Combination of Cardiac Progenitor Cells from the Right Atrium and Left Ventricle provide Synergistic Paracrine Effects for *In Vitro* Cardiac Repair

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Abstract

Cardiovascular diseases, such as ischaemic heart disease, remain the most common cause of death worldwide. With no effective treatment to replace the loss of cardiac tissue following a myocardial infarction, ischaemic heart disease places a significant socioeconomic burden on medicine. Advances in medical research have introduced new potential treatments, of which regenerative medicine such as stem cell therapy seem to be promising for cardiac repair. Various stem cell types have been investigated, which have different effects in cardiac repair. Combination of different cell types have been shown to improve the therapeutic potential which is thought to be due to synergistic or complimentary reparative effects.

Recently, cardiac progenitor cells (CPC) isolated from the right atrial appendage (RAA) and left ventricle have been shown to have different functional effects *in vitro*. Importantly, conditioned media (CM) from CPCs from the right atrial appendage (RAA CPC) have superior cardioprotective effects, while CM from the left ventricle (LV CPC) have superior angiogenic effects. Due to these distinct functional paracrine effects, this study investigated if the combination of RAA CPCs and LV CPCs from the same patient exert synergistic or complimentary paracrine effects for cardioprotection and angiogenesis in an *in vitro* model.

Both RAA CPCs and LV CPCs expressed the mesenchymal cell markers CD90 and CD105, and were predominantly negative for the haematopoietic cell marker, CD34. CPCs were cultured either alone or in combination and exposed to serum deprivation and hypoxic conditions (1% O_2) to stimulate ischaemia. Normoxic conditions (20% O_2) was used as a control. Gene expression of *HIF1A*, *AKT1*, *FGF2* and *PDGFA* were measured to investigate the cellular responses to stimulated ischaemia. *HIF1A* mRNA expression was significantly decreased in the combination group of RAA CPCs and LV CPCs (RAA + LV) cultured in

hypoxia and in hypoxia irrespective of groups. There were no significant differences in the expression of *AKT1*, *FGF2* and *PDGFA*.

To measure the paracrine effects of CPCs, CM was collected following stimulated ischaemia. IGF-1 (pro-survival factor) and VEGF-A (pro-angiogenic factor) concentrations in CM were measured. IGF-1 concentration was comparable across all CPC groups. However, VEGF-A concentration was increased in CM collected from hypoxia, especially in the RAA and RAA + LV hypoxic CM. CM from all CPC groups reduced apoptosis in AC16 cardiomyocyte cell line exposed to stimulated ischaemia. Interestingly, RAA + LV normoxic CM was the only group that significantly increased the expression of HIF-1α protein in AC16 cardiomyocytes exposed to stimulated ischaemia. CM from all CPC groups induced angiogenesis in human umbilical vein endothelial cells and was the highest in the RAA + LV hypoxic CM.

Overall, this study provides evidence that combination of RAA CPCs and LV CPCs may have greater therapeutic effects and have synergistic paracrine effects for cardiac repair. Therefore, *in vivo* studies are warranted to determine if combination of different stem cell types have greater therapeutic potential than single cell therapies.

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a.k.a.	Also known as
AbC	Antibody capture
ACE-I	Angiotensin converting enzyme inhibitor
ACS	Acute coronary syndrome
Ad-MSC	Adipose tissue-derived mesenchymal stem cell
aHIF-1α	Antisense hypoxia inducible factor 1 alpha
ANG	Angiopoietin
ANOVA	Analysis of variance
Anti-anti	Antibiotic-antimycotic
APC	Allophycocyanin
ASC	Adult stem cell
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
a.k.a. FGF2	Fibroblast growth factor 2
BM-MSC	Bone marrow-derived mesenchymal stem cell
BMDC	Bone marrow derived cell
BMI	Body mass index
BMMC	Bone marrow mononuclear cell
BP	Band pass
BSA	Bovine serum albumin
c-Kit	Proto-oncogene c-Kit
c-Myc	Myc proto-oncogene protein
CABG	Coronary artery bypass grafting

CADUCEUS	Cardiosphere-derived autologous stem cells to reverse ventricular dysfunction
CD	Cluster of differentiation
CD105	a.k.a. Endoglin, ENG
CD90	a.k.a. Thymocyte differentiation antigen, Thy-1
CDC	Cardiosphere-derived cell
cDNA	Complementary deoxyribonucleic acid
CKD	Chronic kidney disease
CM	Conditioned media
CO_2	Carbon dioxide
Comp	Compensation
CPC	Cardiac progenitor cell
CSC	Cardiac stem cell
CT	Cycle threshold
CVD	Cardiovascular disease
DEVD	Tetrapeptide sequence of Aspartate-Glutamate-Valine-Aspartate
DM	Diabetes mellitus
DMEM/F12	Dulbecco's modified eagle medium/F12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBM-2	Endothelial basal medium 2
ECG	Electrocardiography/electrocardiogram
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGM-2	Endothelial growth medium 2
ELISA	Enzyme-linked immunosorbent assay

EMT	Epithelial-to-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
EPDC	Epicardium-derived cells
EPO	Erythropoietin
ERK-1/2	Extracellular signal-regulated kinase 1/2
ESC	Embryonic stem cell
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FL	Fluorescent light
Flk1	Foetal liver kinase 1
FMO	Fluorescence-minus-one
FS	Fully stained
FSC	Forward scatter
FSC-A	Forward scatter area
FSC-H	Forward scatter height
GA	Gentamicin/amphotericin B
HbA1c	Glycated haemoglobin
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	Heart failure
HGF	Hepatocyte growth factor
HIF-1	Hypoxia inducible factor 1
HIF-1α	Hypoxia inducible factor 1 alpha
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell

HUVEC	Human umbilical vein endothelial cell
IGF-1	Insulin-like growth factor 1
IGFBP	Insulin-like growth factor binding protein
IHD	Ischaemic heart disease
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iPSC	Induced pluripotent stem cell
Isl1	Islet 1
JAK	Janus kinase
JAK/STAT	Janus kinase -signal transducer and activator of transcription factor
KLF4	Kruppel-like factor 4
KRH	Krebs Ringer Henseleit
LDL	Low density lipoprotein
Lin-	Lineage negative
LLQ	Lower limit of quantification
Log	Logarithm
LP	Long pass
LV	Left ventricle
LV CPC	Left ventricle cardiac progenitor cell
LVEF	Left ventricular ejection fraction
MAGIC	Myoblast autologous grafting in ischaemic cardiomyopathy
MAPK/ERK	Mitogen-activated kinase 1/2 -extracellular signal-regulated kinase 1/2
MI	Myocardial infarction
miR	Micro ribonucleic acid
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger ribonucleic acid

MSC	Mesenchymal stem cell
NO	Nitric oxide
NRT	No reverse transcriptase control
NSTEMI	Non-ST elevation myocardial infarction
NTC	No template control
O_2	Oxygen
Oct-3/4	Octamer binding protein 3/4
OD	Optical density
PBS	Phosphate buffered saline
PCI	Percutaneous intervention
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PE-Cy7	Phycoerythrin-cyanine7
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PI3K/AKT	Phosphatidylinositol 3-kinase -AKT
PKCε	Protein kinase C epsilon
PLGF	Placental growth factor
PMT	Phospho-multiplier tube
POSEIDON	Percutaneous stem cell injection delivery effects on neomyogenesis
PROMETHEUS	Prospective randomised study of mesenchymal stem cell therapy undergoing cardiac surgery
R3-IGF-1	R3-insulin-like growth factor 1
RAA	Right atrial appendage
RAA CPC	Right atrial appendage cardiac progenitor cell
RAAS	Renin-angiotensin-aldosterone system

RCF	Relative centrifugal force
rDNase	Recombinant deoxyribonuclease
REPAIR-AMI	Reinfusion of enriched progenitor cells and infarct remodelling in acute myocardial infarction
RFU	Relative fluorescent units
RISK	Reperfusion injury salvage kinase
RLU	Relative luminescent units
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT	Reverse transcription
Runx2	Runt-related transcription factor 2
SAFE	Survivor activating factor enhancement
Sca-1	Stem cell antigen 1
SCIPIO	Stem cell infusion in patients with severe ischaemic cardiomyopathy
SDF-1	Stromal cell-derived factor 1
SEM	Standard error of mean
SF	Serum-free
SOX2	Sex determining region Y box 2
SP	Side population
SSC	Side scatter
SSC-A	Side scatter area
STAT3	Signal transducer and activator of transcription factor 3
STEMI	ST elevation myocardial infarction
TGF-β	Transforming growth factor beta

TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TTP	Tristetraprolin
UC-MSC	Umbilical cord-derived mesenchymal stem cell
US	Unstained
VAD	Ventricular assist device
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Glossary

Conditioned media (CM)

RAA normoxic CM	CM collected from right atrial appendage (RAA) cardiac progenitor cells cultured in normoxic culture conditions (20% O_2).
LV normoxic CM	CM collected from left ventricle (LV) CPCs cultured in normoxic culture conditions (20% O_2)
RAA + LV normoxic CM	CM collected from a combination of RAA CPCs and LV CPCs cultured in normoxic culture conditions (20% O_2).
RAA hypoxic CM	CM collected from RAA CPCs cultured in hypoxic culture conditions (1% O_2).
LV hypoxic CM	CM collected from LV CPCs cultured in hypoxic culture conditions (1% O_2).
RAA + LV hypoxic CM	CM collected from a combination of RAA CPCs and LV CPCs cultured in hypoxic culture conditions (1% O_2).

Gene expression (mRNA)

RAA normoxia	Messenger ribonucleic acid (mRNA) expression of RAA CPCs cultured in normoxic culture conditions (20% O_2).
LV normoxia	mRNA expression of LV CPCs cultured in normoxic culture conditions (20% O_2).
RAA + LV normoxia	mRNA expression of a combination of RAA CPCs and LV CPCs cultured in normoxic culture conditions (20% O_2).
RAA hypoxia	mRNA expression of RAA CPCs cultured in hypoxic culture conditions (1% O_2).
LV hypoxia	mRNA expression of LV CPCs cultured in hypoxic culture conditions (1% O_2).
RAA + LV hypoxia	mRNA expression of a combination of RAA CPCs and LV CPCs cultured in hypoxic culture conditions (1% O_2).

1 Introduction

Cardiovascular disease (CVD) is the leading cause of mortality globally with an estimated 17.9 million deaths annually. Ischaemic heart disease (IHD), a condition characterised by decreased blood supply to the heart, is the predominant cause of death in CVD accounting for 8.9 million deaths, approximately half (49.7%) of CVD deaths.(1) In IHD, the reduced blood supply to the heart results in ischaemic stress to the myocardium. This eventually leads to myocardial infarction (MI) resulting in reduced cardiac function, as well as death and loss of cardiac cells. In some cases, MI leads to subsequent adverse cardiac remodelling which can progress towards heart failure (HF), an end stage heart disease. Improvements in cardiovascular management has led to a steady decrease in mortality rates and an increase in median age of cardiovascular deaths in patients with CVD in developed countries, which consequently increases the proportion of deaths caused by HF.(2)

Current available treatment can delay progression towards heart failure but is unable to replace the lost cardiovascular cells, which is essential for long-term improvement of cardiac function in patients with IHD and HF. While orthotopic heart transplantation is the only option to achieve this, limited availability of donor hearts makes transplantation a clinically not feasible option.(3)

Stem cell therapy holds great promise for their potential to repair and regenerate cardiac tissue lost in IHD and HF. Currently different stem cell populations are being investigated to identify the best cell source for therapy. Of various stem cell types, cardiac stem cells (CSC) were shown to have better effects on cardiac repair compared to other adult stem cell (ASC) populations and seem to be a promising candidate for cardiac repair.(4) Furthermore, a combination of different stem cell types which have synergistic or complimentary effects may produce greater therapeutic effects than single cell therapies.(5) Improvements in cardiac function following stem cell therapy are attributable to several mechanisms including differentiation of stem cells into cells of the cardiovascular lineage such as cardiomyocytes, endothelial cells and smooth muscle cells, and paracrine mediated effects.(6,7)

A recent study shows that different populations of stem cells in the heart have different functional qualities, with cardiac progenitor cells (CPC) from the right atrial appendage (RAA CPC) have better cell survival potential and CPCs from the left ventricle (LV CPC) have better angiogenic potential, which are major targets for cardiac repair.(8)

1.1 Ischaemic heart disease

1.1.1 Pathophysiology of ischaemia heart disease

Ischaemic heart disease, also known as coronary artery disease, is the most common cause of heart failure in the developed world.(9,10) Atherosclerosis plays an important pathophysiological role in the development of IHD (Figure 1-1).(11,12)

Atherosclerosis involves the remodelling of the arterial wall with the accumulation of sub-endothelial lesions called atherosclerotic lesions or plaques. The pathogenesis of atherosclerosis involves endothelial dysfunction, vascular inflammation and the deposition of lipids, calcium and cellular debris within the tunica intima, the innermost wall of the blood vessel.(13) Endothelial dysfunction occurs due to presence of several risk factors such as dyslipidaemia, hypertension, hyperglycaemia and inflammation, all of which lead to an increased expression of cell adhesion molecules and structural changes, particularly vascular cell adhesion molecule 1 (VCAM-1), which allows platelet activation and infiltration by leucocytes and low density lipoproteins (LDL).(12,14,15) LDL is oxidised in the presence of reactive oxygen species (ROS) and other oxidative pathways, which are then ingested by macrophages to become foam cells.

Foam cells release chemical signals, or cytokines, exacerbating the inflammatory response. This causes chronic inflammation and proliferation of the plaque by recruitment of inflammatory cells, lipids and LDL, migration of smooth muscle from the tunica media and further activation of the endothelium.(14,15)

The atherosclerotic plaque is composed of a necrotic lipid-rich core, inflammatory cells such as foam cells, smooth muscle cells, and calcium deposits. With ongoing chronic inflammation, the plaque develops fibrous tissue and an overlying fibrous cap which can cause stenosis or narrowing the lumen of the artery.(15) Proliferation of the plaque narrows the lumen of the artery, reducing the blood supply. Consequently there is insufficient oxygen supply to the heart in order to meet the high demand of the cardiac tissue leading to ischaemia.(16)

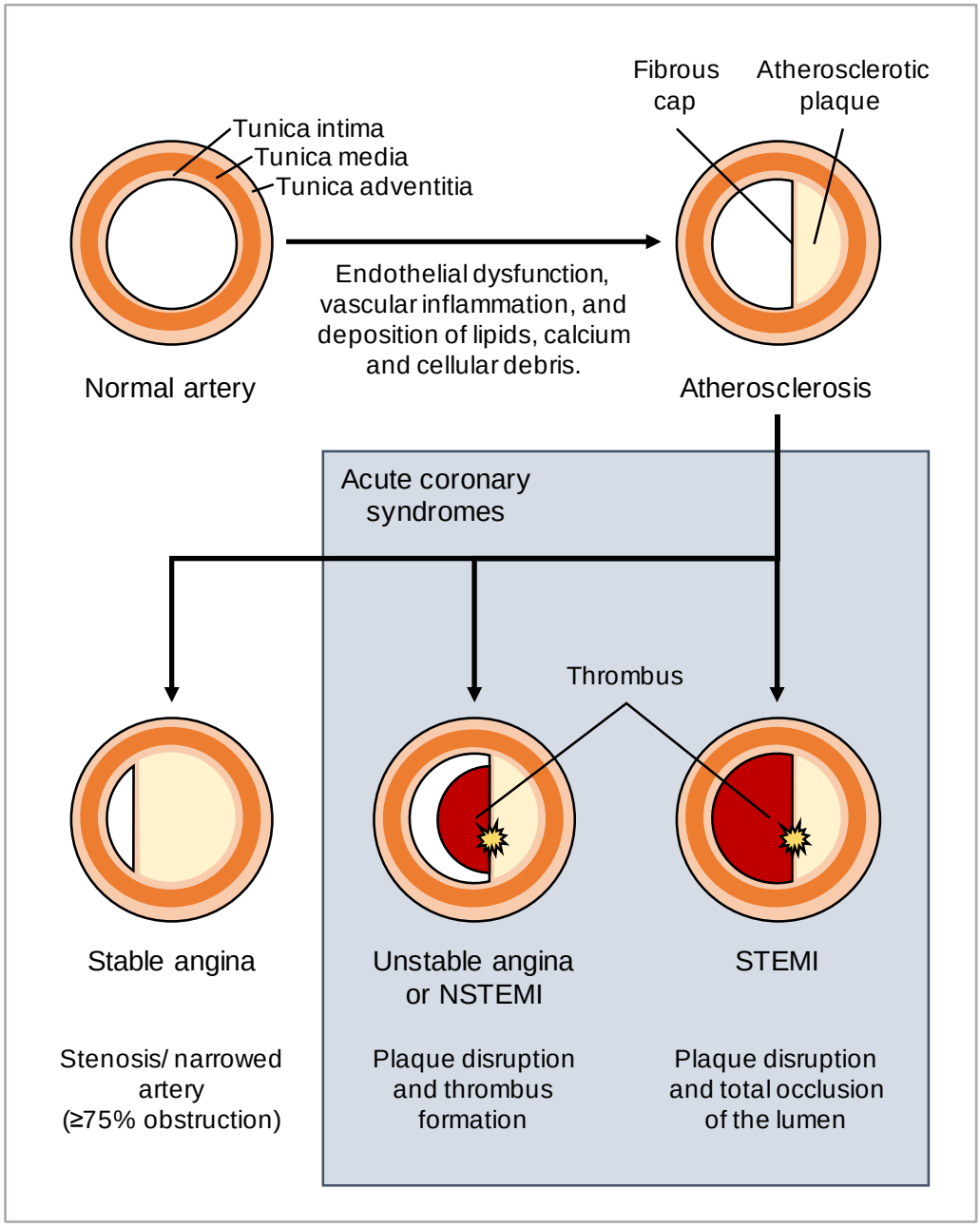


Figure 1-1: Pathogenesis and complications of IHD.
The development of atherosclerosis occurs in the presence of various risk factor. Progression of atherosclerosis can cause obstruction of the lumen of the artery, or more severely, lead to plaque rupture and thrombus formation causing acute coronary syndromes. NSTEMI, non-ST elevation myocardial infarction; STEMI, ST elevation myocardial infarction.

Complications of IHD may manifest as symptoms and signs of varying severity. Stable angina is characterised by episodes of ischaemia induced by increased myocardial demand, as in exercise or stress causing chest pain, usually caused by stenosis. Unstable angina is characterised by ischaemia at rest or minimal exertion.(16,17) Acute coronary syndrome (ACS) is a major complication of IHD, most commonly caused by rupture of the fibrous cap of the atherosclerotic plaque with subsequent formation of a blood clot or thrombus.(11) ACS

includes unstable angina, or MI. MI is characterised by death of the cardiac muscle tissue or myocardial necrosis due to ischaemia.(17) Partial, transient occlusion of the artery causes unstable angina or non-ST elevation MI (NSTEMI), while complete occlusion of the artery causes ST elevation MI (STEMI). NSTEMI and STEMI are characterised based on the changes seen in electrocardiography (ECG) at diagnosis.(17)

Following MI, pathophysiological mechanisms lead to myocardial dysfunction associated with ischaemia, metabolic changes and inflammation. Due to a decrease in oxygen supply, the metabolism of the heart shifts from an aerobic to anaerobic state, which significantly reduces energy/adenosine triphosphate (ATP) production and consequently lactate is produced, resulting in the loss of cellular homeostasis.(18) The loss of cellular metabolism and ischaemic injury causes cell death by necrosis and apoptosis. Necrosis occurs due to the disruption of cellular processes and elicits an inflammatory response, while apoptosis is a programmed cell death mediated by molecular signalling.(19) Due to the changes in cellular metabolism and loss of functional cardiomyocytes, there is decreased cardiac function and contractility of the heart which is acutely compensated by the activation of the renin-angiotensin-aldosterone system (RAAS), sympathetic innervation and production of natriuretic peptide (Figure 1-2).(18,20)

The inflammatory response following MI leads to the recruitment of a cellular infiltrate of neutrophils followed by monocytes/macrophages, which clear cell debris and begins the remodelling process of the heart.(20) In early remodelling (within 72 hours of ischaemic injury), neutrophils degrade the extracellular matrix (ECM). This leads to ventricular dilation and wall thinning later on in the disease process, increasing wall stress on the ventricles.(20) In response to RAAS activation, wall stretch and paracrine factors, late remodelling processes (after 72 hours of ischaemic injury) occur including hypertrophy of non-infarcted myocytes, formation of blood vessels by angiogenesis and collagen deposition and scar formation by myofibroblasts.(20)

1.1.2 Heart failure

Due to the limited regenerative capacity of the heart, the infarcted tissue is repaired by fibrosis, accompanied ventricular dilation and hypertrophy which alters the structural integrity of the heart. The extent of the remodelling process is correlated with infarct size, and is associated with functional consequences such as increased mortality, worse prognosis and complications including heart failure, cardiogenic shock and arrhythmia.(18)

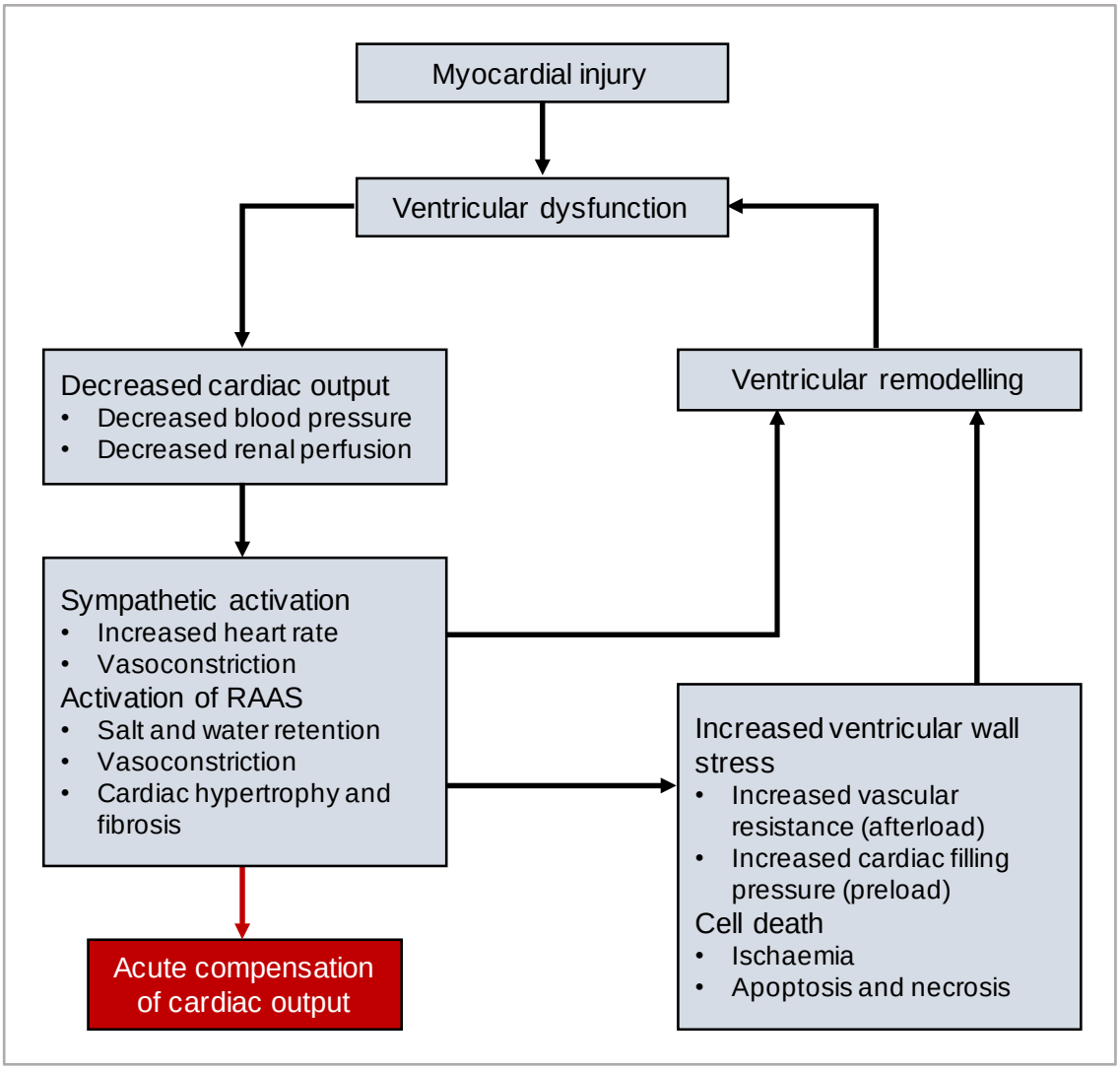


Figure 1-2: Vicious cycle of heart failure.
Compensatory mechanisms improve cardiac function in the short term. These mechanisms cause ventricular dysfunction in the long term causing a progressive loss of cardiac tissue, and deterioration cardiac function. RAAS, renin-angiotensin-aldosterone system.

HF is an end-stage complication of IHD and is characterised by structural or functional abnormalities causing reduced cardiac output or elevated intracardiac pressures. HF is associated with typical symptoms and signs including breathlessness, oedema, fatigue and increased jugular venous pressure.(21) There is a progression in the severity of HF represented as a vicious cycle (Figure 1-2).(10,22) Impaired ventricular function reduces cardiac output. Compensatory mechanisms such as activation of the sympathetic nervous system and activation of the RAAS are activated in response to decreased cardiac output. This causes increased heart rate, systemic vasoconstriction and salt and water retention to improve cardiac function in the short-term. However, the persistent increase in wall stress due to

increased ventricular filling volumes, increased oxygen demand due to increased heart rate and the ongoing ventricular remodelling, subsequent ventricular dysfunction occurs further reducing the cardiac output. Current available treatment slows progression of HF by targeting mechanisms in this 'vicious cycle' but does not address the underlying pathology of lost cardiac tissue causing functional depression of the heart.

1.1.3 Current interventions for IHD

Primary prevention of CVD involves interventions to manage risk factors at both individual and population levels to prevent the development of CVD. While these interventions have reduced CVD associated mortality rates, CVD still remains the leading cause of death worldwide.(1,23)

Management of IHD involves lifestyle modification and pharmacological intervention to control for modifiable risk factors to reduce symptoms and improve prognosis.(16) Modifiable risk factors include smoking, diet, physical activity and management of co-morbidities associated with IHD such as dyslipidaemia, hypertension, diabetes mellitus (DM), and chronic kidney disease (CKD). Lifestyle modifications include reduced exposure to and cessation of smoking, a healthy diet including decreased intake of saturated fat, salt and alcohol, regular physical activity (30 minutes per session, ≥3 times a week of moderate intensity), maintaining or achieving or normal body mass index.(16) Pharmacological interventions to manage risk factors include lipid-lowering drugs such as statins to manage dyslipidaemia and LDL cholesterol, anti-hypertensive drugs such as thiazides, β-blockers and angiotensin converting enzyme inhibitors (ACE-I) to reduce blood pressure to recommended levels (<140/90 mmHg) and for individuals with DM, controlling glucose levels and glycated haemoglobin (HbA1c) (<48-52 mmol/mol).(16)

In the event of an acute MI, time to reperfusion is an essential prognostic factor for the outcome of treatment. Reperfusion is a treatment for restoring blood flow through or around a blocked artery. Reduced time to perfusion is associated with reduced mortality and infarct size. Current reperfusion strategies address the importance of reducing the time to reperfusion.(24) Percutaneous coronary intervention (PCI) and fibrinolysis and, in patients who do not respond coronary artery bypass grafting (CABG) surgery, are indicated as reperfusion/revascularisation strategies of NSTEMI and STEMI.(17,25)

Post-MI treatment involves cardioprotective interventions and secondary prevention of further complications of IHD.(26) Lifestyle changes and pharmacological therapy, including anti-

platelet and anti-coagulant agents to prevent thrombus formation, reduce the risk of further complications and progression of ventricular dysfunction. Revascularization may be indicated in patients with haemodynamic instability by PCI or CABG surgery.(17,26)

The treatment of heart failure aims to improve cardiac function and the patient's quality of life, relieve symptoms, prevent progression of the disease and reduce mortality.(21) Treatment involves improving haemodynamic parameters including ejection fraction, cardiac output and fluid dynamics through various pharmacological interventions. For heart failure which cannot be stabilised by medical therapy, ventricular assist devices (VAD) are indicated as a bridge-to-transplantation therapy for patients awaiting heart transplantation.(21) VADs are mechanical devices that support cardiac function and is an elective surgical treatment. VADs are not viable for long-term use and are associated with major complications such as mechanical failure and death.(27) For end-stage heart failure, the current treatment options are orthotopic heart transplantation, VADs and palliative care.(21) Due to the strict eligibility criteria, limited availability of donor hearts for heart transplantations and the risk of immune rejection, this treatment option is not viable for many patients. Despite the number of available treatment options described above, there is no treatment option that addresses the loss of cardiac tissue in IHD, HF and following a MI. Hence, there is considerable interest in new strategies that promote repair and regeneration of cardiac tissue.

1.2 Stem cell therapy for cardiac repair

Regenerative therapy using stem cells provides a promising adjunct to current treatment with the potential to facilitate regeneration and repair of cardiac tissue by transplantation of stem cells. Current challenges in cell therapy include identifying the ideal stem cell source, optimal delivery method and improving cell survival, retention and engraftment of stem cells into cardiac tissue.(28,29)

Stem cells are defined as undifferentiated cells that can self-renew and have the potential to differentiate into multiple cell lineages, which is known as potency. Progenitor cells are lineage-specific with a more limited differentiation potential and proliferate for a finite number of divisions.(30,31) Embryonic stem cells (ESC) derived from the inner cell mass of an embryo and induced pluripotent stem cells (iPSC) produced by reprogramming somatic cells, are pluripotent cells meaning they can differentiate into the cells of the three germ layers: ectoderm, mesoderm and endoderm.(32) ASCs derived from somatic tissue are

multipotent cells that can differentiate into multiple lineages but have a more limited differentiation capacity.(33) For the successful regeneration of the heart, it is essential to identify stem cells which can differentiate into cardiomyocytes, endothelial cells and smooth muscle cells.

Stem cells have been transplanted into the heart by intracoronary infusion or direct intramyocardial injection, though these delivery routes are associated with limitations.(30) An optimal route of delivery would ensure that sufficient number of stem cells reach the target site, have little to no risk of off-target effects and haematological dissemination, and be minimally invasive.(3) Another limitation of stem cell therapy is the low retention of stem cells following transplantation.(28,30) Transplanted stem cells are subjected to the hostile environment of ischaemic tissue characterised by hypoxia, acidosis, inflammation and oxidative stress.(28,30) Current strategies under investigation include pre-treatment of target tissue, pre-treatment of cells and tissue engineering to improve cell survival and engraftment into cardiac tissue.(33)

Pluripotent stem cells have only just begun progressing towards clinical trials, and there is concern about their safety and complications for transplantation in human studies.(34,35) There have been considerable clinical trials using ASCs which have demonstrated safety, particularly with autologous transplantation, and evidence for improvements in cardiac function and reduction in the remodelling process in post-MI.(36) Currently, ASCs seem to be much more appealing due to their safety compared to pluripotent cells.

The ideal stem cell candidate, or combination of cells, for cardiac repair should be easy to expand *in vitro*, allow autologous transplantation, facilitate cardiomyogenesis (regeneration of cardiomyocytes) and neovascularisation (formation of new blood vessels), and differentiate into cardiac lineages with minimal limitations and complications.(29)

1.3 Pluripotent stem cells

1.3.1 Embryonic stem cells

ESCs are typically derived from the inner cell mass of blastocysts which develop from unused embryos generated from procedures such as in vitro fertilisation.(37) Due to their potential to give rise to all cell types of the body, ESCs have a therapeutic potential for several medical conditions of different body systems.

ESCs have been shown to develop into an foetal liver kinase 1 (Flk1) positive and islet 1 (Isl1) positive CPCs which are present in the early mesoderm during development specific to cardiac lineages which in turn are capable of differentiating into cardiac lineage cells.(38,39) Human ESC-derived cardiomyocytes have been shown to regenerate non-human primate hearts.(40)

However, there are significant limitations to the use of ESCs. Transplantation of undifferentiated human ESCs can cause formation of a teratoma, a heterogeneous tumour containing cells derived from all three germ layers, in mice.(41) Transplantation of ESCs may cause immune rejection.(42) To reduce the consequences of immune rejection of ESCs, immunosuppressive drug regimens and histocompatibility matched ESCs may be used but are clinically challenging.(42,43) To obtain ESCs from the inner cell mass, the embryo is destroyed in the process which raises some ethical and legal concerns.(44)

1.3.2 Induced pluripotent stem cells

The recent discovery of iPSCs is an alternative pluripotent cell to ESCs. iPSCs were first discovered by introducing transcription factors including octamer-binding protein 3/4 (Oct-3/4), sex determining region Y box 2 (SOX2), Myc proto-oncogene protein (c-Myc) and Kruppel-like factor 4 (KLF4) into adult mouse fibroblasts using retroviral vectors.(45) Human fibroblasts were later used to generate iPSCs using a similar method.(46) All four transcription factors described above are important in maintaining pluripotency, cell phenotype and rapid proliferation of ESCs. iPSCs have similar characteristics to ESCs with the ability for unlimited self-renewal and pluripotency, although there are subtle differences which may limit their therapeutic potential.(32,47)

The reprogramming of somatic cells derived from a patient could allow for patient-matched autologous transplantation, eliminating the concern regarding immunogenicity.(32,48) As the generation of iPSCs does not involve the use of embryos, it has less ethical implications compared to ESCs. iPSCs have a promising potential for regenerative medicine though there are challenges that need to be addressed before they can be translated for clinical applications The potential for teratoma formation still remains a concern in iPSCs, which may be more tumorigenic, similar to ESCs.(49,50) Another limitation is that inducing pluripotency could be often inefficient and incomplete with less than 1% of transfected cells becoming iPSCs.(46,48)

1.4 Adult stem cells

Adult stem cells are cells derived from adult somatic tissue and are a readily available source of stem cells. Various populations of ASCs have been characterised for their potential for cardiac regeneration including skeletal myoblasts, bone marrow derived cells, endothelial progenitor cells (EPC), mesenchymal stem cells (MSC) and cardiac stem/progenitor cells. They provide several advantages over pluripotent stem cells as they allow autologous transplantation, are readily available following isolation, relatively safe and are not associated with ethical constrictions.

1.4.1 Skeletal myoblasts

Skeletal myoblasts are one of the most extensively studied ASCs for cardiac repair in patients with IHD. Skeletal myoblasts are suitable for cardiac repair due to their myogenic differentiation potential that can form contractile elements in the heart.(51) Various studies have shown improvements in cardiac function following transplantation of skeletal myoblasts.(52–54) However the myoblast autologous grafting in ischaemic cardiomyopathy (MAGIC) trial, a large scale randomised placebo-controlled phase 1 clinical trial of myoblast transplantation showed no significant improvement in heart function following transplantation of skeletal myoblasts over CABG surgery.(55) Moreover, another study showed that skeletal myoblasts are restricted to the myogenic lineage and cannot differentiate into cardiomyocytes. Importantly they were unable to integrate electromechanically into the myocardium, increasing the risk to cause arrhythmias.(51)

Several mechanisms have been proposed for the generation of arrhythmia. Connexin 43, a gap junction protein, and N-cadherin, an adhesion protein, are essential for electrical-mechanical coupling and forming intercalated discs with cardiomyocytes to allow synchronous contraction.(56) Connexin 43 and N-cadherin are not expressed in differentiated skeletal myoblasts. Overexpression of connexin 43 in skeletal myoblasts improved electrical coupling with cardiomyocytes *in vitro* and *in vivo*.(57,58) However, in a study by Fernandes et al. overexpression of connexin 43 did not significantly decrease arrhythmia following myoblast transplantation *in vivo*.(59) Further studies are therefore required to determine the applicability and feasibility of overexpression of connexin 43.

1.4.2 Bone marrow derived cells

The bone marrow contains a reservoir of haematopoietic and non-haematopoietic stem cells. Due to their ease of accessibility and being abundantly available, bone marrow derived cells

(BMDC) do not require extensive *ex vivo* manipulation.(29,60) Orlic et al. first demonstrated regeneration of the infarcted myocardium by transplantation of BMDCs expressing the stem cell marker, proto-oncogene c-Kit (c-Kit), through differentiation into cardiomyocytes, smooth muscle cells.(61) However, other studies have demonstrated that BMDCs do not transdifferentiate into cells of the cardiac lineage.(62,63) The efficacy and safety of BMDCs has been demonstrated in a number of clinical studies.(60,64,65)

Bone marrow mononuclear cells (BMMC) are an unselected heterogeneous population of stem cells composed of haematopoietic stem cells (HSC), EPCs and MSCs, among others.(33,60) Therapeutic effects of BMMCs were shown in the reinfusion of enriched progenitor cells and infarct remodelling in acute myocardial infarction (REPAIR-AMI), a randomised placebo-controlled study of intracoronary infusion of BMMCs which showed marked improvements in left ventricular function in patients infused with BMMCs.(66)

HSCs isolated from the bone marrow are multipotent stem cells that can give rise to all haematopoietic lineages. Subpopulations of such cells have been isolated from the bone marrow expressing haematopoietic cell markers (cluster of differentiation, CD) such as CD34 and CD133 (formerly known as AC133), which have been shown to promote angiogenesis.(60,67)

EPCs are a subset of HSCs involved in post-natal neovascularisation.(68) EPCs can be isolated from the mononuclear cells of the bone marrow, peripheral blood and umbilical cord blood.(69,70) EPCs are characterised by the expression of a haematopoietic stem cell marker such as CD34 and CD133 and an endothelial cell marker such as vascular endothelial growth factor receptor (VEGFR) or CD31.(71) EPCs can differentiate into mature endothelial cells and are thought to be involved in vascular repair following injury.(71) Due to their ease of accessibility and beneficial effects on neovascularisation, EPCs hold promise for vascular diseases such as peripheral artery disease and IHD.(72)

EPCs can be classified into either; early EPCs and late or outgrowth EPCs. Early EPCs appear 4 to 7 days after culturing mononuclear cells and late EPCs appear after 14 to 21 days of culture and form cobblestone-shaped colonies, the typical appearance of mature endothelial cells.(71) Early EPCs have been shown to augment angiogenesis through paracrine mechanisms but do not directly incorporate into the vasculature while late EPCs have been shown to incorporate into the vasculature but do not exert paracrine mechanisms for angiogenesis.(73,74) These distinct differences in angiogenic effects can be utilised for their therapeutic roles in neovascularisation.

1.4.3 Mesenchymal stem cells

MSCs are multipotent cells distributed throughout the body that can differentiate into mesodermal lineages including chondrocytes, adipocytes and osteoblasts. MSCs derived from the bone marrow (BM-MSC) and adipose tissue (Ad-MSC) being the most investigated.(75) MSCs isolated from umbilical cord (UC-MSC), specifically from the Wharton's jelly and cord blood have recently gained interest, due to their higher proliferative potential, ability to self-renew and lower immunogenicity.(76,77)

Due to the heterogeneity of MSCs, it is difficult to classify these cell as they lack a unique cell marker. The following criteria established by the International Society of Cellular Therapy that outlines the minimal requirements to classify MSCs.(78) MSCs must: be adherent to plastic when cultured under standard conditions; express mesenchymal cell surface markers including CD105, CD73 and CD90 while lacking the expression of cell markers for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR; be able to differentiate into mesodermal lineages.

MSCs have potent immunomodulatory properties by suppressing white blood cells and triggering an anti-inflammatory response.(75) In the phase 1/2, percutaneous stem cell injection delivery effects on neomyogenesis (POSEIDON) trial comparing allogenic and autologous BM-MSC in patients with left ventricular dysfunction due to ischaemic cardiomyopathy, MSCs showed low rates of adverse events including immune rejection, suggesting the use of MSC for allogenic transplantation possible through their immunomodulatory effects.(79)

In the phase 1/2 placebo-controlled, prospective randomised study of mesenchymal stem cell therapy in patients undergoing cardiac surgery (PROMETHEUS) trial, left ventricular ejection fraction (LVEF) increased and scar size was reduced in patients receiving intramyocardial injections of autologous MSCs, demonstrating the efficacy and safety of MSCs.(80)

1.4.4 Cardiac stem/progenitor cells

The heart was once thought to be a post-mitotic organ without the ability to regenerate following ischaemic injury. However, the demonstration of the presence of replicating cardiomyocytes in the heart and the discovery of CSCs have led to a paradigm change.(81,82) CSCs in the adult heart are involved in limited cell turnover and endogenous cardiac regeneration. Low rate of cardiomyocyte renewal (up to 1.9%) has been observed in hearts of

young human adult at 20 years of age.(83) Furthermore, following ischaemic injury, cardiomyocytes are renewed by stem/progenitor cells.(84) The emerging concept of CSCs and their availability in the adult heart for autologous transplantation has elicited overwhelming interest and investigation of these cells. Due to the specific role of CSCs in endogenous cardiac repair and cardiac development, they hold great promise for the regeneration of cardiac tissue.

Different populations of CSCs have been characterised in the heart, of which c-Kit$^+$ cells and cardiosphere-derived cells (CDC) have been the most extensively studied to date.

1.4.4.1 C-Kit$^+$ cells

CSCs were first identified in the rodent heart by the expression of the cell surface marker c-Kit, a common stem cell marker, but negative for haematopoietic lineage markers (Lin-), known as c-Kit$^+$ cells.(81)

Stem cell infusion in patients with ischaemic cardiomyopathy (SCIPIO) was the first randomised phase 1 clinical trial involving the administration of autologous c-Kit$^+$ cells in patients with severe ischaemic cardiomyopathy.(85) C-Kit$^+$ cells were isolated and expanded from the RAA of patients undergoing on-pump CABG surgery. Approximately 4 months after the surgery, c-Kit$^+$ cells were administered to patients in the treatment group by intracoronary infusion in the infarcted regions. No adverse effects were reported in the c-Kit$^+$ cell treated group. In the c-Kit$^+$ cell treated group, LVEF increased from 30.3% before infusion to 38.5% 4 months following infusion ($p=0.001$). In contrast, LVEF did not change during the corresponding time points in the control group which did not receive the stem cell infusion. The beneficial effects of c-Kit$^+$ cell infusion was more pronounced following 1 year after infusion which was associated with a decrease in the infarct size.

Despite these promising results, there has been controversy of the nature of c-Kit$^+$ cells. Sultana et al. states that c-Kit$^+$ cells are actually a population of endothelial cells in the heart rather than resident CSCs.(86) In another study, c-Kit$^+$ cells were shown to have a limited potential to differentiate into cardiomyocytes in mice during aging and after an injury.(87) Nevertheless, the potential benefits from these cells should not be ignored.

1.4.4.2 Cardiosphere-derived cells

CDCs are another type of stem cell from the heart that are extensively studied. CDCs are a heterogeneous population of cardiac cells isolated from cardiospheres, which are cell

aggregates that form when myocardial tissue explants are cultured under specific conditions.(88)

A phase 1 cardiosphere-derived autologous stem cells to reverse ventricular dysfunction (CADUCEUS) trial, in which patients with recent MI with a LVEF of 25-45% and had undergone successful PCI treatment were randomised into the treatment group receiving autologous CDC transplantation and the control group receiving standard treatment.(89) In the CDC treated group, CDCs were isolated from right ventricular endomyocardial biopsies of patients and then infused into an artery related to the infarct area. There were no significant differences in LVEF between patients receiving CDC infusion and patients receiving standard care. However, patients receiving CDC transplantation showed a reduction in infarct size and improved regional contractility.(89)

A study has directly compared the *in vitro* and *in vivo* functional effects of CDCs against BM-MSCs, Ad-MSCs and BMMCs, the cells considered to be the most promising for cardiac repair.(4) Importantly, CDCs showed greater cardiomyogenic differentiation and angiogenic potential than other cell types *in vitro*, as well a balanced paracrine profile. When injected into the infarcted myocardium of mice, CDCs showed superior effects on cardiac repair including greater cell engraftment and *in vivo* differentiation, reduced apoptosis and reduced ventricular remodelling at 3 weeks post-MI. In addition, CDCs increased the LVEF and was the only group that showed significant improvement than the controls. Furthermore, unsorted CDCs had superior functional effects compared to purified c-Kit$^+$ cells after transplantation into the infarcted heart.(4) This evidence along with the results from clinical trials suggest that CDCs are the leading contenders for the ideal stem cell source for efficient cardiac regeneration.

1.4.4.3 Other cardiac stem cell populations

Other endogenous CSC populations include stem cell antigen 1 (Sca-1$^+$) cells and side population (SP) cells. Sca-1$^+$ cells were first isolated from the adult murine heart which express early cardiac transcription factors.(90) SP cells are multipotent progenitor cells isolated from the heart characterised by their ability to exclude the Hoechst dye.(91)

Recent genetic fate mapping studies in mice have revealed populations of progenitor cells that give rise to anatomically distinct areas of the heart.(92) Islet 1 (Isl1) is a cardiac transcription factor expressed in CPCs of the second heart field which contributes to the development of

the right ventricle, atria and outflow tract.(92) Isl1^{+} cells have the ability to differentiate into cardiac lineage cells.(39)

Epicardium-derived cells (EPDC) were identified in the epicardium, a layer of mesothelium covering the heart.(93) The epicardium originates from the proepicardium during development, distinct from the first and second heart field.(92) A subset of epicardial cells undergo epithelial-to-mesenchymal transition (EMT) during development and participate in the development of the myocardium.(93,94) EPDCs have been demonstrated to promote angiogenesis and are a promising candidate for cardiac repair.(95,96)

1.5 Combination cell therapy using different adult stem cell populations for cardiac repair

Since studies have identified functional differences across different stem cell populations, combination of cells that exert complimentary or synergistic effects may have the potential for greater therapeutic effects on cardiac repair than single cell populations.

Early evidence for the clinical relevance of combination cell therapy for cardiac repair was shown using combined transplantation of skeletal myoblasts with BMMCs after myocardial infarction in rats.(97)

Similarly, combined transplantation of skeletal myoblasts with CD133+ cells in a model of chronic IHD improved cardiac function, reduced scar size and apoptosis, and increased angiogenesis in rats.(98) Skeletal myoblasts improve cardiac function by introducing contractile elements, while CD133+ cells increase angiogenesis. The functional benefits of the combined transplantation were mainly attributable to the increased neovascularisation which improved the blood supply for the engraftment of the skeletal myoblasts.

Following combined xenogeneic (from different species) transplantation of BM-MSCs with c-Kit^{+} CSCs in a porcine model of MI improves cardiac function and enhances scar size reduction.(99) Another study showed combined autologous transplantation of BM-MSCs with CSCs in a porcine model of chronic IHD showed similar improvements in cardiac function and scar size reduction.(100) Combined transplantation of CSC/CPCs with saphenous vein pericytes and EPCs, both known for their role in neovascularisation, have reported improvements in cardiac function in a model of MI.(101,102) The effects of combination therapy have been attributed to complimentary or synergistic paracrine profiles of the different stem cell populations, enhancing the therapeutic potential of stem cells.

1.6 Paracrine mechanisms of cell therapy on cardiac repair

Due to the multifunctional properties of stem cells, several mechanisms have been suggested to mediate their beneficial effects on cardiac repair (Figure 1-3). The major mechanisms of cell therapy include replacement of lost cardiac tissue by direct differentiation into functional cardiomyocytes, smooth muscle cells and endothelial and the release of paracrine factors that mediate responses in cardiac repair.(33)

Initially, it was thought that differentiation of stem cells was responsible for improvements seen in cardiac function. Pluripotent stem cells and CSCs can differentiate into cells of the cardiac lineage, although the potential of other ASCs to differentiate into cells of the cardiovascular lineage remains highly controversial.(29,33) Additionally, the functional improvements following stem cell transplantation is not sufficiently explained by differentiation due to the relatively small number of new cardiomyocytes formed.(7,33) This has led to a paradigm change and it is now believed that the majority of the therapeutic effects of cell therapy is due to paracrine-mediated effects.(103,104)

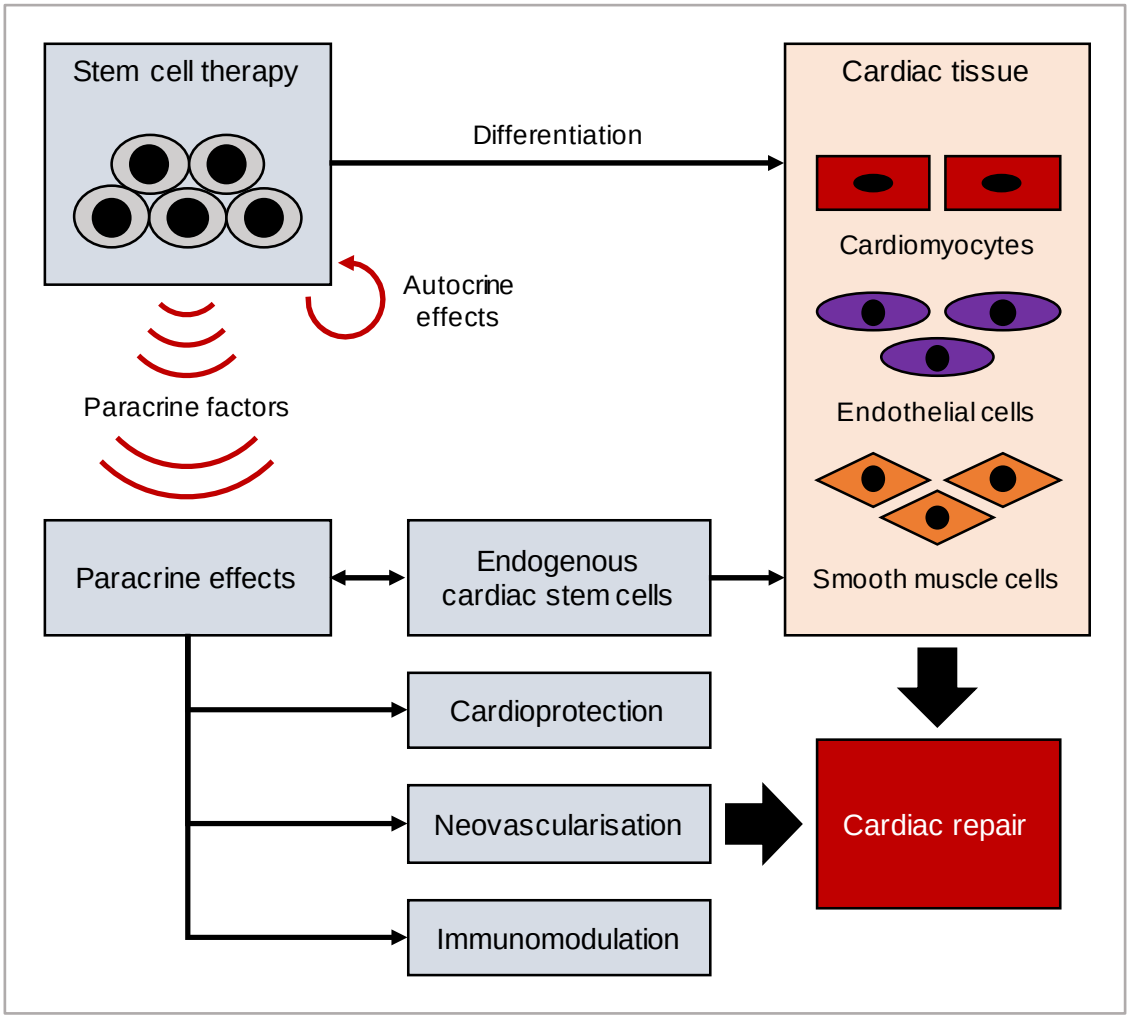


Figure 1-3: Potential mechanisms involved in stem cell therapy.
Differentiation and paracrine effects are the major mechanisms involved in the regenerative effects of stem cell therapy. The release of paracrine factors from transplanted stem cells are thought to mediate the major responses in cardiac repair through paracrine and autocrine effects.

Paracrine factors released by stem cells after transplantation play an essential role in the reparative process of cell therapy. The paracrine mechanism supports the regenerative process by promoting cardioprotection, neovascularisation, immunomodulation and stimulating endogenous stem cells.(33) Of these, cardioprotection and neovascularisation have been the most studied.(7) ASCs secrete a variety of different growth factors, cytokines, chemokines, microvesicles and exosomes to mediate cardiac repair.(7,105) Microvesicles and exosomes are secreted membrane vesicles containing lipids, protein and nucleic acids such as messenger ribonucleic acid (mRNA) and microRNA (miR), involved in cell-cell communication.(105) Prominent paracrine factors secreted by ASCs include insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast

growth factor (bFGF), erythropoietin (EPO), platelet derived growth factor (PDGF), angiopoietins (ANG), and interleukins (IL), among others.(7,106)

Furthermore, the microenvironment of stem cells influences the release of such factors, as in IHD where the ischaemic environment promotes the release of paracrine factors.(103) In addition, secreted factors exert autocrine effects on the stem cells themselves which affect their cell survival, growth and self-renewal properties.(7,106)

1.6.1 Cardioprotection

In the infarcted heart, cell death results from necrosis and apoptosis.(19) Transplantation of stem cells have protective effects on the myocardium and attenuate ischaemic/reperfusion injury, reduce apoptosis and increase proliferation of cardiomyocytes. (7,106)

ASCs such as HSCs, MSCs and CSC/CPCs have been shown to exert anti-apoptotic effects through paracrine-mediated mechanisms.(107–109) Secreted factors by CDCs reduced apoptosis on neonatal rat ventricular myocytes *in vitro*.(110) Similarly, when CDCs were transplanted into murine model of MI, resulting in decreased apoptotic rate at the peri-infarct border.(110)

Cardioprotective effects exerted by paracrine factors are mediated through the activation of several pro-survival pathways (Figure 1-4).(7,111–113) Most paracrine factors released by stem cells activate the reperfusion injury salvage kinase (RISK) pathway which activate the kinases AKT and extracellular signal-regulated kinase 1/2 (ERK-1/2) through phosphatidylinositol 3-kinase -AKT (PI3K/AKT) and mitogen-activated protein kinase 1/2 -ERK-1/2 (MAPK/ERK) signalling.(7,111) Other pathways that are implicated in cardioprotection include survivor activating factor enhancement (SAFE) which activates the signal transducer and activator of transcription 3 (STAT3) through Janus kinase -STAT (JAK/STAT) signalling, and the protein kinase C epsilon (PKCε) pathway.(7,112) These pathways act through various mechanisms to exert cardioprotection including direct anti-apoptotic effects, regulation of gene expression and inhibition of mitochondrial permeability transition pore (mPTP). (7,111,112)

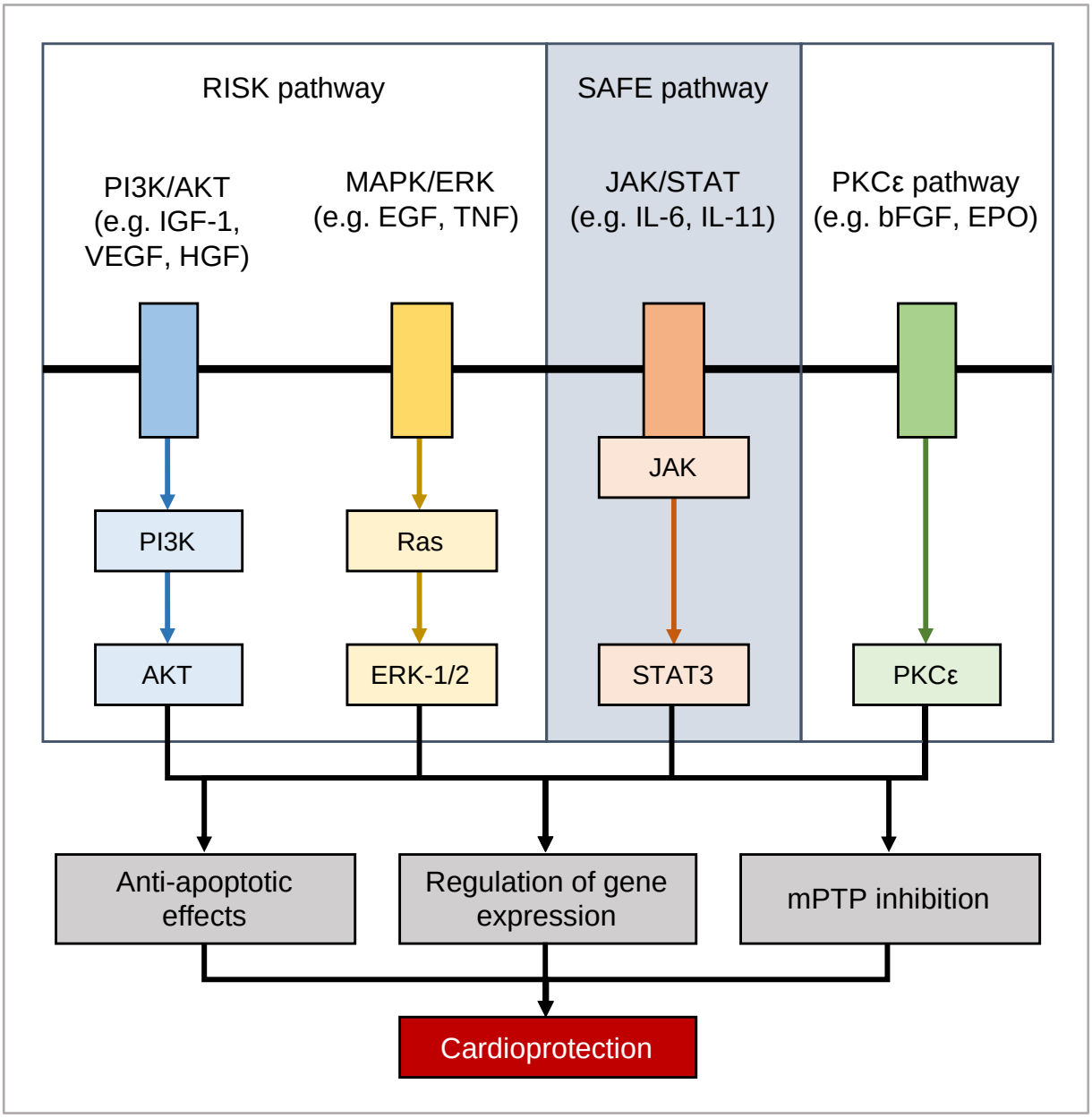


Figure 1-4: Cell signalling pathways involved in cardioprotection.
Various paracrine factors (growth factors, cytokines and chemokines) bind to specific cell receptors to exert cardioprotective effects through cell signalling pathways including the RISK pathway, SAFE pathway and PKCε pathway. RISK, reperfusion injury salvage kinase; SAFE, survivor activating factor enhancement; PI3K/AKT, phosphatidylinositol 3-kinase - AKT; MAPK/ERK, mitogen-activated protein kinase 1/2 -extracellular signal-regulated kinase 1/2; JAK/STAT, Janus kinase -signal transducer and activator of transcription factor; PKCε, protein kinase C epsilon; IGF-1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; TNF, tumour necrosis factor; IL, interleukin; bFGF, basic fibroblast growth factor; EPO, erythropoietin; PI3K, phosphatidylinositol 3-kinase; JAK, Janus kinase; ERK1/2, extracellular signal-regulated kinase 1/2; STAT3, signal transducer and activator of transcription factor 3; mPTP, mitochondrial permeability transition pore.

1.6.2 Neovascularisation

Paracrine factors induce neovascularisation and the formation of new blood vessels. Restoring the blood supply to the ischaemic area is essential to reduce the extent of damage. The mechanisms involved in neovascularisation include: angiogenesis which is the sprouting and proliferation of endothelial cells from pre-existing vessels; vasculogenesis which is the formation of new vessels by fusion and differentiation of stem cells; arteriogenesis which involves the increase in arterial diameter of pre-existing vessels and formation of collateral circulations.(114,115)

Stem cells secrete angiogenic and arteriogenic factors such as VEGF, bFGF, IL-6, HGF, ANG, placental growth factor (PLGF), among others, which have been implicated in neovascularisation.(106,116) Of these, VEGF and bFGF are the most potent angiogenic factors.(117) In addition to the aforementioned factors, EPCs express endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) and secrete nitric oxide (NO) which contribute to neovascularisation.(118)

1.6.3 Immunomodulation

Following ischaemic injury, changes in the microenvironment make it hostile to cells, limiting the engraftment of transplanted stem cells. The release of paracrine factors by stem cells can modulate inflammatory and remodelling processes associated with ischaemic injury establishing a more favourable microenvironment for transplantation.(7,106) This process is well demonstrated in MSCs which secrete various factors to modulate inflammatory cells, anti-fibrotic and anti-inflammatory effects.(119,120)

1.6.4 Stimulating/recruiting endogenous stem cells

Cell therapy has been suggested to mobilise and recruit endogenous and circulating stem/progenitor cells to the site of injury to mediate cardiac repair.(7,106) Studies have shown that transplantation of stem cells can stimulate, recruit and activate of endogenous CSC/CPCs.(121–123) ASCs are known to secrete stromal cell-derived factor 1 (SDF-1), a chemokine involved in mobilisation and recruitment of BMDCs into the peripheral blood with subsequent homing to the site of injury.(124)

1.6.5 Effect of hypoxic preconditioning

Hypoxic preconditioning can promote cell survival of stem cells, reduce apoptotic cell death and increase angiogenesis through upregulation of various paracrine factors through the

transcriptional activity of hypoxia inducible factor 1 (HIF-1).(109,125–127) HIF-1 increases the expression of various paracrine factors including VEGF, EPO, ANG, PDGF, and bFGF.(128–130)

HIF-1 is a transcription factor which plays an important role in the cellular response and adaptation to ischaemia/hypoxia.(130) HIF-1 is a heterodimer composed of an alpha and beta subunit. In normal oxygen conditions, proline residues on the hypoxia inducible factor 1 alpha (HIF-1α) are hydroxylated by HIF prolyl-hydroxylases utilising oxygen as a co-substrate with subsequent degradation by proteasomal pathways. In hypoxic conditions, HIF-1α is stabilised due to the lack of oxygen and the transcriptional action of HIF-1 on hypoxia response elements is increased which mediate cellular responses to hypoxia. HIF-1α can be stimulated by various hypoxia-independent pathways including the PI3K/AKT and PKCε signalling which may be of therapeutic relevance to stem cell therapy.(131,132)

1.7 Differences in right atrial appendage and left ventricle cardiac progenitor cells

Recently it was shown that CPCs derived from different sources of the heart from the same patients exhibited different functional and paracrine properties *in vitro*.(8) Conditioned media (CM) from LV CPCs demonstrated superior angiogenic effects on human umbilical vein endothelial cells (HUVEC), while CM from RAA CPCs demonstrated superior cardioprotective effects on HL-1 cardiomyocytes by reducing apoptosis and increasing proliferation.(8) CPCs used in this study represent a heterogeneous population of CPCs, predominantly expressing mesenchymal cell markers CD90 and CD105. Interestingly, LV CPCs showed a superior angiogenic effect compared to EPCs which are a leading cell source for neovascularisation.(8) The exact reason for these functional reasons are not known although may be due to the distinct functional and developmental characteristics, as well as the different microenvironments, of CPCs from different heart chambers.

Atrial and ventricular cardiomyocytes have functional differences, both in electrophysiological and contractile properties due to differences in gene expression, and morphological and molecular characteristics.(133) During cardiac development, heart chambers arise from distinct regions or cell populations known as the first and second heart fields. The right atrium develops from both the first and second heart fields, while the left ventricle (LV) develops from the first heart field.(92) The microenvironment, or niche, of stem cells influence the function and characteristics of stem cells.(134,135) Furthermore, the

ischaemic microenvironment may influence the epigenetic status of cells.(136,137) Therefore in patients with IHD, the LV which is often subject to the hostile ischemic microenvironment may influence the functional effects and paracrine profiles of LV CPCs through epigenetic modifications. As discussed, several mechanisms may underlie the functional differences between RAA CPCs and LV CPCs which warrants further investigation and may give insight to the therapeutic relevance of specific populations of CPCs isolated from different heart chambers.

1.8 Thesis rationale

The promise of cell therapy for regenerating the functional capacity of the diseased has been shown in various pre-clinical and clinical studies. Although many stem cell populations have been studied, the ideal stem cell has yet to be identified. Cardiac stem cells are involved in heart development, cell turnover and endogenous repair following injury to the heart hence are contenders as the ideal stem cell type. Furthermore, combination of different stem cells that have different reparative effects on the heart can be combined to further increase the efficacy of cell therapy. To date, there are no studies showing the combined effects of CPCs derived from different heart chambers of the same patient on cardiac repair.

As CPCs derived from the RAA and LV exhibit functional differences *in vitro*, combination of these cells may increase the potential for cardiac repair by complimentary or synergistic effects. The different profiles of paracrine factors secreted into the CM by RAA CPCs and LV CPCs may further synergise, stimulate and enhance the secretome of one another.

This study will evaluate the synergistic paracrine effects of a heterogeneous population of CPCs from the RAA and LV of the same patient on *in vitro* cardiac repair. RAA CPCs and LV CPCs will be cultured alone or in combination, in serum deprivation in normoxic and hypoxic conditions. CM collected following serum deprivation will be used for further *in vitro* experiments. The paracrine effects of CM on cardioprotection and angiogenesis will be measured to determine the synergistic paracrine effects of RAA CPCs and LV CPCs on cardiac repair.

1.8.1 Aims

- To determine if combination of RAA CPCs and LV CPCs provide synergistic paracrine effects to mediate cardioprotection and angiogenesis.
- To explore possible mechanisms for synergistic paracrine effects of combination of RAA CPCs and LV CPCs.

1.8.2 Hypotheses

- Combination of RAA CPCs and LV CPCs will have equal or superior paracrine effects through complimentary or synergistic effects compared to single cultures.
- Combination of RAA CPCs and LV CPCs may stimulate and enhance the paracrine signalling and effects by up-regulation of HIF-1.

2 Methods

2.1 Overview of methods

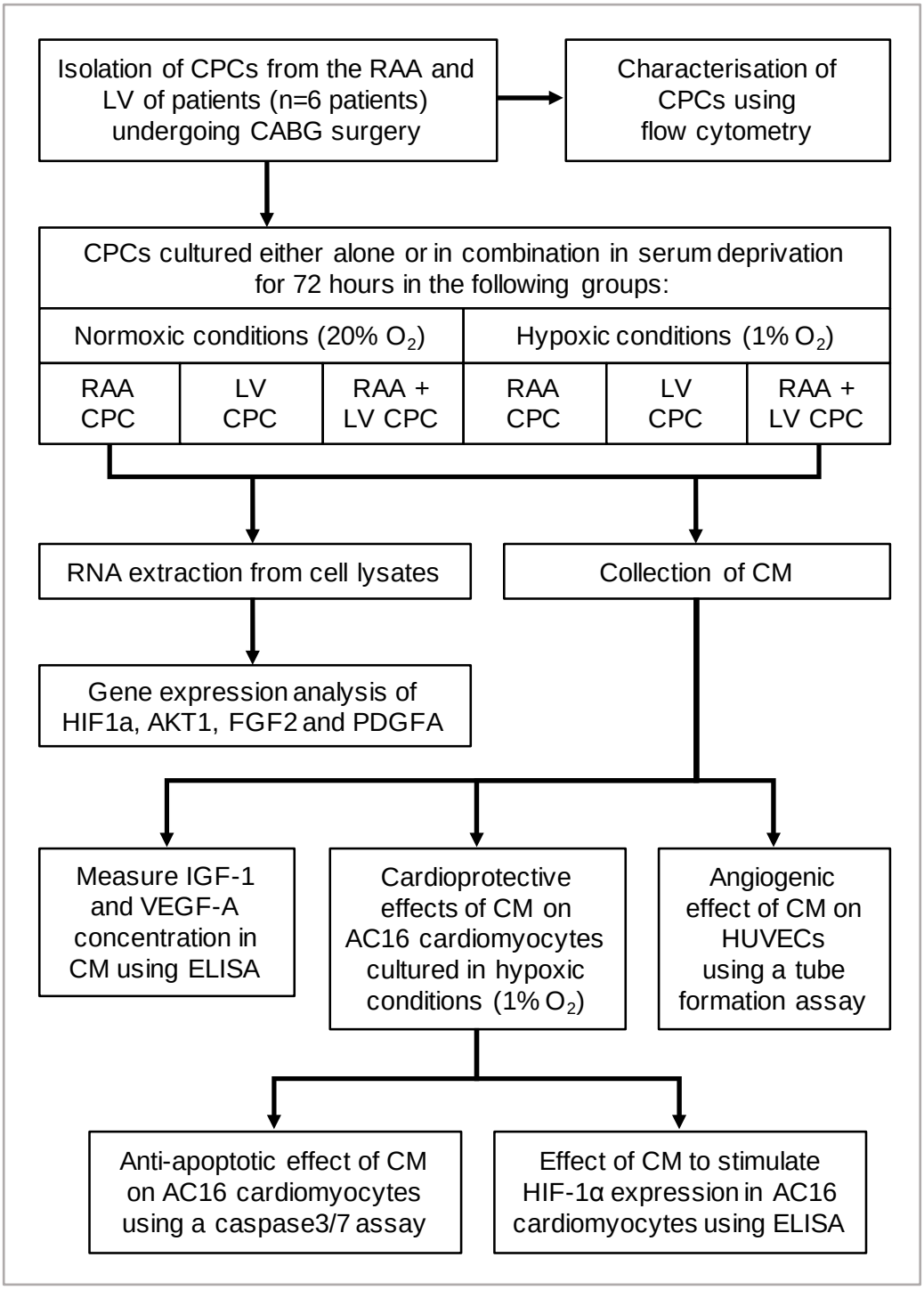
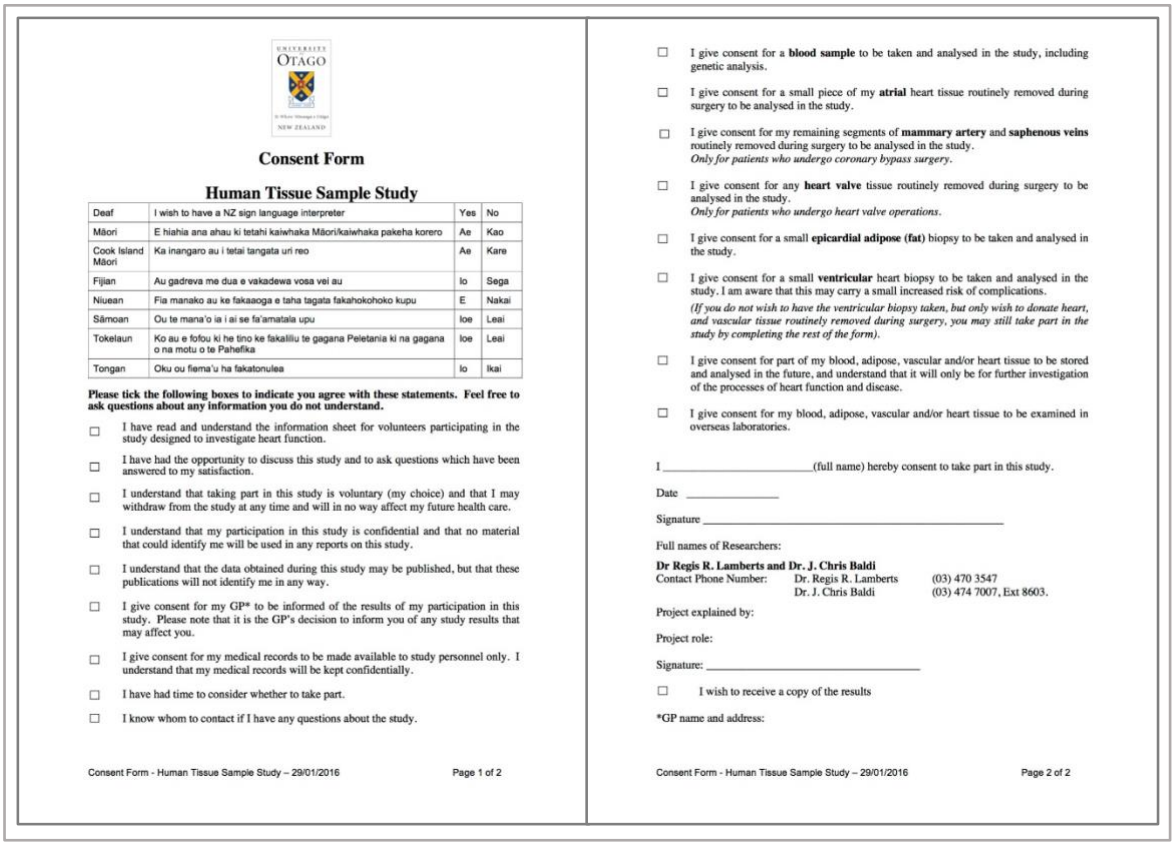


Figure 2-1: Experimental study design.
CPC, cardiac progenitor cell; RAA, right atrial appendage; LV, left ventricle; CABG, coronary artery bypass grafting; CM, conditioned media; ELISA, enzyme-linked immunosorbent assay.

2.2 Ethical approval

The Heart Tissue Sample Study was ethically approved by the Lower South Regional Ethic Committee (ethics reference number: LRS/12/01/001). Patients were informed and consented to the study prior to obtaining tissue sample (Figure 2-2).



Consent Form

Human Tissue Sample Study

Deaf	I wish to have a NZ sign language interpreter	Yes	No
Māori	E hiahia ana ahau ki tetahi kaiwhaka Māori/kaiwhaka pakeha korero	Ae	Kao
Cook Island Māori	Ka inangaro au i tetai tangata uri reo	Ae	Kare
Fijian	Au gadreva me dua e vakadewa vosa vei au	Io	Sega
Niuean	Fia manako au ke fakaaoga e taha tagata fakahokohoko kupu	E	Nakai
Sāmoan	Ou te mana'o ia i ai se fa'amatala upu	Ioe	Leai
Tokelaun	Ko au e fofou ki he tino ke fakaliliu te gagana Peletania ki na gagana o na motu o te Pahefika	Ioe	Leai
Tongan	Oku ou fiema'u ha fakatonulea	Io	Ikai

Please tick the following boxes to indicate you agree with these statements. Feel free to ask questions about any information you do not understand.

- ☐ I have read and understand the information sheet for volunteers participating in the study designed to investigate heart function.
- ☐ I have had the opportunity to discuss this study and to ask questions which have been answered to my satisfaction.
- ☐ I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time and will in no way affect my future health care.
- ☐ I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study.
- ☐ I understand that the data obtained during this study may be published, but that these publications will not identify me in any way.
- ☐ I give consent for my GP* to be informed of the results of my participation in this study. Please note that it is the GP's decision to inform you of any study results that may affect you.
- ☐ I give consent for my medical records to be made available to study personnel only. I understand that my medical records will be kept confidentially.
- ☐ I have had time to consider whether to take part.
- ☐ I know whom to contact if I have any questions about the study.

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- ☐ I give consent for a **blood sample** to be taken and analysed in the study, including genetic analysis.
- ☐ I give consent for a small piece of my **atrial** heart tissue routinely removed during surgery to be analysed in the study.
- ☐ I give consent for my remaining segments of **mammary artery** and **saphenous veins** routinely removed during surgery to be analysed in the study.
  Only for patients who undergo coronary bypass surgery.
- ☐ I give consent for any **heart valve** tissue routinely removed during surgery to be analysed in the study.
  Only for patients who undergo heart valve operations.
- ☐ I give consent for a small **epicardial adipose (fat)** biopsy to be taken and analysed in the study.
- ☐ I give consent for a small **ventricular** heart biopsy to be taken and analysed in the study. I am aware that this may carry a small increased risk of complications.
  (If you do not wish to have the ventricular biopsy taken, but only wish to donate heart, and vascular tissue routinely removed during surgery, you may still take part in the study by completing the rest of the form).
- ☐ I give consent for part of my blood, adipose, vascular and/or heart tissue to be stored and analysed in the future, and understand that it will only be for further investigation of the processes of heart function and disease.
- ☐ I give consent for my blood, adipose, vascular and/or heart tissue to be examined in overseas laboratories.

I _____(full name) hereby consent to take part in this study.

Date _____

Signature _____

Full names of Researchers:

Dr Regis R. Lamberts and Dr. J. Chris Baldi
Contact Phone Number: Dr. Regis R. Lamberts (03) 470 3547
Dr. J. Chris Baldi (03) 474 7007, Ext 8603.

Project explained by:

Project role:

Signature: _____

☐ I wish to receive a copy of the results

*GP name and address:

Consent Form - Human Tissue Sample Study – 29/01/2016 Page 2 of 2

Figure 2-2: Consent form patients signed prior to obtaining heart tissue samples.

2.3 Tissue collection

The RAA and LV were collected from patients (n=6) undergoing on-pump coronary artery bypass grafting (CABG) surgery at Dunedin Public Hospital. Patients with diabetes or undergoing adjunct valve replacement surgery were excluded from the study.

Cardiac tissue was collected in Krebs Ringer Henseleit (KRH) solution, then transferred to a tube containing Hank's balanced salt solution (HBSS) and kept on ice until cell isolation (see chapter 2.4.4).

2.4 Cell culture

All cell culture work was performed utilising aseptic technique in a laminar flow hood. Cells were checked daily by microscopic examination to determine confluency of the cells. Culture media was changed every 2-3 days or subcultured once confluent.

2.4.1 Cardiac progenitor cells

Cardiac progenitor cells were cultured in Ham's F12 complete media containing Ham's F12 nutrient mixture (Gibco, USA) supplemented with 10 ng/mL human basic fibroblast growth factor (Sigma-Aldrich, USA), 0.005 U/mL human erythropoietin (EPO) (Sigma-Aldrich, USA), 10% foetal bovine serum (FBS) (Sigma-Aldrich, USA), and 1X antibiotic-antimycotic (anti-anti) (Gibco, USA).

CPCs were cryopreserved using mesenchymal stem cell freezing media (Merck Millipore, USA).

CPCs at passage 3 to 5 were used for experiments.

2.4.2 AC16 cardiomyocytes

AC16 cardiomyocytes (Merck Millipore, USA) are a proliferating human cardiomyocyte cell line derived from adult human ventricular cardiomyocytes, which were fused with SV40 transformed, uridine auxotroph human fibroblasts devoid of mitochondrial DNA.(138)

AC16 cardiomyocytes were cultured in Dulbecco's modified eagle medium/F12 (DMEM/F12) complete media containing DMEM/F12 media (Gibco, USA) supplemented with 12.5% FBS (Sigma-Aldrich, USA), and 1X anti-anti (Gibco, USA), according to the manufacturer's protocol.

AC16 cardiomyocytes were cryopreserved using DMEM/F12 complete media supplemented with 10 % dimethyl sulfoxide (DMSO).

AC16 cardiomyocytes were used to determine the cardioprotective effects of CM. Cells from passage 22 to 23 were used for experiments (see chapter 2.9 and chapter 2.10).

2.4.3 Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (ATCC, USA) were cultured in endothelial growth medium 2 (EGM-2) (Lonza, USA) containing endothelial basal medium 2 (EBM-2) supplemented with EGM-2 SingleQuots (Lonza, USA) which includes human Epidermal Growth Factor (EGF), vascular endothelial growth factor (VEGF), R3-insulin-like growth

factor-1 (R3-IGF-1) ascorbic acid, hydrocortisone, human bFGF, heparin, FBS, and gentamicin/amphotericin B (GA).

HUVECs were frozen using EGM-2 complete media supplemented with 10% FBS (Sigma-Aldrich, USA), and 10% DMSO.

HUVECs at passage 4 and 5 were used for the tube formation assay to determine angiogenic effects of the CM (see chapter 2.11).

2.4.4 Isolation of cardiac progenitor cells

CPCs were isolated from the RAA and LV tissue through cell dissociation using collagenase II and subsequent seeding under specific culture conditions, as previously described.(8) Primary cells were not sorted and were selected based on specific culture conditions such as adherence to the culture dish in Ham's F12 complete media, hence, represent a heterogeneous population of CPCs from the RAA and LV sample. A minimum of 10 mg of tissue was needed to isolate CPCs. Heart tissue was transferred to a 10-cm dish. Any adipose tissue was removed using scissors. The tissue was then washed twice with HBSS. To determine the size of the culture vessel for plating the cells, tissue was weighed. HBSS was added and the tissue was minced into smaller pieces. The tissue was then placed into a 15-mL tube and allowed to settle on ice. The supernatant was aspirated and fresh HBSS was added. Once the tissue settled, the supernatant was aspirated. 7 mL of 0.04% collagenase II solution (kept at 37°C) was added and transferred to a T25 flask. The tissue was incubated in a shaker-incubator set at 160 RPM at 37°C for 60 minutes. Following incubation, the solution was transferred to a 15-mL tube and kept on ice to allow the tissue to settle. The supernatant was then collected in a fresh tube and kept on ice. 7 mL of fresh collagenase II solution (37°C) was added and mixed with the tissue by repetitive pipetting (x10) and allowed to settle on ice. The supernatant was collected once the tissue had settled and kept on ice.

All steps after tissue dissociation were performed under sterile conditions in a laminar flow hood. The collected supernatant was centrifuged at 300 RCF for 5 minutes. Following centrifugation, supernatant was aspirated, and the cell pellet was loosened by gently tapping the bottom of the tube. The cell pellet was re-suspended in 1 mL Ham's F12 complete media.

Due to the variation in size between RAA and LV samples, cells were seeded onto an appropriately sized plate or flask based on the weight of the tissue to minimise differences in initial seeding density (Table 2-1). Obtaining a sufficient number of viable cells based on the size of the tissue was reproducible, which has been previously validated in the Katare

laboratory.(8) The cells were then incubated in a 5% CO_2/95% air humidified incubator at 37°C for 24 hours. Following 24 hours, cells were supplemented with fresh culture media. Thereafter, culture media was changed every 2-3 days or subcultured when the cells become 90% confluent.

Table 2-1: Table showing the appropriately sized culture vessels used to initially seed cells obtained from the heart tissue.

Weight of the tissue (mg)	Culture dish	Total media volume
10-30	12-well plate	1 mL/well
30-60	6-well plate	2 mL/well
60-150	T25 flask	5 mL/flask
150-300	2 x T25 flask	

RAA CPCs and LV CPCs were subcultured at the same time and at the same seeding density to ensure that cells remained in the same passage. RAA CPCs and LV CPCs showed similar proliferation rates hence reached confluency at a similar time. Due to the limited starting cell number following isolation, cell number was increased during the first few passages by increasing the size of the culture dish between passages. From passage 2 onwards, all CPCs were seeded onto T25 flasks.

2.4.5 Initiation of frozen cells

To initiate frozen CPCs and AC16 cardiomyocytes, the following protocol was used. Cells were quickly thawed in the water bath at 37°C. Once cells were thawed, 1 mL of the cell solution was transferred to a 15-mL tube. 9 mL of complete media was added by slow pipetting to prevent osmotic shock. Cells were then centrifuged at 300 RCF for 5 minutes. The supernatant was aspirated. The tube was tapped, and the cells were resuspended in 1 mL complete media. The cell solution was transferred to the relevant culture vessel then incubated for 24 hours. Following 24 hours, cells were supplemented with fresh culture media.

2.4.6 Cryopreservation of cells

To freeze CPCs and AC16 cardiomyocytes, the following protocol was used. The cell solution was centrifuged at 300 RCF for 5 minutes following trypsinisation. Supernatant was aspirated, and the cell pellet was loosened by gently tapping the tube. Cells were resuspended in freezing media and transferred to a freezing vial. The vial was placed in a cell freezing

container in a -80°C freezer for 24 hours. Following 24 hours, CPCs were stored at -80°C, and AC16 cardiomyocytes in liquid nitrogen at -196°C.

2.4.7 Initiating HUVECs

HUVECs were initiated from frozen aliquots. Prior to thawing cells, 5 mL EGM-2 complete media was added to T25 flasks and placed in a 5% CO_2/95% air humidified incubator for 30 minutes. Cells were quickly thawed in a 37°C water bath. Once thawed, 1 mL of the cell solution was transferred to the T25 flask prepared prior to thawing. Cells were then incubated for 24 hours. Following 24 hours, cells were supplemented with fresh culture media.

2.4.8 Cryopreservation of HUVECs

The cell solution was centrifuged at 200 RCF for 5 minutes following trypsinisation. Supernatant was aspirated, and the cell pellet was loosened by gently tapping the tube. Cells were re-suspended in HUVEC freezing media ($5x10^5$ to $2x10^6$ cells/mL). The solution was transferred to a freezing vial and placed in a cell freezing container in a -80°C freezer for 24 hours and then stored in liquid nitrogen at -196°C.

2.4.9 Subculture

The subculturing protocol between CPCs, AC16 cardiomyocytes and HUVECs differed with respect to the centrifugation, confluency at end of passage, seeding density and the culture vessel that was used to culture the cells (Table 2-2).

Table 2-2: Table outlining differences in culture protocol of the different cell types.

Cell type	CPC	AC16 cardiomyocyte	HUVEC
Centrifugation	300 RCF for 5 minutes	300 RCF for 5 minutes	200 RCF for 5 minutes
Confluency at end of passage	90%	90%	80%
Seeding density/split ratio	4,000 cells/cm^2	Split ratio 1:6	3,000 cells/cm^2
Culture vessel	T25 flask	T75 flask	T25 flask
Total media volume	5 mL	10 mL	5mL

RCF, relative centrifugal force; HUVEC, human umbilical vein endothelial cell.

Once the cells reached confluency, cells were subcultured. Cells were washed with calcium and magnesium free phosphate buffered solution (PBS) (37°C) to remove any traces of serum,

calcium and magnesium which would inhibit the activity of the dissociation agent. PBS was pipetted carefully to the side of the culture vessel taking care not to disturb the cells, and the culture dish was gently rocked. PBS was then aspirated and discarded. TrypLE Express Enzyme (Gibco, USA) (80µL/cm^2 of the culture vessel), a cell dissociation agent, was added to detach the cells. The culture vessel was gently rocked to cover the cell layer and then incubated at 37°C for 5 minutes. The culture vessel was tapped on the side and examined using the light microscope for cell detachment. If cells were less than 90% detached, the culture vessel was incubated for a further 1 minute and re-examined for cell detachment. Once cells were detached, complete media was added at a ratio of 2:1 (complete media: TrypLE) and mixed gently by pipetting to neutralise TrypLE. The cell solution was transferred to a sterile tube and centrifuged. The supernatant was then aspirated. The cell pellet was then re-suspended in 1 mL of complete media. Cells were counted using a haemocytometer. 10 µL of cell solution and trypan blue at a 1:1 ratio was added to the coverslip and counted. The required number of cells were seeded on to a culture vessel and incubated in a 5% CO_2/95% air humidified incubator for 24 hours. After 24 hours, culture media was changed.

2.5 Characterisation of CPCs using flow cytometry

CPCs were characterised using flow cytometry for the expression of mesenchymal stem cell markers, CD90 (thymocyte differentiation antigen, Thy-1) and CD105 (endoglin, ENG), and the circulating haematopoietic progenitor cell marker CD34 as previously described.(8)

CD90 is a heavily glycosylated glycoprotein involved in inflammation, wound healing and cell to cell and cell to matrix adhesion. (139) CD105 is a glycoprotein which interacts with transforming growth factor beta (TGF-β) receptor complex. It is also involved in cytoskeletal organisation, cell morphology and migration, and the development of the cardiovascular system.(139) CD34 is a transmembrane phosphoglycoprotein which is thought to be involved in cell adhesion, differentiation and proliferation.(140)

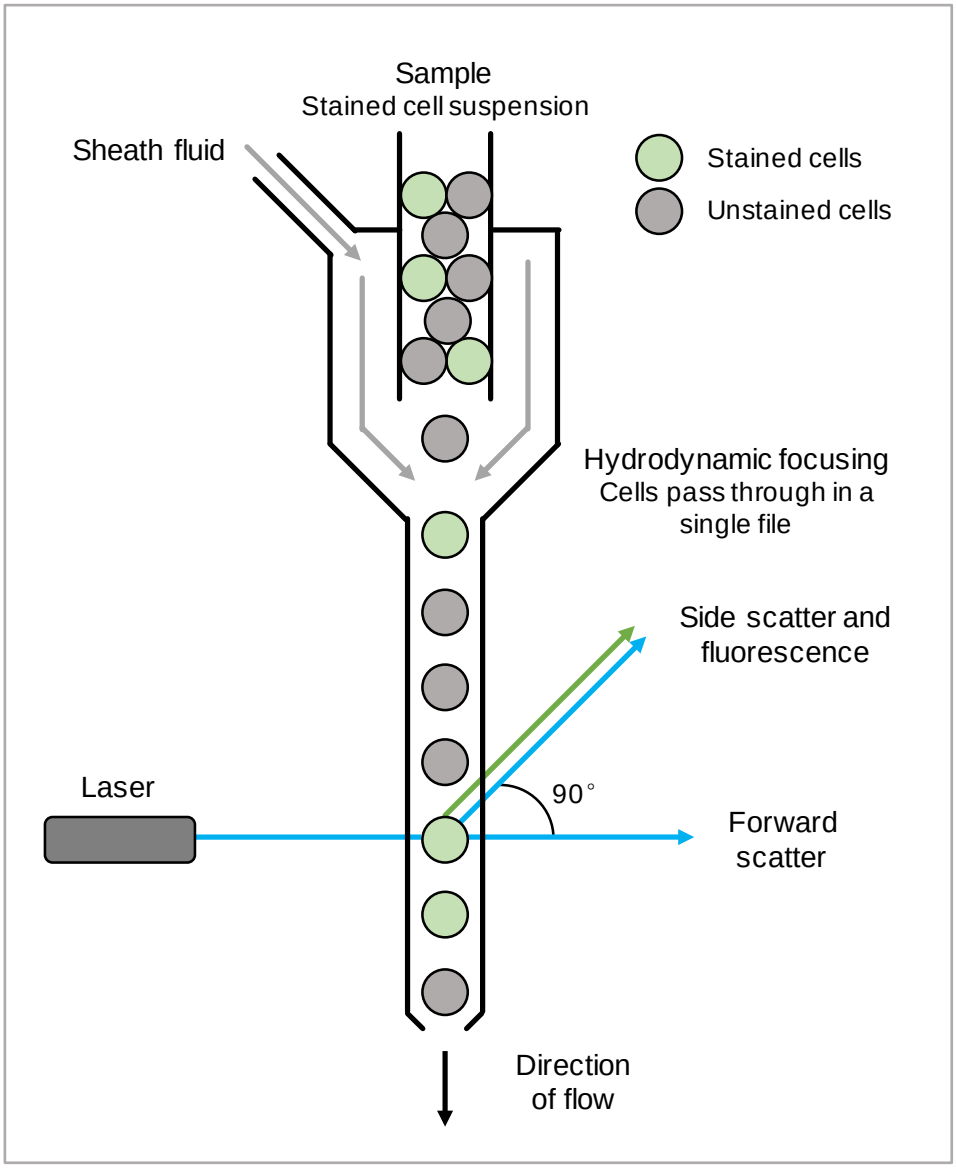

Figure 2-3: Diagram of the flow cytometer.
Cells pass through in a single file by hydrodynamic focusing by the surrounding sheath fluid. As stained cells pass through the laser, the light is scattered in the forward (forward scatter), side (side scatter) directions. The conjugated fluorophore becomes excited by the laser which emits fluorescent light which is detected by the fluorescent light channels.

Flow cytometry is a technique utilised for characterising cells by measuring the fluorescence emitted by fluorophores. Cells are stained with fluorophore-conjugated antibodies or ligands to specific cell markers which are then analysed using a flow cytometer. The flow cytometer allows single cell analysis by hydrodynamically focusing the cell suspension so that the cells pass through the laser one cell at a time (Figure 2-3). When the cell passes through the laser, light is scattered in various directions which is detected by various detectors known as phospho-multiplier tubes (PMT). The forward scatter (FSC) channel detects light scattered in the forward direction, while the side scatter (SSC) channels detect light scattered to the side

(90°). Forward scatter is proportional to cell size and side scatter is proportional to the granularity of the cell. Fluorophores re-emit fluorescent light when excited by a laser with the corresponding excitation wavelength, which is detected by fluorescent light (FL) channels. There are multiple FL channels which detect fluorescent light at different wavelengths. By utilising various fluorophores with different excitation and emission wavelengths, it is possible to analyse multiple cell markers on a per cell basis.

2.5.1 Compensation control

In multi-colour flow cytometry, the use of multiple fluorophores can cause detection of fluorescence outside of the respective FL channels. This is known as the spill-over effect. Therefore, single stained controls are used for compensating for these spill-over effects, controlling for the overlap between channels so that only the fluorophore of interest is detected in the respective channel.

Antibody capture (AbC) beads (Invitrogen, USA) were used for compensation. Positive beads bind to all isotypes of mouse antibody, while the negative beads have no capacity to bind antibodies. Therefore, the use of beads allows clear discrimination between positive and negative populations. For compensation of propidium iodide (PI) (Invitrogen, USA) for cell viability, dead and live cells were used.

2.5.2 Preparation of cells

Cells were subcultured (see chapter 2.4.9). The cell pellet was loosened by gently tapping the tube and resuspending in 1 mL of Ham's F12 complete media. Cells were counted using a haemocytometer. Following cell counting, cells were centrifuged at 300 RCF for 5 minutes. The supernatant was aspirated following centrifugation. The tube was gently tapped, and cells were resuspended in flow cytometry buffer consisting of HBSS supplemented with 3% FBS and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Cells were resuspended at a concentration of $6x10^5$ to $6x10^6$ cells/mL so that the final cell concentration for analysis was approximately $1x10^5$ to $1x10^6$ cells/mL (6-fold dilution). The ideal cell concentration for flow cytometry at data acquisition is $1x10^6$ cells/mL.

2.5.3 Preparation of samples for flow cytometry

Flow cytometry samples were prepared in 5 mL round bottom polystyrene tubes (Corning, USA), according to the tube design shown in the following table (Table 2-3).

Table 2-3: Table showing the tube design for flow cytometry.

Tube number	Tube name	Components
1	Comp CD90 FITC	AbC beads + FITC CD90
2	Comp CD105 PE-Cy7	AbC beads + PE-Cy7 CD105
3	Comp CD34 APC	AbC beads + APC CD34
4	Comp PI positive	Dead cells + PI
5	Comp PI negative	Live cells + PI
6	US1 RAA CPC	RAA CPC
7	FS1 RAA CPC	RAA CPC + antibody solution + PI
8	US2 LV CPC	LV CPC
9	FS2 LV CPC	LV CPC + antibody solution + PI
10	FMO FITC	Cells + CD105 PE-Cy7 + CD34 APC + PI
11	FMO PE-Cy7	Cells + CD90 FITC + + CD34 PE-Cy7 + PI
12	FMO APC	Cells + CD90 FITC + CD105 PE-Cy7 + PI
13	FMO PI	Cells + antibody solution + PI

Comp, compensation; FITC, fluorescein isothiocyanate; PE-Cy7, phycoerythrin-cyanine7; APC, allophycocyanin; PI, propidium iodide; FMO, fluorescence-minus-one control; AbC, antibody capture.

Fifty microlitres of the cell suspension ($3x10^5$ to $3x10^6$ cells) was added to the appropriate tubes. Fifty microlitres of flow cytometry buffer was then added to each tube apart from the fully stained (FS) tubes. Antibody solution was prepared by adding 2 µL each of CD90 FITC, CD105 PE-Cy7 and CD34 APC monoclonal antibody (eBioscience, USA) to 50 µL flow cytometry buffer per every tube that requires antibody solution. Fifty microlitres of the antibody solution was then added to the each of the FS tubes.

The fluorescence-minus-one (FMO) controls were prepared by adding 50 µL of the cell solution to the appropriate tubes. For the FMO PI tube, 50 µL of antibody solution was added. For the other FMO tubes, an additional 50 µL flow cytometry buffer and 2 µL of the appropriate antibodies was added.

For the compensation tubes, 1 drop of AbC beads (component A of anti-mouse AbC bead kit) was added. Thereafter, 2 µL of the appropriate antibody was then added to the compensation

tubes. For the PI compensation tubes, 50 µL of the cell solution and 50 µL of the flow cytometry buffer was added. The tubes were then incubated in the dark on ice for 30 minutes.

The tubes were then incubated in the dark on ice for 30 minutes. For the PI positive tube, cells were incubated in a -80°C freezer for 30 minutes and thawed at room temperature to acquire dead cells prior to the wash step.

To wash the tubes, 2 mL of flow cytometry buffer was added to the tubes containing cells and 3 mL PBS was added to the tubes containing AbC beads. Tubes were then centrifuged at 300 RCF at 4°C for 5 minutes. The supernatant was aspirated, and the wash step repeated for the tubes containing cells. One wash step is sufficient for the beads. Cells were then resuspended in 300 µL flow cytometry buffer and AbC beads resuspended in 300 µL PBS. One drop of negative beads (component B of anti-mouse AbC bead kit) were added to the tubes containing AbC beads. The samples were then vortexed briefly and stored on ice in the dark until data acquisition.

Prior to data acquisition, 0.6 µL of PI (1.0 mg/mL, 1.5 mM) was added to the relevant tubes (final concentration 3 µM) and incubated for 15 minutes.

2.5.4 Data acquisition

Samples were briefly vortexed and loaded on to the Gallios flow cytometer (Beckman Coulter, USA). The corresponding fluorescent light (FL) channels to the fluorophores were included (Table 2-4). Prior to data acquisition, PMT voltages and gain were set to ensure cell populations were not off-scale and there was adequate separation between positive and negative cell populations. A minimum of 10,000 events were acquired from the samples.

Table 2-4: Table showing relevant fluorescent light (FL) channels and their corresponding fluorophores.

FL channel	Fluorophore	Conjugated cell marker	Excitation wavelength (nm)	Emission wavelength (nm)
FL1	FITC	CD90	488	525/20 BP
FL3	Propidium iodide	Viability	488	620/30 BP
FL5	PE-Cy7	CD105	488	755 LP
FL6	APC	CD34	633	660/20 BP

FL, fluorescent light; BP, band pass; LP, long pass

2.5.5 Analysis of flow cytometry data

Data acquired was analysed using FlowJo v10.3 (FlowJo LLC, USA), a flow cytometry analysis software. Compensation controls were used to set up the compensation matrix and applied to all samples prior to gating.

To determine the boundary between positive and negative samples, a negative gating control was used. FMO controls are stained for all fluorophores except for one fluorophore hence can be used to demarcate negative populations. Unstained (US) samples were not used as a negative gating control due to poor alignment with FMO controls, especially in the CD105 PE-Cy7 (FL5) channel (Figure 2-4).

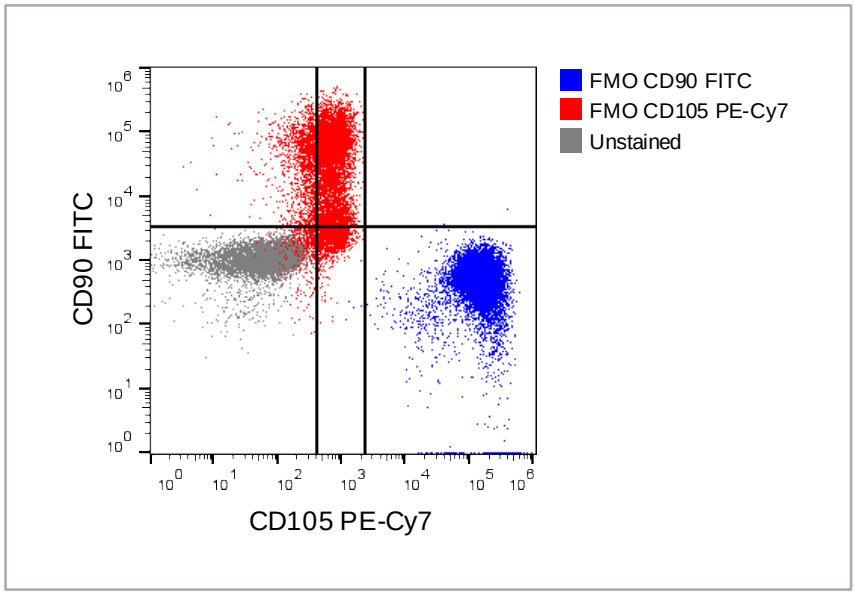

Figure 2-4: Representative bivariate dot plot comparing gates set using FMO controls and unstained samples.
FMO controls were used as the negative gating control due to better representation of the negative population. The gate set using the unstained sample has a lower threshold compared to the gate set by the FMO control for the CD105 PE-Cy7 channel.

CPCs were gated for size, single cells by excluding doublet populations, live cells and the expression of cell markers (Figure 2-5). Cells were gated for size by plotting FSC-area vs. SSC-area. Doublet populations were then excluded using FSC-area vs. FSC-height. Using the negative controls, gates were set for viability (viability PI, FL3 channel), CD34 (CD34 APC, FL6 channel), CD90 (CD90 FITC, FL1channel) and, CD105 (CD105 PE-Cy7, FL5 channel).

The gates set using the negative gating control were then applied to the FS samples. Following doublet exclusion, live cells (PI negative) were gated. Dead cells were excluded

from analysis as they non-specifically bind antibodies which can give false positive results. CD34- cells were then gated from the live cell population to exclude the population of haematopoietic cells. Finally, a bivariate gate was set for CD90 and CD105 from the CD34- population.

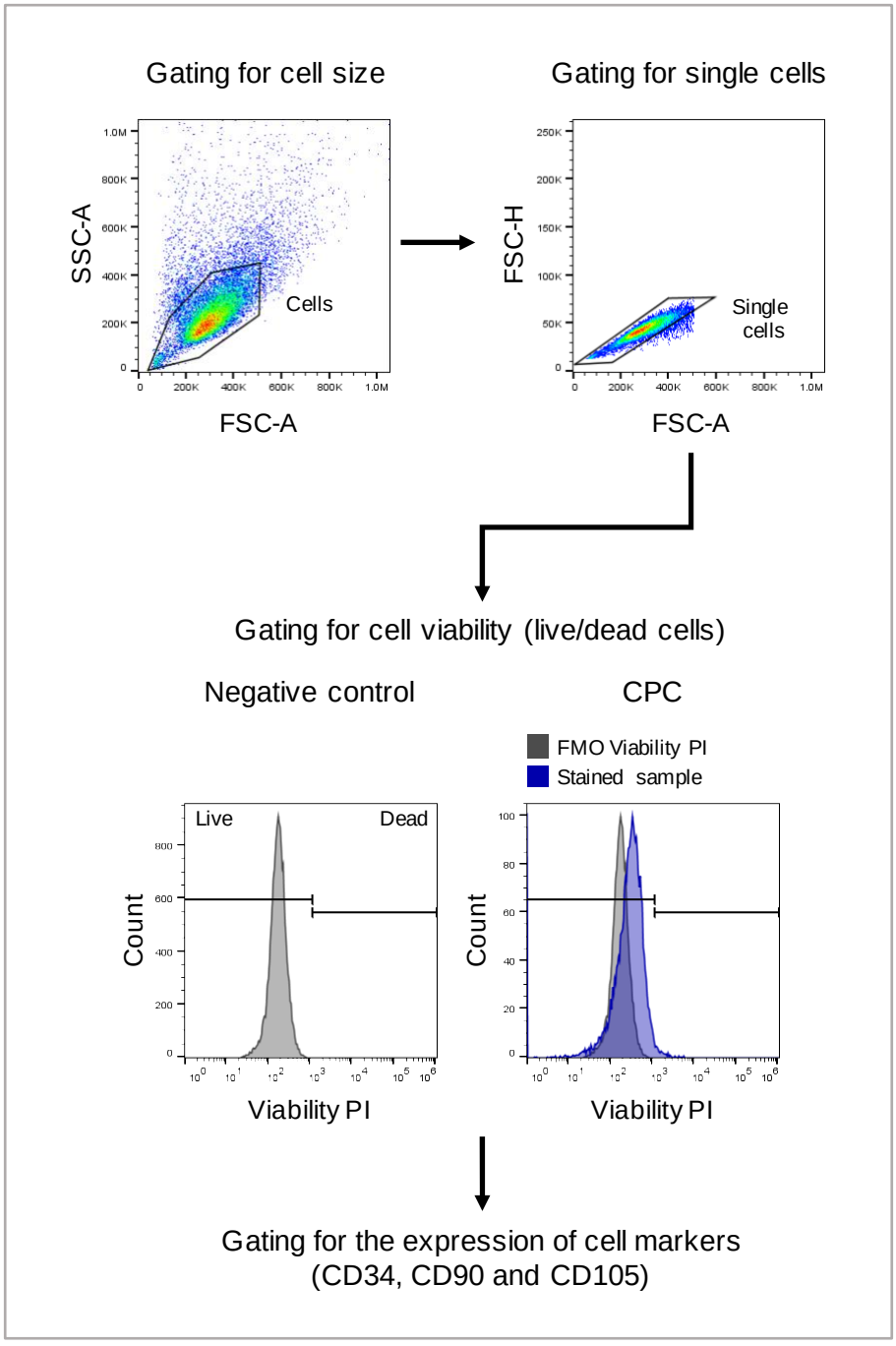

Figure 2-5: Gating protocol for CPCs.
CPCs were first gated for size; doublet populations were then excluded; and live cells were gated prior to analysing the expression of cell markers. RAA CPCs shown in the figure.

2.6 Exposure of serum deprived cardiac progenitor cells to normoxic and hypoxic conditions

RAA CPCs and LV CPCs were either seeded alone or in combination onto T25 flasks at a seeding density of 4,000 cells/cm^2. To mimic the ischaemic environments as in IHD, cells were cultured in serum deprivation in hypoxic conditions (Table 2-5). Normoxic conditions were used as a control.

Table 2-5: Table showing cell number used for serum-free treatment in normoxia and hypoxia.

Culture conditions	Cell groups	Number of cells/T25 flask
Normoxia (20% O_2)	RAA CPC	100,000 cells
	LV CPC	100,000 cells
	RAA and LV CPC (Combination)	50,000 RAA CPC + 50,000 LV CPC
Hypoxia (1% O_2)	RAA CPC	100,000 cells
	LV CPC	100,000 cells
	RAA and LV CPC (Combination)	50,000 RAA CPC + 50,000 LV CPC

Cells were cultured in a 5% CO_2/95% air humidified incubator at 37°C for 24 hours to allow cells to adhere. Following 24 hours, the culture media was changed to serum-free (SF) Ham's F12 media containing Ham's F12 nutrient mixture supplemented with 1X anti-anti. Cells in the normoxia group were cultured in 20% O_2/5% CO_2 humidified incubator for 72 hours while cells in the hypoxia group were cultured in 1% O_2/5% CO_2 humidified incubator for 72 hours.

2.6.1 Collection of conditioned media

Following the experimental period, culture media was collected in a 15-mL tube and centrifuged at 300 RCF for 5 minutes. The cells were washed with PBS and the culture vessel was kept on ice for later ribonucleic acid (RNA) extraction (see chapter 2.6.2). The supernatant (conditioned media, CM) was collected, without disturbing the cell pellet, and aliquoted into sterile tubes. The CM was stored in a -80°C freezer for further experiments.

2.6.2 Collection of lysate for RNA extraction

Following the collection of the CM, RNA was extracted from the cells. 700 µL of TRIzol lysis reagent (Invitrogen, USA) was added to the cells and rocked gently to cover the vessel surface. Cells were then disrupted and homogenised. The lysate was transferred to a microcentrifuge tube and stored in a -80°C freezer until RNA isolation (see chapter 2.7.1).

2.7 Gene expression analysis in cardiac progenitor cells

The mRNA expression of *HIF1A, AKT1, FGF2* and *PDGFA* which encode for the proteins HIF-1α, AKT1, bFGF and PDGF-A respectively. HIF-1α and AKT1 are involved in cell signalling pathways in cardioprotection, angiogenesis and differentiation. bFGF and PDGF-A are secreted by CPCs to mediate their therapeutic effects for cardiac repair.(7)

2.7.1 RNA isolation

RNA was extracted from the lysate prepared after CPCs were subjected to serum deprived conditions in normoxia and hypoxia (see chapter 2.6.2) according to the established protocol in the Katare laboratory. RNA extraction was performed in a fume hood.

The lysates were thawed and incubated at room temperature to allow complete dissociation of the nucleoproteins complex. Thereafter, 140 µL of chloroform was added to the samples. The samples were mixed and incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000 RCF for 15 minutes at 4°C for phase separation. After centrifugation, the mixture separates into an upper colourless aqueous phase, middle interphase and lower red phenol-chloroform phase. The upper aqueous phase containing RNA is transferred to a new microcentrifuge tube, taking care not to disturb other layers.

RNA in the aqueous phase was then precipitated by addition of 350 µL of isopropanol and incubating at room temperature for 10 minutes. The sample was then centrifuged at 12,000 RCF for 10 minutes at 4°C. The resulting RNA pellet was washed twice with 700 µL of 75% ethanol. Following a brief vortex, the RNA containing tube was centrifuged at 7,500 RCF for 5 minutes at 4°C. The supernatant was removed, and the resulting RNA pellet was air dried for 10 to 30 minutes, taking care not to fully dry the pellet. Once the RNA pellet was dried, 25 µL RNase-free water was added, the tube tapped to resuspend the pellet. The tube was briefly centrifuged then incubated on a heat block set at 55-60°C for 15 minutes to allow the pellet to completely dissolve.

RNA concentration and A260/A280 (≥1.8) were measured by loading 2 µL of the RNA solution onto a MaestroNano spectrophotometer (Maestrogen, Taiwan). The RNA sample was then stored at -80°C.

2.7.2 Reverse transcription

RNA samples were thawed on ice. RNA concentration was measured and diluted to a concentration of 100 ng/µL in nuclease free water. Recombinant DNase (rDNase) (Macherey-Nagel, Germany) was added to 20 µL of the diluted RNA sample. The sample was then incubated at room temperature for 15 minutes. Following incubation, rDNase was heat inactivated by incubating the samples on a heat block set at 75°C for 10 minutes. RNA concentration was measured following DNase treatment.

The reverse transcription (RT) was performed to synthesise cDNA using Primescript RT reagent kit (Takara, Japan) according to the manufacturer's protocol. RNA was added to the RT master mix consisting of primescript buffer, primescript enzyme mix, oligo dT primer, random hexamers and nuclease free water in 8 strip tubes (Table 2-6). Nuclease free water was added to the RT master mix as a no reverse transcriptase control (NRT). Tubes were then loaded into a thermal cycler, incubated at 37°C for 15 minutes and then heated to 85°C for 5 seconds to heat inactivate the reverse transcriptase. Samples were cooled to 4 °C and stored at -20°C overnight.

Table 2-6: Final concentration and volume of reagents used for the RT mix for reverse transcription.

Reagents	Final concentration	Volume/reaction
PrimeScript buffer (5X)	1X	2 µL
PrimeScript enzyme mix		0.5 µL
Oligo dT primer (50 µM)	25 pmol	0.5 µL
Random 6 mers (100 µM)	50 pmol	0.5 µL
Total RNA	250 ng/10 µL	250 ng
Nuclease free water		Variable
Total volume		10 µL

2.7.3 Real time polymerase chain reaction

The following day, the synthesised cDNA was diluted 1:1 in nuclease-free water. The polymerase chain reaction (PCR) master mix (Takara, Japan) was then prepared according to the manufacturer's protocol (Table 2-7). PCR primers (Sigma-Aldrich, USA) were reconstituted in Tris-EDTA (TE) buffer. PCR primers have been tested and validated by the manufacturer. 18S was used as a reference gene and was added for every plate used.

Table 2-7: Final concentration and volume of reagents used for the PCR master mix for RT-PCR.

Reagent	Final concentration	Volume/ reaction
SYBR Premix Ex Taq (2X)	1X	5 µL
PCR forward primer (10 µM)	0.2 µM	0.2 µL
PCR reverse primer (10 µM)	0.2 µM	0.2 µL
Nuclease free water		2.6 µL
Diluted cDNA		2 µL
Total		10 µL

Two microlitres of diluted cDNA was aliquoted into a 96 well PCR plate (triplicate/gene). For the negative controls, NRT was aliquoted and nuclease free water was aliquoted as a no template control (NTC). Thereafter, 8 µL of PCR master mix was aliquoted into each well. The plate was then vortexed briefly and loaded onto a PCR thermal cycler. The initial denaturation step was set at 95°C for 30 seconds. Following denaturation, PCR was performed at 95°C for 5 seconds and 60°C for 30 seconds for 40 cycles. The cycle threshold (CT) values were determined using the Step One plus system software.

The fold changes in mRNA expression was calculated manually relative to the RAA normoxia group using the $2^{-\Delta\Delta CT}$ method.(141) ΔCT was calculated by subtracting the mean CT of the reference gene (18S) from each of the mean CT values of the target genes of interest. ΔΔCT was then calculated relative to the RAA normoxia group by subtracting the ΔCT value of RAA normoxia from all the other CPC groups within the gene of interest. Finally, the fold changes in gene expression were calculated by exponentiation of 2 to the -ΔΔCT ($2^{-\Delta\Delta CT}$).

2.8 Enzyme-linked immunosorbent assay (ELISA) for quantification of growth factors in CM

IGF-1 and VEGF-A in the CM were quantified using a commercially available ELISA kit (Elisakit.com, Australia) according to the manufacturer's protocol. Each ELISA kit contains a strip well plate pre-coated with capture antibody, lyophilised protein standard, wash buffer, biotin-labelled detection antibody, streptavidin-horseradish peroxidase (HRP) conjugate, tetramethylbenzidine (TMB) substrate and stop solution.

IGF-1 and VEGF-A are important paracrine factors secreted by stem cells which exert cardioprotective and angiogenic effects respectively.(7)

Lower limit of quantification (LLQ) for both assays were <5 pg/mL (determined by adding 3 standard deviations to the mean optical density (OD) of 5 zero antigen replicates).

2.8.1 Measurement of IGF-1 and VEGF-A

The CM stored in the -80°C freezer was thawed on ice prior to performing the assay.

IGF-1 protein standards were prepared in duplicates ranging from 5000 pg/mL to 78.125 pg/mL in a 2-fold serial dilution in Ham's F12 basal media. VEGF-A protein standards were prepared in duplicates ranging from 2000 pg/mL to 31.25 pg/mL in a 2-fold serial dilution in Ham's F12 basal media.

One hundred microlitres of the standards, zero standard (Ham's F12 basal media) and CM were added to the pre-coated plates in duplicates, sealed and incubated at room temperature for 2 hours. Following incubation, well contents were discarded, and the plate washed 4 times with 250 µL wash buffer per well. Residual wash buffer was removed between each wash step by inverting and taping the plate on paper towels. One hundred microlitres of biotin-labelled detection antibody (200 ng/mL) was added to each well. The plate was sealed and incubated at room temperature for 1 hour. The well contents were then discarded and washed as above. Next, 100 µL of freshly diluted streptavidin-HRP conjugate (500-fold dilution) was added to each well. The plate was then sealed and incubated for 45 minutes. Following incubation, the wells were washed 5 times with wash buffer.

Subsequent steps were performed protected from light. After washing, 100 µL of TMB substrate was added to each well and incubated in the dark for approximately 15 minutes. The development process of the plate (blue colour) was checked every 5 minutes to prevent over development of the plate. To reaction was stopped by the addition of 50 µL of the stop

solution to each well resulting in a colour change from blue to yellow. Finally, OD/absorbance of the plate was measured using a microplate reader set at 450 nm.

For calculation of the results, first the mean of the zero standards was subtracted from the standards and samples. Using GraphPad Prism 7, a standard curve was generated by plotting OD on the y-axis versus log of the standard concentrations on the x-axis. Standard concentrations were converted to base 10 logarithm (Log). A 4-parameter logistic curve was fitted to the standards (R^2 >0.99). The Log(Concentration) of the samples were then interpolated from the standard curve. The concentration of the samples was then calculated by exponentiation of 10 to the Log(Concentration) ($10^{Log(Concentration)}$).

2.9 Effect of CM on apoptosis in cardiomyocytes cultured under hypoxic conditions

The effects of the CM on apoptosis of AC16 cardiomyocytes treated under hypoxic condition was measured using a Caspase-Glo 3/7 assay as previously described.(8) Caspases are a group of proteases which play an important role in various cellular processes involving programmed cell death and inflammation. Caspase -3 and -7 are known as executioner caspases which execute the cellular responses leading to apoptosis.

2.9.1 Caspase-Glo 3/7 and CyQUANT proliferation multiplexed assay

Caspase-Glo 3/7 assay (Promega, USA) was used to quantify caspase -3/-7 activity following culture of AC16 cardiomyocytes in hypoxia for 72 hours. The assay contains a luminogenic substrate with the tetrapeptide sequence DEVD (Asp-Glu-Val-Asp). DEVD is cleaved by caspase 3/7 and aminoluciferin, a substrate for luciferase is released. This allows for the luciferase reaction to occur resulting in the production of light/luminescence.

Basal caspase 3/7 are expressed in cells under normal culture conditions and is proportional to cell number. Therefore, caspase activity was normalised to cell number by multiplexing the caspase assay with a CyQUANT cell proliferation assay (Invitrogen, USA).

For this assay, AC16 cardiomyocytes were seeded onto a 96-well plate at 3,000 cells/well in triplicates and supplemented with 100 µL DMEM/F12 complete media and cultured for 24 hours. After confirming that the cells were attached to the surface, culture media was replaced with new media consisting of 50 µL of CM and 50 µL of SF Ham's F12. Negative control wells were supplemented with 100 µL SF Ham's F12. Cells were then incubated in hypoxia (1% O_2) for 72 hours.

At the end of the experimental period, culture media was removed and 25 µL of PBS was added. Next, 25 µL of caspase reagent (reconstituted according to the manufacturers' protocol) was added to each well. In addition, 25 µL PBS and 25 µL caspase reagent were added to empty wells to be used as blank measurements. Contents of the well were gently mixed on a plate shaker set at 250 RPM for 1 minute. The plate was then incubated at room temperature for 30 to 60 minutes. Following incubation, luminescence was measured using a plate reader.

Following the caspase assay, CyQUANT reagent was prepared at a concentration of 2X and 50 µL was added to each well for a final concentration of 1X. The plate was then incubated at room temperature in the dark for 10 minutes. Following incubation, the fluorescence was measured using a plate reader set at excitation at 480 nm and emission at 520 nm.

Caspase activity (relative luminescent units, RLU) was then normalised to cell number (relative fluorescence units, RFU) (caspase activity/cell number) prior to analysis.

2.10 HIF-1α protein expression in AC16 cardiomyocytes exposed to hypoxia following treatment with CM

2.10.1 Protein extraction from AC16 cardiomyocytes

AC16 cardiomyocytes were plated onto 6-well plates at $5x10^5$ cells/well in triplicates in 1000 µL DMEM/F12 complete media and cultured for 24 hours. After confirming that the cells were attached to the surface, culture media was replaced with new media consisting of 500 µL of CM and 500 µL of SF Ham's F12. Negative control wells were supplemented with 1000 µL SF Ham's F12. Cells were then exposed to hypoxia (1% O_2) for 72 hours.

At the end of the experimental period, protein was extracted from the cells using a cell extraction buffer PTR (HIF1a Human SimpleStep ELISA kit, Abcam, UK) according to the manufacturer's protocol. In brief, culture media was removed, and the cells were washed twice with ice-cold PBS. Cells were then homogenised with 100 µL of ice-cold cell lysis buffer and the lysate was collected and incubated on ice for 15 minutes. The lysate was then centrifuged at 18,000 RCF for 20 minutes at 4°C. The supernatant was transferred to fresh tubes and the pellet discarded. Samples were stored at -80°C until further analysis.

2.10.2 Measuring of protein concentration using Bradford assay

On the day of the ELISA, protein samples were thawed and kept on ice. Protein standards were prepared using bovine serum albumin (BSA) ranging from 1.25 to 25 μg/mL diluted with the Bradford reagent (Bio-Rad, USA). Bradford reagent was used as a blank. Each sample was diluted in Bradford reagent (400-fold dilution). Standards and samples were then briefly mixed by inversion. Next, 100 μL of the standards, blanks and samples were then added to a microplate in triplicates. Absorbance/OD was read using a microplate reader at 595 nm.

The mean of the zero standards was subtracted from the standards and samples. Using GraphPad Prism 7, a standard curve was generated by plotting OD on the y-axis versus standard concentrations on the x-axis. A linear regression was fitted to the standards ($R > 0.99$). The sample concentrations were interpolated from the linear regression. The concentrations were then corrected for the dilution factor. The samples were then diluted to a concentration of 200 μg/mL in cell extraction buffer PTR.

2.10.3 Enzyme linked immunosorbent assay of HIF-1α alpha extracted from AC16 cardiomyocytes

HIF-1α was measured using HIF1a Human SimpleStep ELISA kit (Abcam, UK) according to the manufacturer's protocol.

Standards were prepared from 15 ng/mL to 0.23 ng/mL in a 2-fold serial dilution. Cell extraction buffer was used as a blank. Fifty microlitres of standards, blanks and samples were added to the wells of the ELISA plate in duplicates. Fifty microlitres of antibody solution was then added to each of the wells. The plate was sealed and incubated in a plate shaker set at 400 RPM at room temperature for 1 hour. Following incubation, the well contents were discarded. Wells were washed three times with 350 μL wash buffer PT. Residual wash buffer was removed between each wash step and after the last wash step by inverting and tapping the plate on paper towels.

Subsequent steps were performed protected from light. One hundred microliters of TMB substrate was added to each well and incubated on a plate shaker set at 400 RPM at room temperature for 10 minutes. To stop the reaction, 100 μL of the stop solution was added to each well resulting in a colour change from blue to yellow. Optical density (OD)/absorbance of the plate was measured using a microplate reader set at 450 nm.

Concentration was calculated as previously explained (see chapter 2.8.1). Sample concentrations were then corrected to ng/mL/100 μg of protein.

2.11 Angiogenic effects of CM on HUVECs using a tube formation assay

The angiogenic potential of the CM were measured in HUVECs using a tube formation assay. HUVECs migrate and differentiate to form capillary-like structures which indicates the level/efficacy of angiogenesis following the treatment of interest.

Prior to the assay, matrigel matrix (Corning, USA) and CM were thawed overnight at 4°C. At least 40 minutes prior to the assay, 50 μL of matrigel matrix was added to wells of a 96-well plate. Both the 96-well plate and pipette tips were cooled to 4°C to prevent gelling of the matrix. Caution was taken not to introduce bubbles when adding matrix to the wells as this may disrupt the tube formation. The plate was then incubated at room temperature for 10 minutes and then incubated at 37°C for 30-60 minutes.

HUVECs were resuspended in 1 mL SF EBM-2 media containing EBM-2 basal media (Lonza, USA) supplemented with 1X anti-anti (Gibco, USA) and counted using a haemocytometer. HUVECs were then seeded onto the matrigel-coated wells in triplicates (18,000 cells per well in 50 μL of SF EBM-2). Thereafter, 50 μL of CM was added to the treatment wells. For the negative control, an additional 50 μL of SF EBM-2 was added. For the positive control, HUVECs in 100 μL EGM-2 complete media were seeded in triplicates (18,000 cells per well). The plate was then incubated in a 5% CO_2/95% air humidified incubator at 37°C for 12 hours.

At the end of the experimental period, phase contrast images at 4X magnification were taken using a microscope (Olympus, Japan) fitted with a microscope camera (Lumenera, USA). Images were then analysed using the software Fiji using the angiogenesis analyser plugin to quantify total tube length.(142)

2.12 Statistical analysis

Differences in cell marker expression between RAA CPCs and LV CPCs were compared using paired t-tests.

IGF-1 and VEGF-A concentrations were normalised to a control group (RAA normoxic CM) to account for interpatient variability and allow comparison of the fold changes between the different groups in the same patient. The normalised data was log distributed. Hence,

statistical analysis was performed on the $\log_{10}$-transformed data. Two-way analysis of variance (ANOVA) was used for statistical analysis, followed by a Tukey post-hoc test.

Gene expression data were normalised to RAA normoxia. Two-way ANOVA using $\log_{10}$-transformed data was used to compare between the different groups, followed by a Turkey's post hoc test.

Caspase/CyQUANT data was normalised to the SF Ham's F12 (SF F12) group. One-way ANOVA was performed on the $\log_{10}$-transformed data to compare the CM-treated groups against the control SF F12, followed by Dunnett's post hoc test. Differences between the CM-treated groups were compared using two-way ANOVA, followed by a Turkey's post hoc test.

Differences in HIF-1α protein expression in the CM-treated groups were compared against the negative control SF F12 using one-way ANOVA followed by a Dunnett's post hoc test. Differences between CM-treated groups were compared using two-way ANOVA followed by a Turkey's post hoc test.

Differences in the total tube length from the tube formation assay of the CM-treated groups were compared against the negative control SF EBM-2 and positive control EGM-2 complete (EGM-2 COM) using one-way ANOVA followed by a Dunnett's post hoc test. Differences between CM-treated groups were compared using two-way ANOVA followed by a Turkey's post hoc test.

Data represented as mean ± standard error of mean (SEM). Differences with a p-value ≤ 0.05 were considered statistically significant.

3 Results

3.1 Patient characteristics

The medical history and echocardiographic reports were collected from all patients prior to surgery (Table 3-1). Information such as age, sex, medical history and echocardiogram report were available for each patient. All parameters were comparable across all the patients, except one patient had a reduced ejection fraction (<50 %) (patient number 237).

Table 3-1: Table showing the characteristics from patients where heart tissue was collected and used to isolate CPCs.

Patient number	Age	Sex	BMI	Diabetes	Hypertension	Ejection fraction (%)
237	61	M	30.9	N	Y	44
421	65	M	28.9	N	Y	58.5
436	62	M	27.1	N	Y	53
493	64	F	26.4	N	Y	>60
550	64	M	23.7	N	N	50.5
552	57	M	22.4	N	N	>60

BMI, body mass index.

3.2 Comparable expression of cell surface markers between RAA CPCs and LV CPCs

CPCs were characterised for the expression of CD34, CD90 and CD105. The expression of cell markers was comparable between RAA CPCs and LV CPCs and there were no statistical differences.

Majority of the cells were negative for CD34 population, although due the ischaemic nature of the tissue collected they still expressed CD34 positivity which was especially more in LV CPCs (80.9 ± 5.2% in RAA CPCs vs. 72.4 ± 8.3% in LV CPCs, p=0.298, n=4 patients, Figure 3-1 A and C). This suggests that there are cells that express CD34 within the CPC population, most likely due to their heterogeneity.

CD34 negative population were then used to measure the expression of MSC markers, CD90 and CD105. CD90 expression was higher in RAA CPCs compared to LV CPCs, but this was not statistically significant (72.4 ± 7.1% in RAA CPCs vs. 48.5 ± 17.0% in LV CPCs, p=0.277, n=4 patients, Figure 3-1 B and C). Interestingly, a previous study from our laboratory showed increased expression of CD90 in RAA CPCs.(8) The difference in the current study is possibly due to the smaller sample size due to limited time available for this project. CD105 expression was high in both RAA CPCs and LV CPCs, with nearly all cells expressing the cell marker (99.6 ± 0.12% in RAA CPCs vs. 99.5 ± 0.08% in LV CPCs, p=0.277, n=4 patients, Figure 3-1 B and C). Due to the high expression of CD105, the CD90+CD105+ cells were almost entirely dependent on CD90 expression (72.2 ± 7.1% in RAA CPCs vs. 48.3 ± 16.9% in LV CPCs, p=0.277, n=4 patients, Figure 3-1 B and C).

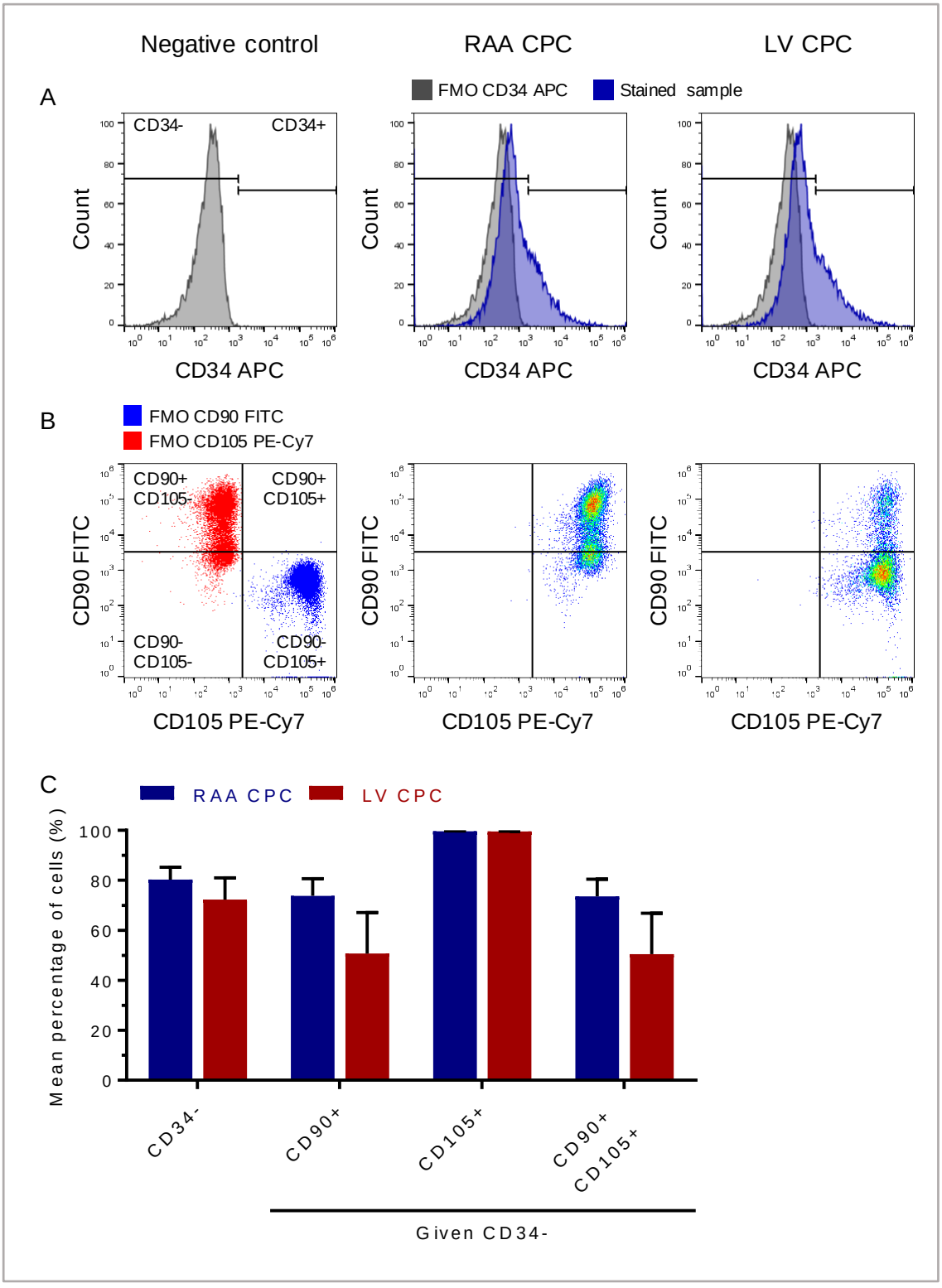

Figure 3-1: Immunophenotype of RAA CPCs and LV CPCs.
A. Representative histograms showing the expression of CD34 (negative control, RAA CPC and LV CPC from left to right). B. Representative bivariate dot plots showing the expression of CD90 (y axis) and CD105 (x axis) within the CD34- population (negative control, RAA CPC and LV CPC from left to right). C. Quantitative bar graph showing the expression of CD34 negative population; CD90+, CD105+ and CD90+CD105+ populations (given CD34-) of RAA CPCs and LV CPCs (n=4 patients). Data represented as mean ± SEM %. Differences between RAA CPCs and LV CPCs were compared using paired t-tests.

3.3 Gene expression following serum deprivation in normoxia and hypoxia

The mRNA expression of *HIF1A*, *AKT1*, *FGF2* and *PDGFA* were measured following serum deprivation of CPCs cultured in normoxia and hypoxia.

3.3.1 Decreased *HIF1A* mRNA expression in hypoxia

Exposure of CPCs to hypoxia reduced the expression of *HIF1A* although the only significant difference was observed in the RAA+LV CPCs cultured in hypoxia when compared to RAA normoxia, LV normoxia and RAA + LV normoxia (0.42 ± 0.17 –fold in RAA + LV hypoxia; vs. 1.0 ± 0 –fold in RAA normoxia, p =0.0357; vs. 1.1 ± 0.17 –fold in LV normoxia, p=0.0272; vs. 1.15 ± 0.36 –fold in RAA + LV normoxia, p=0.0387; n=5 patients, Figure 3-2 A). Other groups failed to exhibit any significant difference.

Interestingly, comparison of *HIF1A* mRNA expression irrespective of the groups showed a significant reduction of *HIF1A* in hypoxia (normoxia vs. hypoxia, p=0.0002, n=5 patients, 3 groups, Figure 3-2 B).

3.3.2 Comparable *AKT1*, *FGF2* and *PDGFA* mRNA expression in CPCs

There were no statistically significant differences in mRNA expression of *AKT1*, *FGF2* and *PDGFA* between any of the CPC groups (Figure 3-2 C-E).

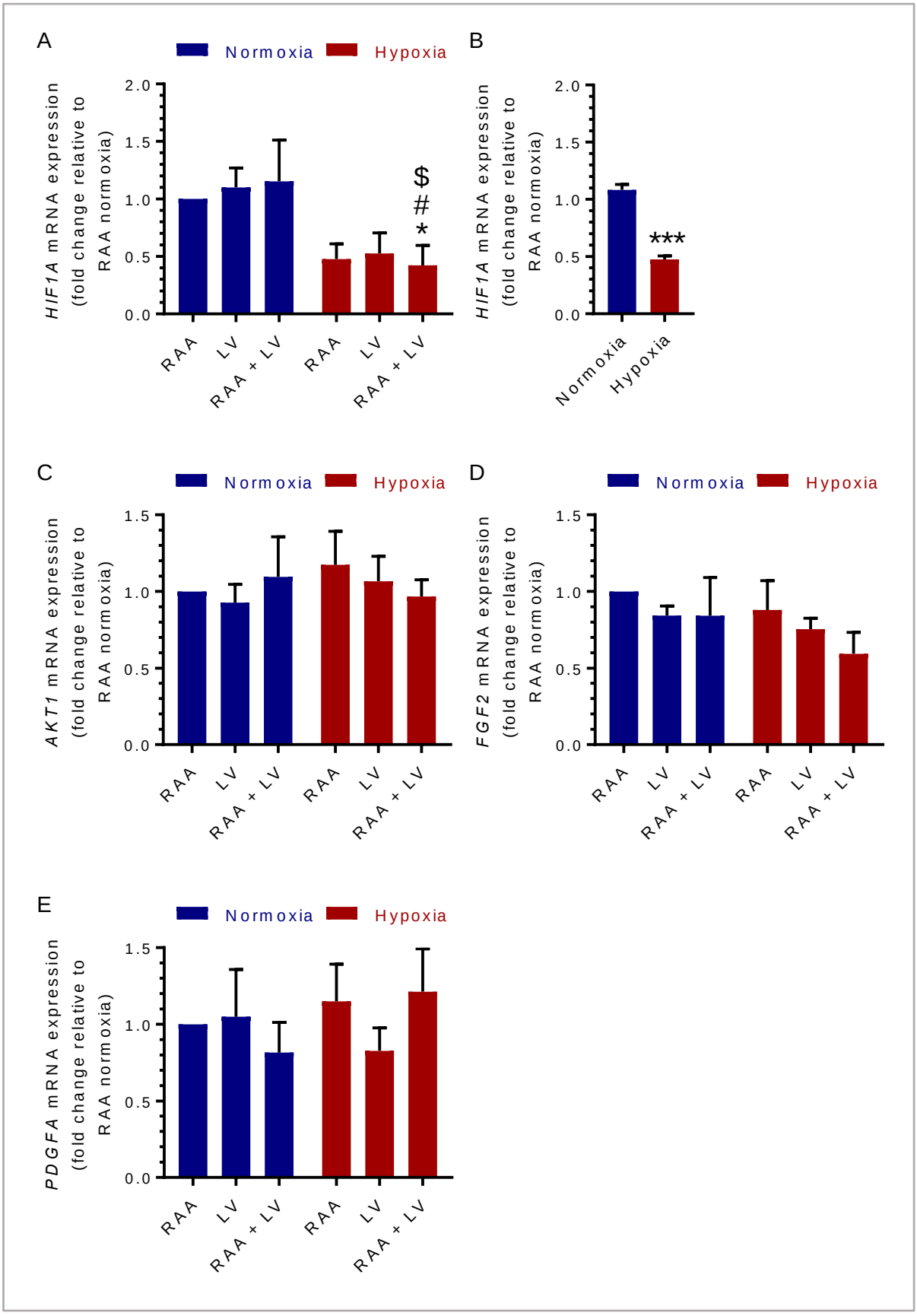

Figure 3-2: Gene expression of CPCs following serum deprivation in normoxic and hypoxic conditions.

A, C, D & E. Quantitative bar graphs showing mRNA expression of CPCs of *HIF1A*, *AKT1*, *FGF2* and *PDGFA*, respectively (n=5 patients, triplicates). Data represented as fold change ± SEM relative to RAA normoxia. Differences were compared using two-way ANOVA followed by a Turkey's post hoc test; where * $p \leq 0.05$ vs. RAA normoxia; # $p \leq 0.05$ vs. LV normoxia; $ $p \leq 0.05$ vs. RAA + LV normoxia. B. Quantitative bar graph showing mRNA expression of *HIF1A* in CPCs cultured in normoxia vs. hypoxia (n=3 groups, triplicates). Data represented as fold change ± SEM relative to RAA normoxia. Differences were compared using two-way ANOVA; where *** $p \leq 0.001$ vs. normoxia group.

3.4 IGF-1 and VEGF-A concentration in CM

IGF-1 and VEGF-A concentration was measured in CM collected following culture of CPCs in normoxic and hypoxic conditions, cultured either alone or in combination, using ELISA.

3.4.1 Comparable secretion of IGF-1 among CM from CPCs

There were no significant differences in the IGF-1 concentration between the different CM. (Figure 3-3 A).

Comparison of IGF-1 concentration irrespective of the groups showed a trend towards reduced IGF-1 concentration in hypoxic CM, although this was not significant (normoxic CM vs. hypoxic CM, p=0.0569, n=6 patients, 3 groups, Figure 3-3 B).

3.4.2 Increased secretion of VEGF-A in hypoxia

VEGF-A secretion was significantly upregulated in the hypoxic CM from RAA CPCs cultured in hypoxia (1.0 ± 0 -fold in RAA normoxia vs. 2.637 ± 0.405 -fold in RAA hypoxia, p=0.0412, n=6 patients). VEGF-A concentration was the highest in the CM combination group (RAA + LV) cultured in hypoxia which was significantly upregulated compared to both the RAA normoxia and RAA + LV normoxia groups (3.428 ± 0.787 –fold in RAA + LV hypoxia; vs. 1.0 ± 0 -fold in RAA normoxia, p=0.0084; vs. 1.241 ± 0.233 –fold in RAA + LV normoxia, p=0.0235; n=6 patients, Figure 3-3 C). VEGF-A concentration in CM was significantly increased in hypoxia groups compared to the normoxia groups (normoxic CM vs. hypoxic CM, p<0.0001, n=6 patients, 3 groups, Figure 3-3 D).

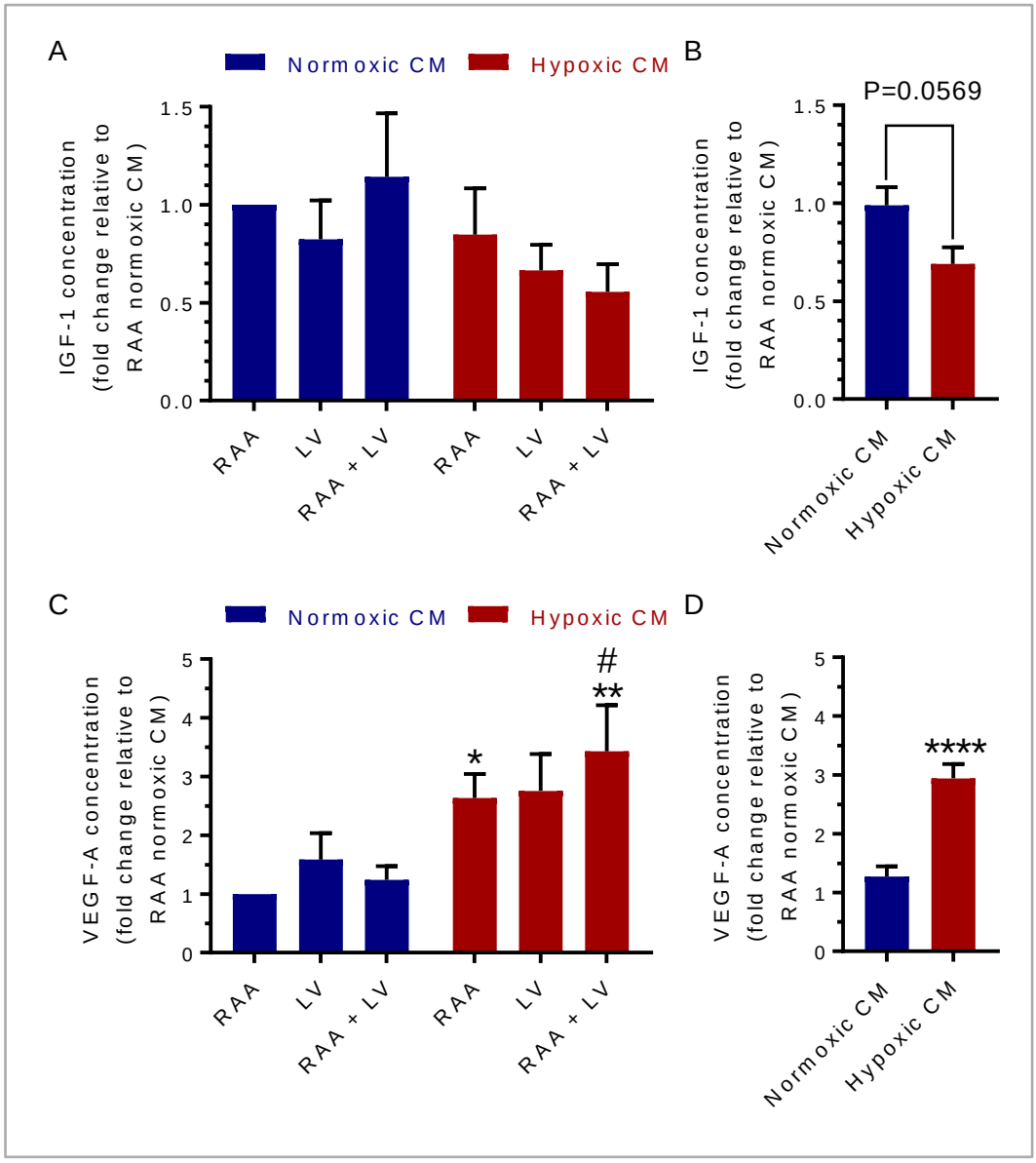

Figure 3-3: IGF-1 and VEGF-A concentration in CM following serum deprivation of CPCs in normoxic and hypoxic conditions.
A and C. Quantitative bar graphs showing IGF-1 and VEGF-A concentration in CM, respectively (n=6 patients, duplicates). Data represented as fold change ± SEM relative to RAA normoxic CM. Differences were compared using two-way ANOVA followed by a Turkey's post hoc test; where * $p \leq 0.05$, ** $p \leq 0.01$ vs. RAA normoxic CM; # $p \leq 0.05$ vs. RAA + LV normoxic CM. B and D. Quantitative bar graphs showing IGF-1 and VEGF-A concentration in CM, respectively, in normoxic CM and hypoxic CM (n=3 groups, duplicates). Differences were compared using two-way ANOVA; where **** $p \leq 0.001$ vs. normoxic CM.

3.5 Anti-apoptotic effects of CM on serum deprived AC16 cardiomyocytes cultured under hypoxia

CM collected from all CPC groups reduced apoptosis in AC16 cardiomyocytes compared to the SF F12 group (RAA normoxic CM, p=0.0004; LV normoxic CM, p=0.0045; RAA + LV

normoxic CM, p=0.0001; RAA hypoxic CM, p=0.021; LV hypoxic CM, p=0.0005; RAA + LV hypoxic CM, p=0.0001; vs. SF F12 group, n=6 patients, Figure 3-4).

There was no statistically significant difference in apoptotic activity among CM-treated groups.

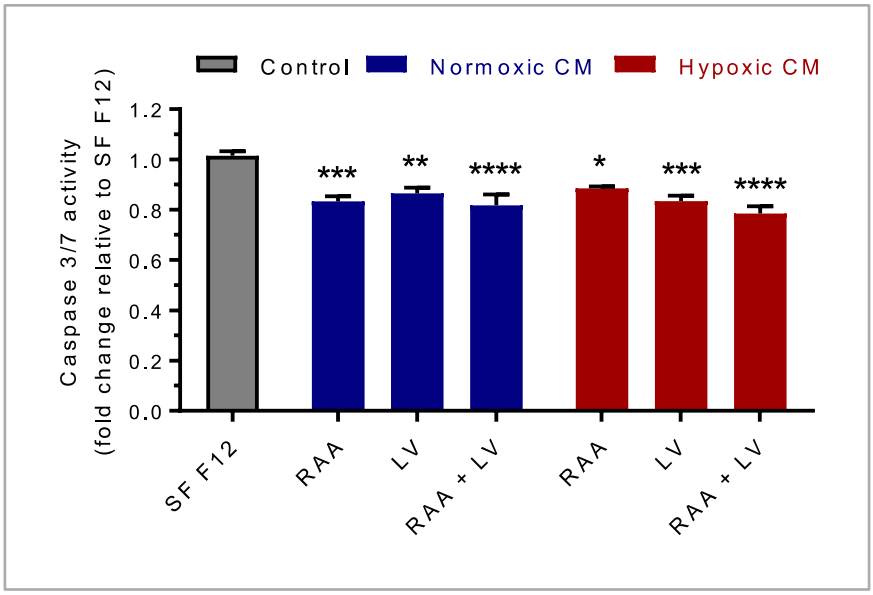

Figure 3-4: Anti-apoptotic effects of CM on serum deprived AC16 cardiomyocytes cultured in hypoxic conditions.
Quantitative bar graph showing caspase 3/7 activity of AC16 cardiomyocytes normalised to cell number (n=6 patients, triplicates). Data represented as fold change ± SEM in caspase 3/7 activity relative to SF F12 group for each individual experiment performed (3 individual experiments performed, n=2 patients/experiment). Differences between CM-treated groups were compared against the control SF F12 group using one-way ANOVA followed by a Dunnett's post hoc test; where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, *** $p \leq 0.0001$ vs. SF F12. Differences among CM-treated groups were compared using two-way ANOVA, followed by a Turkey's post hoc test.

3.6 Normoxic CM from combination of CPCs stimulates HIF-1α protein expression in AC16 cardiomyocytes cultured in hypoxic conditions

All CM-treated groups had a trend to stimulate HIF-1α in AC16 cardiomyocytes following culture in hypoxic conditions, while only the RAA + LV normoxic CM showed a statistically significant increase in HIF-1α protein concentration compared to the SF F12 group (0.18 ± 0.02 ng/mL/100 µg protein in RAA + LV normoxic CM vs. 0.11 ± 0.008 ng/mL/100 µg protein in SF F12 group, p=0.0177, n=5 patients, Figure 3-5).

There were no statistically significant differences in HIF-1α concentration among CM-treated groups.

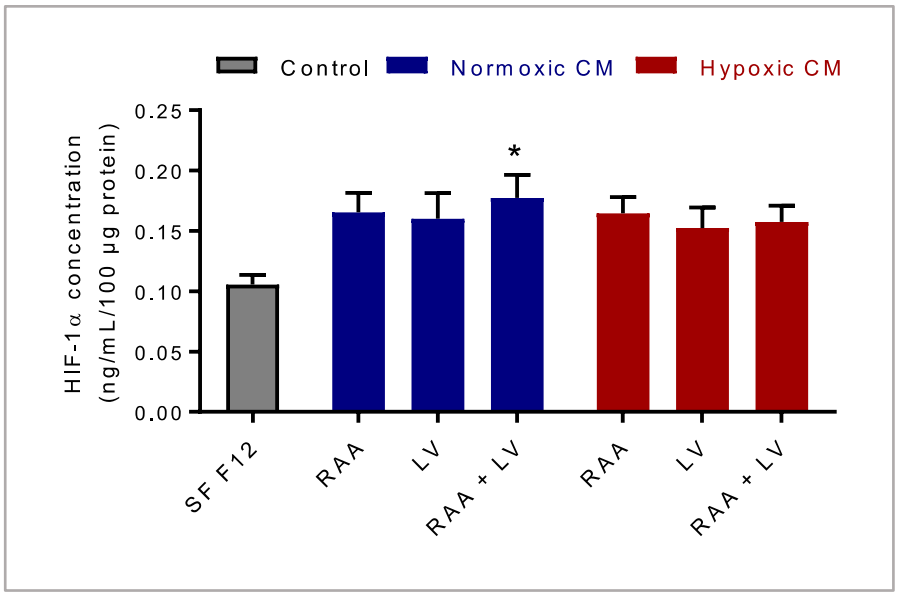

Figure 3-5: Effect of CM to stimulate HIF-1α protein expression in serum deprived AC16 cardiomyocytes cultured in hypoxic conditions.
Quantitative bar graph showing HIF-1α protein concentration (n=5 patients, duplicates). Data represented as ng/mL/100 µg of protein ± SEM. Differences between CM-treated groups were compared against the control SF F12 group using one-way ANOVA followed by a Dunnett's post hoc test; where * $p \leq 0.05$ vs. SF F12. Differences among CM-treated groups were compared using two-way ANOVA, followed by a Turkey's post hoc test.

3.7 Angiogenic effects of CM on tube formation in HUVECs

The effect of CM to improve the angiogenic potential of endothelial cells was estimated by measuring the tube forming ability of HUVECs. All CM-treated groups showed a significant improvement in angiogenesis compared to the negative control SF EBM-2 group. Importantly, this improvement was comparable to the positive control EGM-2 complete group in only the LV hypoxic CM and RAA + LV hypoxic CM (Figure 3-6 B).

Cells treated with CM collected from the RAA + LV CPCs exposed to hypoxia showed a significant increase in angiogenesis compared to the cells treated with CM collected from LV CPCs exposed to normoxia suggesting that RAA + LV hypoxic CM contained the highest levels of angiogenic factors (20713 ± 748.4 µm/field in RAA + LV hypoxic CM vs. 15193 ± 394.4 µm/field in LV normoxic CM, p=0.0326, n=3 patients, Figure 3-6 B).

Comparison of angiogenic effects of normoxic and hypoxic CM irrespective of the groups showed a significant increase in tube formation by hypoxic CM (normoxic CM vs. hypoxic CM, p=0.0069, n=3 patients, 3 groups, Figure 3-6 C).

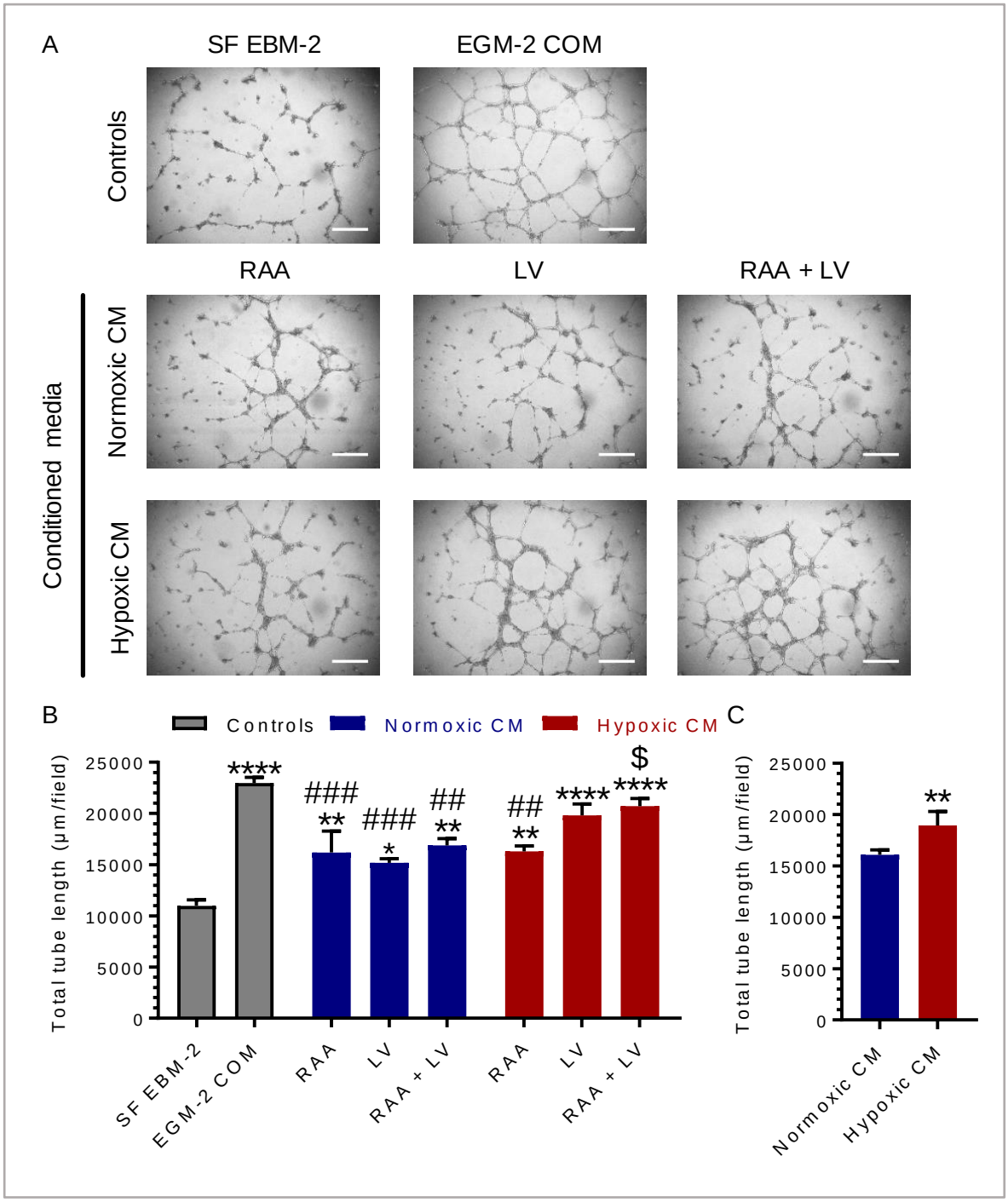

Figure 3-6: Angiogenic effects of CM on tube formation using HUVECs.
A. Phase contrast images showing tube formation in HUVECs taken at 4X magnification 12 hours after initial seeding. Scale bars, 400 µm B. Quantitative bar graph showing the total tube length (n=3 patients, triplicates). Data represented as mean ± SEM µm/field. Differences between CM-treated groups were compared against the controls using one-way ANOVA followed by a Dunnett's post hoc test; where * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$ vs. SF EBM-2; # $p \leq 0.05$; ## $p \leq 0.01$; ### $p \leq 0.001$; vs. EGM-2 COM. Differences among CM-treated groups were compared using two-way ANOVA followed by a Turkey's post hoc test; where \$ $p \leq 0.05$ vs. LV normoxic CM. C. Quantitative bar graph showing total tube length of normoxic CM and hypoxic CM (n=3 groups, triplicates). Differences were compared using two-way ANOVA; where ** $p \leq 0.01$ vs. normoxic CM.

4 Discussion

4.1 Summary of results

There were no significant differences in the expression of cell markers between RAA CPCs and LV CPCs (Table 4-1). CD34 expression in CPCs was higher than anticipated.

The following outlines the results from serum deprivation of CPCs cultured under normoxia and hypoxia (Table 4-2). There were no statistically significant differences in the gene expression of *AKT1*, *FGF2* and *PDGFA* when CPCs were cultured under normoxia and hypoxia. Interestingly, *HIF1A* gene expression was only significantly down-regulated in the combination group cultured in hypoxia and in hypoxia irrespective of groups. Secretion of IGF-1 into CM was comparable among all CPC groups, although there was a decreased trend in hypoxia. However, secretion of VEGF-A was significantly increased by LV CPCs cultured in hypoxia and combination group cultured in hypoxia.

The following outlines the results from functional paracrine effects of CPCs, which were evaluated using the CM collected from serum deprivation of CPCs cultured in normoxia and hypoxia (Table 4-3). Apoptosis was significantly decreased in AC16 cardiomyocytes exposed to serum deprivation and hypoxia when treated with CM from all CPCs groups (normoxic CM and hypoxic CM). HIF-1α protein was only significantly upregulated when treated with RAA + LV normoxic CM, which suggests that the paracrine profile from the combination in normoxia could stimulate HIF-1α.

Angiogenic potential of CM from CPCs as assessed by tube formation using HUVECs, significantly increased when treated with CM from all CPC groups except LV normoxic CM, compared to the negative control where cells were treated with SF EBM-2 media alone. Interestingly, the level of tube formation in HUVECs treated with hypoxic CM from LV CPCs and RAA + LV CPC group were as comparable to the positive control where cells were treated with EGM-2 complete media. RAA + LV hypoxic CM showed the highest angiogenesis which was the only group significantly increased compared to LV normoxic CM.

Together, these results suggest that combination of RAA CPCs and LV CPCs demonstrate better effects on the therapeutic mechanisms of cardiac repair through synergistic or complimentary paracrine effects compared to single cultures.

Table 4-1: Overview of differences in cell markers between RAA CPCs and LV CPCs.

Cell marker	RAA CPC	LV CPC
CD34-	-	-
CD90+	-	-
CD105+	-	-
CD90+CD105+	-	-

- indicates no significant difference.

Table 4-2: Overview of differences in gene expression from serum deprivation of CPCs under normoxia and hypoxia.

Genes expression	Normoxia			Hypoxia		
	RAA	LV	RAA + LV	RAA	LV	RAA + LV
HIF1A	-	-	-	↓	↓	↓↓
Normoxia vs. hypoxia	-			↓		
AKT1	-	-	-	-	-	-
FGF2	-	-	-	-	-	-
PDGFA	-	-	-	-	-	-

- indicates no significant difference; ↓ and ↓↓ indicates degrees of relative differences.

Table 4-3: Overview of differences in functional paracrine effects of CM from CPCs.

Paracrine effects	Normoxic CM			Hypoxic CM		
	RAA	LV	RAA + LV	RAA	LV	RAA + LV
IGF-1 concentration in CM	-	-	-	-	-	-
VEGF-A concentration in CM	-	↑	-	↑↑	↑	↑↑↑
Normoxic CM vs. hypoxic CM	-			↑		
Anti-apoptotic effects	↑	↑	↑	↑	↑	↑
Stimulation/upregulation of HIF-1α	-	-	↑	-	-	-
Angiogenesis	↑↑	↑	↑↑	↑↑	↑↑↑	↑↑↑↑
Normoxic CM vs. hypoxic CM	-			↑		

- indicates no significant difference; ↑, ↑↑, ↑↑↑ and ↑↑↑↑ indicates degrees of relative differences.

4.2 Discussion of results

4.2.1 CD34 expression in CPCs

Previously CD34 was thought to be a specific marker for haematopoietic cells although it is also expressed on non-haematopoietic cells.(140) However, CD34+ cells are also present in the heart as cardiac endothelial precursors, which in additions to CD34, also express the cardiac cell marker, homebox gene *NKX2.5*.(143,144) In this study both RAA CPCs and LV CPCs expressed CD34. Of note, CD34+ cells were present in CPCs isolated from the RAA and LV tissue contain a heterogeneous population of cells including cells from the epicardium. RAA tissue consisted of endocardial, myocardial and epicardial tissue whereas LV tissue consisted of mainly epicardial and myocardial tissue. Similarly, Messina et al. showed the presence of CD34+ cells in CDCs, which like the cells in this study, contain heterogeneous populations of CPCs.(88) Hence, although it was expected that only a minimal expression of CD34+ cells in CPCs from the RAA and LV, due to their heterogeneity presence of CD34+ cells are inevitable.

In addition to the expression of CD34 in the heart, the CD34+ cells may be a result from residual adipose tissue and peripheral blood during CPC isolation from the cardiac tissue. Moreover following ischaemic injury to the heart, cells from the bone marrow (including CD34+ cells) are mobilised into the peripheral blood and it has been suggested that these cells may subsequently engraft into the heart.(145,146)

4.2.2 *HIF1A* gene expression in hypoxia

An unexpected finding in this study was the decreased *HIF1A* gene expression after exposing the CPCs to hypoxia, although only the combination group showed a significant decrease. Studies have shown significant increase in *HIF1A* expression following acute hypoxia (at 4 hours), however, prolonged hypoxia (≥12 hours) showed a decrease in *HIF1A* expression.(147) Several mechanisms have been proposed for this decrease in *HIF1A* following prolonged hypoxia through negative feedback mechanisms to prevent excessive accumulation of HIF-1α. One such mechanism is the upregulation of antisense HIF-1α (aHIF-1α) and miR-429 both of which are increased in hypoxia.(147,148) Further, tristetraprolin (TTP), a mRNA destabilising protein, is also implicated in *HIF1A* mRNA destabilisation following hypoxia.(149) While these factors were not measured in this project due to time constraints, it is possible that CPCs exposed to prolonged hypoxia also activate these factors to reduce *HIF1A* expression.

Interestingly, the decrease in *HIF1A* expression in the RAA + LV CPCs cultured in hypoxia contradicts the level of VEGF-A concentration which was highest in the CM collected from this group. VEGF-A is upregulated in hypoxia through the transcriptional action of HIF-1, which suggests that HIF-1 concentrations were highest in the combination group cultured in hypoxia. HIF-1α protein is heavily regulated post-translationally so no conclusions can be made solely on the mRNA expression.(130) Therefore, the expression of HIF-1α protein in the CPCs after exposing them to hypoxia needs to be measured to observe any definite differences between the groups.

4.2.3 Paracrine factor secretion

IGF-1 concentration was comparable across all CM, although there was a trend towards a decrease in hypoxia (P=0.0569). IGF-1 concentration in CM from MSCs is often increased in hypoxia.(109) In osteoblasts, IGF-1 expression was decreased in hypoxia.(150) This was due to the decrease in Runt-related transcription factor 2 (Runx2) in hypoxia, an osteoblast transcription factor which can activate an upstream response element in the IGF-I gene promoter. In vascular endothelial cells, IGF-1 expression was decreased in hypoxia through the actions of insulin-like growth factor binding protein (IGFBP), a group of carrier proteins for IGF-1 which modulate IGF-1 activity which can be both stimulatory or inhibitory.(151) Although these mechanisms are likely to be cell specific, c-Kit$^+$ CSC/CPCs have been shown to differentiate into osteogenic and vascular lineages.(152) However, these results are not significant and conclusions about IGF-1 secretion by CPCs in hypoxia cannot be made based on current evidence.

VEGF-A concentration was significantly increased in CM collected from CPCs and particularly in the RAA hypoxic CM and RAA + LV hypoxic CM. These results are consistent with literature which show an increase in VEGF-A concentration in hypoxia mediated by its increased transcription by HIF-1.(128) These results suggest that combination of RAA CPCs and LV CPCs have synergistic effects to increase VEGF-A concentration following exposure to hypoxia.

4.2.4 Anti-apoptotic effects of CM on cardiomyocytes

CM from all cell types decreased apoptosis in AC16 cardiomyocytes subjected to serum deprivation and hypoxia due to the paracrine stimulation of various pro-survival pathways. Previously, the anti-apoptotic effects of RAA hypoxic CM were mediated through the PI3K/AKT and PKCε pathways, while LV hypoxic CM mediated these effects through the

PKCε pathway.(8) Anti-apoptotic effects of RAA + LV hypoxic CM were likely mediated through both pathways which may synergise, however, its anti-apoptotic effects were comparable to both the RAA hypoxic CM and LV hypoxic CM.

IGF-1 and VEGF-A both mediate their anti-apoptotic effects through the PI3K/AKT pathway.(7) VEGF-A concentration was significantly higher in RAA hypoxic CM and RAA + LV hypoxic CM which suggests were mediated through the PI3K/AKT pathway. Quantification of paracrine factors in CM involved in the signaling of PKCε pathway such as EPO and bFGF may ascertain the anti-apoptotic effects of LV hypoxic CM and other CM.(7)

Investigation of other pro-survival pathways may identify other mechanisms involved in the anti-apoptotic effects of CM, including the normoxic CM in which the mechanisms are currently unknown.

4.2.5 Paracrine stimulation of HIF-1α through a hypoxia-independent pathway

RAA + LV normoxic CM stimulated the expression of HIF-1α protein in cardiomyocytes cultured in hypoxia. Hypoxia-independent pathways including the PI3K/AKT and PKCε signalling have been shown to enhance the expression of HIF-1α.(131,132) As discussed previously, RAA CPCs and LV CPCs release paracrine factors which act through these pathways. The upregulation of HIF-1α was likely due to the RAA + LV normoxic CM containing paracrine factor able to stimulate the expression of HIF-1α through these various pathways. Further mechanistic studies may elude to the factors and pathways that are involved in stimulation of HIF-1α expression by RAA + LV normoxic CM.

4.2.6 Angiogenic paracrine effects in combination cell therapy

Combination of RAA and LV CPCs hypoxic CM showed the highest levels of angiogenesis. This was likely due to the synergistic or complimentary paracrine effects consisting of the highest levels of angiogenic paracrine factors.

Angiogenesis is increased by VEGF-A, which was significantly increased in only the RAA hypoxic CM and the RAA + LV hypoxic CM. Angiogenesis was second highest in the LV hypoxic CM, although VEGF-A was not significantly increased in the CM. Therefore, increased angiogenesis in this group are likely to be explained by other angiogenic factors that are stimulated by hypoxia.(128) Hypoxic CM significantly increased angiogenesis in HUVECs. This is likely due to the increased secretion of angiogenic factors, especially VEGF, in hypoxia.(128)

Among the CM-treated groups, LV normoxic CM had the lowest tube formations, most likely due to the absence or low levels of angiogenic factors. Recently, a study showed that severe hypoxia can induce quiescence and reduce the vasculogenic potential of CPCs, through the decreased expression of c-MYC.(153) As the left ventricle is often subjected to greater ischaemic stresses compared to the right atrium, LV CPCs may be more senescent and have reduced functional effects on neovascularisation. This result may also be due to the small sample size (n=3), resulting in low statistical power and no conclusions can be made based on the current evidence.

4.3 Strengths of the study

As CPCs were isolated from the same patient, experiments could be performed by using patient-matched samples and at matched passages. This was advantageous to the study as the effects of patient-related factors and inter-patient variations in CPC function could be limited when comparing between the different CPC groups.

CPCs are primary cells which are unable to replicate indefinitely reaching senescence in culture which can affect their phenotype and reparative potential.(154,155) Therefore, RAA CPCs and LV CPCs from each patient were paired for passages to prevent any differences in stem cell function due to differences in passage number.

As this study employed an *in vitro* approach to assess the paracrine effects of CPCs, processes and mechanisms involved in cardiac repair could be analysed in isolation, whereas these processes would occur simultaneously in *in vivo* studies. Ascertaining mechanisms of the functional effects of processes involved in cardiac repair is important to understanding the therapeutic benefits of cell therapy.

4.4 Limitations of the study

4.4.1 Small sample size

Due to constraints in time and resources, and technical difficulties with experiments, the sample size was low, especially for characterising CPCs using flow cytometry (n=4 patients) and measuring angiogenic effects of CM using a tube formation assay (n=3 patients) experiments. The low sample size reduces the power of this study and may have been insufficient to show significant differences between groups.

4.4.2 Inadequate characterisation of CPCs

CPCs were isolated and characterised using collagenase II to release stromal cells containing CPCs from the cardiac tissue. These cells were then cultured and expanded as CPCs. There are several limitations in the methods used to characterise these cells.

Cardiac tissue is composed of several cell types and tissue including cardiomyocytes, smooth muscle cells, endothelial cells, fibroblasts, adipose tissue, and a small population of stem cells. Therefore, the isolation of CPCs is possibly susceptible to contamination. Daily examination of cells was carried out to observe any signs of contamination or changes in cell morphology. Fibroblast contamination was the most concerning as these cells show a similar mesenchymal cell morphology to CPCs. This was previously examined in the Katare laboratory which showed that CPCs stained negative for the cardiac fibroblast marker, fibroblast surface protein 1.(8) Isolation of CPCs was executed using the established Katare laboratory protocol, which showed no fibroblast contamination, and utmost care was taken to eliminate the possibility of contamination in this study.

4.4.3 Serum deprivation and hypoxia to model stimulated ischaemia

The area of the infarct in IHD is characterised by hypoxia, decreased supply of nutrients (serum deprivation), acidosis, and inflammation. Stimulated ischaemia was simplified in this study and only serum deprivation and hypoxia were used to stimulate ischaemia *in vitro*, however, these conditions are sufficient to induce apoptosis and affect hypoxia-induced signalling which were crucial aspects to this study.(156–159)

Furthermore, physiological oxygen levels in the microenvironment of stem cells, which range from 1-5%, should be used to assess stem cell function to better approximate *in vivo* conditions, rather than the normal culture conditions (20% O_2) often used to culture cells.(160,161)

4.5 Implications and clinical relevance

Results from this study has provided evidence that combination therapy may provide synergistic effects suggesting that identification of the effective combination of stem cell which exert synergistic and complimentary effects on cardiac repair could be more beneficial. Although combination of stem cells is appealing, the technical difficulties and invasiveness of

procuring different stem cell population needs to be assessed and compared against the therapeutic benefits of combination therapy.

Preconditioning refers to treatment of stem cells to enhance or modulate their therapeutic actions. Hypoxia increased angiogenic effects of CM from CPCs, thereby suggesting that hypoxic pre-conditioning may be another approach to effectively improve the therapeutic effects of stem cells.

4.6 Future directions

The exact mechanisms for the different functional effects of RAA CPCs and LV CPCs are not clear and warrants further investigation. Paracrine factors released by CPCs are not only limited to cytokines but include exosomes and microRNAs. Assessment of the paracrine profile could deduce mechanisms involved in the paracrine effects of CPCs.

While the *in vitro* results may be promising, *in vivo* studies are needed to confirm the therapeutic effects of RAA CPCs and LV CPCs, as well as their use in combination. Other paracrine mediated effects such as immunomodulation and stimulation of endogenous stem/progenitor cells can be investigated *in vivo*.

Combination of RAA CPCs and LV CPCs increased cardioprotection by reducing apoptosis and increasing HIF-1α expression in cardiomyocytes through paracrine factors. A previous study showed CM from RAA CPCs had the highest cardioprotective effects compared to LV CPCs and EPCs through activation of cell proliferation and reduced apoptotic cell death.(8) Therefore, other cardioprotective effects such as cell proliferation should be investigated to give a better indication of the cardioprotective effects of combination of RAA CPCs and LV CPCs.

The paracrine effects of CPCs on proliferation and migration of endothelial cells were not investigated in this study, which are essential processes for angiogenesis. It was previously shown that CM from RAA CPCs and LV CPCs had superior migration potential on endothelial cells compared to CM from EPCs.(8) Also, other ASC populations can increase proliferation and migration of endothelial cells by releasing pro-angiogenic factors.(7,106) Therefore, it would be interesting to measure the potential of CM on proliferation and migration of endothelial cells using a combination of RAA CPCs and LV CPCs, and to compare cells used in this study to other ASC populations.

The procurement of heart tissue for isolation of CPCs is an invasive procedure, especially for the left ventricle which is not routinely obtained in CABG surgery. It would be interesting to investigate if procurement of heart tissue from less invasive procedures, such as endomyocardial biopsies, have the same functional effects as the CPCs in this study. This would provide a more clinically viable method to obtain CPCs, as well as the possibility to isolate CPCs prior to ischaemic injury to the heart in high-risk individuals.

CPCs used in this study are heterogeneous, which may be similar in composition to CDCs another heterogeneous population of CPCs. Other CPCs in the current literature are often isolated and characterised based on specific cell markers or techniques. Therefore, further characterisation may reveal distinct differences in the composition or population of stem cells in relation to the functional, genetic and developmental differences of the right atrium and left ventricle.

4.7 Conclusion

This study provides evidence that different combination of stem cells has synergistic or complimentary paracrine effects thereby enhancing the therapeutic potential of stem cells. Combination of RAA CPCs and LV CPCs synergistically improved VEGF-A secretion, stimulated HIF-1α expression in cardiomyocytes in hypoxia and increased angiogenesis in endothelial cells by paracrine mechanisms. Stated as part of the 'next-generation' of cell therapy, combination of stem cells is likely to play an important role in the future of stem cell therapy.

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Appendix

Table A1 List of solutions and their composition.

Solution	Composition
Phosphate buffered saline (PBS)	137 mM Sodium chloride 10 mM Sodium monobasic 1.4 mM Potassium phosphate dibasic 2.7 mM Potassium chloride
Hank's balanced salt solution (HBSS)	5.4 mM Potassium chloride 0.3 mM Sodium phosphate hepta hydrate 0.4 mM Potassium monobasic 4.2 mM Sodium bicarbonate 1.3 mM Calcium chloride 0.5 mM Magnesium chloride hexa hydrate 0.6 mM Magnesium sulphate hepta hydrate 137 mM Sodium chloride 5.6 mM D-glucose pH adjusted to 7.4
Krebs Ringer Henseleit (KRH) buffer	116 mM Sodium chloride 4 mM Potassium chloride 1 mM Magnesium chloride 1.8 mM Calcium chloride 25 mM Glucose 10 mM HEPES pH adjusted to 7.4
Tris-EDTA (TE) buffer	100 mM Tris·hydrochloride 1 mM EDTA, pH 8.0
Flow cytometry buffer	HBSS supplemented with • 3% FBS • 10 mM HEPES
Collagenase II solution	1357 units of collagenase II per mL of HBSS

Table A2 List of media used for cell culture experiments.

Media	Composition
Ham's F12 complete media	Ham's F12 nutrient mix supplemented with • 10% FBS • 1X antibiotic-antimycotic (anti-anti) • 10 ng/mL human FGF2 • 0.005 U/mL human EPO
Serum-free Ham's F12 media	Ham's F12 nutrient mix supplemented with 1X anti-anti
DMEM/F12 complete media	DMEM/F12 media supplemented with • 12.5% FBS • 1X anti-anti
AC16 cardiomyocyte freezing media	DMEM/F12 complete media supplemented with 10% DMSO
EGM-2 complete media	EBM-2 supplemented with EGM-2 SingleQuots containing • Human EGF • Vascular endothelial growth factor • R3-IGF-1 • Ascorbic acid • Hydrocortisone • Human FGF2 • Heparin • FBS • GA
Serum-free EBM-2 media	EBM-2 supplemented with 1X anti-anti
HUVEC freezing media	EGM-2 complete media supplemented with • 10% FBS • 10% DMSO

Table A3 List of antibodies used.

Antibody	Type of antibody	Host/ isotype	Species specificity	Manufacturer (Cat no.)
CD34 APC monoclonal antibody	Primary conjugated	Mouse	Human	eBioscience, USA (17-0349-42)
CD90-FITC monoclonal antibody	Primary conjugated	Mouse	Human	eBioscience, USA (11-0909-42)
CD105-PE-Cy7 monoclonal antibody	Primary conjugated	Mouse	Human	eBioscience, USA (25-1057-42)

Table A4 List of primers used for PCR.

Gene	Primer Sequence (5' to 3')	Species specificity	Manufacturer
HIF1A	Forward: AAAATCTCATCCAAGAAGCC Reverse: AATGTTCCAATTCCTACTGC	Human	Sigma-Aldrich, USA
AKT1	Forward: AAGTACTCTTTCCAGACCC Reverse: TTCTCCAGCTTGAGGTC	Human	Sigma-Aldrich, USA
FGF2	Forward: TGGCTTCTAAATGTGTTACG Reverse: GTTTATACTGCCCAGTTCG	Human	Sigma-Aldrich, USA
PDGFA	Forward: TAGGGAGTGAGGATTCTTTG Reverse: CGAGGAATCTCGTAAATGAC	Human	Sigma-Aldrich, USA

Table A5 List of kits used.

Kit	Manufacturer (Cat no.)
AbC anti-mouse bead kit	Invitrogen, USA (A10344)
Caspase 3/7 Glo assay	Promega, UK (G8093)
CyQUANT cell proliferation kit	Invitrogen, USA (C7026)
Human IGF-1 ELISA kit	ELISAkit.com, Australia (0028)
Human VEGF-A ELISA kit	ELISAkit.com, Australia (0035)
PrimeScript RT reagent kit	Takara, Japan (RR037A)
SYBR Premix Ex Taq	Takara, Japan (RR420L)
HIF1a Human SimpleStep ELISA kit	Abcam, UK (ab171577)