Cytoprotective Effects of iPSC Secretome on Ischemia/Reperfusion Injury in Cardiomyocytes

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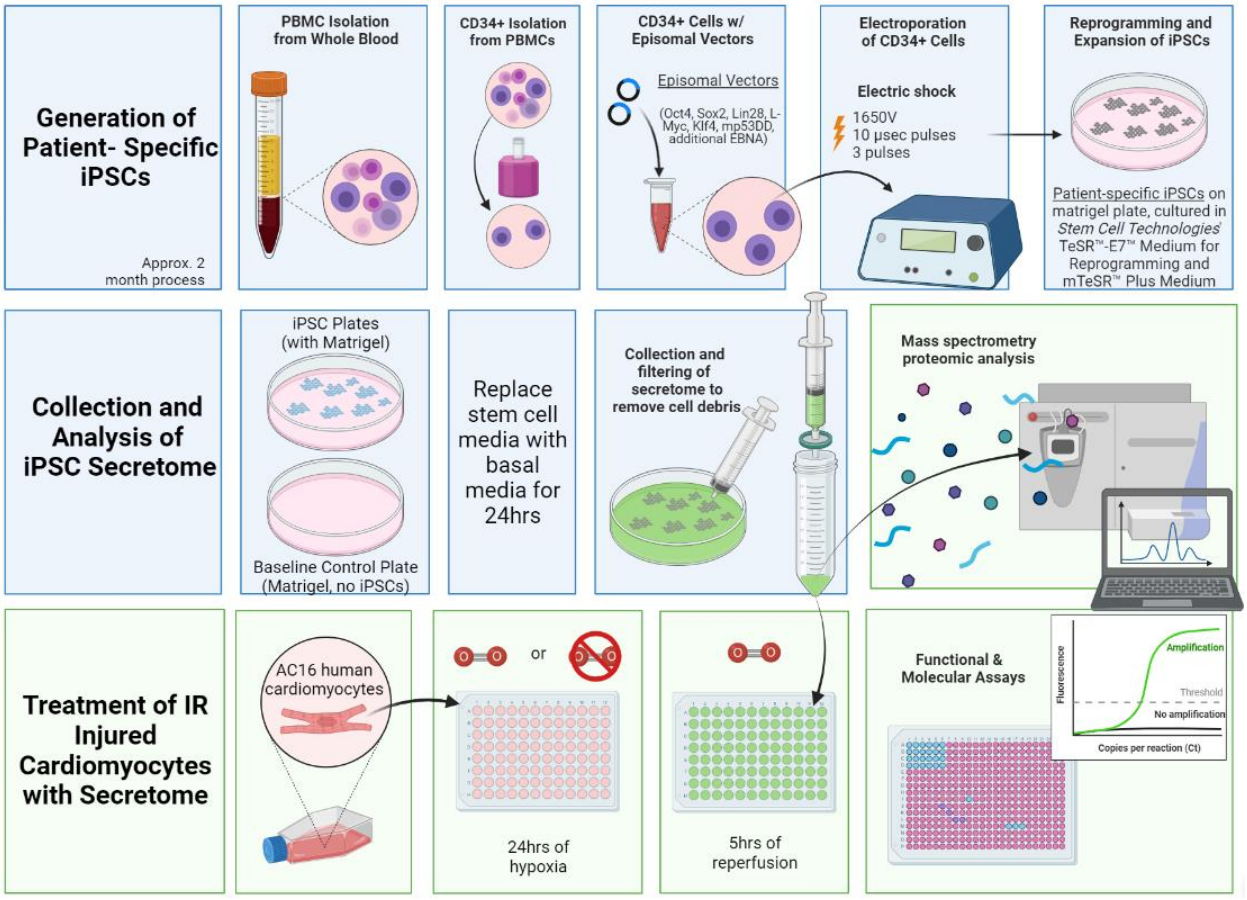
Abstract

Myocardial infarction (MI) often leads to heart failure or death due to the myocardium's inability to regenerate after injury. Many attempts have been made to implant stem cells into infarcted hearts to help induce recovery without meaningful improvements. Now, the focus has shifted towards cell-free therapies that aim to induce cardiac protection from and repair after MI. This study aims to evaluate whether the secretome from induced pluripotent stem cells (iPSCs) serves any cytoprotective effect in cardiomyocytes after an ischemia-reperfusion (IR) injury. We hypothesize that the iPSC secretome will significantly protect cardiomyocytes from cell death, hypertrophy, and calcium overload after IR injury. $CD34^+$ cells were isolated from the blood of healthy donors (HE; n=3) and dilated cardiomyopathy patients (CM; n=3), then reprogrammed into iPSCs prior to secretome collection. AC16 human cardiomyocytes were cultured in hypoxic conditions and reperfused in normoxia to mimic an IR injury. During reperfusion, cells were treated with iPSCs secretome to access the effect of iPSC paracrine factors on cardiomyocyte function. Mass spectrometry was performed on iPSC secretomes to investigate factors that may be responsible for the secretome's effects on AC16 cells. iPSCs secretome was found to decrease cell death and hypertrophy in IR-injured cardiomyocytes. Additionally, intracellular calcium concentrations varied between cardiomyocytes treated with secretomes from HE- and CM-derived iPSC lines. This effect can be explained by differentially expressed proteins in HE-derived iPSC secretomes, which may be an indication of improved contractility rather than calcium overload. This study suggests that iPSC secretome has cytoprotective effects on cardiomyocytes after IR injury, and more research is needed to assess its therapeutic potential after MI.

Graphical Abstract



Résumé

L'infarctus du myocarde (IM) mène souvent à une insuffisance cardiaque ou la mort en raison de l'incapacité du myocarde à se régénérer après une blessure. De nombreuses essais cliniques ont été faites pour implanter des cellules souches dans des cœurs infarcis pour aider à induire la récupération sans améliorations significatives. Aujourd'hui, l'attention s'est déplacée vers les thérapies sans cellules qui visent à induire une protection cardiaque et une réparation après un IM. Cette étude vise à évaluer si le sécrétome des cellules souches pluripotentes induites (CSPi) a un effet cytoprotecteur dans les cardiomyocytes après une lésion d'ischémie-reperfusion (IR). Nous émettons l'hypothèse que le sécrétome CSPi protégera de manière significative les cardiomyocytes de la mort cellulaire, de l'hypertrophie, et de la surcharge calcique après une lésion IR. Les cellules $CD34^{+}$ ont été isolées du sang de donneurs sains (HE ; n = 3) et de patients atteints de cardiomyopathie dilatée (CM ; n = 3), puis reprogrammées dans des CSPi avant la collecte du sécrétome. Des cardiomyocytes humains AC16 ont été cultivés dans des conditions hypoxiques et reperfusés en normoxie pour imiter une lésion IR. Au cours de la reperfusion, les cellules ont été traitées avec le sécrétome CSPi pour accéder à l'effet des facteurs paracrines venant de CSPi sur la fonction des cardiomyocytes. La spectrométrie de masse a été réalisée sur des sécrétomes CSPi pour étudier les facteurs pouvant être responsables des effets du sécrétome sur les cellules AC16. Il a été constaté que le sécrétome des CSPi entraînait une diminution de la mort cellulaire et de l'hypertrophie dans les cardiomyocytes blessés par l'IR. De plus, les concentrations de calcium intracellulaire variaient entre les cardiomyocytes traités avec des sécrétomes de lignées CSPi dérivées de HE et de CM. Cet effet peut être expliqué par des protéines différentiellement exprimées dans les sécrétomes CSPi dérivés de HE, ce qui peut être une indication d'une

contractilité améliorée plutôt que d'une surcharge en calcium dans les cardiomyocytes. Cette étude suggère que le sécrétome CSPi a des effets cytoprotecteurs sur les cardiomyocytes après une lésion IR, et des recherches supplémentaires sont nécessaires pour évaluer son potentiel thérapeutique après un IM.

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Contribution of Authors

Jeremy Zwaig designed research with support from Ida Derish, Kashif Khan, and Renzo Cecere; the majority of experiments were performed by Jeremy Zwaig, with complementary experiments conducted by Ida Derish, Bin Yu, Kashif Khan, and Elise Rody. Jeremy Zwaig analyzed the data and performed statistical analyses, with support from Ida Derish and Kashif Khan. Jeremy Zwaig wrote the thesis, with feedback from Ida Derish and Kashif Khan. Renzo Cecere and Sabah Hussain reviewed the thesis and suggested edits and recommendations.

List of Figures and Tables

List of Abbreviations

Abbreviation	Definition
AC16	human cardiomyocyte
ACAA2	acetyl-Coenzyme A acyltransferase 2
ANK2	ankyrin-B
ATP	adenosine triphosphate
BMP	bone morphogenic protein
BMPR	bone morphogenic protein receptor
BNIP3	Bcl2 interacting protein 3
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CM	cardiomyopathy patient
CSC	cardiac stem cell
CV	crystal violet
DEP	differentially expressed protein
DMEM/F12	Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ESC	embryonic stem cell
EV	extracellular vesicle
FBS	fetal bovine serum
HE	healthy donor
HF	heart failure
HSP	heat shock protein
IP3	inositol-1,4,5-trisphosphate receptors
iPSC	induced pluripotent stem cell
IR	ischemia-reperfusion
IRI	ischemia-reperfusion injury
IWP4	inhibitor of WNT production-4
LVAD	left ventricular assist device
MI	myocardial infarction
MSC	mesenchymal stem cell
NCX1	Na/Ca exchanger
NKA	Na/K ATPase
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	proepicardium

PEC	pre-epicardial cells
PECM	PEC maintenance media
PKC	protein kinase C
PRDX	peroxiredoxin
RA	retinoic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcription-polymerase chain reaction
SFRP1	secreted frizzled-related protein 1
SOD	superoxide dismutase
SRI	sorcin
VEGF	vascular endothelial growth factor

1. Introduction

Heart failure (HF) occurs when the heart is unable to efficiently pump blood throughout the entire body, disrupting other bodily functions. There are approximately 669,600 (3.6%) Canadians over the age of 40 living with HF, which is a 43% increase from the last 13 years ago;[1] this increased incidence poses a significant burden on the healthcare system. Patients with HF often suffer from a decrease in their quality of life and a high rate of mortality after diagnosis.[1] In fact, even in developed countries, the 5-year survival rate for HF patients is frequently below 50%.[2] Some common causes of HF include myocardial infarction (MI), hypertension, coronary artery disease, heart valve disease, arrythmias, congenital heart defect, and myocarditis.[3] During a MI, patients' hearts are subject to ischemia followed by reperfusion, both of which cause cell death and cardiac scarring.[4] Cardiomyocytes, the predominant contractile cell in the heart, are terminally differentiated and cannot repair themselves after an injury, including after MI.[5] Consequently, the heart undergoes physiological changes to accommodate the decrease in functional myocardium. Overtime, these changes become maladaptive and lead to HF.[6] Some medications exist to treat the symptoms of HF, however, there is currently no therapeutic approach that can induce cardiac regeneration. For HF patients, the solution with the best outcome is heart transplantation.[7] Unfortunately, demand for healthy hearts far exceeds supply and many people never reach the top of the transplant list.[8] To alleviate the organ donor shortage and improve patient outcomes, novel therapeutic approaches are needed that improve prognosis and prevent HF after MI.

One approach that has been extensively researched as a potential HF treatment is stem cell therapies, in which, stem cells or their derivatives are implanted into the infarct or border zones of heart failure patient's hearts. Previously, this stem cell implantation has yielded unencouraging

outcomes, as the cells have been unable to integrate into the native tissue and therapies have been unable to improve cardiac function or reduce mortality after MI.[9] Additionally, many stem cell therapies pose a considerable risk of teratoma formation and ventricular arrhythmias.[10]

More recently, researchers have founds that some of the positive effects seen in cardiac stem cell therapies can be attributed to the paracrine effects of the implanted stem cells.[9] Much of the research in cell-free stem cell therapies has been focused on mesenchymal stem cells (MSCs) because of their known secretory profile of factors with anti-apoptotic, pro-angiogenic and endogenous repair effects.[11, 12] While factors secreted by MSCs, also referred to as the secretome, have shown preclinical cytoprotective effects in myocardium after MI, other stem cell sources have been investigated. One notable cell type that merits more investigation is induced pluripotent stem cells (iPSCs), which can be generated in a patient-specific manner with only a small sample of blood. The cytoprotective properties that iPSC secretome exerts on cardiomyocytes after ischemia-reperfusion injury have not been explored.

The aim of this study is to investigate the cytoprotective effects of iPSC secretome on AC16 human cardiomyocytes during oxygen reperfusion after hypoxia. We hypothesize that the iPSC secretome will significantly protect cardiomyocytes from cell death, hypertrophy, and calcium overload after IR injury. Cardiomyocyte cell death, hypertrophy, and calcium overload are frequently seen during MI, leading to maladaptive remodeling, decreased cardiac function, progression to HF, and death. Thus, if iPSC secretome can limit these negative outcomes, then more research should be conducted to assess its viability to serve as a therapeutic treatment, in either an autologous or allogeneic manner.

2. Review of the Literature

2.1. Myocardial Infarction

Acute myocardial infarction (MI) is a leading cause of heart failure and death globally, with a 30-day mortality rate of 13.6%.[13] The main cause of MI is coronary heart disease, which usually occurs as plaque builds up in the coronary arteries in a process called atherosclerosis. The plaques that cause artery wall thickening are typically made of lipids, immune cells, inflammatory cells, smooth muscle cells, extracellular matrix, calcium, and necrotic debris.[14] Additionally, atherosclerosis can be categorized into two phenotypes: stable and unstable plaques.[15] The former has a thick cap that is resistant to rupturing, while the latter has a thinner cap and is more prone to rupturing, releasing prothrombotic factors, and causing intra-vascular thrombosis.[16] Such a thrombus formation can cause an MI by restricting blood flow to cardiac tissue and depleting the heart of oxygen and nutrients.

On a molecular level, a MI causes hypoxia, which disrupts the electron transport chain and decreases the production of adenosine triphosphate (ATP).[4] Consequently, ion pumps are disrupted causing acidosis, calcium overload, hyperosmolarity, impaired enzyme activity and clumping of nuclear chromatin and ribosomal detachment.[4, 17] The combination of these effects ultimately leads to cell death via necrosis, apoptosis, necroptosis and pyroptosis.[17] After a MI, an infarct zone remains in the portion of the heart that was subject to ischemia, defined by its scaring and fibrotic tissue.[18] Additionally, the disruption of calcium homeostasis may lead to changes in cardiomyocyte contractility, as calcium plays an important role in cell excitation–contraction coupling.[19] To accommodate the reduction in healthy myocardium caused by MI, the surviving tissue must adapt. Compensatory physiological changes take place to preserve function and

survival, however, overtime, this cardiac remodeling becomes maladaptive and leads to fibrosis and dysfunction.[6, 20]

2.1.1. Risk Factors

While the onset of a heart attack is often unpredictable, there are numerous factors that present an increased risk of poor cardiovascular health. For example, the incidence of heart failure is higher for men than for women and increases as people age.[21] Some behaviours that present a risk for cardiovascular disease include smoking, physical inactivity, and poor nutrition.[22] Other health conditions that present a risk for poor cardiovascular health include high blood cholesterol, hypertension, obesity, diabetes mellitus, metabolic syndrome, kidney disease, and poor sleep.[22]

2.1.2. Current Treatment and Reperfusion Injury

Once an acute MI occurs, it is vital to promptly restore blood flow to the heart to minimize arrythmias, tissue death, heart failure, and death. The first step in treatment is revascularization, which is being done with increasing frequency via percutaneous coronary intervention, but can also be done by coronary artery bypass grafting when the disease is complex or the patient has diabetes, and the patient does not have co-morbidities that would precludes open-heart surgery.[23-25] Additional antiplatelet and anticoagulant therapies are administered in parallel to surgical and interventional treatments to improve blood flow to the heart and to reduce the risk of thrombus formation at the site of stent placement or balloon angioplasty.[23] The restoration of blood flow through the cardiac vessels is a process called reperfusion.

Paradoxically, despite reperfusion being essential for restoring oxygen and nutrients to the heart, this process has adverse effects on cardiac tissue, causing myocardial death and enlarged infarct size.[26] On a molecular level, reperfusion injuries are caused by an increase in reactive oxygen species (ROS) formation, a disruption in intracellular Ca^{2+}, Na^{+}, and pH distribution, and

an increase in proteolytic activity.[17] Additionally, ROS can directly cause cell death by lipid peroxidation of cellular membranes and can trigger apoptosis by inducing the release of cytochrome c into the cytoplasm.[27, 28]

2.1.3. Disease Progression and Heart Failure

When a MI occurs, the damage to the heart is irreversible due to the adult mammalian heart's insignificant regeneration abilities.[29] In short, the heart is unable to activate a repair mechanism to replace damaged tissue with healthy tissue; thus, restoring optimal cardiac function after an injury, such as MI, is infrequent without medical intervention. One study found that 37% of first-time MI patients were diagnosed with HF upon admission or during their hospitalization, with another 13% dying after their first MI.[30] During a follow-up, the same study found that 76% of patient who survived their first MI were diagnosed with HF within 5 years.[30] Currently, the prevention and treatment options available for HF are limited to pharmacological therapies and invasive surgeries to maintain or improve the performance of cardiomyocytes, however, no therapy exists to regenerate or repair dysfunctional myocardium.

Current Canadian guidelines recommend that HF with reduced ejection fraction (HFrEF) patients are treated with one therapy in each of the following four categories: angiotensin receptor-neprilysin inhibitors, β-blockers; mineralocorticoid receptor antagonists; and sodium glucose transport 2 inhibitors.[31] Under circumstances described by Canadian Cardiovascular Society heart failure (HF) guidelines, sinus node inhibitors, soluble guanylate cyclase stimulators, hydralazine/nitrates, and digoxin should be prescribed in combination with or as a substitute for the therapies listed above.[31] It is also recommended that comorbidities, such as iron deficiency, atrial fibrillation, and functional mitral regurgitation, be treated in parallel to HF treatment.[31] All of these pharmacological interventions aim to improve the management of HF and to minimize its

symptoms, however, none induce cardiovascular repair or lead to sustained improvements in cardiac function overtime.

When pharmacotherapies are ineffective and unable to prevent the progressive decline in the health of HF patients, surgical interventions, such as left ventricular assist device (LVAD) implantation and heart transplantation, are needed.[32] The gold standard for treatment is a heart transplant, which provides patients with an average of 4.9 life-years saved.[33] LVADs are often considered a bridge-to-transplant solution because outcomes are comparatively less effective than heart transplants, however, they still offer HF patients a 1-year survival rate of over 80% and a 4-year survival rate of roughly 50%.[34] Unfortunately, heart transplants are not always available to patients due to a limited donor supply. The severity of this shortage is amplified for each patient when considering the quality of the organ and the match with the recipient, two factors that are critical for heart transplantation.[35] In fact, there are 2,500 heart transplant recipients in the United States each year, which is only a small fraction of patients on the transplant waitlist.[36] Additionally, as with any surgery, there are numerous risks and complications associated with heart transplants and LVAD implants.[36] For these reasons, novel therapies are needed that aim to limit the extent of cardiomyocyte damage during IR injury and that minimize the risk of MI progression to HF.

2.2. Stem Cells

Stem cells are unspecialized cells that have two critical properties: the ability to self-renew, and the potential to differentiate into one or many mature cell types. Stem cells can be classified by the number of different cell types they can develop into. A human zygote is an example of a totipotent stem cells, which can differentiate into both embryonic and extraembryonic structures, thus, any cell type in the human body. Pluripotent stem cells are capable of differentiating into all germ layers, but they cannot differentiate into extraembryonic structures. In development, embryonic

stem cells (ESC) from the inner cell mass are considered pluripotent. Other examples of pluripotent cells include mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs). As these cells specialize further from pluripotent, to multipotent, oligopotent, and unipotent stem cells, they become capable of differentiating into fewer cell lineages (Fig. 1).[37]

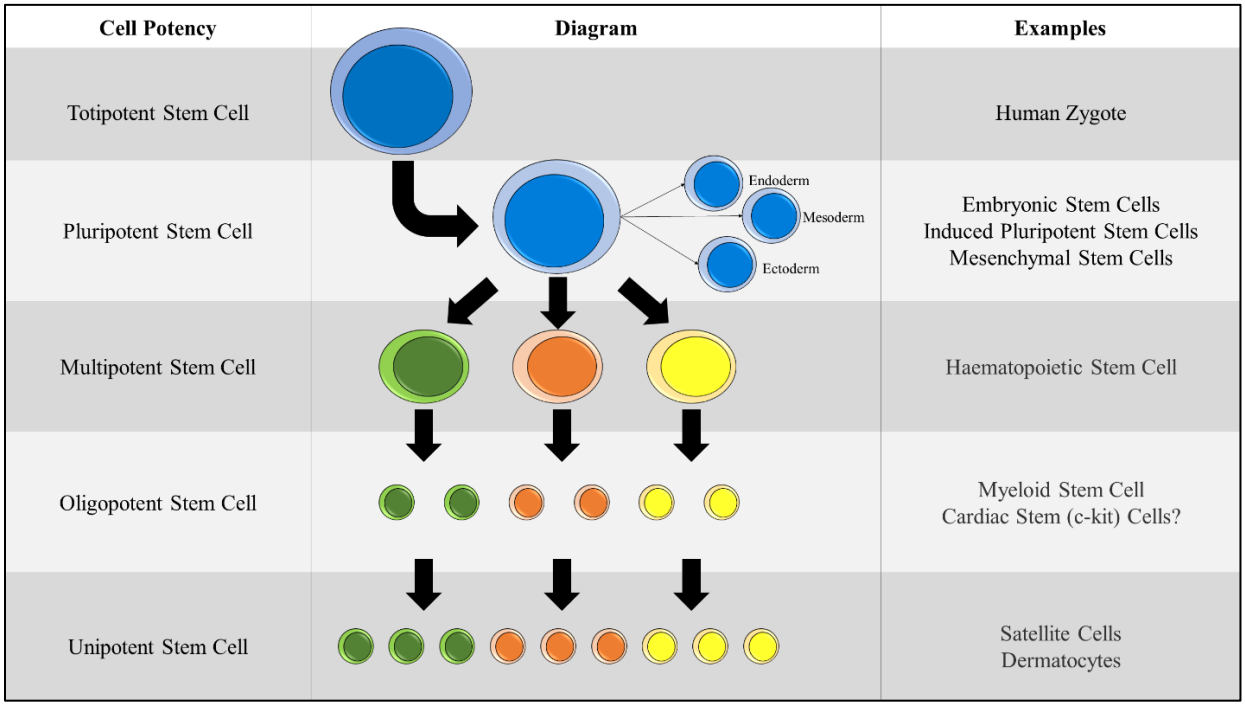

Figure 1: Stem Cell Hierarchy.
Totipotent stem cells can self-renew and differentiate into any embryo and extraembryonic cell type. Pluripotent stem cells can self-renew and differentiate into any embryonic cell type, including those from any germ layer. Multipotent stem cells can self-renew and differentiate into any cell type within a specific tissue lineage (e.g., haematopoietic stem cells can differentiate into all types of blood cells). Oligopotent stem cells can self-renew and differentiate into 2 or more lineages within a specific tissue. Unipotent stem cells can self-renew and differentiate into a single cell type.

In mammals, stem cells can be sourced from different tissues during several stages of development. For instance, stem cells can be isolated from an embryos, however, ethical concerns of using this cell type have been described.[38] Stem cells can also be found in and isolated from mammalian adult tissue; for example human liver stem cells, satellite cells, and haematopoietic

stem cells, all of which are multipotent stem cells that can be found in various amounts in the liver, skeletal muscle, and bone-marrow, respectively.[39-41]

It was speculated for decades that the heart lacks cells with self-renewal capabilities, as there is insufficient cardiomyocyte regeneration after MI in humans to demonstrate functional recovery. In 2003, c-kit$^+$ cells were first described as adult cardiac stem cells (CSC), capable of self-renewal and differentiation into cardiomyocytes, smooth muscle, and endothelial cells;[42] yet, there have been many conflicting results that put into question the characterization of these cells as CSC.[43] For example, some studies have shown that cardiomyocytes can be generated from neonatal nonmyocyte c-kit$^+$ cells but not from adult c-kit$^+$ cells.[44, 45] This finding, however, does not reject the possibility that cells in the adult human heart have the intrinsic ability to replicate. In 2009, carbon-14 that had integrated into cardiac tissue during cold-war nuclear testing was used to assess the age of cardiomyocytes in the heart.[46] Interestingly, the team found that there was up to a 1% annual cardiomyocyte turnover in young people, and a 0.45% in the elderly.[46] These results suggested for the first time that there is a cardiomyocyte subpopulation with endogenous self-renewal abilities and prompted researchers to investigate methods to therapeutically activate this regenerative pathway.

2.2.1. Induced Pluripotent Stem Cells

In 2006, Yamanaka described the use of Oct3/4, Sox2, c-Myc, and Klf4 for reprogramming murine fibroblasts into iPSCs.[47] Since then, numerous methods of generating iPSCs with reliability, including via the introduction of Yamanaka factors into various somatic cells using an episomal vector or a Sendai virus.[48] iPSCs are generally considered easily accessible due the non-invasive methods of fibroblast and peripheral blood mononuclear cells (PBMC) sample collection, and the relatively simple reprogramming methods. Similar to other stem cells, iPSCs can self-renew and

differentiate into all three germ layers, however, they have the added benefit of retaining the genetic makeup of the donor. Consequently, it is possible to generate patient-specific iPSCs for disease modeling, drug testing, and tissue engineering.[49]

2.3. Cardiac Stem Cell Therapies

When it was first discovered that blastocyst-derived embryonic stem cells can differentiate into visceral yolk sac, blood islands and myocardium cells *in vitro*, researchers speculated that this finding would be essential to model early-stage embryogenesis.[50] Since then, discoveries in the field of embryogenesis have made it possible to direct the differentiation of stem cells to specialized cell types by mimicking steps of embryonic developement.[51] Currently, researchers and clinicians alike have demonstrated a great interest in stem cell therapies, which involves using stem cells and their derivatives in medicine for therapeutic applications. More specifically, stem cells have the potential to drastically advance the field of regenerative medicine because they are speculated to be capable of repopulating the heart with viable cells and promoting endogenous tissue repair after organ degeneration or damage.

Treatments for patients with impaired myocardial function, including HF patients, are currently limited to palliative drug regiments and heart transplants. However, stem cell therapies are generating a great deal of optimism because they attempt to treat the underlying cause of cardiac disfunction to improve cardiac function and alleviate the demand for heart transplants. Unfortunately, the enthusiasm for these novel therapies frequently dampens during clinical trials, as favourable outcomes from preclinical results are not replicated in human efficacy trials. The most common and clinically relevant endpoints in these clinical trial include patient death and major adverse cardiac events, however, other surrogate efficacy endpoints are often used as well to understand the effects of each therapy.[52] While some surrogate endpoints, such as reduced scar

size have been found in several studies, other endpoints such as decreased tissue remodeling and improvement in ejection fraction have lacked reproducibility.[52] Notably, no cardiac stem cell therapy has been shown to significantly reduce mortality as of yet.

Fortunately, lessons from previous cardiac stem cell therapy research and trials can be used to guide future efforts. Cardiac stem cell therapies be placed into one of two categories: the remuscularization of the injured myocardium with exogenous cells or the activation of endogenous repair pathways (Figure 2).[53] Both stem cell therapy approaches are discussed in more detail below.

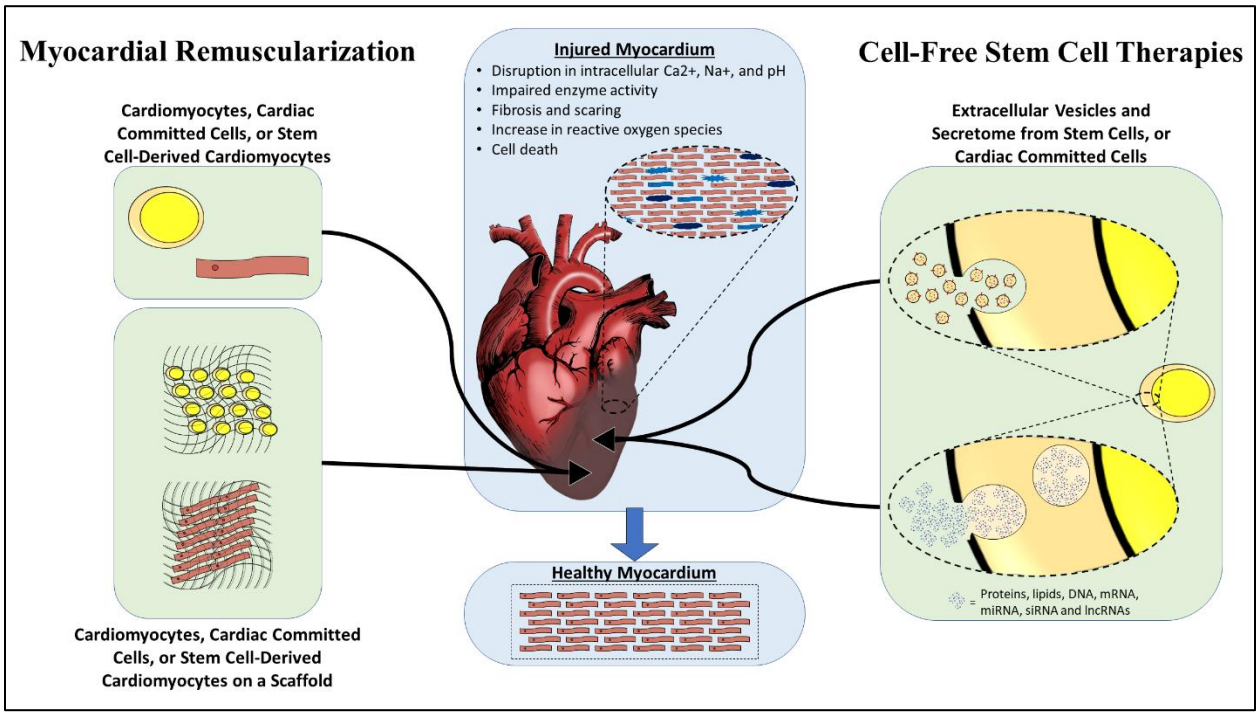

Figure 2: Categories of cardiac stem cell therapies.
To repair myocardium after MI, diverse stem cell therapies are being explored. The implantation of cardiomyocytes and their progenitors into injured myocardium, either by direct injection or in a scaffold, have been investigated for myocardial remuscularization. Cell-free stem cell therapies using extracellular vesicles and secretome from stem cells and cardiac-committed cells are also being investigated.

2.3.1. Myocardial Remuscularization

Clinical trials for cardiac stem cell therapies can vary greatly in their designs, end points, stem cell source, and route of delivery. Some of the first cell therapies attempted to treat HF patients used skeletal myoblasts and haematopoietic stem cells.[54, 55] While these cell types have high proliferative potential, it is now clear that they cannot differentiate into cardiomyocytes, limiting their potential for remuscularization of injured hearts.[53] Another source of stem cells that has been investigated for myocardial remuscularization are ESCs. While ESCs provide researchers with a valuable cell type for disease modeling, the high risk of immune rejection when using allogenic cells remains high.[56, 57] In situations where allogenic cells are used, immunosuppressants are often required for the rest of the patients' lives. When iPSC-derived cardiomyocytes have been used to remuscularize infarcted myocardium in non-human primates, ventricular arrhythmias occur at a staggering rate.[58-60] Ectopic pacemaker-like activity, potentially a consequence of "electrical immaturity" has been identified as the source of graft-induced arrhythmias in these studies.[58, 61] While the use of iPSC-derived cardiomyocytes have a lot of potential for cardiac remuscularization, there are still many hurdles to overcome before they can reliably be used as a stem cell therapy.[62]

Another limitation of early stem cell therapy clinical trials is that they focused on cell injected into and around the infarct tissue, however, poor engraftment frequently limited the success of these trials.[54, 55] To avoid the issue of poor engraftment, other modes of delivery have been used, including cell-loaded patches and hydrogels. A recent study showed that a scaffold to introduce a chitosan and hyaluronic acid hydrogel loaded with amniotic mesenchymal stem cells was capable of repopulating the infarct heart with proliferative cells that differentiated into cardiomyocytes and triggered angiogenesis.[63] It is becoming increasingly clear that biomaterials

will likely be involved to optimize the post-ischemic cardiac microenvironment for stem cell retention and efficiency during implantation.[64, 65] In fact, some teams are working to create a microenvironment that retains a heart's vascular and three-dimensional structure via organ decellularization to generate a whole-heart scaffolds that can be implanted with cardiomyocytes and other cardiac cells.[66-68] Ultimately, these methods require improved strategies for decellularized scaffold reseeding and organ maturation prior to being tested clinically.

Thus far, even the most promising preclinical results have been unable to translate to success in human clinical trials. While there has been promise of advances in the field of cardiac stem cell therapies for the last few decades, the enthusiasm has not dampened. Researchers continue to implement new findings implants from the fields of developmental biology, material science, immunology, and pharmacology into their myocardial engineered, leading to gradual improvements in cardiac remuscularization stem cell therapies.

2.3.2. Cell-Free Stem Cell Therapies

While there has been limited clinical success in remuscularization of the injured myocardium using exogenous cell implants thus far, it has been suggested that some of the observed regenerative effects can be attributed to paracrine factors from the implanted tissues activating of endogenous repair pathways in and around the infarct zone.[69] In an early study, mesenchymal stem cells (MSCs) over-expressing protein kinase B (Akt1) were injected into ischemic rat myocardium and were capable of preventing ventricular remodeling and restoring cardiac function, however, it was unclear whether the effects were due to MSC implantation or paracrine actions.[70] Another team identified that these effects were seen within 72 hours of injection, which suggested to them that the injected stem cells themselves could not have differentiated to muscularize the myocardium

that rapidly.[71] With subsequent experiments, they confirmed that the cytoprotective effect seen in ischemic myocardium after injection of MSCs over-expressing Akt1 was due to paracrine action.[71]

Since then, numerous other studies have shown that extracellular vesicles (EVs) in the conditioned media of MSCs can decrease apoptosis, infarct size, and promote angiogenesis in infarct myocardium.[72-74] Other cell types, including iPSCs, have also been investigated for their secretome's ability to protect the myocardium after MI injury. In fact, it was found that EVs derived from iPSCs had an angiogenic and antiapoptotic effect on murine cardiac endothelial cells.[75] In a MI murine model, these EVs were capable of improving left ventricle function, reducing left ventricle mass, increasing perfusion, preserving viable myocardium, and decreasing apoptosis in the infarct zone.[75] Additionally, iPSC EVs were considered safe because they did not cause to teratoma formation, unlike the iPSC implantation control.[75]

While clinical trials have yet to prove the efficacy of cell-free stem cell cardiac therapies, the evidence is clear that activating endogenous repair pathways will be critical for cardiac regeneration after an MI.

3. Materials and Methods

3.1. Induced Pluripotent Stem Cells (iPSCs) Generation and Maintenance

Peripheral blood was collected from subjects and stored at 4ºC for no more than 12 hours. A modified version of Stem Cells Technology's protocol for Integrated Workflow for the Isolation, Expansion, and Reprogramming of $CD34^+$ Progenitor Cells was used for reprogramming peripheral blood mononuclear cells (PBMCs; STEMCELL Technologies, Inc., DOCUMENT #29801). Briefly, blood samples were mixed with EasySep™ Buffer (STEMCELL Technologies, Inc.), added to a layer of Lymphoprep™ (STEMCELL Technologies, Inc.) in SepMate™ PBMC Isolation Tubes (STEMCELL Technologies, Inc.), and fractionated by centrifugation. The top layer, containing plasma and peripheral blood mononuclear cells (PBMCs), was collected and centrifuged. The PBMC pellet was collected and mixed with fetal bovine albumin (FBS; Gibco) and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich), then was frozen overnight at -80ºC before being transferred to -180ºC for long-term storage. Later, the PBMCs were thawed rapidly at 37ºC, and the media was replaced with EasySep™ Buffer. DNase I and DNase buffer (Invitrogen™, Thermo Scientific) were transiently added to samples to prevent cell clumping caused by nucleic acids in suspension. Next, EasySep™ Human CD34 Positive Selection (STEMCELL Technologies, Inc.) and EasySep™ Dextran RapidSpheres™ (STEMCELL Technologies, Inc.) were added and an EasySep™ Magnet (STEMCELL Technologies, Inc.) was used to isolate $CD34^+$ cells. StemSpan™ Serum-Free Expansion Medium II (STEMCELL Technologies, Inc.) supplemented with StemSpan™ CC100 (STEMCELL Technologies, Inc.) was added to each sample, and cells were reactivated over the span of 7 days, with media changes every two days.

On day 7, cells from each patient were transfected via electroporation using vectors containing Oct4, Sox2, Lin28, Klf4, and L-Myc reprogramming factors from the Epi5™ Episomal

iPSC Reprogramming Kit (Invitrogen™, Thermo Fisher). Electroporation was done on the Neon™ Neon Transfection System, with setting set to 1650 volts, 10 milliseconds, and 3 pulses. Samples were plated onto Matrigel Matrix (Corning) covered plates, and media was gradually replaced with TeSR™-E7™ Medium for Reprogramming (STEMCELL Technologies, Inc.) over the span of 7 days. After approximately 18-25 days, iPSC colonies began to arise. These colonies were mechanically split using 22-gauge needles and replated onto new Matrigel plates. This was repeated for 2-5 passages until over 95% of cells in each well had the morphological characteristics of iPSCs (Figure 2a,b). At this stage, non-iPSC cells were mechanically removed, and Gentle Cell Dissociation Reagent (STEMCELL Technologies, Inc.) was used to chemically split the cells following the manufacturer's instructions. Cells were frozen using Cryostor CS10 (STEMCELL Technologies, Inc.) following the manufacturers instruction. To ensure that the iPSC samples were not contaminated, culture supernatant was collected at P6, and a Mycoplasma PCR Detection Kit (ScienCell™) was used according to the manufacturer's instructions.

3.2. Secretome Collection

The iPSCs from each of 6 cell lines were plated on Matrigel plates between passages P6 and P10 and cultured until plates reached 80-90% confluence. The plates were washed three times with phosphate-buffered saline (PBS). Then, the iPSC plates had their media replaced with serum-free TeSR™-E8™ (STEMCELL Technologies, Inc.) for 24 hours. The supernatant, also called secretome or conditioned media, was collected, filtered through a 0.22 µm pore sized MF-Millipore™ Membrane Filter (Millipore Sigma, Merck), and stored at -20ºC. Another Matrigel plate without cells was also incubated for 24 hours in serum-free TeSR™-E8™ and the media was collected as a baseline control media to ensure that Matrigel was not responsible for any observed results.

3.3. Immunofluorescence

Cultured cells were seeded into Chambered Cell Culture Slides (Corning™ Falcon™). Cells were fixed with 4% paraformaldehyde (PFA; Electron Microscopy Sciences) for 15 minutes, and then washed with PBS. Cells were permeabilized with 0.5% Triton X-100 (Electron Microscopy Sciences) in PBS for 15 minutes at room temperature, then washed in PBS again. Cell were incubated in blocking buffer – 3% Bovine Serum Albumin (BSA) in PBS – for 40 to 60 minutes. Cells were incubated with primary antibodies diluted in 3% BSA PBS at 4ºC overnight. Then, cells were washed with PBS before incubating with dye-conjugated secondary antibody (Invitrogen™, Thermo Fisher) diluted in 3% BSA/PBS at room temperature for an hour. Samples were washed with PBS, then mounted using VECTASHIELD Mounting Media with DAPI (Vector Laboratories Inc.) was added. Slides were visualized on a TH4-100 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

3.4. Reverse Transcription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) and agarose electrophoresis was used to ensure that iPSC lines generated via electroporation were expressing pluripotency markers. RNeasy Plus Mini (QUIAGEN) and Blood/Cell RNA Mini Kit (Geneaid) protocols were used to isolate messenger ribonucleic acid (mRNA) from iPSCs. A pre-existing iPSC line that was generously donated by Dr. Thomas Durcan, and AC16 cardiomyocytes were used as positive and negative controls, respectively. cDNA was generated using SuperScript III First-Strand Synthesis System (Invitrogen™, Thermo Fisher). To see if the genes of interest were being expressed, Platinum™ PCR SuperMix High Fidelity (Invitrogen™, Thermo Fisher) was used with primers for OCT3/4, NANOG, and LIN28 (sequences in Supplemental Table 1). Lamelli buffer 4X

(BioRad) was added to PCR products and analyzed by 1.2% agarose gel electrophoresis using GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific).

3.5. AC16 Human Cardiomyocyte Cell Culture

The AC16 human left ventricular cardiomyocytes (Millipore Sigma) were used for this study. Previously, AC16 cells have been shown to stain positively for several cardiac markers including atrial natriuretic peptide, brain natriuretic peptide, α-catenin, myosin heavy chain 7, troponin I, connexin 43, and GATA4.[76] Cells were cultured in AC16 media, consisting of Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F12; ThermoFisher Scientific) supplemented with 10% FBS and 1% Penicillin-Streptomycin (P/S; Sigma-Aldrich). AC16s were thawed rapidly then combined with 10mL of AC16 media described above. Cells were passaged using trypsin and stored at -180ºC in FBS with 10% DMSO. Experiments were conducted using cells at passages 9 to 13.

3.6. Ischemia-Reperfusion (IR) Injury Model

AC16 cardiomyocytes were plated and cultured until they were >95% confluent. To replicate ischemia *in vitro*, media was replaced with serum-free DMEM/F12 media and incubated with 0% oxygen in a container with an AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan) for 24 hours. Then, normoxic conditions were restored and cardiomyocytes were treated with secretome from one of the 6 iPSC cell lines or baseline control media for 5 hours or 24 hours.

3.7. Assessment of Cardiomyocyte Cell Viability

Crystal violet (CV) assay was used to assess cell survival after IR injury. Cardiomyocytes were seeded in 96-well plates at a seeding density of 5,000 cells. After IR injury, cells were fixed in 4% paraformaldehyde and stained with 100uL of 0.05% crystal violet staining reagent (Sigma). The plates were washed gently with tap water and imaged using a TH4-100 brightfield microscope

(Olympus Corporation, Tokyo, Japan). CV stain was then solubilized with methanol and plates were incubated on a rotator at room temperature for 30 minutes. Experiments were conducted using 6 replicates. Absorbance was measured using a TECAN Infinite© M200 Microplate reader (Tecan Group Ltd, Männedorf,125 Switzerland).

3.8. Assessment of Cardiomyocyte Metabolic Activity

AlamarBlue assay (Fischer) was used to detect cardiomyocytes metabolic activity. Cardiomyocytes were seeded into 96-well plates. After IR injury, cells were incubated in media with 10% AlamarBlue reagent for 4 hours. Experiments were conducted using 6 replicates. Absorbance was measured using a TECAN Infinite© M200 Microplate reader (Tecan Group Ltd, Männedorf,125 Switzerland). Relative metabolic activity was calculated using the following formula:

$$Percentage\ reduction\ = \frac{(117.216) \times A570 - (80.586) \times A600}{(155.677) \times A'600 - (14.652) \times A'570} \times 100\%, \text{where}$$

117.216: molar extinction coefficient of AlamarBlue in the oxidized form at 600 nm

80.586: molar extinction coefficient of AlamarBlue in the oxidized form at 570 nm

14.652: molar extinction coefficient of AlamarBlue in the reduced form at 600 nm

155.677: molar extinction coefficient of AlamarBlue in the reduced form at 570 nm

A570: absorbance of test wells at 570 nm

A600: absorbance of test wells at 600 nm

A’600: absorbance of negative control wells at 600 nm

A’570: absorbance of negative control wells at 570 nm

3.9. Assessment of Cardiomyocyte Hypertrophy

Anti-actin (Life Technologies) was used to visually assess cell size according to the manufacturer's instructions. Briefly, AC16s were plated into in Chambered Cell Culture Slides (Corning™ Falcon™). After IR injury, cells were fixed with 4% PFA (Electron Microscopy Sciences) for 15 minutes, and then washed in PBS. Cells were permeabilized with 0.5% Triton X-100 (Electron Microscopy Sciences) in PBS for 15 minutes at room temperature, then washed in PBS again. Cardiomyocytes were incubated with ActinRed™ 555 ReadyProbes™ reagent (Life Technologies Corporation) and NucBlue® Live Cell Stain ReadyProbes™ reagent (Life Technologies Corporation) for 30 minutes, then, visualized using a X-Cite 120LED Boost High-Power LED illumination System (Excelitas Technologies Corp.) and an Apotome.2 fluorescence microscope (Zeiss). Images were randomly acquired at 20x magnification under a fixed exposure time. Cell area was quantified using ImageJ imaging software ($n > 28$ cells per group).

3.10. Measurement of Intracellular Calcium Concentration

Fluo-4 AM (Invitrogen™, Thermo Scientific) was used to assess calcium handling in AC16 cardiomyocytes after IR injury, either with secretome treatment or without, according to manufacturer's instructions. Briefly, 3μM Fluo-4 AM in AC16 media was added to each well and incubated at 37 °C for 30 minutes. Cell were washed twice with PBS then incubated for 30 minutes in indicator- and phenol-free DMEM/F12 medium to remove any dye that is non-specifically associated with the cell surface and to allow complete de-esterification of intracellular AM esters. Cells were visualized on a Zeiss LSM 780 confocal microscope. Fluorescence intensity was measured for cells ($n=6$ per condition) every 5 seconds for at least 60 seconds.

3.11. Secretome Proteomic Profiling by Mass Spectrometry

For each sample, proteins were loaded onto a single stacking gel band to remove lipids, detergents, and salts. The gel band was reduced with dithiothreitol, alkylated with iodoacetic acid, and digested with trypsin. Extracted peptides were re-solubilized in 0.1% aqueous formic acid and loaded onto a Thermo Acclaim Pepmap (Thermo, 75 μM ID × 2 cm C18 3 μM beads) precolumn and then onto an Acclaim Pepmap Easyspray (Thermo, 75 μM × 15 cm with 2 μM C18 beads) analytical column separation using a Dionex Ultimate 3000 uHPLC at 230 nl/min with a gradient of 2%–35% organic (0.1% formic acid in acetonitrile) over 3 hours. Peptides were analyzed using a Thermo Orbitrap Fusion mass spectrometer operating at 120,000 resolution (FWHM in MS1) with HCD sequencing (15,000 resolution) at top speed for all peptides with a charge of 2+ or greater. The raw data were converted into *.mgf format (Mascot generic format) for searching using the Mascot 2.6.2 search engine (Matrix Science) against human protein sequences (Uniprot). The database search results were loaded onto Scaffold Q+ Scaffold_5.1.0 (Proteome Sciences) for statistical treatment and data visualization.

3.12. Statistical Analysis

One-way ANOVAs were used to determine the significance between treatment and control groups and were followed by *Post-hoc* Dunnett multiple comparisons test to determine statistical significance between selected groups. All data is presented as mean ± SEM. A *t*-test was used to determine the statistical significance of differentially expressed proteins in iPSC secretomes from cardiomyopathy patients compared to healthy donors. Pathway analysis was done using STRING.[77] Results were analyzed at a significance level of $P < 0.05$. Graphs were generated using GraphPad Prism 9 software and Microsoft Excel.

4. Results

4.1. Generation of iPSCs from Blood Sample

Five iPSC lines were derived via electroporation of episomal vectors into PBMCs. Of these lines, 3 of them were generated from the blood of dilated cardiomyopathy patients (CM) and 2 were generated from healthy donors (HE). A sixth line, AIW001-02, was graciously offered by the Thomas Durcan lab, who generated it via retrovirus infection. The age of the blood donors ranged from 39 to 55 years old, with an average age of 45.66 years. Four donors (66.6%) were Caucasian females, while the other two were Black and Asian males (Table 1).

Table 1: Overview of iPSC line profiles.

Patient	Sample ID	Age	Sex (M/F)	Ethnicity	Starting Material	Reprogramming method	Cardiomyopathy?
HE1	AIW001-02	48	F	Caucasian	PBMCs	Retrovirus	No
HE2	HID041004	46	F	Caucasian	PBMCs	Episomal	No
HE3	HID041032	55	M	Black -other	PBMCs	Episomal	No
CM1	HID041019	41	F	Caucasian	PBMCs	Episomal	Yes
CM2	HID041021	45	F	Caucasian	PBMCs	Episomal	Yes
CM3	HID041031	39	M	Asian (East or Southeast Asian)	PBMCs	Episomal	Yes
Average	-	45.66	66% (F)	-		-	50% (Yes)
HE = healthy donor, CM = cardiomyopathy patient, M: male; F: female.							

After 10-25 days after electroporation, iPSC colonies were identifiable (Fig 2a). At that point, cells from these colonies were replated onto new Matrigel plates and cultured. These colonies had a compact morphology and defined borders, with round cells that had high nuclear-to-cytoplasm ratios (Fig 2b), as described by the literature.[78] iPSC cell lines stained for OCT4, SSEA-4, NANOG, and TRA-1-60 (Fig. 2c), and expressed pluripotency markers OCT3/4, NANOG, and LIN28 (Fig. 2d), which confirmed that the cells were indeed iPSCs.

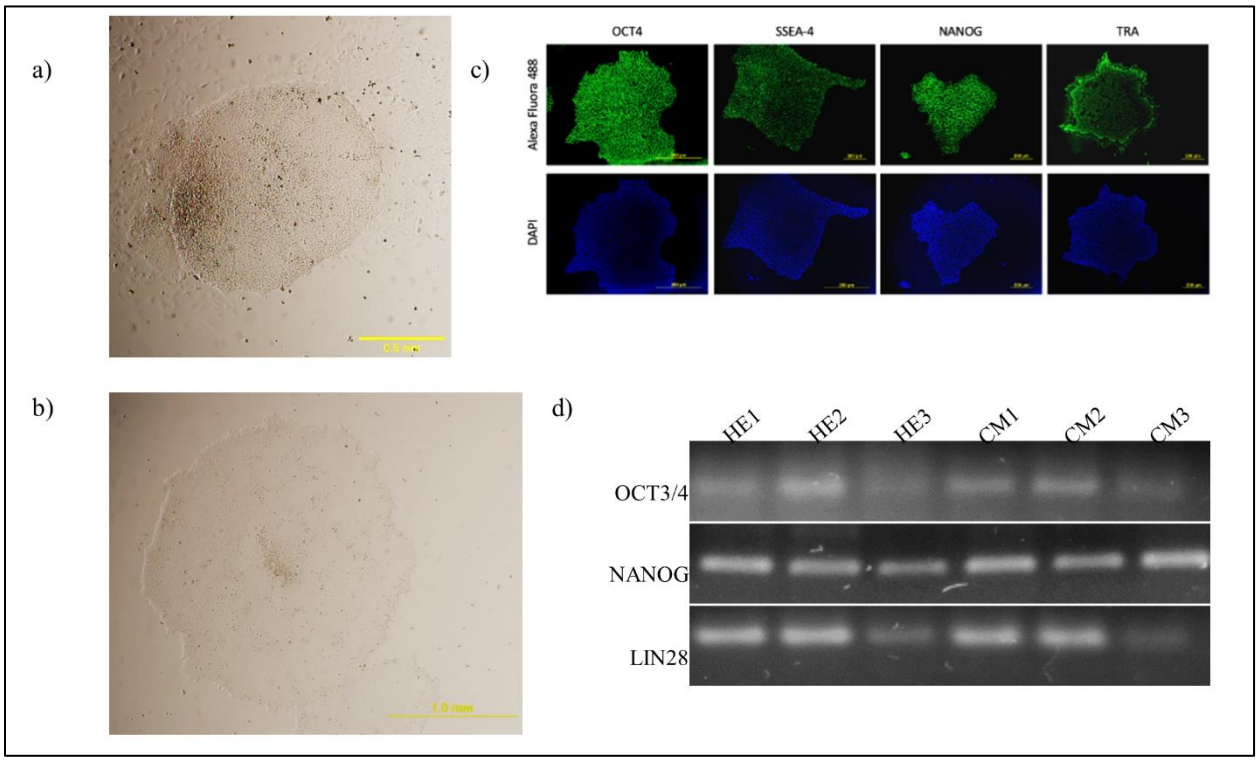

Figure 3: Generation of iPSCs from PBMCs.
(a) Representative brightfield image of iPSC colony forming after electroporation. (b) Representative brightfield image of iPSC colony after mechanical replating. (c) Representative immunofluorescent images of iPSC colonies stained with OCT4, SSEA-4, NANOG, and TRA-1-60. (d) Expression of OCT3/4, NANOG, and LIN28 in iPSC colonies.

4.2. Secretome Promotes AC16 Cardiomyocytes Survival After IR Injury

Previously, 24 hours of ischemia and 5 hours of reperfusion was found to be optimal for inducing IR injury in AC16 cardiomyocytes.[79] In the previous study, cardiomyocytes were cultured with FBS during oxygen reperfusion, while this present study cultured cardiomyocytes with iPSC secretome during reperfusion. Since the reperfusion conditions differed, Crystal Violet (CV) was used to optimize the duration of reperfusion in this experiment. Cardiomyocytes were cultured in 0% oxygen and serum-free media for 24 hours, then oxygen was reintroduced to cell culture concurrently with iPSC secretome or serum-free media without secretome (control media) treatment for 5 or 24 hours. There was a significant increase in cell viability in cells reperfused

with secretome for 5 hours (P <0.0001) and 24 hours (P <0.001), however, it was found that the extended cell culture in control media caused cell damage that could overstate potential benefits of secretome treatment (Supplemental Figure 1). Thus, 24 hours of ischemia and 5 hours of reperfusion in iPSC secretome was used for all experiments.

In CV stained AC16s after IR injury, there was a visible decrease in cell survival and an increase in cardiomyocytes viability in AC16s treated with iPSC secretome (Figure 3a). These changes were quantified by CV absorbance at 540nm. IR injury caused a significant decrease in cell viability (Figure 3b, P < 0.0001), yet iPSC secretome treatment of cardiomyocytes during reperfusion significantly increased in cell survival compared to cells treated with the baseline control media (Figure 3b, P < 0.001). These improvements in cell viability were complemented with increased metabolic activity in secretome-treated cardiomyocytes after IR injury (Figure 3c, P < 0.05).

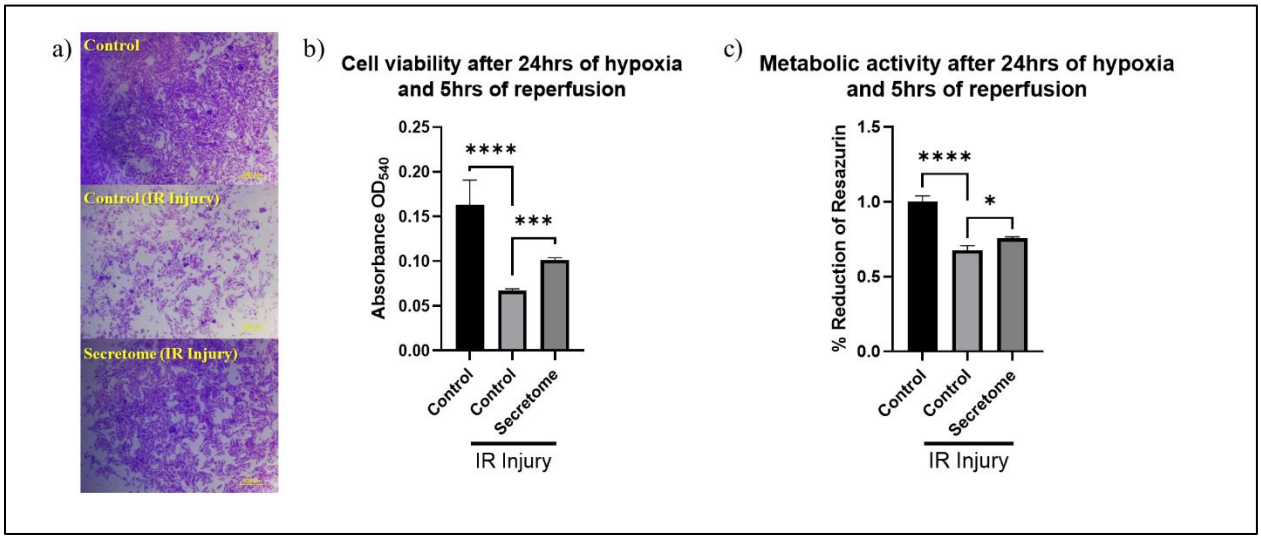

Figure 4: Secretome improves cardiomyocyte viability after IR injury.
(a) Representative brightfield images of cardiomyocytes stained with crystal violet at 10x, after being cultured in normoxia conditions with baseline control media, IR conditions with baseline control media, or IR conditions with secretome treatment. (b) Absorbance of crystal violet stain in cardiomyocytes after IR injury. (c) Relative reduction of resazurin in cardiomyocytes after IR injury. *P < 0.05, ***P < 0.001, ****P < 0.0001, IR = ischemia-reperfusion.

4.3. Secretome Reduces IR-Induced Cellular Hypertrophy

Actin immunostaining was used to assess cardiomyocyte hypertrophy after IR injury, with and without secretome treatment. An increase in cellular area was observed using fluorescent microscopy after 24 hours of hypoxia and 5 hours of reperfusion, and the size of cardiomyocytes decreased in cells treated with secretome (Figure 4a). To quantify the cardiomyocyte hypertrophy, cell area was measured in ImageJ. There was a significant 35.4% increase of in cellular area after IR injury (Figure 4b, $P = 0.05$), and cells that were treated with any of the secretomes during the 5 hours of reperfusion had a significant 19.0% reduction in cell size (Figure 4b, $P < 0.05$). These results suggest that iPSC secretome decreases the hypertrophic effect of IR injury in AC16 cardiomyocytes.

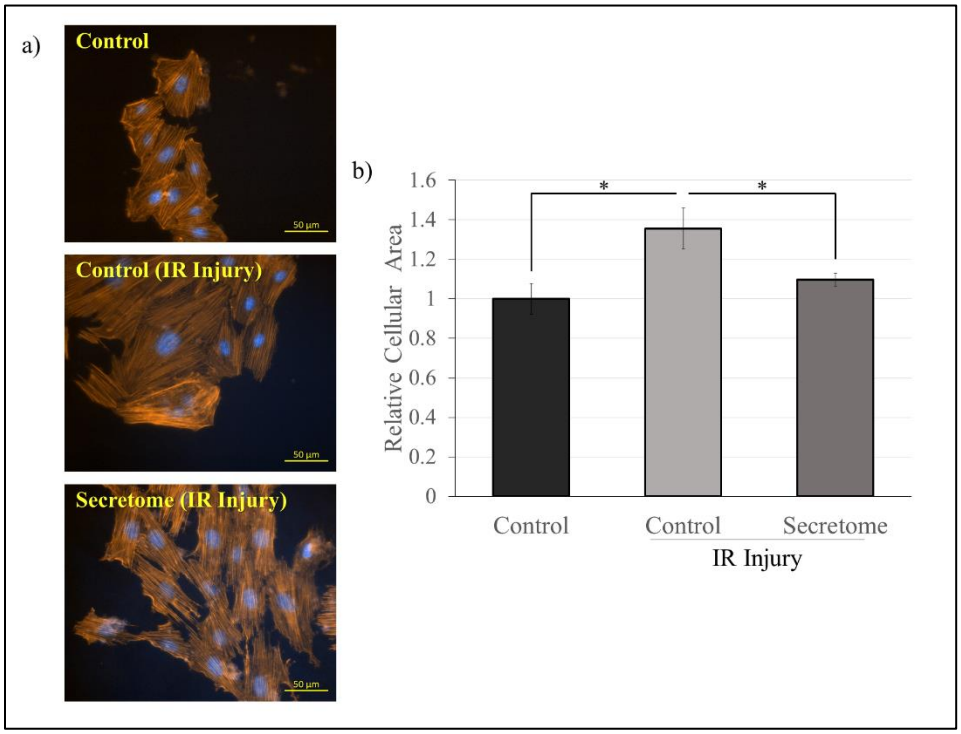

Figure 5: Secretome attenuated the hypertrophic response from IR injury.
(a) Representative fluorescence images of cardiomyocytes stained with ActinRed and NucBlue, after being cultured in normoxia conditions with baseline control media, IR conditions with baseline control media, or IR conditions with secretome treatment. (b) Quantification of cardiomyocyte cellular area. *$P \leq 0.05$, IRI = ischemia-reperfusion injury.

4.4. Secretome Affects Calcium Handling After IR Injury

Cardiomyocytes were treated with Fluo-4 AM after 24 hours of hypoxia and 5 hours of reperfusion in basal media or secretome to visualize and quantify intracellular calcium. Intracellular calcium was visualized under a confocal microscope and fluorescence intensity was measured for at least 60 seconds. The general trend was that cardiomyocytes had an increase in intracellular calcium after IR injury, and the concentration of calcium after secretome treatment was either similar or slightly higher, depending on the secretome used (Figure 5a). Fluorescence intensity significantly increased in cardiomyocytes after IR injury (Figure 5b, $P < 0.001$), however, no significant change in calcium concentration in IR injured cells was measured in iPSC secretome treated cells (Figure 5b).

To assess whether there was a differential effect for secretomes from cardiomyopathy patient iPSC lines and healthy donor iPSC lines on calcium handling, the groups were assessed separately. While secretome from cardiomyopathy patient iPSC lines had no effect on intracellular concentration, secretome from healthy donor iPSC lines significantly increased calcium concentrations in AC16 cardiomyocytes after IR injury (Figure 5c, $P < 0.01$).

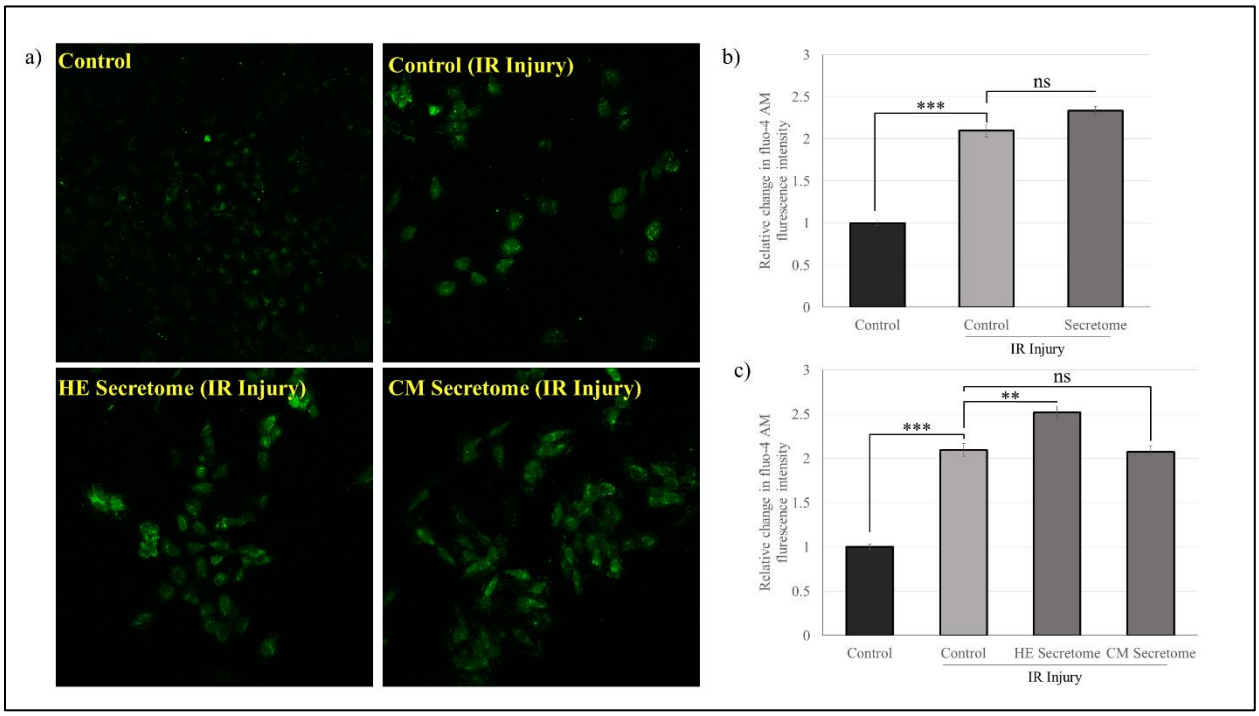

Figure 6: Variable affects of secretome on calcium handling after IR injury.
(a) Representative cardiomyocytes that were cultured in normoxia conditions with baseline control media, IR conditions with baseline control media, or IR conditions with secretome treatment, loaded with Fluo-4 AM to measure intracellular calcium concentration. Relative change in intracellular calcium concentration of cardiomyocytes treated with (b) iPSC secretome, or (c) iPSC secretome specifically from healthy donors and cardiomyopathy patients, grouped separately. **P < 0.01, ***P < 0.001, IR = ischemia-reperfusion injury, ns = not significant, HE = Healthy Donor, CM = Cardiomyopathy Patient.

4.5. Differential Secretome Profiles of iPSC Lines

A proteomic analysis was conducted on secretomes from all 6 iPSC lines that were used for experiments to elucidate the mechanisms by which iPSC secretomes improved cell viability and attenuated hypertrophy in cardiomyocytes after IR injury. This analysis was also used to investigate the differential effects of iPSC secretomes from healthy donors and cardiomyopathy patients on intracellular calcium concentrations in cardiomyocytes. We hypothesized that the secretome profile from healthy donors iPSCs would contain additional factors that regulate

calcium homeostasis. These differentially expressed proteins (DEPs) were then used to assessed whether the increase in intracellular calcium was a positive or negative molecular effect.

In total, 1791 proteins were found, with 72 expressed exclusively in the secretome of iPSC from cardiomyopathy patients, and 416 exclusively expressed in iPSCs from healthy donors (Figure 6a). The remaining 1303 proteins were expressed in the secretomes of both groups, with 139 proteins being expressed at significantly higher levels in one group or the other – mainly expressed in higher levels in the healthy donor lines (Figure 6b, Supplemental Table 1). GO pathway analysis revealed that 391 of the 1303 proteins expressed in both groups contributed to biological pathways involved in response to stress, response to hypoxia, negative regulation of apoptotic process, and detoxification of reactive oxygen species (Figure 6c). Proteins involved in at least 3 of 4 of those pathways are ACAA2, CASP3, ERO1L, GSTP1, HSP90B1, HSPD1, HYOU1, LMNA, P4HB, PRDX2, PRDX3, PRDX5, PSMD10, PSME3, RPS27A, SFRP1, SOD1, SOD2, THBS1 (Figure 6d).

GO pathway analysis also revealed a decrease in a mitochondrial calcium homeostasis biological pathway in iPSC secretomes from cardiomyopathy patients, as compared to the secretomes of lines from healthy donors ($P = 0.0297$). The differentially expressed proteins (DEPs) in this pathway are LETM1, OPA1, PHB2, PMPCB, YME1L1 (Figure 6e). Other proteins involved in intracellular calcium homeostasis, calcium signaling, and regulation of muscle contractions were differentially secreted by iPSCs from healthy donors. Specifically, ANK2, CAB39, CHERP, CKB, CNN1, PRKACA, SRI were identified and are involved in these pathways (Figure 6f).

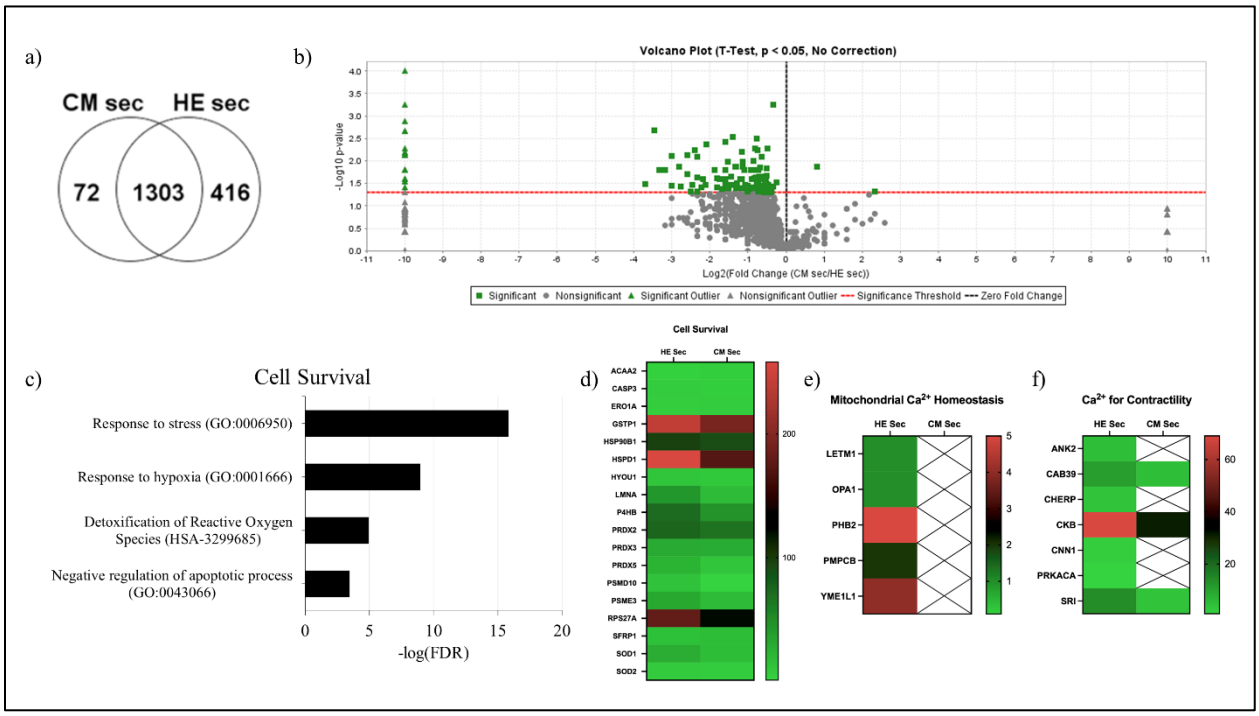

Figure 7: Proteomic profiling of secretomes from healthy donors and cardiomyopathy patients.

(a) Venn diagram comparing identified proteins in secretomes from healthy donors and cardiomyopathy patients. (b) Volcano plot identifying differentially expressed proteins in secretomes from healthy donors and cardiomyopathy patients. (c) GO pathway analysis of iPSC secretome from all iPSC lines revealed the presence of proteins in biological pathways involved in cell survival. (d) Heat maps of differentially expressed proteins involved in cell survival, mitochondrial Ca^{2+} homeostasis, and intracellular Ca^{2+} calcium and contractility. Data is presented as number of peptides.

5. Discussion

5.1. Interpretation of Results

The use of cell-free therapies has been gaining attention for their potential to minimize injury and repair the myocardium after MI. In this study, we showed that the use of iPSC secretome may be an effective tool for improving cardiomyocyte survival after IR injury. Our findings showed that secretome attenuated cell death and cellular hypertrophy in cardiomyocytes after IR injury. Contrarily to our hypothesis, intracellular calcium concentration increased in AC16 cardiomyocytes after secretome treatment, however, this effect was only observed in cells treated with iPSC secretome from healthy donors. Together, these results indicate that iPSC secretome has potential to protect, and potentially repair, cardiomyocytes after an IR injury.

5.2. Cell Survival Proteins of Interest in iPSC Secretome

After a MI, cardiomyocytes cell death due to apoptosis and necrosis lead to progressive cardiac dysfunction and an ventricular maladaptive remodeling.[80] During cardiac development, cardiomyocytes become terminally differentiated and lose their ability to proliferate, which prevents adult hearts from regenerating when cell death occurs, such as after IR injuries.[5] Consequently, preserving cell viability during hypoxic stress and after cellular reperfusion is vital to minimizing cardiac dysfunction. Previous studies have shown that paracrine factors from different stem cells acted to inhibit apoptosis in cardiac cells and improve cardiac function.[81, 82] In this study, decreased cell death was observed in cardiomyocytes treated with iPSC secretome, as compared to baseline control media. Additionally, plates of cardiomyocytes treated with secretome had an increased reduction of resazurin, as measured by the AlamarBlue assay. This assay is frequently used to assess cell viability and metabolism, thus, finding suggests that there were a

greater number of healthy cardiomyocytes in secretome-treated groups.[83] One possible explanation for this finding is that acetyl-Coenzyme A acyltransferase 2 (ACAA2), which was found in iPSC secretomes, has been known to have a role in decreasing BCL2 Interacting Protein 3 (BNIP3)-mediated apoptosis.[84] Additionally, several heat shock proteins (HSP) were identified in the iPSC secretome, specifically from the HSP60, HSP70, and HSP90 families. HSPs are chaperone molecules that support the folding, assembly, and disassembly of proteins, and are frequently upregulated during cellular stress to maintain proper biological function.[85] The cardioprotective abilities of HSPs were originally identified for their ability to protect myocardial function, preserve metabolic functional recovery, and reduce infarct size IR injury.[86-88] Other studies have also shown that treating cardiomyocytes with HSPs during reperfusion, called ischemic post-conditioning, can reduce infarct size, apoptosis, and vascular injury.[89-91] More recently, it was shown that HSP90 is involved in the activation and mitochondrial targeting of protein kinase C (PKC) epsilon, which inhibits apoptosis by the upregulation of Bcl-2 and inhibition of Bax.[92] The presence of HSPs in iPSC secretomes suggests that they may be involved in improved cell viability via PKC epsilon mitochondrial targeting when secretome is used for ischemic postconditioning during IR injury.

Much of the cell death during a myocardial infarction are a consequence of reperfusion after ischemia, which causes an increase in ROS and oxidative stress on the myocardium.[93] In iPSC secretomes from health donors and cardiomyopathy patients, numerous anti-oxidative enzymes were identified that may have played a role in minimizing cellular damage and improving cell viability after IR injury. Specifically, 3 proteins in the peroxiredoxin family (PRDX2, PRDX3, PRDX5) were identified, as well as superoxide dismutase 1 (SOD1) and 2 (SOD2). Peroxiredoxins play a key role in detoxifying peroxides, and a previous study has shown that overexpression of PRDX3 has been found to prevent cardiac remodeling and heart failure after MI in a mouse

model.[94] Moreover, SOD proteins, which were found in iPSC secretome, have been shown to play a role in cardioprotection via ROS reduction.[95, 96] Thus, we propose that iPSC secretome improves cell viability in cardiomyocytes in part by detoxifying reactive oxygen species. Given this finding, future studies should investigate the antioxidative effects of iPSC secretome on cardiomyocytes after IR injury. Investigating the interactions between antioxidative proteins in iPSC secretome could provide a framework for improved attenuation of ROS during MI treatment.

Another key protein that was secreted in each iPSC secretome was secreted frizzled-related protein 1 (SFRP1), a regulator of the Wnt signaling pathway. In fact, SFRP1 and other Wnt pathway antagonist are frequently involved in cardioprotection after MI.[97] This notion was reinforced in a recent study, where exogenous SFRP1 was shown to effectively inhibit cardiomyocyte apoptosis, reduce myocardial fibrosis, inhibit cardiac fibrous hyperplasia, inhibit myocardial remodeling, and improve cardiac function in a murine model.[98] This evidence suggests that Wnt pathway antagonists in iPSC secretome may also play a role in improved cardiomyocyte viability and decreased hypertrophy after MI.

Given the numerous proteins of interest involved in cell survival secreted by iPSCs, we propose that iPSC secretome may be used to improve cell viability in cardiomyocytes. Further investigation is required to elucidate whether this is indicative of an improved prognosis after MI.

5.3. Calcium Homeostasis and Contractility Proteins of Interest in iPSC Secretome

During a MI, intracellular calcium is dysregulated and can lead to calcium overload and cardiomyocyte dysfunction.[4, 17] Conversely, calcium is required for excitation–contraction coupling and proper cardiac function in cardiomyocytes.[99, 100] In this study, iPSC secretome did not significantly affect intracellular calcium concentration; however, when examining secretomes from donors with different cardiac phenotypes separately, a difference was observed. More

specifically, an increase in intracellular calcium was seen in cardiomyocytes treated with secretome from HE iPSCs after IR injury, while CM iPSCs had no effect on calcium concentration. Differentially expressed proteins were identified in secretomes from both groups to assess the mechanisms that might elucidate the dissimilar effects on cardiomyocytes. Additionally, the proteomic analysis was used to investigate whether the increase in intracellular calcium was linked to cardiomyocyte function or dysfunction. Given that intracellular calcium concentrations varied between groups, we hypothesized that proteins involved in calcium homeostasis would be differentially expressed.

Our findings supported this hypothesis, as many proteins identified were involved in calcium ion transport and cellular chemical and ion homeostasis. Interestingly, many of these calcium homeostasis proteins are either directly or indirectly involved in muscle contractions. For instance, sorcin (SRI) is differentially expressed in secretomes from healthy donor iPSC secretomes. This protein interacts with the sarcoplasmic reticulum's ryanodine receptor to release Ca^{2+} and modulate intracellular Ca^{2+} levels.[101] Studies show that SRI is essential for non-arrhythmogenic cardiac contractions, and that the overexpression of SRI enhanced fractional shortening and decreased end-systolic diameter.[102, 103] Both of these studies suggest that SRI, a protein that contributes to calcium homeostasis, is vital for proper cardiac contractility. Thus, it is possible that SRI contributed increasing intracellular Ca^{2+} in cardiomyocytes treated with healthy donor iPSC secretomes in this study, and that this increase is associated with improved contractility.

The ankyrin-B (ANK2) protein is an adaptor protein that interacts with and coordinates the assembly of Na/Ca exchanger (NCX), Na/K ATPase (NKA), inositol-1,4,5-trisphosphate receptors (IP3), ATP-sensitive potassium channel (Kir6.2), and L-type calcium channel $Ca_v1.3$ in

cardiomyocytes.[104-108] In addition to its role in maintaining ion homeostasis, ANK2 has been shown to play a vital role in excitation-contraction coupling.[109] Studies have shown that ANK2 mutations and knockouts can lead to arrythmias and the risk of sudden death.[108-110] This phenomenon can be explained by abnormal localization and a decreased expression of NKA, NCX, and IP3 receptor.[109] Additionally, ANK2-deficient mice have been found to have an increased level of Ca^{2+} sparks, which are associated with cardiac arrythmias.[111] While no studies exist that investigate the overexpression of ANK2, its importance for contractility suggests that ANK2 warrants further investigation as a potential therapeutic for dysregulated cardiac beating after MI.

In this study, intracellular calcium concentrations increased significantly in IR-injured cardiomyocytes treated with secretome from healthy donor iPSCs, while no change was seen in the treatment with secretome from cardiomyopathy patient iPSCs. This finding was supported by the DEPs involved in calcium homeostasis in the healthy donor iPSC secretome. Given that several of these proteins are also vital for proper contractile function in cardiomyocytes, there is reason to believe that this increase in intracellular Ca^{2+} may be a beneficial response to secretome treatment, rather than a sign of calcium overload; however, further research is needed to fully understand the effects of iPSC secretome on calcium handling after IR injury.

5.4. Scope of Secretome Analysis

In recent years, paracrine factors secreted by stem cells and their derivatives have been a focus of cell-free cardiac therapies. Secreted factors from different cell sources include lipids, proteins, deoxyribonucleic acid (DNA), messenger RNA (mRNA), microRNA (miRNA), small interfering RNA (siRNA) and long noncoding RNAs (lncRNAs).[74] This study focuses specifically on proteins secreted by stem cells; however, it is likely that many secreted factors elicit a cytoprotective response in cardiomyocytes after IR injury. In future studies, it could be beneficial to characterize

all factors present in iPSC secretomes, as doing so would create a more complete picture of the nucleic acids and other molecules in secretomes that have a cytoprotective effect on cardiomyocyte after IR injury. Another limitation of this study is the sample size of 6 iPSC lines. With a greater number of CM and HE iPSC lines, other critical proteomic differences between patient groups might emerge that would prove us with even greater insight. Additionally, with a larger sample of iPSC lines, it will be possible to assess differences in secretome from other patient categories. For example, donor age and gender affect the profile of factors secreted by MSCs, however, it is unclear if the same is true for iPSC cell lines derived from patient blood.[112]

5.5. Current Cardiac Model of MI

One limitation of this study is that findings in immortalized cell lines do not necessarily reflect future outcomes in humans. Immortalized cell lines are either tumorous cells that have been isolated from tissue or primary cells that have been artificially generated to proliferate indefinitely.[113] This is starkly different from adult heart tissue that has limited proliferative potential. Additionally, there is a lot of variability in cardiomyopathies, and it is quite difficult to replicate the complexity of a heart attack in immortalized cell lines or animal models. In general, preclinical models of disease do not represent the variability of cardiomyopathies seen in the general population. Consequently, there is often poor efficacy when seeking to translate preclinical findings to clinical trials. Not surprisingly, the efficacy of novel therapies in clinical trials also differs greatly between different human subpopulations.

To overcome this poor clinical translatability, iPSCs and their derivatives have been gaining in popularity as a preclinical model for drug discovery, cardiomyopathy modeling, and tissue engineering. Methods to differentiate iPSCs to cardiomyocytes are well established, and we have been able to generate iPSC-derived cardiomyocytes (iPSC-CMs) with high reliability

(Supplemental Figure 2). The benefit of using iPSC-CMs in preclinical research are plentiful: 1) an abundant number of cells can be generated from cells collected non-invasively, 2) cell lines can better represent the genetic variability in human population, 3) it is possible to conduct assays on many iPSCs lines in parallel to assess the universal applicability of discoveries, and 4) better predictions can be made for which subpopulations can benefit the most from a novel therapy during clinical trials. While this list of benefits is not exhaustive, there are also notable limitations in using iPSC-CMs as a cardiac model in preclinical research.

The primary concern for researchers is that these cells express a fetal phenotype. During cardiac development, many physiological changes occur to cardiomyocytes, both *in utero* and postnatally. As compared to fetal immature cardiomyocytes, adult mature cardiomyocytes have more structured myofibrils, changes in electrophysiology, calcium handling, and contractility, and a transition from glycolysis to oxidative phosphorylation for ATP production.[61] This discrepancy makes modeling cardiomyopathies with iPSC-derived cardiomyocytes difficult due to many cardiovascular diseases manifesting themselves in middle-aged and geriatric patients. To overcome the challenges that arise due to the fetal phenotypes of iPSC-derived cardiomyocytes, researchers have been investigating methods for the maturation of these cells, in many cases, attempting to replicate steps of cardiogenesis that promote maturation *in vivo*.

To develop maturation protocols for iPSC-CMs, it is possible to draw inspiration from developmental biology.[114] It is hypothesized that replicating the signaling and niche conditions of cardiac development *in vitro* with iPSC-CMs may allow for these cells to undergo similar maturation. The greatest challenge is that there are many factors at play during cardiogenesis *in vivo,* and no maturation protocol targeting a single signaling pathway or condition has been able to yield the desired results so far. A multifaceted, multi-step protocol will be needed to produce

iPSC-CMs with a mature, adult-like phenotype. Some of the methods being explored individually and in combination to induce maturation in iPSC-CMs include long-term culture, co-culture, electrical stimulation, and pathway activation.[114, 115]

The heart is generated from three unique tissue types: cardiogenic mesoderm cells, the proepicardium (PE), and cardiac neural crest cells.[116] During development, cells from the PE migrate and attach to the myocardium. Then, PE cells envelop the myocardium and differentiate into several cardiac cell types, many of which are vital for chamber maturation and ventricular muscle growth.[116] Consequently, a team from Massachusetts General Hospital and Harvard University have recently identified that co-culturing iPSC-CMs with iPSC-derived pre-epicardial cells (PECs) induces organizational and contractile changes in cardiomyocytes that are important in maturation.[117] To further validate the use of this maturation method and to assess the feasibility to integrate this method into Dr. Cecere's myocardial regeneration lab at McGill University, I am completing a six-month graduate studentship at the Massachusetts General Hospital. The method for generating PECs was replicated (Supplemental Figure 3), and follow-up studies are being conducted at the time of this thesis publication.

In future work studying the effects of iPSC secretome on IR injured cells, it would be beneficial to replace immortalized cell lines with iPSC-derived cardiomyocytes. Mainly, the effects can be evaluated on a wider range of cardiomyocytes with genetics that match the donors. Several other benefits are a consequence of this primary advantage. Testing iPSC secretome on more cardiomyocyte cell lines derived from a diverse patient population would allow for inference to be made about the universality or subpopulation-specific effects of secretome therapy. Additionally, using iPSC-derived cardiomyocytes for the IR injury would permit the investigation of whether there are differences between autologous versus allogeneic secretome treatments. For

example, IR-injured cardiomyocytes derived from a cardiomyocyte patient could be treated with autologous iPSC secretome and allogeneic iPSC secretome from a healthy donor. If the latter is the only one to have a positive effect, then a patient-specific iPSC secretome treatment should be forgone in favour of an optimal allogeneic iPSC secretome.

6. Conclusion

The work outlined in this study suggests that iPSC secretome provides cytoprotective effects to cardiomyocytes after MI. The improved cell viability and the attenuation of hypertrophy in secretome-treated cardiomyocytes serve as evidence that many factors in iPSC secretome play a role in protecting cardiomyocytes from oxidative stress and cell death caused by IR injury. Moreover, the proteins in iPSC secretome from healthy donors lead to increased intracellular calcium after IR injury, which may be an indication of an increase in contractility strength.

It is vital that novel therapies that can improve prognosis and prevent HF after MI are investigated because of the poor outcomes of HF patients and limitations of heart transplantation surgery. Given the positive outcomes of this study, there is reason to be optimistic that therapeutically targeting several key pathways in parallel after MI would be beneficial to minimizing cardiac damage. The clinical utility of patient-specific secretome is quite limited because it takes quite some time to generate iPSC lines. More research is needed to assess whether iPSC secretomes from certain donors are more cytoprotective than others, and if there are adverse effects of using allogenic iPSC secretome to treat IR injury. Additionally, studying the contribution of each protein factor in the secretome and investigating their interactions with one another could lead to the creation of a generic therapy for MI patients that integrates a combination of proteins frequently found in iPSC secretome.

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8. Supplementary Information

Supplemental Table 1: List of top 20 differentially expressed proteins in secretome from iPSCs from cardiomyopathy patients and healthy donors.

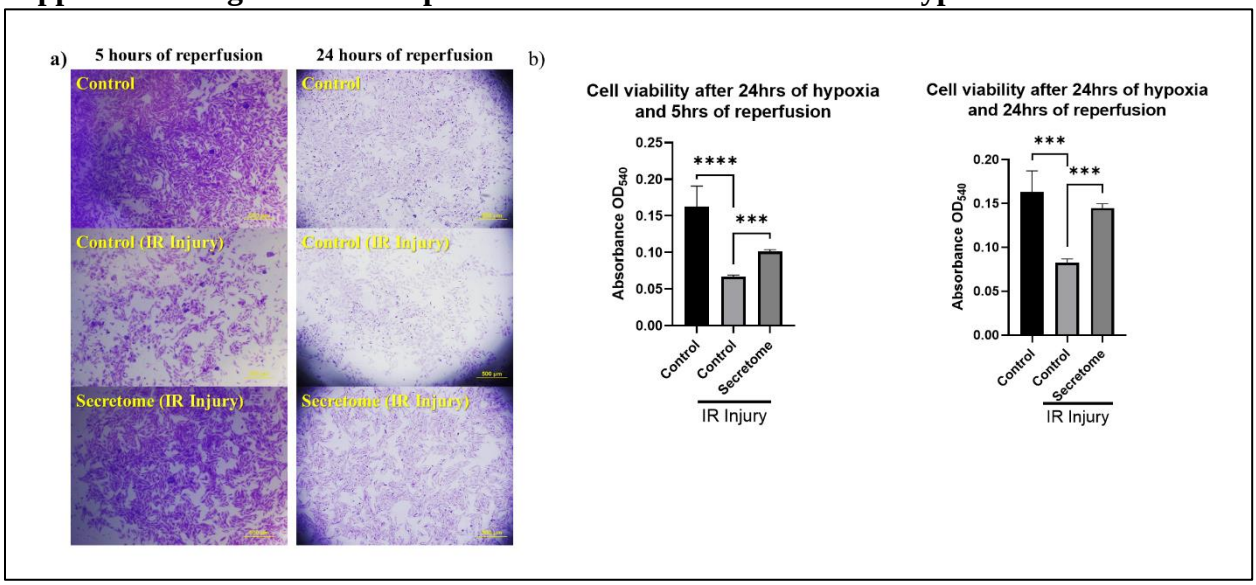
Protein	Description	P-value	Fold-change (CM/HE)
NAP1L1	Nucleosome assembly protein 1-like 1	0.00056	0.8
HMGN1	Non-histone chromosomal protein HMG-14	0.0011	0.2
GTF2F1	General transcription factor IIF subunit 1	0.0013	0.2
UFM1	Ubiquitin-fold modifier 1	0.0013	0.3
PITHD1	PITH domain-containing protein 1	0.0013	0.3
IRF2BPL	Probable E3 ubiquitin-protein ligase IRF2BPL	0.0013	0.3
RRP9	U3 small nucleolar RNA-interacting protein 2	0.0013	0.3
POLR1G	DNA-directed RNA polymerase I subunit RPA34	0.0013	0.3
RPAP3	RNA polymerase II-associated protein 3	0.0022	0.4
PSMD6	26S proteasome non-ATPase regulatory subunit 6	0.0023	0.3
TLE1	Cluster of Transducin-like enhancer protein 1	0.0029	0.2
TXNL1	Thioredoxin-like protein 1	0.0029	0.4
MATR3	Matrin-3	0.0031	0.6
EIF6	Eukaryotic translation initiation factor 6	0.0038	0.3
NUDT5	ADP-sugar pyrophosphatase	0.0053	0.7
EXOSC4	Exosome complex component RRP41	0.0053	0.2
EXOSC2	Exosome complex component RRP4	0.0053	0.2
TRIM28	Transcription intermediary factor 1-beta	0.0058	0.2
NANS	Sialic acid synthase	0.0058	0.2
EIF2A	Eukaryotic translation initiation factor 2A	0.0062	0.2

HE = Healthy Donor, CM = Cardiomyopathy Patient

Supplemental Table 2: List of Taqman Probes used in all qPCR experiments.

Taqman Probe Targets	Catalogue number	Manufacturer	Use
SOX2	Hs04407947_ml	ThermoFisher Scientific	Pluripotency marker
NANOG	Hs04260366_g1	ThermoFisher Scientific	Pluripotency marker
WT1	Hs01103751_m1	ThermoFisher Scientific	PEC marker
TBX18	Hs01385457_m1	ThermoFisher Scientific	PEC marker
TBX5	Hs00361155_m1	ThermoFisher Scientific	Transcription factor, critical in proepicardium development and specification
Beta-actin	Hs01060665_g1	ThermoFisher Scientific	Housekeeping gene
BMPR1A	Hs01034912_g1	ThermoFisher Scientific	Potential chemoattractant receptor
BMPR1B	Hs01010965_m1	ThermoFisher Scientific	Potential chemoattractant receptor
BMPR2	Hs00176148_m1	ThermoFisher Scientific	Potential chemoattractant receptor

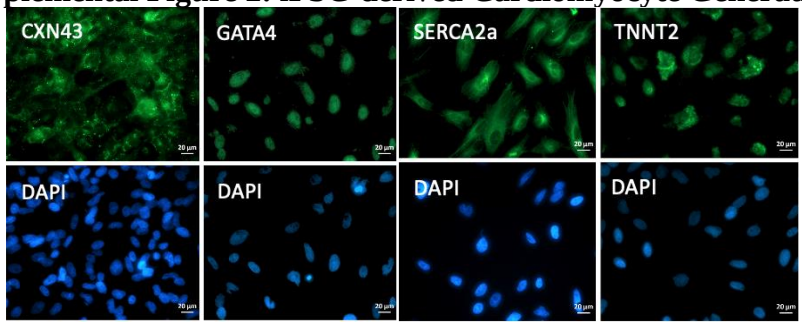
Supplemental Figure 1: Cell reperfusion for 24 hrs after 24 hrs of hypoxia were unviable.



(a) Representative brightfield images of cardiomyocytes stained with crystal violet, after being cultured in normoxia conditions with baseline control media, IR conditions with baseline control media, or IR conditions with secretome treatment. Left panel show 5 hours of reperfusion, and right panel shows 24 hours of reperfusion (b) Absorbance of crystal violet stain in cardiomyocytes after IR injury, with 5 and 24 hours of reperfusion.

Supplemental Figure 2: iPSC-derived Cardiomyocyte Generation and Maintenance.

(a)

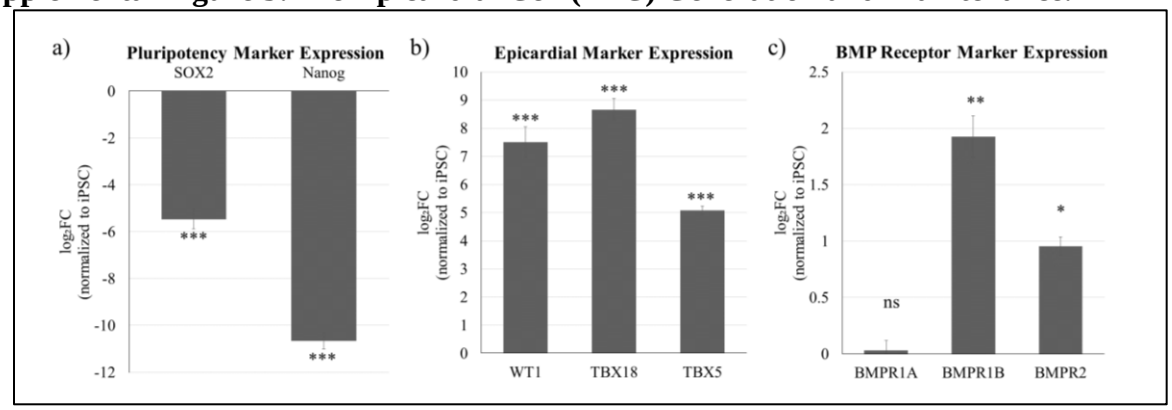


(b) https://drive.google.com/file/d/1_1MN58EP-7FV5NhunTrJDyGYjX8Oi8m8/view?usp=sharing

Characterization of iPSC-derived cardiomyocytes. (a) Representative images for iPSC-derived cardiomyocyte immunostaining, using CXN34, GATA4, SERCA2a, and TNNT2 antibodies. (b) Representative video of beating iPSC-derived cardiomyocyte under brightfield microscopy.

Protocol: Induced pluripotent stem cells were plated on Matrigel plates and cultured in mTeSR Plus until 80-90% confluent. Cells were then treated with 12μM CHIR99021 (Stemgent, San Diego, CA) in Roswell Park Memorial Institute (RPMI) medium supplemented with B27 without insulin (RPMI-INS; Gibco) for 24 hours. The media was replaced with RPMI-INS for 24 hours, and then with RPMI-INS with 5μM Inhibitor of WNT Production-4 (IWP4; Stemgent, San Diego, CA) for 48 hours. After, the media was refreshed with RPMI-INS for 48 hours. Then, RPMI supplemented with B27 (RPMI+B27; Gibco) was used for iPSC-derived cardiomyocyte maintenance, with media changes every 2 days.

Supplemental Figure 3: Pre-Epicardial Cell (PEC) Generation and Maintenance.



RT-qPCR analysis of differentiated PEC at day 7 of differentiation. (a) Log_2 fold change in PEC gene expression of pluripotency markers SOX2 and NANOG was normalized to the level in iPSC. (b) Log_2 fold change in PEC gene expression of epicardial markers WT1, TBX18, and TBX5 was normalized to the level in iPSC. (c) Log_2 fold change in PEC gene expression of BMP receptor markers BMPR1A, BMPR1B, and BMPR2 was normalized to the level in iPSC. The presented adjusted p value in all figures corresponds to the group in the respective column versus iPSCs. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

Differentiation Protocol: iPSCs were treated with 12 μM Stemolecule™ CHIR99021 (Stemgent, San Diego, CA) in RPMI medium supplemented with B27 without insulin (Gibco, Grand Island, NY) (RPMI-INS) for 24 h. Then, the medium was replaced with RPMI-INS for 24 h. At days 2 and 4, the media was replaced with RPMI-INS supplemented 50 ng/ml bone morphogenic protein (BMP) 4 (PeproTech, Rocky Hill, NJ), 5 ng/ml vascular endothelial growth factor (VEGF; PeproTech, Rocky Hill, NJ) and 4μM retinoic acid (RA; Stemgent, San Diego, CA) in RPMI-INS. On day 6, iPSC-derived PECs (iPECs) were either used for experiments or frozen at -80ºC for short-term storage. Thawed iPECs were cultured for 2 days in PEC maintenance media (PECM) prior to experiments to enable the culture to recover. PECM consisted of DMEM/F12 supplemented with 1×insulin-selenium-transferrin (Gibco, Grand Island, NY), 5ng/ml VEGF (PeproTech, Rocky Hill, NJ), 10μM retinol, 4μM RA (Stemgent, San Diego, CA) and 60μg/ml ascorbic acid (Sigma).

RT-qPCR Protocol: RNeasy Plus Mini (QUIAGEN) and Blood/Cell RNA Mini Kit (Geneaid) protocols were used to isolate mRNA from PEC samples. Superscript IV VILO Master Mix (ThermoFisher, #11754050) was used for complementary deoxyribonucleic acid (cDNA) synthesis, and ran on T100™ Thermal Cycler (Bio-Rad) or TGRADIENT PCR system (Biometra) according to manufacturer's protocol. For PECs, Real-Time qPCR was done with TaqMan™ Gene Expression Master Mix (ThermoFisher, cat#4369016) on CFX384™ Real-Time System (Bio-Rad). Gene Expression Taqman probes (Applied Biosystems, Foster City, CA) can be found in Supplemental Table 2.

Results: Upon treating iPSCs with BMP, VEGF, and RA, cells expressed a significant downregulation of pluripotency markers SOX2 and NANOG ($p<0.001$), as well as a significant upregulation of known epicardial markers WT1, TBX18, and TBX5 ($p<0.001$). Both of these

results were consistent with previously described iPSC-derived PECs.[117] Additionally, iPSC-derived PECs expressed increased expression of BMPR1B ($p<0.01$) and BMPR2 ($p<0.05$), as compared to iPSCs. BMPR1A was expressed in PECs, however, no change in expression was observed as compared to iPSCs ($p=0.45$). This aligns with the literature that identified that BMP receptors were present in the proepicardium and that BMP signaling promotes pre-epicardial cell recruitment to the heart during embryogenesis.[118]