Stem Cell-based 3D Bioprinting for Cardiovascular Tissue Regeneration

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Table of Contents

1. Abstract

This chapter describes an overview of the approaches studied for cardiac regeneration as well as their challenges and limitations. Cardiovascular diseases are the greatest cause of death, especially in aging populations. As the adult human heart has limited regenerative capability, researchers are trying novel ways to develop new clinical

approaches to improve cardiac regeneration. Such approaches are cell-free therapy using paracrine factors, cellular therapies using stem cells and various types of 3D bioprinting technologies. Cardiovascular tissue engineering (CTE) has revolutionized the approaches to improve cardiac regenerative medicine by designing 3D bioprinted cardiovascular tissue with or without specialized biomaterials to recapitulate the human heart microenvironment. Engineered heart tissues (EHTs), tissue organoids and cell sheets are cutting-edge techniques and have applications in disease modelling, drug screening, toxicity assays and have the potential for patient-specific models and improving cardiac function and regeneration by *in vivo* transplantation.

Keywords: bioprinting, cardiac patch, stem cells, 3D cell culture, *in vitro* and *in vivo* applications, bioinks, hydrogels

2. Introduction

Cardiovascular disease (CVD) represents the single greatest cause of death in the world, especially in the aging population (Thomas et al., 2018, Joseph et al., 2017, Wang et al., 2016). The increased incidence of CVD has been more recently associated with co-morbidity with other chronic diseases, such as kidney failure and type II diabetes (Stevens et al., 2010, Heidenreich et al., 2011, Manuel et al., 2014, Wang et al., 2016). In the last decade, CVD accounted for nearly one third of all deaths worldwide (Roth et al., 2017, Thomas et al., 2018, Wang et al., 2016). The global disease burden caused by CVD is estimated to include up to 400 deaths per 100,000 in developed countries and is further driven by an unprecedented growing and aging population, with notable increases in ischaemic heart disease (IHD), stroke and heart failure (HF) (Joseph et al., 2017, Roth et al., 2017, Thomas et al., 2018). IHD and strokes are caused by a lack of blood supply and oxygen to the heart or brain, respectively, and are the main CVD contributions to the global disease burden accounting for 8-10% of all deaths in Europe (Joseph et al., 2017, Palomeras Soler and Casado Ruiz, 2010, Wang et al., 2016). Their treatments have greatly advanced over the past three decades, resulting in improved survival rates (Heidenreich et al., 2011, Palomeras Soler and Casado Ruiz, 2010, Thomas et al., 2018). Multiple therapeutic interventions including drugs (such as cholesterol modifiers and anti-hypertensives) and surgical procedures aiming at repairing or bypassing damaged arteries, have greatly reduced the mortality of CVD patients and allowed for lifestyle changes to complement these primary interventions (Deaton et al., 2011, Heidenreich et al., 2011, Roth et al., 2017).

HF is a more complex presentation of CVD, characterized by the heart inability to pump enough blood to meet the body oxygen demand (Ferreira et al., 2019). HF is widely considered to be a chronic phase of cardiac impairment, secondary to other CVD as well as risk factors, including diabetes, obesity and chronic hypertension (Ambrosy et al., 2014, Ziaeian and Fonarow, 2016). This compromises its contractility and leads to heart failure and death as the current treatments options are limited (Go et al., 2013). Palliative drugs, such as ACE inhibition, beta blockade, and diuretics, or the mechanical assist devices (including ventricular-assist devices, or VAD, pacemakers, defibrillators) only delay the progression of heart failure and do not lead to the regeneration of the heart tissue (Lui et al., 2014, Burchfield and Dimmeler, 2008, Li et al., 2009). The gold standard treatment remains a heart transplant, which is available to less than 0.1% of heart failure patients (Gerbin and Murry, 2015). In the next

decade, HF prevalence is estimated to double from 27 million cases worldwide to over 50 million cases, with a 1 in 5 lifetime risk of developing some form of HF (Bui et al., 2011, Ferreira et al., 2019, Ziaeian and Fonarow, 2016). This trend suggests new disease models, treatment options and strategies are vital, as the CVD epidemic continues to grow.

Tissue regeneration in the human body occurs primarily via the recruitment of progenitor cells to replace lost cells through differentiation and proliferation (Go et al., 2013, Günter et al., 2016, Witman et al., 2020). However, adult human heart has limited regenerative capacity as cardiac myocytes lose their ability to proliferate after birth (Li et al., 2009). As the heart does not contain enough stem, precursor, or reserve cells to effectively heal itself after an injury, majority of cardiomyocytes are lost, and the necrotic muscle is replaced with scar tissue(Li et al., 2009, Lee and Walsh, 2016). This compromises its contractility and leads to HF and death as the current treatments' options are limited (Go et al., 2013). In this context, heart regeneration has been the focus of several studies in the past decades, where cardiovascular researchers developed new approaches to deliver cells to the heart, reactivate the endogenous regenerative capacity through paracrine mechanism or by bio-engineering technologies (Lee et al., 2016, Lui et al., 2014, Cambria et al., 2017).

In order to overcome the limited survival of cardiac cells following their delivery in a damaged heart, 3D bioprinting technology for cardiac regeneration has gained increasing popularity in the past decade (Roche et al., 2020b). Cardiovascular tissue engineering (CTE) has emerged to design and manufacture biologically relevant cardiovascular tissues for both research applications with the goal of furthering cardiovascular regenerative medicine, and clinical applications to improve CVD patient outcomes and quality of life. A key component of CTE is accurately recapitulating the human heart microenvironment to promote cell survival, functionality and increase success of potential implantation. Additionally, this process can utilize patient-derived stem cells to improve contractile function and vascularization in bioengineered microtissues with decreased risk of immunological response (Roche et al., 2020b).

3. Molecular, Cellular and Extracellular Approaches to Promote Cardiovascular Regeneration in Humans

This book chapter aims at illustrating a comprehensive overview of the state-of-the-art approaches currently used to promote cardiovascular regeneration (**Fig. 1**). These include cell-based therapies and cardiac tissue engineering approaches to differentiate and proliferate into functional cardiac myocytes and through paracrine effects to mediate cytoprotection, recruit cells, mediate inflammatory response and prevent fibrotic scar tissue formation. Currently, numerous strategies are investigated thanks to the potential beneficial effects of stem cells on

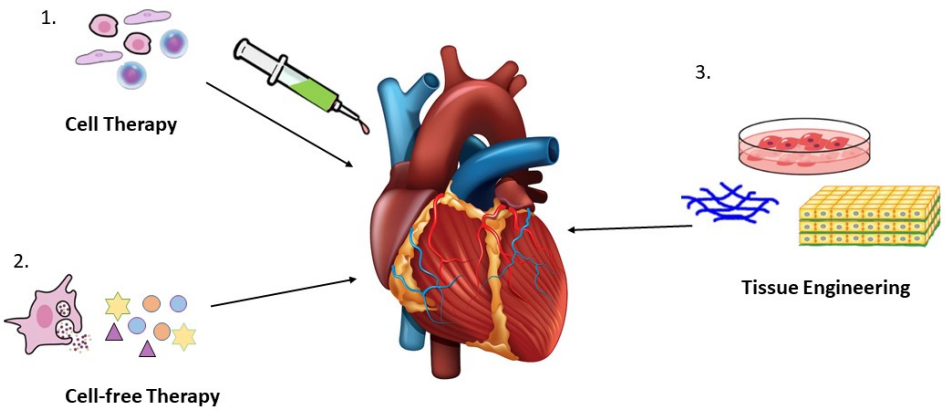

Figure 1. Approaches for cardiac regeneration to treat a damaged heart. These include: (*1*) Cell therapy by transplantation in the myocardium via intracoronary, intramyocardial, intravenous or transendocardial. (*2*) Cell-free therapy secreting paracrine factors such as cytokine, growth factors and microRNAs to improve cardiac regeneration. (*3*) Tissue engineering approaches combining cells with biomaterial such as cell sheets, scaffolds, spheroids to design cardiac patches or injectable materials for transplantation into the infarcted heart.

the failing myocardium in the preclinical or clinical settings. Furthermore, including bio-engineering methods to improve the heart microenvironment and promote cell survival, including the use of stem cells and 3D bioprinting technology. Also, the multiple aspects of CTE work in unison to serve this end; this includes areas such as cell types and culture technique, polymers and biopolymers, material scaffolds, vascularization of tissues, hydrogels, bioprinting and bioinks. This book chapter will thoroughly describe all the possible tools for the improved generation of viable and functional heart tissues for *in vitro* and *in vivo* applications. However, several limitations and key unanswered questions prevent their direct application to humans, which will be highlighted through this manuscript as well.

3.1 – Cell-Free Approaches

From the molecular viewpoint, the beneficial effects of cell therapy mainly function through paracrine mechanisms, they play essential roles during cardiogenesis, cytoprotection, neovascularization and limit inflammatory, profibrotic and apoptosis (Bollini et al., 2011, Gerbin and Murry, 2015, He et al., 2003, Lui et al., 2014). Hence, researchers proposed a novel strategy for heart regeneration and new treatment, that is, cell-free therapy by using paracrine factors (Witman et al., 2020, Noseda et al., 2011). Paracrine factors including growth factors and cytokines are normally released from endogenous cells in the myocardium in response to injury (Burchfield and Dimmeler, 2008). Various types of stem cells such as BMCs, CSCs,

ESs and MSCs have shown to mediate cytoprotection via increased expression of fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), erythropoietin (Epo), and granulocyte-colony stimulating factor (G-CSF) (Cambria et al., 2017, Hansen et al., 2019). For example, Lui et al. (2013) study showed that VEGF-A promotes endothelial specification, engraftment, proliferation and survival of human Isl1+ cardiovascular progenitor cells; hence suggesting a novel approach for vascular regeneration in the ischemic heart (Lui et al., 2014, Lui et al., 2013). The main advantage of this cell-free therapy is its safety, that is, avoid the risks of unlimited cell growth and tumour formation, however, bioactive molecules in the extracellular medium undergo rapid hydrolysis and it is still unclear if they can provide long-term benefit to patients. For example, VEGF lifetime in human blood is less than 30 min (Eppler et al., 2002, Gomzikova and Rizvanov, 2017).

Paracrine factors can be secreted in a spatiotemporal manner and enhance regeneration of cardiac myocytes, but the method of delivery is still to be improved. Various clinical trials have used delivery methods such as intracoronary, intravenous and intramyocardial injection of growth factors but have failed to provide consistent results of significant improvement of myocardial ischemia (Henry et al., 2003, Lui et al., 2014). For example, in the NORTHERN clinical trial, VEGF gene therapy via intramyocardial injection have failed to improve the perfusion of ischemic myocardium (3 and 6 months) (Stewart et al., 2009). Another clinical trial based on neuregulin-1 (rhNRG-1) showed no significant difference from the placebo group however, it showed that the short-term administration of rhNRG-1 treatment improved the cardiac function of chronic heart failure patients by increasing LVEF% and reduced the end-diastolic volume and end-systolic volume (Gao et al., 2010). The poor outcomes of growth factor-based approaches could be due to the lack of controlled release, off-target side effects, inappropriate dosage, and the duration of expression (Cambria et al., 2017, Henry et al., 2003, Lui et al., 2014). More studies are required to find the delivery methodologies with appropriate dosage and appropriate duration of expression of the growth factors as prolonged expression might lead to unwanted side effects (Lui et al., 2014).

Over the past decade, extracellular vesicles (EVs) attracted the interest of studies aiming at regenerating the myocardium as promising tools for the delivery of biologically active molecule to promote new tissue formation (Gomzikova and Rizvanov, 2017, Raik et al., 2018). However, more important matters need to be first addressed such as the type and size of vesicles, their content, high cost and time-consuming isolation procedure as well as their potential immunogenicity (Cambria et al., 2017, Gomzikova and Rizvanov, 2017).

3.2 - Cell-Based Approaches

Stem cells have been the focus of emerging research to heal or replace damaged cardiac tissues (Cambria et al., 2017, Roche et al., 2020b). They are potentially useful in cardiac regeneration as they can self-renew as well as differentiate into multiple types of cells in the body. Various cell types of have been studied due to their regenerative potential. These include skeletal myoblasts (SMs), bone marrow- derived cells (BMCs), and mesenchymal stem cells (MSCs) (Li et al., 2009, Segers and Lee, 2008). Despite promising preclinical studies, the transplantation of these cells displayed heterogeneous clinical outcomes which could be due to differences in design including, cell preparation, delivery route, dose, and follow-up methods (Cambria et al., 2017, Behfar et al., 2014). Due to the inconsistencies of the first-generation cell types, the field has tried to use other cells types known as the second-generation

cell types (**Fig. 2**). This include cardiac stem/progenitor cells (CSCs/CPCs), pluripotent stem cells (embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Cambria et al., 2017). Second-generation cell types have shown to be more efficient and improve therapeutic effects compared to the first generation; assessed by cardiac function, angiogenesis, scar size and improved engraftment but also have some limitations (Rossini et al., 2011, Citro et al., 2014). This section provides an overview of the first-generation and second-generation cell types.

3.2.1 - Skeletal Myoblasts (SMs)

The first type of cell used for cardiac regeneration as cellular therapy was skeletal myoblasts delivered by intramyocardial injection during coronary artery bypass grafting (CABG) surgery (Hansen et al., 2019, Menasché et al., 2001). The attributes of SMs are that they are resistant to ischemia, highly abundant, no potential drawbacks of undifferentiated stem cells and no immune rejection from the patients due to their autologous origin (Li et al., 2009). The clinical trials in both MI and HF by Menasché et al. (2001) showed evidence of positive safety data and excellent engraftment but found concerns of being pro-arrhythmic and discovered a lack of electrophysiology coupling due to N-cadherin and connexin-43 downregulation. Consequently, the investigations using SMs have ceased due to evidence that skeletal myoblast might increase the risk of ventricular arrhythmias (Cambria et al., 2017, Gavira et al., 2006, Hansen et al., 2019).

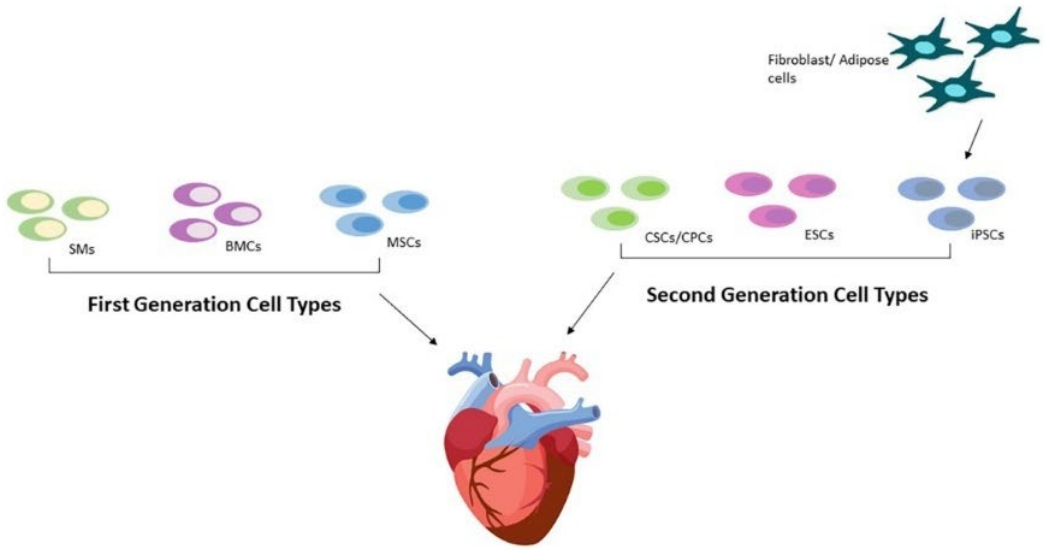

Figure 2. Evolution of Cell Therapy for cardiac regeneration. First-generation cell types such as SMs, BMCs and MSCs showed feasibility and safety outcomes but limited efficacy in clinical setting. Second-generation cell types approaches consist of CSCs/CPCs, ESCs and iPSCs that demonstrated more efficient and improve therapeutic effects on the failing heart.

3.2.2 - Bone Marrow-Derived Cells (BMCs)

BMCs derived from adult bone marrow, give rise to cardiomyocytes vascular endothelium and smooth muscle both *in vitro* and *in vivo* (Ballard and Edelberg, 2007, Li et al., 2009, Orlic et al., 2001). Bone marrow mononuclear cells have mostly been used for the investigations as the cell source that is safe, abundant, and easy to isolate (Cambria et al., 2017, Gerbin and Murry, 2015). The advantages of using BMCs are to avoid ethical and clinical issues. Furthermore, there were prominent results on the preclinical trials using large animal studies, the delivery of BMCs either systemically or intramyocardially have demonstrated an increase of cardiomyogenesis and a significant improvement in heart function (Ballard and Edelberg, 2007, Bollini et al., 2011). Some of the clinical trials showed significant improvement of cardiac function in MI and HF patients, however, others did not find significant beneficial effects of the cell therapy (Nasseri et al., 2014, Schächinger et al., 2004, Tendera et al., 2009). The possible reasons could be due to low cell engraftment and limited differentiation potential; for example, a study by Kajstura et al. (2005) showed that the BMC derived-cardiomyocytes (BMC-CMs) promotes regeneration of the infarcted myocardium, however the developed cardiomyocytes are not fully mature, are poorly coupled and could be oriented to endogenous myocytes (Kajstura et al., 2005, Ballard and Edelberg, 2007).

3.2.3 - Mesenchymal Stem Cells (MSCs)

MSCs also known as bone marrow stromal cells, are adult cells; they are precursors of non-hematopoietic tissues, such as muscle, tendons, bone, fibroblast, and adipose tissue. MSCs are multipotent, have a high expansion rate and have immunomodulatory properties (Ballard and Edelberg, 2007, Cambria et al., 2017, Gerbin and Murry, 2015). Human MSCs are easily isolated from patients which reduce any risk of immune rejection from the patient during transplantation (Li et al., 2009). They have proven to be promising for cardiac repair in numerous pre-clinical trials such as it reversed post-MI remodelling and restored tissue perfusion leading to a significant reduction in infarct size and increases the left ventricle ejection fraction (LVEF) (Mazo et al., 2012, Schuleri et al., 2009, Valina et al., 2007). However, this cell source has raised safety concern in the clinical trials as most MSC studies showed that the cells die within a week or two post-transplantation and little differentiation to cardiomyocytes (Cambria et al., 2017, Gerbin and Murry, 2015, Schuleri et al., 2009). Hence further investigations of the MSCs in clinical trials need to be done with a larger number of patients and have to first fully understand the paracrine mechanisms of MSCs on humans (Cambria et al., 2017).

3.2.4 - Cardiac Stem Cells (CSCs)

Cardiac stem cells (CSCs) and cardiac progenitor cells (CPCs) were first identified in 2003 located in the myocardium upon expression of c-kit; c-kit+ cells indicate the ability to undergo cardiomyogenic differentiation (Beltrami et al., 2003). They are multipotent, self-renewing and have the capacity of forming myocytes, vascular cells and smooth muscle cells (Hansen et al., 2019, Beltrami et al., 2003). Therefore proving that the human heart is capable of regeneration, however, it is likely that the number and function of CSCs and CPCs are impaired and limited with increasing age and due to multiple factors such as CVD, environmental changes and senescent changes within the cells (Ballard and Edelberg, 2007). According to Beltrami et al. (2003), injection of c-kit+ CSCs/CPCs into ischemic heart/post-MI in rats' model reduced the extent of myocardial damage. In a few weeks, there was a recovery of more than 50% of the cardiomyocytes and vascular cells that are normally present in the heart (Beltrami et al., 2003). Nevertheless, the concern regarding c-kit+ CSCs/CPCs is that they have a predilection to differentiate more towards vascular cells rather than cardiomyocytes (Hansen et al., 2019).

Hence, more studies need to be executed to identify the signals require to drive differentiation to specific types of cells (van Vliet et al., 2008). Despite that they are autologous and tissue-specific to cardiovascular lineage, the isolation and expansion of CSCs/CPCs are available in very limited amount (Bollini et al., 2011, Li et al., 2009). Another type of CSCs used are cardiac-derived cells (CDCs), they are derived from myocardial biopsies and cardiac explants in culture to form cardiospheres, they have proven to have positive outcomes in infarcted heart (Cambria et al., 2017, Gerbin and Murry, 2015). For example, CDCs were transplanted via intracoronary delivery in MI pig model, the results showed positive safety data and significant cardioprotection with reduced microvascular obstruction, infarct size and attenuates adverse acute remodelling (Kanazawa et al., 2015). The initial clinical trials with CSCs/CPCs (CDCs) showed to be promising and demonstrated signs of efficacy however most investigations showed that these cells, like BMCs, have minimal long-term engraftment and cardiac differentiation (Bolli et al., 2011, Makkar et al., 2012, Malliaras et al., 2014). Further studies need to be executed to fully elucidate their paracrine mechanisms of cardiac repair and with a larger cohort (Cambria et al., 2017).

3.2.5 - Embryonic Stem Cells (ESCs)

ESCs are derived from blastocyst taken from day 4 to 6 before the implantation into the uterine wall. They are pluripotent, that is, they are capable to form all cell types in the body (Hansen et al., 2019, Lee and Walsh, 2016). ESCs provide a renewable source of cardiomyocytes for basic research and pharmacological testing (He et al., 2003). Mouse ESCs were first derived in 1988 and human ESCs were derived in 1998 however research with ESCs has been controversial since the derivation of ESCs destroy the embryo that raises ethical concerns (Evans and Kaufman, 1981, Lee and Walsh, 2016, Thomson et al., 1998). Mouse and human ESCs can be easily differentiated into cardiomyocytes and human-derived cardiomyocytes have shown to improve the function of infarcted rodent heart and non-human primates' hearts (Lee and Walsh, 2016). However, their clinical use is limited, ES-derived cardiomyocytes could lead to immunogenic and teratogenic side effects and increase the risk of ventricular arrhythmias (Bollini et al., 2011). Despite that, ESCs can direct the specific cellular differentiation pathway for different cell types, there is lack of understanding of the molecular and genetic signals that regulate cell proliferation and differentiation (Ballard and Edelberg, 2007). Therefore, more studies are required to avoid contamination of undifferentiated ESC as the inherent risk of residual undifferentiated stem cells could induce teratoma formation. Proper quality control measures must be executed to minimize the risk of the formation of tumour (Nussbaum et al., 2007).

3.2.6 - Induced Pluripotent Stem Cells (iPSCs)

iPSCs can be obtained from differentiated cells including cardiac fibroblast and adult somatic cells, which are re-expressed by reprogramming four genes, Oct3/4, Sox2, c-Myc, and Kfl4 (Qian et al., 2012, Takahashi et al., 2007, Takahashi and Yamanaka, 2006). IPSCs are an alternative to ESCs as they display similar characteristics while avoiding the ethical tensions. They are pluripotent, self-renewing (in culture) and can differentiate into cardiac lineage (Cambria et al., 2017, Takahashi and Yamanaka, 2006). Derived iPSCs from human somatic cells have been one of the most remarkable discoveries in cardiovascular research as they can be derived from patients with complex genetic defects and create disease models (Braam et al., 2009). Human iPSCs (hiPSCs) can be an autologous cell source for cardiac repair and increase cardiac function as they can differentiate into functional cardiomyocytes (Yan and Singla, 2013, Zhang et al., 2009). For example, Ye et al. (2014)

research demonstrated hiPSC- derived cardiomyocytes (hiPSC-CMs) co-cultured with endothelial cells, and smooth muscles transplanted intramyocardially in a MI porcine model improved LV function, myocardial metabolism, arteriole density, and reduced infarction size and cell death without inducing ventricular arrhythmias (Ye et al., 2014). Nevertheless, hiPSC-CMs are less mature than adult cardiomyocytes, based on the ultrastructure, electrophysiological and metabolically characteristics which can be improved in long-term cultures; however, the ideal level of maturation still need to be found (Jonsson et al., 2012, Kamakura et al., 2013). The limitation of the autologous hiPSCs approach is the financial feasibility as the process of obtaining patient-specific somatic cells, reprogramming them to iPSCs then differentiating into cardiomyocytes as well as doing expensive quality control experiments, could take over four months and doing this for each patient is cost-prohibitive (Gerbin and Murry, 2015). Further tests need to be done to address any rejection or teratoma formation, and iPSC method requires the use of viral vectors before advancement into clinics (Li et al., 2009, Cambria et al., 2017).

Table 1. Cell therapy types to treat heart diseases

	Cell types	Type of Delivery	Advantages	Disadvantages	References
First-generation	Skeletal Myoblasts (SMs)	Intramyocardial injection	-Positive data and excellent engraftment	-Higher rate of arrhythmias	(Gavira et al., 2006, Menasché et al., 2001)
	Bone Marrow-Derived cells (BMMCs)	Delivered either systemically or intramyocardially	-Safe, abundant, and easy to isolate	-Limited differentiation	(Kajstura et al., 2005, Ballard and Edelberg, 2007)
	Mesenchymal Stem Cells (MSCs)	LV injection	-Multipotent -Reduce any risk of immune rejection	- Little differentiation to cardiomyocytes -Minimal cell engraftment	(Mazo et al., 2012, Schuleri et al., 2009, Valina et al., 2007)
Second generation	Cardiac stem cells (CSCs)	LV injection	- Multipotent, self-renewing -Reduce myocardial damage	-Limited amount -Minimal cell engraftment	(Bolli et al., 2011, Doppler et al., 2013)
	Embryonic Stem Cells (ESCs)	Intramyocardial injection	- Pluripotent, self-renewing Improve cardiac function	-Ethical concerns -Increase risk of ventricular arrhythmias	(Evans and Kaufman, 1981, Lee and Walsh, 2016, Thomson et al., 1998)
	Human induced pluripotent stem cells (hiPSCs)	Intramyocardial injection	-Pluripotent, self-renewing -Same benefits as ESCs	-Less mature than adult cardiomyocytes	(Jonsson et al., 2012, Kamakura et al., 2013)

3.3 – 2D vs 3D Cultures

Cardiac cells are typically cultured either as 2D monolayers or more recently, 3D cell cultures, such as spheroids, engineered heart tissues and cell sheets. Both cell culturing techniques include advantages and disadvantages, though previous downsides of 3D culturing are easing in recent years.

Monolayer cell cultures are cheap with access to high throughput assays but often display biological activity that deviates from the *in vivo* response (Duval et al., 2017, Mathur et al., 2015). This is largely due to access to transformed cell lines, genetically modified cells that lower semblance to *in vivo* counterparts but allow unrestricted proliferation. For this reason, transformed cells are often used as the foundation for drug discovery and cardiotoxicity studies (Novakovic et al., 2014). Primary cardiac cells derived from animals or humans are difficult to isolate and therefore not a reliable source for CTE, though the experiments conducted with such cells may be considered necessary as proof-of-concept studies (Zimmermann and Eschenhagen, 2003). While useful for looking at certain biochemical, genetic and functional mechanisms, 2D cultures ultimately lack compete physiology such as cell-cell and cell-ECM interactions. This is especially significant when recapitulating the human heart microenvironment as factors such as electrical conductivity and mechanical contraction are unique to the heart to serve specialized functions, generating and carrying the cardiac action potential and acting as a pump for the cardiovascular system, respectively.

3D cell cultures and technologies are the response to this drawback of 2D cardiac models and are rapidly gaining recognition for their potential to model heart tissues and diseases (Roche et al., 2020b, Duval et al., 2017, Jang, 2017, Günter et al., 2016, Fitzgerald et al., 2015). EHTs have been used through numerous studies as a therapeutic tool as they have been demonstrated to improve cardiac function following myocardial injury. Another EHT-application is for disease modelling using the patient-specific hiPSC-SM to evaluate mutations, drug screening and individual risk of a patient such as drug-induced side effects (Katare et al., 2010, Zimmermann et al., 2006, Naito et al., 2006, Eder et al., 2016). Also, compared to 2D cultures, EHTs can be a promising model to study cardiac function and contractility, and are accessible to perform all types of evaluations as a cardiac muscle tissue in the heart. This includes contraction kinetics, rhythm and rate, genetic and protein analyzes, and histological analyzes of semithin, paraffin or ultrathin sections (Hirt et al., 2015, Eder et al., 2016). While there are limitations in 2D cell systems in term of viability, proliferation, differentiation and function of cardiomyocytes; the advantages of using EHTs are that they are easy to execute and provide a great quality of research outcome as well as allow long-term experimentation and repeated measurements under steady and controlled conditions (Katare et al., 2010, Eder et al., 2016).

Cells can be grown in scaffolds, scaffold-free or matrices environment aiming to mimic the ECM aspects of the heart; for example, biomaterial scaffolds such as collagen and fibrin; provide a 3D environment for cells to attach, interact with each other and conduct electrical signals (Vunjak Novakovic et al., 2014, Eschenhagen and Mummery, 2014). Cardiac myocytes cultured in 3D often employ a biomaterial such as a hydrogel or biocompatible polymer to mimic the ECM, providing a 3D architecture for cell to interact in all spatial dimensions, both with other cells and their environment. This allows for fine-tuning of the microenvironment by modifying properties such as elasticity, stiffness, conductivity and porosity (Novakovic et al., 2014). These are core aspects of CTE as the utility of 3D culturing and bioengineering to simulate blood flow, observe contractile forces and relaxation velocity in cardiac myocytes with variable mechanical and electrical cues are the tools necessary to create a complex and accurate microenvironment (Hirt et al., 2012, Bouten et al., 2011, Wang and Hill, 2010,

Fitzgerald et al., 2015, Ryan et al., 2016). With the increase in controllable parameters there is also an increase in complexity. Lack of standardised protocols compared to 2D culturing means experimental design is more demanding and without high-throughput testing.

Cell spheroids can be generated in several ways, both with and without a scaffold to support the development of the spheroid depending on the method chosen such as hanging-drop or low surface adhesion plates (Sharma and Gentile, 2021, Zuppinger, 2016). Spheroids display phenotypes that are conditional or absent in 2D cultures such as contractile activity in cardiac myocytes and T-tubule formation with endothelial cells. Engineered heart tissues were first introduced by Eschenhagen et al. (1997) and are still utilized today in several variants. These tissue constructs contain cells seeded onto a biomaterial scaffold (see below) and is subjected to mechanical forces, aligning cells along the force lines (Eschenhagen and Mummery, 2014). Cell sheets utilize temperature sensitive surfaces to culture monolayers of cells that can be detached as a sheet of cells and continuously stacked over each other, resulting in a thick sheet of cells (Sakaguchi et al., 2013). Cell sheets are seeing increasing work in vascularized tissue studies, which will be covered in greater detail below.

3.4. – Biomaterials for Cardiovascular Tissue Engineering: Polymers, Scaffolds & Hydrogels

Advancements in cell therapies have allowed the direct introduction of cells to the damaged heart to ascertain any therapeutic benefits of long-term regenerative effects. However, these studies have demonstrated a consistent deficit in exogenous cell survival following transplantation (Menasche, 2011, Oh et al., 2016, Yanamandala et al., 2017). As biomaterials have demonstrated a utility in increasing cell retention, survival and proliferation, various natural and synthetic polymers are now being explored to enhance current therapies and innovate novel approaches as scaffolds for recapitulating human heart physiology.

Typically, *in vitro* tissue engineering methods involve the use of porous scaffolds to either a. provide structural support for a diseased tissue or b. transfer cells to the damaged tissue (Bouten et al., 2011, Giraud et al., 2007, Jawad et al., 2007). Determining a biomaterial for CTE is largely dependent on the specific goal of the research or clinical outcome; if a construct is to regenerate a tissue over a longer period of time, a highly porous structure is necessary that allows nutrients to properly exchange between cells and their environment while promoting tissue vascularization (Chen et al., 2007). Each biomaterial currently used in tissue engineering has its own profile of benefits and drawbacks inherent in the properties of the material when applied to cardiac cells and tissues.

3.4.1 – Natural Biomaterials

Naturally-derived biomaterials are desirable options when attempting to recapitulate human physiology as they can be used to produce biomimetic organ scaffolds (Wang et al., 2020b, Huyer et al., 2015). Well-characterised biomaterials such as alginate, chitosan, gelatin and decellularized extracellular matrix (dECM) are commonly used in CTE due to their strong profile of benefits with relatively small drawbacks. Alginate, generally sourced from brown

algae, can generate highly porous structures in the form of a hydrogel that allows high cell seeding numbers at physiologically relevant cell densities with little-to-no immunological response (Dar et al., 2002). Chitosan is a polysaccharide derived from the crustacean exoskeleton polymer chitin, the latter can be used to generate a number of scaffolds and hydrogels when combined with other polymers (Ahmadi et al., 2015, Lam and Wu, 2012). Liu et al. (2012) reported chitosan hydrogels may improve the myocardial environment following myocardial infarction (MI) via reactive oxygen species regulation and recruiting chemokines. Gelatin is produced from denaturing the ECM protein collagen, resulting in a bioactive protein capable of enhancing cell-scaffold interactions (Kharaziha et al., 2013). For this reason, gelatin and collagen-based scaffolds are commonly utilized in the production of cardiac patches that have seen success in multiple animal studies of regenerating damaged cardiac tissue following MI (Serpooshan et al., 2013, Zimmermann et al., 2006). Silk fibroin (SF) has been also explored as a natural source to 3D bioprint cardiac cells, but his intrinsic properties prevent its use by itself (Vettori et al., 2020). A more recent method of producing favourable scaffolds for cardiac cells is isolating the ECM from tissue without damaging, while removing any cells normally found within (Gilbert, 2012, Ott et al., 2008). This can be performed for example by enzymatically treating human cadaver hearts to recover human cardiac ECM and re-seed with patient-specific cells, without fear of major immunological response (Guyette et al., 2014). Natural biomaterials generally perform well in CTE. However, they are less mechanically stable, possess variable biodegradation rates and are difficult to tweak when possible.

3.4.2 Synthetic Biomaterials

Synthetic biomaterials generally offer favourable and diverse properties at the cost of less semblance to *in vivo* tissue. Plastics such as polylactic acid (PLA) and polyglycolic acid (PGA) have already been widely used in surgical therapies as products such as sutures and stents. Synthetic biomaterials are primarily used as structural scaffolds for cell seeding specific components such as the heart valves engineered on PLA, PGA and polycaprolactone (PCL) scaffolds (Theus et al., 2019). As synthetic compounds, tweaking the properties is possibly by chemically altering structure and combining multiple polymers together for novel desirable features. Recently, conductive and elastic properties have been added to polymers with promising results (Balint et al., 2014, Sales et al., 2007, Stella et al., 2008, Zhao et al., 2017). Spearman et al. (2015) reported a polymer blend of conductive polymer polypyrrole (PPy) and PCL was assessed for cardiac cell sheet development and yielded electrical resistance resembling human heart tissue. Similarly, (Davenport Huyer et al., 2016) suggested the potential application of a novel polymer, named 124 polymer, that yielded elastic properties mimicking adult heart myocardium and supported rat cardiac cell attachment as a scaffold comparable to PLA scaffolds. Furthermore, the 124 polymer elastic properties can be modified before polymerisation and before UV cross-linking. The advancement in synthetic biomaterials is the foundation for innovating novel semi-synthetic scaffolds that incorporate the tissue modelling of natural biomaterials with the versatility of synthetics.

3.4.3 – Hybrid Biomaterials

No individual biomaterial, natural or synthetic, can faithfully recapitulate the human heart microenvironment (Shapira et al., 2016). Hybrid biomaterials, natural biomaterials that have either been biochemically altered or combined with a synthetic biomaterial, can perform a vast range of functions in CTE. Though this does not provide a single hybrid biomaterial that perfectly mimics the cardiac microenvironment, it does provide the foundation for future work. Park et al. (2005) developed a hybrid scaffold with improved capacity to seed and attach cells using the synthetic biomaterials poly-lactic-co-glycolic-acid (PLGA) and poly(DL-lactide-co-caprolactone) in conjunction with collagen coating to incorporate binding factors found in natural ECM, and found the hybrid scaffold promoted cardiac tissue contractile and metabolic performance when compared to either biomaterial alone. Since the Park et al. (2005) study, approaches with similar methodologies have reported promising results, such as Sapir et al. (2011) and Rai et al. (2013) utilising alginate and poly-glycerol-sebacate (PGS), respectively, bound with additional cell adhesion binding domains found naturally on collagen, fibronectin and laminin. These types of hybrid scaffold are vital to the development of functional cardiac patches for clinical use due to their ability to maintain cell viability at high populations while promoting cardiac gene expression and metabolic activity (Shapira et al., 2016).

The physical characteristics of scaffolds can also be enhanced utilising composites of natural and synthetic biomaterials to tweak mechanical properties to closely resemble *in vivo* myocardium (Shapira et al., 2016). Engineered scaffolds require mechanical features to support cell viability, proliferation and function of contractile tissue that will not weather when introduced to the native myocardium which is constantly beating. Hybrid biomaterials address this challenge by incorporating biomaterials with stiffness and elasticity akin to the *in vivo* myocardium supplemented with natural ECM proteins. Kai et al. (2011) utilized electrospinning, a method to produce fibres at nanoscale using electrical force, to mix gelatin and PCL resulting in improved cardiac myocyte attachment and alignment compared to electrospun PCL fibres alone. Kharaziha et al. (2013) electrospun gelatin and PGS at varying ratios and chemically cross-linked the resulting matrix resulting in a hybrid gelatin matrix that demonstrated elasticity like native myocardium and improved cardiomyocyte contraction. Similarly, PGS has been used as a core for a scaffold with a gelatin, fibrinogen or collagen shell for cell adhesion, all resulting in improved elastic properties and increased expression of cardiomyocyte contractile proteins troponin-T and α-actinin (Ravichandran et al., 2011, Ravichandran et al., 2013). Furthermore, cardiac dECM hydrogels are desirable but lack properties such as appropriate stiffness and degradation rates. In response to this, Lee et al. (2017) developed a gelatin hydrogel with tuneable stiffness and degradation via varying degrees of vinyl sulfone polymerization and reported stiffness at 9kPa resulted in improved cardiomyocyte network formation and contractile velocity with enhanced α-actinin and connexin-43 expression.

The development of natural, synthetic and hybrid scaffolds have advanced CTE by demonstrating novel methods to both produce and combine biomaterials that can better mimic *in vivo* tissue properties to recapitulate aspects of the heart microenvironment. The next limitation of optimising the performance of engineered cardiac tissues, is providing a supporting vascular network for oxygen and nutrients transport.

3.5 – The Vascularization Problem

During *in vitro* cell culture, nutrients can be easily supplied to cells, though once an engineered tissue is implanted this becomes severely limited without vasculature in close proximities to cells. CTE builds upon research already aimed at restoring function to ischemic tissue by innovating and testing methods of inducing vasculogenesis, formation of new blood vessels *de novo*, and angiogenesis, formation of new blood vessels from existing vasculature, primarily focusing on the latter. While biomaterial scaffolds have advanced greatly, allowing for clinically relevant tissues to be produced, vascularization remains a great challenge in the field of tissue engineering. An adult human heart houses approximately 10% of the total capillaries in the body, resulting in a densely vascularized structure (Stoker et al., 1982). Therefore, an engineered cardiac tissue requires not only a degree of vascularization to survive in the host, but a highly dense vascular network capable of meeting the metabolic demands of *in vivo* cardiac tissue.

Current strategies for promoting vascularization in engineered tissue primarily consists of either: tissue grafts that are progressively vascularized by the host, or pre-vascularizing tissue constructs (Roche et al., 2021, Esser et al., 2019). There is a large body of evidence that demonstrates a host's vascular system will slowly extend into a non-vascular construct following transplantation, largely dependent on the presence of vessels at the implantation site (Riegler et al., 2015, Tiburcy et al., 2017, Zhang et al., 2001). Therefore, damaged myocardium is an unideal region for relying on endogenous vascularization alone due to poor pre-existing vasculature. The addition of vascular endothelial cells has previously demonstrated self-assembly into tubular structures without external stimuli, and are capable of fusing with host vasculature after transplantation (Stoehr et al., 2016). Though this is promising, endogenous vascularization both with and without endothelial cells takes days at minimum while cell death without oxygen occurs in minutes. Omentum tissue has been explored as another biomaterial to support vascular myocardial regeneration (Wang et al., 2020a). A systematic review by Wang et al. (2020a) reported bioengineered cardiac tissue with omentum support improved cell retention and induced angiogenesis in transplanted tissue. Pro-angiogenic growth factors, notably vascular endothelial growth factor (VEGF), has seen utility in both 2D and 3D cultures to stimulate vascular formation, though the vessels are immature and risk leakage of plasma resulting in further complications (Esser et al., 2019, Fagiani and Christofori, 2013, Visconti et al., 2010). Additionally, extensive VEGF is known to be the cause of vascular tumours in multiple tissue types. While these strategies can increase the rate of endogenous vascularization, other strategies have explored prolonging cell survival in unfavourable conditions (Don and Murry, 2013). Cocktails of enzymes and factors such as caspase inhibitors and insulin-like growth factor-1, have seen success in rats by giving cardiomyocytes protection from a range of cell death mechanisms (Laflamme et al., 2007). It is important to note that while these appear promising for CTE, this strategy is yet to be clinically verified in large scale trials.

Pre-vascularization may be equally as important as endogenous vascularization for engineered cardiac tissues (Roche et al., 2020b). The premise with pre-vascularization is similar to endogenous vascularization, to reduce the amount of time transplanted tissues are without vasculature and subject to harsh conditions such as hypoxic environments. Multiple biomaterial scaffolds mentioned previously have demonstrated formation of vascular networks when seeded with endothelial cells and some have incorporated dECM to produce complex vascularization that fused with endogenous vasculature following transplantation (Ott et al.,

2008, Yang et al., 2014). Other cell vascularization methods include spheroids and cell sheets. Spheroids are a round 3D clusters of cells that can be pre-vascularized when subjected to certain growth methods. Cardiac spheroids can be pre-vascularized by culturing cardiac endothelial cells, fibroblasts and myocytes. Vascularized cardiac spheroids have previously been used to study the heart microenvironment and have been proposed as a method to improve transplanted tissue survival (Caspi et al., 2007, Polonchuk et al., 2017). Cell sheets are a scaffold free, monolayer culture technique, utilising temperature sensitive surfaces to allow cells to detach while maintaining cell-cell connections and ECM (Masuda and Shimizu, 2016). Culturing cells in this manner allows for multiple layers of cell sheets to be produced that can be harvested for tissue implantation. Multiple studies have established cardiac cell sheets as promising for therapeutic applications as they can be pre-vascularized with endothelial cells co-cultured with cardiac myocytes that anastomose to host tissue after transplantation (Sakaguchi et al., 2013, Sekine et al., 2008, Sekiya et al., 2006). More recently, newer techniques to pre-vascularize cardiac tissue have been explored. Song et al. (2020) utilized cell sheets with reprogrammed fibroblasts via known cardiac cell-fate factors (Gata4, Mef2c, Tbx5, Hand2, Myocd) on nanoporous PLGA membranes with layers of endothelial cells between cell sheets. This approach proved effective in implantation on the epicardium and preventing adverse cardiac remodelling in rat hearts post-myocardial infarction. Another method of vascularization is the combination of 3D printing technology with cells and biomaterials called bioprinting.

3.6 – 3D Bioprinting of Heart Tissues

Like all technologies, different bioprinting methods have advantages and disadvantages (Serpooshan et al., 2017). Extrusion based bioprinting dispenses material continuously in a pre-defined shape using either pneumatic or mechanical forces. This technique is the most common and accordingly the least expensive. Additionally, extrusion bioprinting allows rapid printing times and can print extremely high cell densities such as those required for cardiac tissue implants and patches. However, material choice is somewhat limited as highly viscous bioinks are required and therefore high printing pressure can disrupt cell viability. Kolesky et al. (2016) used pneumatic bioprinting to construct preformed vascular networks followed by lining with human umbilical vein endothelial cells (HUVECs) resulting in thick vasculature that was maintained for 6 weeks. Inkjet bioprinting involves the release of fluid droplets at precise locations via thermal or acoustic forces. This method yields high print resolutions as low as 20µm, is compatible with a large range of bioinks and results in high cell viability. Because there are low pressures on the bioink, delicate cell types can be used, but this comes at the cost of lower structural integrity and therefore lower cell densities can be printed. Xu et al. (2009) used primary feline and H1 cardiomyocytes on a controlled porosity alginate hydrogel with viable cell populations, indicating inkjet bioprinting may be useful in engineering designed cardiac tissues. Stereolithography is unlike the previous methods as instead of placement of material, the construct is hardened via light from a vessel of fluid containing photoactive polymers. This method is rapid and removes physical stress on the cells and bioinks resulting in moderately high resolutions as low as 50µm and high cell viability. The range of materials accessible are extremely limited as photoactive polymers are a requirement and are further restricted by an ultraviolet wavelength to activate the polymer that does not harm the cells (Theus et al., 2019). Stereolithography bioprinting has been used to generate patient specific models to assist in surgical planning and to create vascularized tissue constructs from

photoactive polymers with modifiable elasticity and tensile strength (Baudis et al., 2010, Dankowski et al., 2014).

Bioprinted cardiac tissues have been generated to mimic several features of the cardiac microenvironment for both *in vitro* and *in vivo* applications (Duan, 2017, Roche et al., 2020b). These applications include modelling for diseases, drug screening and potential transplantation to replace or support the regeneration of damaged myocardium. The vascularization problem previously discussed has seen progress with the advent of bioprinting by generating vascular networks through several methods including vascular structures via simultaneous bioprinting of cells and biomaterials; addition of angiogenic factors in bioprinted constructs; and bioprinting of channel-based constructs for pre-fabricated vascular networks (Duan, 2017). Previously generated spheroids have been bioprinted, used as building blocks and subsequently fused into vascular constructs with a range of cell types including human smooth muscle cells, human dermal fibroblasts and more importantly cardiac fibroblasts, endothelial cells (EC) and iPSC-CM (Kolesky et al., 2016, Roche et al., 2020b, Wang et al., 2018, Ong et al., 2017). Ink-jet bioprinters can be utilized to deposit biomaterial scaffolds and EC simultaneously to form microvasculature scaffolds allowing EC proliferation into tubular structures with clinically relevant cell viabilities and maintained structural integrity of vasculature (Cui et al., 2018, Yeung et al., 2019). Angiogenic growth factors have been explored in bioprinted constructs with some success. HUVECs cultured in VEGF before bioprinting with iPSC-CM were reported to integrate with host vasculature when transplanted in mice (Maiullari et al., 2018). Furthermore, VEGF slowly released into scaffolds (demonstrated with both Matrigel and alginate) promotes vessel formation and CD31 expression, which similarly have seen promising results in mice transplants (Kuss and Duan, 2017). Clinically relevant constructs require vasculature that supports flow throughout the entire structure. Bioprinting uniquely offers this feature of manufacturing complex and organized networks to promote nutrient, waste and oxygen transport (Jia et al., 2016). This can be accomplished by bioprinting a hydrogel containing a removable internal structure, yielding hollow networks that can be populated with ECs to mimic *in vivo* vasculature (Lee et al., 2014, Bertassoni et al., 2014). Additionally, direct bioprinting of perfusable constructs is possible using multiple print-heads containing an outer cross-linkable hydrogel (e.g. Gel-MA) and an inner head with the appropriate cross-linking solution (Duan, 2017, Jia et al., 2016).

Contractility of bioprinted cardiac tissues remains a challenge to overcome before wide-use of clinically relevant constructs are readily available (Roche et al., 2020b). Cardiac spheroids from iPSC-CM already have been demonstrated to spontaneously contract, but are limited by immature phenotypes (Polonchuk et al., 2017, Birket and Mummery, 2015, Gentile, 2016). Improving the environment of iPSC-CM demonstrates an increase in cell contractility and contributes to overall cardiac tissue development (Wang et al., 2018). Strategies to improve bioprinted cardiac environments include addition of conductive polymers for electrical propagation support and elastic polymers for mechanical support unique to the myocardium (Jiang et al., 2017, Mawad et al., 2016, Baudis et al., 2010, Davenport Huyer et al., 2016). Though these biomaterials have been used in CTE previously, optimising such biomaterials for bioprinting is possible and slowly progressing but still a work-in-progress (Mehrotra et al., 2019, Roche et al., 2020b).

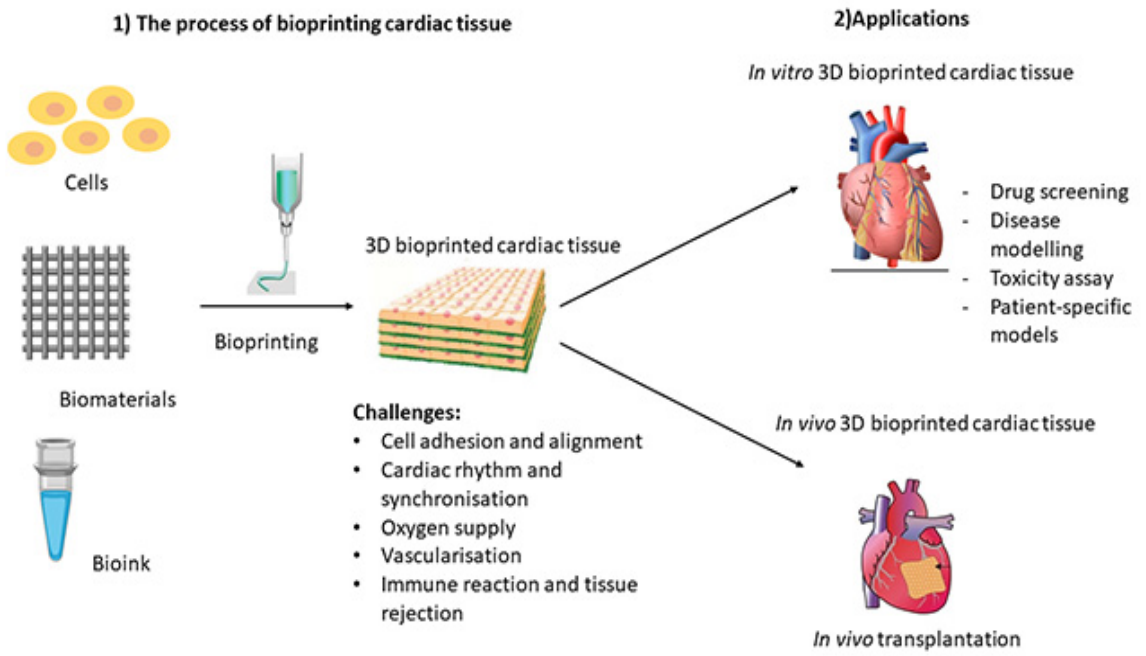

Figure 3. Applications of 3D bioprinted cardiac tissues. 1) The process of bioprinting cardiac tissue, the challenges of EHTs are cell adhesion and alignment, the cardiac tissue contractility and vascularization, oxygen supply, and can cause tissue rejection and immune reaction during transplantation. 2) The applications of 3D bioprinted cardiac tissue for *in vitro* testing are drug screening, disease modelling, toxicity assays and patient-specific models. For *in vivo* testing, 3D bioprinted cardiac tissue are transplanted on ischemic *in vivo* models to promote cardiac regeneration.

3.6.1 – *in vitro* testing of 3D bioprinted cardiac tissues

Cardiac bioprinting provides an alternative approach to regenerate infarcted heart by integrating cardiac cells and 3D biomaterials/ biomaterial-free. The application of the cardiac bioprinting has shown promises as an option to create functional cardiac tissue to regenerate or replaced damaged tissue in the myocardium (Wang et al., 2018, Noor et al., 2019). The bioengineered cardiac tissue can mimic the structural, physiological, and functional features of native myocardium but also, can be used for disease modelling of myocardial infarction, ischemia-reperfusion injury and heart failure (Cui et al., 2018, Maiullari et al., 2018). This approach allows a 3D structure of the complex arrangement of cells and ECM, supporting the cells and enhance their reorganization into functional cardiac tissues (Noor et al., 2019, Birla and Williams, 2020).The engineered cardiac patch has to undergo *in vitro* maturation and testing before being transplanted onto the defected heart. For example, in this research, the *in vitro* testing done on the 3D cardiac patch were maturation testing and vascularization testing (Jang et al., 2017). Jang et al. (2017) developed a 3D pre-vascularized myocardium patch using cardiac progenitors and MSCs with the decellularized extracellular matrix (dECM) bioink, they showed that dECM enhanced structural maturation of cells and promote vascular formation as well as the functionality of cells for tissue regeneration. Also, other *in vitro* tests are done on the 3D cardiac patch for quality control and to look at the cardiac patch structural, mechanical, and electrical properties. The tests performed are flow cytometry, immunohistochemistry,

immunofluorescence, cell viability assays and optical-electrical mapping (Jang et al., 2017, Maiullari et al., 2018, Ong et al., 2017, Tijore et al., 2018).

3D bioprinted cardiac tissue approach could give the possibility to have patient-specific tissue models that could be used to test therapeutic schemes, help in clinical diagnosis and treatment of diseases through replacement of the injured tissues (Jang, 2017). Considerable limitations in currently available in vitro and in vivo models of myocardial infarction are related to their limited ability to recapitulate the complex pathophysiology typical of the human heart tissue and their inability to directly translate from the bench to the bedside (Sharma et al., 2021). Furthermore, a study demonstrated that 3D cardiac tissues derived from hiPSC-CMs coated with extracellular matrix (fibronectin and gelatin nanofilm) have the potential to be used as a drug screening system for drug discovery and cardiotoxicity assay (Takeda et al., 2018). However, 3D tissue culturing requires not only the biological factors but also, the mechanical and electrical simulations as they are required to accustom the engineered cardiac tissues to their new functions. Such functions consist of contraction, reception, delivery of blood and electrical signalling (Alonzo et al., 2019).

Bioreactors or microfluidic "organ-on-chips" devices provide precise control by mimicking the various mechanical and chemical factors *in vivo* and, recapitulate the cellular microenvironment of the heart and monitor the critical parameters such as pH, nutrient supply and oxygen level (Jin et al., 2015, Zhang et al., 2017, Alonzo et al., 2019). Bioreactors and microfluidic devices promote and maintain cellular morphology and cell-specific functionality of the 3D biofabricated tissue but also prevent shear stress and control the flow rates (Paez-Mayorga et al., 2019, Visone et al., 2018). These devices allow the bioprinted tissue to develop a perusable microvascular network which is fundamental for treating ischemic diseases (Qasim et al., 2019). For example, Zhang et al. (2016) encapsulated endothelial cells with microfibrous hydrogel bioink which was then seeded with human induced pluripotent stem cells (HiPCS)-Cardiomyocytes/neonatal rat cardiomyocytes to get spontaneous and synchronous contractions. The 3D engineered endothelialized myocardial tissues were then added to a microfluidic perfusion bioreactor to evaluate cardiovascular toxicity (Zhang et al., 2016). Hence, they demonstrated that the organ-on-a-chip model could be used as drug screening and could act as a 3D organ model system to improve treatment efficacy, but more research needs to be done to find the ideal device for the 3D bio fabricated cardiac tissue.

The limitation is that many biofabricated cardiac tissues are either not conductive for perfusion or do not translate properly from bioprinter to bioreactors/microfluidic systems. Therefore, the development of cardiac engineered tissues that are nurtured in those devices, are not yet suitable for animal models or clinical use (Smith et al., 2018). For example, the perfusion bioreactor design is able to generate and cultured biofabricated/bioprinted vascular networks from human mesenchymal stem cells (hMSCs) cocultured with fibroblast, however, transferring those vascular networks to an animal or patient lead to the destruction of those delicate networks (Ball et al., 2016, Smith et al., 2018). The goal is for the bioprinted cardiac tissues to be cultured and transferred from the setting (bioreactor/microfluidic systems) to be used for clinical purposes.

3.6.2. – *in vivo* testing of 3D bioprinted cardiac tissues

The recent advancements of 3D bioprinted cardiac tissues have showed great promises for cardiac regeneration in ischemic *in vivo* models after the engraftment of bioprinted cardiac patches in small animals such as mice and rats (Roche et al., 2020b, Cui et al., 2018, Yeung et

al., 2019, Ong et al., 2017, Maiullari et al., 2018, Roche and Gentile, 2020b). In particular, in vivo models of permanent left anterior descending (LAD) ligation seem suitable for the preclinical testing of 3D bioprinted patches for cardiac regeneration (Roche and Gentile, 2020a). Research of cardiovascular tissue regeneration using bioprinting mainly focuses on the vasculature, myocardium and heart valves (Cui et al., 2018). To fully investigate whether the cardiac patches improve cardiac function in ischemic models, the *in vivo* tests done are echocardiography, hemodynamics, histological analyzis on the infarct size and fibrosis, cell viability, flow cytometry, gene expression and protein analyzes (Jang et al., 2017, Maiullari et al., 2018, Yeung et al., 2019, Ong et al., 2017).

Jang et al. (2017) developed a 3D-vascularized stem cell patch with cardiac progenitor and MSCs using heart tissue-derived dECM (hdECM) bioink to mimic tissue-specific ECM composition. The cardiac patches were implanted subcutaneously in mouse model. The results showed that the developed 3D stem cell patch promoted significant vascularization and tissue matrix formation in the infarcted heart model *in vivo* **(Fig 4A).** The patch also shown to improve cardiac functions, reduced cardiac hypertrophy and fibrosis and increased migration from patch to infarct area (Jang et al., 2017). In another research, Maiullari et al. (2018) integrated the use of 3D bioprinting (PEG-Fibrinogen) with iPSC-derived cardiomyocytes and HUVECs, and the 3D bioprinted cardiac patch was subcutaneously implanted in mice models. They demonstrated that the bioprinted pre-vascularized stem cell patch can effectively develop vasculature in the transplanted tissues and support the host`s vasculature. Also, it can provide cardiac niche-like microenvironment which leads to beneficial results for cardiac repair (Maiullari et al., 2018). However, more pre-clinical studies using bioink and hydrogel are needed to gain an understanding of their potential to effectively treat myocardial infarction before translating them into human clinical trials (Alonzo et al., 2019). Furthermore, the use of biomaterials faces challenges such as adverse host responses, that is, inflammatory response, immunogenicity, but also fibrous tissue formation, biomaterial degradation and toxicity of the biomaterial products that could affect the long term function of the 3D bioprinted cardiac tissue construct (Norotte et al., 2009, Ong et al., 2017).

Consequently, other pre-clinical studies are using 3D bioprinted biomaterial-free cardiac tissue on *in vivo* model and are yielding satisfactory outcomes. For example, Ong et al. (2017) bioprinted tissue spheroids composed of hiPSC-CMs, fibroblast and endothelial cells into myocardial patches **(Fig 4B)**. These cardiac patches were implanted onto rat models showing vascularization and engraftment into native rat myocardium, suggesting the therapeutic regenerative potential of this 3D biomaterial-free method (Alonzo et al., 2019, Ong et al., 2017). But the limitation of this biomaterial free cardiac patch is that the heart rate of the rats was different from the cardiac patches. Hence more studies need to be executed and extended to understand the effect of the cardiac patch in rat myocardium and the long-term regenerative potential in the damaged heart before being clinically studied on humans. Wang et al. (2018) demonstrated that the omentum can be used to support the engraftment of cardiac patches to overcome those challenges. Yeung et al. (2019) implanted the 3D bioprinted biomaterial-free cardiac patch followed by the omentum patch into rat myocardial infarction model resulting in an increased in blood vessels, smaller scar area and improvement of the cardiac function (via echocardiography study) **(Fig 4C)**. Concluding that the 3D bioprinted biomaterial–free cardiac patches have the potential to improve regeneration in cardiac tissue, promote angiogenesis and reduce scar tissue formation in the infarcted area followed by the omentum

patch (Yeung et al., 2019). But the limitation of this biomaterial free cardiac patch is that the heart rate of the rats was different from the cardiac patches. Hence more studies need to be executed and extended to understand the effect of the cardiac patch in rat myocardium and the long-term regenerative potential in the damaged heart before being clinically studied on humans. Also, further studies are required to understand the omentum mechanism to improve functional cardiac benefit which is crucial for clinical translation (Wang et al., 2020a). Despite the improvement and new avenues of 3D bioprinted cardiac tissue, limitations remain in *in vivo* models such as, poor vascularization and not synchronous contractile activity (Roche et al., 2020b).

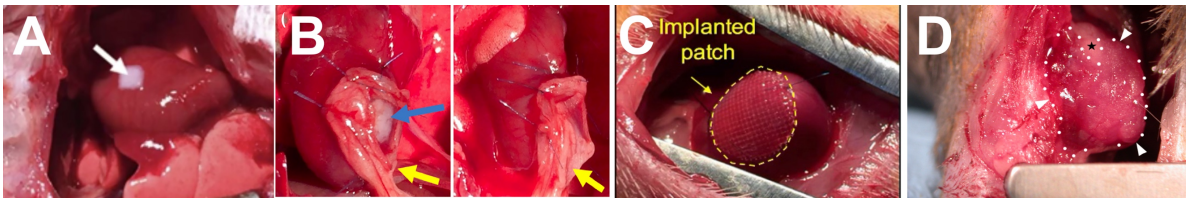

Figure 4. Types of 3D bioprinted cardiac patch for *in vivo* implantation. A) 3D bioprinted pre-vascularized stem cell sheet patch using hdECM bioink with hCPCs and human MSCs on the mouse heart (Jang et al., 2017). B) 3D bioprinted biomaterial-free cardiac patch made from spheroids of co-cultured hiPSC-CM:FB:EC onto the rat heart (Ong et al., 2017). C) 3D bioprinted biomaterial-free cardiac tissue followed by omentum patch. Cardiac patch made from spheroids of co-cultured hiPSC-CM:FB:EC (blue arrow) and omentum patch (yellow arrow) implanted in the rat model (Yeung et al., 2019). D) 3D bioprinted hydrogel-based cardiac patch was transplanted on the mouse heart (hydrogel made of alginate 4% and gelatine 8% in media) (Roche and Gentile, 2020b).

4. Discussion

In the past decades, cardiovascular regeneration has been the focus to improve cardiac function and prevent heart failure in ischemic adult heart. New approaches such as reactivating the endogenous regenerative capacity through paracrine mechanisms and deliver cells to the heart are undergoing pre-clinical studies and clinical studies. Cell-free therapies have given researchers a potential strategy for regenerating tissue endogenously, yet further studies are required. Notably, most studies require further validation, safety confirmation for the method of delivery, appropriate dosage and ideal duration of expression of the paracrine factors as prolonged expression might lead to unwanted side effects. On the other hand, approaches utilising cells and biomaterials have seen mixed results. Investigation of some cellular therapies involving SMBs have ceased due to potential complications while other stem-cell based therapies that is, MSCs, BMCs, CSCs, and iPSCs are all still being considered for their clinical potential. However, the current limitation of cell-based therapies is their limited amount and little differentiations to cardiomyocytes. More studies need to be done to elucidate their paracrine mechanisms for cardiac repair and with a larger cohort. HiPSCs in particular, have propelled cell-based therapy and CTE forward as an abundant source of previously lacking cardiomyocytes. Furthermore, HiPSCs can be differentiated into functional cardiomyocytes and be utilized to study patient-specific disease model but is cost-prohibitive. Furthermore, iPSCs are less mature than adult cardiomyocytes and the optimum level of maturation still need to be found. The major concerns of cell therapies are the patient`s immunological response,

teratoma formation caused by pluripotent stem cells and arrhythmias. These concerns have been somewhat addressed via numerous strategies such as patient-derived iPSC and biomaterials to support regeneration of myocardium that have limited-to-no immune response. However, with the cell-based and cell-free approaches, more studies need to be executed to understand the long-term regenerative potential and their mechanism effects to improve cardiac function and improve cardiac myocytes proliferation.

CTE has allowed the use of each component, that is, cell types, biomaterials, bioinks and bioprinting as well as growth factors to recapitulate the microenvironment of the human heart. Leading the ability to design and manufacture cardiovascular tissues that could improve CVD patient outcome and quality of life. The tools and concepts currently studied and utilized in CTE are beginning to transition from scientific research to clinical applications (Roche et al., 2020a, Wang et al., 2020b, Birla and Williams, 2020, Theus et al., 2019). This is undoubtedly due to the multi-disciplinary actions of biology and engineering that have yielded several avenues to explore methods of cardiac regeneration using 3D printing. Bioprinting has emerged as a technology that stands out, largely due to the incorporation of aspects from across the field of CTE and the possibility of producing functional cardiac tissues with complex architecture and reproducibility. Currently there are challenges to be answered before the promise of fully functional bioprinted cardiac tissues can be realized. Tailor-made bioinks that uniquely suit the cardiac environment are still some time away from recapitulating cardiac physiology in terms of electrical signalling and physical properties specific to the cardiac niche. Furthermore, engineered heart tissues currently in development need validation from large scale *in vivo* studies to determine the best method of properly integrating with host vasculature after transplantation. At present, both pre-vascularization and activation of endogenous vascularization mechanisms have been promising. Contractility, both synchronicity and magnitude, of bioprinted tissues is another hurdle that must be overcome before therapeutic interventions like bioprinted cardiac patches can be readily available.

5. Conclusions

The future of cardiac engineering requires a more integrated approach to recapitulate the niche environment of both the physiological myocardium and the pathophysiological. Unique and complex bioinks that are tailored to providing these biochemical and structural intricacies and highly specialized biofabrication technologies such as advanced 3D culturing platforms and bioprinters capable of ultra-high resolutions while maintaining high cell viability and populations are significant milestones for translating research to clinical practice and supportive therapy to truly regenerative.

The 3D bioprinted cardiac tissue could replace *in vitro* 2D cultures model system and could be used as disease modelling, drug and toxicity testing. Furthermore, bioprinted cardiac patch have been studied in small animals and have shown that they have therapeutic regenerative potential. However, the limitations of grafting 3D cardiac patches on small animals are poor vascularization and not synchronous contractile activity. Omentum could be used to overcome those challenges as well as support the engraftment of cardiac patches. However, further studies need to be done before bioprinted cardiac tissues can be tested clinically on humans.

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