EFFECTS OF HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS ON *IN VITRO* FUNCTIONS OF AGED MOUSE CLONOGENIC CARDIAC CELLS

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by

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LIST OF SYMBOLS

%	Percentage
n	Nano
^{o}C	Degree Celsius
V	Voltage
α	Alpha
β	Beta
κ	Kappa
μ	Micro

LIST OF ABBREVIATIONS

ACS	Acute coronary syndrome
AEC	Animal ethics committee
AT	Ammonium Triton X-100
bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
BMMNCs	Bone marrow mononuclear cells
BMSCs	Bone marrow mesenchymal stem cells
BSA	Bovine serum albumin
CCs	Cardiac c-kit cells
CDCs	Cardiosphere derived cells
CFSE	Carboxyfluorescein Succinimidyl Ester
CSp	CardioStem sphere
cTnI	Cardiac troponin I
CXCR4	C-X-C Chemokine Receptor Type 4
ECM	Extracellular matrices
ESCq-FBS	Embryonic Stem Cells Qualified FBS
ESCs	Embryonic stem cells
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FT	Fibrinolytic therapy
HGF	Hepatocyte growth factor
HO-1	Heme oxygenase-1
IGF	Insulin growth factor
IGF-R1	Insulin growth factor receptor 1

IL6	Interleukin 6
IL8	Interleukin 8
Isl-1	Islet 1
ISCT	International Society for Cellular Therapy
ITS	Insulin-Transferrin-Selenium Reagent
LIF	Leukaemia inhibitory factor
LV	Left ventricular
LVAD	Left ventricular assisted device
LVEF	Left ventricular ejection fraction
MACS	Magnetic activate cell sorting
MI	Myocardial infarction
MSCs	Mesenchymal stem cells
NSTEMI	Non-ST-elevation myocardial infarction
PBS	Phosphate Buffer Solution
PCI	Percutaneous coronary intervention
PE	Phycoerythrin
PSCs	Pluripotent stem cells
RT	Room Temperature
ROS	Reactive oxygen species
SDF-1	Stromal-derived Factor-1
SDS	Sodium Dodecyl Sulphate
SFRP1	Secreted Frizzled Related Protein-1
SP	Side population
ST	SDS Triton X-100
STEMI	ST-elevation myocardial infarction
UA	Unstable angina
VEGF	Vascular endothelial growth factor

KESAN-KESAN SEL INDUK MESENKIMA JELI WHARTON MANUSIA KE ATAS FUNGSI *IN VITRO* SEL-SEL JANTUNG KLONOGENIK TIKUS YANG TUA

ABSTRAK

Kajian menunjukkan bahawa fungsi-fungsi sel stem yang tua bertambah baik selepas terdedah kepada persekitaran sistemik yang muda *in vivo*. Memandangkan penyakit jantung biasanya mempengaruhi pesakit-pesakit yang telah berumur, kebolehupayaan sel-sel mesenkima (MSC) dari jeli Wharton manusia dalam mempertingkatkan fungsi-fungsi sel kardiak c-kit (CCs) *in vitro* masih tidak jelas. Tujuan penyelidikan ini adalah untuk mengaji kesan-kesan MSCs terhadap fungsi-fungsi CCs yang tua. CCs diasingkan daripada jantung tikus C57/BL6N yang berumur 1- dan 18-bulan, dikultur bersama MSCs melalui sentuhan sel-sel, atau dipisahkan dengan penggunaan Transwell. Stemness, kinetik pertumbuhan, panjang telomer relatif dan aktiviti telomerase CCs yang tua dinilai berbanding dengan CCs yang muda (yCCs) serta CCs yang tua (aCCs) tanpa pengkulturan bersama MSCs. Bagi menguji keberkesanan matriks ekstra sel daripada MSCs, CCs dikultur atas matriks ekstra sel MSCs yang telah dirawat dalam media kardiogenik. Ujian penentuan pertumbuhan dan tekanan oksidatif dilaksanakan bagi penilaian kesan matriks esktra sel MSCs ke atas CCs. Semua maklumat dianalisa dengan ANOVA. aCCs primari menunjukkan kadar klonogenik yang lebih rendah berbandingkan yCCs (9.5 ± 2.9% vs. 21.2 ± 4.4%; $p < 0.05$). Selepas pertumbuhan klonogenik, hanya sel dengan $CD90^{Pos}CD140a^{Pos}CD166^{Neg}$ diperolehi daripada aCCs, di mana ia berbeza dengan phenotype daripada yCCs ($CD90^{Neg}CD140a^{Neg}CD166^{Pos}$) dengan menggunakan flow cytometri. aCCs klonogenik mempunyai panjang telomer relatif yang setara dengan yCCs. Namun demikian, ekspresi gen *Gata4*, *Nkx2.5* dan *Sox2* aCCs klonogenic adalah rendah, dengan kadar perubahan 2.4, 3767.0, 4.9 masing-masing. Selain itu, keupayaan aCCs klonogenik untuk membentuk sfera adalah rendah (4 ± 1 *vs.* 64 ± 19 spheres; $p < 0.05$) dan gagal berbeza secara spontan kepada sel-sel jantung dan endotelium. Pengkulturan dengan MSCs melalui sentuhan sel meningkatkan kadar penghijrahan aCCs

sebanyak 54.6 ± 4.4%, berbandingkan pengkulturan aCC dalam Transwell (42.9±2.6%) dan tanpa MSCs (44.7 ± 2.5%, $p < 0.05$). Pengkulturan sel melalui sentuhan sel dan Transwell menambahbaikkan pertumbuhan sel-sel yang tua sebanyak 15.0% dan 16.4% dengan menggunakan carboxyfluorescein succinimidyl eater (CFSE) selama 3 hari ($p < 0.05$). Maklumat-maklumat ini mencadangkan bahawa MSCs dapat mempertingkatkan kinetik pertumbuhan aCCs. Keberkesanan pembezaan CCs kepada sel-sel jantung dan endotelium tidak ketara dalam kesemua kumpulan. Namun, pengkulturan aCCs ke atas matriks ekstra sel MSCs meningkatkan daya tahan aCCs kepada tekanan oksidatif berbandingkan kontrol, dinilai berdasarkan ujian penentuan sel-sel yang hidup setelah rawatan hidrogen peroxida (26.3 ± 0.8% *vs.* 24.4 ± 0.4%; $p < 0.05$). Jantung yang berusia mempunyai sekumpulan CCs yang mengekalkan kepanjangan telomer dan boleh diasingkan berdasarkan keupayaan pembaharuan. Namun, CCs ini tidak dapat berfungsi seperti CCs yang muda. Pertumbuhan kinetic dan pembezaan CCs tua dapat dipertingkatkan sedikit setelah dikultur bersama MSCs melalui sentuhan sel-sel.

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ABSTRACT

Exposure of aged stem cells to young systemic environment has shown to improve their functions *in vivo*. While heart disease commonly affects elderly patients, it is unclear if biologically young Wharton's jelly-derived mesenchymal stem cells (MSCs) can improve the functions of aged cardiac c-kit cells (CCs) *in vitro*. This study examined the effects of MSCs on the functions of aged CCs. CCs were isolated from 1- and 18-month-old C57BL/6N mice and were co-cultured with human MSCs with direct cell-cell contact or separated with a Transwell insert. Stemness, growth kinetics, relative telomere length and telomerase activity of the aged CCs were evaluated in comparison with both young (yCCs) and aged CCs (aCCs) without MSC co-culture. To test the effects of extracellular matrices (ECM) produced from MSCs, CCs were cultured on ECM-derived from MSCs treated with cardiogenic medium. Proliferation and oxidative stress assays were performed to evaluate the effect of MSC-derived ECM on CCs. All data were analysed using ANOVA. The primary aCCs showed significantly lower clonogenicity compared to yCCs (9.5 ± 2.9% vs. 21.2 ± 4.4%; $p < 0.05$). Following clonogenic expansion, only $CD90^{Pos}CD140a^{Pos}CD166^{Neg}$ cells were expanded in the aCCs, which were different from the phenotype of yCCs ($CD90^{Neg}CD140a^{Neg}CD166^{Pos}$) as assessed by flow cytometry. Clonogenic aCCs showed comparable telomere length to yCCs. However, these cells showed lower *Gata4, Nkx2.5* and *Sox2* gene expressions, with changes of 2.4, 3767.0, 4.9 folds, respectively. These cells presented a lower sphere formation capability (4 ± 1 *vs.* 64 ± 19 spheres; $p < 0.05$) and did not spontaneously differentiate into cardiomyocyte and endothelial lineage. Direct co-culture of both cells increased aCC migration which repopulated 54.6 ± 4.4% of the gap area as compared to aCCs with MSCs in Transwell (42.9 ± 2.6%) and aCCs without MSCs (44.7 ± 2.5%, $p < 0.05$). Both direct and Transwell co-culture

improved proliferation in aCCs by 15.0% and 16.4%, respectively as traced using carboxyfluorescein succinimidyl ester (CFSE) for 3 days ($p < 0.05$). These data suggest that MSCs can improve the growth kinetics of aCCs. No difference was observed across all groups regarding their differentiation capability to form cardiomyocyte, endothelial and smooth muscle cells. However, ECM-derived from MSC conferred aCCs with enhanced resistance to oxidative stress as compared to control, measured based on viability post-H_2O_2 treatment (26.3 ± 0.8% *vs.* 24.4 ± 0.4%; $p < 0.05$). CCs retained long telomere length are present in aged heart and can be obtained based on their self-renewing capability. However, these cells are functionally compromised. The growth kinetics and cardiac differentiation of these cells are minimally enhanced by MSCs, and this requires cell-cell contact.

CHAPTER 1

INTRODUCTION

The heart has been known as post mitotic organ for decades. This dogma has been changed as a group of cardiac stem cells can be found in the adult heart (Beltrami *et al.*, 2003). These cells have been isolated from rat (Beltrami *et al.*, 2003), mouse (Smith *et al.*, 2014), swine (Ellison *et al.*, 2011), and human (Bearzi *et al.*, 2007, He *et al.*, 2011). Studies showed that cardiac stem cells expressing c-kit can regenerate infarcted hearts in animal models (Beltrami *et al.*, 2003, Hong *et al.*, 2014, Kazakov *et al.*, 2015), protect surviving cardiomyocytes from apoptosis (Kawaguchi *et al.*, 2010, Ellison *et al.*, 2011), promote angiogenesis and myogenesis (Di Siena *et al.*, 2016). The beneficial effect of c-kit has been further expanded to first human clinical trial, which showed improvement in left ventricular ejection fraction (LVEF) and decreased in scar tissues (Bolli *et al.*, 2011, Chugh *et al.*, 2012).

Nonetheless, ageing reduced the number of c-kit cardiac stem cells in patients (Hu *et al.*, 2014) and caused shortening in telomere length over time (Ellison *et al.*, 2013). These cells acquired senescent phenotype and their functions were impaired (Lewis-McDougall *et al.*, 2019). Since autologous stem cell transplantation is preferred, the efficacy of cardiac stem cells in heart regeneration from patients of old age group has been a concern. This prompted us to investigate if cardiac c-kit cells (CCs) could be isolated from aged mouse heart. If they do, were they similar in their phenotypes and functions? All these will be covered in Chapter 4.

Recent studies showed the synergistic effect of bone marrow mesenchymal stem cell (BMSCs) on c-kit cardiac stem cells. BMSCs were able to engraft and differentiate to cardiomyocytes, recruit resident c-kit cardiac stem cells to the infarct region and stimulate cardiomyocyte cell cycling (Hatzistergos *et al.*, 2010). Administration of combined BMSCs and c-kit cardiac stem cells showed improvement in heart function in swine (Williams *et al.*, 2012, Karantalis *et al.*, 2015) and rat model (Bao *et al.*, 2017). However, the function of

BMSCs have been shown to deteriorate with age (Stolzing *et al.*, 2008). Hence, combination stem cell therapy using BMSCs may not be ideal if the c-kit cardiac stem cells were also isolated from elderly patients.

Study showed that by exposing aged mice to young microenvironment, the aged mice regain youthful characteristics (Conboy *et al.*, 2005). Wharton's Jelly MSCs represent the youngest adult stem cells, and lack of ethical issues (Stolzing *et al.*, 2008, Fong *et al.*, 2011, Scheers *et al.*, 2013). Therefore, this study aimed to evaluate the effect of this biologically young human Wharton's Jelly mesenchymal stem cells (MSCs) on the function of aCCs, which will be covered in Chapter 4 and 5. The first chapter is dedicated to provide information on CCs, its contribution in stem cell therapy, and how these scientific evidences guided the design and rationale behind this study. The results of this study will bring in new ideas and future research in establishing critical role of c-kit CCs in cardiac regenerative therapy among elderly patients.

CHAPTER 2

LITERATURE REVIEW

2.1 Cardiovascular Disease Is the Number One Killer Disease Worldwide

Cardiovascular disease remains the number one killer disease worldwide, accounting for one-third of total death (WHO, 2016). The mortality rate reached 17.9 million of deaths in 2015 (Roth *et al.*, 2017), and is predicted to exceed 23.6 million by the year of 2030 in United States (Benjamin *et al.*, 2018). The increase in prevalence of the disease will be a burden to the economy as a result of increase expenditure in medical treatment, of which is estimated to reach $749 billion in 2035 from $204.8 billion in 2014 (Benjamin *et al.*, 2018). Furthermore, the prevalence of cardiovascular disease increases with age in both males and females according to National Health and Nutrition Examination Survey 2011-2014 (Figure 2.1) (Benjamin *et al.*, 2018).

In Malaysia, the estimated age standardised death rates in the year of 2012 declined by 22.6% and 19.1% for male and female, respectively. This is mainly due to the improvement in medical facilities and health awareness among the residents (WHO, 2014). The death rate continues to stay on top of all causes of death, which accounts for about 36% of total deaths in 2014 worldwide (Figure 2.2) (WHO, 2014).

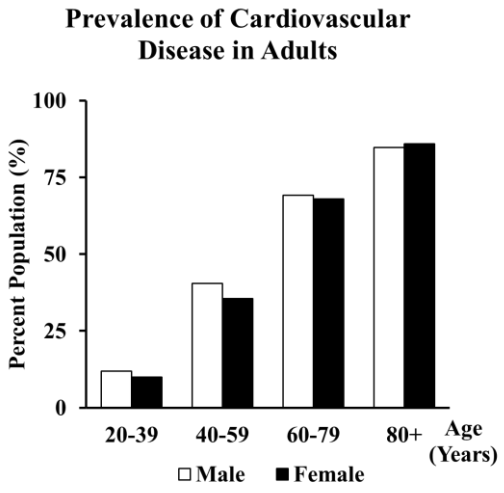

Figure 2.1: Prevalence of cardiovascular disease in United States (Source: National Centre for Health Statistics and National Heart, Lung, and Blood Institute) (Benjamin *et al.*, 2018)

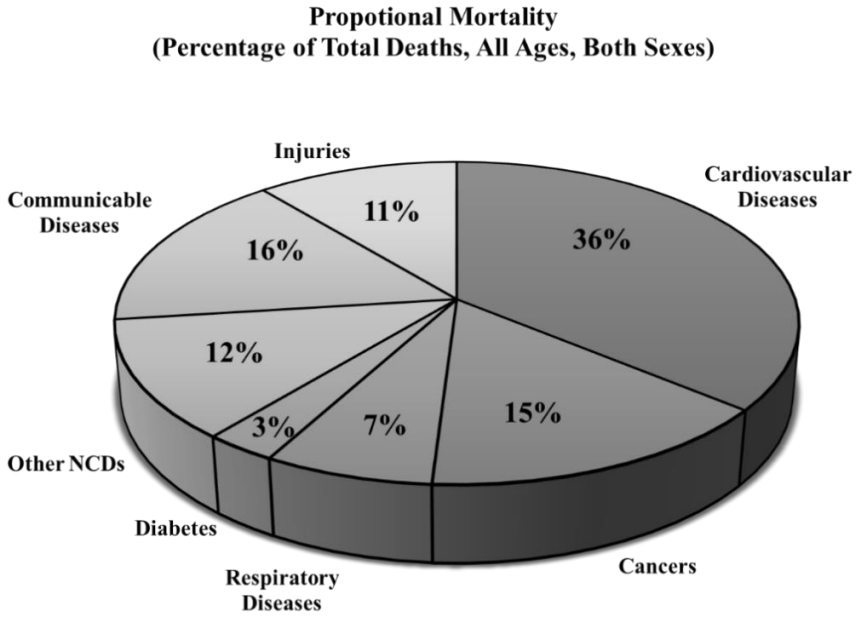

Figure 2.2: Proportional Mortality Rate of Cardiovascular Diseases from Total Deaths in 2014 (Source: World Health Organisation) (WHO, 2014).

2.2 Pathophysiology of Myocardial Infarction and Heart Failure

The most common form of cardiovascular disease is myocardial infarction (MI) (Benjamin *et al.*, 2018), pathological condition which is manifested by the lack of blood flow and oxygen following blockage of coronary vessels (Gabriel-Costa, 2018). Following MI, cardiomyocytes suffered from ischaemia, which weakens and damages its membrane integrity, causing intracellular content to release to the surrounding and finally die of necrosis and apoptosis (Orogo and Gustafsson, 2013). To compensate the loss of cardiomyocytes in the infarcted region, existing cardiomyocytes undergo hypertrophy to preserve tissue volume after infarction (Rubin *et al.*, 1983, Ginzton *et al.*, 1989). Sympathetic nervous system and the renin-angiotensin-aldosterone system are also being activated to help re-establishing the cardiac output and blood pressure of the heart (Triposkiadis *et al.*, 2009, Zucker *et al.*, 2014, Hartupee and Mann, 2017). However, continuous activation of these systems resulted in maladaptive compensatory mechanism and lead to heart failure (Braunwald, 2013, Gabriel-Costa, 2018). The first phase of tissue repair is the infiltration of leukocytes to the infarct site to remove dead cells. The fibroblasts are then activated by post-infarcted inflammatory cytokines which

progressively repopulate the infarcted region, and ultimately lead to scar tissue formation (Kawaguchi *et al.*, 2011, Frangogiannis, 2012, Turner and Porter, 2013). This event causes regional wall motion abnormality and left ventricular (LV) remodelling. If left untreated, patients will ultimately suffer from heart failure (Thygesen *et al.*, 2012).

In clinics, MI can be identified through clinical and pathological presentations, elevated necrosis biomarkers and medical devices such as electrocardiogram (ECG) or echocardiography (Thygesen *et al.*, 2007, Thygesen *et al.*, 2012). Patients with MI often develop ST-segment/T-wave changes or left bundle branch block and pathological Q waves. Acute coronary syndrome (ACS) is caused by rupturing of an atherosclerotic plaque, with subsequent platelet aggregation and thrombus formation (Kumar and Cannon, 2009). In clinical settings, ACS is classified into ST-elevation MI (STEMI), non-ST-elevation MI (NSTEMI) and unstable angina (UA) based on symptoms and clinical presentation for immediate treatment strategies (Kumar and Cannon, 2009).

Patients with STEMI are characterised by the presence of thrombus that is rich in fibrin, which completely occludes a coronary artery and causes transmural ischaemia (Agewall, 2008). Patients often undergo immediate reperfusion therapy to restore blood flow of the occluded coronary. Other treatments include pharmacological intervention with thrombolytic medication such as streptokinase and plasminogen activators in conjunction with percutaneous coronary intervention (Agewall, 2008). In contrast, NSTEMI patients are presented with platelet rich clot that partially occludes a coronary artery causing non-transmural ischaemia (Agewall, 2008). UA patients are presented with unstable plaque that can cause platelet aggregation, resulting in the formation of platelet-rich thrombus and ultimately thrombosis upon plaque rupture (Roe *et al.*, 2001). Early cardiac catheterisation or surgical bypass grafting is used to restore blood flow while pharmacological treatments include antiplatelet and antithrombotic drugs for treating the symptoms.

2.3 Current Treatment for Cardiovascular Diseases

2.3.1 Reperfusion Therapy

Reperfusion therapy helps restoring blood flow after MI. Currently available reperfusion therapy includes fibrinolytic therapy (FT) and primary percutaneous coronary intervention (PCI). PCI could improve the outcomes in patients with STEMI, provided it can be delivered in a timely manner (Antman *et al.*, 2004). The 'door-to-balloon' time of < 90 min after onset of MI increases the chances of survival following PCI (Boersma *et al.*, 1996, De Luca *et al.*, 2004, Antman, 2008). FT serves as an alternative therapy for PCI for patients with a delay up to 12-hr after the onset of symptoms (Pinto *et al.*, 2011). Most procedures are now performed with drug-eluting stents to reduce restenosis rates (Htay and Liu, 2005, Kastrati *et al.*, 2007). Although blood flow restoration salvages patient's life after MI, this therapy does not replace the lost cardiomyocytes.

2.3.2 Pharmacological Treatment

Advances in pharmacological intervention have led to the discovery of various cardio-protective drugs such as 1. statin which attenuates inflammatory response following MI (Patti *et al.*, 2010, Han *et al.*, 2018), 2. β-blockers to reduce heart rate and blood pressure, hence, reducing the myocardial workload and oxygen demand (Yusuf *et al.*, 1985, Kjekshus, 1986, Kezerashvili *et al.*, 2012) and 3. The angiotensin converting enzyme inhibitor decreases the production of angiotensin II, which then lowering blood pressure and attenuating LV remodelling (Khalil *et al.*, 2001, Susan *et al.*, 2005, Oh *et al.*, 2016). Nevertheless, current pharmacological strategies could only reduce the pain and minimise symptoms of the infarcted patients (Ramsdale *et al.*, 1982). In addition, patients might develop unknown adverse drug effect that may affect the functions of other organs, such as lung (Huang *et al.*, 2013), liver (Bhardwaj and Chalasani, 2007, Russo *et al.*, 2014, Bellosta and Corsini, 2018) and kidney (Adhiyaman *et al.*, 2001, Hörl, 2010). The effectiveness of cardio-protective drugs is also affected by the presence of other diseases, such as diabetes (Gyberg *et al.*, 2015). Although

these medications help to alleviate the symptoms of MI, they do not replace the dead cardiomyocytes.

2.3.3 Ventricular Assist Device Implantation

Current standard of left ventricular assisted device (LVAD) is performed through standard median sternotomy (Slaughter *et al.*, 2009). The implanted pumps allow the flowing of blood from left ventricle to ascending aorta and this is controlled by an external system (Slaughter *et al.*, 2009). LVAD prevents bradycardia and supports the heart function in potentially lethal ventricular arrhythmias (Ponikowski *et al.*, 2016). Hypertrophied cardiomyocytes were reversed in patients treated with LVAD for more than 6 months, with reduction in cardiomyocyte size and DNA damage response (Canseco *et al.*, 2015). The development of a much less invasive LVAD by miniaturising the assisted device and thus avoid the need for sternotomy has improved early survival (Schechter *et al.*, 2015). However, the failure of implanted devices in patient were mainly due to increased risk of device failure and infection after installing the device (Eckman and John, 2012, Ponikowski *et al.*, 2016, Kormos *et al.*, 2017).

2.3.4 Whole Heart Transplantation

Whole heart transplantation is the only curative option for end-stage heart failure. Patients receiving whole heart transplantation have achieved long-term survival for up to 20 years (Deuse *et al.*, 2008, Roussel *et al.*, 2008). However, limited number of organ donors, risk of rejection, and the complex surgical procedure make the procedure the least favourable to pursue (Tonsho *et al.*, 2014).

2.4 Stem Cells

Stem cells are primitive cells, which are capable of self-renewal, clonogenic and able to differentiate into specialised cell types (Gage *et al.*, 1995, Sobhani *et al.*, 2017). They can be classified based on their differentiation plasticity: totipotent, pluripotent, multipotent and unipotent. Totipotent cells can give rise to all types of cells in the body as well as

extraembryonic placental cells. Pluripotent cells can give rise to almost every cell in an organism. Multipotent cells can give rise to limited more than one cell types, while cells with unipotent capability can only give rise to one cell type (Sobhani *et al.*, 2017). The most commonly known stem cell types are pluripotent stem cells. Pluripotent stem cells can be obtained from inner cell mass of an embryo, which possess the ability to form an organism (Evans and Kaufman, 1981). Furthermore, stem cells can also be isolated from adult tissues. These cells are mainly multipotent or unipotent, where they can give rise to a more specific and committed cell type based on their origins (Sobhani *et al.*, 2017). Stem cells divide asymmetrically to a more committed daughter cells and also primitive cells, to ensure stemness and self-renewal (Knoblich, 2008, Gómez-López *et al.*, 2014).

2.4.1 Pluripotent Stem Cells

Embryonic stem cells (ESCs) have been long discovered and shown to be able to give rise to basically every cell type in the body. Nonetheless, the fact that this cell type has to be isolated from inner cell mass of an embryo leads to ethical debates (Lalit *et al.*, 2014). The discovery of induced pluripotent stem cells (PSCs), which was first described by Yamanaka *et al.* (Takahashi and Yamanaka, 2006), could be derived from genetically reprogrammed adult fibroblast cells with four pluripotent factors (*Oct4*, *Sox2*, *Klf4*, *cMyc*). Induced PSCs share similar properties with ESCs. They are pluripotent and could be provided as off-the-shelf products. Various methods have been published to differentiate PSCs to cardiac lineages (Laflamme *et al.*, 2007, Kattman *et al.*, 2011, Lian *et al.*, 2013). Preclinical transplantation of PSC-derived cardiomyocytes demonstrated cardiac function improvement in rat (Laflamme *et al.*, 2007), pig (Kawamura *et al.*, 2012) and monkey (Shiba *et al.*, 2016). Although no tumour formation has been observed, the risk of teratoma formation from undifferentiated PSCs remains as the major concern (Mora *et al.*, 2017). Furthermore, the transplantation of PSC-derived cardiomyocytes could contribute to ventricular arrhythmias (Shiba *et al.*, 2016) and also risk of rejection if allogeneic cells were used (Lu *et al.*, 2013).

2.4.2 Adult Stem Cells

Adult stem cells are undifferentiated cells that can proliferate, self-renew and multipotent. They are present in small number in different organs such as skeletal muscle (Formigli *et al.*, 2010), bone marrow (Behfar *et al.*, 2014) and heart (Beltrami *et al.*, 2003). The ability of these adult stem cells to replace cell lost have drawn the attention of scientist to investigate the effect of various adult stem cells for treating MI as they can be easily isolated and expanded *in vitro* (Beltrami *et al.*, 2003, Formigli *et al.*, 2010, Behfar *et al.*, 2014). Adult stem cells are the only stem cell type thus far that has shown successes in engraftment, differentiation, and improvement in heart function following transplantation.

2.5 Adult Stem Cells for Heart Regeneration

2.5.1 Skeletal Myoblasts

Skeletal myoblasts have been tested in human trials (Table 1.2) (Menasche *et al.*, 2001, Menasche *et al.*, 2003, Smits *et al.*, 2003, Siminiak *et al.*, 2004, Dib *et al.*, 2005, Siminiak *et al.*, 2005, Gavira *et al.*, 2006, Menasche *et al.*, 2008, Durrani *et al.*, 2010) because they can form contractile elements when in contact with cardiomyocytes *in vitro* (Durrani *et al.*, 2010). They can be isolated as satellite cells from skeletal muscle (Formigli *et al.*, 2010) and these cells can be expanded *in vitro* from patient's biopsied muscle within two to three weeks (Menasche *et al.*, 2003). Transplantation of autologous skeletal myoblast to patients with severe ischaemic cardiomyopathy showed no perioperative complications following cell transplantation (Menasche *et al.*, 2003). This is followed by mixed results in other skeletal myoblast-treated patients (Smits *et al.*, 2003, Siminiak *et al.*, 2004, Dib *et al.*, 2005, Gavira *et al.*, 2006). Furthermore, skeletal myoblasts also failed to develop intercalated discs following transplantation (Ferreira-Cornwell *et al.*, 2002) and limited by the incidence of ventricular arrhythmias following myoblast transplantation, due to propagation of different action potentials between skeletal myoblasts and host cardiomyocytes (Menasche *et al.*, 2003, Abraham *et al.*, 2005). In another randomised phase II Myoblast Autologous Grafting in Ischaemic Cardiomyopathy (MAGIC) trial, which demonstrated no improvement in left

ventricular ejection fraction (LVEF) (Menasche *et al.*, 2008). Similarly, a double-blinded, randomised controlled study (MARVEL-1) also showed higher risk of ventricular tachycardia following skeletal myoblast intramyocardial injection in patients with MI (Povsic *et al.*, 2011). These findings limit the use of skeletal myoblast in treating the damaged heart. The list of clinical trials using skeletal myoblasts was shown in Table 2.1.

Table 2.1: List of Clinical Trials Using Skeletal Myoblasts for Heart Regeneration

Clinical Trials	Number of Patients	Duration (Months)	Changes in LVEF (%)	References
Menasche *et al.* (2003)	9	10.9	+8%	(Menasche *et al.*, 2003)
Smits *et al.* (2003)	5	6	+7%	(Smits *et al.*, 2003)
Siminiak *et al.* (2004)	10	12	+6%	(Siminiak *et al.*, 2004)
Dib *et al.* (2005)	30	24	+8%	(Dib *et al.*, 2005)
Gavira *et al.* (2006)	12	12	+20%*	(Gavira *et al.*, 2006)
Menasche *et al.* (2008)	97	6	+4%	(Menasche *et al.*, 2008)

*Significant improvement in LVEF ($p < 0.05$)

2.5.2 Bone Marrow-derived Mononuclear Cells

Bone marrow is a home to multiple stem cell populations with regenerative potential, namely the bone marrow mononuclear cells (BMMNCs), mesenchymal stem cells (MSCs) and haematopoietic stem cells (HSCs). Orlic *et al.* first reported events of neomyogenesis in a mouse model of MI following intramyocardial injection of bone marrow cells (Orlic *et al.*, 2001). This study was then sparked tremendous interest in using the BMMNCs from bone marrow aspirate to treat damaged myocardium and soon initiated first cell therapy in human trials due to its high availability and feasibility (Behfar *et al.*, 2014). Various human clinical trials have been performed to test the functional efficacy of BMMNCs in heart repair.

Stauer *et al.* (2002) demonstrated that intracoronary administration of autologous BMMNC in human improved cardiac function (Strauer *et al.*, 2002). Intracoronary

administration of autologous BMMNCs also showed improvement in global LVEF in acute MI (Wollert *et al.*, 2004). Similar results have also been reported in other randomised controlled trials (Assmus *et al.*, 2002, Schachinger *et al.*, 2004, Afzal *et al.*, 2015). Intracoronary administration of enriched $CD133^{Pos}$ BMMNCs was demonstrated to be safe in COMPARE-AMI trial (Mansour *et al.*, 2010), able to improve LV performance, increase myocardial perfusion, and enhance cell viability in acute MI patients (Bartunek *et al.*, 2005). Although BMMNCs has been shown to be safe (Strauer *et al.*, 2002, Janssens *et al.*, 2006, Mansour, 2016), the outcomes showed mixed results (Dawn *et al.*, 2009). Some studies suggest that BMMNCs has not been beneficial to the damaged heart (Janssens *et al.*, 2006, Meyer *et al.*, 2006, Gowdak *et al.*, 2008, Meyer *et al.*, 2009, Nowbar *et al.*, 2014). TIME randomised trial (Traverse *et al.*, 2012) and Late TIME trial (Traverse *et al.*, 2012) were performed to elucidate if duration of injection affects the outcome of BMMNCs in heart regeneration. Both studies showed no improvement in LVEF regardless of time of injection after acute MI. Similarly, intracoronary administration of autologous BMMNCs in REGENERATE-AMI trial demonstrated reduction in infarct size but no improvement in LVEF (Choudry *et al.*, 2016). Some key clinical trials which were conducted are summarised in Table 2.2.

Table 2.2: List of Clinical Trials Using Bone Marrow-derived Mononuclear Cells for Heart Regeneration

Clinical Trials	Number of Patients	Duration (Months)	Changes in LVEF(%)	References
TOPCARE-AMI (2002)	20	4	+8.5%*	(Assmus *et al.*, 2002)
TOPCARE-AMI (2004)	59	4	+8.0%*	(Schachinger *et al.*, 2004)
BOOST (2004)	60	6	+6.7%*	(Wollert *et al.*, 2004)
BOOST (2006)	60	6	+5.9%	(Meyer *et al.*, 2006)
REPAIR-AMI (2006)	204	4	+10.5%*	(Schachinger *et al.*, 2006)
LEUVEN-AMI (2006)	67	4	+3.3%	(Janssens *et al.*, 2006)
ASTAMI (2006)	97	6	+3.1%	(Lunde *et al.*, 2006)
TCT-STAMI (2006)	20	6	+4.8%*	(Ge *et al.*, 2006)
TOPCARE-CHD (2007)	121	3	+1.8%*	(Assmus *et al.*, 2007)
Gowdak (2008)	10	12	+4%	(Gowdak *et al.*, 2008)
FINCELL (2008)	80	6	+4%*	(Huikuri *et al.*, 2008)
HEBE (2008)	26	12	+2.2%*	(Hirsch *et al.*, 2008)
BOOST (2009)	60	61	-2.5%	(Meyer *et al.*, 2009)
ASTAMI (2009)	100	36	+1.8%	(Beitnes *et al.*, 2009)
REGENT (2009)	200	6	+3%*	(Tendera *et al.*, 2009)
Traverse (2010)	40	6	+6.2%	(Traverse *et al.*, 2010)
BONAMI (2010)	101	3	+3.3%	(Roncalli *et al.*, 2011)
REPAIR-AMI (2010)	204	24	+4.7%*	(Assmus *et al.*, 2010)
FOCUS-HF (2011)	30	6	+4.5%*	(Perin *et al.*, 2011)
HEBE (2011)	200	4	+3.8%*	(Hirsch *et al.*, 2011)
Late-TIME (2011)	87	6	+0.5%	(Traverse *et al.*, 2011)
TOPCARE-AMI	55	60	+11%*	(Leistner *et al.*, 2011)
TIME (2012)	120	6	+3.2%	(Traverse *et al.*, 2012)
Antoinitsis (2012)	9	12	+21.2%*	(Antonitsis *et al.*, 2012)
FOCUS-CCTRN (2012)	92	6	+1.4%	(Perin *et al.*, 2012)
SWISS-AMI (2013)	200	4	+1.4 (For early injection) +1.1% (For late injection)	(Surder *et al.*, 2013)

*Significant improvement in LVEF ($p < 0.05$)

2.5.3 Bone Marrow-derived Mesenchymal Stem Cells

Bone marrow cells are composed of haematopoietic or non-haematopoietic cells. Bone marrow-derived mesenchymal stem cells (BMSCs) are the multipotent precursors of non-haematopoietic lineages and possess the ability to differentiate into adipose, bone, cartilage, skeletal muscle, neural, and other cell types (Dominici *et al.*, 2006). BMSCs can be harvested from bone marrow, they are proliferative *in vitro*, and can be stored for long term use, or served as an off-the-shelf product. Several studies documented that BMSCs were able to differentiate into cardiomyocytes *in vitro* with the use of demethylating agent such as 5-azacytidine (Xu *et al.*, 2004, Antonitsis *et al.*, 2007), or through co-culture with cardiomyocytes (Cai *et al.*, 2012a). Furthermore, these cells expressed low MHC class I and lacked MHC class II (Schu *et al.*, 2012), thus making BMSCs as a better allogeneic cell source for heart regeneration as compared to many other cell types.

Clinically, intracoronary infusion of BMSCs shortly after acute MI has been shown to be safe and feasible for transplantation, and able to improve LV function (Chen *et al.*, 2004, Kim *et al.*, 2018a) for up to five years of follow-up (Rodrigo *et al.*, 2013). In POSEIDON-DCM trial (Hare *et al.*, 2012), patients were randomised to either allogeneic or autologous BMSCs intravenously and followed for up to 12 months (Hare *et al.*, 2012). The study showed that greater improvement in LV function in non-ischaemic dilated cardiomyopathy patients was observed in allogeneic as compared to autologous BMSCs (Hare *et al.*, 2017). More importantly, allogeneic BMSC transplantation did not exert immune response in patients (Hare *et al.*, 2017). MESAMI phase I pilot study was conducted to introduce BMSCs through intramyocardial injection in patients with chronic ischaemic cardiomyopathy (Guijarro *et al.*, 2016). They observed significant improvements in LVEF at 12-month follow-up albeit with a smaller sample size of ten (Guijarro *et al.*, 2016).

In Phase II placebo-controlled randomised MSC-HF trial, patients suffered from heart failure who received a high number of autologous BMSCs through intramyocardial administration showed greater functional improvement in the ischaemic heart after 12 months,

suggesting a positive correlation between cell dose and disease severity (Mathiasen *et al.*, 2015). In the TRIDENT study, low dose (20 million) and high dose (100 million) cells were tested by transendocardially administered to patients with ischaemic cardiomyopathy for up to 12 months (Florea *et al.*, 2017). Subjects who were given high dose BMSCs showed significant improvement in LVEF, which suggests that the dose of administered BMSCs does dictate the functional outcome (Florea *et al.*, 2017). Another study which administered 25, 75, or 150 million cells of allogeneic bone marrow mesenchymal precursor cells in chronic heart failure patients in a phase II trial by transendocardial injection. Chronic heart failure patients administered with 150 million cells showed significant reduction in major adverse cardiac events at 12-months followed up despite no significant improvement in LVEF was observed (Perin *et al.*, 2015). This suggests high dose of BMSC administration is necessary for gaining greater therapeutic benefit in damaged heart. Table 2.3 lists the major human clinical trials of using BMSCs in heart regeneration.

Table 2.3: List of Clinical Trials Using Bone Marrow-derived Mesenchymal Stem Cells for Heart Regeneration

Clinical Trials	Number of Patients	Duration (Months)	Changes in LVEF(%)	References
Chen (2004)	69	6	+18%*	(Chen *et al.*, 2004)
Hare (2009)	53	6	+6.5%	(Hare *et al.*, 2009)
POSEIDON (2012)	30	13	+1.65% (Allogeneic) +2.3% (Autologous)	(Hare *et al.*, 2012)
PROMETHEUS (2014)	6	18	+10.1%*	(Karantalis *et al.*, 2014)
SEED-MSC (2014)	80	6	+1.6%*	(Lee *et al.*, 2014)
TAC-HFT (2014)	65	12	+7.6%	(Heldman *et al.*, 2014)
MSC-HF (2015)	55	6	+5.0%*	(Mathiasen *et al.*, 2015)
MESAMI (2016)	10	12	+6.3%*	(Guijarro *et al.*, 2016)

*Significant improvement in LVEF ($p < 0.05$)

2.5.3(a) Indirect Effects of BMSCs in Cardiac Therapy

The BMSCs can secrete a wide array of cytokines, chemokines and growth factors (Mirotsou *et al.*, 2007, Markel *et al.*, 2008). Conditioned medium derived from BMSCs attenuated cardiac fibroblast proliferation through down-regulation of genes regulating cellular proliferation and inhibition of type I and III collagen synthesis *in vitro* (Ohnishi *et al.*, 2007). Paracrine factors secreted by BMSCs exerted anti-apoptotic effects on cultured cardiomyocytes and endothelial cells under conditions that mimic ischaemia in mice model with acute MI (Iso *et al.*, 2007). Secreted factor such as vascular endothelial growth factor (VEGF) is primarily present in conditioned medium generated from BMSCs, which is responsible for cardioprotection and angiogenic effects in MI rat heart (Gao *et al.*, 2007). This observation is further supported using VEGF- and hepatocyte growth factor- (HGF-) overexpressing murine BMSCs, which showed improvement in ventricular ejection function and reduction in scar size (Deuse *et al.*, 2009).

Recently, BMSC-derived extracellular vesicles, including exosomes and microvesicles have been investigated. Exosomes secreted from GATA4 overexpressing BMSCs are anti-apoptotic and could protect heart function through miRNAs-mediated activation of cell survival signalling pathways (Yu *et al.*, 2015). BMSCs released extracellular vesicles upon hypoxia, promoted neoangiogenesis and preserved cardiac function following intramyocardial injection into acute MI rat model (Bian *et al.*, 2014). In acute MI rat model, administration of BMSC exosomes can enhance cardiac function and promote neovasculogenesis (Teng *et al.*, 2015).

2.5.3(b) Direct Transdifferentiation of BMSCs into Cardiomyocytes

Mechanism of which transplanted BMSCs transdifferentiate into cardiomyocytes has been proposed. BMSC can differentiate into cardiomyocyte *in vitro* when induced by 5-azacytidine, although the differentiation is extremely rare under physiological conditions (Martin-Rendon *et al.*, 2008, Mu *et al.*, 2011). The combination of angiotensin II and 5-azacytidine can further promoting BMSC differentiation into cardiomyocyte-like cells (Xing

et al., 2012). Engrafted human BMSCs in the mice myocardium could differentiate into cardiomyocytes (Toma *et al.,* 2002). However, the BMSCs-derived cardiomyocytes still retained their stromal phenotype and could not become functional cardiomyocytes *in vitro* despite expressing cardiac-specific markers (Rose *et al.,* 2008). In another study, BMSCs-overexpressing Akt transplantation has superior effect in engraftment within the infarcted myocardium. Authors observed a rare event whereby BMSC fused with cardiomyocytes as well as low rate of BMSC transdifferentiation (Noiseux *et al.,* 2006). *In vitro* co-culture model of human BMSCs and neonatal rat ventricular cardiomyocytes has demonstrated active cell fusion between these two cells (Shadrin *et al.,* 2015). While these hybrids showed electrophysiological properties and evidence of cytoplasmic content exchanges, there was no fusion of nucleus being observed (Shadrin *et al.,* 2015). This event might be closely related to gap junctional coupling between BMSCs and cardiomyocytes, at which the inhibition of cardiac specific transcription factors NKX2.5 and GATA4 reduced with cell-cell gap junction inhibition (Lemcke *et al.,* 2017). However, these hybrid cells are not proliferative, and the event of fusion is extremely rare (Shadrin *et al.,* 2015).

2.6 Endogenous Cardiac Stem Cells

Mammalian heart was once believed to be a terminally differentiated organ, with no regenerative capacity. Although majority of the cardiomyocytes are permanently withdrawn from the cell cycle, emerging evidence demonstrates that cardiomyocyte proliferation exists albeit at a very low rate (Bergmann *et al.,* 2009, Senyo *et al.,* 2013), with a turnover rate of about 1% (Bergmann *et al.,* 2009) or 4-10% (Senyo *et al.,* 2013). Other study showed a 3.4-fold increment in the number of cardiomyocytes from 1 to 20 years of age based on cell cycle activity (Mollova *et al.,* 2013). Cardiomyocyte turnover rate as measured by thymidine analogue iododeoxyuridine method, however, reported as high as 22% of the cardiomyocytes are replaced and renewed annually (Kajstura *et al.,* 2010b). In addition, the genomic C-14 method demonstrated the number of cardiomyocytes remain constant over the lifetime starting 1 month after birth (Bergmann *et al.,* 2009). There was limited cardiomyocyte turnover, with

the cardiomyocyte renewal of 1% turning over annually at the age of 20 (Bergmann *et al.*, 2009).

Despite differences reported in the rate of cardiomyocyte turnover, consensus was reached that the heart is not a post-mitotic organ (Bergmann *et al.*, 2009, Senyo *et al.*, 2013). In new-born mice, the generation of new cycling cardiomyocytes decreased dramatically upon delivery (Walsh *et al.*, 2010). However, the authors revealed that postnatal cardiomyocyte generation is a result of a short proliferative burst day 14. This means that postnatal cardiomyocytes retain the potential to proliferate but activity surged at day 14 to contribute to the final cardiomyocyte number in mice via insulin growth factor-1/insulin growth factor-1-receptor/Akt (IGF-1/IGF-1-R/Akt) pathway (Naqvi *et al.*, 2014).

Nevertheless, adult mammalian heart has limited regenerative capability and ageing can affect cardiomyocyte turnover (Bergmann *et al.*, 2009). The estimated cardiomyocyte turnover rate is 1% at the age of 25 years old and declines to 0.45% at the age of 75 (Bergmann *et al.*, 2009). Studies showed that the heart of one-day-old mouse can be fully regenerated after MI induction (Porrello *et al.*, 2011, Haubner *et al.*, 2012). However, the regeneration capacity is lost in seven-day-old MI mouse as evidenced by scarring in the heart which is similar to the adult after 21 days (Porrello *et al.*, 2011, Haubner *et al.*, 2012). In a case study, the heart of a human new-born child with severe MI due to coronary artery occlusion was able to recover completely (Haubner *et al.*, 2016). This again suggests that inert heart regeneration is greatly dependent on age (Bergmann *et al.*, 2009, Porrello *et al.*, 2011, Haubner *et al.*, 2012). To investigate the difference in dividing myocytes in the normal and diseased heart, Kajstura *et al.* collected human hearts from patients 19 to 104 years old and investigated the magnitude of myocyte regeneration (Kajstura *et al.*, 2010a). They observed inverse relationship between young myocytes and ageing, with 0.69% and 0.89% of the young myocytes declines per year in women and men, respectively. The declines were associated with an increased number of senescent cells (Kajstura *et al.*, 2010a). Therefore, the search for suitable cell candidates for treating infarcted heart continues.

As the loss of cardiomyocytes post-MI could not be replaced merely by pharmacology interventions, stem cell therapy offers hopes to reconstitute infarcted heart either by secreting cardioprotective paracrine factors integrate with the target tissues and differentiate into functional cardiomyocytes to replenish the pool of cardiac muscle cells. Studies have shown that the heart harbours a group of cardiac stem cells that can be harvested, purified, expanded *in vitro* and finally implanted into patient own heart in autologous transplantation (Beltrami *et al.*, 2003, Bearzi *et al.*, 2007, Bolli *et al.*, 2011). Cardiac stem cells have been isolated by different groups based on their surface marker expression (SCA-1 (Oh *et al.*, 2003) and c-kit (Beltrami *et al.*, 2003)), functional properties such as the ability to efflux dye Hoechst 33342 (Side population) (Pfister *et al.*, 2005), and the factor-stimulated self-aggregated 3-dimensional multicellular clusters (a.k.a. cardiospheres) (Messina *et al.*, 2004).

2.7 Types of Cardiac Stem Cells

2.7.1 SCA-1 Cardiac Stem Cells

Oh *et al.* first reported a population of SCA-1-expressing cells from murine adult heart, which was distinct from haematopoietic origin as they lacked CD34, CD45 markers. These cells expressed cardiac transcription factors such as GATA4 and MEF2C (Oh *et al.*, 2003), and could differentiate into cardiomyocytes *in vitro* in the presence of 5-azacytidine or oxytocin (Matsuura *et al.*, 2004). When these cells were transplanted into mice intravenously, cells that also co-expressing chemokine receptor type 4 (CXCR4) migrated to the injured myocardium and differentiated into cardiomyocytes following MI (Oh *et al.*, 2003, Oh *et al.*, 2004). Cardiac stem cells co-expressing SCA-1 and WT1 in the mouse heart have been demonstrated to give rise to *de novo* cardiomyocytes that structurally and functionally integrate with resident heart muscle after MI (Smart *et al.*, 2011). Chong *et al.* (2011) have recently demonstrated that a population of SCA-1 and PDGFRα positive cells derived from proepicardium of mouse heart has the capacity for clonogenic propagation, long term *in vitro* growth, and multilineage differentiation both *in vitro* and *in vivo* (Chong *et al.*, 2011). Nonetheless, SCA-1 positive cardiac stem cells in human have not been identified so far.

Human SCA-1-like cardiac cells has been shown to express early cardiac transcription factors such as GATA4, and NKX2.5 and could achieve better cardiac differentiation upon 5-azacytidine treatment (Smits *et al.*, 2009).

2.7.2 Side Population Cells

Functionally, cardiac stem cells can be isolated based on the ability to efflux metabolic dyes through overproduction of ATP-binding cassette transporters (ABCG2 or MDR-1). ABCG2 protects and regulates homeostasis and function of cardiac side population (SP) cells (Pfister *et al.*, 2008). Loss of ABCG2 expression in *Abcg2* knockout mice lead to reduction in proliferation capacity and increased cell death (Pfister *et al.*, 2008). SP cells were identified based on the ability to efflux dye Hoechst 33342 (Goodell *et al.*, 1996), and their presence ranged from 0.03% to 3.5% of total mononuclear cardiac cells (Pfister *et al.*, 2005). SP cells express SCA-1 but not haematopoietic markers such as CD34 and CD45 (Martin *et al.*, 2004, Pfister *et al.*, 2005). Pfister *et al.* demonstrated the presence of two types of SP cells based on the surface marker CD31 (Pfister *et al.*, 2005, Liang *et al.*, 2010). They showed that only cardiac SP cells that does not express CD31 could differentiate into functional cardiomyocytes *in vitro* (Liang *et al.*, 2010). When these cells were co-cultured with neonatal cardiomyocytes, they expressed cardiac differentiation markers, suggesting the ability of these cells to form cardiomyocytes (Hierlihy *et al.*, 2002). Furthermore, SCA-1-expressing cardiac cells could migrate from non-ischaemic myocardium into the infarcted area where they differentiated into both cardiomyocyte and endothelial-like myocardium (Liang *et al.*, 2010) through up-regulation of CXCR4 (Oyama *et al.*, 2007), a receptor for the chemotactic cytokine known as stromal derived factor 1 (SDF-1) (Liang *et al.*, 2010, Liang *et al.*, 2011).

2.7.3 Cardiospheres

Cardiospheres are characterised by a mixed population of cardiac cells that grow as three dimensional (3D) clusters *in vitro* (Messina *et al.*, 2004). They are derived from the outgrowth cells from atrial or ventricular explant which were then induced with factors to aggregate and form spherical structures (Messina *et al.*, 2004). They expressed endothelial

(KDR/FLK-1, CD31) and stem cell (CD34, c-kit, SCA-1) markers (Messina *et al.*, 2004). They could spontaneously differentiate into cardiomyocytes and endothelial cells, which were sphere size and culture time dependent. These cardiospheres were self-adherent cell clusters that can grow as monolayer culture on plastic surface and were termed cardiosphere-derived cells (CDCs) (Messina *et al.*, 2004). CDCs are a mixture of stromal, mesenchymal and progenitors which are comprised of c-kit, CD31 and CD34 cells (Davis *et al.*, 2009). They are clonogenic, capable of long-term self-renewal, and can commit to cardiac, smooth muscle and vascular lineages both *in vitro* and *in vivo* (Messina *et al.*, 2004, Smith *et al.*, 2007). Similar to SCA-1-expressing cells, these cells also expressed CXCR4 under hypoxic condition and could migrate to injured myocardium following intravenous injection (Tang *et al.*, 2010). In view of its promising regenerative capability in animal studies, autologous CDCs were first tested in Phase I clinical trial (CADUCEUS), on patients with acute MI. The outcome showed reduced infarct size and increased viable heart mass at six months. However, no difference in LVEF was observed as shown in Table 2.4 (Makkar *et al.*, 2012). Furthermore, CDCs were also found to possess the angiogenic potential and can secrete variety of growth factors including angiopoietin, basic fibroblast growth factor (bFGF), HGF, IGF-1 and VEGF (Li *et al.*, 2012).

Table 2.4: List of Clinical Trials Using Cardiosphere-derived Cells for Heart Regeneration

Clinical Trials	Number of Patients	Duration (Months)	Changes in LVEF(%)	References
CADUCEUS (2012)	25	6	+5.4%	(Makkar *et al.*, 2012)
CADUCEUS (2014)	25	12	+5.4%	(Malliaras *et al.*, 2014)

*Significant improvement in LVEF ($p < 0.05$)

2.7.4 Islet-1 Cardiac Progenitor Cells

Laugwitz et al. (2005) first described the presence of LIM homeodomain transcription factor Islet-1 (ISL-1) cardiac progenitor cells in the heart of rat, mouse and human (Laugwitz *et al.*, 2005). These cells were mainly found in neonatal heart (Cai *et al.*, 2003), forming

outflow tract, right ventricle, and atrial (Cai *et al.*, 2003). To recapitulate developmental precursors in the embryonic heart, embryonic derived ISL-1 cardiac progenitor cells expressing NKX2.5 and FLK1 were shown to differentiate into cardiomyocytes, smooth muscle, and endothelial cells (Moretti *et al.*, 2006). Despite limited *in vitro* expansion, ISL-1 positive ventricular progenitor cells have been shown to differentiate into functional ventricular muscle tissues (Domian *et al.*, 2009). ISL-1 cardiac progenitor cells persisted in the proximal outflow tract during adulthood (Genead *et al.*, 2010) but their number was rare with only 500-600 cells being detectable in the myocardium of a one to five day old rat (Laugwitz *et al.*, 2005). Furthermore, the ISL-1 expression gradually lost the differentiation capacity with age (Weinberger *et al.*, 2012).

2.8 c-kit Cardiac Stem Cells

2.8.1 Characteristics of c-kit Cardiac Stem Cells

Cardiac stem cells were first identified in the adult rat heart (Beltrami *et al.*, 2003). These cells were known to be clonogenic, able to self-renew, and multipotent (Kawaguchi *et al.*, 2010). Attempts to isolate cardiac stem cells had also been extended to mice (Smith *et al.*, 2014), canine (Linke *et al.*, 2005) and human (Bearzi *et al.*, 2007, He *et al.*, 2011) following the report, based on the surface marker c-kit (Beltrami *et al.*, 2003, Ellison *et al.*, 2013, Smith *et al.*, 2014). Study have shown that constitutive expression of c-kit promotes cardiac repair through enhanced angiogenic and myogenic response after injury in mice (Di Siena *et al.*, 2016). Likewise, absence of c-kit signalling in the heart worsened cardiac remodelling after MI (Di Siena *et al.*, 2016). The isolated c-kit cardiac stem cells from the transgenic mice constitutively expressing c-kit also showed higher growth potential, clonogenicity, and cardiomyocyte differentiation as compared to c-kit cardiac stem cells from wild type mice *in vitro* (Di Siena *et al.*, 2016). Kawaguchi *et al.* (2010) isolated a group of clonogenic c-kit cardiac stem cells that expressed high levels of GATA4 and found that these cells have better potential in heart regeneration by increasing cardiomyocyte survival and contractility through IGF-1/IGF-1R/Akt pathway (Kawaguchi *et al.*, 2010).

Smith *et al.* (2014) demonstrated the freshly isolated c-kit cardiac stem cells and showed that they expressed SCA-1, CD90, CD140a, and CD166 but negative for haematopoietic markers such as CD34 and endothelial markers such as CD31 (Smith *et al.*, 2014). The cells also expressed MSC-related markers such as CD29, CD44, CD105, and CD90 (Gambini *et al.*, 2011). Some studies suggested that the isolated c-kit cardiac stem cells were heterogeneous, and can be divided into two distinct populations based on their differentiation potency, namely the myogenic or the vasculogenic cardiac stem cells (Hosoda, 2012). Myogenic cardiac stem cells are more committed to differentiate into myocyte lineage and they expressed c-kit but not KDR (Hosoda, 2012). Whereas, vasculogenic cardiac stem cells expressed both c-kit and KDR, and found to reside mainly in the vascular niche of the vessel wall, which derive vascular endothelial or smooth muscle cells (Hosoda, 2012).

These cells also showed expression of cardiac transcription factors such as GATA4 and NKX2.5 (Kawaguchi *et al.*, 2010, Smith *et al.*, 2014). These two markers are early markers of early cardiac committed cells. The crosstalk between these two cardiac transcription factors are critical for early cardiogenesis (Durocher *et al.*, 1997). SOX2 has been known to maintain cell pluripotency (Park *et al.*, 2012). Cardiac c-kit cells also expressed SOX2, suggesting that the cells are partially committed to cardiac lineage but at the same time maintaining cell pluripotency (Smith *et al.*, 2014, Leong *et al.*, 2018). TERT encodes for telomerase reverse transcriptase, a core catalytic subunit of telomerase enzyme, helps protecting chromosome ends from shortening by adding small repeated DNA to the ends of chromosomes, thus, maintaining telomere integrity (Nugent and Lundblad, 1998, Osterhage and Friedman, 2009, O'Sullivan and Karlseder, 2010). This is also one of the important genes indicating stemness of cells.

2.8.2 Cardiac Stem Cell Niche and Activation

2.8.2(a) Niche and Location

The niche of c-kit cardiac stem cells is mostly found in the atria and apex, the region with low haemodynamic stress surrounded by differentiated myocytes (Leri *et al.*, 2005,

Bearzi *et al.*, 2007). These cells can divide symmetrically and asymmetrically, but asymmetrical division is more predominant to maintain the pool of stem cells while deriving a more committed daughter cell (Urbanek *et al.*, 2006, Bearzi *et al.*, 2007, Hosoda, 2012). The stem cells that reside in the hypoxic niche microenvironment are mostly quiescent (Sanada *et al.*, 2014), and oxygen is required for re-activation of the quiescent cells to re-enter cell cycle. The quiescent stem cells within the hypoxic niche could be stimulated to re-enter cell cycle by injury (Sanada *et al.*, 2014), be it the infarction (Docshin *et al.*, 2018) or drug induced cardiotoxic injury such as isoproterenol (Ellison *et al.*, 2013).

Ellison *et al.* (2011) showed that IGF-1 and HGF are key growth factors to activate c-kit cardiac stem cells, and administration of the two factors in combination with cardiac stem cells promote cardiac repair in infarcted swine heart (Ellison *et al.*, 2011) with profound increased bromodeoxyuridine (BrdU) labelled cardiac stem cells and new cardiomyocyte formation (Ellison *et al.*, 2011).

2.8.2(b) Function of c-kit Cardiac Stem Cells

Intravenous infusion of adult c-kit cardiac stem cells ameliorates LV remodelling in mice model by reducing fibrosis, cardiomyocyte hypertrophy and increasing cardiomyocyte density in the LV myocardium (Kazakov *et al.*, 2015). c-kit regulates the homeostasis between pro- and anti-angiogenic proteins in the LV myocardium towards pro-angiogenic mediators to increase endothelial cell density (Kazakov *et al.*, 2015). Thus, the transplantation of c-kit cardiac stem cells can ameliorate oxidative stress in cardiomyocytes and non-cardiomyocytes after stem cell transplantation (Kazakov *et al.*, 2015). Intracoronary infusion of autologous cardiac stem cells improves regional and global LV function by promoting cardiac and vascular regeneration in swine (Bolli *et al.*, 2013). The transplanted cells expressed cardiac specific markers in the infarcted region, suggesting cardiac stem cells can be differentiated into cardiomyocytes (Bolli *et al.*, 2013).

Cardiac stem cells are able to differentiate into cardiomyocytes, smooth muscle and endothelial cells (Beltrami *et al.*, 2003). Cardiac troponin I is a marker to identify mature cardiomyocytes (Bedada *et al.*, 2014). *In vitro*, the differentiated cardiomyocytes were identified by cardiac troponin I, which has been widely used to confirm cardiac lineage commitment (Beltrami *et al.*, 2003, Kawaguchi *et al.*, 2010). Smooth muscle actin is present on actin cytoskeleton of smooth muscle cells (Lehman and Morgan, 2012), while von Willebrand factor is an endothelial cell marker (Zanetta *et al.*, 2000). These markers have been used to characterise cardiac stem cell commitment to smooth muscle and endothelial lineages *in vitro* (Beltrami *et al.*, 2003, Ellison *et al.*, 2013, Smith *et al.*, 2014). Other cardiac-related markers at gene level such as *Myh6* and *Myh7* are also widely used for determining cardiac lineage specification (Plaisance *et al.*, 2016). *Myh6* encodes for α-myosin heavy chain, which is important for structural organisation (Epp *et al.*, 1993, Posch *et al.*, 2011). The loss of *Myh6* led to embryonic lethality (Jones *et al.*, 1996). β-myosin heavy chain is encoded by *Myh7*. The shift of expression from *Myh6* to *Myh7* in mice was related to severe cardiovascular stress due to maladaptive response (Krenz and Robbins, 2004). Mutation of this gene causes patients with hypertrophic cardiomyopathy (Laredo *et al.*, 2006).

2.8.3(c) Engraftment

Although promising results have been obtained from the recent human clinical trial using patients' own cardiac cells for heart repair (Bolli *et al.*, 2011, Chugh *et al.*, 2012), the survival of c-kit cardiac stem cells following transplantation are poor and thus limiting the treatment outcome (Hong *et al.*, 2014). Using mice model, Hong *et al.* performed intracoronary c-kit cardiac stem cell administration in coronary occluded mice followed by reperfusion. They found that more than 85% of c-kit cardiac stem cells that were present during the five min of administration were lost by 24-hr, and only about 3.5% of cardiac stem cells being spotted at day seven, for which the number of transplanted cells declined continuously (Hong *et al.*, 2014).

Encouraging results that demonstrated c-kit cardiac stem cells improved global function of LV in animal models have led to the very first human clinical trial - Phase I, randomised, open-label, single-center trial of Stem Cell Infusion in Patients with Ischaemic CardiOmyopathy (SCIPIO) (Bolli *et al.*, 2011), conducted on patients with ischaemic cardiomyopathy. Briefly, c-kit cardiac stem cells were isolated from the right atrial appendage of patients undergoing open heart surgery for coronary artery bypass grafting. Harvested c-kit cardiac stem cells underwent expansion *in vitro* and were then infused back into the donor heart via intracoronary injection. The results indicated the potential use of autologous transplantation of c-kit cardiac stem cells in improving the regional and global heart function. The study demonstrated the improvement of LVEF by 7.6% within 4 months (Bolli *et al.*, 2011) and 13.7% at 12 months (Chugh *et al.*, 2012) following transplantation (Table 2.5).

Table 2.5: List of Clinical Trials Using Cardiac Stem Cells for Heart Regeneration

Clinical Trials	Number of Patients	Duration (Months)	Changes in LVEF (%)	References
SCIPIO (2011)	23	4	+8.2%*	(Bolli *et al.*, 2011)
SCIPIO (2012)	33	4 and 12	+7.6%* (4 months) +13.7% (12 months)	(Chugh *et al.*, 2012)

*Significant improvement in LVEF ($p < 0.05$)

2.9 Ageing

2.9.1 Telomere Length

According to Hayflick limit of cell division, every single cells have limited capability in division, which is determined by telomere attrition, or shortening (Shay and Wright, 2000). Telomeres are located at the ends of chromosomal DNA with thousands of tandem repeats of the TTAGGG sequence, which function to protect the chromosome ends from DNA damage, degradation and thus maintain cellular and DNA stability during replication (Fyhrquist and Saijonmaa, 2012, Nguyen *et al.*, 2016). However, telomere length is shortened by 30-150 base pairs with each cell division. Upon reaching critical telomere length, cells will become senescent, along with activation of cell cycle inhibitor arrest pathways, via $p16^{INK4a}$ (Avolio *et al.*, 2014), p21 or p53 (Blackburn, 2000). Nonetheless, the telomere shortening process can be