OPTIMISATION OF INDUCED PLURIPOTENT STEM CELLS DERIVATION FROM HUMAN CORD BLOOD AND ITS APPLICATION FOR CARDIAC REGENERATION IN CRYOINJURED RAT MODEL

FATIN FAZRINA BINTI ROSLAN

UNIVERSITI SAINS MALAYSIA

2024

OPTIMISATION OF INDUCED PLURIPOTENT STEM CELLS DERIVATION FROM HUMAN CORD BLOOD AND ITS APPLICATION FOR CARDIAC REGENERATION IN CRYOINJURED RAT MODEL

by

FATIN FAZRINA BINTI ROSLAN

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

August 2024

ACKNOWLEDGEMENT

First and foremost, all praises to Allah and His blessing throughout the completion of this study. I express gratitude to my main supervisor, AP Dr. Tan Jun Jie, for consistently pushing me beyond my limits, maximizing my potential, and encouraging me to see the bigger picture. His unwavering support has been instrumental in the completion of my studies. Thank you to AP Dr. Tan Jun Jie for the grant that supported this project and provided financial assistance for the stipend throughout my study. The industrial grant from Cryocord and the Matching Grant USM Research University Grants (RUI) are acknowledged for chapter 3. Chapter 4 is supported by the Fundamental Research Grant Scheme (FRGS) grant, and the recent achievement by him as a recipient of the honourable Dr. Ranjeet Bhagwan Singh Research Grant (RBS) supports my study for chapter 5. I also sincerely appreciate my co-supervisor, Dr. Mohd Nor Azim Ab Patar, whose words of wisdom have been a great source of inspiration, guidance, and advice throughout my studies. I thank Dr. Kumitha Theva Das for her continuous support in my studies and personal life. She is a go-to person for advice. Special thanks to Dr. Zarina Thasneem Zainudeen for her guidance in processing cryopreserved cord blood. I acknowledge the valuable assistance of Dr. Ooi Jer Ping and Yu Yuexin in PV loop data analysis. Their dedication, even during weekends and public holidays in the surgery room, is admirable. I also appreciate Dr. Azlina and the SPH team's continuous support and kindness. Thanks also to AP Dr Bakiah, Dr Siti Hawa Ngalim, and Dr Ooi Cheong Hwa, who supported me indirectly.

I sincerely thank CryoCord Premier Stem Cell Bank Malaysia for funding this project and providing cord blood samples. Additionally, I appreciate Genomax

Technologies Sdn. Bhd. for the NovoCyte Flow Cytometer Systems and Bio-Diagnostics Sdn. Bhd., who trained me to use the Neon Electroporation System.

To my awesome colleagues teaming up with Yu Yuexin, Wang Meng Meng, Cui Can, Chung Chun Chen and Xu Yaping: big thanks for just being there through all my ups and downs, and for all the kindness you have thrown my way. A massive thank you for all the time and care you have showered on me, literally keeping me alive here: Rajayaashini, Aliaa Arina, Sarminiy, and Miao Xinzhi. Here is to each of you rocking it in your research and having a super bright future! Cheers!

This thesis is lovingly dedicated to my parents, Roslan Kasim and Faridah Abd Rahman, because their love and support have been my anchors. Huge thanks to my family-in-law, Jamaludin Omar, and Norizan Noor Mohamed for always understanding me and continuously supporting me. To my siblings, you are always the MVPs in my life! Saving the best for last, big thanks to my husband, Mohamad Fikri Jamaludin, for simply being the incredible person you are. Thanks for all the strength you share with me and for being the best friend I could ever ask for. And to myself, hoping that curiosity and courage will be with you to face new adventures!

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
β	Beta
μ	Micro
V	Voltage
K	Thermal conductivity
Wm	Watts per meter

LIST OF ABBREVIATIONS

CB	Cord Blood
CMs	Cardiomyocyte
CHIR	Glycogen synthase kinase-3β inhibitor
CO	Cardiac output
cTnT	Cardiac troponin T
CVDs	Cardiovascular diseases
EMT	Epithelial-Mesenchymal Transition
hESCs	Human embryonic stem cells
hiPSCs	Human induced pluripotent stem cells
hiPSC-CMs	HiPSC-derived cardiomyocytes
hiPSC-PECs	HiPSC-derived pre-epicardial cells
LDL	Low-density lipoprotein
Sca-1	Stem cell antigen-1
BMP-4	Bone morphogenic protein 4
VEGF	Vascular endothelial growth factor
RA	Retinoic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid,
Rocki	ROCK inhibitor Y-27632 dihydrochloride
Tra-1-60	Tumor related antigen 160/Podocalyxin
SSEA-4	Stage specific embryonic antigen 4
cTnT	Cardiac Troponin T
mLC2v	Ventricular myosin light chain-2.
TH	Tyrosine hydroxylase-like
SOX17	SRY-box transcription factor 17
FoxA2	Forkhead box protein A2 (FOXA2)
BDNF	Brain-derived neurotrophic factor
GDNF	Glial cell line-derived neurotrophic factor

LIST OF APPENDICES

Page

PENGOPTIMUMAN SEL INDUK PLURIPOTEN DARI DARAH TALI PUSAT MANUSIA DAN APLIKASI UNTUK REGENERASI JANTUNG DALAM MODEL KECEDERAAN KRIO TIKUS

ABSTRAK

Terapi kardiomiosit dianggap satu-satunya pilihan untuk mengisi semula kehilangan miokardium, tetapi terdapat cabaran dalam mencapai penambahan berkesan kardiomiosit berfungsi yang boleh diterjemahkan kepada manfaat klinikal. Kajian ini bertujuan untuk mengkaji penggunaan dan fungsi sel pra-epikardium dalam terapi kardiomiosit secara in vivo Darah tali pusat disimpan beku digunakan untuk menjana sel induk pluripoten terinduksi (hiPSC) dari sel stem matang manusia untuk memanfaatkan 'kemudaan' sel janin. HiPSC yang dihasilkan juga digunakan untuk kardiomiosit (CM) dan sel pra-epikardium (PEC) dengan kecekapan protocol pembezaan yang tinggi. Untuk mengesahkan identiti PEC pada kardiomiosit, kedua-dua CM dan PEC yang dibezakan telah dikultur bersama selama 6 hari, serta percambahan dan peranan isyarat Hippo telah dinilai. PEC ($1x10^6$ sel/100 µl) juga digabungkan dengan CM ($1x10^6$ sel/100 µl) untuk merawat jantung tikus yang tercedera selepas 1 bulan. Fungsi jantung dinilai menggunakan analisis hemodinamik gelung isipadu tekanan Millar. Perbezaan antara kumpulan dianggap signifikan apabila $p < 0.05$ menggunakan ANOVA. Sel CD34 yang dibekukan berjaya diprogram semula menjadi HiPSC dengan ekspresi penanda pluripoten yang konsisten. HiPSC yang dihasilkan menunjukkan keupayaan pembezaan, membentuk kardiomiosit, neuron dopamin, dan sel endodermal awal. Kultur bersama MSC meningkatkan percambahan sel CD34 yang dibekukan ($9.7 \pm 0.1 \times 10^5$ vs. $2.7 \pm 0.3 \times 10^5$, $p < 0.0001$, $n = 6$), dan proses ini tidak menjejaskan keupayaan mereka untuk menghasilkan

HiPSC yang berkualiti. Kultur bersama PEC/CM menunjukkan peningkatan proliferasi CM, disahkan oleh pengaktifan semula ekspresi YAP dan TAZ dan disokong oleh ekspresi positif cTnT/EdU bagi penanda percambahan, selaras dengan perencat MST1/2 XMU-MP1. Rawatan dengan PEC/CM tidak meningkatkan fungsi jantung tikus yang cedera dengan ketara. Walau bagaimanapun, kumpulan yang dirawat dengan CM menunjukkan dua hasil keanjalan ventrikel kiri (Ees) yang berbeza, iaitu hasil yang sangat berfaedah hingga sangat memudaratkan. Kesimpulannya, sel CD34 yang berasal dari darah tali pusat beku telah digunakan untuk penjanaan HiPSC, dan pengembangan dengan MSC tidak mengubah kualitinya. PEC menjejaskan percambahan CM, dan kesan ini dimediasi oleh penurunan pengawalan laluan isyarat Hippo. Rawatan dengan PEC tidak meningkatkan fungsi jantung dengan ketara dalam terapi CM untuk jantung tikus yang cedera selepas 1 bulan, menunjukkan keperluan untuk analisis mendalam mengenai faedah pemberian bersama sel-sel ini secara in vivo.

OPTIMISATION OF INDUCED PLURIPOTENT STEM CELLS DERIVATION FROM HUMAN CORD BLOOD AND ITS APPLICATION FOR CARDIAC REGENERATION IN CRYOINJURED RAT MODEL

ABSTRACT

Cardiomyocyte therapy was considered the only option to repopulate myocardial loss, but challenges existed in achieving effective replenishment of functional cardiomyocytes that could translate into clinical benefits. This study aimed to examine the use and function of pre-epicardial cells in cardiomyocyte therapy in vivo. Cryopreserved cord blood was used to generate human-induced pluripotent stem cells (hiPSCs) to harness the 'youthfulness' of foetal cells. The generated hiPSCs were also used to produce cardiomyocytes (CMs) and pre-epicardial cells (PECs) with high differentiation efficiency using established protocols. To confirm the effects of PECs on cardiomyocytes, both differentiated CMs and PECs were co-cultured for 6 days, and the proliferation and role of Hippo signaling were assessed. PECs ($1x10^6$ cells/100 µl) were also combined with CMs ($1x10^6$ cells/100 µl) to treat cryoinjured rat hearts after 1 month. Cardiac functions were assessed using Millar Pressure Volume Loop cardiac hemodynamics analysis. Differences between groups were considered significant when $p < 0.05$ using ANOVA. Frozen CD34 cells were successfully reprogrammed into hiPSCs with consistent expression of pluripotent markers. The generated hiPSCs demonstrated multilineage differentiation ability, forming cardiomyocytes, dopamine neurons, and early endodermal cells. MSC co-culture increased the proliferation of frozen CD34 cells ($9.7 \pm 0.1 \times 10^5$ cells vs. $2.7 \pm 0.3 \times 10^5$ cells, $p < 0.0001$, $n = 6$), and this process did not compromise their ability to produce quality hiPSCs. PEC/CM co-culture showed increased CM proliferation,

confirmed by the reactivation of YAP and TAZ expression and corroborated by cTnT/EdU-positive expression of proliferation markers, consistent with MST1/2 inhibitor XMU-MP1. Treatment with PEC/CM did not significantly improve the cardiac functions of cryoinjured rat hearts. Nonetheless, the CM-treated group exhibited two divergent left ventricular elastance (Ees) outcomes, with results ranging from highly beneficial to markedly detrimental. In conclusion, frozen cord blood-derived CD34 cells were used for hiPSC generation, and expansion with MSCs did not alter their quality. PECs affected CM proliferation, and this effect was mediated by the downregulation of the Hippo signaling pathway. Treatment with PECs did not significantly improve cardiac function in CM therapy for cryoinjured rat hearts after 1 month, suggesting the need for an in-depth analysis of the benefits of co-administering the cells in vivo.

CHAPTER 1

INTRODUCTION

Heart failure (HF) is characterised by an irreversible decline in functional myocardium, affecting approximately 64 million patients worldwide (Savarese *et al.*, 2023). While heart transplantation remained the primary treatment, challenges such as donor shortages and the requirement for lifelong immunosuppression significantly restricted its widespread use. Despite advancements in pharmacological therapy that improved symptom alleviation in myocardial infarction, these treatments could not restore dead myocardium, leaving most infarcted hearts with non-functioning scar tissue, ultimately leading to failure (Heallen *et al.*, 2019). Addressing these obstacles called for innovative cell therapeutic strategies to enhance the management of this prevalent and debilitating cardiovascular condition.

Cell replacement therapy, investigated in numerous clinical trials, yielded limited and disappointing outcomes (Pompilio *et al.*, 2015). Around twenty years ago, reliance on adult stem cells for cardiac regeneration became apparent. However, their limitations were evident as they proved incapable of generating a substantial number of functional new cardiomyocytes within the injured area, as revealed in vivo (Michler, 2018). The observed effects were primarily attributed to paracrine impact, particularly aiding angiogenesis, but not to the production of new cardiomyocytes (Guo *et al.*, 2020). Subsequently, researchers adopted an alternative approach by employing human embryonic stem cells (hESCs). The successful engraftment of human embryonic stem cell-derived cardiomyocytes (hESC-CMs) demonstrated their capacity to remuscularise substantial portions of the infarcted monkey heart (Chong *et al.*, 2014). However, ethical concerns arose due to the use of hESCs, which involved the destruction of embryos, sparking debates on their moral status.

Seminal advancements in cardiac differentiation of human induced pluripotent stem cells (hiPSCs) provided the capability to recreate definitive stages of cardiac development in vitro (Funakoshi *et al.*, 2021; Tan *et al.*, 2021). Despite notable progress in applying human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), these cells displayed an immature phenotype characterised by underdeveloped organisation and electromechanical function (Karbassi *et al.*, 2020). A range of approaches had been implemented to promote the potential of hiPSC-CMs, but an effective regimen had yet to be established. This highlighted significant gaps in our understanding, as the recapitulation of cardiac development remained incomplete.

The strategy of integrating cardiomyocytes with non-myocyte cells, such as endothelial cells, smooth muscle cells, and fibroblasts, appeared as a promising approach to enhance the structural and functional aspects of hiPSC-CMs (Kim *et al.*, 2010; Giacomelli *et al.*, 2017; Pasquier *et al.*, 2017). Some non-myocyte cells originate from epicardial-related cells, particularly pre-epicardial cells (Tan *et al.*, 2021). During embryonic heart development, the epicardium played a pivotal role, giving rise to crucial coronary smooth muscle cells for vasculature and myocardial fibroblasts for proliferation and compaction (Gittenberger-de Groot *et al.*, 1998; Vrancken Peeters *et al.*, 1999; Guadix *et al.*, 2006; Cai *et al.*, 2008).

The proepicardial organ, a transient structure, gave rise to the epicardium and played a crucial role in heart development. Epicardial coverage of the entire heart was initiated at E11 and was completed by the end of E16 (Hirakow, 1992). The epicardium, the outermost layer of the heart, is a source of non-myocyte cells crucial in shaping the heart. In rats, epicardial migration began at E9.5, resulting in the

complete coverage of the outer surface of the heart by E10.5 (Wiesinger *et al.*, 2021). A distinct subset of epicardial cells underwent epithelial-to-mesenchymal transition (EMT), leading to the formation of a mesenchymal layer between the epicardium and myocardium, aided by paracrine effects. A study by Gittenberger et al. proved that inhibiting epicardial outgrowth resulted in abnormalities affecting the compact myocardial layer, myocardialisation of cushion tissue, looping, septation, and coronary vascular formation. Utilise the distinctive properties of pre-epicardial cells showed potential in addressing cardiac damage and supporting myocyte structure and function (Gittenberger-de Groot *et al.*, 2000). Beyond their contribution to myocardial mass development, proepicardial cells also undergo EMT to generate vascular smooth muscle cells, cardiac fibroblasts, and cardiac progenitors.

Nevertheless, adult epicardial cells differed from their foetal counterparts, lacking features seen in embryos. Foetal epicardial cells progressed faster through EMT than adult epicardial cells (Cao and Poss, 2018). Epicardial cells derived from hESCs and hiPSCs exhibited characteristics similar to foetal epicardial cells. The generation of pre-epicardial cells (PECs) from hiPSCs represented a recent and significant milestone in heart research (Bargehr *et al.*, 2019; Tan *et al.*, 2021). The derivation of epicardial cells from hiPSCs enabled exploration at an earlier primitive stage, such as the pre-epicardium. Based on their described capabilities, including roles in myocardial expansion, coronary development, and derivation of cardiac interstitial cells, PECs were likely to play a crucial role in treating injured hearts. However, the successful retention of PECs as functional epicardial cells with regenerative and repair potentials, such as cardiac muscle proliferation, had not been conclusively proven. If exploited in a directed manner, these PEC characteristics might serve as vital support cells to mediate myocardial healing and shape the structure and function of the heart.

Furthermore, reported that the co-culture of epicardium-derived cells and neonatal murine cardiac muscle cells upregulated tight junctions, contractile protein expression, cardiac muscle alignment, and functional contractility in vitro (Weeke-Klimp *et al.*, 2010). Recent studies highlighted intrinsic signalling pathways, such as the Hippo signalling pathway, as key players in enhancing cardiomyocyte proliferation. The Hippo Signalling pathway, crucial in cardiac regeneration and tissue homeostasis, involves Hippo kinases (MST1/2 and LATS1/2) and the YAP/TAZ complex. In its inactive state, the Hippo pathway activated YAP and TAZ, thereby facilitating cell proliferation and inhibiting apoptosis through the regulation of target genes. Understanding the function of pre-epicardial cells in suppressing the Hippo signalling pathway in ventricular cardiomyocytes played a central role in this research exploration. This project aimed to decipher the key biochemical cues from pre-epicardial cell signalling in hiPSC-cardiomyocyte co-culture, aiming to elucidate the intricate interplay of the Hippo pathway in cardiomyocyte proliferation.

The primary approach for salvaging damaged myocardium involved cardiomyocyte therapy, to replenish the infarcted area, maintain cell viability, and synchronising contraction with the host heart to prevent remodelling. Strategies to enhance the engraftment and integration of injected hiPSC-CMs included coadministration with non-myocyte support cells. The hypothesis suggested that hiPSC-PECs enhanced cardiomyocyte survival, engraftment, and integration. This study aimed to examine the effects of hiPSC-PECs on hiPSC-CMs therapy in cryoinjured rat hearts.

This study also aimed to validate the hypothesis that cord blood with a low cell count could undergo expansion in a mesenchymal stem cell (MSC) co-culture and later

be reprogrammed into hiPSCs. Cord blood (CB) is a valuable source of hematopoietic stem cells (HSCs), being young and relatively disease-free, which reduces the risk of pathogen transmission compared to other adult cell sources. The practice of cryopreserving cord blood for future use was supported by over 40,000 successful HSC transplants in children and adults, including treatments for non-malignant and malignant haematological diseases (Mayani *et al.*, 2020). Regrettably, a substantial proportion approximately 36% to 39% of the total collected CB was discarded, incurring substantial economic costs, depleting resources, and wasting precious samples, including donor units representing rare blood types or diseases (Tan, 2017).

To diversify downstream applications from cord blood, reprogramming and generating hiPSCs from these samples inevitably proved the concept of reuse rather than discarding the collected cord blood. This approach allowed the differentiated hiPSCs to generate cardiomyocytes and pre-epicardial cells, which could then be tested in vitro for their role in the Hippo signalling pathway and in an in vivo study involving the co-injection of hiPSC-CMs/PECs in a cryoinjured rat model. These differentiated cells were used to explore the potential of pre-epicardial cells to enhance the efficacy of cardiomyocyte therapy, potentially leading to significant advancements in the treatment of heart failure.

1.1 Problem Statement

Human induced pluripotent stem cells (hiPSCs) offered a differentiation potential similar to embryonic stem cells, enabling the study of developmental processes. However, hiPSC-derived cardiomyocytes (hiPSC-CMs) showed an immature phenotype in terms of structural integrity and functionality. This highlighted gaps and the need for effective treatments. For successful reconstruction of injured

myocardium, which required complex cellular and structural arrangements, the strategy involved integrating hiPSC-CMs with non-myocyte cells. The importance of epicardial formation in heart development was well established. The iPS technology generated pre-epicardial cells, but their application and use in cell therapy after injury were still unknown. The Hippo signalling pathway plays a role in heart development during epicardium formation. This study aimed to understand the signalling related to hiPSC-derived pre-epicardial cells (hiPSC-PECs) functions and how these cells influenced ventricular myocytes to compact and proliferate at a specific developmental stage. This understanding would help modulate the cells to achieve desired effects in cell therapy under controlled conditions.

1.2 General Objective

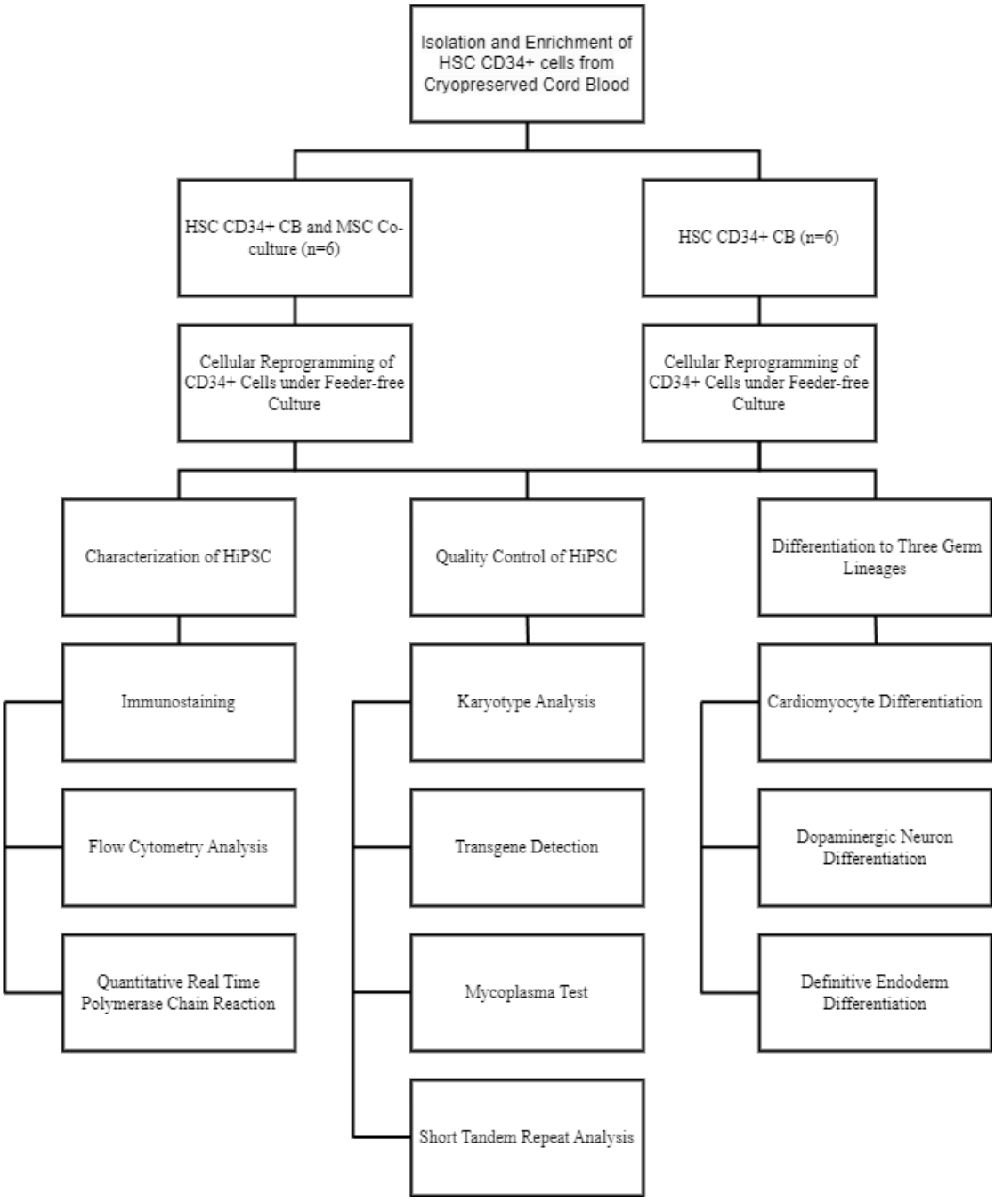
This project aims to examine the use and function of pre-epicardial cells in cardiomyocyte therapy in vivo.

1.3 Specific Objective

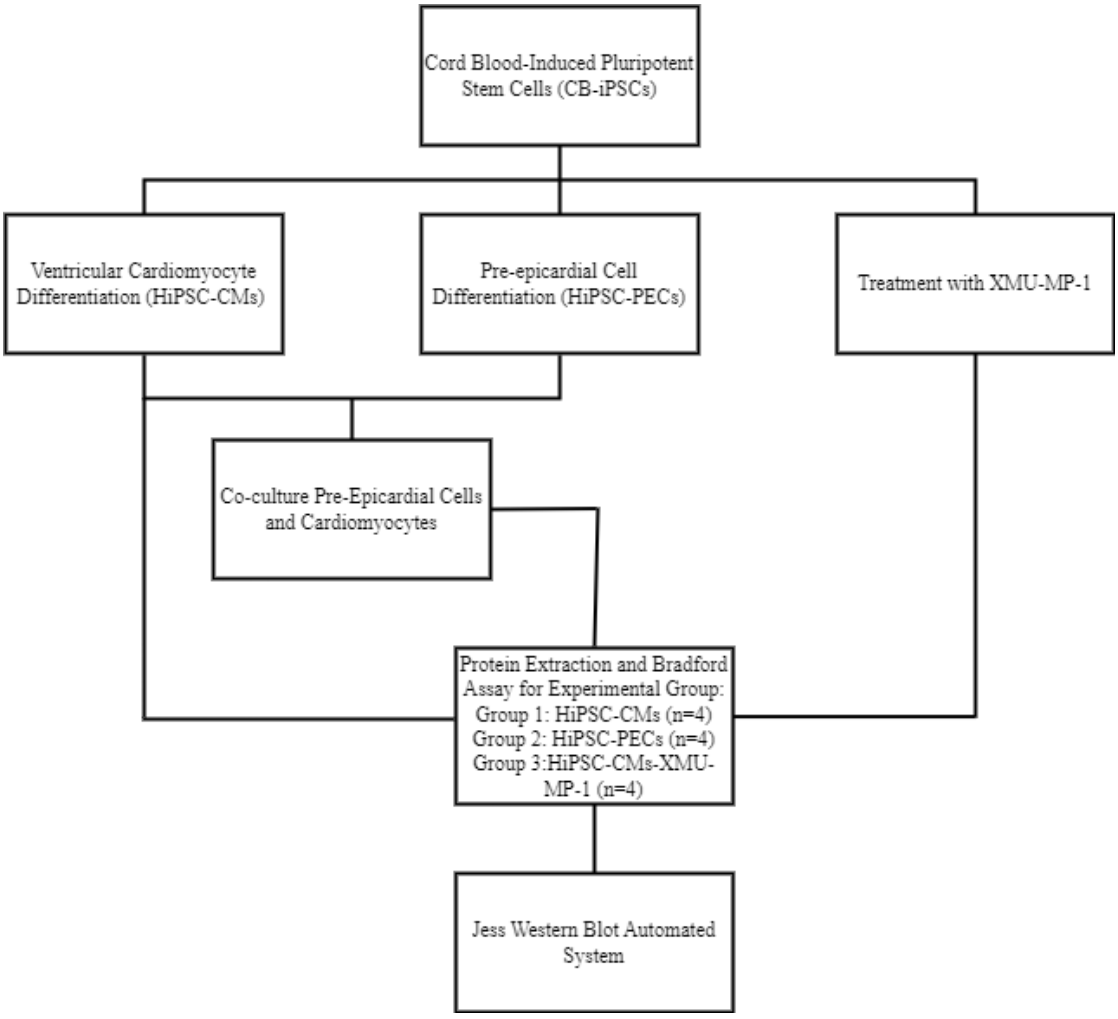
1. To investigate the effects of MSC co-culture on the cryopreserved cord blood $CD34^+$ cells and their reprogramming efficacy.
2. To examine the role of the Hippo signalling pathway in pre-epicardial cell/cardiomyocyte interaction in co-culture in vitro.
3. To evaluate the effect of co-administered pre-epicardial cells and cardiomyocytes on treating cryoinjured rat hearts.

1.4 Flow Chart

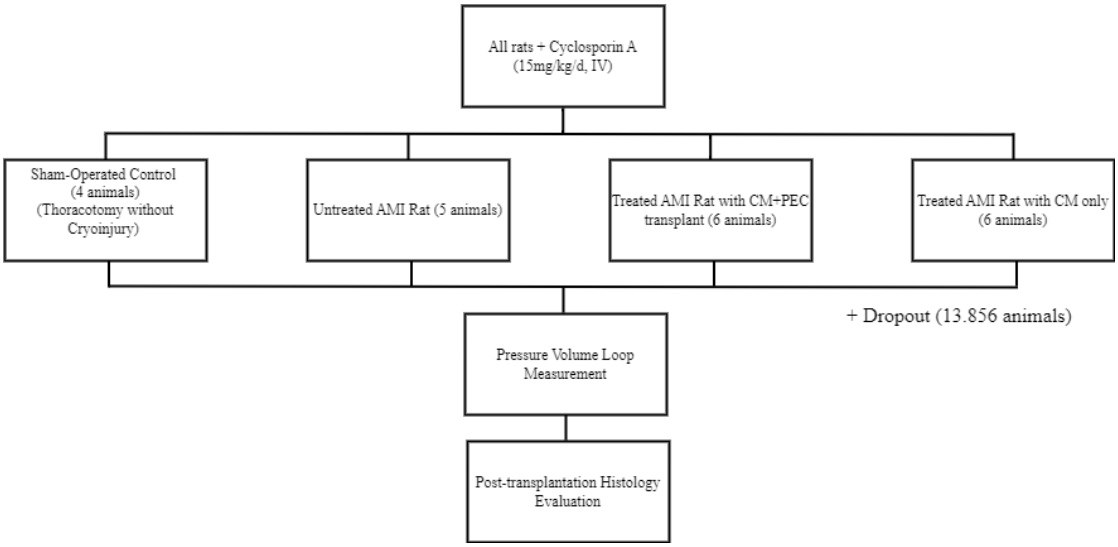
(A) REPROGRAMMING OF MESENCHYMAL STEM CELLS EXPANDED CORD BLOOD-DERIVED CD34+ CELLS TO GENERATE HUMAN-INDUCED PLURIPOTENT STEM CELLS



(B) **HIPPO SIGNALING PROMOTED HIPSC-CARDIOMYOCYTE PROLIFERATION BY CO-CULTURING WITH HIPSC-PRE-EPICARDIAL CELLS**



(C) **CO-ADMINISTERED HUMAN INDUCED PLURIPOTENT STEM CELLS-DERIVED CARDIOMYOCYTES/PRE-EPICARDIAL CELL INTO CRYOINJURED RAT HEARTS**



CHAPTER 2

LITERATURE REVIEW

2.1 Cardiovascular Disease Persists as the Primary Cause of Deaths

Cardiovascular diseases (CVDs) have persistently remained the leading cause of global mortality for several decades (Lindstrom *et al.*, 2022). Globally, the number of deaths caused by CVDs has increased from 12.1 million in 1990 to 20.5 million in 2021, constituting about one-third of all deaths. According to the study by Sacco et al., there is a predicted 34% relative increase in CVD mortality among men and 30% among women aged 30-70 from 2013 to 2025, assuming current risk factors and population growth persist (Sacco *et al.*, 2016). Despite this, women have a higher prevalence rate of CVD than men, while men face higher disability-adjusted life years and mortality rates between ages 25-39, comparable to women aged 30-39 (Sun *et al.*, 2023). These disparities are attributed to high systolic blood pressure, high body mass index, and elevated low-density lipoprotein cholesterol levels.

Global disparities in CVD mortality persist, with low- and middle-income countries experiencing higher rates than their high-income countries. High-income countries anticipate a potential -19% reduction in men and -16% in women deaths by 2025 (Sun *et al.*, 2023). Similarly, in Malaysia, the trend indicates a higher risk of cardiovascular events in the rural population compared to the urban population (Noor Hassim *et al.*, 2016). Income is a significant social determinant that influences health at the national and household levels. A study on American adults highlighted the impact of family income, particularly on individuals with lower income (Minhas *et al.*, 2023). Notably, this group exhibited higher rates of smoking, marijuana use, limited access to

healthcare, and lower educational attainment compared to those with higher family incomes.

The World Heart Federation Report 2023 highlighted modifiable risk factors contributing to CVD deaths in 2021(WHF, 2023). Elevated LDL cholesterol, linked to 3.8 million deaths, underscores the need for precise cholesterol management. Additionally, high fasting plasma glucose, contributing to 2.3 million deaths, sheds light on the complex link between diabetes and cardiovascular health. Moreover, air pollution causes 4.8 million deaths. The combination of high body mass index, tobacco use, low physical activity, and elevated blood pressure collectively leads to numerous fatalities. Addressing these factors globally requires tailored interventions and comprehensive public health strategies, urging nations to priorities cost-effective, population-wide approaches and foster public-private partnerships across sectors. Therefore, an urgent call is made to develop and implement effective, precisely targeted strategies for preventing CVDs, particularly in low- and middle-income countries.

2.2 Heart Regeneration

Since the last century, the heart has been classified as a post-mitotic organ, supported by evidence of neonatal cell-cycle arrest in rodents, fostering the prevailing belief that postnatal cardiomyocytes were inherently incapable of re-entering the cell cycle (Soonpaa *et al.*, 1996). Nevertheless, studies on the integration of carbon-14, generated by nuclear bomb tests during the Cold War, into DNA to establish the age of cardiomyocytes in humans have revealed cardiomyocyte renewal (Bergmann *et al.*, 2009). A discernible growth capacity is reported in human adult mammals, albeit exceptionally low. The turnover is less than 1% of cardiomyocytes replaced annually at age 20, declining further to 0.4% per year by age 75 (Bergmann *et al.*, 2009). In mice

labelled with 15N-thymidine, the reported turnover rate was approximately 0.76% per year (Senyo *et al.*, 2013). Similarly, in humans using the mitotic marker phosphorylated histone H3 (pH3), reported rates ranged from 0.04% to 1.6% per year (Mollova *et al.*, 2013). Despite the limited annual re-entry of cardiomyocyte cell cycles, the challenge of replenishing the heart with an adequate number of cardiomyocytes to restore function after injury persists. The limited annual re-entry of cardiomyocytes into the cell cycle presented a significant challenge in replenishing the heart with sufficient cardiomyocytes to restore function after injury. Therefore, selecting suitable animal models was essential for studying cardiomyocyte regeneration and exploring potential therapeutic interventions. Zebrafish and urodele amphibians proved especially valuable in this context due to their natural ability to fully regenerate their hearts even after removing a substantial portion (20-50%) of the apical myocardium (Oberpriller and Oberpriller, 1971; Poss *et al.*, 2002). In contrast, mature cardiomyocytes typically exhibited a low turnover rate, reflecting their limited regenerative capacity under normal conditions. This limitation often resulted in cardiomyocyte necrosis, myocardial fibrosis, and eventually heart failure. For example, research demonstrated that new cardiomyocytes were generated from existing ones at an estimated rate of about 0.76% per year in young adult mice (Senyo *et al.*, 2013). Although this rate decreased with age, it increased approximately four-fold after myocardial injury in the border region, suggesting that regenerative pathways were activated in response to the injury.

Further supporting the need for varied animal models, a neonatal rat myocardial infarction model revealed that both male and female rats were capable of generating endogenous neocardiomyogenesis following injury (Wang *et al.*, 2020). Additionally, a study employing a modified rodent model found that adapting a 10 μL pipette tip by cutting it to an internal diameter of 0.48 mm enabled accurate resection of the LV apex

with high homogeneity and reproducibility. This modification was practical for studying the structural, functional, and molecular mechanisms of cardiac regeneration in both neonatal mice and rats and demonstrated the cardiac regenerative potential and functional recovery of the hearts (Bei *et al.*, 2023). Moreover, Wang et al. established a neonatal leporine myocardial infarction (MI) model and discovered that neonatal rabbits could naturally regenerate their hearts after a P1 MI, achieving normal cardiac geometry and function within 3 weeks after recellularisation (Wang *et al.*, 2022). Ideally, in preclinical settings, therapy was first tested in small animal models due to their short life cycle and low maintenance costs. However, given the differences in heart anatomy and size, studying large animal models remained necessary, as they more closely resembled human physiology. The studies involving large animals were discussed in a recent review paper published by our group (Yu *et al.*, 2023). In the context of tissue engineering, methods such as decellularisation removed cells from tissues, leaving a structural extracellular matrix (ECM). This ECM was then repopulated with cells in a process called recellularisation to restore tissue function and prepare it for potential transplantation (Sesli *et al.*, 2018) (Skepastianos *et al.*, 2023).

2.2.1 Adult Stem Cell

For more than twenty years, adult stem cells have been at the forefront of efforts to regenerate cardiomyocytes and repair damaged myocardial tissue, particularly after events like myocardial infarction. Despite the observed angiogenic activity attributed to their paracrine effects, there is a lack of evidence supporting the idea that adult stem cells actively contribute to generating new contractility cardiomyocytes (Garbern and Lee, 2022). In the study by Oh et al., $Sca1^+$ cells were initially proposed as a potential source of cardiomyocytes in adult mouse hearts (Oh et al., 2003) were discredited by many researchers who confirmed that $Sca1^+$ cells do not contribute to cardiomyocytes

but rather act as endothelial precursors (Neidig et al., 2018; Vagnozzi et al., 2018). Similarly, c-kit was reported to be more like an endothelial precursor, as c-kit^{+} cardiac cells do not retain the ability to differentiate into cardiomyocytes (Zaruba et al., 2010; Jesty et al., 2012). In addition, bone marrow appears to be a superior type of adult stem cell, with approximately 80 clinical trials performed (Pompilio et al., 2015). These trials attribute the effects to paracrine actions on the heart rather than the direct generation of new cardiomyocytes (Michler, 2018). Although some studies report no significant risk of arrhythmias, the improvement of left ventricular systolic function is still very low (Abdel-Latif et al., 2007; Afzal et al., 2015). Furthermore, the differentiation of MSCs into cardiomyocyte-like cells did not appear to contribute significantly to the generation of cardiomyocytes; instead, it is mediated through paracrine activities (Guo et al., 2020). Despite the inability of adult stem cells to differentiate into spontaneously beating cardiomyocytes for enhanced contractility upon injury, they exhibit notable improvements in angiogenesis. In conclusion, these cells demonstrate their supportive role in cardiomyocytes by generating non-cardiomyocyte cells that contribute to vascularisation.

2.2.2 Embryonic Stem Cell

In the pursuit of mimicking embryo development for an enhanced understanding of heart development, researchers are actively engaged in studying cells intricately connected to embryos, including the isolation of hESCs. The differentiation potential of hESCs initiates with fertilisation, where specialised sperm and oocyte fuse to form a zygote (Shahbazi *et al.*, 2019). Subsequently, this zygote undergoes cleavage, resulting in the formation of a multicellular morula by day 3 and transforming into blastocysts by day 5. Within the blastocyst, the inner cell mass (ICM) contains pluripotent cells that can differentiate into three primary germ layers: the ectoderm, endoderm, and

mesoderm (Thomson *et al.*, 1998; Murry and Keller, 2008; de Sousa Lopes and Mummery, 2014) . The ectoderm, the outermost layer, originates from the epiblast layer and later develops into the integumentary, nervous, and pituitary systems. Mesoderm forms within or in the middle of epiblast and hypoblast layers, eventually differentiating into the musculoskeletal, cardiovascular, reproductive, and connective tissue. The endoderm, the innermost layer, is derived from the epiblast layer and gives rise to organs, such as the lungs, gastrointestinal tract, liver, pancreas, bladder, thyroid, and parathyroid glands. The formation of these germ layers is a crucial aspect of organogenesis in the human body, leading to the development of various tissues and organs (Thomson *et al.*, 1998).

The utilisation of hESCs offers a unique opportunity to investigate cell differentiation, closely mimicking early developmental conditions. Moreover, the hESCs cardiac differentiation protocol provides a means to optimise the process through the incorporation of various pathways. In an early protocol, hESCs cells were cultured in suspension plated to form embryoid bodies (EBs) aggregates in KO-DMEM and 20% FBS, resulting in approximately 8.1% of spontaneously contracting cardiomyocytes (Kehat *et al.*, 2001). The utilisation of growth factors such as Activin A, FGF2, and BMP4 resulted in an improvement in cardiac differentiation, leading to a significant increase in spontaneously contracting cardiomyocytes in both embryoid bodies (23.6%) (Burridge *et al.*, 2007) and monolayer (30%) (Laflamme *et al.*, 2007). An initial experiment with only a small molecule, SB203580, a p38 mitogen-activated protein kinase inhibitor, displayed 22% efficiency (Graichen *et al.*, 2008). However, when combined with prostaglandin I2 (PGI2), it had a negative impact on differentiation, reducing cardiac efficiency to over 10%, suggesting that insulin may also negatively impact differentiation (Xu *et al.*, 2008). Furthermore, the incorporation of growth factors like Activin A, FGF2, and BMP4 complementary with a small molecule of WNT inhibition such as dickkopf homolog 1 (DKK1) increased cardiac efficiency to 50% in ES-Eb culture (Yang *et al.*, 2008) while in monolayer ES showed better results with 50-70% (Kattman *et al.*, 2011). Then, modification to the protocol for WNT inhibitor was made by replacing DKK1 with small molecule IWR-1 (Willems *et al.*, 2011); however, the result showed that efficiency was not good, approximately 30%. Notably, a reported study highlights a production scale surpassing one billion cells per hESC-CMs batch, with successful engraftment observed in a primate model of heart failure (Chong *et al.*, 2014). This optimisation serves as a benchmark for future comparisons with hiPSCs in subsequent studies.

However, the ethical concerns associated with hESCs stem from their derivation, involving the destruction of human embryos by various methods, such as mechanical dissection (Ström *et al.*, 2007), laser dissection (Turetsky *et al.*, 2008), immunosurgery (Cowan *et al.*, 2004), microdissection (Khan *et al.*, 2018), and minimised trophoblast cell proliferation (MTP) (Khan *et al.*, 2018). The ethical discourse centres on the moral status of the embryo, prompting debates on whether the early-stage embryo warrants the same moral considerations as a developed human being, influenced by philosophical, religious, and cultural perspectives.

2.2.3 Induced Pluripotent Stem Cell

In the laboratory, hiPSCs possess pluripotent capabilities similar to hESCs achieved through reprogramming of adult somatic cells (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Puri and Nagy, 2011). The choice of adult somatic cells in reprogramming does not significantly impact hiPSC variability. For instance, a study that tested fibroblast and blood cells from the same donor showed no major variability between the produced hiPSCs, in terms of both reprogramming and differentiation efficiency (Kyttala *et al.*, 2016). However, hiPSCs may retain residual epigenetic memory, potentially influencing differentiation toward the tissue of origin ((Kim *et al.*, 2011; Sanchez-Freire *et al.*, 2014). Moreover, hiPSCs derived from cells of genetically different donors revealed significant differences in the hiPSC differentiation propensity (Kajiwara *et al.*, 2012; Kyttala *et al.*, 2016).

To enhance differentiation capability, Yamamoto's study found that iPSCs maintain differentiation potential with a glycolytic pathway-supporting medium, showing high hromodomain-helicase-DNA-binding protein 7 (CHD7) expression, but lose potential with a mitochondrial-supporting medium, displaying reduced CHD7 levels (Yamamoto *et al.*, 2022). Mutations in CHD7 are associated to the onset of

CHARGE syndrome, named after its defining symptoms: Coloboma (eye abnormalities), Heart defects, Atresia of choanae (blockage of nasal passages), Retardation of growth and development, Genital anomalies, and Ear anomalies (Zhoulideh and Joolideh, 2023). Optimal culture conditions involve selecting a glycolytic pathway-supporting medium, using single-cell suspensions during passage, and seeding on an extracellular matrix with less potent cell-binding capabilities. This reduces variability in differentiation potential by eliminating spontaneously differentiated cells (Yamamoto *et al.*, 2022), paving the way for precise iPSC differentiation using growth factors, small molecules, cytokines, and mechanical signals.

The shift from using hESCs to hiPSCs for cardiomyocyte differentiation is primarily due to ethical considerations, as hiPSCs can be generated from adult cells, avoiding the ethical concerns associated with using embryos. Lian et al. demonstrated that hiPSCs exhibit a similar efficiency to ESCs in a monolayer layer (Matrigel), achieving an 85% cardiac efficiency. Furthermore, their study revealed the initial introduction of the RPMI/B27 combination, later modified by excluding insulin for the first 5 days of differentiation, leading to the development of the GSK3β and WNT inhibitor small molecule differentiation protocol (Lian *et al.*, 2012; Lian *et al.*, 2013). Over time, the field has shifted from using complex to adopting simpler and more reliable protocols. Presently, small molecules like CHIR99021 play a crucial role in improving the WNT/b-catenin pathways, along with effective WNT inhibitors such as IWP-2, IWP-4 (Lian *et al.*, 2012; Lian *et al.*, 2013), XAV939 (Wang *et al.*, 2011; Minami *et al.*, 2012; Funakoshi *et al.*, 2021), KY0211 (Minami *et al.*, 2012) and WNT-C59 (Liang *et al.*, 2020).

These advancements have facilitated the investigation of cardiomyocyte subtypes, including ventricular, atrial, and pacemaker subtypes, to enhance our understanding of the complexity of heart structure without genetic modification involved, as summarised by (Lyra-Leite *et al.*, 2022). In the study during days 3-5 of cardiac mesoderm differentiation, adding retinoic acid (RA) favoured the atrial subtype (Stefanovic and Zaffran, 2017). The differentiation of ventricular cardiomyocyte (CM) subtypes relies on WNT signalling, as confirmed by other studies (Burridge *et al.*, 2014; Karakikes *et al.*, 2014; Funakoshi *et al.*, 2021). Despite promising developments, CMs derived from hiPSCs, differ significantly from those of adult cardiomyocytes. These differences encompass cell morphology, mitochondrial quantity, sarcomere structure, calcium transients, and electrophysiology (Karbassi *et al.*, 2020).

Addressing the challenge of hiPSC-CMs involves co-culturing these cells with non-myocyte cells such as fibroblasts, endothelial cells, and smooth muscle cells, yielding positive outcomes (Ye *et al.*, 2014). In vivo transplantation demonstrated the efficacy of a patch composed of human cardiomyocytes and a non-myocyte combination of endothelial cells and fibroblasts, showing vascularisation and integration with the host rodent coronary circulation (Stevens *et al.*, 2009). Furthermore, enhancing cardiomyocyte function is evident in the co-culture of cardiomyocytes with Akt-activated endothelial cells. This results in improved efficiency of the cardiomyocyte differentiation protocol and better intercellular coupling, ultimately leading to improved chronotropy and synchrony (Pasquier *et al.*, 2017). A similar trend was observed by Kim et al. in their study on human embryonic stem cell-derived cardiomyocytes (hESC-CMs), where early isolation of these cells from embryoid bodies without non-cardiomyocyte interaction hindered the development of ion channels and electrophysiological maturation (Kim *et al.*, 2010). Some non-myocyte

cell types, descendants of epicardial cells, markedly enhance the structural and electromechanical function of hiPSC-CMs.

2.3 Pro-Epicardium as Descendent Cells of Non-Myocyte

The epicardium is the outermost layer of the heart, formed through the active migration of mesothelial cells originating from the proepicardial organ adjacent to the septum transversum at E9.0-E9.5 in mouse embryonic development or at approximately 4 weeks post-conception in humans (Risebro *et al.*, 2015). The pro-epicardial cells exhibit a predominant migration through the pericardial fluid, facilitating their colonisation of the looped heart. This migratory process further extends to establishing a distinct layer of subepicardial mesenchyme. The migration and expansion of human $WT1^{+}$ epicardial cells forms a single-layered flat epithelium known as the epicardium, which establishes a connection to the myocardium around E11.0 in mouse embryos or during week 5 post-conception in humans (Duim *et al.*, 2016). Within this layer are epithelial-like cells, and a subset of them delaminates to invade the myocardium, contributing to the formation of vascular smooth muscle cells (SMCs) and cardiac fibroblasts (Quijada *et al.*, 2020).

In embryonic heart development, the epicardium plays a pivotal role, giving rise to crucial coronary smooth muscle cells for vasculature and myocardial fibroblasts for proliferation and compaction (Gittenberger-de Groot *et al.*, 1998; Vrancken Peeters *et al.*, 1999; Guadix *et al.*, 2006; Cai *et al.*, 2008). The failure to form the epicardium results in non-compaction of the heart, myocardial wall thinning, reduced vasculature, and an inability for the embryo to survive to birth. A study by Gittenberger et. al. demonstrated that inhibiting epicardial outgrowth results in abnormalities impacting the compact myocardial layer, myocardialisation of cushion tissue, looping, septation, and

coronary vascular formation. Utilising the distinctive properties of pre-epicardial cells shows potential in addressing cardiac damage and supporting myocyte structure and function (Gittenberger-de Groot *et al.*, 2000).

Beyond its contribution to myocardial mass development, proepicardial cells can undergo EMT to generate cardiac fibroblasts, vascular smooth muscle cells, and cardiac progenitors. The foetal epicardial cells progressed faster through EMT than adult epicardial cells (Cao and Poss, 2018). Several pre-epicardial differentiation protocols mimicking early developmental models have been explored. Epicardial cells derived from human embryonic stem cells (hESCs) (Witty *et al.*, 2014; Iyer *et al.*, 2015; Bao *et al.*, 2016; Guadix *et al.*, 2017; Zhao *et al.*, 2017; Bargehr *et al.*, 2019) and human induced pluripotent stem cells (hiPSCs) (Witty *et al.*, 2014; Iyer *et al.*, 2015; Bao *et al.*, 2016; Guadix *et al.*, 2017; Zhao *et al.*, 2017; Tan *et al.*, 2021) exhibited characteristics similar to foetal epicardial cells (Wiesinger *et al.*, 2021) summarised in Figure 2.1.

Based on their described capabilities, such as roles in myocardial expansion, coronary development, and derivation of cardiac interstitial cells, pre-epicardial cells (PECs) are likely to be instrumental in treating the injured heart. Successful retention of PECs as functional epicardial cells, possessing regenerative and repair potentials, is crucial. If exploited in a directed manner, these PEC characteristics may serve as important support cells to mediate myocardial healing in vivo. Moreover, evidence shows that co-culture of epicardium-derived cells and neonatal murine cardiomyocytes upregulated tight junctions, contractile protein expression, cardiomyocyte alignment, and functional contractility both in vitro and in vivo (Weeke-Klimp *et al.*, 2010). In the study involving hESC-derived epicardial cells and cardiomyocytes, a substantial augmentation in graft cardiomyocyte proliferation rates was observed, leading to a 2.6-fold increase in cardiac graft size (Bargehr *et al.*, 2019). This approach not only

increased vascularisation in both the graft and host but also demonstrated improved systolic function compared to hearts subjected to cardiomyocytes alone or epicardial cells alone, or vehicle administration. The investigation involving hiPSC-derived pre-epicardial cells (PECs) demonstrates that co-culturing induces dense aggregation of cardiomyocytes (CMs), resulting in a connected beating syncytium characterised by heightened contractility and improved calcium handling (Tan *et al.*, 2021). Furthermore, PECs secrete insulin-like growth factor 2 (IGF2), affirming their role in stimulating CM proliferation within the co-culture environment. In three-dimensional PEC-CM spheroid co-cultures, the development of outer smooth muscle cell layers around cardiac micro-tissues emphasises the significant supportive role of PECs as non-myocytes in facilitating cardiomyocyte function and organisation.

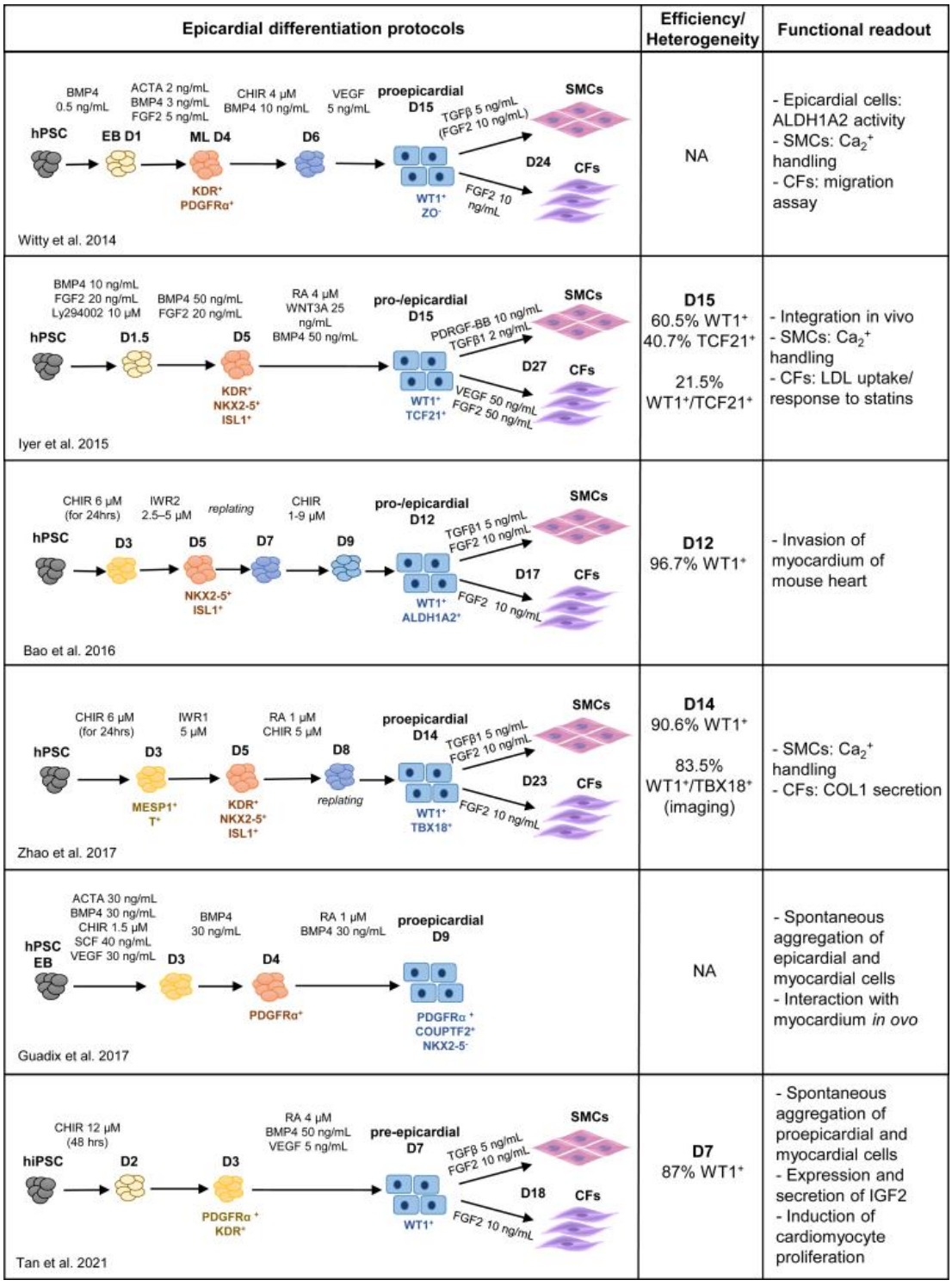


Figure 2.1 Summary of Epicardial Differentiation Protocol

Figure 2.1 was adapted from (Wiesinger *et al.*, 2021). The protocols listed in Figure 2.1 discussed the timeline of epicardial differentiation, involving various sets of small molecules and/or growth factors used to induce hiPSCs to differentiate into epicardial cells.

2.4 The Role of Hippo Signaling Pathway in Cardiac Regeneration

The Hippo pathway plays a crucial role in cardiac regeneration, promoting myocyte proliferation (Xin *et al.*, 2013) and regulating heart size by inhibiting WNT signalling (Heallen *et al.*, 2011). Key components of the Hippo pathway include a kinase cascade involving Hippo kinases (MST1/2 and LATS1/2) and a transcriptional co-activator complex comprising YAP and TAZ. Activation of the Hippo pathway induces cytoplasmic retention of YAP, while its inactivation facilitates YAP translocation into the nucleus, promoting cellular proliferation (Zou *et al.*, 2020). Phosphorylated YAP favours cytoplasmic retention: phosphorylation at Serine 127 promotes binding to 14-3-3 proteins, confining YAP to the cytoplasm. Conversely, phosphorylation at Serine 397 primes YAP for subsequent phosphorylation by casein kinase 1 (CK1), initiating polyubiquitination and YAP degradation (Figure 2.2).

A small molecule inhibitor of the STE20 family kinases, serine/threonine kinases MST1 (STK4) and MST2 (STK3), suppresses Hippo pathway signalling and increases YAP activity in neonatal rat cardiomyocytes, suggesting a potential therapeutic role in attenuating cardiac hypertrophy (Triastuti *et al.*, 2019). Furthermore, re-activation of YAP activity in induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) through XMU-MP-1 enhances cellular survival and proliferation while mitigating apoptosis in response to oxidative stress (Bui *et al.*, 2023).

The regulatory influence of the Hippo pathway extends beyond cardiomyocytes to non-myocytes such as fibroblasts (Xiao *et al.*, 2018) and epicardial cells (Singh et al., 2016), influencing vascular cells throughout various stages of cardiac development and regeneration.