm^2). This trend was also observed in the impaired LV group (Figure 3.2B), where the LV chamber had the highest number ($p<0.05$) of c-kit^{pos} cells (85±3/mm^2), compared to RA (56±1/mm^2), LA (68±1/mm^2) and RV (68±2/mm^2) (Figure 3.2B). The RA had significantly ($p<0.05$) lower c-kit^{pos} cells compared to the LA (68±1/mm^2), RV (68±2/mm^2) and LV (85±3/mm^2).

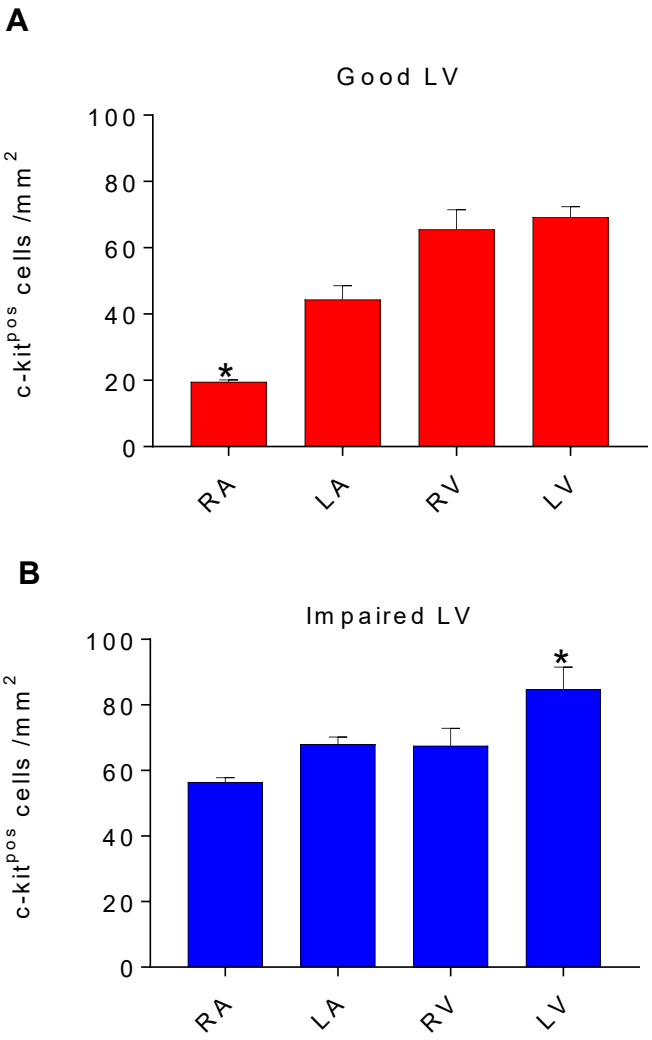


Figure 3. 3 c-kit^{pos} cells in all four chambers of the heart.
Quantification of c-kit^{pos} cells across all four chambers of human myocardium. (A) Good LV patients. * denotes $p<0.05$ vs all chambers, (B) Impaired LV patients. * denotes $p<0.05$ vs all chambers. Data is Mean ±SEM, n=5 patients per chamber.

3.2.3 Quantification of MDR1^{pos} cells in all four chambers of the human heart from good and impaired LV patients.

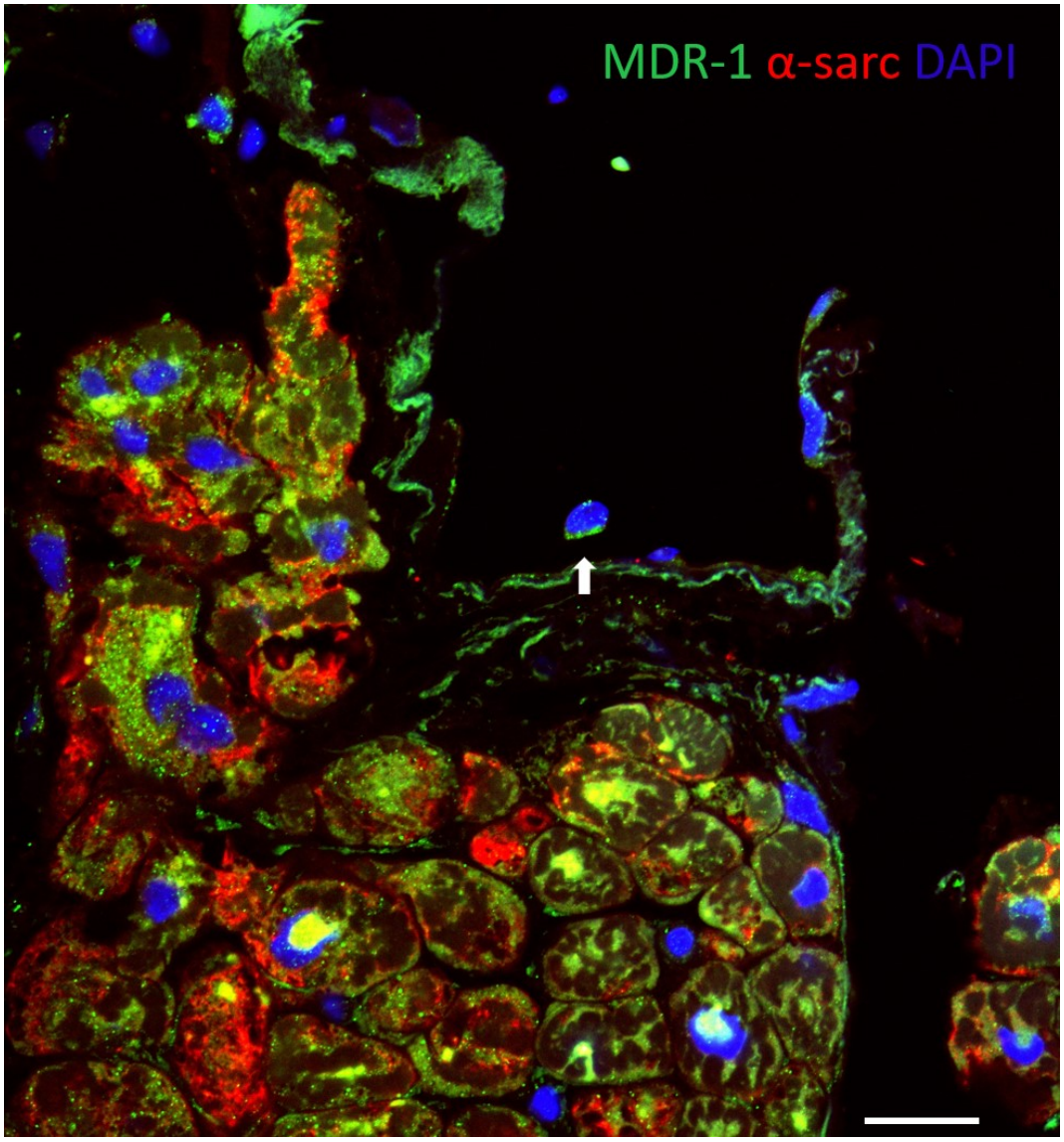


Figure 3. 4 Identification of MDR-1^{pos} CPCs cells. Representative confocal image showing an MDR-1^{pos} (green) cell indicated by the white arrow within the myocardial/endocardial region. Cardiomyocytes are stained in red (α-sarcomeric actin). Nuclei are stained in blue by DAPI.. Scale = 20μm

Similar to the c-kit^{pos} cell distribution, in good LV patients, the LV chamber had significantly ($p<0.05$) more MDR-1^{pos} cells (66±2/mm^2) than RA (18±1/mm^2), LA (45±1/mm^2) and RV (54±2/mm^2) (Figure 3.4A). Moreover, the RV chamber had significantly ($p<0.05$) higher number of MDR-1^{pos} cells (54±2/mm^2) compared to LA (45±1/mm^2) and RA (18±1/mm^2) (Figure 3.4A), In the impaired LV group, the RA (101± 4/mm^2) and RV (99±4/mm^2) had significantly ($p<0.05$) more MDR-1^{pos} cells compared to LA (86±1/mm^2) (Figure 3.4B). The LV had (92±3 /mm^2) MDR1^{pos} cells, which showed no significant differences to the other chambers.

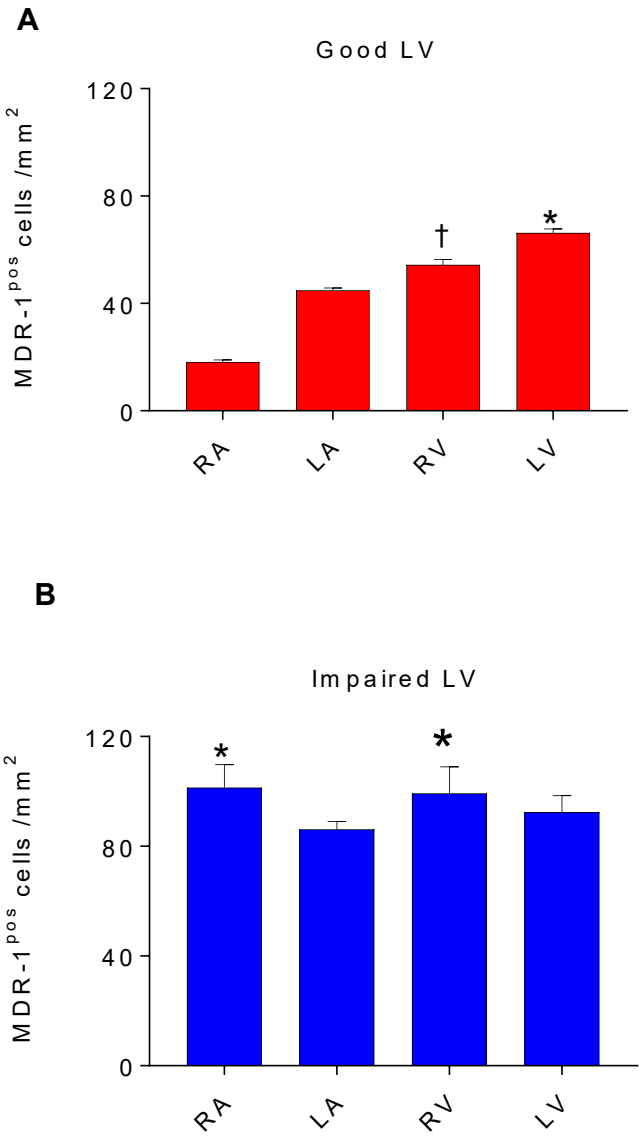


Figure 3. 5 MDR-1^{pos} cells in all four chambers of the heart.
Quantification of MDR-1^{pos} cells across all four chambers of human myocardium. Data is mean ±SEM, n=5 per chamber. (A) good LV patients. * denotes significant differences ($p<0.05$) vs all chambers, † denotes significant differences ($p<0.05$) vs RA and LA. (B) impaired LV patients. * denotes significant differences ($p<0.05$) vs LA .

3.2.4 Comparison of the overall distribution of c-kitpos cells and MDR1^{pos} cells between good and impaired LV patients.

The impaired LV function group had significantly ($p<0.05$) greater c-kit^{pos} cells (69± 2/mm^2) compared to the good LV group (50± 5/mm^2) (Figure 3.5A). Moreover, the overall number of MDR-1^{pos} cells (95± 2/mm^2) in the impaired LV group was significantly higher ($p<0.05$) compared to the good LV group (46± 4/mm^2) (Figure 3.5B).

3.2.5 Comparison of the overall distribution of c-kitpos cells or MDR1^{pos} cells between atria and ventricle.

Overall the ventricles had significantly ($p<0.05$) higher number of c-kit^{pos} cells than atria in both good (67±3/mm^2 vs 32±5/mm^2) (Figure 3.6A) and impaired (76±3/mm^2 vs 62±2/mm^2) LV groups (Figure 3.6B). For MDR1^{pos} cells, the ventricles of the good LV group showed significantly ($p<0.05$) more (60±2/mm^2) MDR-1^{pos} cells than the atria (31±4/mm^2) (Figure 3.7A). There were no differences in the number of MDR1^{pos} cells between ventricles and atria for the impaired LV function group (Figure 3.7B).

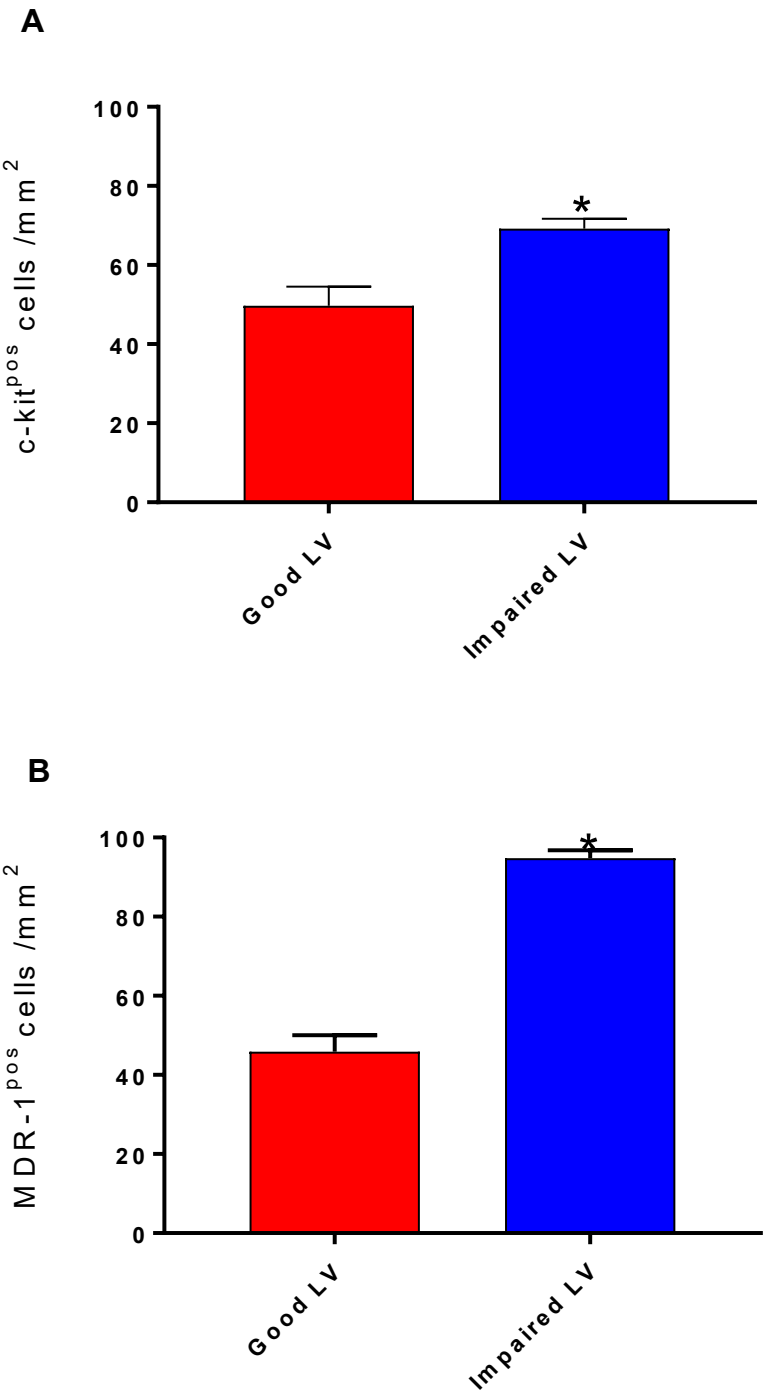


Figure 3. 6 Overall distribution of c-kit^{pos} and MDR-1^{pos} cells in Good LV vs Impaired LV. The overall abundance of c-kit^{pos} (A) and MDR-1^{pos} (B) cells were compared between good LV and impaired LV group. Data is mean ±SEM, n=20 per group (samples from each chamber taken from 5 patients). * denotes significant differences ($p<0.05$) vs good LV.

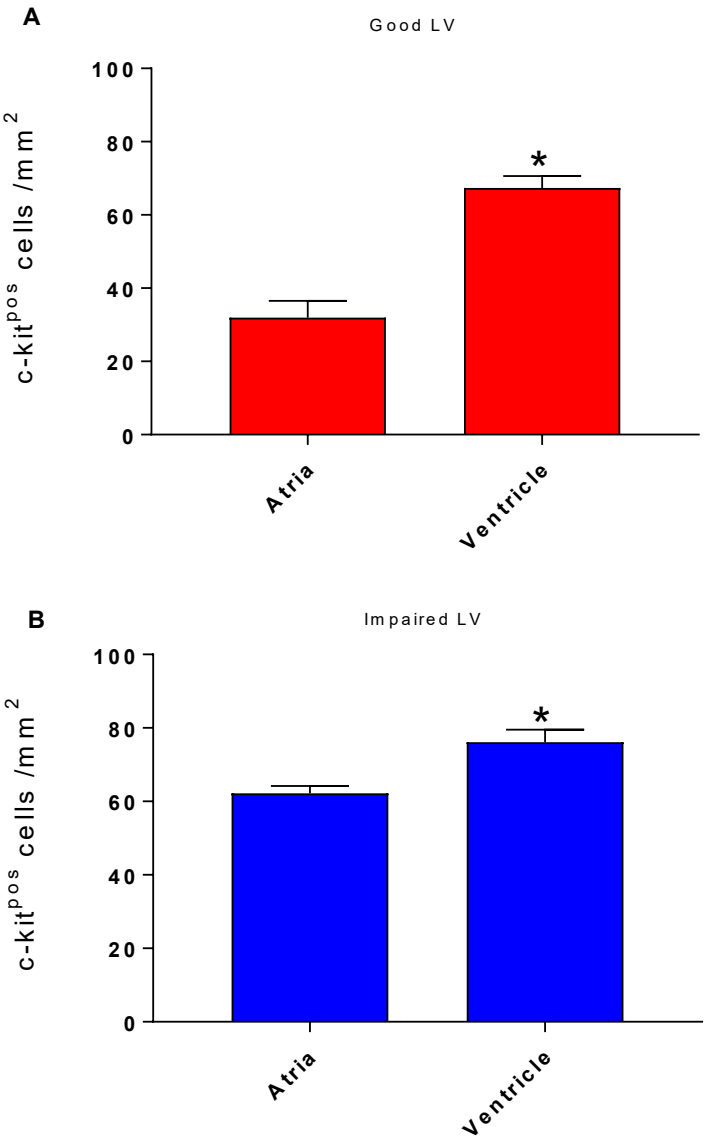


Figure 3. 7 Overall distribution of c-kit^{pos} cells in Atria and Ventricle.
The overall abundance of c-kit^{pos} cells was compared between Atria (A) and Ventricle (B). Data is mean ±SEM, n=10 per group (samples from each chamber taken from 5 patients). * denotes significant differences ($p<0.05$) vs atria.

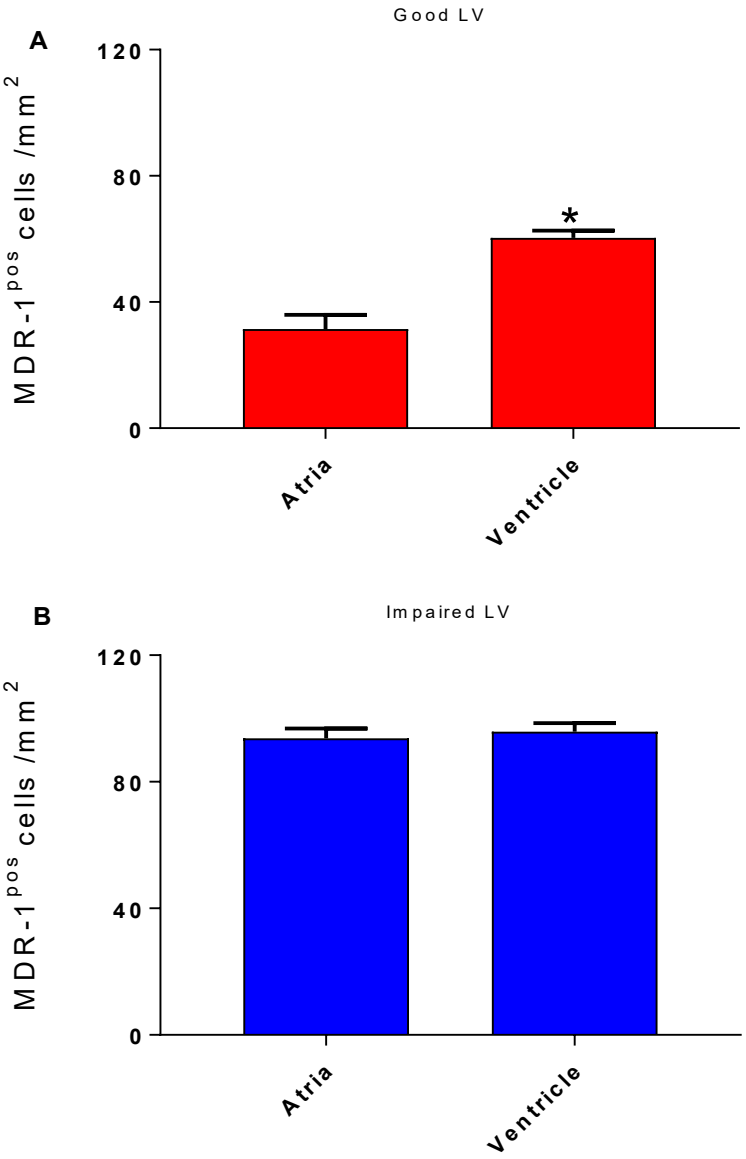


Figure 3. 8 Overall distribution of MDR-1^{pos} cells in Atria and Ventricle. The overall abundance of MDR-1^{pos} cells was compared between Atria (A) and Ventricle (B). Data is mean ±SEM, n=10 per group (samples from each chamber taken from 5 patients). * denotes significant differences ($p<0.05$) vs atria.

To summarise, the main findings were:

- The Left Ventricle harbours the greatest number of c-kit^{pos} cells, and the RA harbours the least c-kit^{pos} cells, regardless of good or impaired LV function.
- The number of MDR1^{pos} cells is greatest in the LV and least in the RA for good LV patients. However, the LA harbours the least MDR1^{pos} cells in impaired LV patients.
- Impaired LV patients harbour increased number of c-kit^{pos} cells and MDR1^{pos} cells, compared to good LV patients.
- The Ventricle harbours increased number of c-kit^{pos} cells, regardless of good or impaired LV function.
- The Ventricle harbours a greater number of MDR1^{pos} cells in good LV patients. There were no differences between atria and ventricle for MDR1^{pos} cells in impaired LV patients.

3.2.6 Quantification of c-kit^{pos} tryptase^{pos} mast cells in all four chambers of the human heart from good and impaired LV patients.

In the good LV group, the c-kit^{pos} tryptase^{pos} mast cells (Figure 3.8) are more abundant in LV (39± 4/mm^2) compared to RA (13± 1/mm^2), LA (20± 2/mm^2), RV (36± 2/mm^2 (Figure 3.9A)). RA and LA chambers have significantly ($p<0.05$) less c-kit^{pos} mast cells compared to RV and LV chambers.

The LV chamber (47± 1/mm^2) in impaired LV group (Figure 3.9B) also had significantly ($P<0.05$) more abundant c-kit^{pos} mast cells compared to RA (29± 0/mm^2), LA (36± 1/mm^2) and RV (37± 2/mm^2). RA had significantly ($p<0.05$) fewer mast cells compared to other chambers. In addition, significantly ($p<0.05$) fewer mast cells were present in LA compared to RV and LV chambers.

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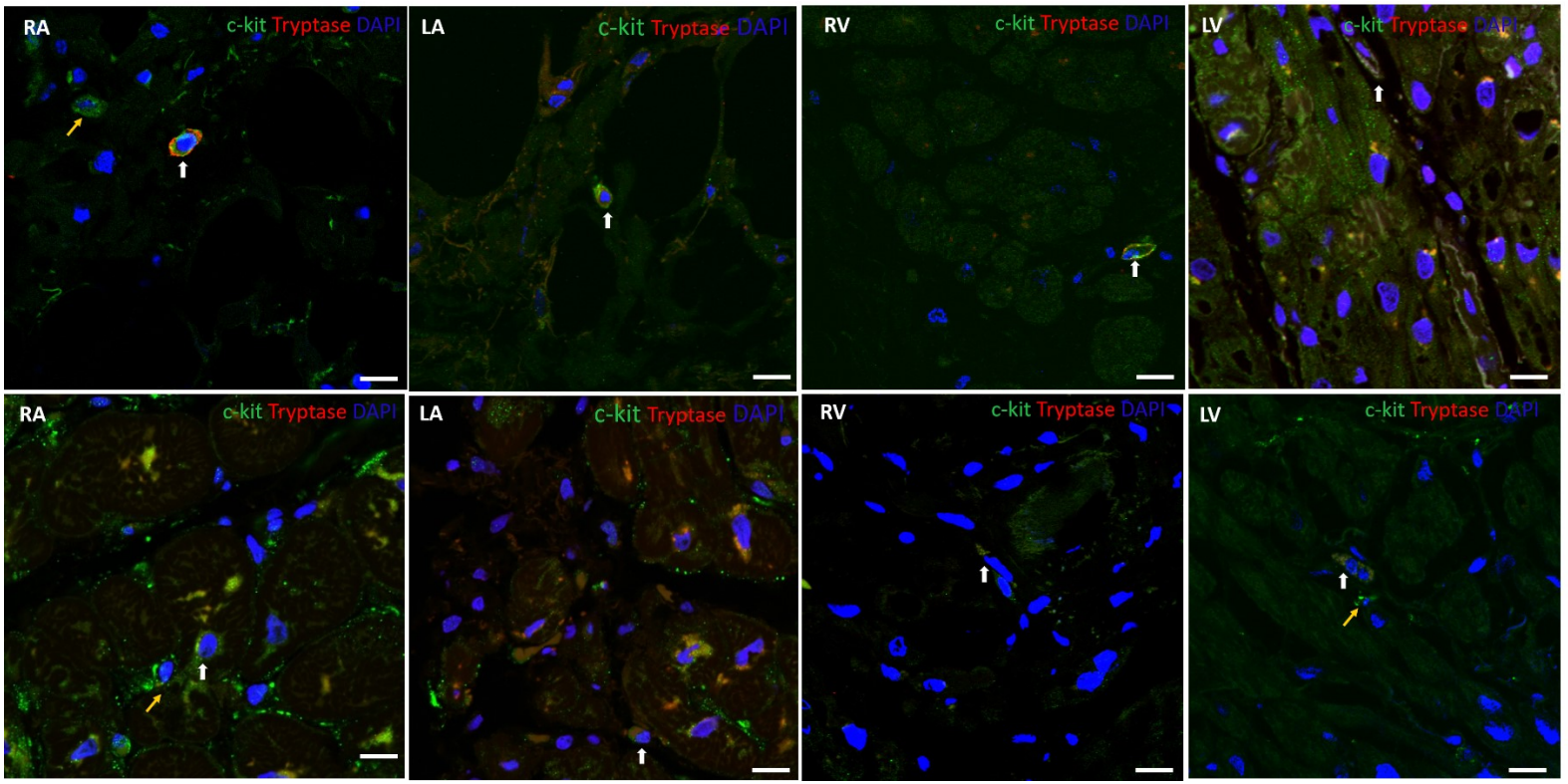

Figure 3. 9 Identification of c-kit^{pos} Mast cells. Representative confocal images show c-kit^{pos} (green) tryptase^{pos} (red) mast cell, indicated by the white arrow within the myocardial/ endocardial region. Also, a c-kit^{pos} (green) tryptase^{neg} (red) CPC is indicted by a yellow arrow. Nuclei are stained in blue by DAPI. Scale = 10μm. Top Row – Cardiac chambers of good LV; Bottom Row- Cardiac chambers of Impaired LV.

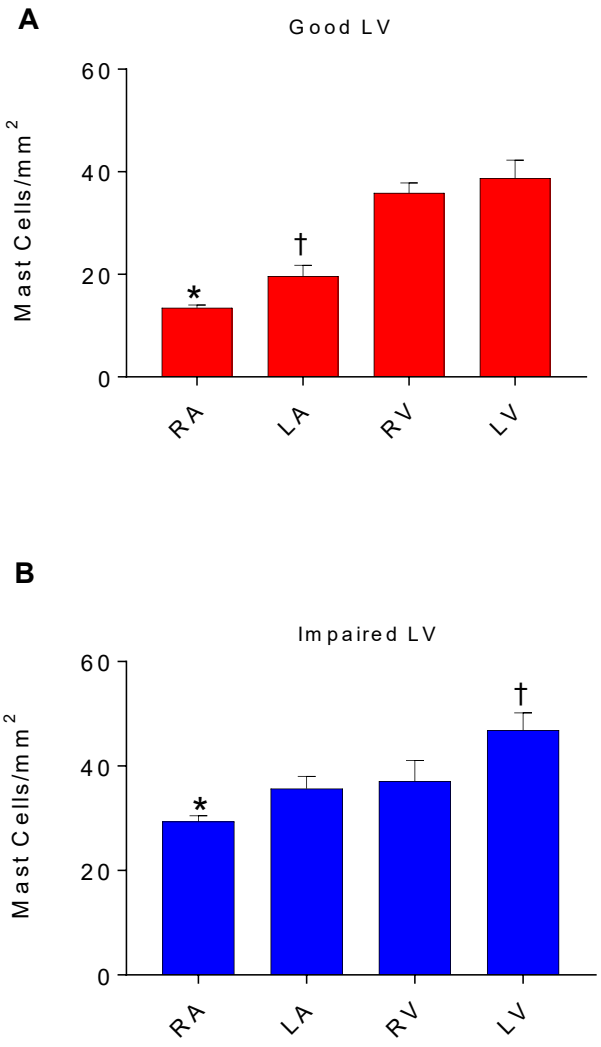

Figure 3. 10 c-kit^{pos} tryptase^{pos} mast cells in all four chambers of the heart. Quantification of c-kit^{pos} tryptase^{pos} cells across all four chambers of human myocardium. Data is mean ±SEM, n=5 per chamber. (A) good LV patients. * denotes significant differences ($p<0.05$) vs RV and LV, † denotes significant differences ($p<0.05$) vs RV and LV. (B) impaired LV patients. * denotes significant differences ($p<0.05$) vs all chambers. † denotes significant differences ($p<0.05$) vs LA and RV.

3.2.7 Quantification of MDR1^{pos} tryptase^{pos} mast cells in all four chambers of the human heart from good and impaired LV patients.

In good LV group, the MDR-1^{pos} mast cells (Figure 3.10) were more abundant in LV (44±3/mm^2) than RA (13± 0/mm^2), LA (32± 2/mm^2) and RV (39± 2/mm^2 (Figure 3.11A)). RA had significantly ($p<0.05$) less MDR-1^{pos} mast cells compared to all other chambers. LA also had significantly less ($p<0.05$) MDR-1^{pos} mast cells than LV.

In the impaired LV group (Figure 3.11B), the distribution of MDR-1^{pos} mast cells was similar between RA (51± 3/mm^2), RV (50± 3/mm^2), LV (52± 2/mm^2). The LA chamber (40± 1/mm^2) had significantly ($p<0.05$) low number of MDR-1^{pos} mast cells compared to other chambers.

In good LV group, the MDR-1^{pos} mast cells were more abundant in LV (44±3/mm^2) than RA (13± 0/mm^2), LA (32± 2/mm^2) and RV (39± 2/mm^2 (Figure 3.11A)). RA had significantly ($p<0.05$) less MDR-1^{pos} mast cells compared to all other chambers. LA also had significantly less ($p<0.05$) MDR-1^{pos} mast cells than LV.

In the impaired LV group (Figure 3.11B), the distribution of MDR-1^{pos} mast cells was similar between RA (51± 3/mm^2), RV (50± 3/mm^2), LV (52± 2/mm^2). The LA chamber (40± 1/mm^2) had significantly ($p<0.05$) low number of MDR-1^{pos} mast cells compared to other chambers.

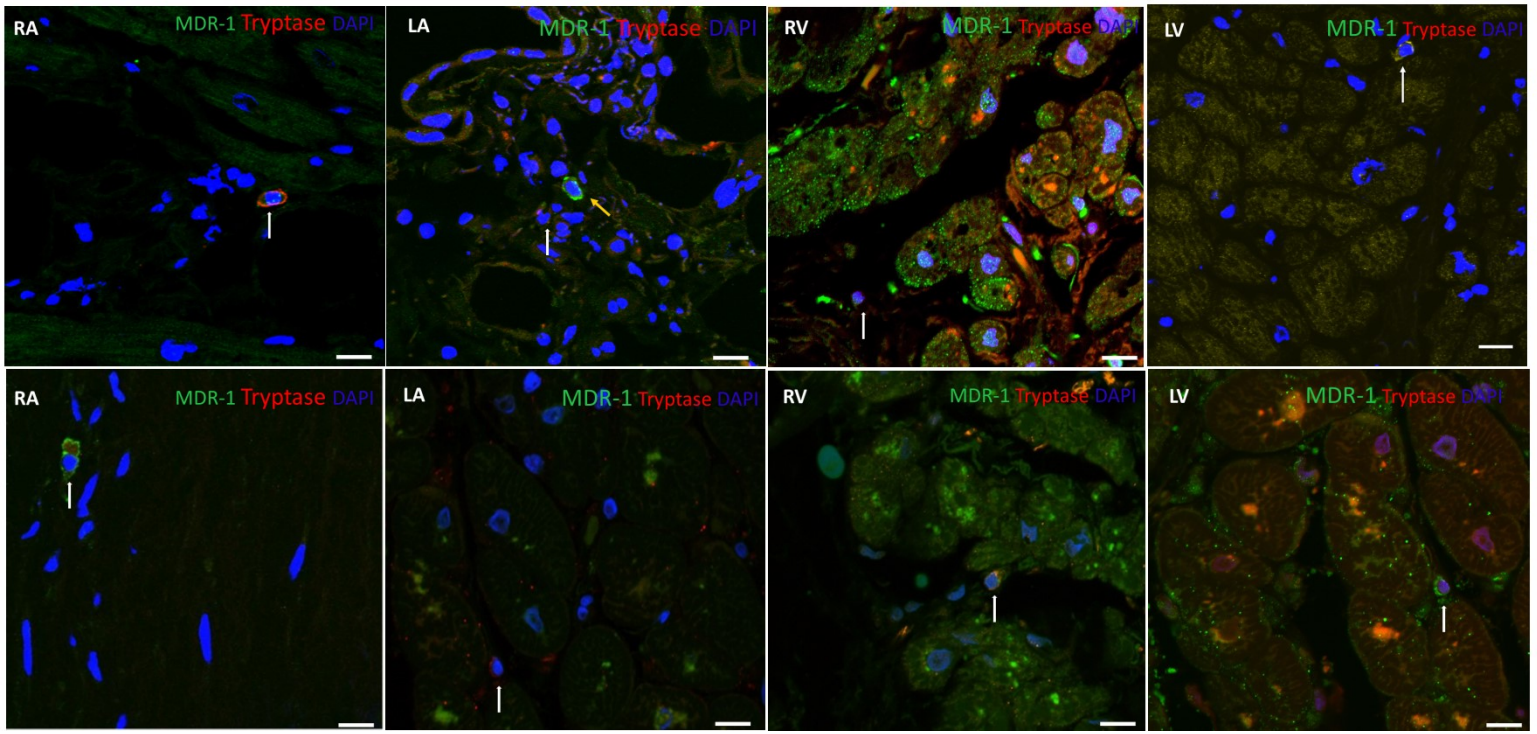

Figure 3. 11 Identification of MDR-1^{pos} Mast cells. Representative confocal images show MDR-1^{pos} (green) tryptase^{pos} (red) mast cells, indicated by the white arrow within the myocardial/endocardial region. Also, an MDR-1^{pos} (green) tryptase^{neg} (red) CPC is indicted by a yellow arrow. Nuclei are stained in blue by DAPI.. Scale = 10μm. Top Row – Cardiac chambers of good LV; Bottom Row- Cardiac chambers of Impaired LV.

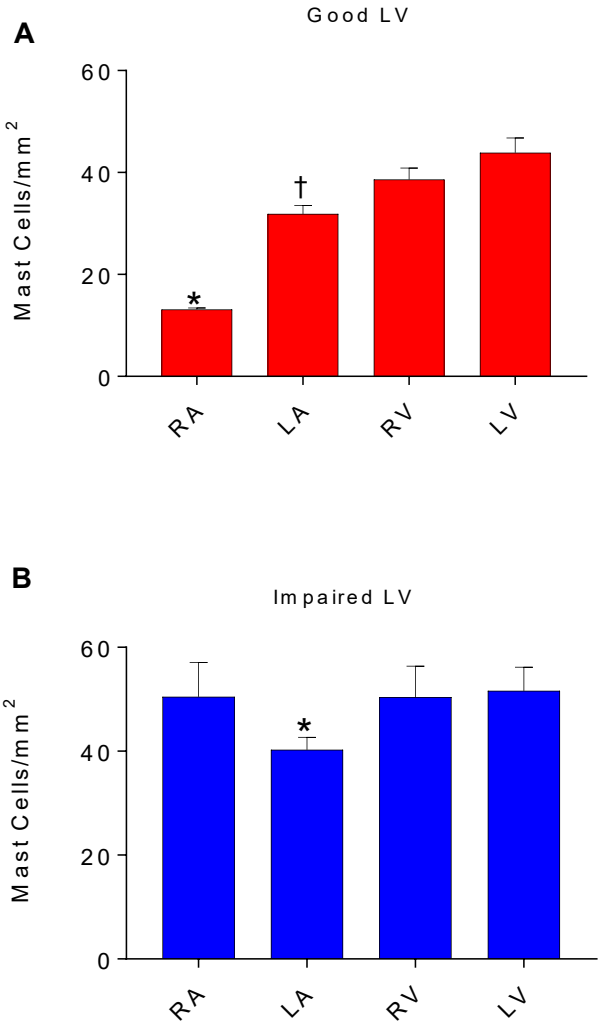

Figure 3. 12 MDR-1^{pos} tryptase^{pos} mast cells in all four chambers of the heart. Quantification of MDR-1^{pos} tryptase^{pos} mast cells across all four chambers of human myocardium. Data is mean ±SEM, n=5 per chamber. (A) good LV patients. * denotes significant differences ($p<0.05$) vs all chambers, † denotes significant differences ($p<0.05$) vs LV. (B) impaired LV patients. * denotes significant differences ($p<0.05$) vs all chambers.

3.2.8 Comparison of the overall distribution of c-kit^{pos} and MDR1^{pos} tryptase^{pos} mast cells between good and impaired LV patients.

The impaired LV function group had significantly higher ($p<0.05$) c-kit^{pos} mast cells (37± 2/mm^2) compared to the good LV group (27± 3/mm^2) (Figure 3.12A). The MDR-1^{pos} mast cells (48± 1/mm^2) was significantly ($p<0.05$) higher in impaired LV group compared to good LV group (32± 3/mm^2) (Figure 3.12B).

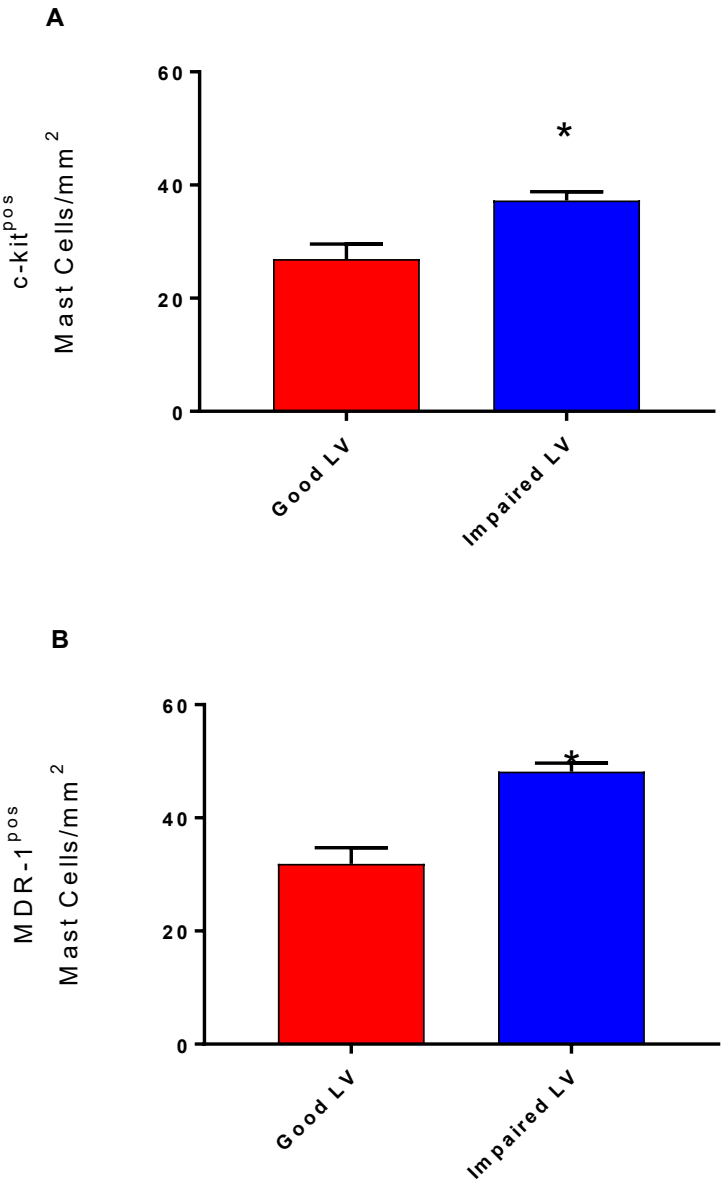

Figure 3. 13 Overall distribution of c-kit^{pos} tryptase^{pos} and MDR-1^{pos} tryptase^{pos} mast cells in Good LV vs Impaired LV. The overall abundance of c-kit^{pos} (A) and MDR-1^{pos} (B) mast cells were compared between good LV and impaired LV group. Data is mean ±SEM, n=20 per group (samples from each chamber taken from 5 patients). * denotes significant differences ($p<0.05$) vs good LV.

3.2.9 Comparison of the overall distribution of c-kitpos and MDR1pos tryptasepos mast cells between atria and ventricle.

The distribution of c-kit^{pos} mast cells was significantly ($p<0.05$) higher in ventricles compared to atria in both good (37±2/mm^{2}vs 17±1/mm^2) (Figure 3.13A) and impaired (42±2/mm^2 vs 33±1/mm^2) LV groups (Figure 3.13B). The distribution of MDR-1^{pos} mast cells was also higher in the ventricles compared to atria in both good (41±2/mm^2 vs 23±3/mm^2) (Figure 3.14A) and impaired (51±2/mm^2 vs 45±2/mm^2) LV groups (Figure 3.14B). In the good LV group, the distribution MDR-1^{pos} mast cells were statistically significant ($p<0.05$).

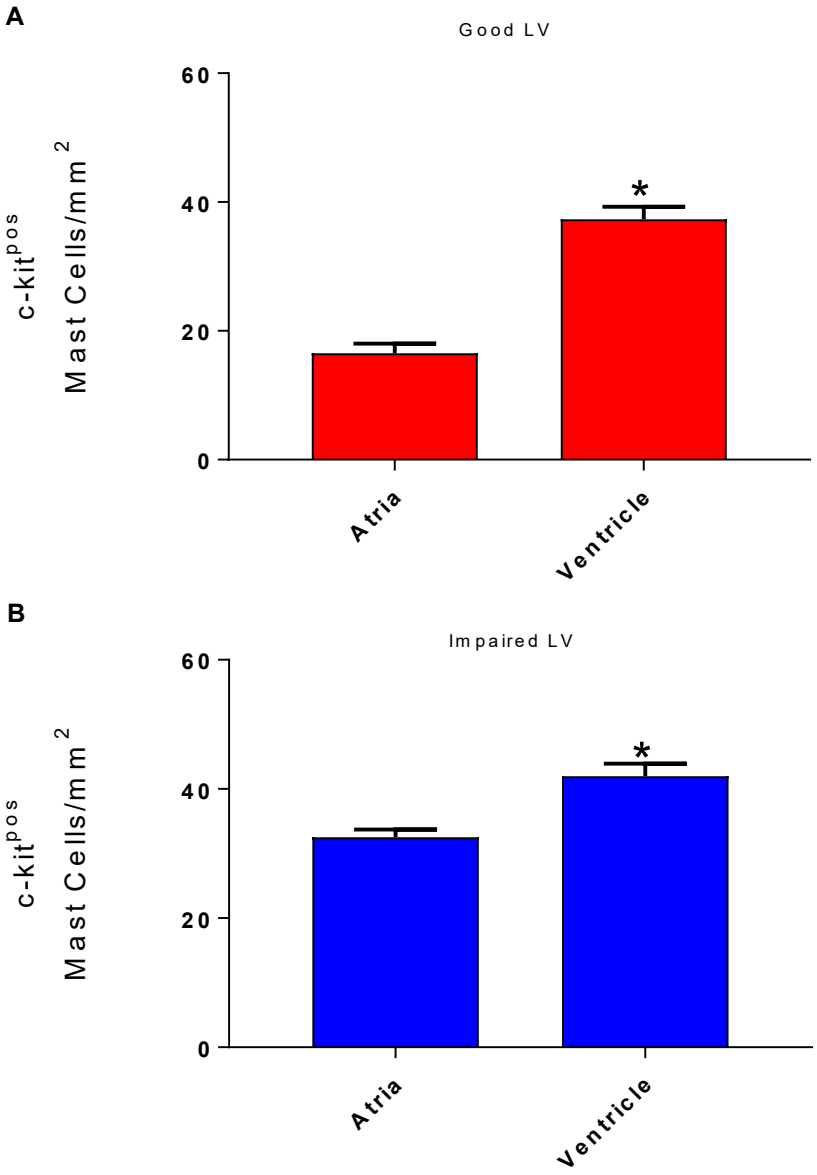

Figure 3. 14 Overall distribution of c-kit^{pos} tryptase^{pos} mast cells in Atria and ventricle. The overall abundance of mast cells was compared between Atria (A) and Ventricle (B). Data is mean ±SEM, n=10 per group (samples from each chamber taken from 5 patients). * denotes significant differences ($p<0.05$) vs atria.

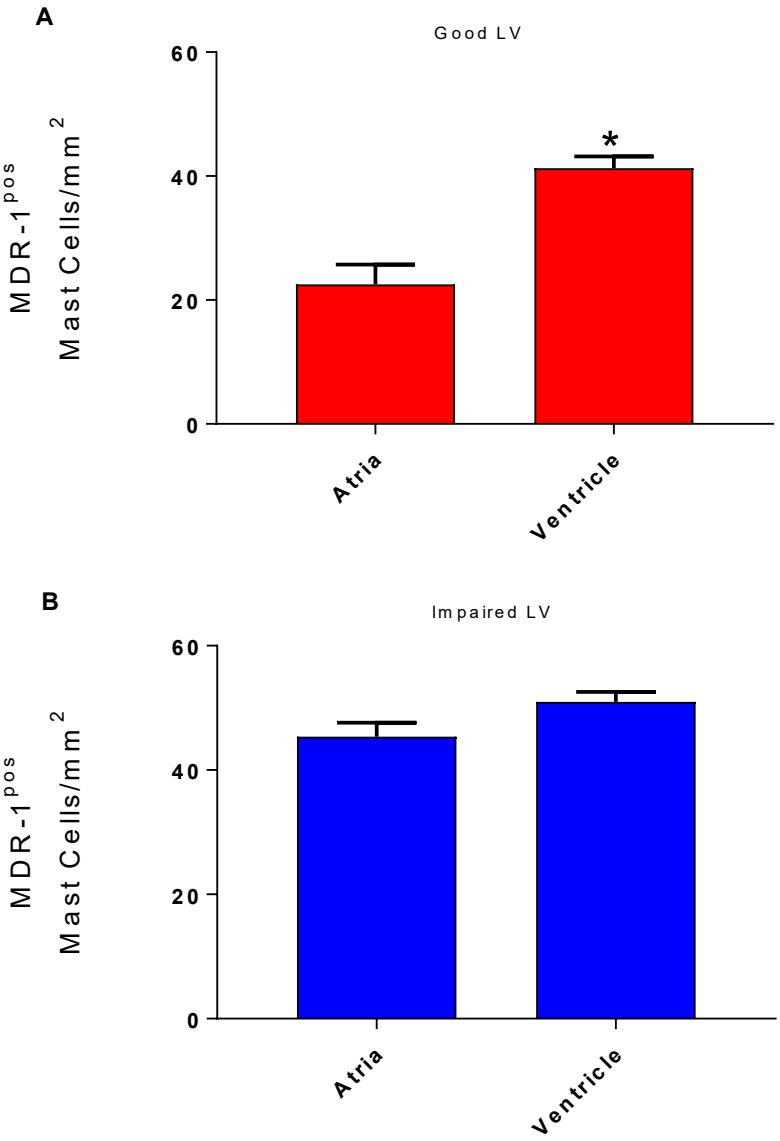

Figure 3. 15 Overall distribution of MDR-1^{pos} tryptase^{pos} mast cells in Atria and ventricle. The overall abundance of mast cells was compared between Atria (A) and Ventricle (B). Data is mean ±SEM, n=10 per group (samples from each chamber taken from 5 patients). * denotes significant differences ($p<0.05$) vs atria.

To summarise, the main findings were:

- The Left Ventricle harbours the greatest number of c-kit^{pos} mast cells, and the RA harbours the least c-kit^{pos} mast cells, regardless of good or impaired LV function.
- The number of MDR1^{pos} mast cells is greatest in the LV and least in the RA for good LV patients; however, the LA harbours the least MDR1^{pos} mast cells in impaired LV patients.
- Impaired LV patients harbour increased number of c-kit^{pos} mast cells and MDR1^{pos} mast cells, compared to good LV patients.
- The Ventricle harbours increased number of c-kit^{pos} mast cells, regardless of good or impaired LV function.
- The Ventricle harbours a greater number of MDR1^{pos} mast cells in both good LV and impaired LV patients.

3.2.10 Quantification of c-kit^{pos} tryptase^{neg} CPCs in all four chambers of the human heart from good and impaired LV patients.

In good LV patients, the number of c-kit^{pos} CPCs were higher in LV (31± 4/mm^2) compared to RA (6± 0/mm^2), LA (25± 3/mm^2) and RV (30± 5/mm^2 (Figure 3.16A)). RA had significantly ($p<0.05$) less c-kit^{pos} CPCs compared to other chambers. In impaired LV group (Figure 3.16B), LV (38± 2/mm^2) had significantly ($p<0.05$) more c-kit^{pos} CPCs than RA (27± 0/mm^2), LA (32± 0/mm^2) and RV (30± 2/mm^2). RA had significantly ($p<0.05$) less c-kit^{pos} CPCs compared to LA and LV.

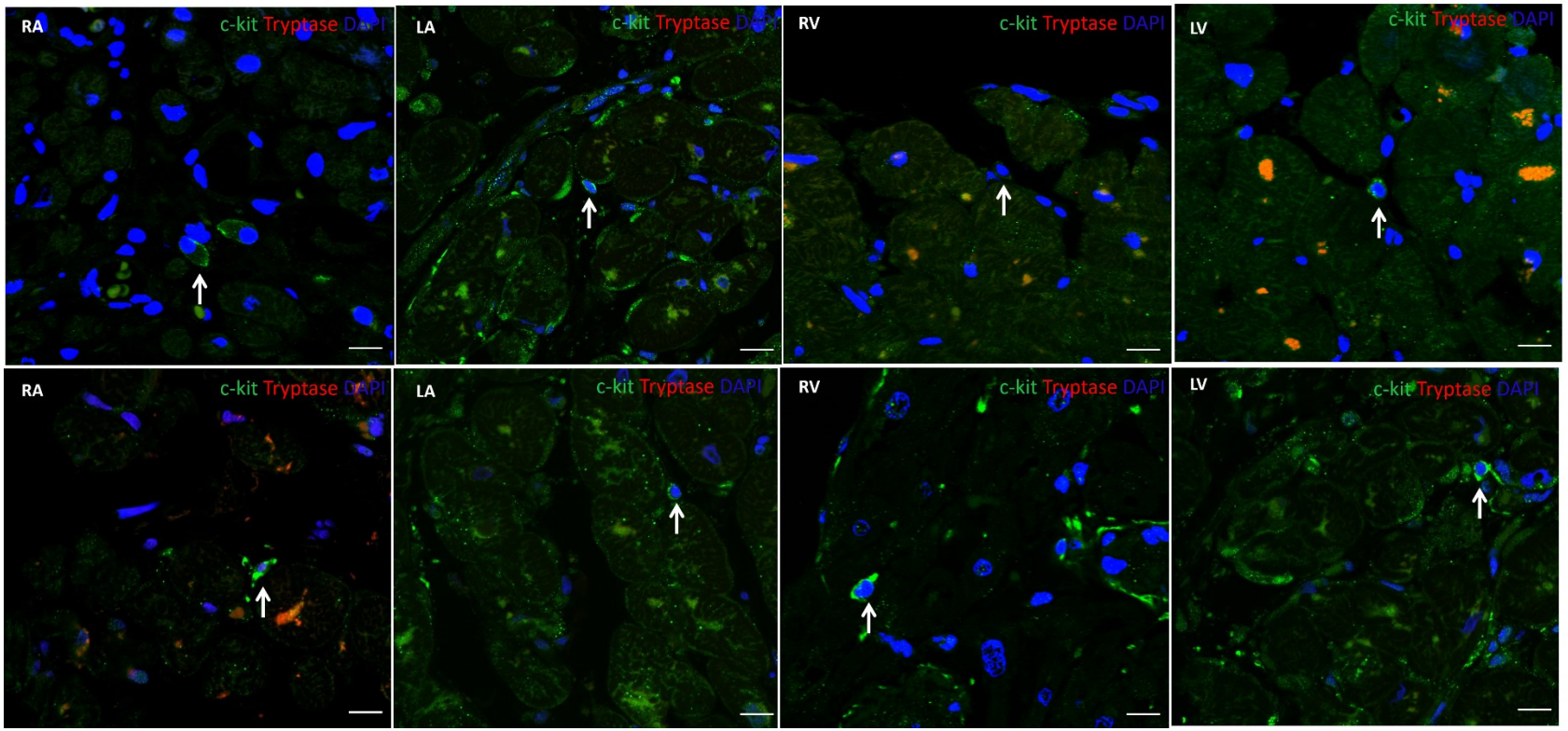

Figure 3. 16 Identification of c-kit^{pos} CPCs. Representative confocal images show c-kit^{pos} (green) tryptase^{neg} (red) CPCs, indicated by the white arrow within the myocardial/endocardial region . Nuclei are stained in blue by DAPI.. Scale = 10μm. Top Row – Cardiac chambers of good LV; Bottom Row- Cardiac chambers of Impaired LV.

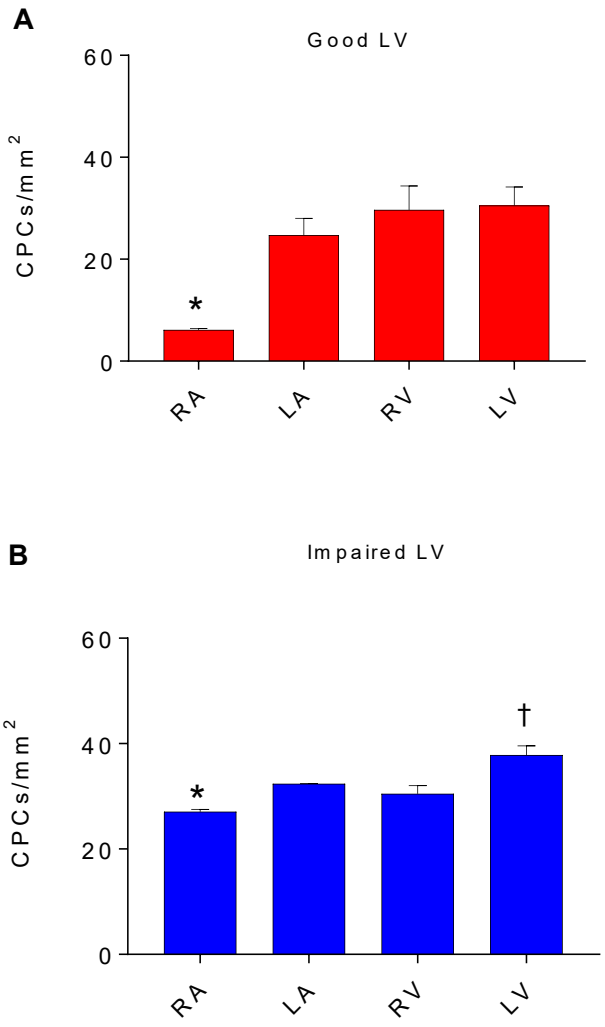

Figure 3. 17 c-kit^{pos} tryptase^{neg} CPCs in all four chambers of the heart. Quantification of c-kit^{pos} tryptase^{neg} cells across all four chambers of human myocardium. The data is represented as mean ±SEM, n=5 per chamber. (A) good LV patients. * denotes significant differences ($p<0.05$) vs all chambers. (B) impaired LV patients. * denotes significant differences ($p<0.05$) vs LA and LV. † denotes significant differences ($p<0.05$) vs all chambers.

3.2.11 Quantification of MDR1^{pos} tryptase^{neg} CPCs in all four chambers of the human heart from good and impaired LV patients.

The MDR-1^{pos} CPCs were more abundant in LV (22± 2/mm^2) compared to RA (5± 1/mm^2), LA (19± 3/mm^2) and RV (16± 2/mm^2); within the good LV group (Figure 3.18A). RA had significantly ($p<0.05$) less MDR-1^{pos} CPCs compared to other chambers. In impaired LV group (Figure 3.18B), RA (51± 1/mm^2) and RV (49± 3 /mm^2) had similar distribution of MDR-1^{pos} CPCs compared to LA (46± 1/mm^2) and LV (41± 1/mm^2). The RA and RV had significantly ($p<0.05$) more MDR-1^{pos} CPCs compared to the LV chamber.

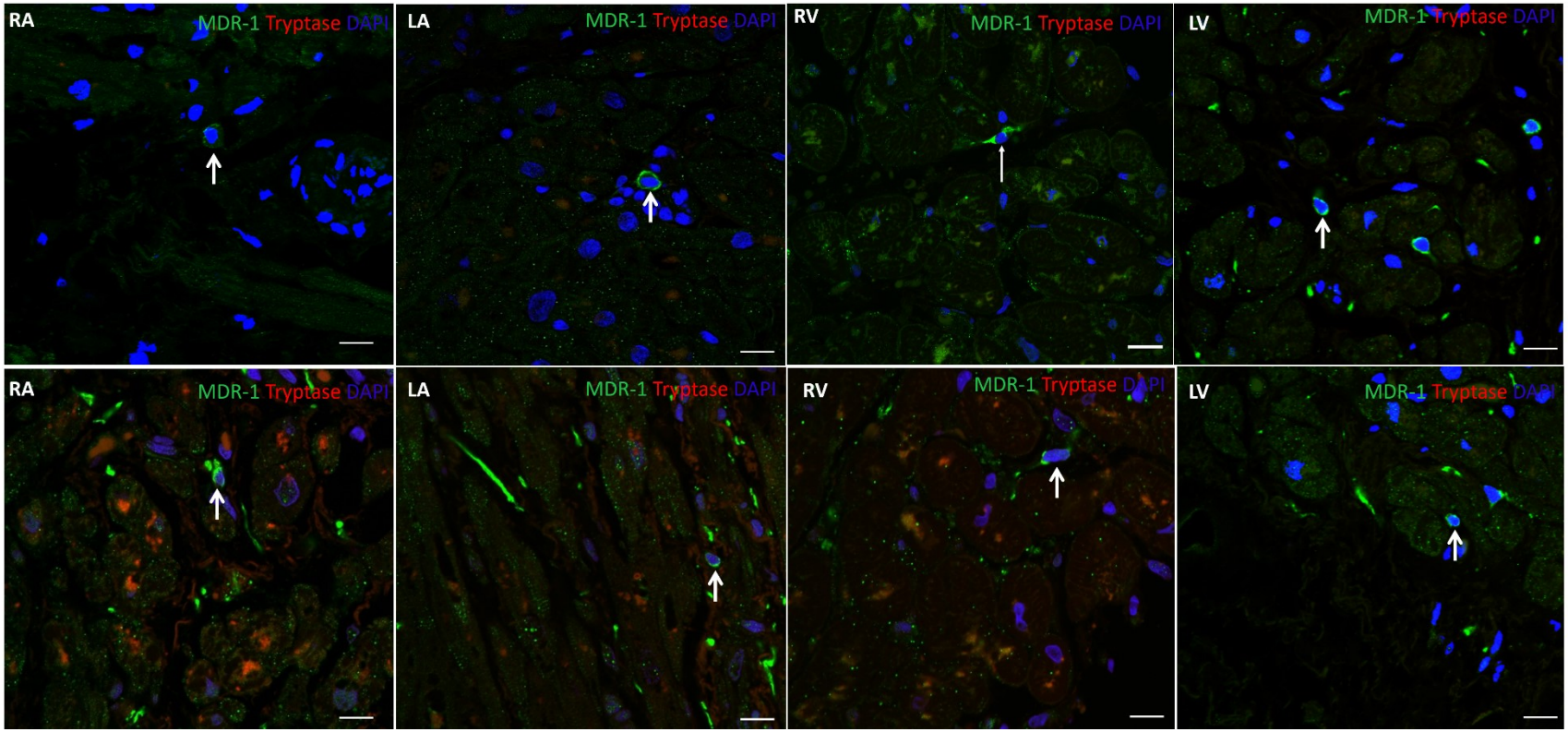

Figure 3. 18 Identification of MDR-1^{pos} CPCs. Representative confocal images show (A) MDR-1^{pos} (green) tryptase^{neg} (red) CPCs; indicated by the white arrow within the myocardial/endocardial region. Nuclei are stained in blue by DAPI.. Scale = 10μm. Top Row – Cardiac chambers of good LV; Bottom Row- Cardiac chambers of Impaired LV.

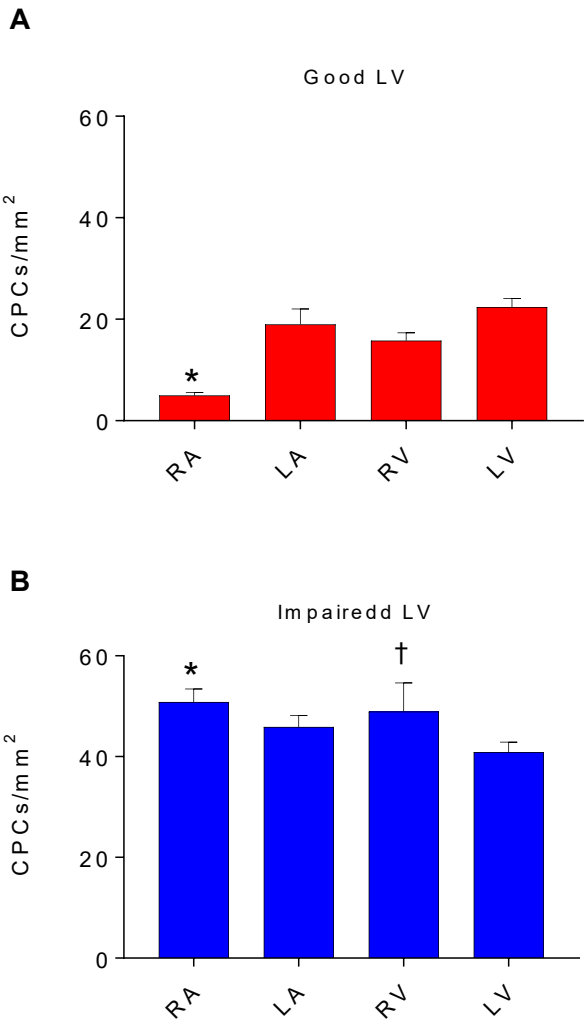

Figure 3. 19 MDR-1^{pos} tryptase^{neg} CPCs in all four chambers of the heart. Quantification of MDR-1^{pos} tryptase^{neg} CPCs across all four chambers of human myocardium. The data is represented as mean ±SEM, n=5 per chamber. (A)Good LV patients. * denotes significant differences ($p<0.05$) vs all chambers. (B) impaired LV patients. * denotes significant differences ($p<0.05$) vs LV. † denotes significant differences ($p<0.05$) vs LV.

3.2.12 Comparison of the overall distribution of c-kit^{pos} and MDR1^{pos} tryptase^{neg} CPCs between good and impaired LV patients

Overall, the c-kit^{pos} (32± 1/mm^2 vs 23± 3/mm^2) (Figure 3.19A) and MDR-1^{pos} (47± 1/mm^2 vs 16± 2/mm^2) (Figure 3.19B), CPCs were significantly higher ($p<0.05$) in impaired LV group than good LV group respectively.

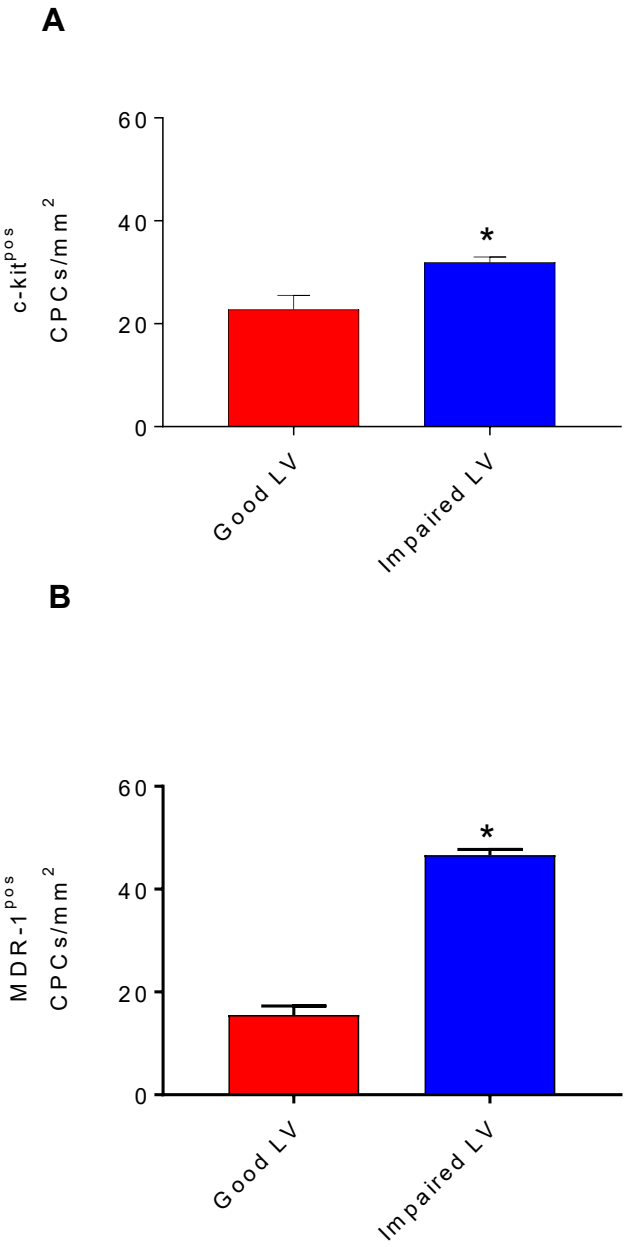

Figure 3. 20 Overall distribution of c-kit^{pos} tryptase^{neg} and MDR-1^{pos} tryptase^{neg} CPCs in Good LV vs Impaired LV. The overall abundance of c-kit^{pos} (A) and MDR-1^{pos} (B) CPCs were compared between good LV and impaired LV group. Data is mean ±SEM, n=20 per group (samples from each chamber taken from 5 patients). * denotes significant differences ($p<0.05$) vs good LV.

3.2.13 Comparison of the overall distribution of c-kit^{pos} and MDR1^{pos} tryptase^{neg} CPCs between atria and ventricle.

The distribution of c-kit^{pos} CPCs was significantly ($p<0.05$) higher in ventricles compared to atria in both good (30±3/mm^2 vs 15±3/mm^2) (Figure 3.20A) and impaired LV (34±2/mm^2 vs 30±/mm^{2}1) group (Figure 3.20B). However, the distribution of MDR-1^{pos} CPCs was significantly($p<0.05$) higher in the ventricle (19±2/mm^2) compared to the atria (12±3/mm^2) in the good LV (Figure 3.21A) group only. In the impaired LV (Figure 3.21B) group, the distribution of the MDR-1^{pos} CPCs was marginally higher in the atria (48±1/mm^2) than the ventricle (45±2/mm^2).

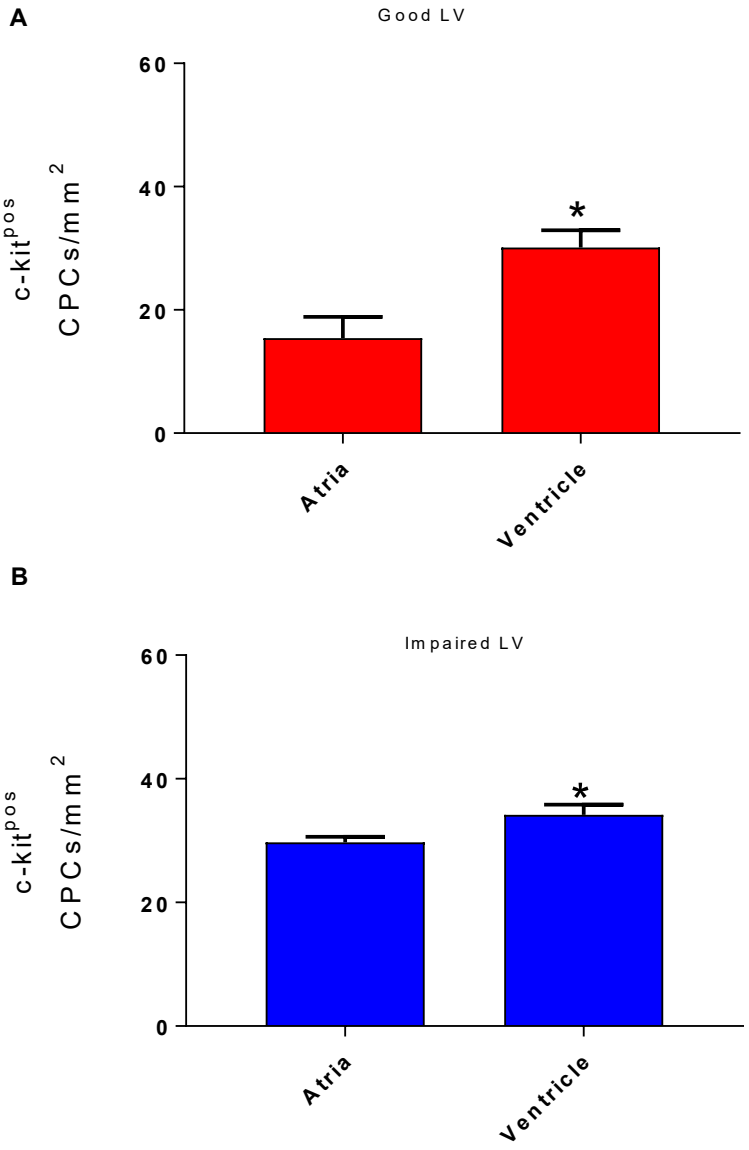

Figure 3. 21 Overall distribution of c-kit^{pos} CPCs in Atria and ventricle. The overall abundance of c-kit^{pos} CPCs was compared between Atria (A) and Ventricle (B). Data is mean ±SEM, n=10 per group (samples from each chamber taken from 5 patients). * denotes significant differences ($p<0.05$) vs atria.

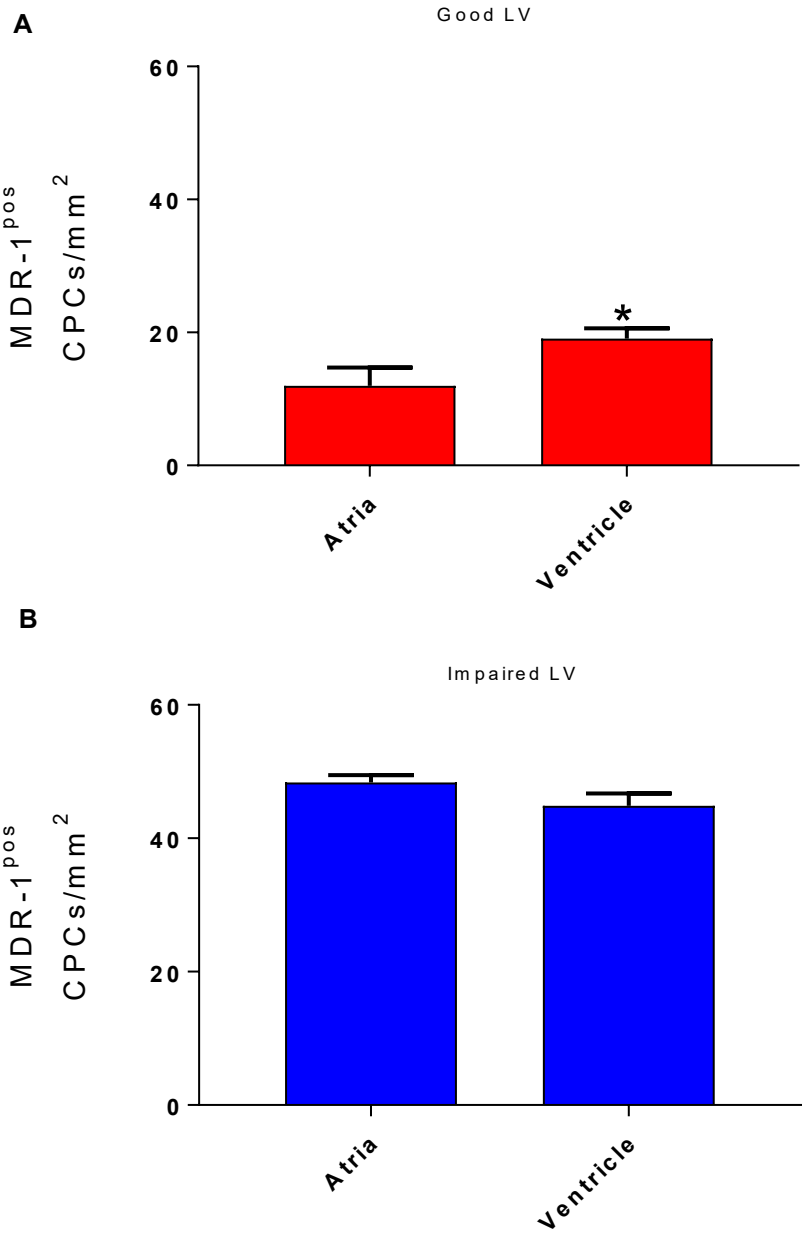

Figure 3. 22 Overall distribution of MDR-1^{pos} CPCs in Atria and ventricle. The overall abundance of MDR-1^{pos} CPCs was compared between Atria (A) and Ventricle (B). Data is mean ±SEM, n=10 per group (samples from each chamber taken from 5 patients). * denotes significant differences ($p<0.05$) vs atria.

To summarise, the main findings were:

- The Left Ventricle harbours the greatest number of c-kit^{pos} CPCs, and the RA harbours the least c-kit^{pos} CPCs, regardless of good or impaired LV function.
- The number of MDR1^{pos} CPCs are greatest in the LV and least in the RA for good LV patients. However, MDR1^{pos} CPCs are greatest in the RA and least in the LV in impaired LV patients.
- Impaired LV patients harbour increased number of c-kit^{pos} CPCs and MDR1^{pos} CPCs, compared to good LV patients.
- The Ventricle harbours increased number of c-kit^{pos} CPCs, regardless of good or impaired LV function.
- The Ventricle harbours a greater number of MDR1^{pos} CPCs in good LV patients. However, the atria had more MDR1^{pos} CPCs than the ventricle in impaired LV patients.

3.3 Discussion

3.3.1 Quantification of CPCs in all four chambers of the human heart from good and impaired LV patients.

In this study, the distribution of eCPCs from all four chambers obtained from a single patient at the time of surgery was assessed and compared to the distribution of the eCPCs between normal and impaired LV group.

It was noticeable that during confocal analysis that CPCs were distributed more in the myocardial layer within the interstitial space. Some regions within the section had no nuclei; this is possibly due to underlying fibrotic response to myocardial injury. Furthermore, it may also represent damage that occurred during biopsy or sectioning (I performed all sectioning) of the samples, due to the relatively small myocardial sample size,

Consistently the number of mast cells and the CPCs were distributed more in the ventricle than atria. Moreover, the LV chamber had more CPCs when compared to other chambers. This finding is similar for both good and impaired LV group. However, the mast cells and CPCs are more in abundance in the impaired LV group compared to the good LV group.

These findings are different from the outcomes that have been reported before. The c-kit^{pos} CPCs have been identified in a variety of species (Messina et al. 2004; Ellison et al. 2011) including that of humans (Bearzi et al. 2007 and Arsalan et al. 2012). Characteristically these cells have reported to have ~1 eCPC per 1,000 cardiomyocytes or 45,000 human CPCS per gram of tissue (Torella et al. 2007). In 2008, Pouly et al. used immune histochemical analysis of biopsies derived from right atrial appendages and right-side septum of patient undergoing heart transplant and ischemic cardiomyopathy. They found the right-sided septum to harbour more c-kit^{pos} cells when compared to RAA. Another study by Itzhaki-Alfia A et al. (2009), reported a higher number of c-kit^{pos} and Islet-1^{pos} in RA (24±2.5% and 7%) compared to other chambers (LA- 7.3±3.5%, RV-4.1±1.6%), and LV-9.7±3%; P=0.001). In this study, 113 biopsies were obtained 94 patients from discarded tissues from the

surgery. They did not lineage sort for their CPCs. However, their finding of an increased number of c-kit^{pos} cells in the impaired LV group correlates with our findings.

In 2012 Arsalan et al., isolated lineage negative CPCs from endocardial samples of RA, LA and LV from patients undergoing cardiac surgery. They showed that the prevalence of c-kit^{pos} cells in both atria (4.90 ± 1.29%) was about 5 times greater than in the left ventricle (0.62 ± 0.14%, P = 0.035).

Further study by Dixit et al. (2017) examined the progenitor cells derived from the RA, LV and peripheral blood and compared their functional differences. They showed the c-kit^{pos} CPCs population to be <1%, as previously described and showed higher abundance in LV compared to RA (0.87 ±0.45% vs 0.5±0.2%).

CPCs have been shown to play a crucial role in cardiac homeostasis (Nadal-Ginard B et al. 2014). Using various rodent models with diffuse myocardial injury c-kit^{pos,} CPCs have been shown to restore cardiac function through regeneration of lost cardiomyocytes and when the CPCs were ablated this reparative process was not observed Ellison et al. (2013). Hence this may explain why more CPCs cells were observed in the impaired ventricle.

The MDR-1^{pos} cells mostly had a similar trend to c-kit^{pos} cells. This observation suggests that they are of the same cell population, and their expression of the surface markers may vary according to their physiological/differentiation state.

Interestingly as with our finding, Patella et al. (1998) showed more mast cells in the impaired LV group compared to normal LV. The distribution of cardiac mast cells is also higher in ventricle than atria and more abundant in impaired LV group compared to good LV group. It has been estimated that on average there is a fourfold increase in CPCs in the failing heart; 80% of which are mast cells that express CD45 (KuboH et al 2008). This finding may be related to the innate response of the myocardium to the loss of function in the impaired LV group.

From our study, the age is the only patient characteristic, apart from LV impairment, that could correlate with the above observations. The age of the impaired LV population was significantly higher when compared to good LV patients. Several

studies have shown age and disease to affect the characteristics of CPCs (Cesselli et al. 2011; Lewis-McDougall F et al. 2019). These changes have been attributed to cellular senescence through various mediators. Accordingly, in this study, the impaired LV group had more CPCs and mast cells compared to the normal LV group as a possible response to cardiac homeostasis. The study by Lewis-McDougall F et al. (2019) showed that despite the increased in CPCs within aged failing hearts; the CPCs were shown to be dysfunctional. The other patient demographics factors were insufficient in numbers to associate a correlation with our findings. However, patient medical history, history of smoking, atrial fibrillation, previous myocardial infarction and the use of cardioprotective drugs, such as β-blockers and statins can alter the frequency of c-kit^{pos} progenitors in the heart (Gambini E et al. 2012).

4. Isolation, clonogenicity and proliferation of CPCs isolated from all four chambers of the human heart.

4.1 Introduction

The understanding of the characteristics of CPCs that have been evaluated to date (Ellison et al. 2014, Smith A et al. 2014) has mainly come from animal work. In addition, several groups have isolated CPCs from human myocardium but limited mainly to the RA chamber (Bearzi et al. 2007; Monsanto et al. 2017; Itzhaki-Alfia A et al. 2009). These studies have shown the isolated CPCs to maintain a stable phenotype over time and proliferate and differentiate into different cardiac lineages (smooth muscle, endothelial and cardiomyocyte (CM)) (Bearzi et al. 2007; Ellison et al. 2013; Smith et al. 2014; Vicinanza C et al. 2017; Pouly et al. 2008; Mauretti et al. 2017).

Characteristically these cells have been reported to be ~1 eCPC per 1,000 cardiomyocytes or 45,000 human CPCs per gram of tissue (Torella et al. 2007). In 2008, Pouly et al. used immune histochemical analysis of biopsies derived from right atrial appendages and right-side septum of patients undergoing heart transplant and ischemic cardiomyopathy. They found the right-sided septum to harbour more c-kit^{pos} cells when compared to RAA. Another study by Itzhaki-Alfia A et al. (2009), reported a higher number of c-kit^{pos} and Islet-1^{pos} in RA (24±2.5% and 7%) compared to other chambers (LA- 7.3±3.5%, RV-4.1±1.6%), and LV-9.7±3%; *P*=0.001). However, these cells were not lineage sorted to characterise the isolated cells.

Further study by Dixit et al. (2017) examined the progenitor cells derived from the RA, LV and peripheral blood and compared their functional differences. They showed the c-kit^{pos} CPCs population to be <1%, as previously described and showed higher abundance in LV compared to RA (0.87 ±0.45% vs 0.5±0.2%).

The isolation of the CPCs from the myocardium has been achieved by enzymatic dissociation or by explant culture technique. During the enzymatic digestion process,

the myocardial sample is exposed to proteolytic enzymes such as collagenase, which breaks down tissue compartments releasing small cells. This technique is well described by Smith et al. (2014) for rodent hearts. However, Bearzi et al. described both enzymatic and explant cultures techniques to isolate CPCs from human myocardium. When compared to enzymatic isolation process, the explant technique has been shown to yield less heterogeneous cell populations, while demonstrating higher proliferative rates and cell viability from relatively smaller biopsy samples (Salehinejad P et al. 2012; Yoon JH et al. 2013; Hilkens P et al. 2013).

Once isolated, the cells are further purified using fluorescent or magnetic antibody tags by FACS or MACS, respectively (Zhu & Murthy et al. 2013). The MACS separation purity has been reported at around 75% (Zhou et al. 2013) and provides more structural integrity to the isolated cells compared to FACS along with higher output and faster separation (~10^{11} cells/hour vs 10^{7} cells/hour). However, with FACS techniques, a high level of purity of the cell population (>95%) can be achieved. c-kit^{pos}CD45^{neg}CD31^{neg} CPCs have been isolated using MACS by others, including our group (Bearzi et al. 2007; Ellison et al. 2013; Smith et al. 2014; Vicinanza et al. 2017).

Our lab group has characterised the phenotype of the CPC as described previously. The isolation of CPCs, according to this phenotype, has enabled us to understand the fundamental biological principles and their application towards therapeutic goals.

One of the essential features of CPC is the ability to generate clones. This inherent characteristic allows sufficient numbers to be created and expanded in-vitro without losing the regenerative capacity and differentiation potential (Ellison-Hughes & Lewis. 2017). Supplementation of various growth factors to the culture media has also allowed successful propagation of clonal c-kit pos CPCs and CDCs, without compromising their phenotype, differentiation potential or genomic stability (Itzhaki-Alfi A et al. 2009; Ellison-Hughes et al. 2011). Previously, clonogenic CPCs have been shown to reside in niches within the myocardium (Beltrami et al. 2003). A recent study by Lewis-McDougall FC et al. (2019); showed that CPCs isolated from hearts of older patient to have reduced proliferation, clonogenicity and differentiation potential. More importantly, the findings of single CPC derived clones from young and old patient were indistinguishable with regards to morphology, senescence,

multipotency, self-renewing ability and differentiation potential. CPCs has been shown to play a crucial role in cardiac homeostasis (Nadal-Ginard B et al. 2014). Using various rodent models with diffuse myocardial injury CPCs have been shown to restore cardiac function through the regeneration of lost cardiomyocytes and when the CPCs were ablated this reparative process was not observed (Ellison et al. 2013).

Despite this volume of evidence regarding human CPCs, so far no study has extensively characterised the CPCs derived from all four chambers of the human heart or compared the characteristics of CPCs derived from normal and impaired LV function patients; to understand their basic characteristics .

The specifics aim for this study are:

1. To isolate c-kit^{pos}CD45^{neg}CD31^{neg} CPCs from all four chambers of the heart from patients with normal and impaired LV function using either enzymatic or explant culture technique.
2. To assess and compare the clonogenicity and proliferation, of human CPCs from different cardiac chambers using in vitro assays.
3. To compare the clonogenicity and proliferation of human CPCs isolated from patients with normal and impaired LV function.

4.2 Results

4.2.1 Isolation of CPCs from myocardial samples using enzymatic technique.

As described in the Methods, both enzymatic and explant culture techniques have been described and validated as a method of isolating CPCs from tissue biopsies. The choice of method primarily depends on the weight of the sample. Initially, I used the enzymatic approach to learn and gain competency in isolating CPCs. I enzymatically digested three samples obtained from right atrial appendage (RAA, n=1) and left atrial appendage (LAA, n=2). The number of CPCs isolated using the enzymatic technique was 19,366 ± 7050; counted using a hemocytometer (Table 4.1). During this learning curve, I managed to achieve ~85% purity in isolating c-kit^{pos}CD45^{neg} CPCs (Table 4.1; Figure 4.1).

Table 4. 1 c-kit^{pos} CPCs isolated using enzymatic digestion.

Sample	Site	LV Function	Weight of sample (g)	Number of CPCs	% Purity
1	RAA	Good	0.596	27500	74.3
2	LAA	Impaired	0.277	15600	86.1
3	LAA	Impaired	0.652	15000	90

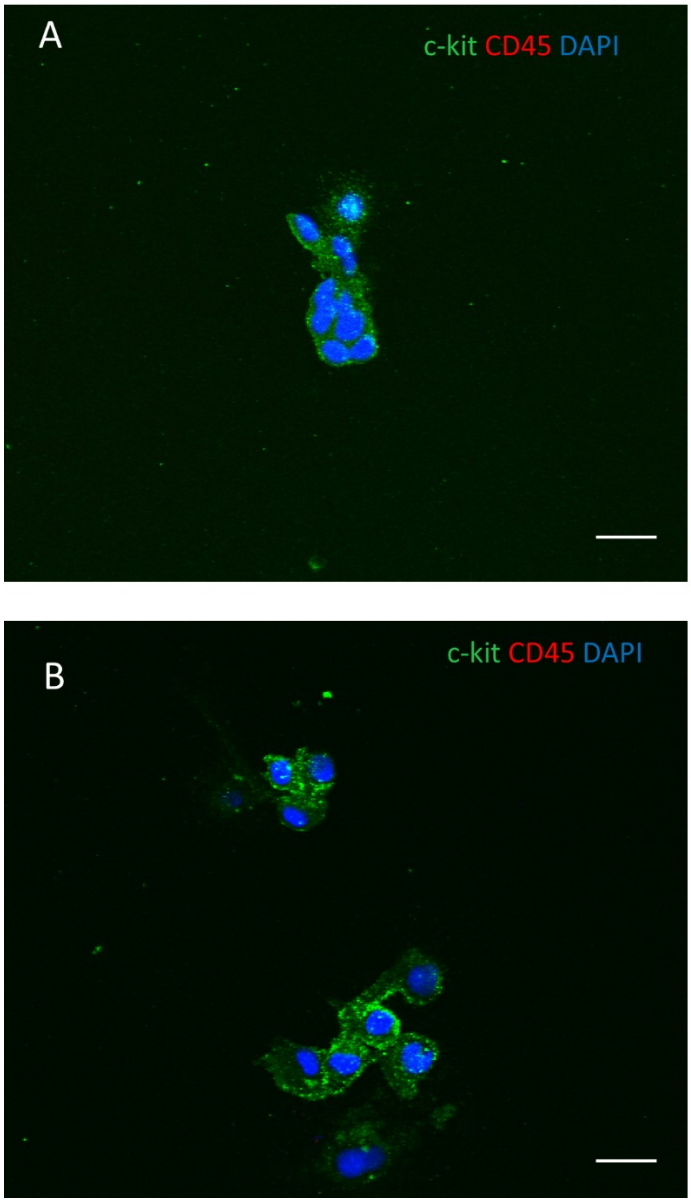

Figure 4. 1 Identification of c-kit^{pos} CPC isolated using enzymatic digestion. CPC isolated (A and B) from right atrial appendages stained by IHC for c-kit (green), CD45 (red) and DAPI (blue). Scale = 20μm.

4.2.2 Isolation of CPCs from myocardial samples using explant culture technique.

All four-chamber biopsies were taken from good LV patients (n=5) and impaired LV patients (n=5) (Table 4.2). The atrial samples were received as a larger mass when compared to ventricular samples. To keep a consistent sample size, the Tru-Cut needle was used (as described in methods) on the atrial biopsies to obtain a representative sample size across all the chambers.

Explant culture technique was used to extract cells from each biopsy (Figure 4.2A&B). At day six in culture, the outgrowth cells obtained from the explant for each chamber were MACS sorted for c-kit^{pos}CD45^{neg}CD31$^{neg.}$ Although we achieved a high yield of CPCs, there was significant contamination of DDR2+ fibroblasts in the culture (Figure 4.3A). I, therefore, proceeded with a further purification step to remove the fibroblasts. After MACS sorting for ckit^{pos}CD45^{neg}CD31^{neg,} the cells were plated on a plastic flask for 20 minutes to allow fibroblasts to stick to the plastic. After this period, the supernatant containing the unattached cells (the CPCs) were transferred to CELLstart™ CTS™ coated flasks for expansion. The supernatant containing the unattached cells had no fibroblasts present and were ckit^{pos}CD45^{neg}CD31^{neg} CPCs (Figure 4.3B). The cells that stuck to the plastic were confirmed as fibroblasts, being positive for DDR2 (Figure 4.3C).

The samples used for explant isolation had a mean weight of 0.005g for both good and impaired LV group. The inter chamber comparison for the average number of CPCs per gram of tissue obtained also showed no statistical significance for both good and impaired LV group. However, the average number of CPCs per gram of tissue is significantly ($p<0.05$) higher in good LV group when compared to impaired LV group (Table 4.3).

Table 4. 2 Weight of the biopsy obtained from each patient

Sample Number	LV Function	Right Atrium (mg)	Left Atrium (mg)	Right Ventricle (mg)	Left Ventricle (mg)
1	Good	270	288	5.16	5.22
2	Good	251	266	4.89	5.32
3	Good	302	279	5.12	5.16
4	Good	266	273	5.03	5.19
5	Good	199	229	5.21	4.98
6	Impaired	268	302	5.16	4.79
7	Impaired	225	229	5.15	5.12
8	Impaired	245	215	4.99	5.15
9	Impaired	206	312	5.26	6.03
10	Impaired	259	283	5.18	5.12

Table 4. 3 Weight of the sample used for explant, Mean±SEM number of CPCs isolated per gram of tissue for each cardiac chamber from good and impaired LV.

Cardiac Chamber	Average Weight of Sample used for Explant (g)	Average No of CPCs /g of tissue (following purification step)	
		Good LV	Impaired LV
RA	0.005	29825 ± 1106	20748 ± 1500
LA	0.005	27748 ± 696	25995 ± 1450
RV	0.005	28394 ± 709	19948 ± 1344
LV	0.005	36554 ± 1350	22348 ± 1266

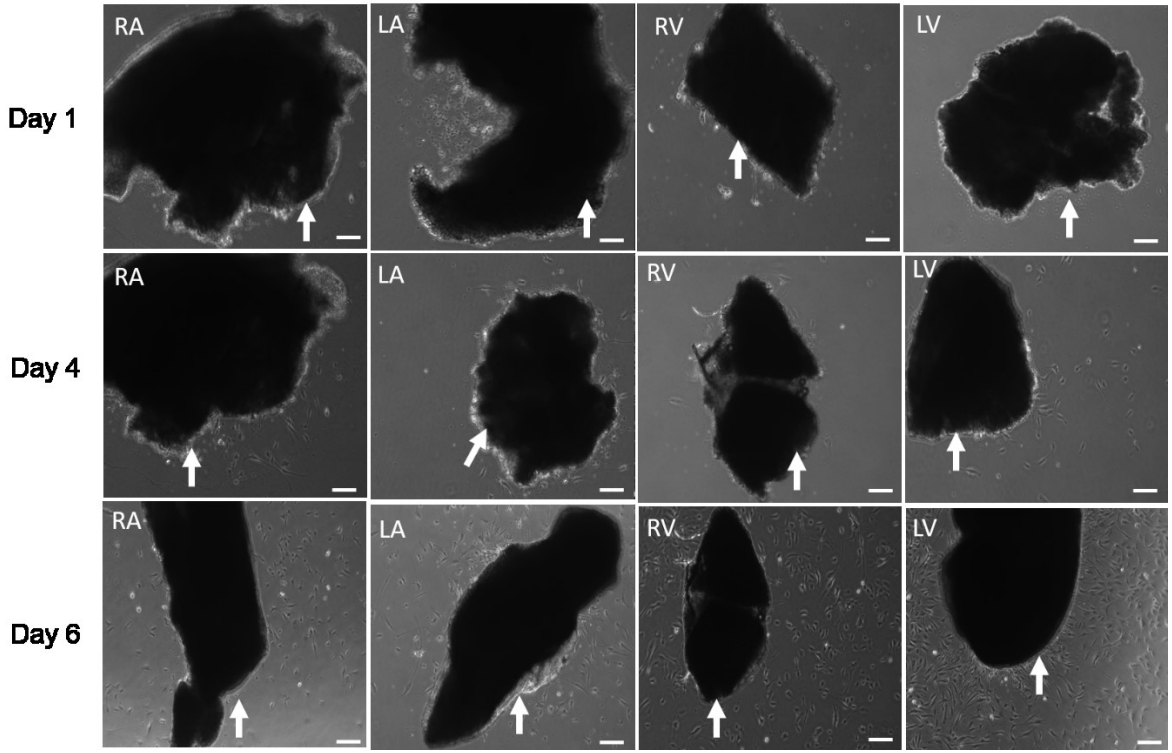

Figure 4. 2A Outgrowth of cells from biopsy during explant culture-Good LV. Each well had 5 pieces of of myocardial tissue (white arrow) were plated in a 6 well fibronectin coated plates (A). Outgrowth of cells from the myocardial biopsy pieces at day1, day 4 and day 6 was observed in all four chambers through transmitted light microscope. Scale = 200µm

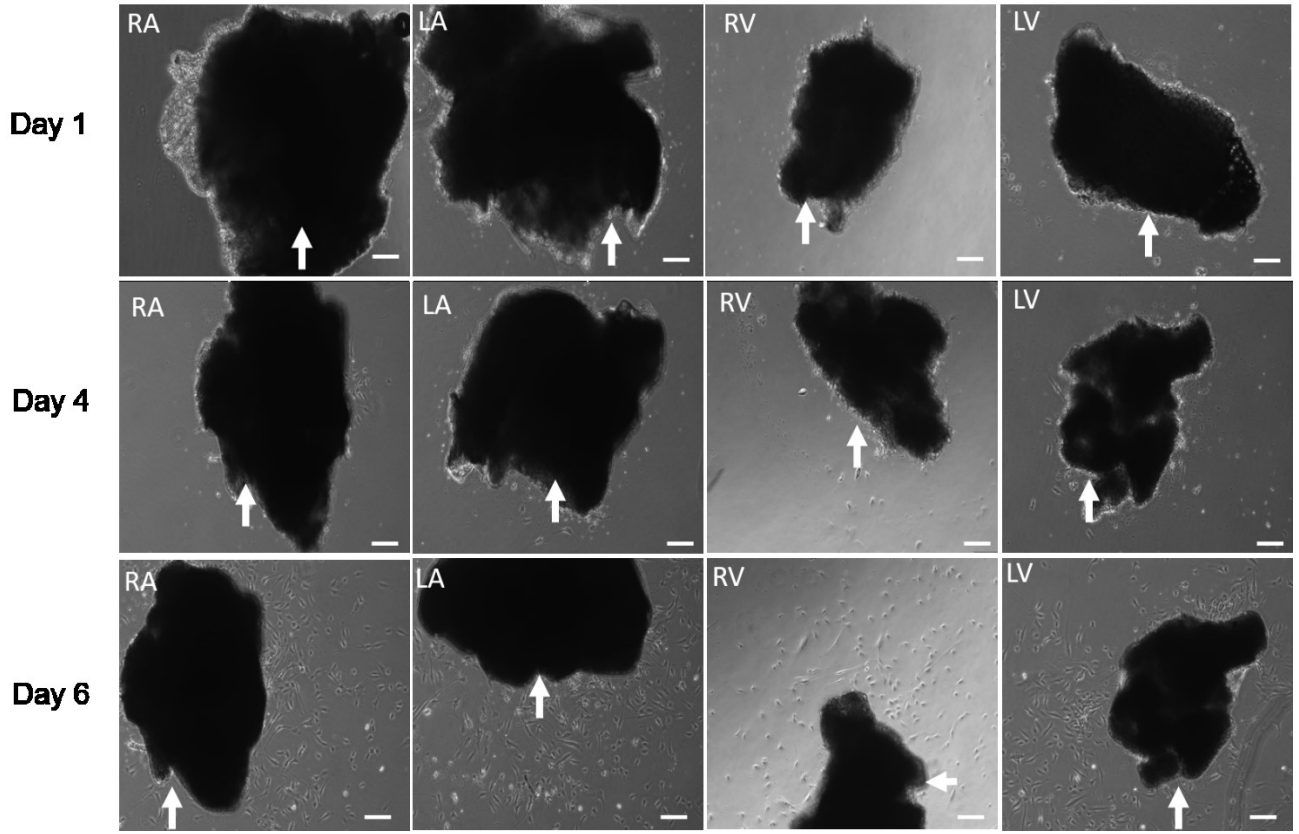

Figure 4. 2B Outgrowth of cells from biopsy during explant culture-Impaired LV. Pieces of myocardial tissue (white arrow) were plated in a 6 well fibronectin coated plates (A). Outgrowth of cells from the myocardial biopsy pieces at day1, day 4 and day 6 was observed in all four chambers through transmitted light microscope. Scale = 200μm

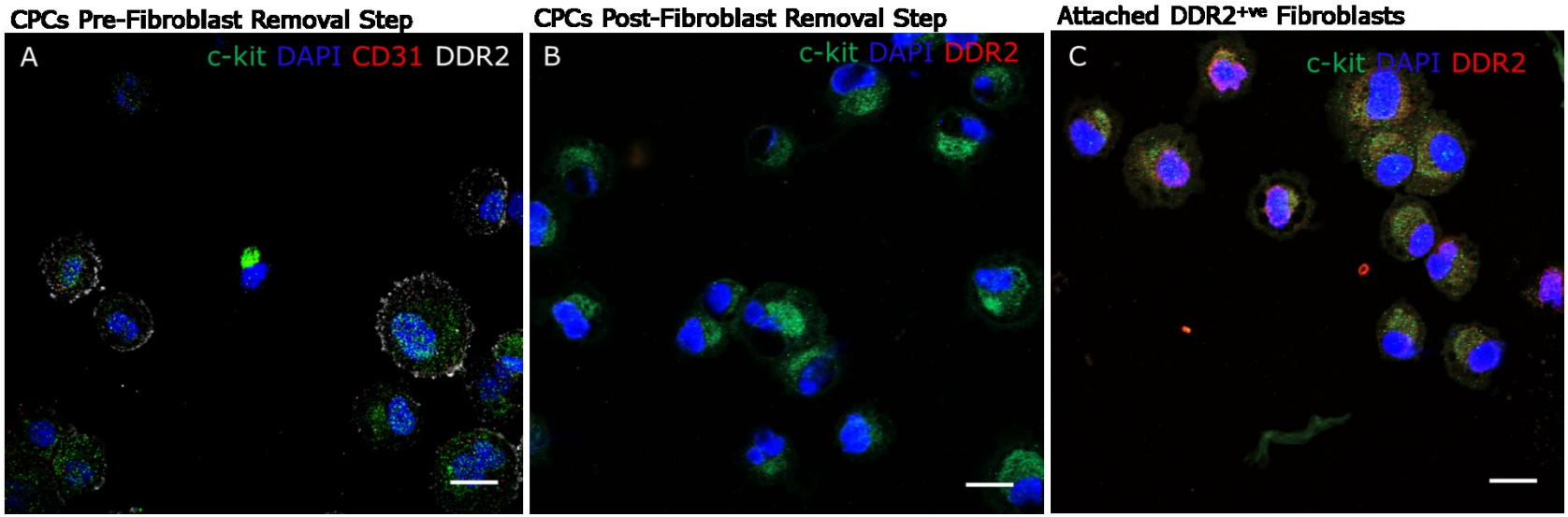

Figure 4. 3 Purification of CPCs derived from explant culture of Fibroblasts. (A) Isolated c-kit^{pos} (green), CD31^{neg} (red) CPCs showing contamination with DDR2+ (white) fibroblasts. B) IHC of the c-kit^{pos} CPCs from the supernatant following the fibroblast removal step showing no evidence of DDR2+ (red) fibroblasts. (C) IHC of the attached cells from the plastic flask showed predominant DDR2+ (Red) positive fibroblasts. DAPI stain nuclei blue. Scale = 20μm

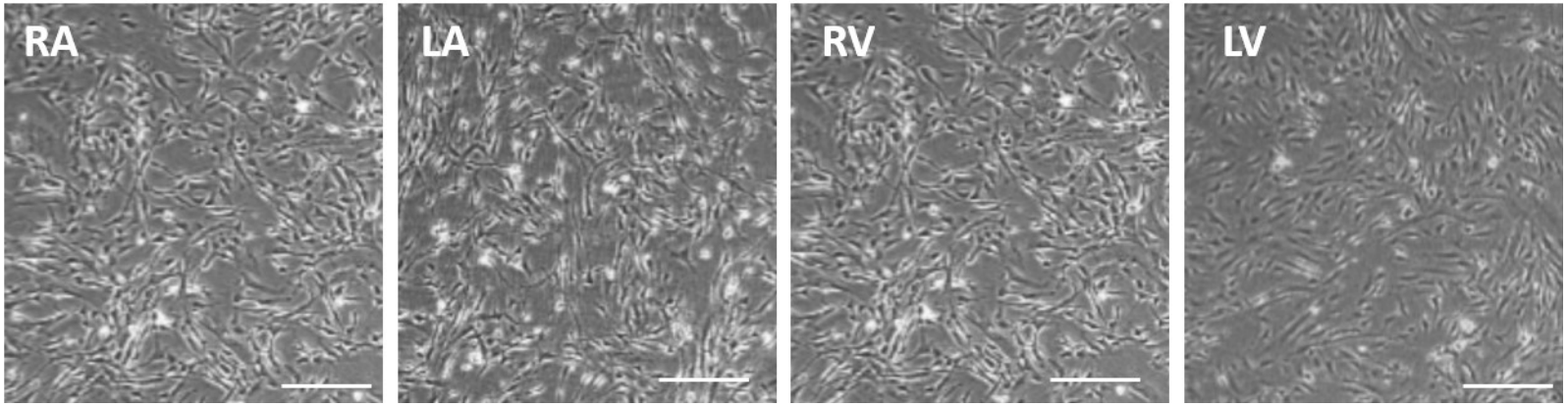

Figure 4. 4A Phase contract microscopic images-Good LV. Isolated CPCs in culture for each cardiac chamber, taken at day 6. Scale=500μm.

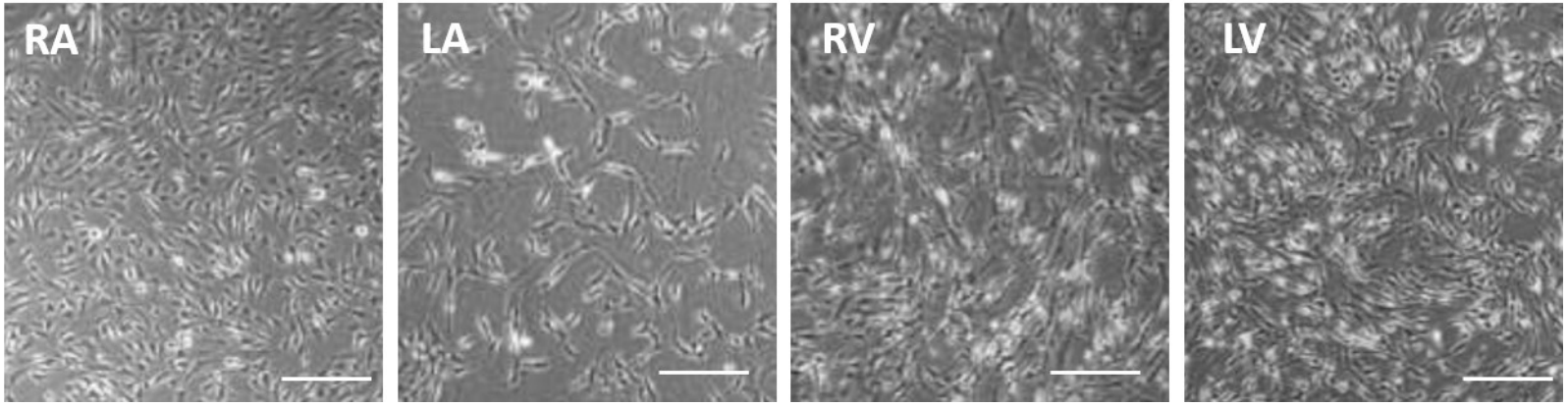

Figure 4. 4B: Phase contract microscopic images-Impaired LV. Isolated CPCs in culture for each cardiac chamber, taken at day 7. Scale=500μm.

4.2.3 Cellular morphology of the isolated CPCs from all four chambers.

Our observations have shown the isolated CPCs to be generally small and well rounded. These characteristic phenotypes are consistent with the description published by our lab group (Figure 4.3).

Largely, the cells displayed a homogeneous cellular growth thorough out the culture period for all four chambers. On average, it took 6.8 days to reach confluence for the CPCs derived from good LV and 7.5 days for CPCs derived from impaired LV (Figure 4.4 A&B).

To summarise, the main findings were:

- c-kit^{pos} CD45^{neg}CD31^{neg} CPCs can be isolated using both enzymatic and explant culture techniques.
- Isolated CPCs can be further purified to remove fibroblasts effectively by using the fibroblast adherence to plastic removal technique.
- Explant culture technique can be used to isolate CPCs from small biopsies taken from all four chambers of the human heart.

4.2.4 Clonogenicity of human CPCs isolated from patients with normal and impaired LV function.

At passage 5, through serial dilution, single c-kit^{pos}CD45^{neg}CD31^{neg} CPCs from good LV patients (n=5) and impaired LV patients (n=5) were deposited into 96 well cloning plates (n=3). IHC confirmed that CPCs maintained their phenotype as described previously (Figure 4.5). The single CPC went onto form clonal colonies of high-density cells (Figure 4.6). Three of the fastest-growing, small rounded, phase bright CPC colonies from each patient were picked up and expanded further.

Amongst good LV patients (Figure 4.7A), there was no significant difference in CPC clonogenicity between cardiac chambers RA (58±5%), LA (39±6%), RV (55±12%) and LV (63±11%). Amongst impaired LV patients (Figure 4.7B), the CPCs derived from the RV chamber (34±3%) had significantly decreased ($p<0.05$) clonogenic capability compared to the LV (49±3%).

Overall, the ability to generate single CPC-derived clones was significantly ($p<0.05$) greater in the good LV (54±5%) group, compared to the impaired LV (43±2%) group (Figure 4.8). There were no differences in clonogenicity between atria and ventricle for good (48±5% vs 59±8%) and impaired LV function (45±4% vs 42±3%) (Figure 4.9).

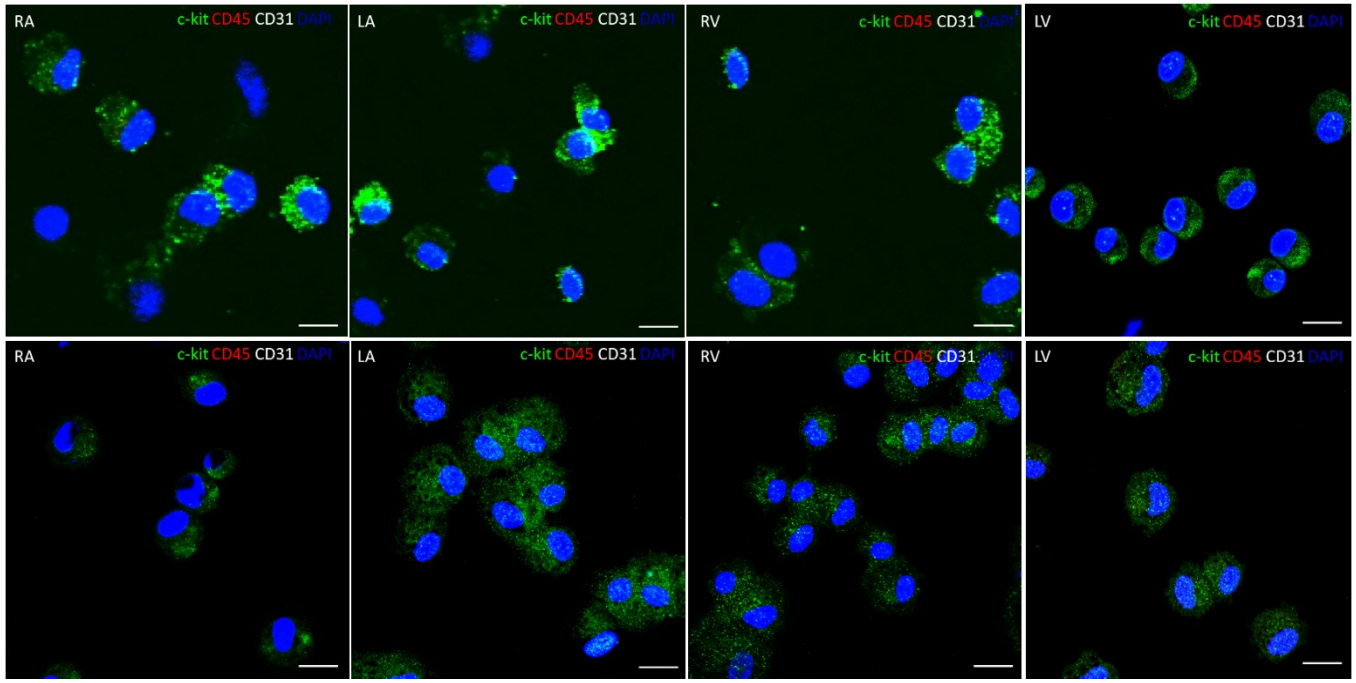

Figure 4. 5 Characterisation of the isolated c-kit^{pos} CPCderived from explant culture before Clonogenicity Experiment. Top row- CPCs from Good LV patients, Bottom row- CPCs from impaired LV patients. IHC for c-kit (green), CD45 (red) CD31 (white) and DAPI (blue). Scale = 20μm

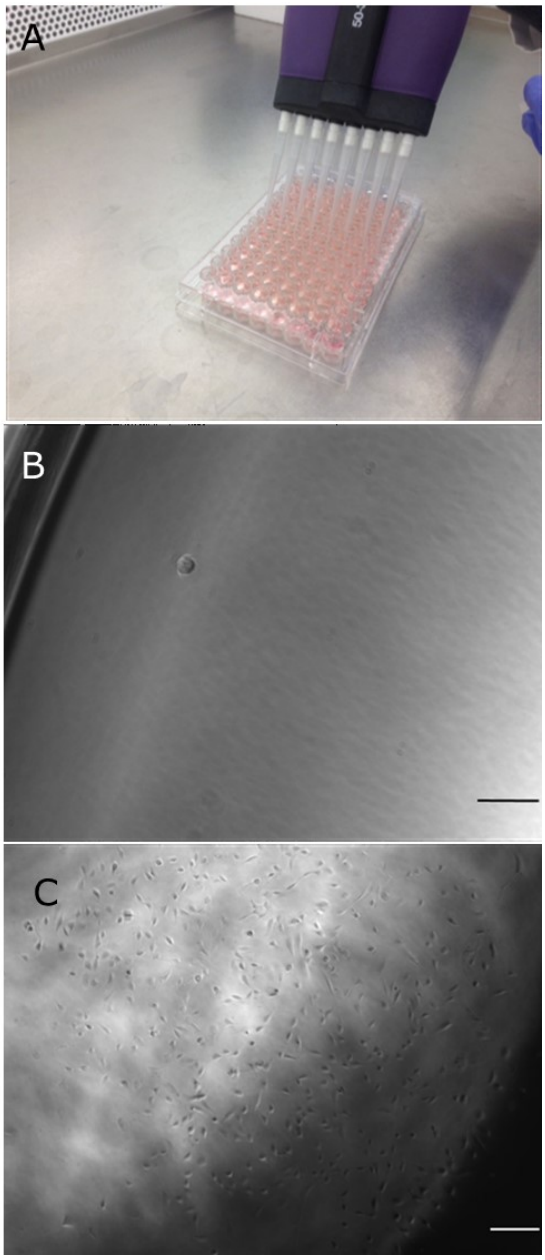

Figure 4. 6 c-kit^{pos} CPC clones derived from single CPC. Through serial dilution, a single CPC of isolated CPC was deposited into a well of a 96 well plate (A). Transmitted light microscope showing the single CPC (B) and its clonal expansion at day 4. Scale= 500μm.

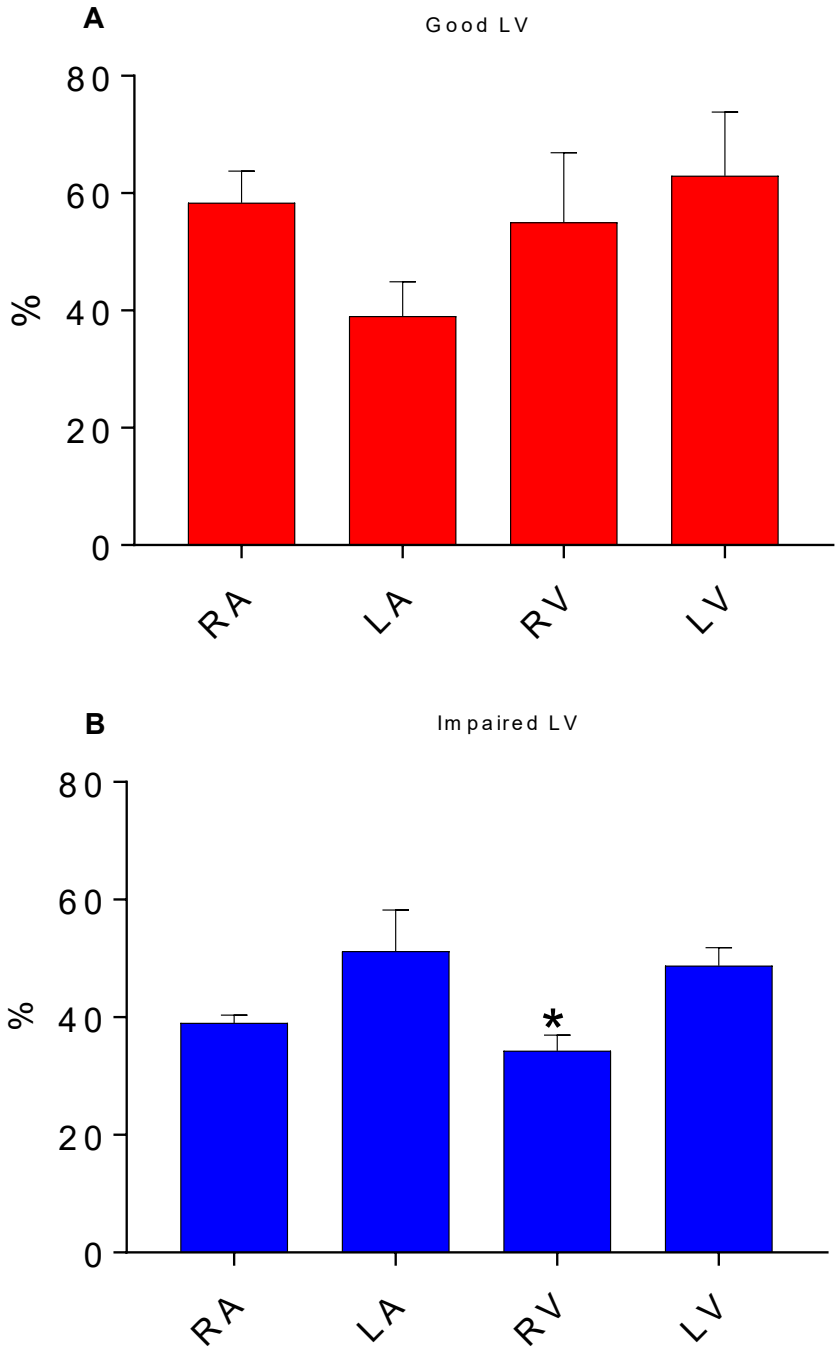

Figure 4. 7 Inter-chamber comparison of CPC clonogenicity. The ability to generate clones from a single CPC for each chamber for good LV (A) impaired LV (B) groups. Data are Mean ±SEM, n=5 per chamber. *denotes $p<0.05$ vs. LV.

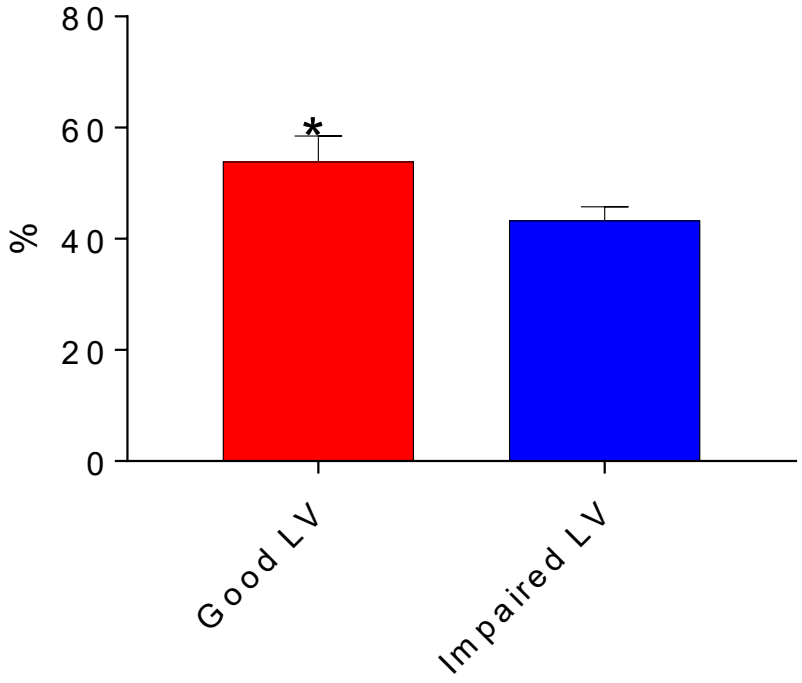

Figure 4. 8 Overall comparison of CPC clonogenicity capabilities between Good LV and Impaired LV. The ability to generate clones from a single CPC for good LV and impaired LV is compared. Data is Mean ±SEM, n=20 per group. *denotes $p<0.05$ vs. Impaired LV.

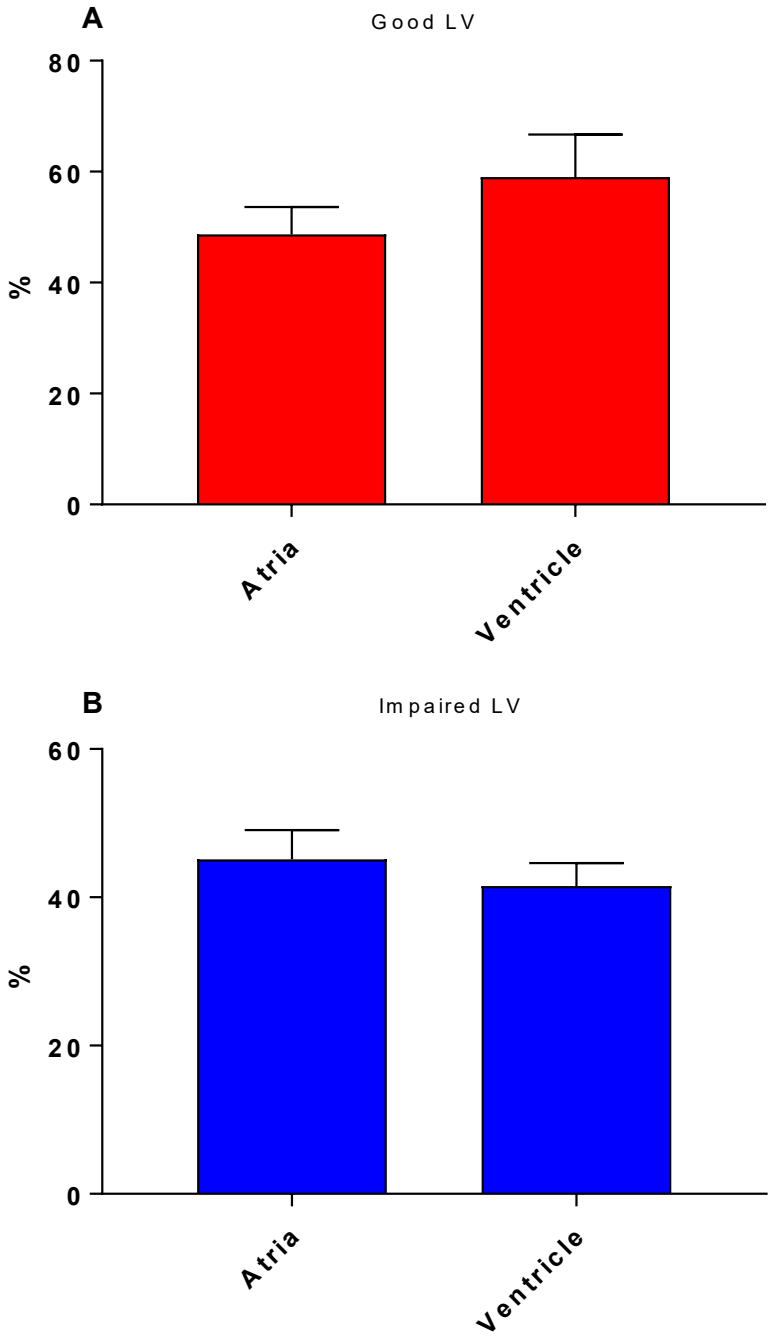

Figure 4. 9 Comparison of CPC clonogenicity between Atria and Ventricle. The ability to generate clones from a single CPC for both atria and ventricle for good LV (A) impaired LV (B) groups were compared. Data is Mean ±SEM, n=10 per chamber.

To summarise, the main findings were:

- In Good LV group, there were no significant differences in CPC clonogenicity between chambers.
- In the impaired LV group, the RV chamber CPCs showed a significant reduction in clonogenicity compared to the LV chamber.
- Overall the CPCs from the good LV group showed a significant increase in clonogenicity compared to the impaired LV group.

4.2.5 Proliferation of human CPCs isolated from patients with good normal and impaired LV function

The clonally derived CPCs from both good and impaired LV patients used for proliferation showed stable phenotype as described before (Figure 4.10)

At passage 5, the proliferation of c-kit^{pos}CD45^{neg}CD31^{neg} clonally derived CPCs were assessed using BrdU incorporation in vitro assay over 24 hours for good LV (Figure 4.11) and impaired LV patients (Figure 4.12). The number of BrdU^{pos} CPCs were compared to baseline (received BrdU for 30 mins) and represented as a percentage of total cells.

Amongst good LV patients, CPCs isolated from the RV chamber (64±4%) showed a significantly ($p<0.05$) decreased proliferation, compared to CPCs isolated from the LV chamber (80±2%) (Figure 4.13A). Amongst impaired LV patients, CPCs isolated from the RA chamber (64±6%) showed significantly decreased ($p<0.05$) proliferation, compared to CPCs isolated from the LV chamber (73±3%) (Figure 4.13B).

Overall, the proliferation of CPCs was similar between good (71±2%) and impaired LV (69±2%) patients (Figure 4.14). The proliferation of CPCs for atria and ventricle for both normal LV (70±2% and 68±3%) and impaired LV (72±3% and 71±2%) patients were also similar (Figure 4.15).

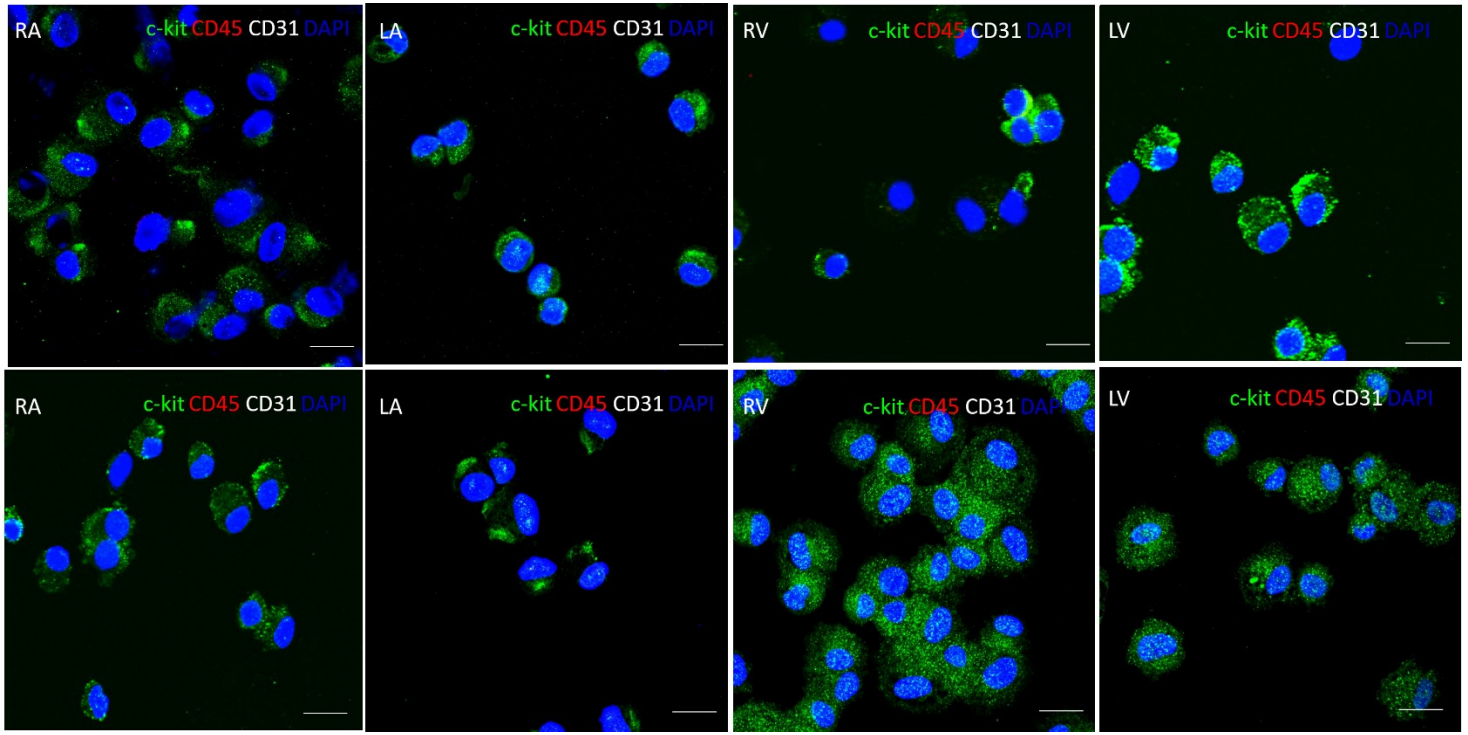

Figure 4. 10 Characterisation of the isolated c-kit^{pos} CPC before Clonogenicity Experiment. Top row- CPCs from Good LV patients, Bottom row- CPCs from impaired LV patients. IHC for c-kit (green), CD45 (red) CD31 (white) and DAPI (blue). Scale = 20µm.

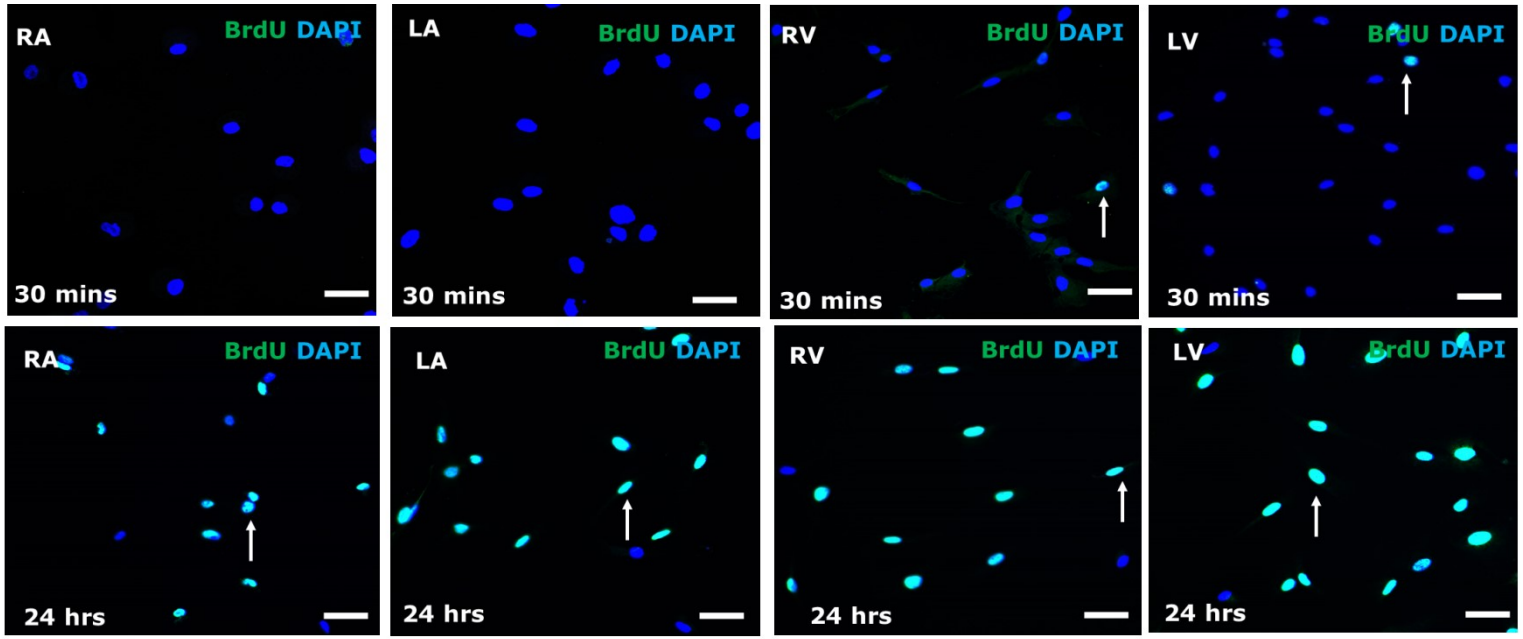

Figure 4. 11 Assessment of proliferation of isolated human CPCs- Good LV. Proliferation was assessed through the BrdU incorporation in vitro assay over 24 hours of CPCs. $BrdU^{pos}$ (green) and DAPI (blue) indicated by the white arrow at baseline 30 minutes; and at 24 hours. Scale = 20μm.

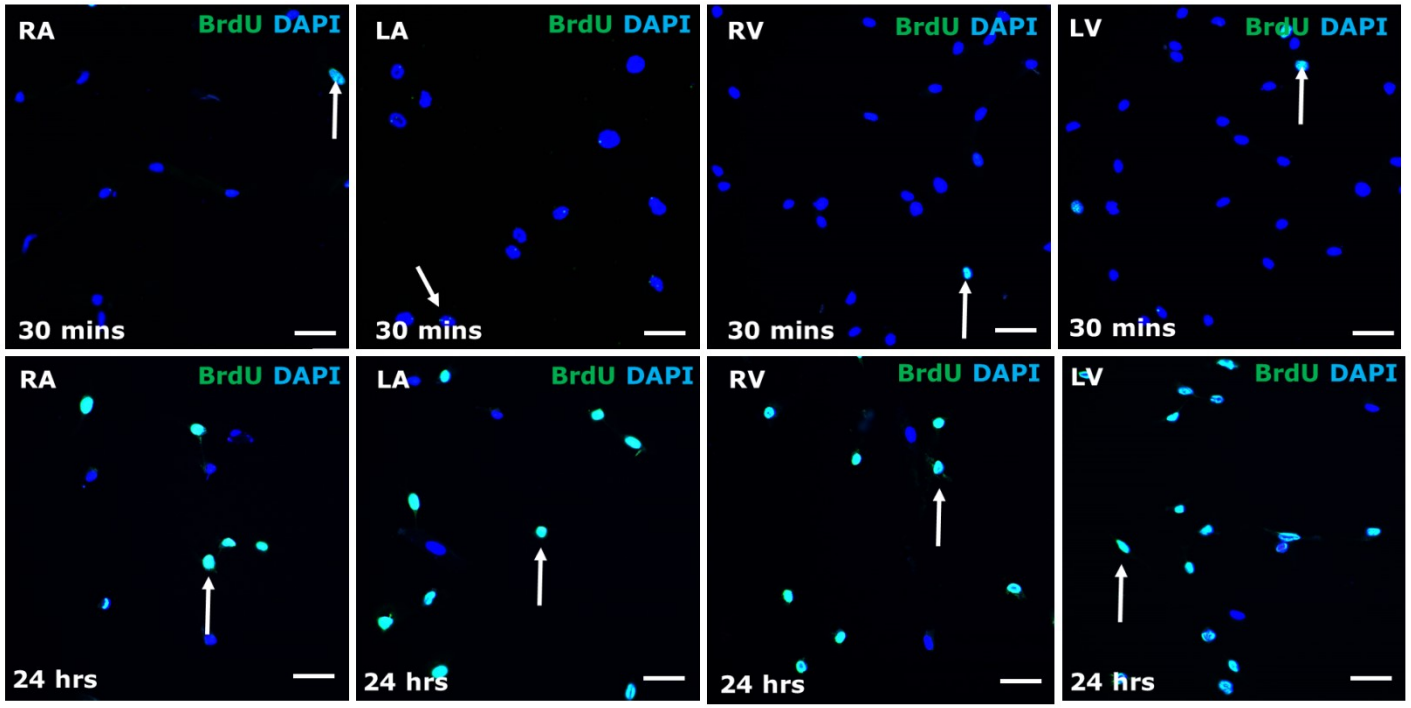

Figure 4. 12 Assessment of proliferation of isolated human CPCs- Impaired LV. Proliferation was assessed through the BrdU incorporation in vitro assay over 24 hours of CPCs. $BrdU^{pos}$ (green) and DAPI (blue) indicated by the white arrow at baseline 30 minutes; and at 24 hours. Scale = 20μm.

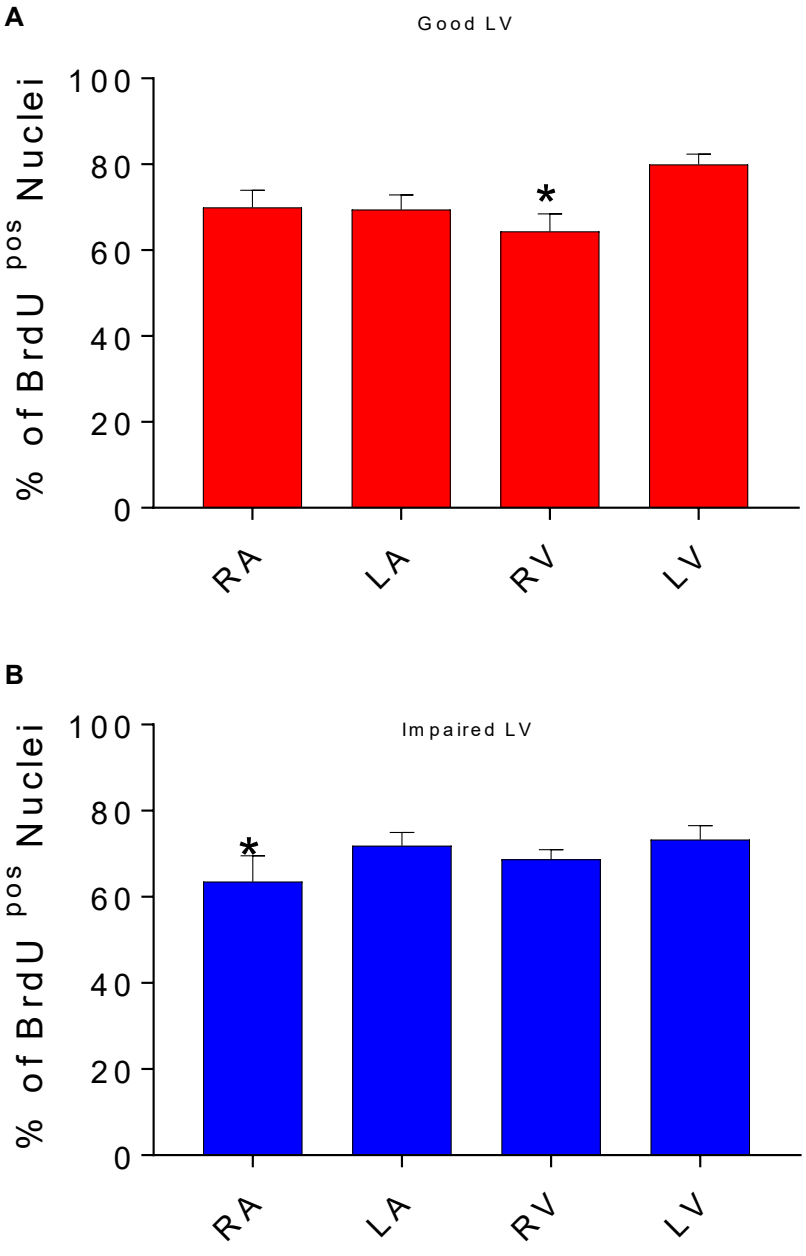

Figure 4. 13 Inter chamber Comparison of CPC proliferation. Inter chamber comparison of the CPC proliferation for good LV (A) and impaired LV groups (B). Data are Mean ±SEM, n=5 per chamber. In good LV group (A), * denotes $p<0.05$ vs. LV. In impaired LV group (B), * denotes $p<0.05$ vs. LV

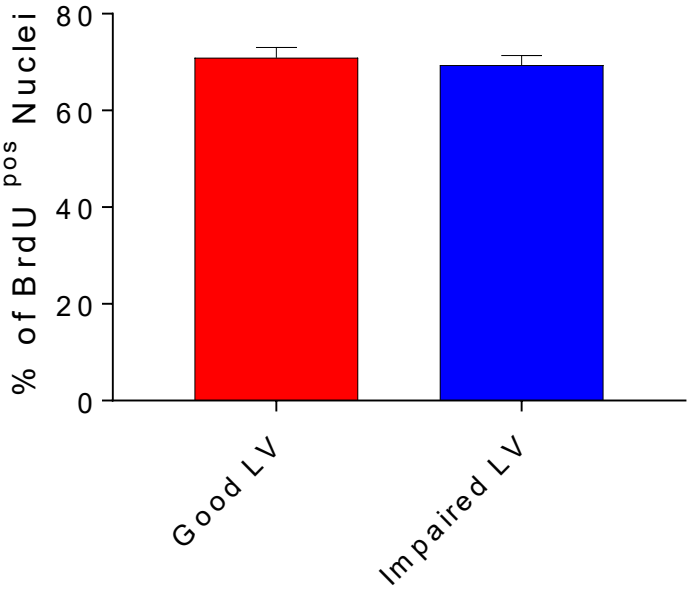

Figure 4. 14 Overall Comparison of CPC proliferation between Good and Impaired LV. The overall assessment of CPC proliferation between good and impaired LV function. Data are Mean ±SEM, n=20 per chamber.

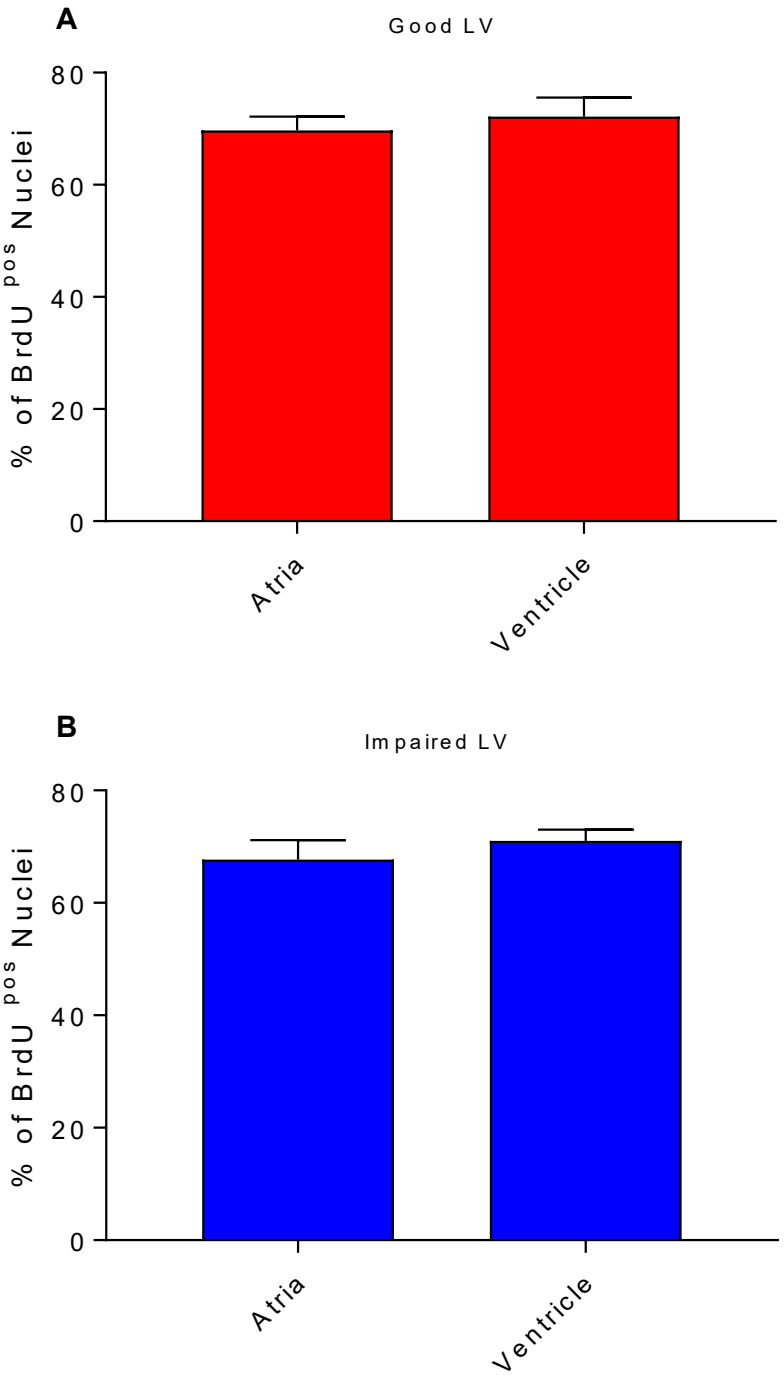

Figure 4. 15 Comparison of CPC proliferation between Atria and Ventricle. The comparison of the CPB proliferation between atria and ventricle for good LV (A) and impaired LV (B). Data is Mean ±SEM, n=10 per chamber.

To summarise, the main findings were:

- CPC proliferation was significantly decreased in the RV compared to LV in good LV patients.
- CPC proliferation was significantly decreased in the RA compared to LV in impaired LV patients.
- There was no significant difference in CPC proliferation between good and impaired LV patients.
- There was no significant difference in CPC proliferation between atria and ventricle regardless of if they were derived from good or impaired LV function groups.

4.3 Discussion

4.3.1 Isolation of human CPCs isolated from patients with normal and impaired LV function

Despite achieving a high yield of c-kit^{pos} CPCs (90% purity) using the enzymatic approach, I decided to use explant culture technique to the isolate CPCs as the biopsy obtained from the ventricle chambers were too small (~5.1mg for RV and ~5.2mg for LV) for enzymatic isolation. Therefore, I used the explant culture technique to isolate the CPCs from each cardiac chamber to be consistent with the isolation process.

Although there has not been a comprehensive comparison made between the explant techniques versus enzymatic isolation methods, there are several advantages and disadvantages to both techniques. Two isolation methodologies have been used to isolate CPCs from both human and murine hearts (Beltrami AP et al. 2003; Messina E et al. 2004, Cesselli D et al. 2011; Smith A et al. 2014; Vicinanza et al. 2017). Following this procedure, the isolated cells are further characterised to yield CPCs based on transcriptional or surface markers.

When compared to enzymatic isolation process, the explant technique has been shown to yield less heterogeneous cell populations, while demonstrating higher proliferative rates and cell viability from relatively smaller biopsy samples (Salehinejad P et al. 2012; Yoon JH et al. 2013; Hilkens P et al. 2013). These findings have been mainly attributed to undamaged tissue pieces with intact extracellular matrix (ECM) and release of cytokines and growth factors, which are relevant for cellular expansion (Hynes RO et al. 2009; Jing W et al. 2010). The effectiveness of the enzymatic process is mostly dependent on the type and concentration of the enzyme used for dissociation. The use of enzyme leads to the breakdown of ECM, resulting in lower yield with prolonged higher doubling time of the cells (Karahuseyinoglu S et al. 2007 and Seshareddy K et al. 2008). Bearzi et al. (2007) compared the two techniques and reported a 41% success in isolation of CPCs using enzymatic and 59% success using explant techniques. The weight of

the biopsy sample determines the choice of either enzymatic digestion or explant methodology.

Generally, all the samples contained all layers of the myocardium and were obtained on full cardiopulmonary bypass (CBP). All but one patient in this study underwent CABG. The other patient aged 77 in the impaired LV group, underwent mitral valve repair and LAAO for atrial fibrillation. There was a significantly high number of CPCs isolated from good LV (30630 CPCs/g) samples compared to impaired LV samples (22260 CPCs/g). However, there was no statistical difference observed in the inter-chamber comparison. The CPCs derived from the impaired LV group on average took longer to reach confluence when compared to CPCs from good LV (7.5 vs 6.8 days), but this was not statistically significant.

These observed differences could be attributed to several factors, including the quality of the samples, the impact of CPB on endogenous CPCs, age, and disease. The association between atrial fibrillation and cardiac fibrosis is well documented (Reese-Petersen Al et al. 2020). The cardiac fibrosis destroys the normal cellular architecture and therefore, may affect the number and the quality of the CPCs. Several studies have shown that CPB activates systemic inflammatory response and cause morbidity (Fujii et al. 2020). This impact on endogenous CPCs characteristics is largely unknown. However, it is widely documented that ageing and disease can alter the number and CPC behaviour (Cesselli D et al. 2011; Devalla et al. 2018; and Lewis-McDougall F et al. 2019) due to alteration in expression for markers such as p16INK4A, SA-β-gal with truncated telomeres. The activation of the inflammatory response to CPB may contribute to the ongoing cellular changes that occur due to ageing and disease.

A further sub-analysis of our patient demographics shows that all but one of the patients were on a statin (3hydroxy-3-methylglutaryl coenzyme A- HMG-CoA reductase) for their hypercholesteremia and ACE (Angiotensin-converting enzyme) inhibitors for hypertension. Both medications have reduced significant morbidity and mortality for cardiac patients. Statins have been shown to reduce cardiac mortality and morbidity by lowering LDL cholesterol significantly. However, their positive pleiotropic effects have also been reported through mediators that regulates endothelial progenitor cells (Assmus B et al. 2003; Sandhu et al. 2017). In addition

to this, ACE inhibitors which work on the renin-angiotensin mechanism have also been shown to enhance the characteristics of endothelial progenitor cells through modulation intracellular signalling pathways (Ahmadian E et al. 2015).

It would appear that the number of CPCs isolated using explant technique from each cardiac chamber is multifactorial and may be altered in the diseased heart.

4.3.2 Clonogenicity of human CPCs isolated from patients with normal and impaired LV function

Early on Bearzi et al. (2007), showed isolated CPCs could be cloned with an efficiency of ≈0.7% for CPCs derived from the enzymatic approach. In 2004 Messina et al., took atrial and ventricular samples from patients undergoing cardiac surgery and generated cardiospheres (CS) by explant culture technique. They showed CS to be made up of clonally derived cells. In addition, CS derived from single cells were shown to be 1-10% clonally efficient. It is worth remembering that CS is composed of only 1% of c-kit^{pos} cells. However, our experiments showed increased clonal efficiency of CPCs with ≈54% for good LV and ≈43% for impaired LV patients. These experiments were conducted at P5 using CPCs derived from explant culture technique, and the CPCs were shown to maintain their phenotype as described earlier on. Further studies have also confirmed that these CPCs are cable of maintaining stable phenotypic characteristics in culture (Lewis-McDougall F et al. 2019; Ellison et al. 2013 and Vicinanza et al. 2018). Furthermore, the overall clonal efficiency was better for good LV group compared to impaired LV group (≈54% vs ≈43%), and the source of the CPC (atria or ventricle) did not appear to affect the ability to generate clones. These findings have not been previously reported.

In the present study, the good LV patients were relatively younger (average age 56 years) compared to patients within the impaired LV group (average age 71 years). Age and disease have been shown to be a critical determinant of CPCs functional characteristics. Various studies (Chimenti et al. 2003; Torella et al. 2004; Gonzalez et al. 2008; Cesselli et al. 2011; Lewis-McDougall F et al. 2019) have demonstrated a high level of senescent markers, impaired telomerase activity and telomerase erosion to be associated with ageing and disease. Indeed, CPCs isolated from aged and diseased patients express high levels of p16^{INK4a} and functionally these cells have been shown to be non-cycling and non-differentiating (Rolle IG et al. 2020; Cesselli et al. 2017; Torella D et al. 2006).

A further study by Cesselli et al. (2011), which examined the CPCs derived from the donor and explanted hearts during transplantation, showed shortened telomeres (up to 25%) with two-fold increased expression of p16^{INK4a} and p21 senescent markers

within the aged and diseased group, compared to the donor hearts. The CPCs obtained from aged and diseased hearts in their study showed three times reduction in clonal efficiency compared to CPCs derived from the donor hearts.

In addition, data from our lab have shown increased senescent CPCs with increased age, and CPCs isolated from aged (>70 years) patients shown decreased clonal efficiency, proliferation and differentiation potential. However, single CPC-derived clones from young, middle-aged, and old subjects were indistinguishable in terms of morphology, senescence, multipotency, self-renewing transcript profile, and differentiation (Lewis-McDougall F et al. 2019). These findings suggest that CPCs age and become senescent in a stochastic, non-autonomous manner. Furthermore, they show that senescent CPCs have SASP that adversely affect cycling competent non-senescent cells, making them more senescent. The above findings may explain why some of the cells deposited in the 96 well plates did not clonally expand and may also explain the differences observed between cells derived from good and impaired LV patients.

Age and disease related changes to CPCs characteristics have also been demonstrated in animal models. Gonzalez et al. (2008), took CPCs from the rat heart and showed chronological ageing is related to telomerase attrition, and by using BrdU labelling, they demonstrated cycling competent CPCs to exist in the aged-senescent heart.

In conclusion, in line with previous findings that show decreased clonal efficiency of CPCs isolated from aged and diseased patients, the present study observed a 20% reduction in clonal efficiency in the impaired LV group, compared to the good LV group. This reduction is most likely due to the increased age and disease of the impaired LV patients, compared to the good LV group.

4.3.3 Proliferation of human CPCs isolated from patients with good normal and impaired LV function

The rate of proliferation of clonally derived CPCs within good and impaired LV group was ≈71% vs ≈70%. The proliferation rate was similar between good vs impaired LV group and between atria vs ventricles regardless of LV function. Although the proliferative rate of different cardiac chambers has not been described before; the impact of disease and ageing in relation to CPC proliferation has been documented.

Bearzi C et al. (2007), isolated CPCs from biopsies obtained from healthy human hearts (without cardiovascular disease at the time of autopsy) and patients undergoing cardiac surgery. The generated single cell-derived clones had a proliferative rate of 90 ± 7% after 5 days in culture. Unfortunately, no comparison between inter-chamber or good vs impaired LV were made, unlike this study.

Lewis-McDougall et al. (2018) showed that CPCs isolated from older (77-86 years) subjects had decreased ($P<0.05$) proliferation compared to CPCs isolated from middle-aged (34-62 years) subjects. However, the single CPC derived clones were shown to have comparable characteristics for proliferation and differentiation. These finding further supports our observations.

A study by Walravens A et al. (2018), which compared cellular characteristic of human myocardial derived cardiac progenitors (c-kit^{pos} CPC) derived from single clones and cardiosphere-derived cells (CDC) from young (y) and adult (a); showed higher proliferative rate for yCPCs compared to aCPCs.

Several other studies also showed the age as a crucial determinant in proliferative ability. These studies showed the proliferative rates to be highest in CPCs derived from fetal and neonates; with age (increased to 13 years) related decline in proliferative ability (Mishra A 2011; van Vliet P et al. 2011). Although in our study we did not compare the age in relation to proliferation, our CPCs were isolated from an aged population, and the patients in the Impaired LV group were older than the patients in the good LV group. However, we did not observe any differences in CPC proliferation between good and impaired LV function. This finding may be related to the way the CPCs were isolated using explant culture technique than via enzymatic

approach. It has been previously shown that explant culture minimises cellular damage and increase viability and proliferation. Cells derived from the explant technique have been shown to contain fewer heterogeneous cell population and demonstrate increased proliferation and cell viability in comparison to enzymatic methodology. These findings are likely to be due to the existence of intact ECM, which protects the cells from mechanical and enzyme induced stress. In addition to this, the release of growth factors and cytokines into the culture media may also provide a favourable environment for the cells (Salehinejad P et al. 2012; Yoon JH et al. .2013; Mushahary D et al. 2018). On further examination, such cells expressed high levels of mitosis and genes related to cell cycle (Sotiropoulou P A et al. 2006).

This study showed that clonally derived CPCs from a single cell irrespective of the cardiac chamber or cardiac function is far more likely to be stochastic. These cycling competent cells are not affected by age or disease and therefore, potentially can maintain a stable phenotype.

5. Cardiospherogenesis and differentiation potential of CPCs isolated from all four chambers of the human heart.

5.1 Introduction

The ability to differentiate into all three cardiac lineages; cardiomyocytes, smooth muscle and endothelial cells is an essential feature of CPCs and defines them as multipotent stem/progenitor cells (Beltrami et al. 2003; Ellison et al. 2013). Cardiospheres are typically multicellular clusters (20–150 μm cellular spheres) derived from clonally expanded cells in suspension and, which can contribute to multiple lineages as first described by Beltrami et al. (2003).

Further study by Smith et al. (2014) showed that clonally derived CPCs could differentiate into beating cardiomyocytes when grown in differentiating media containing specific growth factors that target TGFβ and Wnt signalling pathways. In 2013, Ellison et al. showed that injecting clonally derived CPCs into the myocardium following an infarction can replace up to 20% of the cardiomyocytes within the infarcted zones; contributing to the improvement of LV function.

This particular characteristic allows the clonally derived CPCs to be manipulated at various stages using specific growth factors to direct differentiation.

In this study, clonally expanded CPCs from all four chambers from both good and impaired LV patients were used to generate cardiospheres. We then evaluated the differentiation potential for endothelial, smooth muscle and cardiomyocyte lineages by plating the generated cardiospheres in a differentiation media.

The specifics aim for this study were:

1. To assess and compare the cardiospherogenesis of human CPCs from all 4 cardiac chambers.
2. To compare the cardiospherogenesis of human CPCs isolated from patients with normal and impaired LV function.
3. To assess and compare the differentiation potential of CPCs from all 4 cardiac chambers.
4. To compare the differentiation potential of CPCs isolated from patients with good and impaired LV function.

5.2 Results

5.2.1 Cardiospherogenesis of CPCs isolated from all 4 chambers.

50 000 CPCs derived from single clones were platted on bacteriological dishes, and the number of cardiospheres were quantified at day 6 and expressed as the average number of cardiospheres/mm^2 for each chamber (Figure 5.1A&B). Irrespective of cardiac function, cardiospheres generated from LV chamber were noticeably larger when compared to cardiospheres derived from other cardiac chambers.

In both the good and impaired LV patient groups, CPCs from the LV (Good, 9±1.4; Impaired,7±0.3/mm^2) chamber showed a significant increase ($p<0.05$) in the ability to generate cardiospheres compared to the other chambers (RA (Good, 6±1; Impaired,4±0.2 /mm^2), LA (Good, 5±0.5; Impaired,4±0.6 /mm^2) and RV (Good, 5±1; Impaired,4±0.3 /mm^2) (Figure 5.2)). The overall ability of CPC cardiospherogenesis was significantly increased ($p<0.05$) in the good LV group (6±0.6), compared to the impaired LV group (5±0.4) (Figure 5.3). In the good LV group, there were no differences in CPC cardiospherogenesis between atria (5±0.4) and ventricles (7±1) (Figure 5.4). In the impaired LV group, the ventricles (5±0.5) showed a significant increase in ($p<0.05$) cardiospherogenesis, compared to atria (4±0.3) (Figure 5.4).

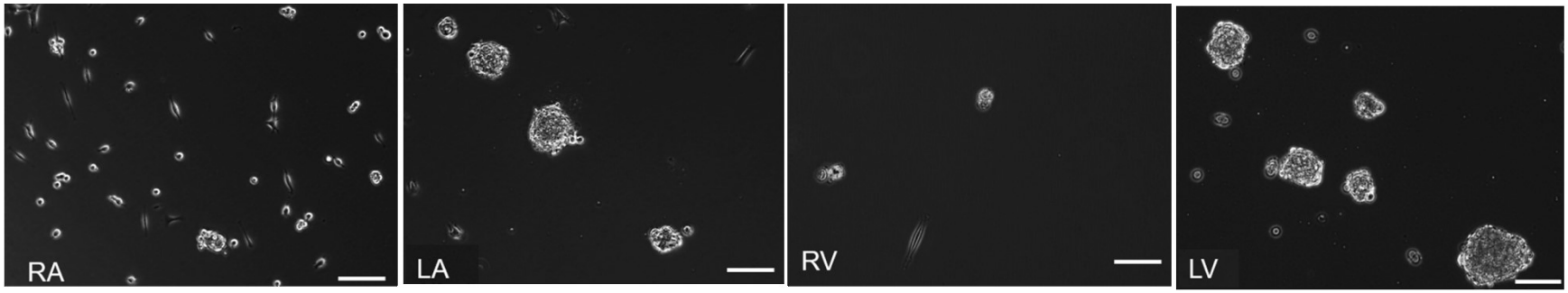

Figure 5. 1A Cardiospherogenesis of CPCs from each chamber-Good LV. Observation of cardiospherogenesis from all four chambers (good LV) under transmitted light microscope at day 6. 50000 cells were plated per dish. Scale =100μm.

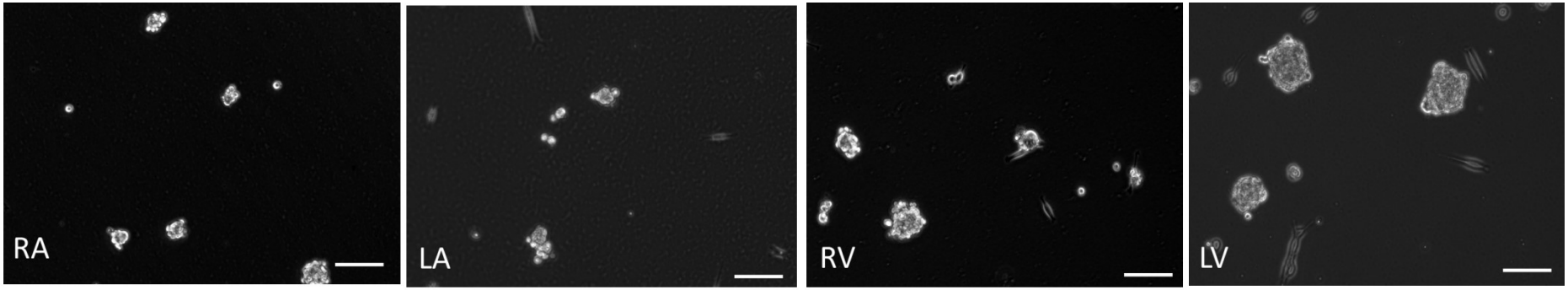

Figure 5. 1B Cardiospherogenesis of CPCs from each chamber-Impaired LV. Observation of cardiospherogenesis from all four chambers (good LV) under transmitted light microscope at day 6. 50000 cells were plated per dish. Scale =100μm

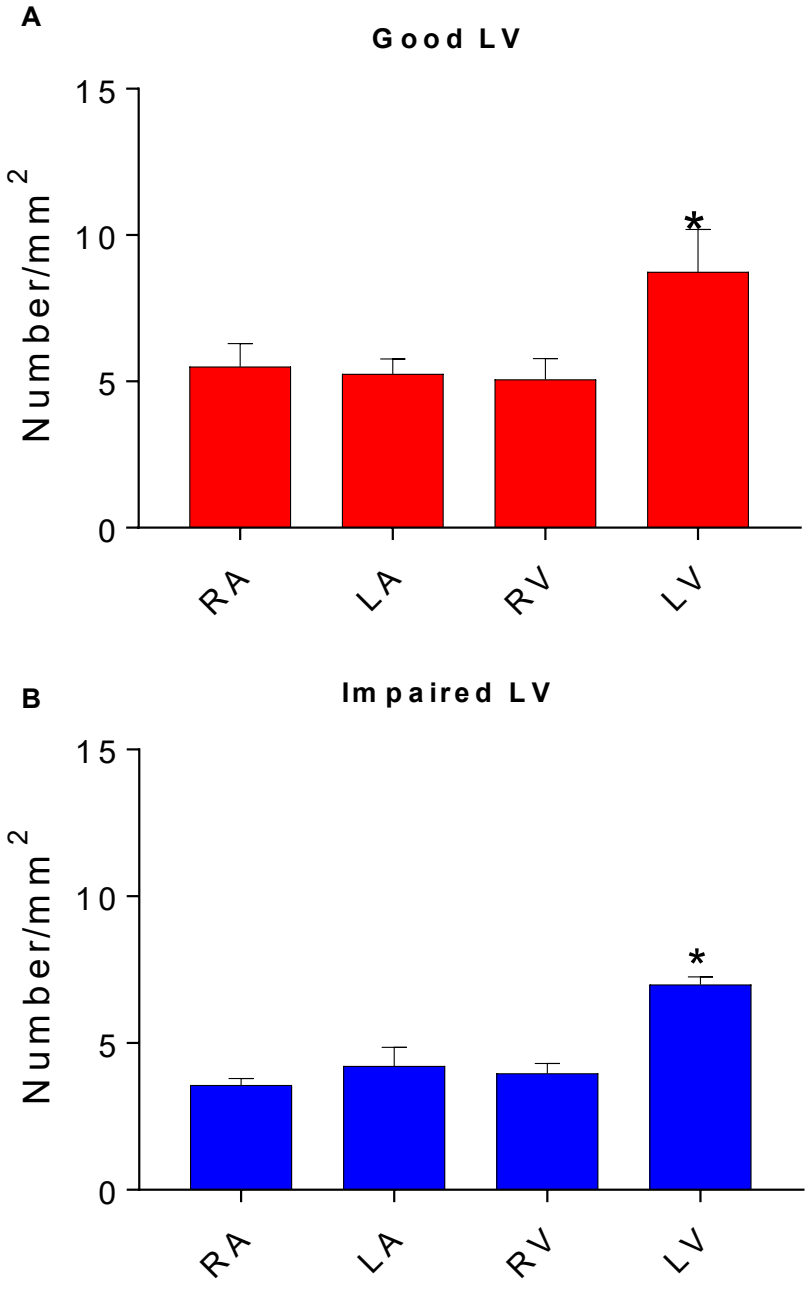

Figure 5. 2 Inter chamber Comparison of Cardiospherogenesis of CPCs. Cardiospherogenesis of CPCs isolated from each chamber from good LV (A) and impaired LV patients (B). * denotes $p<0.05$ vs. all other chambers. Data are Mean ±SEM, n=5 per chamber.

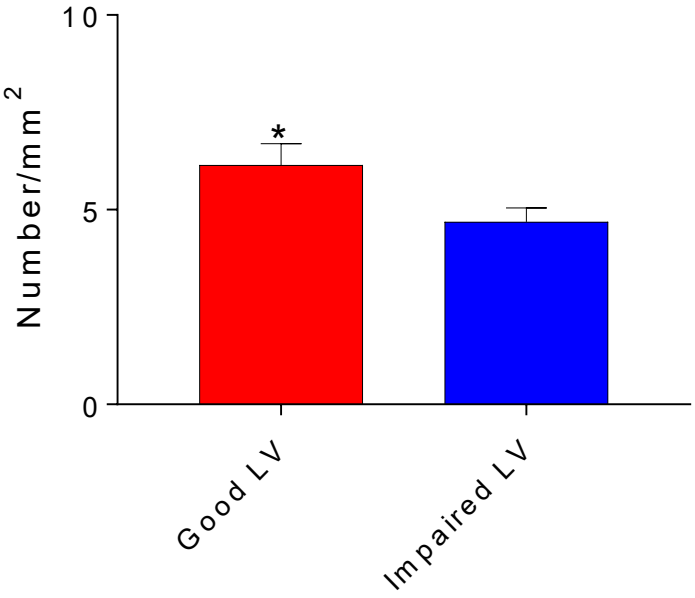

Figure 5. 3 Overall Cardiospherogenesis of CPCs assessment: Good LV vs Impaired LV. The overall comparison of Cardiospherogenesis between good and impaired LV function. The data is represented as mean ±SEM, n=20 (samples from each chamber taken from 5 patients). * denotes a significant difference ($p<0.05$) vs impaired LV.

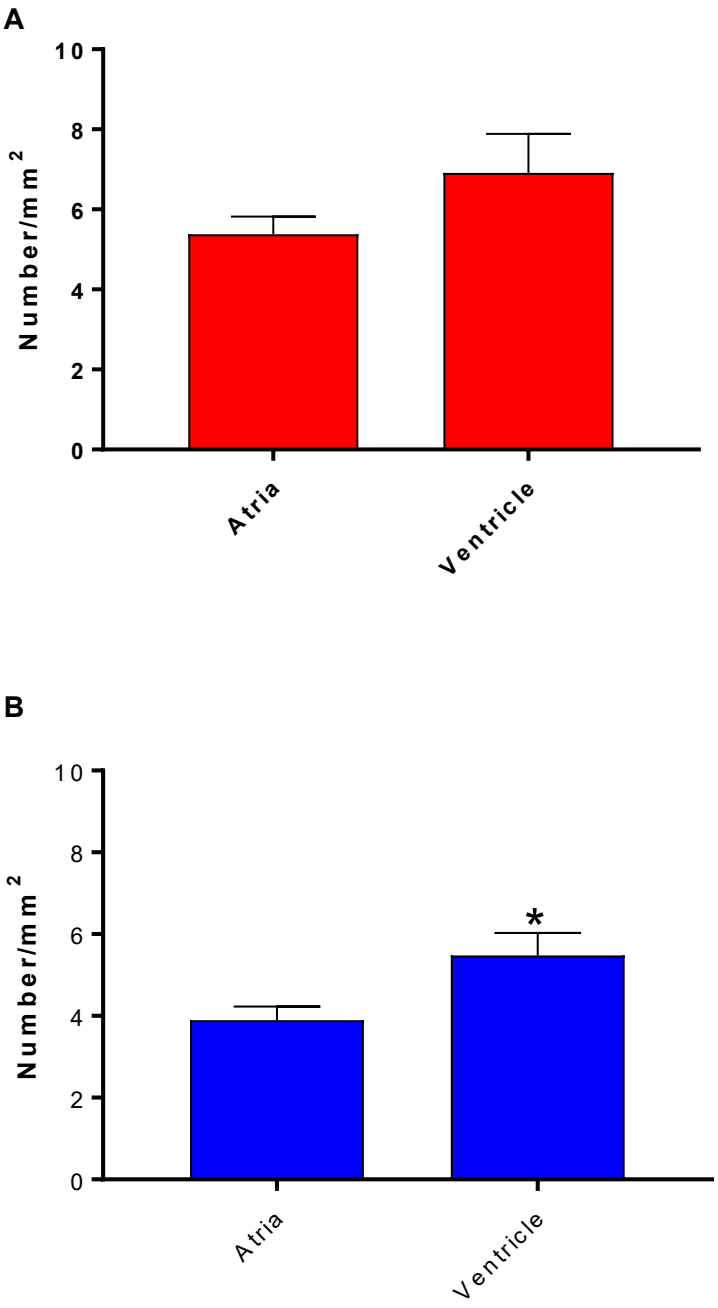

Figure 5. 4 Comparison of Cardiospherogenesis of CPCs: Atria vs Ventricle. Comparison of cardiospherogenesis between atria and ventricle. (A) Good LV; (B) impaired LV. The data is represented as mean ±SEM, n=10 per chamber (samples from each chamber taken from 5 patients). * shows a significant difference ($p<0.05$) vs atria.

To summarise, the main findings were:

- CPCs from the LV chamber showed increased cardiospherogenesis, compared to the other cardiac chambers, regardless of good or impaired LV function.
- Overall, CPCs from good LV patients showed increased cardiospherogenesis, compared to impaired LV patients.
- In the impaired LV, CPCs showed increased cardiospherogenesis in the ventricle compared to CPCs from atria.

5.3 Differentiation of CPCs into the three cardiac lineages

It is well documented that CPCs are multipotent and capable of differentiating into all three cardiac lineages: cardiomyocytes, smooth muscle and endothelial cells. The progeny of a single CPC generated cardiosphere has been shown to express biochemical markers of cardiomyocytes, smooth muscle and endothelial cells (Messina E et al. 2004; Bearzi et al. 2007; Vicinanza et al. 2017).

We used the generated cardiospheres from all four chambers of the human heart to assess for multipotency using a spontaneous differentiation protocol as decribed in methodology (Figure 5.5).

5.3.1. Differentiation of CPCs into the cardiomyocyte lineage

Representative images of Nkx2.5/ α-sarcomeric actin expressed CPCs from good LV patients and impaired LV patients are shown on figures 5.6 and 5.7 respectively.

Following 8 days in cardiomyogenic differentiation media, in good LV patients, the expression of Nkx2.5 showed no significant difference between cardiac chambers (LV chamber (4±0.9AU), RA (3±0.6 AU), LA (3±0.3 AU) and RV (2±0.1 AU)). However, this expression of Nkx2.5 CPCs cardiospheres was significantly ($p<0.05$) higher in the LV chamber (11±1.1 AU) from impaired LV patients compared to all the other cardiac chambers (RA (4±0.4 AU), LA (4±0.5 AU) and RV (3±0.6 AU) (Figure 5.8)).

The expression for α-sarcomeric actin (Figure 5.9) was significantly ($p<0.05$) increased in CPC cardiospheres from the LV (Good, 30±3.6 AU; Impaired, 60±2.3 AU) compared to all the other cardiac chambers for both good and impaired LV patient groups (RA (Good, 16±2.2 AU; Impaired, 23±4.9 AU), LA (Good, 19±2 AU; Impaired, 30±2.5 AU) and RV (Good, 9±2.4 AU; Impaired, 35±4 AU).

Overall, the expression of Nkx2.5(Good, 5±0.8 AU; Impaired, 3±0.3 AU) and α-sarcomeric actin (Good, 37±4 AU; Impaired, 18±2 AU) was

significantly ($p<0.05$) increased in CPC cardiospheres from impaired LV patients, compared to good LV patients (Figure 5.10).

There was no difference in Nkx2.5 expression between atria (3±0.3 AU) and ventricles (3±0.6 AU) for good LV patients. However, in the impaired LV group, the ventricles (7±1.4AU) showed a significantly ($p<0.05$) increased expression of Nkx2.5, compared to atria (4±0.3AU) (Figure 5.11).

There was no difference in α-sarcomeric actin expression between atria (17±1.5 AU) and ventricles (20±4 AU) for good LV patients. However, the α-sarcomeric actin expression was significantly ($p<0.05$) increased in the ventricles (48±4.7 AU) of impaired LV patients, compared to atria 27±2.8 AU) (Figure 5.12).

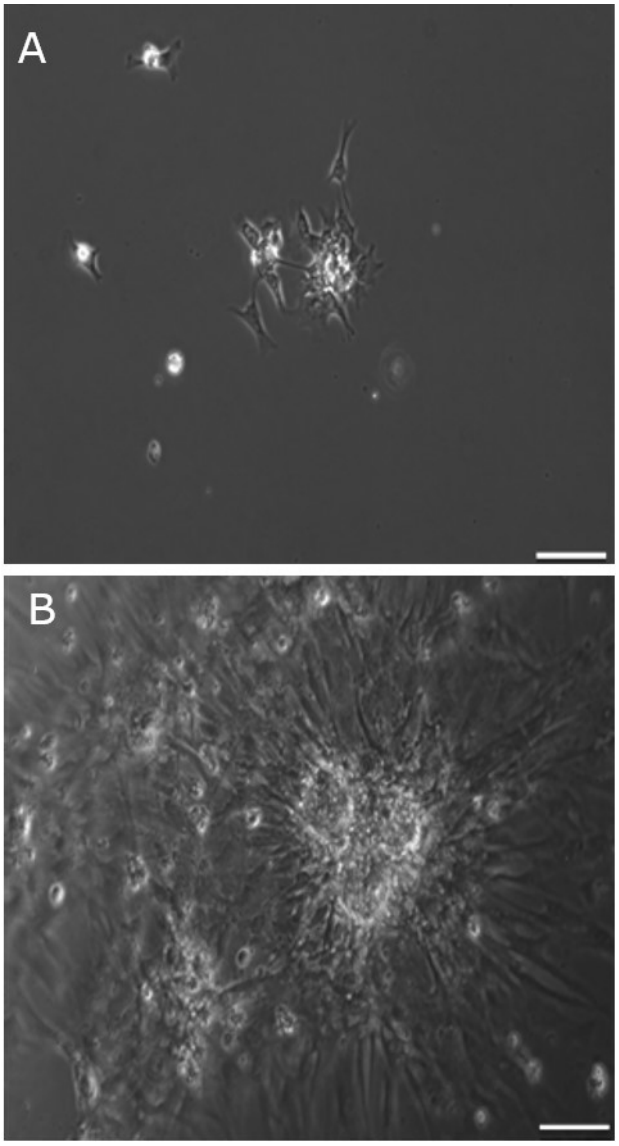

Figure 5. 5 Differentiation of CPC-derived cardiospheres. The generated cardiospheres were plated in differentiation media on laminin coated dishes. Transmitted light microscope showing the outgrowth of cells from the cardiospheres at (A) Day 2 (B) Day 7. Scale= 100μm.

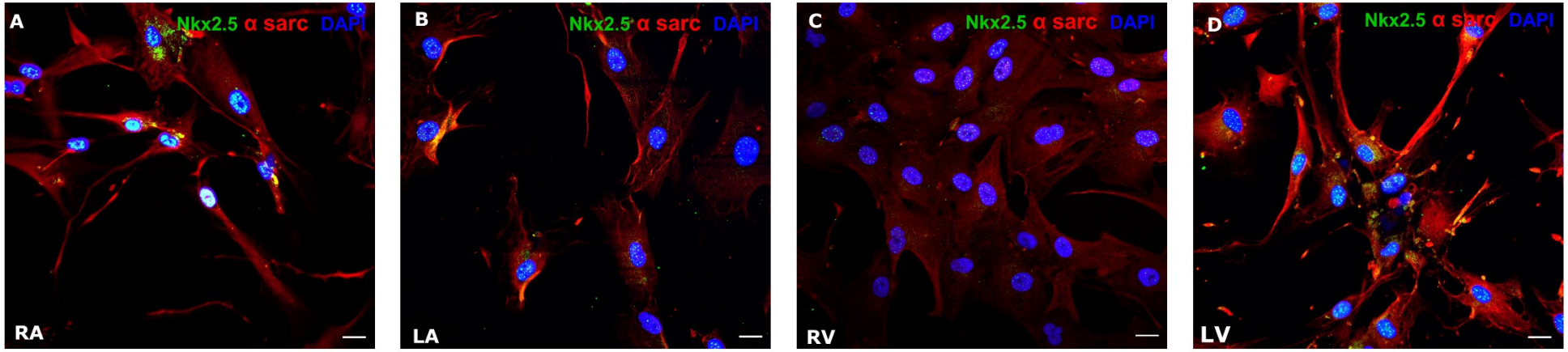

Figure 5. 6 Nkx2.5 and α-sarcomeric actin expression-Good LV. IF staining of differentiated cells from each cardiac chamber (A-D) after plated in cardiomyogenic differentiation media for 8 days (Good LV). Green spots in nuclei – Nkx2.5; red- α sarcomeric actin and the nuclei were counterstained with DAPI (blue). Scale = 20μm.

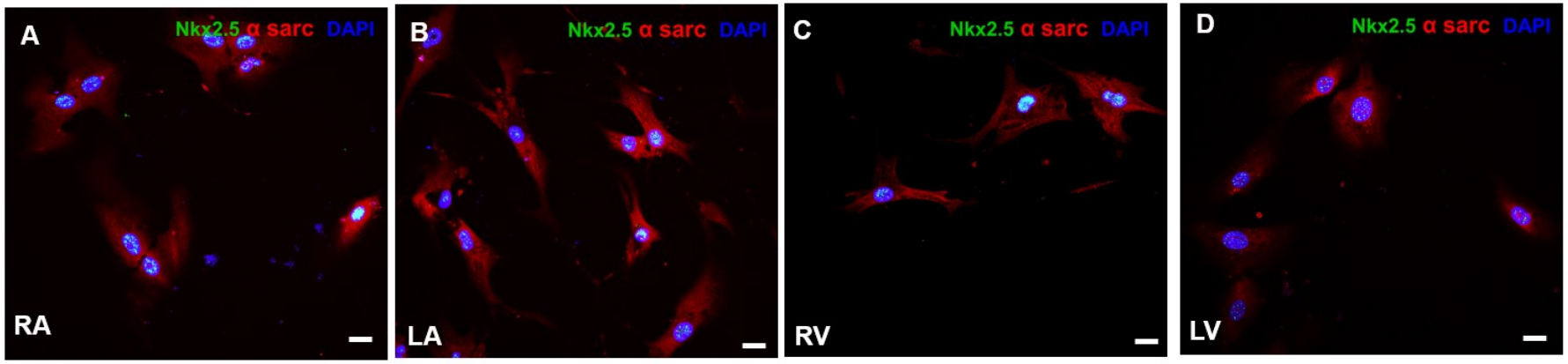

Figure 5. 7 α-sarcomeric actin expression-Impaied LV. IF staining of differentiated cells from each cardiac chamber (A-D) after plated in cardiomyogenic differentiation media for 8 days (Good LV). Green spots in nuclei – Nkx2.5; red- α sarcomeric actin and the nuclei were counterstained with DAPI (blue). Scale = 20μm.

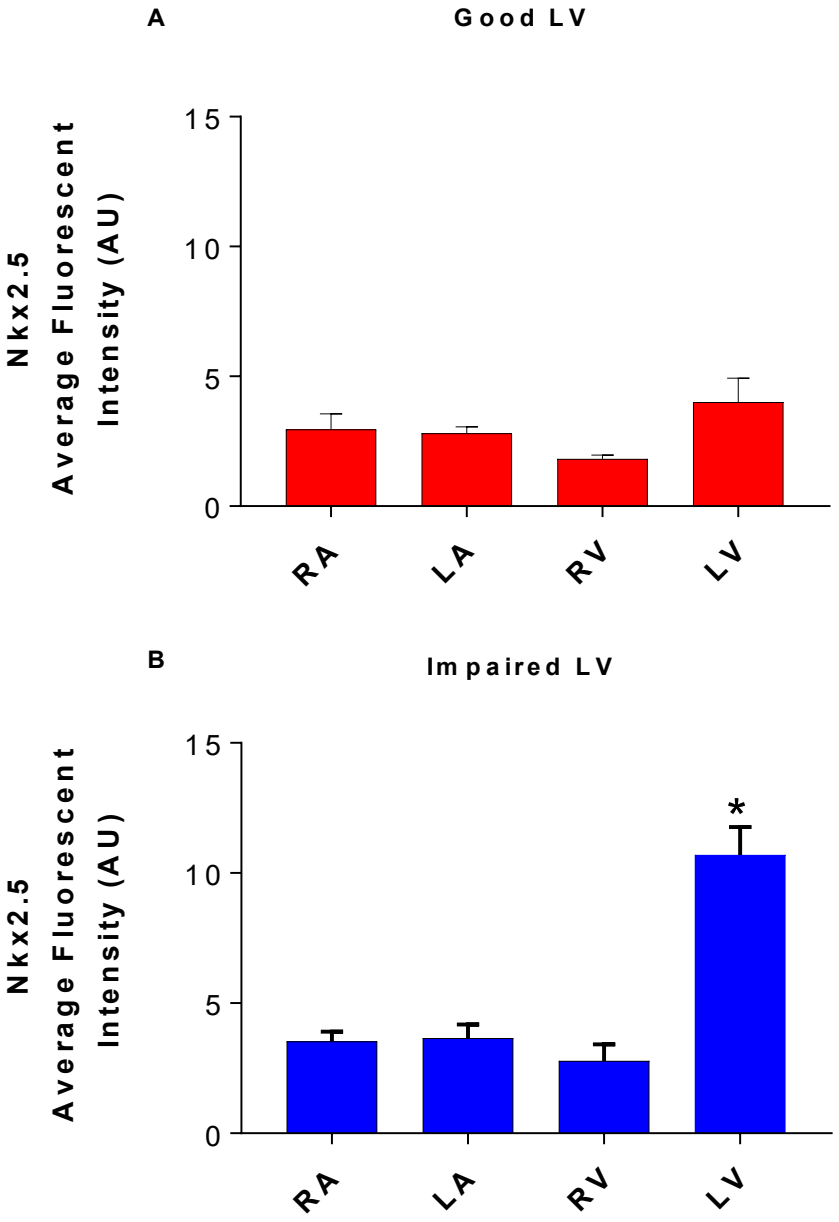

Figure 5. 8 NKx-2.5 expression of CPC cardiospheres following 8 days in cardiomyogenic differentiation media. Inter chamber Comparison NKx2.5 expression in both Good (A) and Impaired (B) LV. The data is represented as mean ±SEM, n=5 per chamber. * shows a significant difference ($p<0.05$) between LV and other chambers.

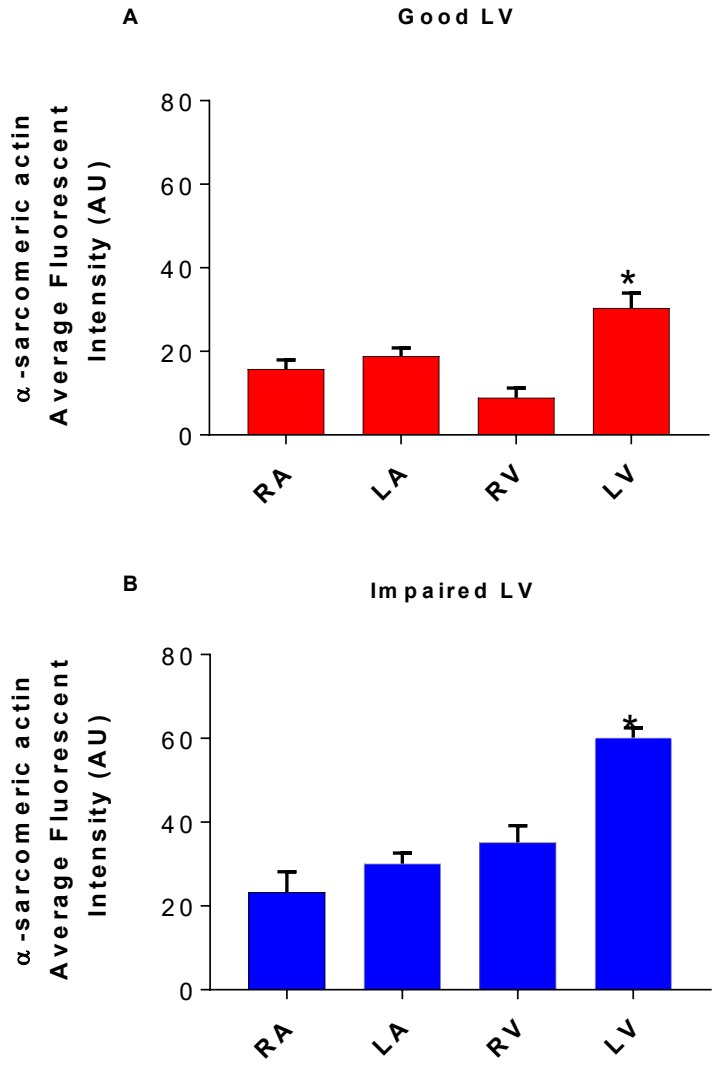

Figure 5. 9 α-sarcomeric actin expression of CPC cardiospheres following 8 days in cardiomyogenic differentiation media. Inter chamber Comparison α-sarcomeric actin expression in both Good (A) and Impaired (B) LV. The data is represented as mean ±SEM, n=5 per chamber. * shows a significant difference ($p<0.05$) vs all **chambers.**

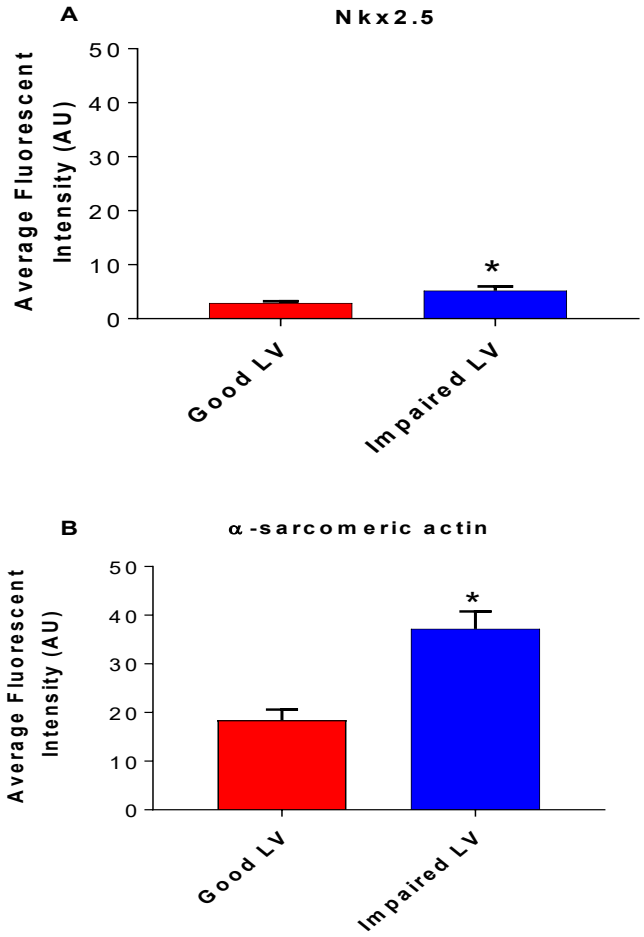

Figure 5. 10 Nkx2.5 and α-sarcomeric actin expression of CPC cardiospheres following 8 days in cardiomyogenic differentiation media between good and impaired LV. Comparison of Nkx2.5 (A) and α sacomeric actin (B) expressed cardiomyocytes was made between good and impaired LV. The data is represented as mean ±SEM, n=20 per group (samples from each chamber taken from 5 patients). * shows a significant difference ($p<0.05$) vs good LV.

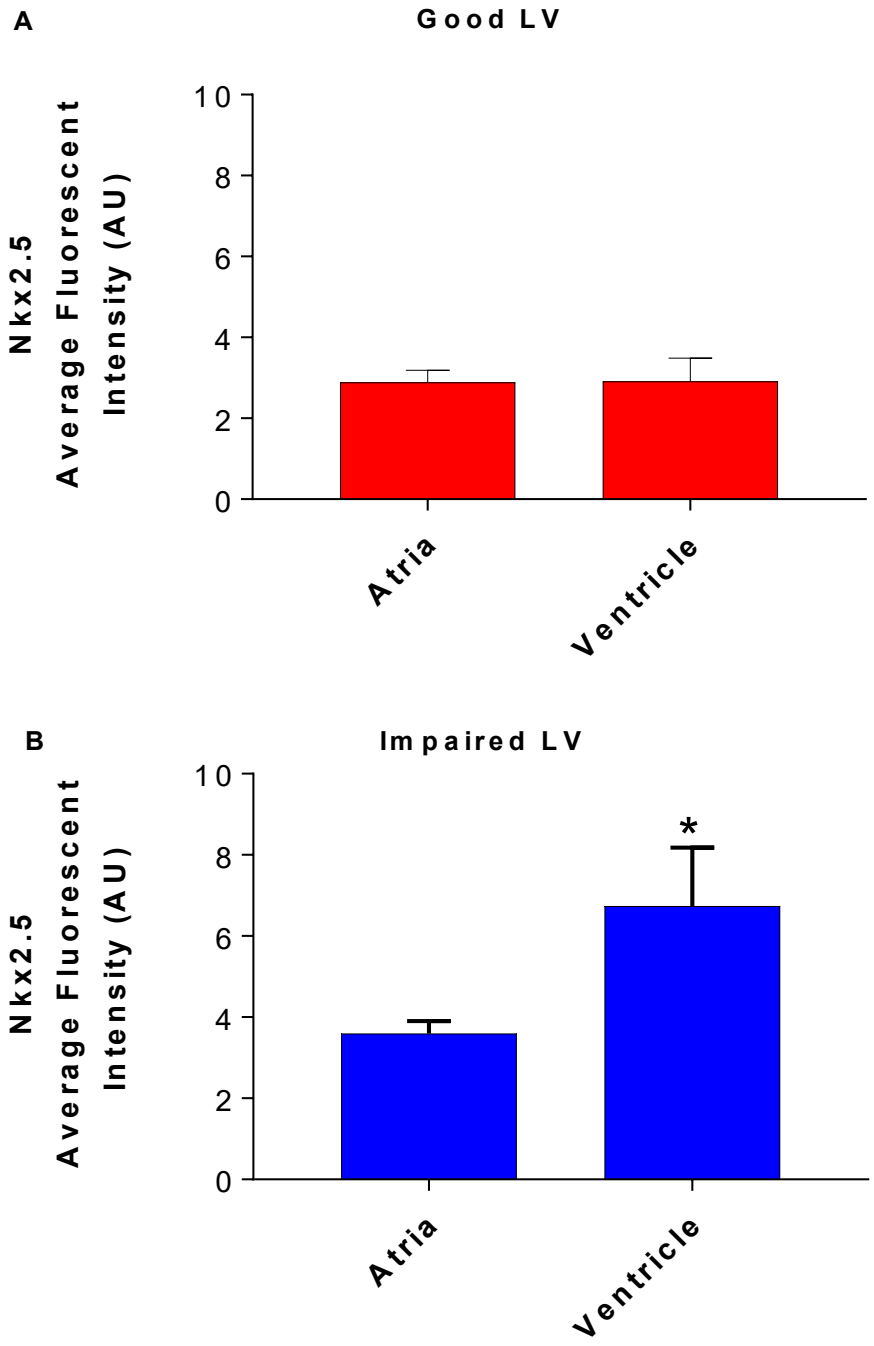

Figure 5. 11 Overall comparison of Nkx2.5 expressed cardiomyocytes between atria and ventricle. Comparison of Nkx2.5 expressed cardiomyocytes was made between atria (A) ventricle (B). The data is represented as mean ±SEM, n=10 (samples from each chamber taken from 5 patients) per chamber. * shows a significant difference ($p<0.05$) vs atria.

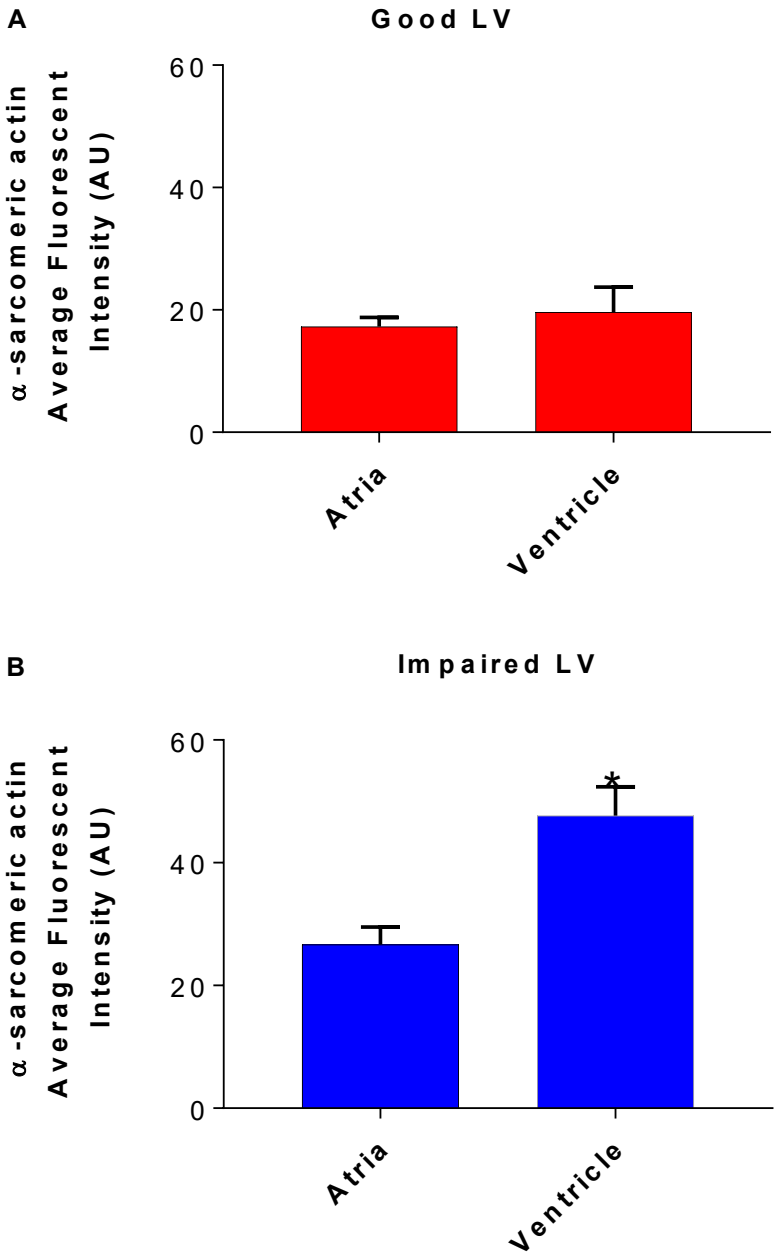

Figure 5. 12 Overall comparison of α- sarcomeric actin expressed cardiomyocytes between atria and ventricle. Comparison of α-sarcomeric actin expressed cardiomyocytes was made between atria (A) ventricle (B). The data is represented as mean ±SEM, n=10 (samples from each chamber taken from 5 patients) per chamber. * shows a significant difference ($p<0.05$) vs atria.

The primary outcomes of this section shows:

- CPC cardiospheres differentiate into the cardiomyocyte lineage.
- In the impaired LV group, Nkx2.5 expression in CPC cardiospheres from the LV chamber was increased, compared to the other cardiac chambers
- Expression of α-sarcomeric actin in CPC cardiospheres derived from the LV chamber was significantly increased, compared to the other cardiac chambers regardless of LV function.
- Overall, Nkx2.5 and α-sarcomeric actin expression were significantly higher in impaired LV group compared to the good LV group.
- The ventricles from the impaired LV group showed a significantly greater Nkx2.5 and α-sarcomeric actin expression compared to the atria.

5.3.2. Differentiation of CPCs into the endothelial cell lineage

Representative images of vWF expressed CPCs from good LV patients and impaired LV patients are shown on figures 5.13 and 5.14 respectively.

In the good LV patients, the CPC cardiospheres from the LA (24±3.4) showed a significantly ($p<0.05$) increased vWF expression, compared to all other cardiac chambers (RA (7±1.3 AU), RV (4±0.8 AU) and LV (4±1.0 AU) (Figure 5.15)). There were no differences between chambers (RA (10±3 AU), LA (9±0.2 AU), RV (14±3 AU) and LV (12±1.0 AU)) for vWF expression in CPC cardiospheres from the impaired LV patient group (Figure 5.15).

Overall, there was no difference between good (10±2 AU) and impaired LV (11±1 AU) patients for vWF expression of CPC cardiospheres (Figure 5.16).

In Good LV patients, vWF expression was significantly ($p<0.05$) increased in CPC cardiospheres from the atria (15±3 AU), compared to the ventricle (4±1 AU) (Figure 5.17). There were no differences for vWF expression between the atria (10±2 AU) and ventricles (13±2 AU) for the impaired LV group.

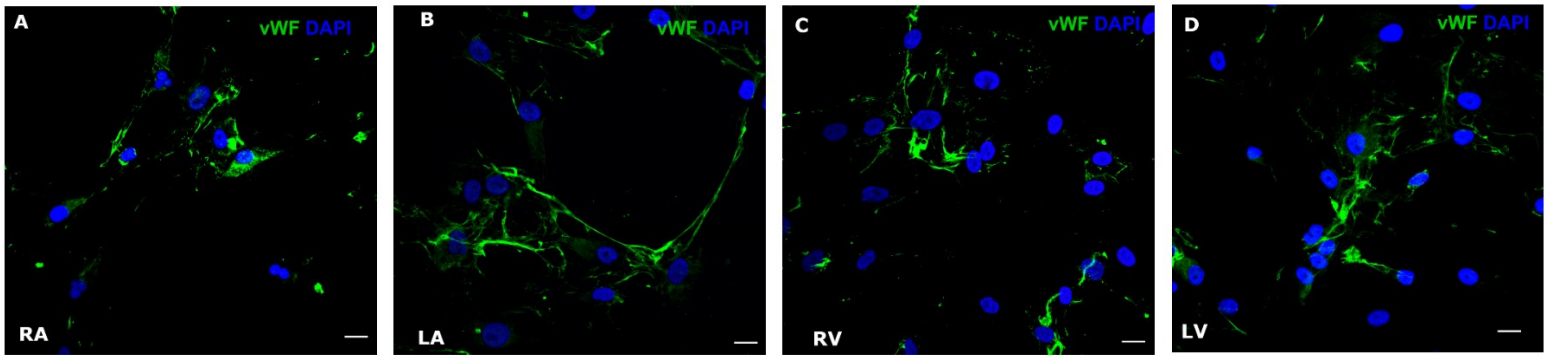

Figure 5. 13 vWF expression-Good LV. IF staining of differentiated cells after plated in endothelial differentiation media for 8 days. Green – vWF; nuclei were counterstained with DAPI (blue). Scale = 20μm.

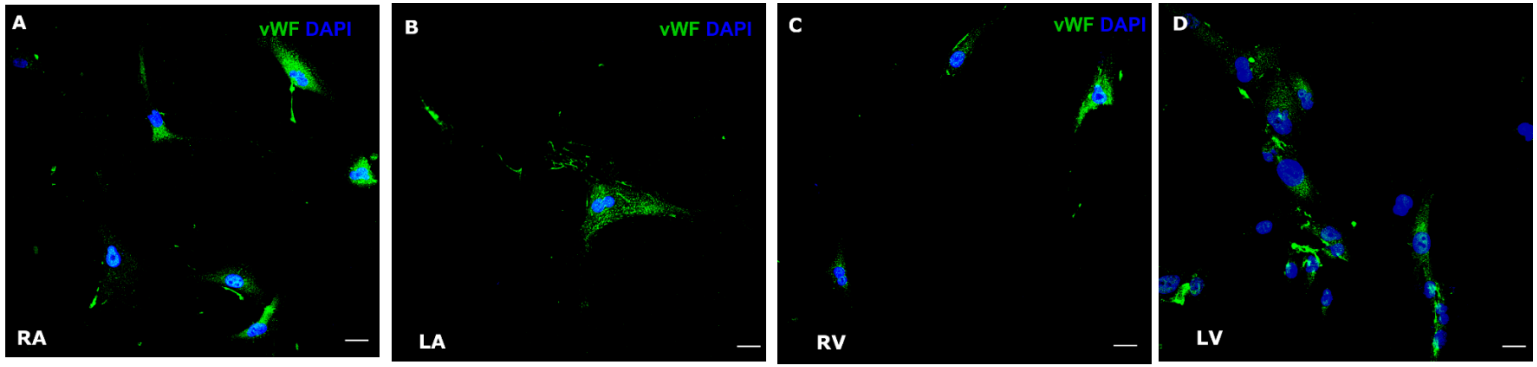

Figure 5. 14 vWF expression-Impaired LV. IF staining of differentiated cells after plated in endothelial differentiation media for 8 days. Green – vWF; nuclei were counterstained with DAPI (blue). Scale = 20μm.

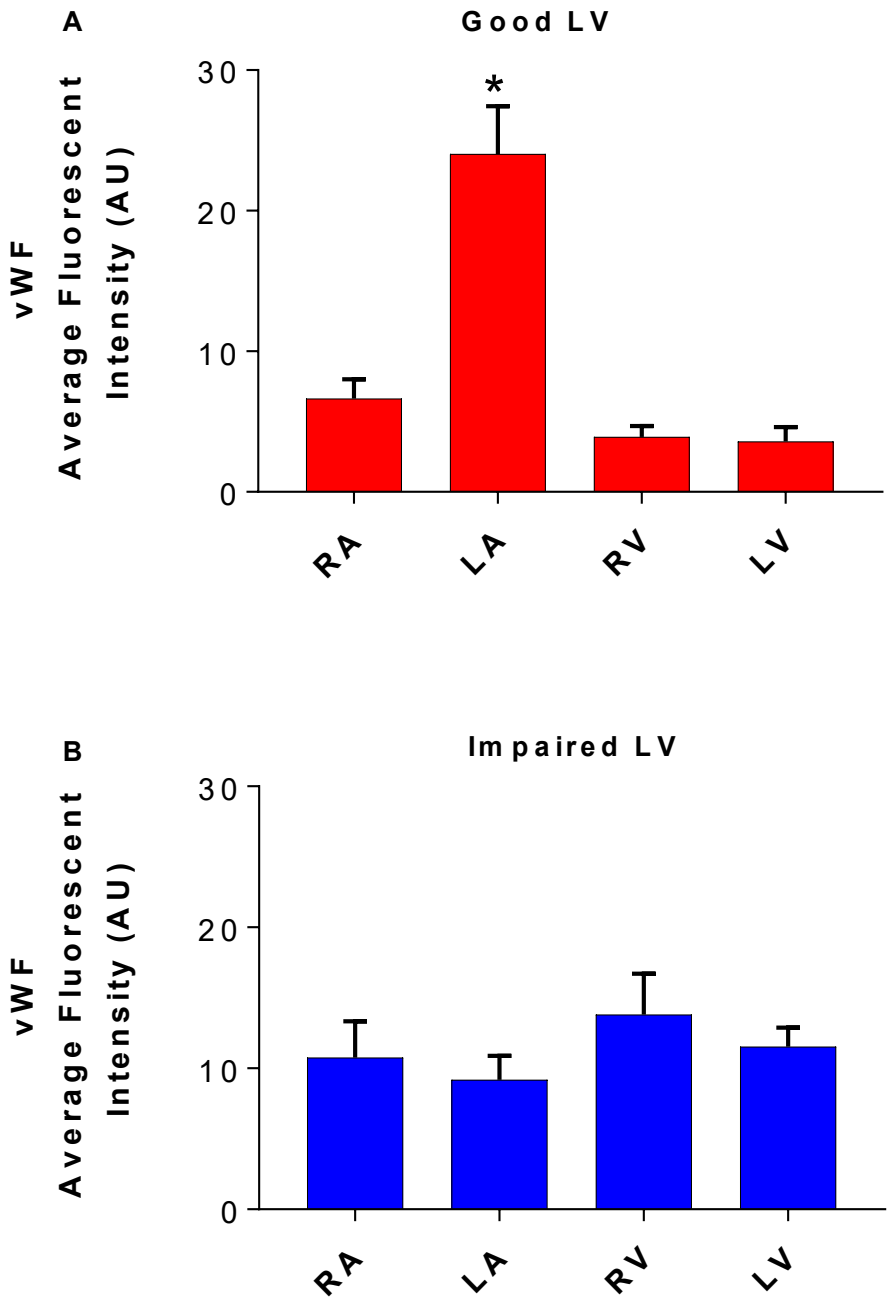

Figure 5. 15 Inter chamber Comparison of vWF expression – Good (A) and Impaired LV (B). Inter chamber Comparison vWF expressed endothelial cells. The data is represented as mean ±SEM, n=5 per chamber. * shows a significant difference ($p<0.05$) vs all chamber.

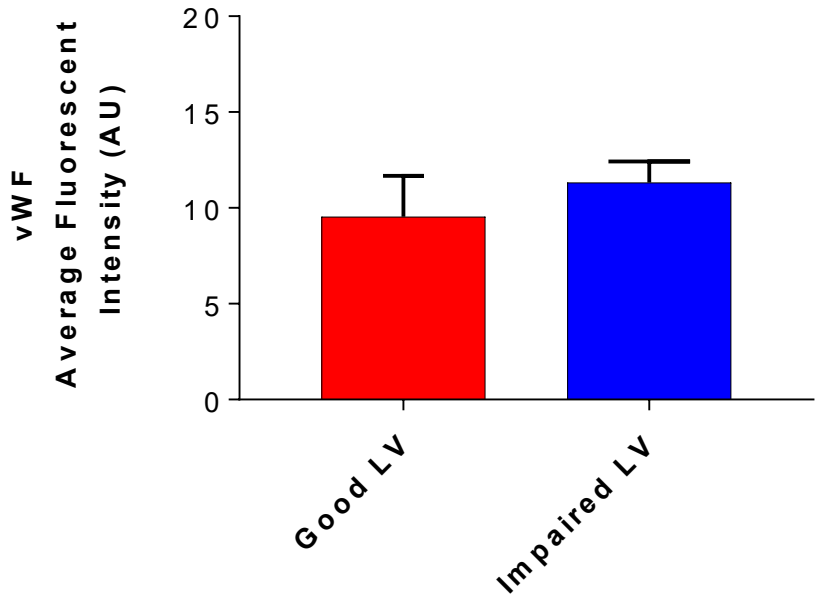

Figure 5. 16 Overall comparison of vWF expressed cells between good and impaired LV. Comparison of vWF expressed endothelial was made between good and impaired LV. The data is represented as mean ±SEM, n=20 per group (samples from each chamber taken from 5 patients).

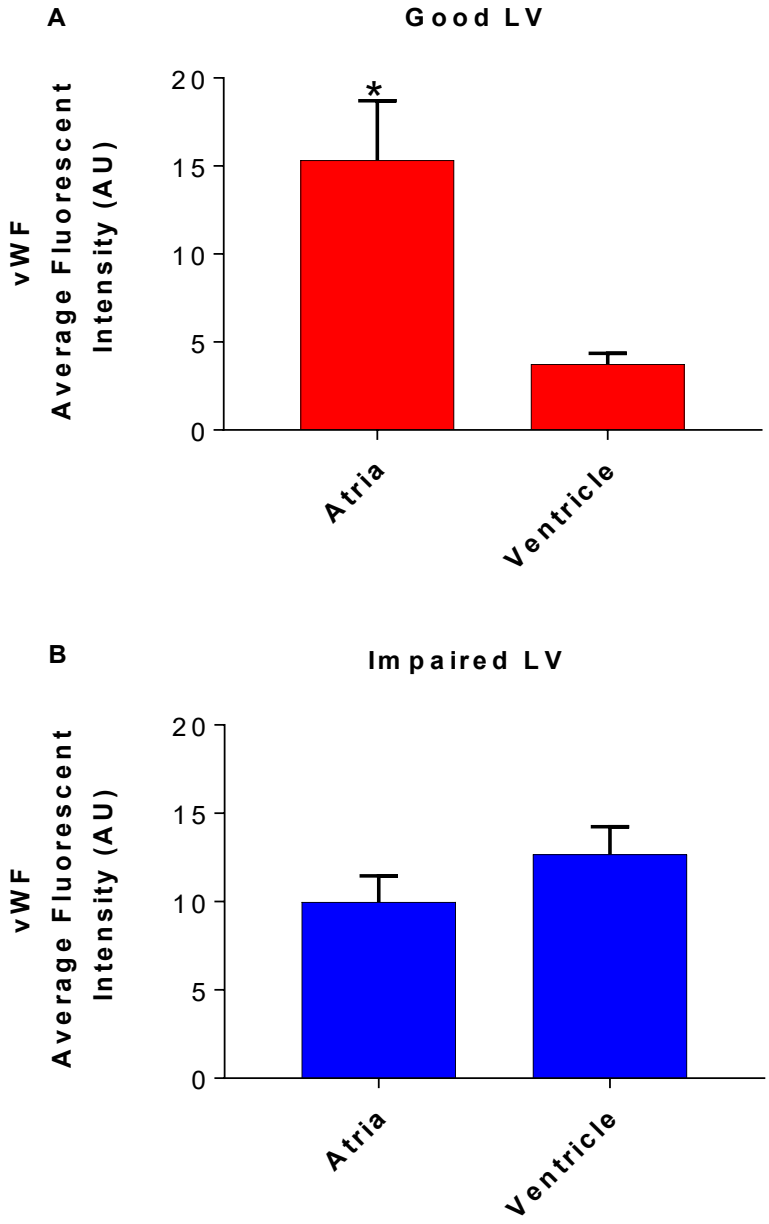

Figure 5. 17 Overall comparison of vWF expressed cells between atria and ventricle. Comparison of vWF expressed endothelial cells was made between atria (A) ventricle (B). The data is represented as mean ±SEM, n=10 (samples from each chamber taken from 5 patients) per chamber. * shows a significant difference ($p<0.05$) vs ventricle.

The main outcomes of this section shows:

- vWF expression in the LA chamber was significantly increased, compared to the other cardiac chambers in good LV group.
- Overall, there was no statistical difference in vWF expression between good LV group and impaired LV group.
- In good LV group, the vWF expression was significantly greater in the atria compared to the ventricle.

5.3.3 Differentiation of CPCs into the smooth muscle cell lineage

Representative images of calponin expressed CPCs from good LV patients and impaired LV patients are shown on figures 5.18 and 5.19 respectively.

In both good LV and impaired LV patients, the calponin expression of CPC cardiospheres from the LV (Good LV, 8±2 AU; Impaired LV 18±2 AU) was significantly ($p<0.05$) increased, compared to other cardiac chambers (RA (Good LV, 2±0.2; Impaired LV 10±1.7), LA (Good LV 3±0.2 AU; Impaired LV 5±1.1 AU) and RV (Good LV 4±0.9 AU; Impaired LV 11±1.5 AU) (Figure 5.20)).

Overall, calponin expression in CPC cardiospheres was significantly ($p<0.05$) increased in the impaired LV group (11±1.3 AU) compared to the good LV group (4±0.7 AU) (Figure 5.21).

In both good and impaired LV patients, calponin expression was significantly ($p<0.05$) increased in the ventricle (Good LV 6±1.2 AU; Impaired LV 15±1.7 AU), compared to the atria (Good LV 2± 0.2; AU Impaired LV 7± 1.3 AU) (Figure 5.22).

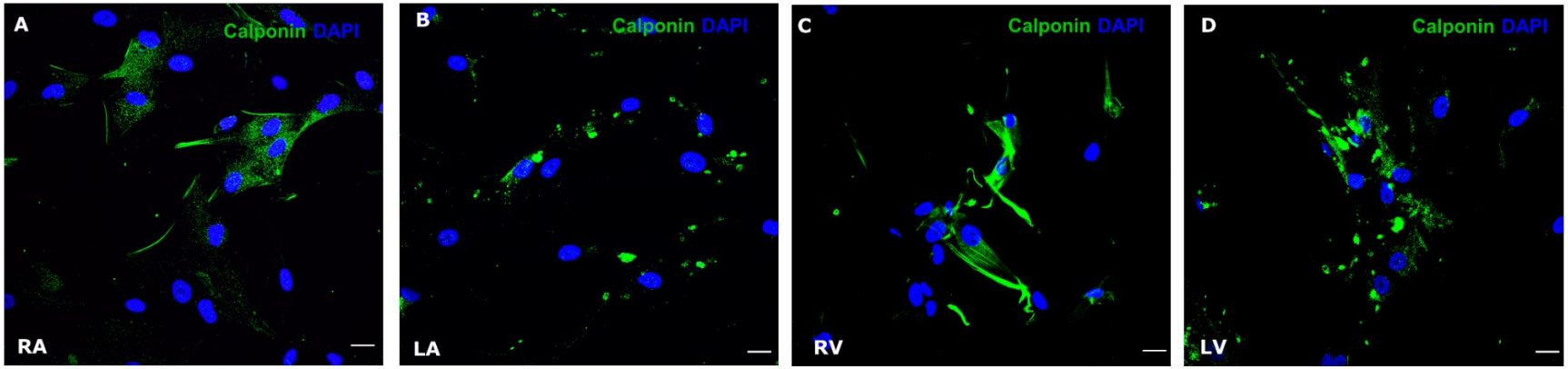

Figure 5. 18 Calponin expression-Good LV. IF staining of differentiated cells from good LV group after plated in smooth muscle differentiation media for 8 days. Green – calponin; nuclei were counterstained with DAPI (blue). Scale = 20μm

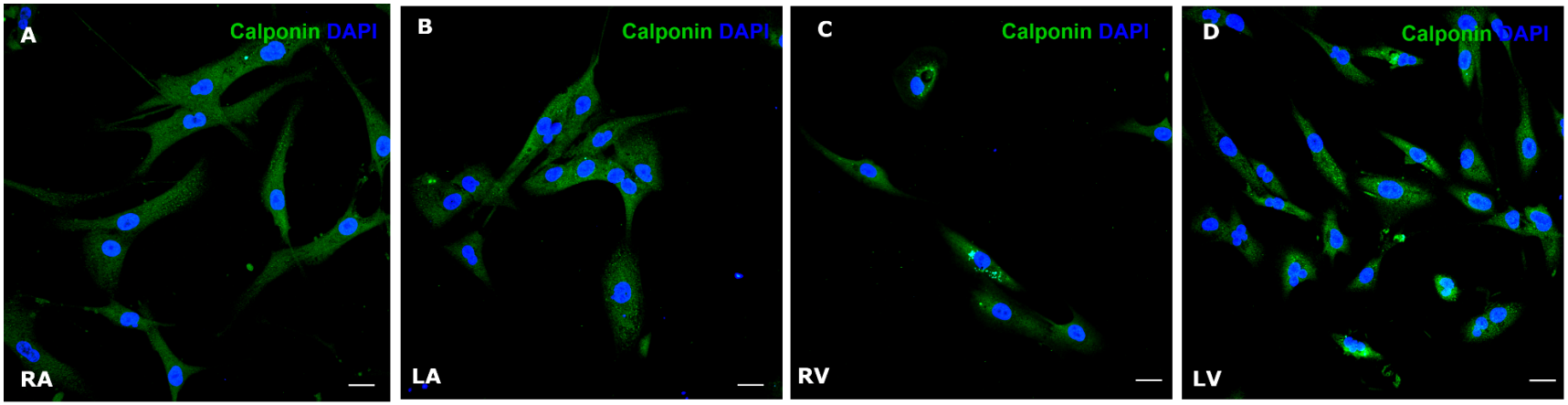

Figure 5. 19 Calponin expression-Impaired LV. IF staining of differentiated cells from good LV group after plated in smooth muscle differentiation media for 8 days. Green – calponin; nuclei were counterstained with DAPI (blue). Scale = 20μm

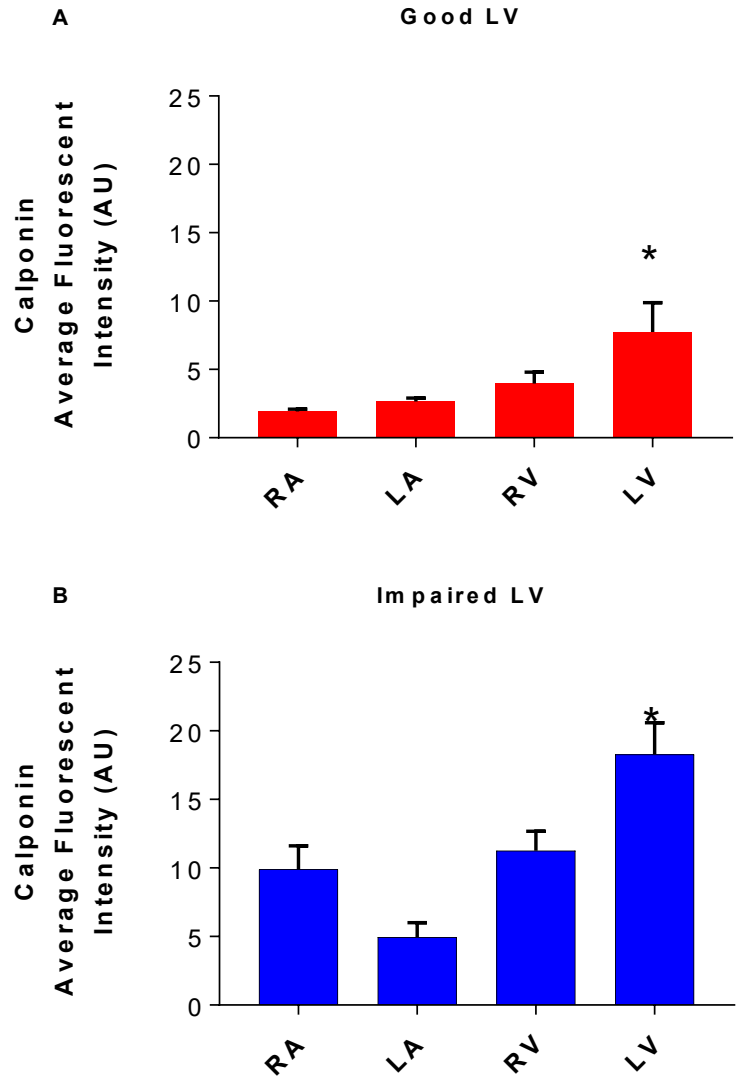

Figure 5. 20 Inter chamber comparison calponin expressed cells. Inter chamber comparison calponin expressed smooth muscle cells was made between good (A) and impaired LV (B). In good LV group - * denotes a significant difference ($p<0.05$) vs RA and LA . In impaired LV group- * denotes a significant difference ($p<0.05$) vs all chamber. The data is represented as mean ±SEM, n=5 per chamber.

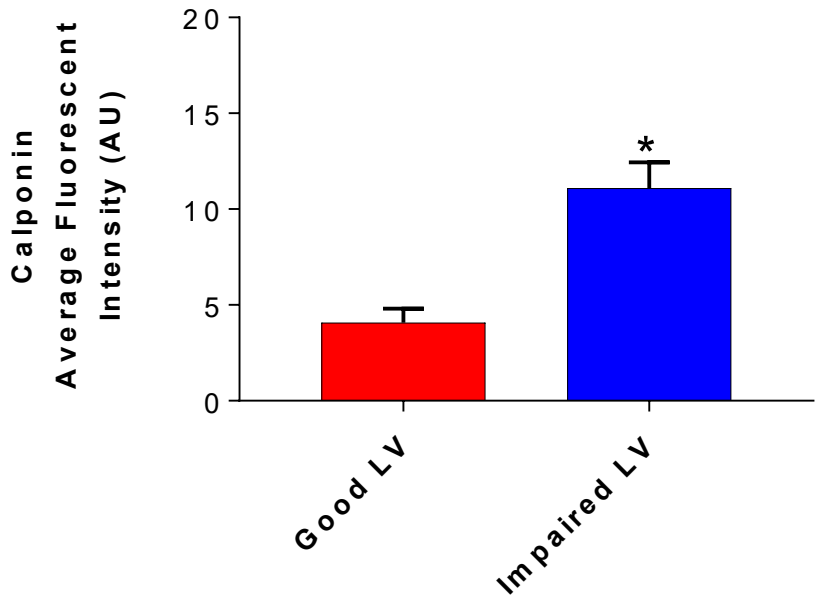

Figure 5. 21 Overall comparison of calponin expressed cells between good and impaired LV. Comparison of calponin expressed smooth muscle cells made between good and impaired LV. The data is represented as mean ±SEM, n=20 per group (samples from each chamber taken from 5 patients). * shows a significant difference ($p<0.05$) vs good LV.

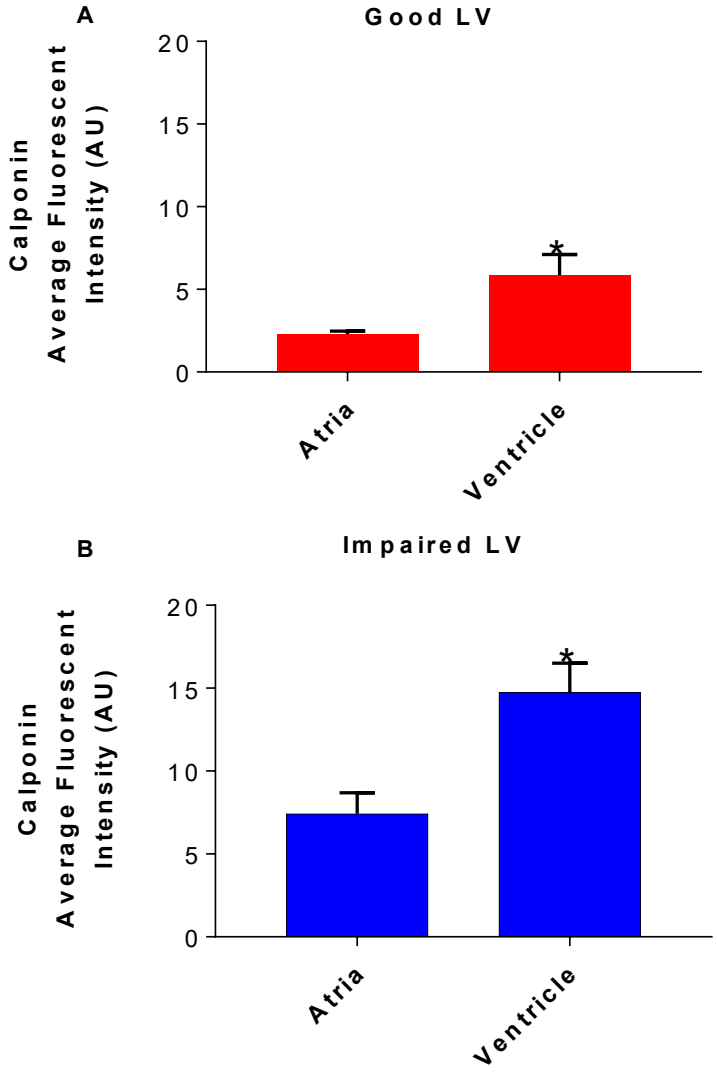

Figure 5. 22 Overall comparison of calponin expressed cells between atria and ventricle. Comparison of calponin expressed smooth muscle cells was made between atria (A) ventricle (B). The data is represented as mean ±SEM, n=10 (samples from each chamber taken from 5 patients) per chamber. * shows a significant difference ($p<0.05$) vs atria.

The primary outcomes of this section show:

- Calponin expression in the LV chamber was significantly increased, compared to the other cardiac chambers regardless of LV function.
- Overall, calponin expression in CPC cardiospheres was significantly higher in impaired LV group compared to the good LV group.
- The ventricles from the impaired LV group showed a significantly higher calponin expression in CPC cardiospheres compared to the atria regardless of LV function.

5.4 Discussion

5.4.1 Cardiospherogenesis of CPCs isolated from all 4 chambers.

The term “cardiospheres” was coined by Messina et al. after isolating undifferentiated cells that formed clusters; which resembled that of neurospheres derived from neuronal progenitor cells (Reynolds BA et al. 1992). In this study, cells were isolated from human atrial (postnatal) and ventricular biopsies; as well as murine hearts. They showed that within 2-3 days, the cells had formed cardiospheres with variable sizes (20-150 μm). They are made up of cardiac CPCs with its core cells demonstrating high stemness capabilities. When attached, cells from the cardiospheres spontaneously differentiate into cells expressing markers of all three cardiac lineages, including, cardiomyocytes, smooth muscle cells and endothelial. Furthermore, they were also shown to be clonogenic with ability to proliferate.

However, unlike this study, they did not compare the ability of each cardiac chamber to generate cardiospheres or assess this ability between normal and impaired ventricles. To date, no study has made such a comparison.

In this study, we demonstrate that cardiospheres can be generated from CPCs isolated from all four chambers of the human heart. Furthermore, we showed the CPCs derived from LV chamber are approximately twice as likely to generate cardiospheres with larger cardiospheres compared to other chambers regardless of LV function. Unlike other studies, we also showed that overall, the CPCs derived from good LV could generate cardiospheres more efficiently than CPCs from impaired LV group.

It has been shown that the generation of cardiospheres decreases with age, disease and is dependent on sample source (Mishra R et al. 2010). This may explain our finding of higher cardiosphereogenesis in good LV compared to Cardiospheres derived from impaired LV patient samples. It is also worth pointing put that the impaired LV samples were derived from old aged patients, and the good LV sample was derived from a younger patient population. These findings were consistent with the findings of Lewis-McDougall et al. 2019. They examined the effects of ageing and disease on isolated CPCs from young and old patients with heart disease. They

demonstrate increased expression of senescent phenotype with increasing age and with the disease. In particular, they also observed that CPCs isolated from younger patients (aged 34-62) generated significantly more and greater size of cardiospheres compared to the CPCs derived from older patients (aged 76-77). They went on to conclude that CPCs isolated from elderly patients with failing heart is dysfunctional with impaired cardiosphereogenesis. These findings strongly corroborate the above results noted in our study. From our findings, the LV chamber regardless of the LV function and CPCs derived from good LV patients, are far more efficient at generating cardiospheres and therefore might be the best source to isolate multipotent CPCs.

5.4.2 Differentiation of CPCs into the three cardiac lineages

The ability to differentiate into the three cardiac lineages (cardiomyocytes, endothelial cells and smooth muscle cells) is an essential hallmark of CPCs (Beltrami et al. 2003; Bearzi C 2007; Ellison et al. 2013). These characteristics have also been demonstrated in cardiosphere derived cells, where the cells undergo spontaneous differentiation into recognised lineages as described before(Messina et al.; Smith A et al. and Ellison et al. 2013). However, these effects have not been studied in CPCs isolated from each cardiac chamber, nor have the comparisons been made between cells derived from good and impaired LV patients.

Bearzi et al. (2007), who isolated CPCs from human myocardium showed that in differentiating medium, the human CPC clones were able to differentiate into all three lineages (myocytes, SMCs, and ECs) and through electrical stimulation, at 1Hz they were able to induce contractility. Further in vivo study using clonogenic human CPCs, where the CPCs were injected into rat myocardium following infarction, showed new myocardial regeneration along with neovasculature formation. By using real-time PCR, they confirmed the presence of human transcription factors for cardiomyocytes, SMC and EC genes (Bearzi et al. 2007). Similar findings have also been reported by other studies using CPCs derived from various rodent models and humans (Itzhaki-Alfia et al. 2009; Beltrami et al. 2003; Bearzi C et al. 2007; Ellison et al. 2013; Cesselli D et al. 2011).

Itzhaki-Alfia et al. (2009) reported that CPCs derived from human RA, although not statistically significant, were more capable of differentiation into the cardiomyocyte lineage, compared to CPCs derived from LA chamber. Indeed, at one-week post-transplantation of the CPCs derived from RA were shown to be positive for human sarcomeric actin and early sarcomere formation. At one-month post-transplantation, they demonstrated some of the transplanted cells to express cardiac actin and intercalated disc structures. However, in our study, we have compared the multipotency of the CPCs derived from all four cardiac chambers in both good and impaired LV groups. Comparison of this kind has not been made before. We assessed the ability of CPCs isolated from all four chambers of the heart in both good and impaired LV to differentiate into each cardiac lineage spontaneously.

Due to the limited plasticity of CPCs, the *in vitro* differentiation of these cells still remains a challenge. To date, various methodologies using several differentiation factors including TGF-β, oxytocin, dexamethasone, 5-Azacytidine, Ascorbic Acid and retinoic acid have been explored.However, their efficacy is still very much debated (Malandraki-Miller et al. 2018).

Lim JY et al. 2007, showed TGF-β1 to be a crucial player in cardiac differentiation through induction of Nkx2.5, which is a cardiac TF. Furthermore, TGF-β1 was shown to facilitate the differentiation of atrial derived Sca-1+ CPCs into functioning CMs (Goumans et al. 2007). These cells, as well as beating showed cross striations and gap junction communication. . Techniques which enhance the proliferation of the committed progenitors to improve the efficacy of CM generation is highly desirable but the signals which regulates the replication/differentiation of the CPCs is still lacking. Our lab group has successfully differentiated cloned c-kit^{pos} CPCs to generate beating CM through the addition of oxytocin, BMP2/4, TGF-β, and DKK1 at various stages in the differentiation protocol. (Smith AJ et al 2014; Vicenza et al. 2018; Lewis-McDougall F et al. 2019).

One of the characteristics of CPCs is to be able to differentiate into all three cardiac lineages, as discussed in the introduction. We wanted as a baseline using spontaneous differentiation protocol to understand this characteristic of the isolated CPCs from each cardiac chamber and compare such characteristics of CPCs between CPCs derived from good and impaired LV patients. Although the spontaneous differentiation not efficacious (Batalov I et al. 2015 and Rajala et al. 2011), such evaluation will allow crucial understanding of the isolated CPCs.

In this study, both α-sarcomeric actin^{pos} cardiomyocyte precursor cells and calponin expressed smooth muscle cells were significantly higher in the LV chamber regardless of the LV function status. The expression of Nkx-2.5^{pos} cells was significantly higher in the LV chamber within the impaired LV group. Overall, the expression of α-sarcomeric actin, Nkx-2.5 and calponin were significantly higher in the impaired LV group compared to good LV. However, the vWF expression was higher in the LA chamber of the good LV group and showed no significant difference between chambers for the impaired LV group.

There is some evidence to suggest that the disease process can alter the CPCs activity and regulate differentiation. Urbanek et al. (2005); report an 85-fold and a 25-fold increase in differentiation into all three cardiac lineages in CPCs derived from myocardium following acute and chronic infarcts respectively. In another study, it was shown that differentiation into all three cardiac lineages was comparable between CPCs derived from old explanted hearts and young donor hearts (Cesselli D et al. 2011). More recently, it was shown from the data of patients undergoing cardiac surgery that CPCs isolated from older patients and diseased patients to have impaired differentiation capabilities despite increased accumulation (Lewis-McDougall F et al. 2019). They showed the CPCs derived from older patients to have more senescent phenotype with a high expression for markers such as p16INK4A, SA-β-gal with truncated telomeres. More importantly, their study demonstrates that single CPC derived clones irrespective of age and disease were homogenous in terms of multipotency and differentiation.

The behaviour of the isolated CPCs is related to their genetic profile and gene expression. The complex dynamic process that regulates various genes and TFs is crucial for cardiogenesis and influenced by their epigenetic memory (Mauritz et al. C 2008 and Devalla et al. 2018).

As shown before (Cesselli D et al. 2011; Devalla et al. 2018 and Lewis-McDougall F et al. 2019) it is possible to clonally select cycling competent CPCs from a single CPC, irrespective of age and disease. Such CPCs have been shown to retain their

functional capabilities and are potentially able to repair diseased myocardium depending on their epigenetic memory.

Our study has identified, isolated and propagated clonally derived CPCs from all four chambers of the human heart and made a comparison of these characteristics between good and impaired LV patients. We have shown that clonally derived CPCs can differentiate into all three cardiac lineages, and this ability is altered in good and impaired LV. Understanding such fundamental characteristics and its relevant biology can enable us to plan an effective CPCs based regenerative therapy.

6 DISCUSSIONS

6.1 Introduction

This thesis focused on two objectives. The first objective was to identify, quantify and characterise CPCs in human myocardial samples taken from all 4 cardiac chambers. The second objective assessed whether human CPCs isolated from the four cardiac chambers of patients with normal LV function behave in the same way, exhibiting similar growth and multipotency in vitro compared to human CPCs isolated from patients with impaired LV function.

It was hypothesised that: There would be more CPCs in the atria, compared to ventricles; human CPCs isolated from patients with impaired LV function would have decreased proliferative capacity, clonogenicity, and multipotency potential compared to human CPCs isolated from patients with normal LV function; there would be no differences in growth and multipotency of the human CPCs isolated from the four cardiac chambers.

6.2 Identification and Quantification of CPCs in human myocardial sample obtained from all four chambers of the heart in patients with normal and impaired LV function.

The existence of both c-kit^{pos} and MDR-1^{pos} CPCs in human myocardium has been widely documented (Messina E et al. 2004; Bearzi et al. 2007; Itzhaki-Alfia A et al. 2009; Nadal-Ginard B et al. 2014), and the exhaustive evidence from work done in rodent models and other species have also shown the heart to contain such CPCs, which behave as stem/progenitor cells and are capable of regenerating new myocardium *in vivo* (Beltrami et al. 2003; Ellison et al. 2013; Smith AJ et al. 2014; Vicinanza C et al. 2017). Despite this evidence, the distribution, stem cell properties and differentiation potential of endogenous CPCs in all four cardiac chambers of the human heart, and how these are affected by LV function has not been characterised extensively.

The cardiac CPCs have been shown to have a variety of surface markers (Scalise et al. 2019) which tends to overlap. It is plausible that such phenotypic variation of expression may represent the same CPC at a different time frame in its development (Scalise M et al. 2019; Vicinanza C et al. 2017).

This study, as previously described (Torella et al. 2007), found CPCs within the interstitial space of the myocardium taken from all four chambers of the human heart. Our quantification analyses revealed that in patients that had good LV function, the RA chamber harboured less CPCs (~5 times), compared to the other chambers and in the impaired LV group, the CPCs frequency was significantly higher (~2 times) in the LV chamber when compared to other chambers. Moreover, overall, the impaired LV group had a greater number of CPCs than the normal LV function group. The density of the CPCs (1 cell per 1000 myocytes or 50,000 hCPCs per gram of tissue) is reported to be similar in both rodents and humans (Torella et al. 2007; Vicinanza C et al. 2017). Contrary to our findings, the distribution of the CPCs was shown to be higher in the atria than the ventricle (Sarvanakumar et al. 2013), however, the previous data were obtained from rodent models. A study by Itzhaki-Alfia A et al. (2009), which made no comparison between normal and impaired LV function, claimed the human right atrium harboured more CPCs, compared to the other chambers. However, these were c-kit^{pos} cells and were not lineage sorted to purify for c-kit^{pos} CD45^{neg} tryptase^{neg} CPCs. It has been reported that only 10% of cells that are positive for c-kit are lineage negative (Vicinanza C et al. 2017).

The finding of greater numbers of CPCs in the hearts of patients with impaired LV function, compared to patients with normal LV function has also been observed in previous studies. These data may be explained by the cardiac homeostatic process and the effects of ageing. Indeed, the hearts of patients who had died of acute infarction undergoing transplantation harboured more CPCs than patients who had chronic heart failure needing a transplant. (Urbanek K et al. 2005). This increase was notably higher in acute (7.5 times higher than undiseased) syndromes compared to the chronic disease process (3.5 times higher). This large pool of CPCs has contained more senescent CPCs, expressing the senescence-associated marker p16^{INK4a}.

In the present study, the cohort of patients within the good LV group was relatively young (mean age of 56.4), compared to those in the impaired LV group (mean age of 71). In addition to the injury-induced homeostatic response, the age of the patient can interact with disease status can also alter the number and potency of CPCs (Cesselli et al. 2011; Cheung T.H et al. 2013). Recently this was demonstrated on animal models by Castaldi et al. (2017). In a study which examined biopsies taken from the elderly population with dilated cardiomyopathy without any coronary disease, compared to age-matched control patients with normal left ventricular function, there was a two-fold increase in CPCs in aged hearts with the disease compared to the control (Chimenti C et al. 2003). A further study by Cesselli et al. (2011) evaluated the impact of ageing and CHF on CPCs. In this study, both ageing and disease were shown to be associated with telomere shortening, reduction in CPCs telomerase activity, increased frequency of telomere-induced dysfunction foci within CPCs and higher expression of $p16^{INK4A}$ and $p21^{CIP1}$. Moreover, a study by Mohsin et al. (2013), showed that CPCs isolated from multiple patients with heart failure had characteristic differences in growth rate, telomere length and senescence marker expression. This large pool of CPCs that are generated in response to ageing and disease are highly dysfunctional (Cesselli et al. 2018; Lewis-McDougall et al. 2019). Epigenetic profiling of HSCs between old and young showed modified genetic markers with subsequent changes in gene expression, which support self-renewal with loss of differentiation (Sun d et al. 2014). This evidence further strengthens and explain our observations.

Previous work by Kubo H et al. (2008) showed CPCs to increase by four-fold in diseased heart, but 80% of these cells were shown to be mast cells. Taken together with previous reports, the present findings further corroborate that there are increased CPCs in the hearts of patients with impaired LV function. It is possible that the complex interaction between ageing, disease and homeostasis at the cellular level alters the CPC activity directly and indirectly and therefore, alters CPC distribution and potency (Rolle IG et al. 2020).

The identification and quantification of CPCs in the human myocardial sample is further complicated by patient-specific factors, including their past medical history, smoking history, AF and medication history, including statins and beta-blockers. In a

study by Gambini et al. (2012), which compared patient demographics, along with clinical variables to CPC number; showed several interesting outcomes. This study showed a direct correlation between beta-blocker usage and number of CPCs. Smoking, AF and previous MI showed an inverse relationship to CPC enrichment. In our study, we could not assess the impact of these important factors, as they are too low in numbers. However, in our study, these factors were relatively matched for the good and impaired LV groups.

The present study showed that the left ventricular chamber, irrespective of LV function,to harbour the greatest number of c-kit^{pos} CPCs compared to other cardiac chambers. Hence, the LV chamber may provide a better source of CPCs isolation.

6.3 Characterisation and Comparison of growth, clonogenicity and differentiation of human CPCs isolated from each of the four chambers of the heart, taken from patients with impaired or normal LV function.

Potten and Loeffler have described certain characteristics that a cell should have to be classified as a stem/progenitor cell. Indeed, stem cells are "undifferentiated cells capable of 1) proliferation, 2) self-maintenance, 3) production of a large number of differentiated progenies, 4) regeneration of the tissue after injury, and 5) flexibility in the use of these options" (Potten & Loeffler, 1990).

The cardiac stem/ progenitor cells represent ~1% of the cell population, which are capable of cardiomyogenesis (Vicinanza et al. 2017). The characteristics of the CPC population have been specified by our lab group (Ellison et al. 2013; Smith AJ et al. 2016; Vicinanza et al., 2017) to be c-kit^{pos}, CD31^{neg}, CD45^{neg} and Tryptase^{neg}. Furthermore, it is prudent that it is distinguished from cardiac c-kit^{pos}CD31pos (endothelial) and CD45^{pos} and Tryptase^{pos} (mast) cells.
However, the potential role of this CPCs in cardiomyogenesis has been questioned by several groups (van Berlo et al. 2014; Li et al. 2018). Further analysis of their methodology indicates inadequate mouse model, which does not tag or more importantly, lineage trace the CPCs as described above (Vicinanza et al. 2017).

Despite the vast amount of research that is published about human cardiac CPCs; none have assessed the basic characteristics according to the phenotype described by our lab group. This present study performed a comprehensive analysis of the functional characteristics of the CPCs isolated from each chamber of the heart, from patients with either normal or impaired LV function.

The isolated CPCs demonstrated stem/progenitor characteristics in terms of generation of single cell-derived clonogenicity, proliferation, ability to generate cardiospheres and differentiation capabilities into all three cardiac lineages: cardiomyocytes, smooth muscle and endothelial cells. These data are in agreement with previous studies (Messina E et al. 2004; Bearzi et al. 2007; Torella D et al. 2007; Itzhaki-Alfia A et al. 2009).

The present study found that CPCs isolated from the normal LV function patients had better capabilities to generate single cell-derived clonal colonies when compared to the impaired LV function group (≈54% vs ≈43%), and only the CPCs derived from RV chamber showed reduced clonogenicity compared to the LV chamber (34±3% vs 49±3%). Nevertheless, these clonal rates are better than what is reported. Previously, Bearzi et al. (2007) showed the clonal efficiency to be ≈1-10% amongst single CPC derived clones.

The previous studies mentioned above primarily used the enzymatic isolation process, in contrast to compared to this work. The CPCs derived from the explant culture methodology are less likely to be affected by the proteolytic stress and subsequent potential cellular damage that follows the enzymatic isolation process. These CPCs are more likely to be cycling competent cells, which is aided by the intact ECM and the production of necessary growth factors during the culture process (Dergilev KV et al. 2016).

Ageing and disease have been shown to influence the functional characteristics of CPCs (Gonzalez et al. 2008; Cesselli et al. 2018). Recently, Lewis-McDougall et al. (2019) showed that CPCs derived from the hearts of aged (>70 years) patients who were about to undergo cardiac surgery were dysfunctional, exhibiting decreased proliferation, clonogenicity, and differentiation, compared to CPCs isolated from the hearts of middle-aged patients. However, the characteristics of the single cell-derived clones from aged and young patients were not different with regards to morphology, senescence, multipotency, self-renewing transcript profile and differentiation. Our results also indicate better clonal efficiency in good LV patients, who were relatively young compared to patients from the impaired LV group. Our subsequent experiments using clonally expanded CPCs also confer similar observations, to the one described by Lewis-McDougall et al. 2018.

In our study, the proliferation of the clonally derived CPCs from a single cell was unaffected by LV function and was comparable between atria and ventricle. The CPCs from the LV chamber showed increased capacity to generate cardiosphereogenesis irrespective of good or impaired LV function.

Another important hallmark of CPCs is their ability to differentiate into all three cardiac lineages; cardiomyocytes, smooth muscle and endothelial cells. This important characteristic has been reported by many studies (Itzhaki-Alfia et al. 2009; Beltrami et al. 2003; Bearzi C 2007; Ellison et al. 2013; Cesselli D et al. 2011; Lewis-McDougall et al. 2018).

As with cardiac development, the pluripotent stem cell differentiates through various stages (Figure 6.1), including CPCs before becoming cardiomyocytes, capillaries and arteries(Aguilar-Sanchez et al. 2018; Cianflone et al. 2019). This differentiation process is regulated by various transcription factors. Therefore, through the manipulation of these transcription factors, CPCs could be directed to a particular lineage type (Aguilar-Sanchez et al. 2018; Cianflone et al. 2019). Furthermore, the metabolic adaptation that occurs through ageing and disease; should not be overlooked, as these factors alter the underlying transcription profile. To this cause, various cell types and differentiation protocols are used to assess their differential capabilities (Malandraki-Miller et al. 2018).

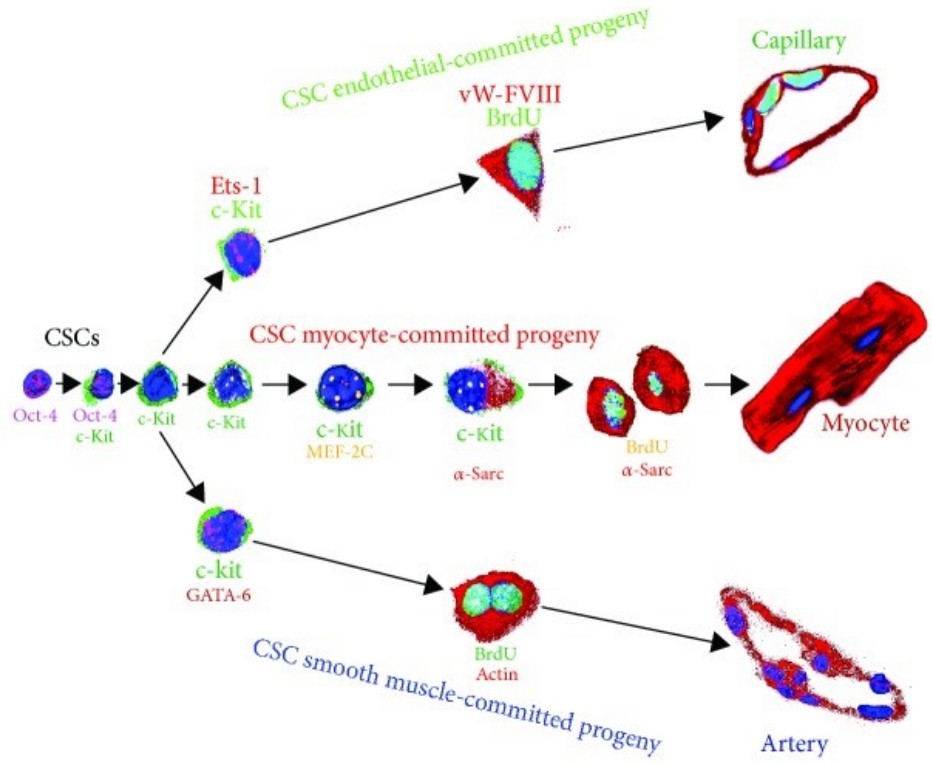

Figure 6. 1 Developmental cardiac markers for stages of cell differentiation. Adapted from Cianflone et al. 2019

Although no inter-chamber comparison of differentiation capabilities has not been reported, Ellison et al. 2013; shown CPCs derived from a single cell to be multipotent and differentiate into all three lineages. Furthermore, when these cells are injected into the myocardium following an infarction; they replace up to 20% of cardiomyocytes and improve overall LV function.

The present study describes inter-chamber variability in the clonally derived CPCs ability to differentiate into the three lineages described, and this characteristic is altered by impaired LV function. This finding is possibly as a result of metabolic adaptation that occurs during injury and ageing, which ultimately regulates the differentiation capabilities, through modification of transcription factors (Malandraki-Miller et al. 2018; Cesselli et al. 2018).

The results of this study indicate, the potential of CPCs as cell-based therapy to depends on several factors, including cellular retention and cell survival, which is estimated at 1% of donor cell at four weeks. The long-term cell survival of these cells has been problematic, compounded by the unfavourable inflammatory

environment mediated by injured tissues and along with possible epigenetic induced changes that occurs in culture (Aguilar-Sanchez C et al. 2017). However, the results from our study indicate that CPCs derived from LV chamber using explant culture technique, that are clonally expanded, might provide a better source of cells, which could lead to more effective cell-based therapies.

To summarise, the four cardiac chambers are unique in both cellular and functional characteristics. The CPCs isolated from each cardiac chamber are likely to show some subtle differences in characteristics. This, along with the disease of the myocardium, alters the isolated CPC activities. The present study provided a much-needed analysis to understand the inter-chamber variability and its associated functional characteristics.

7 LIMITATIONS OF THE STUDY

The findings outlined in this thesis provide a further understanding of the number and dynamics of CPCs within each cardiac chamber and between hearts with normal or impaired LV function. It shows that each cardiac chamber has CPCs that are capable of generating clones, proliferate, form spheroid bodies in suspension and differentiate into the three cardiac lineages; cardiomyocytes, smooth muscle and endothelial cells. In addition, the results from the study demonstrate that these functional characteristics are altered when compared between chambers and between good and impaired LV function. However, if I were to repeat this study, I would address the limitations identified below for a more thorough and in-depth analysis of each cardiac chamber and characterisation of the CPCs.

The design of the study, methods used to accomplish the aims and analysis contributes to the limitations of the study.

This study, in its entirety, was carried out by myself. I am a cardiac surgical trainee with a major interest in cardiovascular research related to clinical translation in HF. Despite having basic lab experience during medical school time, undertaking this PhD was a significant learning point at every level. Therefore naturally, the learning curve took considerable time before optimally working within the lab. Initial few months were spent optimising protocols and learning various lab techniques to prepare me for the experiments ahead. A significant portion of the time was spent learning confocal microscopy.

Patient selection:

Due to patient recruitment, the average age of the patient population in the normal LV function group was low compared to impaired LV group. Several studies, as discussed above, show age and disease to play a critical role to CPC homeostasis. Hence, an aged and sex-matched patient population sample would have enabled a more reliable comparative analysis. Furthermore; if the study had recruited patients undergoing transplant, the comparison between good, impaired LV and poor LV group could have been made.

Hence, more patients population that is matched for age and sex with a homogeneous distribution of demographics will allow better statistical interpretation and conclusion.

CPC location and distribution within all four chambers of the human heart.

The anatomy of the heart is highly complex. The biopsy obtained from each cardiac chambers is very small and may not be representative of the cardiac chamber. Although we were able to analyse all four chambers of the heart, the results need to be interpreted cautiously as biopsies were taken at a particular point in each chamber.

The tissue sections should also be analysed for evidence of cardiac fibrosis, as fibrosis is a hallmark of cardiac disease. The histological analysis of this would allow us to map the CPC location more precisely.

Isolation, clonogenicity, proliferation of CPCs isolated from all four chambers of the human heart.

Isolated CPCs could also have been analysed using flow cytometry for other CPC markers before proceeding with functional in vitro assays. This additional test would have further strengthened the study.

A more thorough assessment of chamber specific atrial and ventricular myosin light chain expression (i.e. MLC-2a for atrial specificity and MLC2v-for ventricular specificity) of differentiated progeny would have allowed us to determine whether there were any underlying chamber specific characteristics of the CPCs derived from each chamber.

The functional aspect of this study only focused on clonally derived cells. A comparison between original CPCs and clonally derived CPCs would have provided a valuable insight into the functional characteristic.

Cardiospherogenesis and differentiation potential of CPCs isolated from all four chambers of the human heart.

This study assessed clonally derived CPCs for their ability to generate cardiospheres and spontaneously differentiate into all three lineages. Although this provides a useful analysis, a comprehensive comparison could have been made between isolated CPCs and clonally derived CPCs. The ability of CPCs from each chamber to generate secondary and tertiary cardiospheres could also have been evaluated.

In addition to the biochemical markers used to assess differentiation; other biochemical markers of cardiomyocytes (GATA-4, MEF2C, α-cardiac actinin, troponin I, troponin T, cardiac myosin heavy chain, cx43), smooth muscle cells (GATA-6, α-smooth muscle actin) and endothelial (Ets1 and CD31) could have provided a more robust assessment. I also could have undertaken some functional assays to determine whether the CPC-differentiated progeny were functional, i.e. cardiomyocyte beating assay (Smith et al. 2014).

8 CONCLUSION

This is the first study that has examined the phenotypic characteristics and dynamics of the CPCs derived from all four chambers of the human heart and compared these properties between the chamber and between normal and impaired LV function patients.

Our results show LV chamber to harbour increased CPCs, and it was shown that increased numbers of CPCs exist in the hearts of patients with impaired LV function. Furthermore, we have identified, isolated and propagated clonally derived CPCs from all four chambers of the human heart. The CPCs derived from the LV chamber can generate more cardiospheres than other chambers. Furthermore, the cardiomyogenic and smooth muscle expressions are higher in the LV chamber irrespective of the LV function.

Understanding such fundamental characteristics of the CPCs derived from the cardiac chambers and its relevant biology can enable us to plan an effective CPC-based regenerative therapy.

9. FUTURE DIRECTIONS

This thesis gives a basic understanding of CPCs isolated from each cardiac chamber, and the results indicate LV chamber as a potential source of CPCs for cellular therapy, but this requires further validation in an animal model.

Using an established animal model such as the MI-regeneration NSG mouse model; the clonally derived CPCs from all four cardiac chambers can be used used to evaluate the regenerative potential and make a comparison between good and impaired LV derived CPCs. This in-vivo analysis will identify the most suitable source of CPCs for successful myocardial regeneration protocols that will allow better cellular retention and lead to effective cardiomyogenesis.

10. REFERENCES

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