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Repopulation and Stimulation of Porcine Cardiac Extracellular Matrix to Create Engineered Heart Patches

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ABSTRACT

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Heart failure is the main cause of death for both men and women in the United States. The only proven treatment for patients with heart failure is heart transplantation. The goal of this research is to create patches of tissue that could mimic the function of the native heart to repair the damaged portions of the heart. In this study, whole porcine hearts were decellularized to create a 3D construct that was recellularized with cardiomyocytes (CM) differentiated from human induced pluripotent stem (IPS) cells. At day 4 of differentiation, IPS-derived CMs were implanted onto cardiac extracellular matrix (cECM) and ten days after recellularization, the cells started to beat spontaneously. After implantation, the progenitor CMs continued to proliferate and populate the cECM. A live/dead assay showed the potential of the cECM as a scaffold suitable for recellularization. Confocal microscopy images were taken to evaluate the organization of the cells within the matrix and the impact of the cECM on the growth and maturation of the CMs. Representative cardiac Troponin T (cTNT) and vimentin immunostaining images of CMs derived from iPSCs, on cECM and on standard cell culture plates showed that the cECM allowed the cells to organize and form fibrils with the fibroblasts, compared with CMs cultured in regular culture plates. The timeline of implantation of the cells was a key factor for the development of the heart tissue constructs. Progenitor CMs seeded onto cECM showed better organization and the ability to penetrate 96 μm deep within the collagen fibers and align to them. However, mature CMs seeded onto the matrix showed a disorganized network with very reduced interaction of CMs with fibroblasts, forming two different layers of cells; CMs on top of fibroblasts. In addition, the depth of penetration of the mature CMs within the matrix was only 20 μm. To evaluate the impact of the addition of support cells to the CM monolayer cultures, CMs were co-cultured with human umbilical vein endothelial cells (HUVEC) and it was demonstrated that at ratios of 2:1 HUVEC:CM the beating rate of the CMs was improved from 20 to 112 bpm, additionally, the CM monolayer cultures showed a more synchronized beating pace after the addition of HUVECs. Pharmacological stimulation was performed on CM monolayer cultures using norepinephrine as a stimulator and the results showed that the beating pace of the CMs was improved to 116 bpm after 5 minutes of drug exposure. For future studies, inosculation of the tissue constructs could be performed with the incorporation of membrane proteins to understand the mechanotransduction of the cells. As a preliminary study, the action of dual claudins was evaluated with HUVEC cultures and the results showed the potential of these membrane proteins in the healing of the damaged cell membrane.

Keywords: heart, decellularization, recellularization, cardiac extracellular matrix, induced pluripotent stem cells, differentiation, cardiomyocytes, human umbilical vein endothelial cells, stimulation, norepinephrine.

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1. Introduction

Heart failure, a disabling and frequently fatal disease, is the main cause of death for both men and women in the United States. Approximately 18 patients die each day because of this pathology while waiting for a transplant, and more than 5 million individuals currently suffer from heart failure. The treatment of heart disease requires a novel therapeutic strategy to reduce mortality and improve the quality of life for heart patients.

Heart disease often results in myocardial infarction. This occurs when blood flow stops to a part of the heart and the muscle does not receive a supply of nutrients and oxygen, causing irreversible cell damage. Several attempts have been made to mimic the environment and function of the native human heart to treat heart diseases. The functional contractile cells that populate the heart muscle have very limited capacity for regeneration after birth, and therapies for myocardium regeneration following heart disease and failure are in urgent need. Around one billion cardiac cells are lost after a heart attack, diminishing the pumping function of the heart [1]. The only proven treatment for patients whose cell damage progresses to heart failure is heart transplantation. However, the demand for donor hearts suitable for transplant is much higher than the supply, creating long waiting lists and leading to patient death in most cases. In addition to the lack of donor organs, the immune response after transplantation represents another problem, and in some cases the complete rejection of the new organ is possible.

Heart tissue engineering is an alternative that holds great promise for those treatments based on the reconstruction of patient-specific cardiac muscle. Tissue engineering techniques based on using a patients' own cells could resolve the immune response problem because the non-immunogenicity of autologous tissues reduces the likelihood of rejection by the recipient's body after transplantation. Regenerative medicine has appeared as a potential alternative to heart transplants for heart disease patients [2], with the implementation of scaffolds from biomaterials that can support and create an environment for the heart cells to grow and develop similarly as in the native human heart. Decellularized scaffolds provide a natural environment, free of immunogenic factors because of the absence of DNA, and preservation of the heart native structure which is suitable for cell culture and proliferation [3] could be a potential solution to treat heart diseases.

Repopulation of heart tissue has been studied for several years. Induced pluripotent stem (IPS) cells have unique characteristics such as capacity of proliferating indefinitely and the potential of differentiating into several type of cells. Cardiomyocytes (CMs) are the cells that perform the beating function in the heart. They can be derived from IPS cells following specific cell differentiation protocols with the same potential as the CMs found in the native human heart [4]. Various studies have demonstrated complete differentiation of IPS cells into cardiomyocytes that express the cardiac phenotype as earlier as day 4 post-differentiation and develop the beating function similarly as in the human heart [5], and further implantation in heart tissue could be possible. Some studies have shown that CMs can be implanted in cardiac tissue patches created from decellularized rat and pig hearts to improve differentiation of the cells [6]. However, the CMs obtained from these differentiation protocols are not completely mature and show immature characteristics in morphology, cytoskeletal proteins, and ion channel expression and organization

[7]. The lack of these characteristics leads to a desynchronized beating function, and it is an issue that must be overcome.

In addition, along with CMs, several types of cells are found in the human myocardium. These cells include human cardiac fibroblasts, smooth muscle cells, and endothelial cells among others provide support to the CMs. Some studies have proven that coculture of CMs with these support cells improve engraftment and functional characteristics in *in vitro and in vivo* models compared to monolayers of CMs alone [8]. The endothelial cells have a leading role in the human organism as the promoters of angiogenesis and vascularization. [9]. The portion of the heart that is affected after a heart attack usually forms a scar and the pumping function of the heart is decreased severely. Endothelial cells have been studied and several publications have demonstrated the healing potential of, and the promotion of angiogenesis by HUVECs in diverse types of damaged tissue. These cells secrete specific growth factors to promote cell proliferation and vascular development to help reinstate reendothelialization of different tissues of the heart that are lost after infarction. The survival of CMs and their contractility depend on appropriate blood supply. In the mature myocardium, each CM is in physical contact with at least one capillary blood vessel [10]. The addition of supporting cells, such as HUVECs, has proven to have an influence on the improvement of the contractility rate of the CMs, as well as, on the synchronization of the pace of the CMs.

One of the biggest challenges in the recellularization of decellularized pig hearts is to get the cells to beat at synchronized rates similar to the human heart. To accomplish this goal, mechanical, electrical and pharmacological stimulation of the cells can be performed. In the human heart, the cells responsible for adapting and adjusting the beating rate of the contractions are located in the sinoatrial node and are called pacemaker cells. These cells are able to set up

and maintain the pace of the entire heart muscle preventing arrythmias or other beating disorders, allowing the blood to be pumped correctly to the rest of the body. Since the movements of the cardiac muscle are involuntary, they are controlled by the sympathetic branch of the nervous system. The membrane of the cardiac cells has G protein coupled receptors, specifically β-adrenergic receptors responsible for the excitatory response to specific neurotransmitters such as norepinephrine, which predominates in the sympathetic pathways that control the cardiac muscle contractions. The alteration of the release of norepinephrine helps to set the pace and the force of the cardiomyocyte contraction in response to different events such as exercise or fear. The implementation of pharmacological stimulation *in vitro* using drugs such as norepinephrine could provide a solution to obtain more mature and synchronized CMs that could potentially be used to populate decellularized scaffolds such as porcine cECM.

This approach, along with the addition of support cells to the *in vitro* CMs cultures, could be implemented in the creation of an engineered heart to enhance the function of the beating heart tissue and provide a step further in the discovery of a regenerative therapy that can improve and enhance the life of heart patients.

2. Literature Review

2.1 Whole Heart Decellularization

Biologic scaffold materials composed of extracellular matrix (ECM) are typically obtained in a process that involves decellularization of tissues and organs. Different protocols have demonstrated the ability to completely decellularize tissues or organs by using physical, chemical or enzymatic agents to lyse cells and remove them from the tissue [11]. Removing cellular remnants may prevent an immune response while preserving the underlying structure [12,13]. Preliminary studies have shown the feasibility of creating complete decellularized rat hearts to be used as scaffolds to support and promote cardiac cells [14].

Porcine hearts are similar to human hearts in terms of anatomy, size and protein expression, and they can provide an ideal scaffold for engineering human-size hearts. The ECM is a three-dimensional meshwork of proteins and polysaccharides that imparts structure and mechanical stability to tissues. It is composed of a set of 367 proteins that represent the core components, and mediate a map for physical interactions [15]. It has been demonstrated that cardiac extracellular matrix (cECM) scaffolds derived from decellularized pig hearts can support the attachment and growth of human endothelial cells and fibroblasts [3].

In order to obtain a cECM that can complete this function, DNA must be almost completely removed from the matrix to ensure a non-immunogenic response [16]. In addition to this, ECM components such as collagen and GAGs must remain in the matrix to provide support

for the attachment of cardiac cells. Cytotoxicity tests must be completed to ensure blood compatibility of the decellularized heart tissue. There are different assays based on human blood hemolysis that have been developed to measure the remaining cytotoxicity of the cECM.

The chemicals used to decellularize a tissue can have different effects on the tissue ultrastructure, mechanical behavior and biochemical composition, and may affect the host response [17]. Thus, it is important to remove the residual chemicals after the decellularization process to reduce the possibility of producing a cytotoxic cECM. Momtahan *et al* (2016) developed a method to examine the cytotoxicity of acellular cECM. The hemolysis assay used in their study directly measured the cell death caused by the detergents used in the decellularization process. The study showed that detergents such as Triton-X and SDS, which are commonly used in decellularization protocols, need to be removed to obtain a non-cytotoxic cECM that can be viable for implantation. The hemolysis assay is a method that can be used to examine the cytotoxicity of a tissue or scaffold due to residual detergents.

2.2 Cell Culture, Differentiation and Recellularization

2.2.1 Cardiomyocyte Differentiation from IPS Cells

Human embryonic stem cells have the potential to differentiate into all of the cell types of the body and may be useful as a source of cells for transplantation or tissue engineering [18]; however, their use is often perceived as unethical. Cardiomyocytes can also be created from the differentiation of IPS cells that are created from an adult patient's own cells – an ethical source of cells. However, current cardiac differentiation protocols exhibit variable success across different IPS lines. These protocols usually use a cECM in combination with growth factors to promote cardiogenesis [5]. It has been proven that dynamic cECM promotes epithelial-

mesenchymal transition of human IPS cells and complemented growth factor signaling to enable robust cardiac differentiation [19]. Lu *et al* (2013) reported that multipotential cardiovascular progenitor cells migrate, proliferate, and differentiate into cardiomyocytes after 20 days of perfusion into cECM. They showed that cECM promoted cardiomyocyte proliferation, differentiation, and myofilament formation from the repopulated human multipotential cardiovascular progenitor cells [6].

Burridge *et al* (2014) reported complete differentiation of IPS cells into cardiomyocytes. The protocol (CDM3) they used to differentiate the cells consisted of three components: basal medium (RPMI 1640), L-ascorbic acid 2-phosphate, and rice-derived recombinant human albumin. Cardiomyocytes produced under their protocol were positive for cardiac markers troponin T-positive (TNNT2) and α-actinin. Contraction of the CMs began at days 7-9 of culture and minimal cell death was observed. The characterization of differentiated CMs showed that at days 15-20 the cells had an atrial-like action potential, and at days 30-35 of culture CMs demonstrated the phenotype of ventricular-like cells. Although this protocol produces immature CMs, the cells progressed from an unspecified cardiomyocyte precursor phenotype to a predominantly ventricular phenotype.

CMs obtained from differentiation protocols are usually in an immature stage and cannot carry the beating function as well as mature CMs do in the adult heart. Mature CMs may better reflect the physiology of the adult heart and therefore be more useful in disease modeling. There are several ways to promote the maturation of CMs derived from IPS cells [20]. Yang *et al* (2014) demonstrated that after applying electrical stimulation and mechanical loading on the intermediate CMs, the cells displayed higher conduction velocity, contraction force and increased calcium transient kinetics. In addition to these stimulation methods, pharmaceutical

stimulation could be considered to improve the synchronized beating function. Altogether, these development characteristics can enhance the beating function of the CMs in the cECM.

2.2.2 Coculture of Cells

In the native human heart, the CMs contribute to the beating function, but the cardiac muscle needs other support cells to achieve the correct function of the heart. There are several types of cells that contribute to the overall beating function, such as endothelial cells, cardiac fibroblasts, and smooth muscle cells. Several authors have demonstrated that the behavior of the CMs is influenced by the presence of these support cells. For example, Polonchuk *et al* (2017) showed that the ratio of coculture of different populations of support cells, specifically endothelial cells and cardiac fibroblasts is critical to successfully model the behavior of the human heart [8]. Adult CMs comprise 30% of the total cell population in the native human heart, while endothelial cells represent 10%, and cardiac fibroblasts the remaining 60%. The authors showed that primary CMs do not survive in culture for extended periods without supporting cells. Endothelial cells play a role in forming the vasculature of the heart, while cardiac fibroblasts appear to play a supportive role for the vascular network formation.

2.2.3 Human Umbilical Vein Endothelial Cells (HUVECS) for Recellularization of Cardiac ECM

The main goal for all processes involving heart tissue engineering is to be able to create an organ that can be transplanted into patients with heart failure. To do this, the engineered heart must have the same behavior and functionality as a native human heart. To accomplish that, in

addition to the differentiation and repopulation of cECM with cardiomyocytes derived from IPS cells, the use of HUVECs could help to achieve this goal. It has been reported that HUVECs express brain derived neurotrophic factor (BDNF) as well as other growth factors that enable axonal growth in skeletal muscle [21].

Another growth factor, vascular endothelial growth factor (VEGF), which is also expressed by HUVECs, promotes angiogenesis in damaged tissues and facilitates the crosstalk between neural and vascular systems [22,23]. This communication between the neural and vascular systems may allow the heart tissue to function as well as a native heart, and could further contribute to the creation of a transplantable heart from decellularized pig hearts. Other studies have demonstrated the role of HUVECs in the reendothelialization of different tissues of the body to promote cell proliferation and vascular development [24,25,26].

Shvartsman *et al*. (2014) reported that VEGF, in addition to other growth factors, promotes the innervation of skeletal muscles after ischemic injuries. VEGF has been identified as a potential component that aims at restoring neural functions in damaged tissues. In addition, nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF) have roles in blood vessel growth and maturation in skeletal muscles. In this study, researchers demonstrated that HUVECs secrete NGF, GDNF, and VEGF, which are essential in the revascularization of injured tissue. These growth factors also promote axonal growth in tissues and help to maintain the crosstalk between the neuromuscular and the vascular systems.

Even though the biotechnology that uses HUVECs as precursors of vascularization processes in tissues hasn't been studied in heart tissue, the research that has been done in other types of tissue from the human body could help to develop a similar technology that can be applied in engineered hearts.

2.2.3.1 HUVECS Culture

Several methods have been reported to cultivate HUVECs and promote angiogenesis in damaged tissue [27]. Hadjizadeh *et al* (2017) reported successful angiogenesis using multilayer surface-modified polymer fibers, and two different methods of cell seeding by HUVECs. They proposed a protocol for culture of the HUVECs. The cell culture protocol consisted of culture medium (M199) containing sodium bicarbonate (2.2mg/mL), sodium heparin (90µg/mL), penicillin/streptomycin (100 U/100µg/mL), 10% fetal bovine serum (FBS), L-glutamine (2mM), and endothelial cell growth supplement (ECGS). The cells were kept in an incubator at 37°C and 5% CO_2. In addition, they studied two different methods of cell seeding by HUVECs: In the first one, HUVECs, fibrin gel matrix and fibers were combined simultaneously. They found that by using this method, angiogenesis did not occur after 10 days of culture period. In the second method, the HUVECs were seeded in a sandwich between two layers of fibrin gel matrix with or without fibroblast cell monolayer over the fibrin gel. The study showed micro-vessel formation after day 5 of the culture period and concluded that the presence of fibroblasts facilitated the endothelial progression.

Sanchez-Muñoz *et al* (2015) studied the angiogenesis of skin using HUVECs and human adipose mesenchymal stem cells (hADMSCs). The protocol that they proposed for the culture of HUVECs consisted of M199 medium, and supplements composed of 10% FBS, 10 U/mL heparin, and 2.5 µg/mL endothelial cell growth factor (ECGF). The cells were kept in an incubator at 37°C and 5% CO_2. The culture medium was changed 3 times a week and the cells were used at 3-4 passages. They proposed 3 different models for angiogenesis of the tissue. The first model contained hADMSCs, the second model contained HUVECs and the third model contained HUVECs and hADMSCs. All of the three models also contained fibroblasts and

keratinocytes, which express VEGF that aim in the angiogenesis. Researchers observed that capillary-like structures (small channels) were formed only in model 3, and the cells proliferated and adhered to the entire tissue layer. Several growth factor levels, including VEGF, hepatocyte growth factor (HGF), and platelet-derived growth factor-AB (PDGF-AB), increased by 3-, 4- and 3-fold, respectively, at day 15 of cell culture.

2.3 Stimulation of Cardiac ECM

Several approaches have been pursued to improve the beating function of the CMs implanted in heart tissue patches and accelerate their maturation. The different approaches to mature IPS-derived CMs implanted in cECM that can be considered are mechanical, electrical, and pharmacological stimulation. The combination of these types of stimulation in a 3D matrix has produced CMs that acquire a physiological cell hypertrophy, mature their contractile apparatus, and improve Ca^{2+} handling properties [28].

2.3.1 Mechanical Stimulation

The ultimate goal of tissue engineering is to be able to create implants similar to native tissue. It is essential to use physiological stimuli to improve the quality of the tissue constructs. Mechanical stimulation of cardiac tissue patches leads to a maturation of immature CMs differentiated from IPS cells. Mature CMs cultivated *in vitro* must exhibit similar functions to CMs in the adult myocardium. The cardiac environment is highly mechanically active with spontaneous contractions and stretching can be used to mimic these conditions in the heart tissue constructs [29]. It has been demonstrated that application of mechanical stretch improves

contractile function, alignment of CMs along the stretch axis, and gene expression of CM markers [30].

Lux *et al* (2015) accomplished maturation of CMs implanted in tissue constructs by mechanically stretching the cECM during cultivation of CMs isolated from rat hearts. Mechanical stimulation was used to alter passive muscle loading. It is commonly utilized to mimic the most important aspects of the way CMs work in the heart. In this study, researchers used a matrix consisting of a combination of the small intestine submucosa from pigs with preserved mesenteric arterial and venous pedicles – named Biological Vascularized Matrix (BioVam). They seeded the CMs isolated from rat hearts into the matrix and prepared it for mechanical stretch. The initial experiments were conducted at 2%, 5%, 10%, and 20% stretch to observe the impact of the mechanical stimuli on the contractile function of the matrix. They found that 2% stretch did not induce any changes in the contractile function while 10% and 20% stretch decreased the contractile function of the cardiac constructs. Thus, all experiments were conducted at 5% uniaxial cyclic stretch stimuli (2.2 mm) at 1 Hz for 48 hours starting at day 8 of culture. An analysis at the cellular level demonstrated a strong alignment of the cells parallel to the direction of applied mechanical stimulation which increased the contractile function in the cardiac constructs.

2.3.2 Electrical Stimulation

Cardiac function depends on the appropriate timing of contraction in the different regions of the heart. To maintain these functions, electrical activity in each region is adapted to its specialized function. In the adult human heart, CMs are classified as ventricular-, nodal-, and atria-like cells based on their action potentials. IPS-derived CMs from various protocols consist

of a mixture of the three different phenotypes with ventricular-like cells being the predominant class of CMs. As mentioned before, IPS-derived CMs produced *in vitro* exhibit an immature state and need to achieve maturation. Cell culture in combination with electrical stimulation could enhance the maturation and ameliorate the beating function of the CMs.

Mature mammalian myocardium exhibits both a positive force-preload relationship and a positive force-frequency (FFR) relationship, which are important for the contractile performance of healthy myocardium. Heart failure occurs when both mechanisms are lost in combination with fundamental alterations in excitation-contraction coupling. Despite the evidence of organotypic structural and molecular maturation, cECM repopulated with CMs have not shown a positive FFR. The positive FFR is dependent on the maturity of the intracellular calcium stores, the sarcoplasmic reticulum, and T-tubulation. Heart muscle development and maturation in the human body depend on electro-mechanical inputs, so by supporting auxotonic contracting function through mechanical loads and electrically stimulating at frequencies observed in neonatal hearts, the beating rate of cECM seeded with CMs could be enhanced [31].

Godier-Furnemont *et al* (2016) achieved maturation function of engineered heart muscle (EHM). They proposed a mechanism of maturation consisting of a combination of mechanical and electrical stimulation within the culture protocol. They constructed EHM from mixtures of collagen type I, Matrigel and neonatal rat heart cells. They stimulated the EHM with auxotonic contractions at frequencies between 2 and 6 Hz. EHM was stimulated first just with auxotonic contractions and at day 8 of culture, spontaneous beating frequencies of 1.23 Hz were observed. This proved that incorporation of mechanical stimulation in culture improves the beating function of the EHM. After day 8 of culture, electrical stimulation was incorporated. Frequencies of 2, 4 and 6 Hz were delivered to the EHM. After 5 days of electro-mechanical stimulation at 4

Hz, a marked drop in spontaneous beating frequency – an indicator of maturation was observed. This study also demonstrated a positive FFR in the EHM electro-mechanically stimulated at 4 Hz, demonstrating that stimulation is crucial for functional maturation. CMs stimulated at 4 Hz also showed calcium transients similar to CMs derived from a 13-day old rat myocardium. The sarcoplasmic reticulum calcium storage and release capacity was enhanced in samples stimulated at 2 and 4 Hz, demonstrating maturation in myocardial function.

2.3.3 Pharmacological Stimulation

CMs derived from IPS cells have an enormous potential for therapeutic applications. However, their immature stage after differentiation restricts their potential. Various secretions in the human body (e.g. hormones) are essential for optimal heart growth and development. Some scientists have proven that hormones such as Tri-iodo-L-thyronine (T3) aid in the development of a healthy heart [32]. T3 is a hormone that represses expression of fetal genes in neonatal CMs to enhance normal cardiac maturation. Abnormally high T3 levels in humans can lead to various complications such as decreased cardiac output, growth restriction, neuropathologies and tachycardia.

Yang *et al* (2014) showed how CMs treated with T3 increased their size, anisotropy and sarcomere length. They also demonstrated that the cells had lower proliferative activity, higher contractile force generation, enhanced calcium handling properties and increased mitochondrial respiration capacity. Their results showed that the cells increased their size from 604 μm^2 to 991 μm^2, demonstrating that CMs exhibited a more mature morphology when they were treated with T3. The importance of cell size is reflected in the impulse propagation, maximal rate of action potential depolarization and total contractile force. The researchers in this study also proved that

the contractile force was improved by treatment with T3. The twitch force increased from 7.5 nN/cell to 12.3 nN/cell. CMs treated with T3 displayed shorter time to peak contraction and significantly decreased relaxation time. This study suggested that the hormone T3 promotes the maturation of CMs derived from IPS cells by the improvement of morphological and functional characteristics of CMs.

Other molecules produced by the human body could contribute to the development of the morphological and functional characteristics of CMs derived from IPS cells [33]. Catecholamines, such as epinephrine and norepinephrine, are hormones produced by the medulla of the adrenal gland and they help in the activation of sympathetic responses in the human body, particularly inotropic and chronotropic responses. Those responses are involved in changes of beating rate by the heart. The β-receptors are responsible for the responses and the changes in cardiac frequency in the heart, particularly β_1-receptors. Drugs that interact with these receptors are called β-blocking drugs, and aid in altering the beating cardiac function. Isoproterenol is an example of a β-blocker and is a potent vasodilator that, along with epinephrine and norepinephrine, can help modulate the beating heart.

Fan *et al* (2016) demonstrated that CMs exposed to short periods of pharmacological stimuli with epinephrine or norepinephrine increased their contraction rate, reaching a maximum at 5 min, followed by a slow decline. In contrast, the exposure of CMs to the drugs for time periods of 60 minutes did not increase the contraction response. Their results indicate that continuous exposure of the cells to the stimulator caused functional desensitization of CMs and prevented the contraction rate to increase. The exposure of the CMs to these stimulation drugs could enhance the contraction rate of the CMs; therefore, the potential of the cells to beat within the cECM could also improve. This specific type of stimulation in combination with other

stimulating mechanisms enhance the possibility to obtain patches of cECM that could mimic the function of the human heart.

2.4 Summary

There are many aims that must be achieved to obtain a scaffold that can function as the human heart does. First, the cECM obtained from the decellularization of whole animal hearts must meet specific characteristics. For example, DNA must be removed from the tissue to avoid host rejection. Also, the detergents used to 'wash' the heart must preserve the collagen meshwork to make a functional cECM. Whole decellularization of pig hearts has accomplished these characteristics [3].

Second, the cECM needs to be repopulated with CMs that can carry the beating function the same way CMs do in the human heart. Human CMs finish their proliferation process at 7 months of age, so when myocardial infarction occurs, the cells in the heart are not able to heal and regenerate. Various protocols have been developed to completely differentiate IPS cells into CMs. However, most of the CMs obtained in these protocols produce immature CMs that cannot perform the beating function correctly, and desynchronized beating tissue patches are observed. Some authors have proposed alternative approaches to mature the CMs lines produced in differentiation protocols [20]. Mechanical loading and electrical stimulation have been proposed as pathways to produce mature CMs [20]. Pharmaceutical stimulation could also be considered as a method to mature CMs [33].

Lastly, rapid and sufficient blood perfusion is necessary for the integration and survival of *in vitro* bioengineered tissue constructs. This is also important when looking at the possibility

of implantation of tissue patches into a living organism or complete organ transplant. HUVECs have been demonstrated to be capable of secreting several growth factors that are essential in the angiogenesis or blood vessel formation of the tissues in the human body, as well as, provide support for the CMs in the heart muscle allowing them to perform their function better. These cells require special culture protocols that researchers have developed. The expression of these growth factors promotes angiogenesis in injured tissues that can also connect the vascular and nervous system providing the support necessary for the CMs to perform their function correctly.

3. Objective

The objective was to define first the conditions for recellularization of decellularized porcine cECM scaffolds with CMs differentiated from human peripheral blood mononuclear cell-derived (PBMC) IPS cells to derive patches of functional heart tissue that mimic the native human heart. Second, the improvement of the beating function of the CM monolayers in coculture with HUVECs and pharmacological stimulation using adrenergic drugs.

3.1 Tasks

1. Demonstrate that the CMs express better attachment characteristics when seeded on cECM at the progenitor stage.
    a. Recellularization of tissue patches with progenitor and mature cardiomyocytes
        i. Culture of progenitor (day 4 post differentiation) and mature (day 15 post differentiation) stage CMs on cECM to evaluate the differences in attachment characteristics of the cells in both scenarios.
2. Demonstrate that the beating function of the CM monolayers can be increased from 10 to 70-120 beats per minute in a synchronized manner.
    a. Coculture of CMs with HUVECs
        i. Ratios of HUVECs and CMs of 1:1 and 2:1.

b. Pharmaceutical stimulation of the CMs

    i. Norepinephrine in concentrations of 10 and 20 μM in intervals of 10 minutes.

c. Mechanical stimulation prototype

3.1.1 Rationale and Hypothesis

In the native human heart, the CMs contribute to the beating function, but the cardiac muscle needs other support cells to achieve the correct function of the heart. Primary CMs do not survive in culture for extended periods without supporting cells. The addition of HUVECs to the cultures can improve the beating function of the CMs. The timeline for the recellularization of the tissue constructs with the cells is also an important factor in obtaining beating patches with improved functionality to be transplanted to a patient. Moreover, the exposure of the cells to drugs such as norepinephrine a known neurotransmitter that plays a significant role in the improvement of the heart rate, can also contribute to increase the synchronized beating function of the cells.

4. Recellularization of Cardiac Extracellular Matrix

Several attempts have been made to create a regenerative therapy that could mimic the environment and function of the native human heart to treat heart failure diseases. Although the differentiation of IPS cells into CMs has been done for several years [2,3,14,31,34] with positive results regarding the beating function of the cells, the implementation of biomaterials such as decellularized scaffolds to create beating constructs must be done. In this study, the effectiveness of porcine cardiac extracellular matrix (cECM) as a supporting scaffold for cardiomyocytes (CMs) differentiated from human induced pluripotent stem cells (iPSCs) was demonstrated. Acellular cECM was produced in an automated, pressure-controlled whole heart decellularization apparatus [3] and human iPSCs generated from peripheral blood monocytes [4] were used for CM differentiation. Differentiated CMs on cECM exhibited improved phenotype maintenance, elongation, arrangement, and beating functions compared to CMs cultured in regular cell culture plates, and CMs seeded onto cECM 15 days after differentiation.

4.1 Materials and Methods

4.1.1 Decellularization of Porcine Hearts and Tissue Preparation

Whole porcine hearts from 6-month-old female Yorkshire Cross swine were harvested from a local abattoir following approved protocols for safety and animal care. In order to obtain acellular cECM, decellularization of hearts was achieved using an automated pressure-controlled apparatus, similarly to studies previously published by Momtahan *et al* [3]. Following

decellularization, the hearts were dissected to separate the left ventricle, and a cryostat machine (HM525 NX, Thermo Scientific®, Pittsburgh, PA) was used to obtain round slices (10 mm diameter, 300 µm thickness) of decellularized cardiac tissue. The samples were mounted on 10 mm diameter glass coverslips (Agar Scientific, Stansted Essex, UK) and placed in a 48-well plate containing 1 ml of 70% Ethanol solution. The tissue samples were treated with 1 ml of antibiotic solution containing 100 U/mL penicillin, 100 µg/mL streptomycin, 25 µg/mL amphotericin B in 75% ethanol to prevent bacterial and fungal contamination. The plates were incubated at room temperature for 3 h in a rotary shaker, and each well was washed 3 times with 1 mL sterile Dulbecco's phosphate-buffered saline (DPBS, Gibco™). Thereafter, the tissue culture plates were centrifuged at 200 x g for 4 minutes to remove trapped bubbles, and 500 µL of Fetal Bovine Serum (FBS, Gibco™) were added to each well. The plates were stored at 4°C for up to two weeks.

4.1.2 IPS Cell Culture and Cardiomyocyte Differentiation

Human IPS cells reprogrammed from peripheral blood monocytes (PBMCs) cryopreserved at -190°C were obtained from the University of Utah Cardiovascular Research and Training Institute (CVRTI, Salt Lake City, UT). The cells were thawed and seeded in 6 well-plates coated with a DPBS -Vitronectin (VTN-N, Gibco™) solution (5.5µl VTN-N per ml DPBS). Cell cultures were maintained with TeSR™-E8™ Medium (StemCell Technologies™) in an incubator (Panasonic® MCO-19M-UV) at 37°C, 5% CO_2, and medium changes were performed daily. Cell passage was performed at 90% confluency using StemPro® Accutase® (Gibco™) as a dissociation reagent to detach the cells from the plate, and the culture plates were incubated for 5-6 min. Cell suspensions were centrifuged at 800 RPMs for 5 minutes, and 1:4

dilutions were adjusted to reach confluency every 3-4 days. To improve cell survival and attachment, Rho-associated protein kinase (ROCK) inhibitor (RevitaCell™, Gibco™) was added to the cell suspensions for the first 24 h, at every passage and after thawing.

Cardiomyocyte differentiation was performed once the IPS cells reached 90% confluency. TeSR™-E8™ Medium was replaced with Cardiomyocyte Differentiation Medium A (StemCell Technologies™) supplemented with Corning® Matrigel® (10µL/mL). On day 2, the medium was replaced with Cardiomyocyte Differentiation Medium B (StemCell Technologies™) and on days 4 and 6, the medium changes were performed with Cardiomyocyte Differentiation Medium C (StemCell Technologies™). At day 8, small areas of beating cells were visible, the medium was replaced with Cardiomyocyte Maintenance Medium (StemCell Technologies™) and renewed every 2 days.

4.1.3 Recellularization of Decellularized Cardiac Extracellular Matrix

On day 4 of differentiation, the healthiest CMs were dissociated from the culture plates using TrypLE™ Enzyme (Gibco®). One ml of enzyme was added to each well and aspirated immediately to assure just enough enzyme to be in contact with the cells. The cell cultures were incubated for 5 min at 37°C in 5% CO_2. The cell monolayer was resuspended in Cardiomyocyte Differentiation Medium C supplemented with ROCK inhibitor (RevitaCell™, Gibco™). A cell counter was used to count the cells and the cell suspensions were diluted to $1x10^6$ cells/ml. The tissue plates were incubated for 1 h at 37°C prior to seeding, and 1 ml of cell suspension was added to each well. Medium change was performed one day after the seeding to prevent the cells from becoming dependent on the ROCK inhibitor. Thereafter, the medium was changed every two days as directed by the manufacturer. To evaluate the survival of the cells on the matrix, a

live/dead assay (Biotium) was performed at days 7 and 20 post recellularization on tissue constructs following the protocol from the manufacturer. Depending on the cell line, the tissue constructs started to beat 10-15 days post-seeding and the beating function of the cells was visually controlled and quantified. To evaluate the impact of the cECM on the cells (maturation and organization) the CMs were also seeded onto the matrix following the same protocol at days 10-15 post differentiation depending on the health of the cells.

4.1.4 Histology and Immunofluorescence Imaging

Recellularized ECM and 2D monolayer samples were washed with phosphate buffered saline (PBS, Gibco^{TM}) and fixed with 4% paraformaldehyde at room temperature for 10 min (2D monolayers) or 60 min (cECM). Samples were further washed three times with PBS solution and permeabilized with a PBS solution containing 0.2% Triton® X-100 (Fisher Scientific) for 30 min (2D monolayers) or 60 min (ECM). Afterwards, samples were blocked for 60 min using a PBS solution containing 2% fetal bovine serum (FBS, Sigma Aldrich, St. Louis, MO). Samples were then incubated overnight at 4°C in a PBS solution containing 2% FBS, monoclonal anti-cardiac Troponin T (cTnT, RV-C2, 1:50, F5D; 1:50; Developmental Studies Hybridoma Bank, DSHB), and monoclonal anti-Vimentin (V6630, 1:200, Sigma Aldrich, St. Louis, MO). The following day, the samples were washed three times with PBS and incubated for 60 min with a goat anti-mouse IgG2b Alexa Fluor® 647 (A21241, 1:500, Invitrogen Eugene, OR), and goat anti-mouse IgG1 Alexa Fluor® 488 (A21121, 1:500, Invitrogen Eugene, OR), diluted in a PBS solution containing 2% FBS. Following the three PBS washes, the samples were mounted using ProLong Gold Antifade Reagent (Invitrogen Eugene, OR). A two-track protocol was used to obtain 3D-stacks using a Zeiss LSM 880 Airyscan confocal microscope (Carl Zeiss, Jena, Germany)

equipped with a 20x lens. Images were processed using Imaris software (v6.1.0, Bitplane AG, Zurich, Switzerland).

4.2 Results

4.2.1 Recellularization of Decellularized cECM

At day 4 of differentiation, the progenitor CMs cell cultures were passaged onto cECM to continue and complete the differentiation process. The cells started to beat 15 days post seeding on the matrix, contrary to what regularly happens when the cells are differentiated in cell culture plates, in which they beat after 8-10 days of differentiation. A live/dead staining assay was performed 7 and 20 days post seeding (11 and 24 days post differentiation) to evaluate the cytotoxicity of the matrix, and the results are shown in Figure 1.

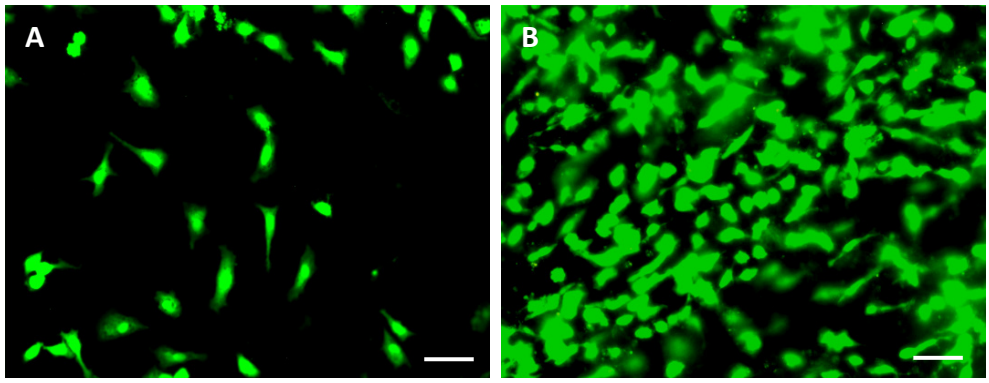

Figure 1. Live/dead Assay performed on cECM populated with CMs at days (A) 7 post seeding and (B) 20 post seeding. Scale bars represent 100 µm.

In a progenitor stage, CMs continued to proliferate up to day 15 post differentiation when they started to attain a more mature phenotype and stop proliferation. For the recellularization of the cECM, one million progenitor CMs were seeded onto matrix as previously described. Figure

1A shows the cells 7 days after seeding, at which point, CMs were in a progenitor stage and they continued to expand and populate the cECM. When the cells reached a more mature phenotype (after day 20 post seeding), they stopped growing and proliferating, nevertheless the cells had expanded creating a bigger and denser population within the cECM up to ~95% of its area (Fig. 1B). The beating function was recorded for the samples and the cells registered a beating rate of 13 bpm at day 7 post seeding and 44 beats per minute at day 20 post seeding. The live/dead assay showed the survival of the CMs on the tissue matrix after recellularization proving that the cECM provides a suitable environment for cell growth with no cytotoxic effects for the cells.

Confocal microscopy images were taken to evaluate the organization of the cells within the matrix and the impact of the cECM on the growth and maturation of the CMs. Representative cTNT and vimentin immunostaining images of CMs derived from iPSCs, on cECM and on standard cell culture plates are shown in Figure 2. The cells showed improved arrangement and organization when differentiated onto cECM (Fig. 2A and 2B, white arrows) compared with CMs differentiated in regular cell culture plates (Figure 2C and 2D). Cardiomyocytes (TNNT2, red) differentiated on the porcine cECM oriented into longitudinal fibrils that interdigitated with longitudinal strands of fibroblasts (Vimentin, green) in the same direction of the collagen fibers of the cECM. By contrast, cardiomyocytes differentiated on standard cell culture plates associated into 2D clusters/monolayers of beating cells, without any specific orientation (Figure 2 C, D).

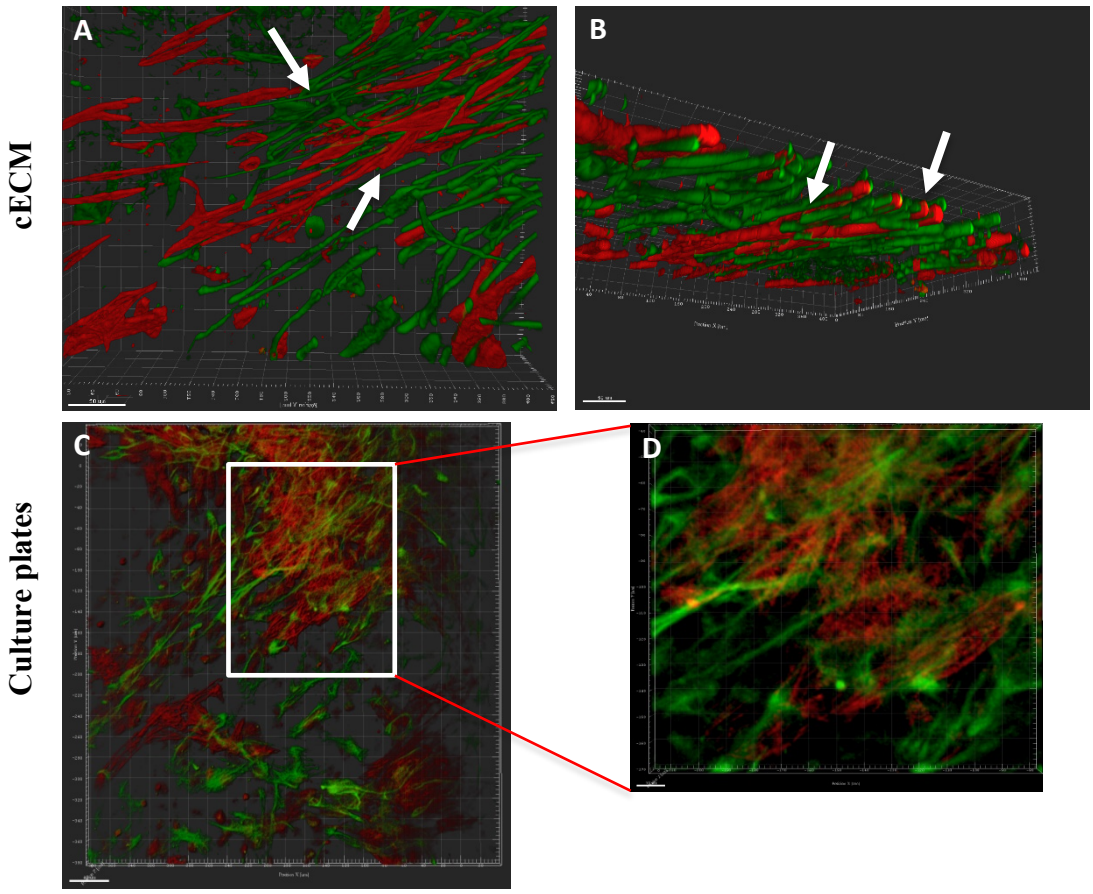

Figure 2. Cardiomyocytes differentiated on decellularized porcine cECM formed longitudinally oriented fibrils. 3D reconstructed confocal images of cardiomyocytes differentiated on decellularized porcine cECM (A, B) and 2D monolayers differentiated on a standard cell culture plate (C, D); see Materials and Methods for details. Image in D is magnified region denoted by white box in C. Confocal images were obtained using Zeiss LSM 880 Airyscan confocal microscope, 20X. Scale bar represents 50 µm.

To evaluate the impact of the cECM on the maturation and organization of the cells, the CMs were seeded onto the matrix as progenitors (4-5 days post differentiation) and as fully differentiated CMs (mature cells, 15 days post differentiation). Confocal microscopy images were taken and figures 3 and 4 show the results of the cultures for both scenarios.

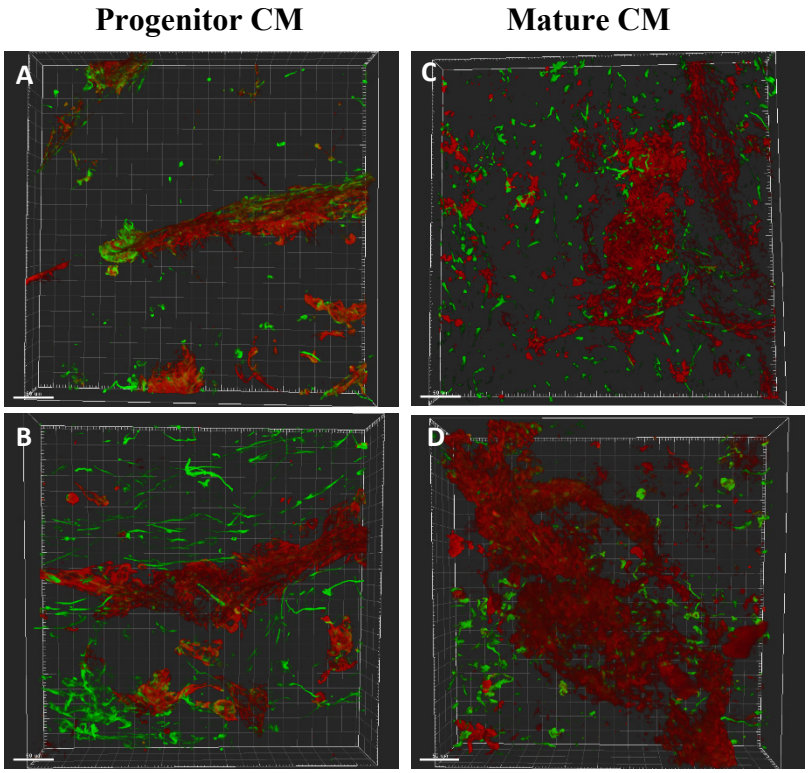

Figure 3. 3D reconstructed confocal images of cardiomyocytes differentiated on decellularized porcine cECM show a better organization (A, B) when seeded as progenitors, compared with cardiomyocytes seeded on decellularized porcine cECM (C, D) as mature cells; see Materials and Methods for details. Confocal images were obtained using Zeiss LSM 880 Airyscan confocal microscope, 20X. Scale bar represents 50 µm.

When the CMs were seeded as progenitors, the organization of the cells (Fig 3A and 3B) was better. The cells interacted and formed a network with longitudinal strands of fibroblasts and appeared to intercalate with them. On the contrary, when the CMs were seeded as mature cells, they presented a different pattern than fibrils (Fig. 3C, and 3D); instead they formed clusters of cells on top of the fibroblasts, and no significant interaction was observed between the CMs (red) and the fibroblasts (green) compared to when progenitors CMs were seeded onto the cECM. The

pattern observed with seeded mature CMs was similar to the one observed when the CMs were differentiated and kept in regular culture well plates (Fig 2C and 2D).

Another factor that is important when comparing the behavior of the cells at different seeding timelines is the ability of the CMs to penetrate deep and between the collagen fibers of the cECM. To evaluate this issue the depth of penetration of the CMs in the cECM was measured on confocal microscopy images.

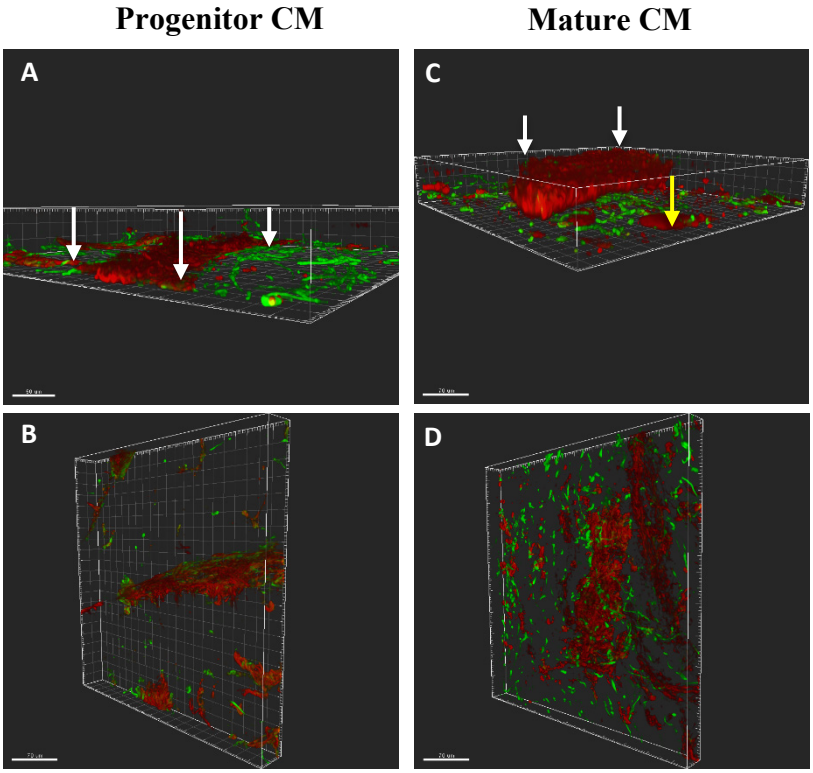

Figure 4. 3D reconstructed confocal images of cardiomyocytes differentiated on decellularized porcine cECM showed a deeper penetration within the matrix (A) when seeded as progenitors, compared with cardiomyocytes seeded on decellularized porcine cECM (B and C) as mature cells; see Materials and Methods for details. Confocal images were obtained using Zeiss LSM 880 Airyscan confocal microscope, 20X. Scale bar represents 50 µm (A) and 70 µm (B, C).

Progenitor CMs seeded onto the cECM were able to penetrate deeper within the matrix compared with mature CMs. The mechanism of migration of the cells is thought to be mostly by gravity following the tortuous path of the pores and gaps through the tissue construct. Progenitor CMs are single cells and this characteristic also gives them the facility to move through the collagen fibers of the matrix. Contrary, the fully differentiated CMs organize in clusters, connected to each other through transmembrane proteins and that hinders the ability of the cells to migrate within the collagen matrix. The penetration of the cells was measured in 3 different samples, 4 measurements in each one (n=12) for both the progenitor and mature CMs. The progenitor CMs were able to penetrate the matrix 96.39±8.34 µm (Fig 4A, white arrows), while the mature CMs were able to penetrate only 20.13±4.5 µm (Fig 4, C white arrows). In Figure 4C, there was a small portion of CMs (red) that penetrated deeper when seeded as mature CMs (yellow arrow); 56 µm; however, this was not significant when comparing that small portion with the overall penetration ability of the mature CMs. Comparing the thickness of the cECM which was 300 µm and the ability of the cells to penetrate the cECM in both scenarios, the progenitor cells penetrated 32.1±2.8% of the depth of the matrix while the mature cells were able to penetrate only 6.7±1.5%.

4.3 Discussion

The decellularized porcine cECM used as a scaffold for cardiac tissue regeneration is a promising biotechnology for the creation of recellularized beating tissue patches to treat heart failure disease. The initial experiment proved that the cECM is a suitable environment for cell growth and development. Following recellularization at early stages of differentiation, the CMS derived from human IPS cells continued to proliferate, populating the matrix up to 95% of its

area. The cells seeded onto the cECM were able to completely differentiate into fully mature CMs and were able to perform their beating function correctly. Cardiomyocytes registered a beating rate of 13 bpm at day 7 post seeding and 44 beats per minute at day 20 post seeding. The beating function of the cells diminished once they were seeded on tissue because they need time to adapt to the dense collagen environment and migrate through the collagen fibers. It is harder for the cells to acquire a good beating function rapidly, and that is the reason why they take longer to beat stronger when the tissue is recellularized.

In addition to the survival and ability of the cells to grow and complete differentiation within the cECM, the alignment of the cells was better and similar to the native human heart when the cells completed the differentiation process on cECM as shown in figure 2A and 2B. Cardiomyocytes were able to interact with the fibroblast and form fibrils that are similar to those found in the human heart. In contrast, when the cells were grown and differentiated on regular culture plates (Fig 2C and 2D), they did not show an organized pattern in fibrils, nor interaction with the fibroblasts. Instead, they formed a disorganized network of 2D clusters or monolayer of beating cells without any specific orientation.

Another important finding from this experiment was the impact of seeding time of the cells onto the matrix. When the CMs were seeded onto the matrix as progenitor CMs, as they grew and differentiated, they were able to organize in fibrils along with the fibroblasts. They showed a specific orientation and were able to contract the collagen fibers of the matrix while performing their beating function correctly. By contrast, the cells seeded as mature CMs were not able to organize in fibrils and interact with the fibroblast and the collagen fibers of the matrix as well as the progenitor CMs (Fig. 3A and 3B). Instead, they formed 3D clusters in a ball-shape

on top of the fibroblasts creating layers of cells without any significant interaction, with the CMs on top of the fibroblasts (Fig. 3C and 3D).

Finally, the timeline of seeding also affected the ability of the CMs to burrow within the cECM. Progenitor CMs were able to complete their differentiation on the matrix and that gave them the ability to burrow deeper and organize themselves in a better orientation within the collagen fibers of the matrix. The cells were able to populate the 300 µm thick matrix down to 96.39±9.34 µm of its depth when seeded as progenitors, while mature CMs were only able to penetrate the matrix 20.13±4.5 µm of its depth. No significant interaction was observed between the fibroblasts and the CMs; instead, the CMs formed a layer on top of the fibroblasts and more than half of the population of cells showed no interaction between them (Fig. 4D). The best approach was to allow the progenitor CMs induced from IPS cells to finish their differentiation process in the cECM. Since the cells as progenitors have the capacity to proliferate, it is not only possible for them to burrow within the collagen fibers of the cECM, but also to organize themselves and create better connections with the fibroblast cells in the vicinity.

5. Coculture of Cardiomyocytes and HUVECS

In a human heart, the CMs provide the beating function of the organ; however, these cells need support from other type of cells such as endothelial cells to perform their function correctly. In this study, the improvement of the beating rate of CMs was demonstrated after the addition of HUVECs to the 2D monolayer CM cultures at different ratios. Additionally, CMs showed a more synchronized pace after the addition of the HUVECs to the CMs cultures. The improvement of the function of the cells was done by analyzing videos, and the beating rate was improved when CMs were cocultured with HUVECs.

5.1 Materials and Methods

5.1.1 HUVECS Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Applications, Inc. (San Diego, CA). Cell culture and expansion was performed in T-75 flasks using Endothelial Growth Medium (Cell Applications Inc). The cells were thawed and resuspended in Endothelial Growth Medium (15 ml per flask) and incubated at 37°C in a 5% CO_2 incubator. The medium was changed one day after seeding to remove traces of DMSO, and thereafter, fresh medium was added to the cell cultures every other day. Once the cell population reached ~60%, the medium was doubled, and when confluency reached ~90%, the cells were passaged using Trypsin/EDTA (Cell Applications Inc.) as a dissociation reagent following the protocol provided by the manufacturer. The cells were centrifuged at 220 x g for 5 minutes, 1:3

dilutions were adjusted to reach confluency every 4 days, and the medium change was performed every other day.

5.1.2 Coculture of Cardiomyocytes and Human Umbilical Vein Endothelial Cells.

On day 15 after differentiation started, HUVECs were added to the CM monolayer cultures in ratios of 2:1 and 1:1 HUVEC:CM which are similar to ratios in the native human heart [8,10], and the cardiomyocyte maintenance medium was supplemented with VascuLife® VEGF Endothelial Cell Culture Medium (LifeLine Cell Technology) at the same ratios (complete medium). The passage of the HUVECs from the T-75 flask to the tissue samples was performed following the protocol provided by the manufacturer. The cells were counted using a Mini Automated Cell Counter (Moxi™, ORFLO Technologies®) and the dilutions were adjusted to reach the desired concentration. The cells were resuspended in complete medium and seeded onto the CM cultures. The medium was changed every other day and the beating function of the cells was visually observed every 24 hours.

5.2 Results

5.2.1 Coculture of Cardiomyocytes and HUVECS

In the native human heart, there are several types of cells beside cardiomyocytes. Although the CMs are responsible for most of the beating function of the heart muscle, these other types of cells, known as support cells, play an important role in the correct function of the heart. Endothelial cells are one of those type of support cells, and in this study these cells were used to help improve the beating function of the tissue patches. For the first observation, the

coculture was performed in regular cell culture plates. HUVECs were added in a 2:1 HUVEC:CM ratio 15 days post differentiation and the beating function was observed and recorded every 24 hours for 8 days. The beating function of the cells was improved by 59% just 24 hours after the addition of HUVECs. However, after 72 hours of coculture, the beating function was further improved only by 5% compared with the beating pace registered after 24 h of treatment. Contrary to this, after 120 hours of coculture, the cells improved their beating rate up to 112 bpm (77% compared with pre-coculture samples). After 192 hours of coculture, the cells stopped beating but they remained attached to the plate. Figure 5 shows a diagram of the beating function of the cells. Black markers show the CMs beating rate at 0, 24, 72 and 120 hours post coculture with HUVECs. The orange markers show the behavior of the cells and their beating rate 48, 96, 144 and 192 hours post coculture.

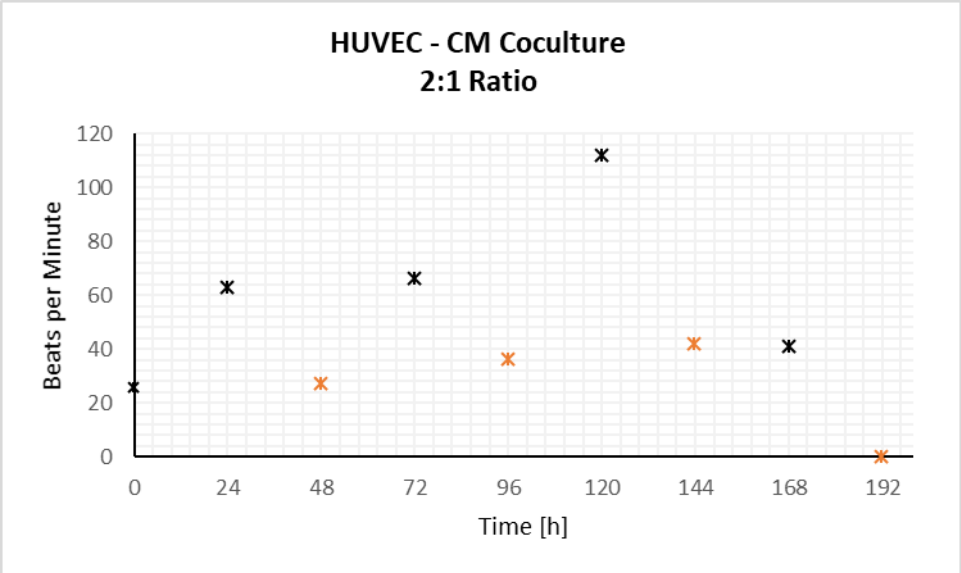

Figure 5. Beating rate of CMs-HUVECs cocultured cells 2:1 ratio. Orange markers represent the behavior of the cells at feeding times, black markers represent the behavior of the cells between feeding times.

The second observation was performed at culture ratios of 1:1 HUVEC:CM on culture plates containing CMs on day 15 post differentiation. The beating function of the cells was recorded every 24 h for 8 days and the results are shown in Figure 6.

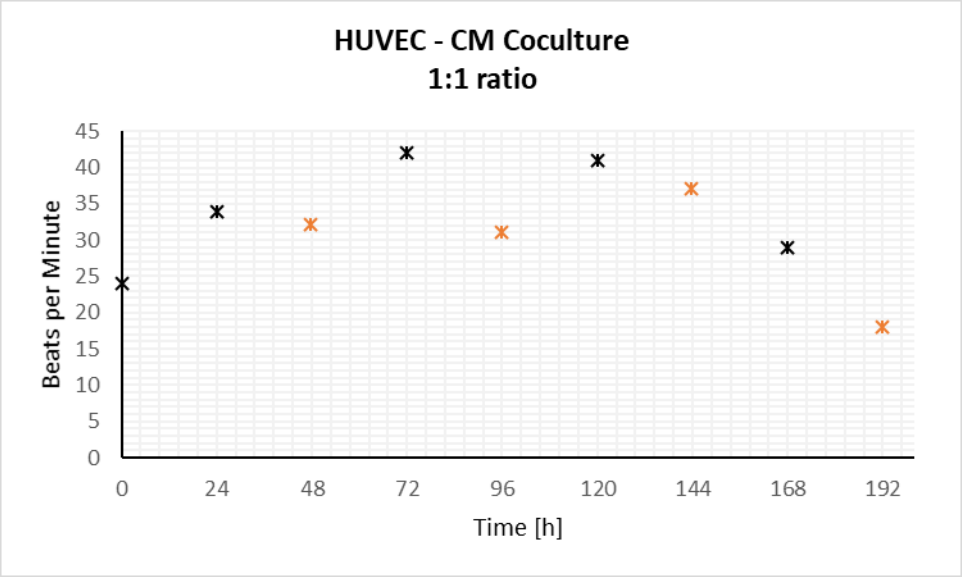

Figure 6. Beating rate of CMs-HUVECs cocultured cells 1:1 ratio. Orange markers represent the behavior of the cells at feeding times, black markers represent the behavior of the cells between feeding times.

After 24 hours of the addition of the HUVECs to the CM culture, the beating rate was improved by 29%. The maximum pace (42 bpm) was found 72 hours after the HUVEC addition with an increment of 43% compared to pre-coculture samples. After 120 hours of HUVEC implantation, the beating rate recorded was similar to the maximum; however, after 120 h the pace started to decline, and the beating function diminished to 18 bpm by day 8.

5.3 Discussion

For both observations with the two different ratios, it is interesting that when a medium change was performed, the beating rate of the cells declined (orange markers in Fig. 5 and 6). This decline in the pace of CMs is explained by the fact that the cells needed time to reabsorb nutrients from the fresh medium that was fed to them; therefore 24 hours after the medium change, once the cells assimilated the needed nutrients of the fresh medium, the beating function was improved again. It is important to highlight that the medium changes were performed outside of the incubator, thus the temperature of the cells changed, and that is another reason that may explain the behavior of the cells at those feeding times.

The results were much better when the ratio of coculture was 2:1. The beating rate started to improve just after 24 h of coculture and the maximum observed at this condition was 112 bpm. Additionally, the cells showed a more synchronized pace after the addition of the HUVECs to the culture. However, the cells cultured at 1:1 ratio showed a slower beating rate compared with the results obtained in the first observation. Even though the cells showed a similar trend, by beating faster 120 h after the implantation of the HUVECs, the difference between the pace of the cells cultured at 2:1 ratio compared with the cells cultured at 1:1 ratio at the same time was 63%, suggesting that a 2:1 ratio of coculture is better for the CMs behavior and functionality.

One of the major challenges with experiments done *in vitro* is to keep the cells beating for longer periods of time. CMs do not proliferate once they reach a mature phenotype. Therefore, they tend to stop their beating function and die due to the conditions of *in vitro* culture. For both observations, the cells slowed their beating function after 6 days of coculture and stopped beating completely by day 8 (23 days post differentiation). One of the biggest reasons for this was the limited oxygen and nutrient supply when the cells were cultured *in vitro*.

Even though endothelial cells are responsible for blood vessel formation, the lack of characteristics similar to the native human heart environment prevented the cells from performing their natural and normal function. Lastly, there are more types of cells present in the native human heart such as cardiac fibroblasts and smooth muscle cells; to obtain a construct that can better mimic the function of the native heart, all these cells must be added to the CM cultures.

6. Pharmacological Stimulation of 2D Monolayer CM Cultures

When CMs are cultured *in vitro*, unsynchronized beating function is one of the major problems. The cells do not behave the same way that happens in the native heart, and this is one of the biggest challenges to overcome in order to create a regenerative therapy that could assist in the healing process of heart disease.

Some studies have been published that demonstrated how the addition of norepinephrine to CMs leads to an improvement in the beating function, as well as in the contraction force of the cells [33]. In this experiment, the CMs cultured in monolayers were stimulated with norepinephrine at different concentrations. The cells were exposed to short periods of drug stimuli, and the results were recorded, showing that the beating function of the cells increased, compared to CMs without stimuli.

6.1 Materials and Methods

Cardiomyocytes were cultured as previously described in cell culture plates. At day 21 post differentiation, when the cells attained a mature phenotype, pharmacological stimulation was performed. Cardiomyocytes were stimulated with a solution of norepinephrine (NE, Sigma Aldrich®) at two different concentrations (10 and 20 μM) to evaluate the impact of the drug on the cells. The stimulator medium was prepared by diluting the NE in Cardiomyocyte Maintenance Medium (StemCell Technologies™) at the desired concentrations. The cells were

beating weakly before stimulation was performed, and the rate was recorded before NE stimuli by placing the cells in the microscope and recording their contraction. The stimulation assay was performed following a protocol published by Fan *et al* [33]. Stimulator medium was added to the cells for 10 minutes and the contraction of the cells was recorded (first round). After that, fresh medium (without NE) was added to the cultures and the cells were observed for 60 minutes to evaluate the impact of the drugs in the cells. Following the 60-minute period without NE, stimulator medium was added again to the cells for 10 minutes (second round) and the contraction response of the cells was recorded again. Another observation was done by keeping the cells with stimulator medium for 60 minutes following the first period of stimulation, after which the contraction rates were recorded again for 10 minutes.

6.2 Results

The first observation was performed with cells cultured on regular culture plates (6 well-plates) using a 10 μM stimulator medium. The pre-stimulation rate was recorded before the addition of the drugs and the cell beating rate was 38 bpm (Figure 7). The Stimulator medium was added to the cell cultures and the first observation was recorded 5 minutes after the addition of the drugs. The CMs expressed a beating rate of 114 bpm (Fig 7, red arrow), an increment of 67% when compared with the beating rate of the cells before the stimulation. A second measurement was recorded 10 minutes after the addition of the NE and it showed a beating rate of 68 bpm. The beating function decreased by 40% compared with the measurement recorded 5 minutes after the stimulator was added to the cells, but the pace was better when compared with the pre-stimulation rate. After the first round of stimulation, the medium was changed to remove the stimulator and the beating function was recorded twice: 10 and 20 minutes after the removal

of the stimulator. The first measurement showed 74 bpm and the second one 50 bpm. The beating rate decreased 56% when compared with the pace of the cells that were in contact with the stimulator for 5 minutes (114 bpm). After the stimulator was re-added to the cell cultures for an additional 10 minutes, the beating rate sped up again reaching a pace of 106 bpm (Fig 7, green arrow).

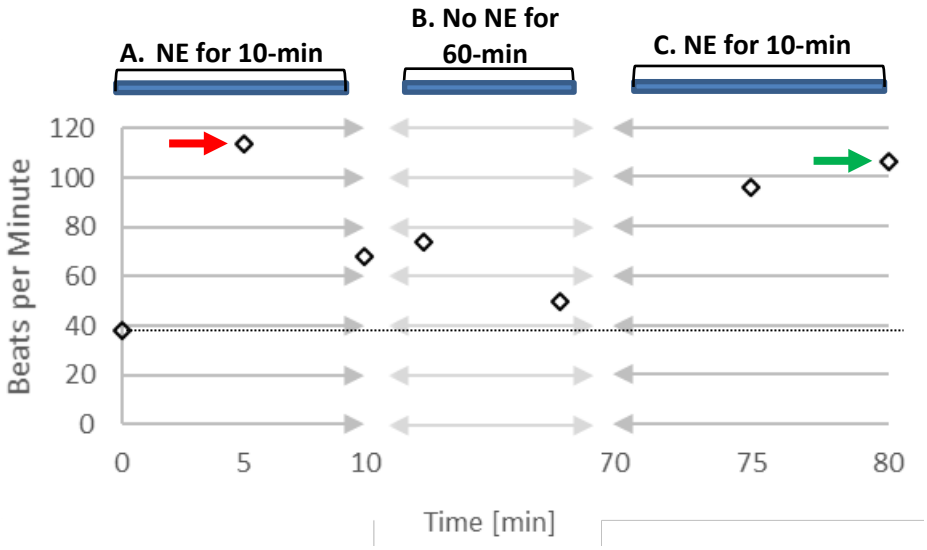

Figure 7. Stimulation results with 10 µM (A) 10-min stimuli, (B) no stimuli for 60-min and (C) 10-min stimuli. Red arrow shows bpm peak after first round of stimulus. Green arrow shows bpm peak after second round of stimulus. Dotted line is plotted at the pre-stimulation rate as a reference.

The second observation was carried out similarly, but the cells were treated with 20 µM NE stimulator; additionally, the recordings were performed in shorter intervals, 3 times in each stimulation round (Figure 8). The CMs were beating at a pace of 26 bpm before the stimulation with NE was performed (pre-stimulation rate, dash line on Fig. 8). Once the stimulator medium was added to the cell cultures the contraction rate was recorded 5 minutes after the addition of the drugs in intervals of 2 or 3 minutes. The first recording showed an increase of 21% of the

contraction rate of the cells, and the peak was found to be at 5 minutes (Fig 8, red arrow) of drug stimulation with an increment of 59% in comparison with the pre-stimulation rate. After the 10-minute stimulation period, the stimulator medium was removed and changed for regular CM medium. The cells were placed back in the incubator and the contraction rate was recorded. The cells exhibited a contraction rate close to the pre-stimulation rate, with a change of only 7%. After the 60-minute period without stimuli, the cells were stimulated again with NE and the functional beating was recorded once again. CMs exhibited an increment in the contraction rate; this time with a peak at 80 minutes (Fig 8, green arrow). The change in the contraction rate was 62% (69 bpm) compared to pre-stimulation rate (26 bpm).

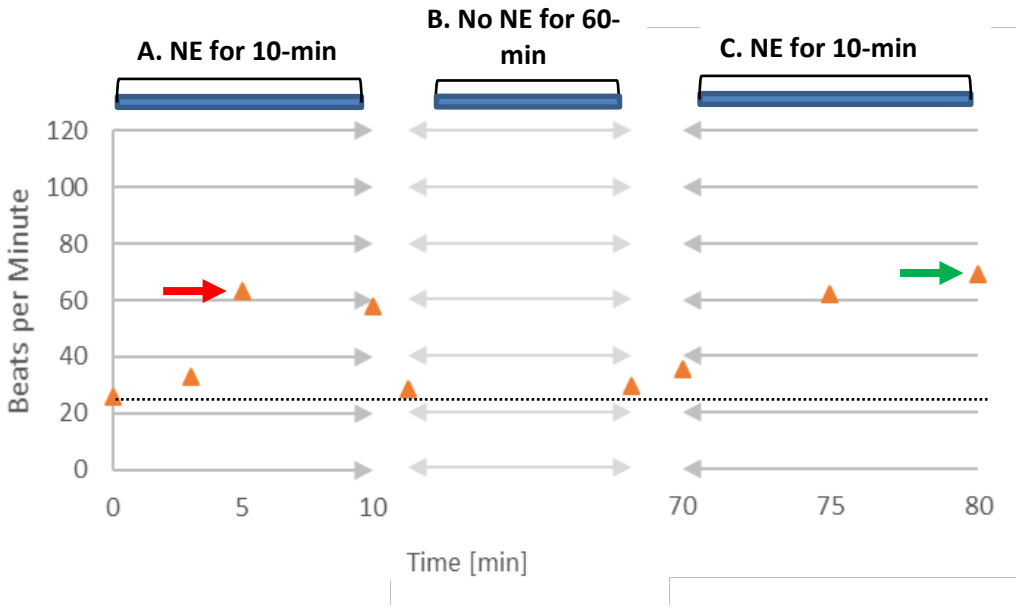

Figure 8. Stimulation results with 20 µM (A) 10-min stimuli, (B) no stimuli for 60-min and (C) 10-min stimuli. Red arrow shows bpm peak after first round of stimulus. Green arrow shows bpm peak after second round of stimulus. Dotted line is plotted at the pre-stimulation rate as a reference.

6.3 Discussion

Figure 9 shows the results for both pharmaceutical stimulation assays plotted together.

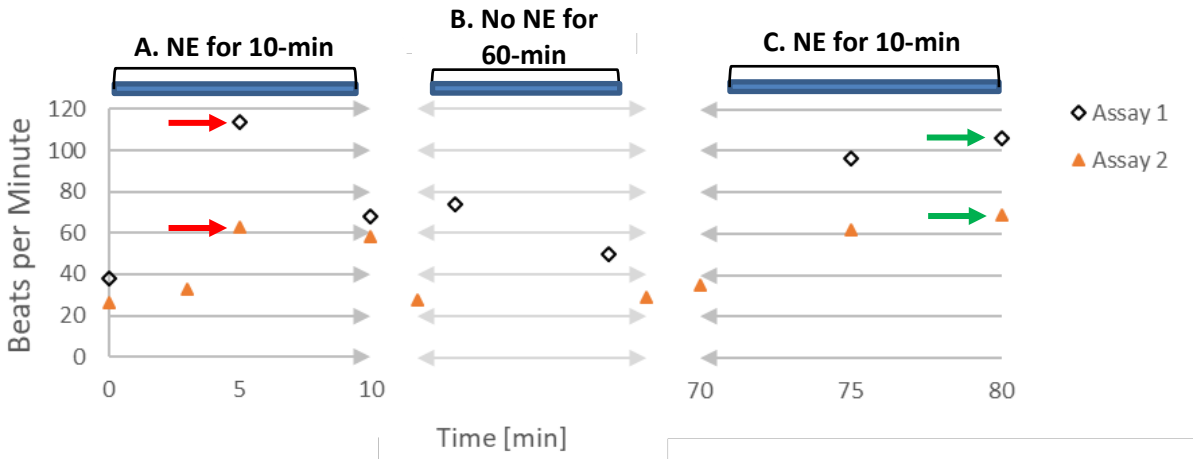

Figure 9. Stimulation of CMs with NE. (A) 10-min period stimuli, (B) 60-min period without stimuli, and (C) 10-min period stimuli. Assay 1 was performed with stimulator 10 µM NE and Assay 2 was performed with stimulator 20 µM NE.

For the first assay during the first round of stimulation, the cells exhibited a better contraction rate when treated with 10 µM than cells treated with higher concentration of stimulator (20 µM, second assay). The highest pace recorded in the assay performed with 10 µM NE was 116 bpm, after 5 minutes of stimulation (Fig. 9A red arrow). When the stimulator was added after a 60-min period of non-stimuli, the increase in the CM beating rate was similar to the initial response (Fig. 9C red arrow). However, for the second assay the pace decreased compared with the results obtained from the first assay (Fig. 9A green arrow). The cells exhibited a slower beating rate, being 69 bpm, the highest pace achieved by the cells after the second round of stimulation (Fig. 9C green arrow). These results led to several observations. First, the concentration of the drugs creates a response from the cells. The cells exhibited improved contraction rates when the concentration of the stimulator medium was 10 µM as observed

previously by Fan *et al* [33]. In contrast, the measurements made in both assays after 5 min of stimulation showed that the beating rate of the cells decreased by 45% (114 bpm, 5 min after stimulation with 10 μM NE versus 63 bpm, 5 min after stimulation with 20 μM NE) when the stimulator concentration was doubled during assay 2. Second, we hypothesize that the temperature of the cells is an important variable that affects the beating function of the CMs. The temperature of the CMs in the human heart is 37°C; taking the culture plates out of the incubator several times may decrease the temperature of the cultures and therefore, their beating function. This hypothesis arose based on the results obtained from the first assay, when the cells were only taken outside of the incubator to record the beating rate twice instead of three times as done in the second assay. Third, for both assays, the 60-min period without stimuli showed a decrease in the beating function (Fig. 9 B), which was slower for assay 1 and faster for assay 2; but no improvement in the beating function was observed. The last observation suggests that the cells do need the stimuli of the drugs to improve the beating function. Norepinephrine is an antagonist released by the sympathetic system in the human body. This antagonist binds to the beta receptors (β_2) located in the membrane of the cells and the response is to increase the beating function of the CMs when the pace slows down.

7. Inosculation of Engineered Heart Tissue

The creation of heart tissue engineered constructs is only the beginning step in the development of a regenerative therapy to treat heart failure disease. The remaining challenge is the establishment of an efficient communication between the beating patch with the host and to maintain this connection to ensure the survival of the heart tissue constructs. This survival can be ensured by creating a vascularized tissue that can connect to the host. Vascularization may be achieved by the stimulation of angiogenesis or the inosculation of preformed microvascular networks within the implants to the host microvasculature [35]. See Figure 10.

In all types of human tissues, the endothelial cells are responsible for the creation of the vasculature. These cells are connected to each other by tight junctions that enable the communication between cells. There are specific proteins that are part of the tight junctions known as Claudins which are located in the membrane of the cells. These proteins are the most important components of the tight junctions, and are responsible for the establishment of the paracellular barrier that controls the flow of molecules in the intercellular space between the cells of the epithelium [36]. Endothelial cells express claudins 1, 2, 3, 4, 7, 9 and 14. Most of the processes that occur inside a cell require energy, and the cells have three sources: chemical, electrical, and mechanical. Mechanical forces are translated into biochemical activity of the cell and involves the interaction of many structural components including the extracellular matrix, the cytoskeleton, the lipid bilayer, and the intracellular organelles [37].

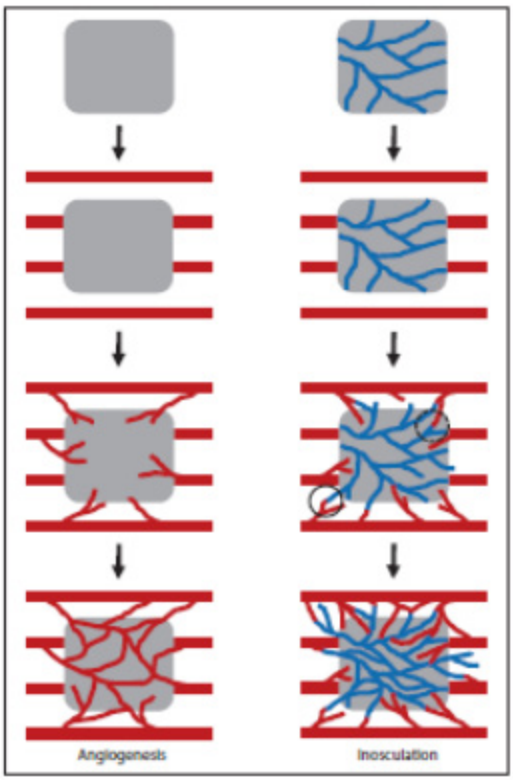

Figure 10. Angiogenesis (left) vs. Inosculation (right). In angiogenesis, new blood vessels sprout and grow into the implant (gray). During inosculation (circles), existing blood vessels (blue) connect to host blood vessels (red).

Tight junctions and specifically, their membrane proteins known as claudins, are capable of interacting with all the mentioned cellular sites to transduce mechanical forces. Inosculation can improve the angiogenic host tissue response and accelerate the directed growth of microvessels from the host microvasculature towards the implants and potentially eliminate the rejection of the tissue constructs by the host.

In this preliminary study, the potential that claudins have in healing the membrane of damaged HUVECs was demonstrated. Future studies are required to better understand the restoration of the mechanotransduction machinery through the introduction of soluble dual

surface chimeric claudins, as this restoration can be turned into advantages in the process of inosculation of tissues.

In our initial experiment with endothelial cells, HUVECS (Cell Applications, Inc.) were cultured in T-75 flasks using Endothelial Cell Growth Medium (Cell Applications Inc). The cells were thawed, resuspended in Endothelial Growth Medium (15 ml per flask) and incubated at 37°C in a 5% CO_2 incubator. The medium was changed one day after seeding to remove traces of DMSO, and thereafter, fresh medium was added to the cell cultures every other day. Once the cell population reached ~90%, the cells were passaged using Trypsin/EDTA (Cell Applications Inc.) as a dissociation reagent following the protocol provided by the manufacturer. The cells were passaged onto a 12-well plate and grew to confluency. The monolayer was wounded using a 200 µL pipette tip by applying just enough pressure to damage the monolayer but not scratch the surface of the well plate. Pictures were taken to identify the wound gap in each well using a light transmission microscope. For the positive test, the dual surface claudins (chCLDNs)[38] were added to the HUVEC medium at a concentration of 1 µM, and for a negative control, single surface claudins (chCLDNs with "one end") were added to the HUVEC medium at the same concentration. One extra observation was performed only with HUVEC medium. The cells were incubated for 3h, 6h and 24h at 37°C in a 5% CO_2 incubator, and pictures were taken at each interval of time at the same spot every time to evaluate the gap closure for each observation.

Preliminary Results

After the wound was made on the monolayers, pictures were taken using a light transmission microscope, and measurements of each gap (3 samples; 3 times each one) were recorded before the addition of the dual (test) and single (control) chCLDNS. Gap measurements

were made 3, 6, and 24 hours after the addition of the proteins to assess their effects on the closure of the wounded monolayers.

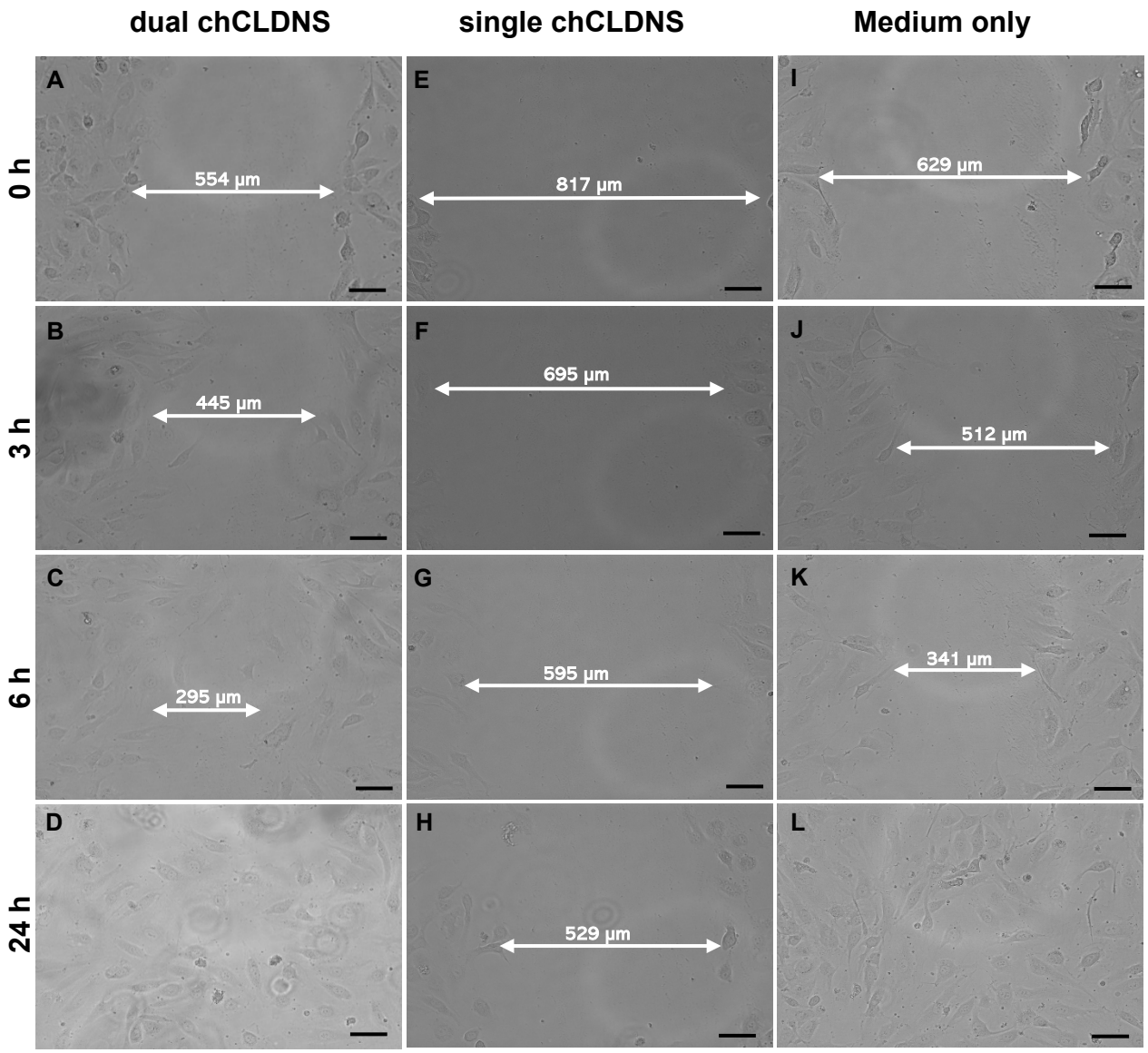

Figure 11. Wound healing assay. (A), (E), (I) wounded HUVEC monolayer before addition of proteins, (B), (C), (D) cells treated with dual chCLDNS 3, 6 and 24 h after treatment, (F), (G), (H) cells treated with single chCLDNS 3, 6 and 24 h after treatment and (J), (K), (L) cells treated with HUVEC medium only. Scale bars represent 100 µm.

The results from this preliminary experiment indicated that there was an influence of the chimeric claudins on the membrane proteins of the cells. The images obtained 3h after the addition of the dual chCLDNS showed that the monolayer wound began to close when compared with the original gap length (Fig. 11B). The average length of the original wound was 554 µm and the length recorded 3h after the addition of the proteins was 445 µm. The wound closed about 23%±9% of its original length. After six hours of treatment the closure was 295 µm, 48%±9% of the original gap length (Fig 11C).

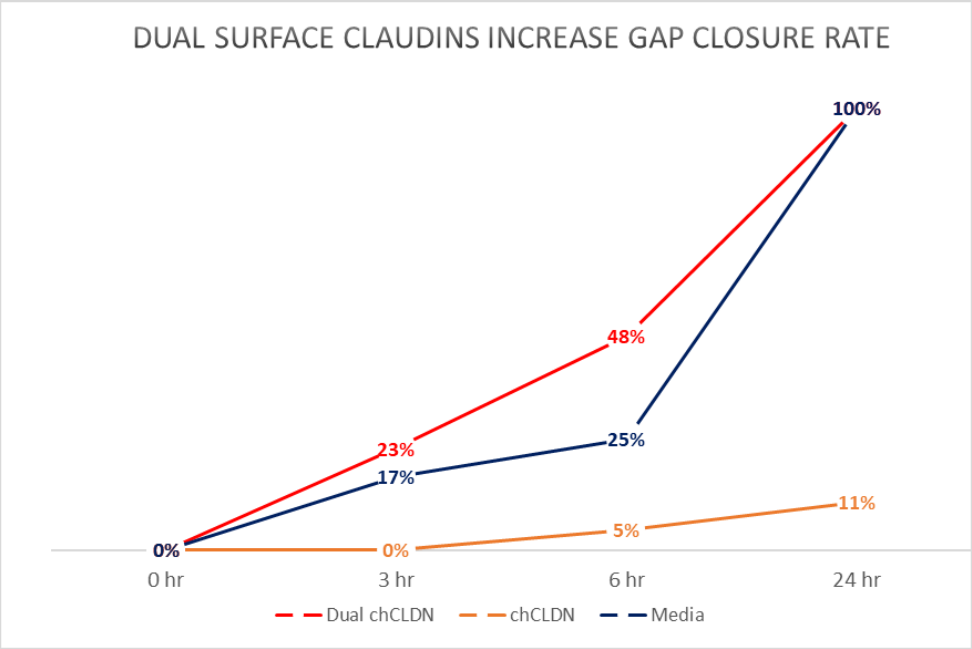

Figure 12. Dual surface claudins increase the gap closure rate in wounded cells in comparison with cells treated with single surface claudins and only cell culture medium.

For the final stage of the experiment, the data were recorded 24 h after the addition of the proteins. For this stage of the experiment, both the gap on the positive control and on the samples treated with medium closed completely. However, in the negative control, the gap closure was

just 11% (Fig. 10H, Fig. 12) suggesting that the cells do need protein with both endings to be able to completely heal and close the wound. The samples treated with just regular cell medium closed completely after 24 h due to the natural proliferation function of the cells.

Discussion

It is important to understand how every tissue works in the human body. The heart muscle needs more than cardiomyocytes to perform its job. Trying to mimic the behavior of the heart muscle must include all the connections and the environment that the native human heart has. In addition to including support cells, the connections made by those cells need to be understood. For this preliminary experiment, the hypothesis is that the single surface claudins occupied sites on the membrane preventing the cell from reestablishing the connections with the cells in the vicinity, and that is why the gap on the negative controls did not close completely.

Additionally, the cells treated with dual surface claudins were able to heal and completely close the wound, suggesting that claudins with both ends are necessary in the healing process of the membrane of the cells because they aid in reestablishing the communication with the cells in the vicinity. Comparing these observations with the results obtained when the wound was treated only with medium, the conclusion is that the cells closed the wound in the experiment including only medium because they proliferated and populated the space. Better observations must be done to understand the behavior of the cell membrane and the responses of the membrane proteins when the single surface claudins were added to the samples. The introduction of the inosculation concept in the research of regenerative therapies in the cardiovascular field could promise better and faster results in obtaining *in vitro* models that can be incorporated into *in vivo*

models to develop a therapy that can deliver better results in the treatment of heart failure disease.

8. Thesis Synopsis

8.1 Conclusions

Several concepts need to be considered when evaluating the possibility of creating a new regenerative therapy that can help patients suffering from heart failure disease. The heart is the main motor of the human body; it is responsible to deliver the blood that carries oxygen and vital nutrients for the correct function of every single tissue and cell that composes the human anatomy. The correct functioning of the heart is vital for correct function of the human body; therefore, the therapies targeted to aim in the healing of the damaged heart need to be very specific and almost perfect. This raises a lot of questions and leads to different scenarios of how regenerative medicine can be applied to help heart patients now and in the near future.

In chapter 5, the viability of creating a beating tissue construct by recellularization of decellularized porcine cECM was demonstrated. For this study several conclusions were made. First, it was shown how the IPS cells-derived CMs were able to grow and complete the differentiation process to fully mature CMs on the decellularized porcine cECM. Additionally, the cECM was shown to be a scaffold suitable for cell development, growth and expansion, without any cytotoxic effects. The populated cECM continued to beat for up to 90 days, with beating rate up to 113 bpm, creating a heart tissue construct that could be incorporated as a regenerative therapy that could aim toward the healing of the damaged heart of patients suffering from heart failure disease.

Second, and one of the major findings in this study is that the cECM provided an exceptional environment for CM development, not only because it does not have any cytotoxic effect on the cells, but also because the CMs are able to organize in fibrils and create connections with the fibroblasts, setting-up an oriented network of fibroblasts and CMs similar what is observed in the native human heart.

Third, the conditions of the implantation of the CMs onto the matrix have an important role in the creation of the beating tissue constructs. The cells were able to penetrate and create an organized network within the cECM when they finished the differentiation process in the matrix. The implantation was performed at day 4 of differentiation when the CMs were in a progenitor stage, meaning that they still had the ability to proliferate and organize themselves within the collagen fibers of the cECM. However, when the differentiation process was carried out in regular culture plates and the recellularization of the cECM was done with mature CMs, once the implantation occurred, the cells had lost the ability to proliferate. In addition, their ability to burrow and interact with the collagen fibers of the matrix was decreased; thus, the CMs formed layers of cells on top of the cECM instead of burrowing deep inside the matrix. These layers did not interact with the fibroblasts as well as when the implantation was done with progenitor CMs, and that led to a non-oriented network of cells that affected the beating function of the CMs, leading to desynchronized beating patterns within the matrix, and therefore a defective beating tissue construct that would not be suitable for heart tissue regeneration.

In chapter 6, the potential of the addition of support cells such as HUVECs to the 2D monolayer cultures to improve the beating function of the cells was demonstrated. In the native human heart, along with CMs, there are several types of cells such as endothelial cells and fibroblasts that are responsible for the correct functioning of the heart tissue. In this study,

HUVECs were added to the 2D monolayer culture of CMs to evaluate the impact of these cells in the CMs and their functioning, and several conclusions arose. First, the HUVECs added in correct ratios (2:1 HUVEC:CM) improved the beating function of the CM cultures. The beating rate recorded improved after 24 h of treatment by 59%, and after 120 h of coculture the cells expressed a beating pace of 112 bpm (77% improvement compared to cell culture with only CMs). However, when the coculture ratio was of 1:1 HUVEC:CM, the results were worse. The beating function improved only 41%, 42 bpm being the maximum pace.

Second, it was observed that at feeding times, when the medium of the cell cultures was changed, the beating function diminished. Since these experiments were done *in* vitro, the beating function of the cells changed drastically and slowed down because the cells needed time to adapt to the fresh medium. The medium change was performed outside of the incubator, at room temperature and that influenced the beating rate of the cells, contrary to what happens *in vivo*, when the nutrients are delivered to the cells with no change in temperature.

Finally, the support cells (in this case the endothelial cells), along with the ratio of coculture play an important role in the correct functioning of the CMs, and it is reflected in their beating rate being the 2:1 ratio (HUVEC:CM coculture), a better approximation of the behavior of the cells in the native human heart.

Another approach that is commonly used to speed up the pace of the cells and improve their beating rate is the addition of drugs in the culture medium. Cardiomyocytes have beta receptors (β_2) located in their membrane. In the presence of antagonist drugs such as Norepinephrine, there is an improvement in the CM beating rate. In chapter 7, the impact of pharmacological stimulation performed on the CMs was shown. From this portion of the study, one can conclude that the CMs cultured *in vitro* need the action of the drugs on its membrane to

correctly perform their beating function. Norepinephrine, a neurotransmitter, is secreted by the adrenal glands and targets several tissues including the heart. In the native human heart, the sympathetic branch of the nervous system is responsible to adjustments in the beating pace of the heart by the action of norepinephrine; it increases the beating rate when needed to keep up the normal pace of the heart.

Also, the amount of drug that the cells should be exposed to is limited to a concentration of 10 μM. When the cells were exposed to that concentration, the beating rate was improved from 30 bpm to 116 bpm after 5 minutes of drug stimulation. However, when the CMs were exposed to a higher concentration of drugs (20 μM) the beating rate was only improved to 69 bpm. Even though the beating rate for the second observation falls into the regular beating rate of an adult human at rest, these observations were performed *in vitro* in regular culture plates, and a faster pace will be required when the cells are passaged onto the cECM due to the difficulties of the CMs to beat faster and stronger within the matrix.

8.2 Recommendations for Future Work

In chapter 7, the inosculation of the heart tissue constructs was proposed. The preliminary study done with HUVECs suggested the possibility of incorporating surface proteins found in the membrane of the cells. The experiment was performed with HUVECs because of the simplicity of the culture protocol, and the availability of the dual claudins at BYU. The endothelial cells used in this study belong to the family of cells that possess in their membrane this particular claudin protein. The presence of endothelial cells in the cardiac cultures is essential for the correct functionality of the CMs, being also an important consideration when creating the 3D cardiac tissue constructs using decellularized cECM for future studies. As endothelial cells play

an essential role in the angiogenesis of the tissues, vascularization may be achieved by the stimulation of angiogenesis or the inosculation of preformed microvascular networks within the implants to the host microvasculature, moving this tissue engineering technology one more step toward patient treatments.

The mechanotransduction events that occur inside the cell remain unknown and the understanding of these processes could lead to new findings that can improve the creation of a more complete cardiac regenerative therapy. As endothelial cells have these proteins in their tight junctions, the CMs have proteins in their gap junctions known as connexins that allow the communication between cells and the transferring of the electrical impulses responsible for the creation of the contraction of the cells. Understanding the biology at the membrane of the cells could lead to more findings related to the limited capacity of the CMs to heal in the cardiac tissue.

Mechanical stretching has been proven to be a useful resource to obtain contractile cardiac tissue patches with cell alignment similar to the organization in the human heart muscle. The incorporation of the preliminary mechanical prototype designed and assembled in the laboratory (Fig. 13) to stretch and stimulate the 3D beating tissue construct, is important to obtain a stronger and more mature beating patch that could mimic the function of the native human heart tissue.

The recommendation is to mechanically stretch the tissue construct that has been populated not only with CMs but also with other support cells such as endothelial cells and cardiac fibroblasts. In addition, electrical stimulation must be included for future studies, since it has been proven that electrical stimuli result in the maturation of the CMs and in the alignment of them. Based on the literature, I propose a mechanical stimulation performed by cyclical

stretch of 5% to 8% for 48 hours and electrical stimulation with frequencies in the range of 1-1.67 Hz.

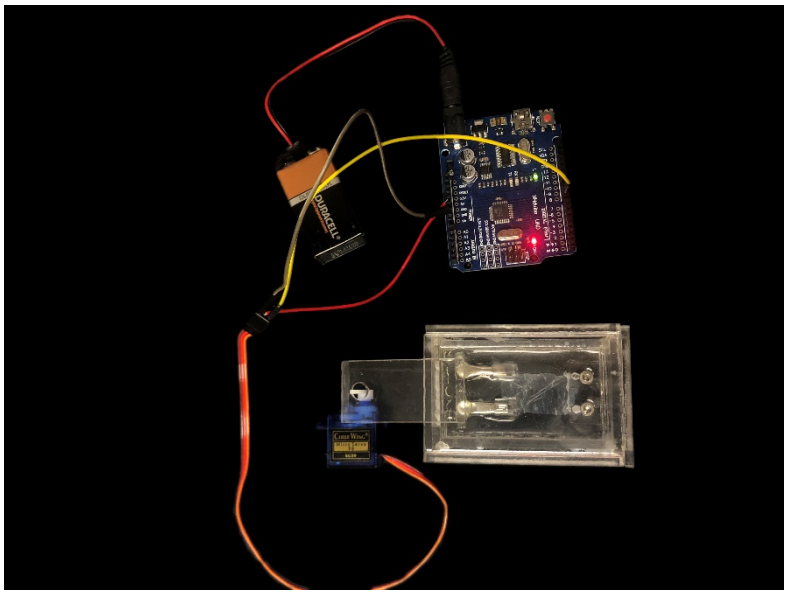

Figure 13. Mechanical Stretcher Prototype Built in Our Lab.

References

[1]. Coulombe, Kareen LK, et al. "Heart regeneration with engineered myocardial tissue." Annual review of biomedical engineering 16 (2014): 1-28.

[2]. Sekine, H., et al. (2008). "Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts." Circulation 118(14 suppl 1): S145-S152.

[3]. Momtahan, N., et al. (2015). "Automation of pressure control improves whole porcine heart decellularization." Tissue Engineering Part C: Methods 21(11): 1148-1161.

[4]. Riedel, M., et al. (2014). "Functional and pharmacological analysis of cardiomyocytes differentiated from human peripheral blood mononuclear-derived pluripotent stem cells." Stem Cell Reports 3(1): 131-141.

[5]. Burridge, Paul W., et al. "Chemically defined generation of human cardiomyocytes." Nature Methods 11.8 (2014): 855-860.

[6]. Lu, T.-Y., et al. (2013). "Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells." Nature Communications 4.

[7]. Barbuti, A., et al. (2016). "Human derived cardiomyocytes: A decade of knowledge after the discovery of induced pluripotent stem cells." Developmental Dynamics 245(12): 1145-1158.

[8]. Polonchuk, Liudmila, et al. "Cardiac spheroids as promising in vitro models to study the human heart microenvironment." Scientific reports 7.1 (2017): 7005.

[9]. Lamalice, L., et al. (2007). "Endothelial cell migration during angiogenesis." Circulation Research 100(6): 782-794 [Ch5- 5.1].

[10]. Garzoni, Luciana R., et al. "Dissecting coronary angiogenesis: 3D co-culture of cardiomyocytes with endothelial or mesenchymal cells." Experimental cell research 315.19 (2009): 3406-3418.

[11]. Crapo, P. M., et al. (2011). "An overview of tissue and whole organ decellularization processes." Biomaterials 32(12): 3233-3243.

[12]. Poornejad, N., et al. (2016). "Efficient decellularization of whole porcine kidneys improves reseeded cell behavior." Biomedical Materials 11(2): 12.

[13]. Poornejad, N., et al. (2016). "The impact of decellularization agents on renal tissue extracellular matrix." Journal of Biomaterials Applications 31(4): 521-533.

[14]. Ott, H. C., et al. (2008). "Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart." Nature medicine 14(2): 213-221.

[15]. Cromar, G. L., et al. (2012). "Toward a systems level view of the ECM and related proteins: A framework for the systematic definition and analysis of biological systems." Proteins-Structure Function and Bioinformatics 80(6): 1522-1544.

[16]. Keane, T. J., et al. (2012). "Consequences of ineffective decellularization of biologic scaffolds on the host response." Biomaterials 33(6): 1771-1781.

[17]. Momtahan, N., et al. (2016). "Using Hemolysis as a Novel Method for Assessment of Cytotoxicity and Blood Compatibility of Decellularized Heart Tissues." ASAIO Journal 62(3): 340-348.

[18]. Levenberg, S., et al. (2002). "Endothelial cells derived from human embryonic stem cells." Proceedings of the National Academy of Sciences 99(7): 4391-4396.

[19]. Zhang, J. H., et al. (2012). "Extracellular Matrix Promotes Highly Efficient Cardiac Differentiation of Human Pluripotent Stem Cells The Matrix Sandwich Method." Circulation Research 111(9): 1125-1136.

[20]. Yang, X., et al. (2014). "Engineering adolescence." Circulation Research 114(3): 511-523.

[21]. Grasman, J., et al. (2016). “Human Endothelial Cells Secrete Neurotropic Factors That Direct Axonal Growth”. Tissue Engineering Part A, 22(S1): Abstract 158, page S-43, Oral Presentation, TERMIS, Dec 11, San Diego, CA

[22]. Shvartsman, D., et al. (2014). "Sustained delivery of VEGF maintains innervation and promotes reperfusion in ischemic skeletal muscles via NGF/GDNF signaling." Molecular Therapy 22(7): 1243-1253.

[23]. Coultas, L., et al. (2005). "Endothelial cells and VEGF in vascular development." Nature 438(7070): 937-945.

[24]. Sanchez-Munoz, I., et al. (2015). "The Use of Adipose Mesenchymal Stem Cells and Human Umbilical Vascular Endothelial Cells on a Fibrin Matrix for Endothelialized Skin Substitute." Tissue Engineering Part A 21(1-2): 214-223.

[25]. Yuan, C. Y., et al. (2015). "Coculture of Stem Cells from Apical Papilla and Human Umbilical Vein Endothelial Cell Under Hypoxia Increases the Formation of Three-Dimensional Vessel-Like Structures in Vitro." Tissue Engineering Part A 21(5-6): 1163-1172.

[26]. Slupsky, J. R., et al. (1998). "Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40." Thrombosis and Haemostasis 80(6): 1008-1014.

[27]. Hadjizadeh, A. and D. Mohebbi-Kalhori (2017). "Interfacial self-assembly of endothelial cells toward angiogenic network formation in the composite hydrogel culture systems." Journal of Bioactive and Compatible Polymers 32(1): 61-73.

[28]. Nunes, S. S., et al. (2013). "Biowire: a platform for maturation of human pluripotent stem cell-derived cardiomyocytes." Nature Methods 10(8): 781-787.

[29]. Shradhanjali, A., et al. (2015). "Cardiomyocyte Stretching for Regenerative Medicine and Hypertrophy Study." Tissue Engineering and Regenerative Medicine 12(6): 398-409.

[30]. Lux, M., et al. (2016). "In vitro maturation of large-scale cardiac patches based on a perfusable starter matrix by cyclic mechanical stimulation." Acta Biomaterialia 30: 177-187.

[31]. Godier-Furnemont, A. F. G., et al. (2015). "Physiologic force-frequency response in engineered heart muscle by electromechanical stimulation." Biomaterials 60: 82-91.

[32]. Yang, X., et al. (2014). "Tri-iodo-l-thyronine promotes the maturation of human cardiomyocytes-derived from induced pluripotent stem cells." Journal of molecular and cellular cardiology 72: 296-304.

[33]. Fan, X., et al. (2016). "Cardiac β 2-Adrenergic Receptor Phosphorylation at Ser 355/356 Regulates Receptor Internalization and Functional Resensitization." PloS one 11(8): e0161373.

[34]. Ieda, M., et al. (2010). "Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors." Cell **142**(3): 375-386.

[35]. Laschke, M. W., and M. D. Menger. "Vascularization in tissue engineering: angiogenesis versus inosculation." European Surgical Research 48.2 (2012): 85-92.

[36]. Van Itallie, C. M. and J. M. Anderson (2006). "Claudins and epithelial paracellular transport." Annu. Rev. Physiol. **68**: 403-429.

[37]. Friedl, Peter, Katarina Wolf, and Jan Lammerding. "Nuclear mechanics during cell migration." Current opinion in cell biology 23.1 (2011): 55-64.

[38]. Mizrachi, D. et al. Making water-soluble integral membrane proteins in vivo using an amphipathic protein fusion strategy. Nat Commun 6, 6826, doi:10.1038/ncomms7826 (2015).