UNIVERSITY OF CALIFORNIA, IRVINE

The role of the cardiac microenvironment during cardiomyocyte development and maturation

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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Dissertation Committee:
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2016

DEDICATION

To my boyfriend (Daniel) and family (Mom, Dad, Chelsea).

Thank you for always believing in me and telling me to chase my dreams.

And to my Grandparents and Mom for their brave journey to immigrate to America for better opportunities for their children.

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CURRICULUM VITAE

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Life Science Immersion Program Certification *2014*

University of California, Irvine, CA *2005-2009*
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POSITIONS/EMPLOYMENT

University of California, Irvine, Irvine, CA
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- Created thick prevascularized cardiac tissue from human induced pluripotent stem cells and cardiac extracellular matrix (cECM) and determined the role of blood vessels and cECM in cardiomyocyte development and maturation
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California Institute for Regenerative Medicine
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PUBLICATIONS

Publications

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2. Welch-Reardon, K., Fong, A., Hughes, C. et al, Angiogenic sprouting is regulated by endothelial cell expression of Slug (Snai2), Journal of Cell Science, 2013
3. Newman, A., Fong, A., Hughes, C. et al, Analysis of Stromal Cell Secretomes Reveals a Critical Role for Stromal Cell-Derived Hepatocyte Growth Factor and Fibronectin in Angiogenesis, Arterioscler Throm Vasc Biol, 2013
4. Fong, A. and Lock, L., Neurotrophins Increase Mouse Embryonic Stem Cell Proliferation, Journal of Undergraduate Research, 2009. (Online press) (Poster and Oral presentation)

Poster

1. Fong, A., Lopez, M., Heylman, C., George, S., Hughes, C., Adult cardiac ECM promotes induced pluripotent stem cell-derived CM maturation. ISSCR's Annual Conference. 2015
2. Fong, A., Lopez, M., George, S., Ngo, T., Gershon, P., Hughes, C., Cardiac Extracellular Matrix Enhances Cardiomyocyte differentiation from iPSC. ISSCR's Regional Forum, "Stem Cells in Translation." 2013
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4. Fong, A., Lopez, M., Hughes, C., Prevascularized Thick Cardiac Tissue. 3rd International Symposium on LifeChips "Empowering human health with technology." 2012

LEADERSHIP, MEMBERSHIPS & CERTIFICATIONS

- UCI's Activate to Captivate Communications Certification Program *2015*
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- 2013 Business Plan Competition at UCI's School of Business | **Team Leader** *2013*
  - Won 3rd Place for Campus-Wide Division and Life Sciences Invitational Award
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HONORS/AWARDS & RESEARCH SUPPORT

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- 2013 Business Plan Competition at UCI's Paul Merage School of Business *2013*
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ABSTRACT OF THE DISSERTATION

The role of the cardiac microenvironment during cardiomyocyte development and maturation

By

Ashley Heather Fong

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2016

Professor Christopher C.W. Hughes, Chair

The use of induced pluripotent stem cell (iPSC)-derived cardiomyocytes (CM) has great potential for developing novel therapies to treat cardiovascular disease (CVD). Unfortunately, CM derived from pluripotent stem cells (PSC) are immature, displaying characteristics of fetal CM. Immature PSC-derived CM show automaticity (spontaneous beating), which can lead to potential arrhythmic events and thus these cells would not be safe for use in transplantation. They also lack mature cell ion channels and so drug screening using these cells would also be ineffective. Consequently, it is imperative to create strategies to generate mature PSC-derived CM, and we have focused our studies on the cardiac microenvironment's role in this process.

There is evidence that components within the cardiac microenvironment contribute to CM development *in vivo*, and our studies investigated the impact of cardiac extracellular matrix (ECM) and endothelial cells (EC) on human iPSC-derived CM development and maturation. Specifically, we generated native decellularized cardiac ECM from bovine heart tissue and compared the mechanical properties and ECM fiber distribution in ECM from fetal and adult

tissue. Normally, CM differentiation is performed on Matrigel, ECM derived from a mouse tumor. When compared to Matrigel, the iPSC-derived CM differentiated on cardiac ECM showed enhanced expression of CM development and functional genes. Maturation was further enhanced by culture in 3D versus 2D matrix as evidenced by increases in CM maturation markers, calcium signaling, and responsiveness to drugs. We also discovered that EC promote iPSC-derived CM maturation during EC/CM co-culture and that the maturation is mediated, in part, by notch signaling and endothelin-1. Taken together, these data identify a critical role for the cardiac microenvironment components: 3D culture, cardiac ECM, and EC in regulating iPSC-derived CM development and maturation, and provide a novel strategy to generate mature iPSC-derived CMs to be used in treatments for CVD.

CHAPTER 1

Introduction

CARDIOVASCULAR DISEASE

In the United States, heart disease is the leading cause of death. According to the National Center for Health Statistics, cardiovascular disease (CVD) was responsible for the mortality of 796,494 individuals in 2013, and as of 2015, there are 85.6 million adults in the U.S. living with CVD. In addition to the U.S., CVD is also the leading cause of death in the world and is responsible for 17.3 million deaths each year and it is expected to rise to 23.6 million deaths in 2030.[1] From these statistics, it is evident how disastrous CVD is to our global population and why we should place great resources and effort to combat this disease.

CVD encompasses the diseases of the heart and blood vessels. The most common kind of CVD is coronary artery disease, which includes myocardial infarction. Other CVDs include stroke, congenital heart disease, hypertensive heart disease, cardiomyopathy, arrhythmia, and heart failure. Currently, treatments for these diseases range from medication to surgery. Many risk factors contribute to CVD including genetic background and most importantly lifestyle such as physical activity, diet, and tobacco/alcohol use. In response, the American Heart Association promotes healthy living through "Life's Simple 7" which is to get active, control cholesterol, eat better, manage blood pressure, lose weight, reduce blood sugar and stop smoking. These

lifestyle changes can drastically reduce the risk for CVD, however new advanced therapies need to be developed in order to eradicate CVD completely.

Myocardial infarction

Myocardial infarction, also known as a heart attack, is the most prevalent type of CVD. The ischemic event caused by a blockage within the heart's coronary artery, by blood clots or atherosclerotic plaques, results in the death of millions to billions of cardiomyocytes,[2] inflammation,[3] and negative left ventricle remodeling.[4] The left ventricle wall thickness will decrease significantly diminishing the heart's ability to function, and scar tissue will form as a result of fibrosis. The fibrotic tissue is rich in collagen, thereby decreasing the heart's ability to contract and pump blood.[5] Ultimately, myocardial infarction impairs the heart's function and novel therapies are needed to repair the damaged myocardium.

Currently, the first line of treatment after a myocardial infarction is to administer thrombolytics, or "clot-busting" drugs within the first 2-12 hours.[6] In order to relieve the blockage, patients may undergo a coronary angioplasty where a catheter delivers an expandable balloon to widen the vessel to allow blood flow.[7] In some cases, a stent will be administered to keep the artery open and prevent further blockage. However, if the blockage can not be removed a coronary artery bypass graft surgery is needed resulting in open heart surgery where the surgeon attaches a healthy vessel to bypass the blocked coronary artery allowing continued flow of oxygenated blood throughout the heart.[8]

Although the blood flow is restored, the damaged myocardium is unable to regenerate and ultimately undergoes fibrosis potentially leading to heart failure.

Heart failure

Heart failure is a condition where the heart can not adequately pump enough blood throughout the body and significantly impacts patients' quality of life. The inability to pump blood may stem from thinning or thickening of the walls of the heart as a result of myocardial infarction or heart failure.[9] Currently, heart failure is treated with medicines such as ACE inhibitors, Beta blockers, or Digoxin, however if the disease worsens medical procedures and surgery will be needed.[10] Patients may be given pacemaker or an implantable cardioverter defibrillator which will correct unsychronized pumping or irregular heart beat.[11] Consequently, if severe heart failure symptom persist, despite previous treatments, the patient may need a mechanical heart pump such as a left ventricular assist device or ultimately a heart transplant.[12] Due to the shortage of hearts available for transplantation, the development of novel therapies is needed in order to regenerate the myocardium and restore the heart's function.

PLURIPOTENT STEM CELL

Pluripotent stem cells (PSC) have the ability to proliferate in the undifferentiated state and differentiate into the three germ layers of the embryo. Specifically, PSC are derived from blastocyst-staged embryos to generate embryonic stem cells (ESC) or somatic cell reprogramming to generate induced pluripotent stem cells (iPSC). The triad of transcription factors, Oct4, Sox2, and Nanog, work as an interconnect system to maintain

pluripotency and when disrupted differentiation occurs.[13-15] Conversely, the microRNA-145 regulates Oct4, Sox2, Klf4 and represses pluripotency in hESC.[16] Another important characteristic of PSC is increased telomerase activity which enables the cells to proliferate indefinitely without shortening of the telomeres.[17] Since PSC have the ability to self-renew and differentiate into any cell of the human body, they provide an invaluable tool to use for biology and regenerative medicine research.

PSC can be studied *in vitro* and provide limitless applications ranging from studying development to treating diseases. In order to be able to use these cells successfully in the lab, the PSC must be routinely monitored to ensure pluripotency and the absence of chromosome abnormalities.[18] Multiple assays are used to determine the cells' pluripotent state including measuring alkaline phosphatase activity, *in vitro* differentiation capacity into the three germ layers, *in vivo* teratoma formation, and protein expression of Oct4, Sox2, Nanog, SSEA-3, and SSEA-4.[19, 20] Because PSC proliferate indefinitely, there is high probability the PSC will obtain chromosome abnormalities and therefore should be assessed regularly to maintain normal karyotype.[21, 22] In order to maintain pluripotency, the PSC need to be cultured with specialized medium containing basic fibroblast growth factor for human PSC or leukemia inhibitory factor for mouse PSC. The PSC's unique characteristics provide endless possibilities to utilize these cells for basic science research and treating diseases.

Embryonic Stem Cells

Embryonic stem cells (ESC) are derived from cells within the inner cell mass of the pre-implantation embryo, and were first isolated from mice in 1981.[23, 24] The 50-150 cells of the inner cell mass are removed resulting in the destruction of the embryo and cultured *in vitro* on mouse embryonic fibroblasts (MEF) to generate ESC lines. In 1998, James Thomson and colleagues successfully isolated the pluripotent cells from the inner cell mass of a 4-5 day old human blastocyst and cultured the cell *in vitro* to generate the first human ESC (hESC) lines.[21] The hESC cell lines generated include H1, H13, H14, H7, and H9, and these lines are still being used by stem cell scientists all around the world. Since the generation of hESC requires the destruction of human embryos, an alternative type of PSC to use for research is greatly desired.

Induced Pluripotent Stem Cell

In 2006, Yamanaka and colleagues developed the ability to reprogram somatic cells into induced pluripotent stem cells (iPSC), which have similar characteristics to hESC, such as unlimited, undifferentiated proliferation and ability to differentiate into cells of the adult body.[25, 26] Additionally, the mouse iPSC are capable of germline transmission demonstrating that iPSC can contribute to the embryo proper.[27] Originally, iPSC were generated by using retrovirus to induce expression of Oct3/4, Sox2, c-MYC, and Krüppel-like factor 4 (KLF4) in adult human dermal fibroblasts, and after 3-4 weeks grown on irradiated MEFs, the cells organized into tightly packed colonies resembling hESC colonies.[25, 26] The iPSC have similar features of hESC including morphology, proliferation, surface antigens, gene expression, epigenetic signatures of pluripotent

transcription factors, telomerase activity, in addition to the ability to generate teratomas and differentiate into the three germ layers.[26]

After the discovery of iPSC, many groups have discovered alternative ways to reprogram somatic cells into iPSC besides using retroviral integration of Yamanaka's factors (Oct3/4, Sox2, c-MYC, Klf4). Besides using the traditional somatic cell, fibroblasts, other groups have been able to generate iPSC from beta cells,[28] stomach cells,[29] adipose stem cells,[30] and keratinocytes[31] demonstrating the universal capacity to reprogram somatic cells of any identity. In addition, a wide range of viruses have been successful at generating iPSC including lentivirus,[32] adenovirus,[33] Adeno-Associated Virus (AAV),[34] and Sendai virus.[35] In addition, other groups have demonstrated that the transfection of microRNA or RNA/proteins of Yamanaka factors can also reprogram somatic cells into iPSC. More recently, studies have discovered that cocktails of small molecules are capable of generating iPSC and thereby help create a more defined system which is needed when developing biologic therapies for the clinic.[36, 37]

The most important aspects of the discovery of iPSC are the ability to reprogram a patient's own cells, thereby creating autologous cell lines that can be used for transplantation and create disease-specific cell lines to model and study human diseases *in vitro.* Currently, Japan is creating a repository of iPSC lines that will match 98% of the populations HLA markers therefore creating an autologous iPSC bank that can be used for transplantation therapies, thereby avoiding the need for immune suppression. Major progress has been made in creating disease-specific lines that phenocopy the disease

pathology *in vitro* thereby enabling scientist and clinicians to study the disease in the lab and develop novel therapies to correct the diseased cells. Overall, the discovery of iPSC has revolutionized our ability to study human development and to implement regenerative medicine applications to treat diseases.

CARDIOMYOCYTE DIFFERENTIATION

The discovery of PSC has opened the flood gates to a multitude of studies generating protocols to differentiate PSC into specific terminally differentiated cells, such as neurons,[38] beta-cells,[39] hepatocytes,[40] and CMs.[41-44] Originally, human PSC differentiation required the removal of bFGF, the addition of high percentage of fetal bovine serum, and the formation of embryoid bodies (EB), which are three-dimensional aggregates of PSC grown in suspension and then re-plated onto 2D surfaces. After 20-30 days, the cells will undergo spontaneous differentiation and result in <1% CM within the differentiated cell population.[45] Due to the low induction rate, Mummery and colleagues generated a differentiation approach that utilizes information gained from developmental studies in which the anterior endoderm plays a critical role in cardiac induction.[46] Specifically, cardiac induction is initiated by the co-culture of hESC with END-2 cells, a visceral endoderm-like cells. Furthermore when the co-culture is combined with the removal of serum[47] or addition of small molecule inhibitor to p38 MAP kinase,[48] the cardiac differentiation results in 20-25% CMs.

The critical role of anterior endoderm in cardiac induction is largely mediated by transforming growth factor-beta (TGF-β) superfamily.[49, 50] The Laflamme group has

focused on two members of the TGF-β superfamily, activin A and BMP4 and generated a monolayer direct differentiation protocol that yields 30-98% CMs. Additionally, a protocol generated by the Keller group also utilizes TGF-β signaling in addition to modulation of canonical Wnt signaling. Specifically, Wnt signaling has been shown to be inhibitory of cardiac differentiation during the later stages of induction.[51, 52] Furthermore, the PSC are formed into EBs and cultured with activin A, BMP4, and bFGF, followed by Wnt inhibition by dickkopf homolog 1 (DKK1), and cultured in 2D thereby resulting in 40-90% CMs.[52]

The potential use of PSC for clinical application has focused our attention on creating defined culture conditions for differentiation, and therefore transition from recombinant proteins to small molecules, as well as the elimination of fetal bovine serum. Lian and colleagues generated a CM direct differentiation protocol that is completely defined, serum-free, and growth factor-free and focuses on temporal modulation of Wnt signaling. Specifically, the PSC are cultured with glycogen synthase kinase 3 (GSK3) inhibitor (CHIR99021) and Wnt inhibitor (IWP2), which after 14 days results in 80-98% CM.[43] By using a defined system for cardiac differentiation, we are creating functional CMs that can be used safely for cell-based therapeutic application without the worry of contamination from non-human components thereby being consistent with good manufacturing practices.[53]

APPLICATIONS OF PLURIPOTENT STEM CELLS

The discovery of PSC has provided researchers the opportunity to use these cells as tools for applications ranging from studying human development to treating diseases.[54, 55] Because of their unique characteristics of unlimited proliferation and pluripotency, scientists can perform *in vitro* studies that are not feasible in humans or informative using animal models.[56] Therefore, the use of human PSC creates a platform for disease modeling, drug screening, and regenerative medicine approaches to treat diseases through cell-based therapies such as cellular transplantation and tissue engineering applications.

Disease modeling

The ability to use PSC to create disease models enables researchers to study human disease pathology and find novel treatments that could be evaluated for efficacy *in vitro*.[56] Historically, diseases have been studied using animal models ranging from rodents to larger animals such as non-human primates. Mice are the go-to animal for modeling human disease because of ease of creating mutations within transgenic mouse lines that mimic the human disease pathology.[57] The replication of disease phenotypes in mice allows researchers to test novel drugs and therapies to understand the efficacy of the treatment without harm to humans. However, there are many differences between mice and humans with the most prominent being mice hearts beat at 600 beats per minute while human hearts beat at 70 beats per minute.[56] Additionally, many studies have shown that mice and human differ in the immune systems.[58, 59] Although animal models may have similar symptoms to human disease, it may not accurately recapitulate the entire disease pathology nor a human's response to the treatment. Lastly, studies with non-

human primates better mimic the human response because of similar anatomy and genetics, however they are extremely expensive and not practical to do on a large scale, and therefore using PSC for disease modeling is an ideal alternative.

Specifically, PSC disease models are generated through multiple techniques including the derivation of hESC lines from diseased embryos and most commonly through development of iPSC lines from cells of diseased patients. Many diseases have been modeled by PSC such as myeloproliferative neoplasm,[60] congenital megakaryocytic thrombocytopenia,[61] and early onset long QT syndrome.[62] More recently, genome editing techniques such as CRISPR and TALEN have made it feasible to correct mutations within diseased iPSC lines thereby demonstrating the clinical utility of this approach to treat diseases.[63]

In particular, Timothy Syndrome (TS) is caused by a missense mutation in the L-type calcium channel CaV1.2, and patients develop a multisystem disorder including long QT syndrome, immune deficiencies and autism.[64, 65] Yazawa and colleagues reprogrammed dermal fibroblasts from TS patients into iPSCs and differentiated the diseased iPSC into CM. The TS iPSC-derived CMs displayed irregular contraction, excess calcium influx, prolonged action potentials, and abnormal calcium transients, all defects that are most likely attributed to mutated CaV1.2.[66] Furthermore, researchers determined that the addition of roscovitine, a drug known to inactivate CaV1.2, restored the electrical and calcium signaling of the TS iPSC-derived CMs.[66] By using disease and patient-specific iPSC as a drug screening platform also known as ‘clinical trial in a dish’, scientists

employ precision medicine to discover novel pharmaceutical compounds more accurately.[67]

Drug screening

Pharmacological studies are used to investigate drugs and their effects on the body in order to determine medicinal value when applied to specific injury or disease. Currently, chemical compound libraries containing thousands to millions of small molecules are screened in a high throughput fashion to find candidate compounds that exhibit a specific biological interaction and can be further investigated in the clinic for therapeutic utility.[68] In the past, the most common models scientists used for screening drugs were animal models or *in vitro* studies using Chinese Hamster Ovary (CHO) cells or human embryonic kidney (HEK) cells. The problem with using these models is their inability to accurately predict cardiac and liver toxicity because animal models have different physiology than humans. Instead, we should use human CM or liver cells for the evaluation process. During the 1990's, many non-cardiovascular drugs were pulled from clinical use for having the adverse side effect of QT interval prolongation, inducing patients' arrhythmia and premature death.[69] The prolongation of the electrocardiographic QT interval was a result of the drug's inhibition of the hERG channel (human ether-a-go-go-related gene) also known as the voltage-gated potassium channel Kv11.1.[70, 71] As a result, the focus on hERG channel activity has become a standard in the preclinical evaluation of drugs. For this reason, the hERG channel was cloned into CHO cells to create a more relevant *in vitro* screening assay. However, since CHO cells do not express the other sodium, potassium, and calcium channels present in human CMs, this screening

method could not accurately predict cardiotoxicty, and therefore better models are needed.

The discovery of using human PSC to generate disease models provides the opportunity to more accurately evaluate the efficacy and toxicity of novel compounds. In particular, hESC and iPSC-derived CMs have been shown to accurately predict drug responses and cardiotoxcicity.[72, 73] Another advantage to using iPSC to generate disease models is the ability to retain the genetic information of the patient, including disease mutations and genetic background, that ultimately impact the efficacy of a drug.[74] And by testing drugs that are suitable for the specific patient populations, we are using precision medicine to uncover safe and effective compounds to use to treat diseases. The only limitation to using PSC-derived CMs for drug screening and toxicity studies are that these CMs have an immature phenotype, resembling fetal CM rather than adult CM.[75-77] Because the immature CMs lack expression of critical channels of adult CM, they may not accurately predict how adult CMs will respond,[73] thus, finding strategies to promote maturation of PSC-derived CMs is of paramount importance.

Cellular transplantation

In addition to disease modeling and drug screening, PSC can be used to develop regenerative medicine approaches such as cellular transplantation to treat diseases. Specifically, PSC derivatives can be used to replace the loss of cells resulting from myocardial infarction, spinal cord injury, diabetes, Alzheimer's Disease, multiple sclerosis, Huntington's Disease, macular degenerative disease and countless other

diseases. When focusing on CVD, many studies have transplanted PSC-derived CMs into infarcted rodent hearts resulting in many beneficial therapeutic effects including decreased scar formation, left ventricle wall thickening, and increase in ejection fraction.[41, 78-85] A majority of the studies investigating transplantation of PSC-derived CMs have concentrated on using small animals such as mice and rats, however the feasibility of this type of therapy on larger animals needs to be proven in order to demonstrate true clinical utility.

Although the use of rodents has provided proof-of-concept that transplanting CMs has beneficial effects, a mouse heart is a fraction of the size of the human heart. For this reason, we need to investigate transplanting into a larger animal and determine the feasibility to scale up production to create enough PSC-derived CMs for transplantation. The rodent studies would transplant millions of CMs while larger animals need billions of CMs.[41, 82] Another vital difference is that mice hearts beat 500-700 BPM which is almost 10 times faster than the human's heart beat.[86] This difference in beat rate can potentially mask side effects such as tachycardia and arrhythmias and therefore inaccurately predict the clinical outcome.[82, 87] In response, Chong and colleagues transplanted hESC-derived CMs into an infarcted non-human primate and discovered after two weeks the graft was perfused with host blood, integrated with host's electromechanical system, and generated new muscle after 3 months.[82] Furthermore, researchers observed ventricular arrhythmias and reasoned that the arrhythmias were attributed to the transplantation of immature hESC-derived CMs. In order to move forward with safer PSC-derived CM transplantation, we need to develop strategies to

mature CMs that will eliminate the dangers of arrhythmias and other aspects resulting from the iPSC-derived CM's immature phenotype. Overall, cellular transplantation of PSC-derived CMs shows great promise in treating the damaged myocardium.

Tissue Engineering

Another regenerative medicine application to treat CVD is tissue engineering. Specifically, researchers can engineer 3D tissues to replace damaged or diseased tissue, and PSC provide a robust resource to use when generating thick tissues. The advantage of using tissue engineering approaches over cellular therapies is that the majority of cells do not survive after cellular transplantation due to the absence of survival signals, lack of vasculature, inflammation, and anoikis.[88] Many cells die from anoikis, also known as programmed cell death caused by the detachment from surrounding extracellular matrix (ECM), which is the result of transplanting single cells without matrix support.[89, 90 91, 92] Therefore the generation of cardiac tissue comprised of both matrix and multiple cell types is needed to provide the appropriate microenvironment to ensure survival of the transplanted cells and maintain tissue integrity after transplantation.

When engineering 3D tissues there are multiple approaches to achieve specific cell patterning and they include creating cell sheets that are stacked,[85] bioprinting,[93] and embedding cells in 3D matrix for self-assembly. Masumoto and colleagues generated cardiac tissue sheets (CTS) using temperature responsive plates comprised of human iPSC-derived CMs, EC and vascular mural cells. The CTS were layered into 3-4 cell layer sheet *in vitro* and transplanted into an athymic nude rat sub-acute MI model

resulting in improved cardiac function.[85] Recently, the Cho group implemented the use of decellulraized ECM and cells as the bioink that an inkjet printer uses to form pre-patterned tissues.[93] The printer nozzel distributes the cells and matrix to form a 3D tissue construct by generating scaffold structures via a layer-by-layer process.[94] Ultimately, the most common type of tissue engineering approach is to embed cells into a 3D matrix and allow for self-assembly.[95, 96]

These tissue engineering approaches require the use of synthetic or biologic scaffolds to create the 3D architecture to provide support for embedded cells. Specifically, synthetic materials such as polyethylene glycol, polyhydroxyethylmethacrylate, polylactide-glycolic acid, and poly(N-isopropylacrylamide) can be easily altered to provide predicable mechanical and chemical properties.[97] However, synthetic scaffolds do not possess the chemical composition or specific cues of biological scaffolds. These ECM scaffolds include fibrin, collagen, fibronectin, and native ECM from decellularized tissues. Since studies have determined that the mechanical and chemical composition of the scaffold greatly impacts CM differentiation, maturation, and survival,[98-100] it is imperative to choose the appropriate scaffold for tissue engineering approaches.

An advantage of engineering tissues for transplantation is the ability to add multiple cell types into a scaffold and allow for self-assembly. These cell types include PSC-derived cardiac cells such as CMs, endothelial cells (EC), and support cells such as fibroblasts. Previous studies have implicated that the co-culture of CM with EC improves graft survival and function,[85, 101] indicating the importance of the presence of non-CM cells

types during graft transplantation. Furthermore, the addition of EC and fibroblasts to the cardiac tissue enables prevascularization – the process by which EC form microvessel networks *in vitro* in a three dimensional matrix supported by human stromal cells.[102] Our previous work demonstrates that a 7 day prevascularization promotes anastomosis and blood flow throughout the tissue within 24 hours after transplantation into a SCID mouse.[102] If the tissue is not prevascularized then blood flow is not observed until 5-7 days, a rate that is too slow for survival of metabolically active tissue by simple diffusion. The prevascularization ensures that the thick cardiac tissue that is transplanted will anastomose with host vasculature and ensure the survival of the transplanted graft.

Several groups have engineered cardiac tissue comprised of CMs, ECs, and stromal cells (triculture) and demonstrated graft viability after transplantation.[101, 103] Studies have shown that scaffold-free prevacularized human patches comprised of hESC-derived CMs, EC, and fibroblasts are able to improve implantation and survival of grafts resulting in an approximately 10 fold increase in graft size when compared to non-vascularized patches in rats.[104] Also of important note, the grafts were perfused with rat blood cells. The cardiac graft's survival is dependent on the development of its vasculature and the graft's ~50 fold increase in the number of vessels was dependent on the stromal cells' ability to secrete proangiogenic factors.[105, 106] Similarly, the triculture was seeded into a biodegradable scaffold of 50% poly-L-lactic acid and 50% polylactic-glycolic acid and upon transplantation onto a rat heart the graft survived, contained infiltrated rat vasculature, and was perfused with host blood.[81, 103] Other scaffolds have been used for tissue engineering such as cardiac ECM; it has been shown to support the cell types of the

triculture as well as demonstrate tissue remodeling.[107] Furthermore, Tulloch and colleagues determined that preconditioning the multicellular cardiac tissue constructs through cyclic stretching improved the graft's contraction force, alignment of cells, and survival after transplantation.[101] The use of multiple cell types (EC and stromal cells) when generating 3D tissues for transplantation will improve graft survival by promoting anastomosis with the host vasculature, resulting in establishment of a blood supply to support transplanted cells.

Tissue engineering applications have great promise to treat CV disease as the use of multiple cell types (PSC-derived CM, EC, stromal cells) and matrix should support the survival of the transplanted tissue construct. However, a concern when deriving cardiac tissue such as myocardium is to ensure the transplanted CMs are capable of contracting at the appropriate forces needed to assist the heart in pumping blood effectively. Currently, CMs derived from PSC sources are immature and can not produce the appropriate force contraction needed for *in vivo* applications.[108] For this reason, we are in dire need for creating strategies to mature PSC-derived CMs so they can be safely and effectively used for cell-based transplantation and drug screening applications to treat CVD.

CARDIOMYOCYTE MATURATION

Currently, the methods to induce CMs from PSC generate immature CM displaying characteristics of fetal CM. The immature characteristics are defined by lack of gene and protein expression of ion channels,[102, 109] disorganized sarcomere structure,[110] decreased size,[111] differences in cell morphology,[110] and electrophysiological properties[73, 102, 112] that

mimic fetal CM.[41, 99, 109] Problems arise in using immature CM for transplantation due to potential arrhythmogenic risk caused by immature CM having a higher propensity for automacity (spontaneous beating)[113] and their inability to function properly and handle an *in vivo* cardiac load.[88, 114, 115] Furthermore, the immature CM's lack expression of specific ion channels will negatively impact predictability of how adult CMs will respond during drug screening and cardiotoxicity studies. Because the use of immature CMs presents safety and efficacy concerns for transplantation and drug screening, it is crucial to identify strategies to generate mature CMs that show characteristics of adult CM.

Many groups have been investigating the specific developmental cues responsible for CM maturation and found a multitude of stimuli that induce maturation *in vitro*. Specifically, mechanical stimulation such as cyclic stretching and micropatterning promote PSC-derived CM maturation.[96, 101, 116, 117] Other factors capable of inducing CM maturation include, the addition of triiodothyronine (T3 hormone),[118] long-tem culture,[119] forced expression of Kir2.1,[120] 3D culturing,[73, 121] cardiac ECM,[122] and signals from non-CM cells.[102, 123] Due to the large number of stimuli that influence CM maturation, we will focus our discussions on the impact from EC and cardiac ECM.

EC influence on CM maturation

Recent studies have provided evidence that EC provide cues to instruct embryo patterning, organ differentiation, and postnatal tissue remodeling independent of blood flow.[124] Currently, there is limited evidence that EC play a role in CM maturation, however it is worth investigating because EC play a significant role in maturation of the

cells of the liver and pancreas. It has been well established that EC promote islet cell maturation.[125, 126] For example, during the last stage of the differentiation of hESC into beta cells, EC can promote beta cell maturation by co-culturing the two cells types together.[127] In addition to beta cell maturation, growth factors produced by EC (such as IL-6 and hepatocyte growth factor) modulate development and maturation of hepatocytes.[128] Most importantly, there is evidence that CMs respond to proteins synthesized by EC. Sano and colleagues determined that IL-6 mediates cardiac hypertrophy in rodent CMs.[129] Of note, during ES differentiation into CMs EC regulate CM development through EphrinB2 and EphB4 signaling.[130] In addition, vascular endothelial growth factor stimulates EC to secrete IL-6,[39, 40] and therefore it is plausible this can be a mechanism to induce hypertrophy. Although hypertrophy can be considered part of postnatal development and for this reason is linked to maturation, it also plays a role in pathological responses. These data indicate that EC play a role in CM maturation.

Recently, Lee and colleagues found direct evidence that EC induce maturation of mouse and human ESC-derived CM. Specifically, EC co-cultured with hESC-derived CMs resulted in improved maturity and upregulation of the microRNAs, miR-125b-5p, miR-199a-5p, miR-221, and miR-222 (miR-combo).[123] The increase in the microRNAs promoted increased expression of CM maturation markers, more negative resting membrane potential, and improved sarcomere alignment and calcium handling. However, the researchers were unable to determine how the EC induced the upregulation of microRNAs within the CM. Therefore, future studies are needed to understand the exact mechanism of how EC promote CM maturation.

Cardiac ECM influence on CM maturation

There is evidence that cardiac ECMs play a role in CM development and since ECM plays a role in hepatocyte[47] and ovarian follicle maturation,[46] it is reasonable to predict that cardiac ECM will play a role in CM maturation. Dequach and colleagues found that during CM induction from hESC, CM maturation (increased cell number per cluster and expression of desmoplakin) was promoted when porcine cardiac ECM is used as a substrate.[131] During hESC induction into CM, native cardiac ECM has been shown to enhance CM development and maturation through increased striation patterning of cardiac troponin and connexin 43 expression.[132] ECM stiffness affects the maturation and differentiation of immature rat CMs demonstrated by changes in force contractions.[99] Lastly, integrins are known to play a role in CM development and maturation because integrins are the primary link between cells and the matrix.[133] Overall, the published studies indicate that cardiac ECM may play a role in CM maturation.

Structure and morphology

Many studies report that PSC-derived CM display an immature phenotype that closely resembles characteristics of fetal CM. As a result of hypertrophic growth, adult CMs are known to be 30-40 fold larger than original size[134] and are cylindrical in shape.[135] Previous studies have found that cyclic stretch of immature hESC-derived CM resulted in greater cell elongation compared to non-stretched CM.[116] Another hallmark characteristic of adult CMs is the presence of traverse tubules (t-tubules), which extend into the CM's interior to allow for more rapid access to extracellular calcium,[136] however, many groups demonstrate that PSC-derived CMs have very few or lack t-tubules.[137]

Several months after birth, human CM stop proliferating and become multinucleated,[134] and CM exhibit polyploidy.[138] Furthermore, fetal CM exhibit unorganized and shorter sarcomeres (1.6 μm) compared to sarcomeres from adult CMs that are 2.2 μm in length and highly organized.[119] Zhang and colleagues found that 3D culturing, compared to 2D, improved hESC-derived CM maturation, as demonstrated by longer sarcomeres (2.09 vs. 1.77 μm).[121] Therefore previous studies indicate multiple stimuli that can generate CM with structure and morphology more closely resembling adult CM rather than fetal CM.

Gene and protein expression

A mature CM will exhibit adult CM gene and protein expression of ion channels, calcium handling proteins and sarcomeric proteins.[109, 139] The different developmental stages of CMs have differential expression of ion channel proteins, thereby modulating changes in ion channel activity.[140] Some examples of proteins that are highly expressed in adult CM, and therefore indicate CM maturation, are sarcoplasmic reticulum calcium ATPase 2a (SERCA2a), calsequestrin (CSQ), Triadin, Junctin, $NaV_{1.5}$, and $Kir_{2.2}$.[102, 109] There is evidence suggesting that non-CM may influence hESC-derived CMs electrophysiology properties as well as expression of specific intracellular calcium-handling proteins and ion channels thereby indicating CM maturation.[102] Furthermore, Zhang and colleagues demonstrated that T3 hormone is capable of increasing expression of cTnT, αMHC, CASQ2 and SERCA2 within PSC-derived CMs,[121] further demonstrating that external stimuli are capable of inducing increased expression of ion channels and calcium handling proteins similar to adult CM.

Electrophysiology

Electrophysiology signaling is one of the most well studied characteristics of CM. Many ion channels work in a fine-tuned manner to generate the cardiac action potential and promote the calcium signaling cascade that leads to contraction. Common tools and methods used to monitor the electrophysiological signaling are patch clamp, microelectrode arrays, and voltage sensitive dyes.[141] Each individual ion channel (potassium, sodium, calcium) contributes to specific currents, and therefore the immature CM's reduced or lack of expression of specific channels will greatly affect the action potential.[142, 143] Specifically, adult CMs display hyperpolarized maximum diastolic potentials, increased action potential amplitudes, and faster upstroke velocities compared to immature CM.[119] Kim and colleagues discovered that co-culturing non-CM with hESC-derived CMs promotes increased V_{max} and action potential amplitude to levels closer to adult CM.[102]

Calcium signaling

Calcium signaling within CMs is critical to their function by enabling electrical and mechanical coupling thereby triggering the CM's contraction. Fetal CM and adult CM vary greatly in their calcium signaling. Adult CM utilize calcium-induced calcium signaling for contraction, a process where the cardiac action potential activates the CaV1.2 channel to release calcium into the cell, and the calcium influx triggers the release of calcium from the sarcoplasmic reticulum (SR), which initiates the

contraction.[144] Conversely, fetal CMs depend on Na^{+}-Ca^{2+} exchange (NCX) to release calcium into the cell for contraction because immature CMs lack a functional SR.[145]

Calcium signaling within the CM can be visualized using calcium-sensitive fluorescent dyes (Fluo-4, etc)[146] or engineered genetic reporters such as GCaMP6f.[147] In addition to our data, several reports have also demonstrated that immature PSC-derived CM display decreased calcium transient amplitude, max upslope, and max downslope in addition to increase in time to peak and time to decay.[118, 119] However stimuli such as cyclic stretching or long term culturing can increase CM calcium signaling and kinetics and thereby shorten the calcium cycle duration characteristic of adult CM.[116, 119] Since improper calcium signaling can lead to potential arrhythmic events, it is important to ensure PSC-derived CM calcium signaling resembles that of adult CM.

Contractile Force

Within the heart, the CM function is to contract with appropriate forces in order to pump blood effectively throughout the body. However, contractile force is one of the least studied parameters of immature and mature CM. A majority of the studies that investigate the contractile forces focus on neonatal rat CM and have determined that they generate forces between 0.1-54 mN/mm^2.[136, 148] Moreover, the contractile force of human hESC-derived CM averaged to 5.7 ± 1.1 nN/cell and is dependent on hESC-CM purity.[121] More recently, Yang and colleagues determined human PSC-derived CMs within a collagen construct generated 0.08 $mN/mm^{2,}$ which is approximately 550 fold less than the adult human myocardium.[118, 149] Therefore, future studies should focus on CM contractile force

and find strategies to induce PSC-derived CMs to produce forces similar to the adult myocardium.

Drug responses

Due to the large differences between mature and immature CM as described above, it is not surprising that immature CMs respond to drugs differently than mature CMs. It is well established that beta-adrenergic signaling is responsible for positive isotropic (contractility force) and chronotropic (beat rate) effects of CMs.[150] The Hescheler group determined that the beta-adrenergic agonist isoproterenol induced a dose-dependent increase in the frequency of spontaneous beating, but did not significantly alter the contractile force.[150] The lack of increased isotropy highlights how immature CMs do not respond to drugs in the same manner as adult CM. Another study investigated the affect of 3D culturing of hESC-CM within fibrin patches and discovered the hESC-CMs within the patch became more mature. Next, they evaluated the patches response to isoproterenol and found that the CMs within the 3D patches exhibited significant positive inotropy (1.7-fold force increase from immature CM),[121] thereby demonstrating the differential response between immature and mature CMs. Many drugs used to treat a variety of diseases ranging from blood pressure to depression target sodium, potassium, and calcium channels within the heart and often lead to life-threatening arrhythmias.[151, 152] Therefore, performing drug screening on immature CMs will not accurately predict the outcome that would normally happen in the adult heart because the immature CMs have reduced or lack expression of many ion channels that are targeted by the drugs.[73, 109]

For this reason, it is imperative to develop mature CMs that can be used to accurately predict outcomes of drug screenings and cardiotoxicity studies.

SUMMARY

Currently, it is not possible to regenerate damaged myocardium in CVD patients. Fortunately, the development of iPSC provides great promise in generating new therapies for CVD because of their ability to differentiate into CMs, provide an unlimited cell source, and generate autologous cell lines for transplantation. Furthermore, PSC-derived CMs can be used for many applications to study and treat CVD, including disease modeling, drug screening, tissue engineering, and cell-based therapy. Unfortunately, CMs generated from PSC display an immature phenotype resembling fetal CMs rather than adult CM and the use of immature CM leads to safety concerns when used for transplantation and the inability to accurately predict outcomes of drug screening and cardiotoxicity studies. Therefore, many groups, including ourselves, are investigating potential strategies to promote CM maturation, through manipulation of their local microenvironment. Ultimately, we strive to develop robust protocols that generate mature PSC-derived CMs that can be safely and effectively used to treat CVD.

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CHAPTER 2

Cardiac Extracellular Matrix Enhances Cardiomyocyte Differentiation of Human Induced Pluripotent Stem Cells (iPSC)

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ABSTRACT

Cellular therapies using cardiomyocytes (CM) differentiated from pluripotent stem cells have great promise in treating heart disease. Numerous protocols exist for differentiating stem cells into CMs, mostly using soluble factors such as inhibitors, recombinant proteins or conditioned medium. However, the majority of these cardiac differentiation protocols use the substrate coating Matrigel – an extracellular matrix (ECM) derived from mouse Engelbreth-Holm-Swarm tumors, consisting primarily of laminin, collagen IV, and entactin. Importantly, the ECM proteins used as substrate coatings have not been well studied in the context of cardiac differentiation in monolayers and therefore we tested the hypothesis that naturally-derived cardiac ECM would be a better substrate for cardiac differentiation than tumor ECM. We generated cardiac ECM by detergent-mediated decellularization of bovine heart tissue. We measured the effects of the substrate coatings by comparing cardiac ECM to Matrigel in their ability to affect CM differentiation of human iPSC. We find that over a 21-day differentiation period, relative to Matrigel, cardiac ECM significantly increases the intensity of the expression of Gata4 (4 fold), Myocyte-specific enhancer factor 2C (23-fold), Insulin gene enhancer protein 1 (11-fold),

Tbx20 (5-fold), and cTNT (22-fold). Overall, the gene expression signature suggests that cardiac ECM improves CM differentiation compared to Matrigel. These studies suggest a protocol for improving cardiac differentiation that may yield CM with greater functionality.

INTRODUCTION

Heart disease is the leading cause of death in the US. Currently, the only types of therapies to treat cardiovascular disease are pharmaceutical drugs, replacement of valves, stents, surgery and ultimately a heart transplantation. However, none of these treatments are able to replace the damaged myocardium in order to improve the heart's overall function. There has been great interest in using pluripotent stem cells (PSC) to treat the damaged muscle because of the cells' ability to differentiate into cardiomyocytes (CM), provide unlimited cell sources, and create autologous cells for transplantation. There have been many studies using animal models from rodents to primates that have used human embryonic stem cell (hESC) and induced pluripotent stem cell (iPSC) derived CMs to treat myocardial infarction resulting in beneficial therapeutic effects.[1-8] Therefore the use of pluripotent stem cells (PSC)-derived CMs may provide great benefit in developing new therapies to treat heart disease.

Due to the great potential of PSC-derived CMs in treating heart disease, many protocols have been generated for *in vitro* CM differentiation of PSC. These protocols include derivation from embryoid body differentiation, co-culturing with END-2 cells,[9] and most commonly, various combinations of recombinant proteins and small molecules.[10-13] The majority of these CM differentiation protocols use Matrigel as the substrate coating; Matrigel is the basement membrane matrix extracted from Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in ECM proteins.[14] Additionally, other ECM proteins, such as gelatin, collagen, fibronectin, are also used as substrates for CM differentiations.[15-17]

The variations of ECM proteins used as substrate coating for cardiac differentiation may greatly impact the development of CM.

Many studies have demonstrated that ECM plays an important role in cellular differentiation and maturation.[18-20] Therefore the substrate coating used during *in vitro* differentiation can greatly impact the cell's development, suggesting the importance of using the appropriate type of ECM. Recently, there has been interest in using coating substrates that mimic the biochemical composition of native ECM such as ECM derived from decellularized tissues.[21-23] In particular, the differentiation of CM from ESC was enhanced when seeded onto cardiac ECM compared to liver ECM, thereby suggesting that the linage commitment and differentiation is dependent on the tissue-specific origin of the ECM.[24] During the CM differentiation of hESC, the embryoid bodies were embedded into a cardiac ECM hydrogel resulting in improved differentiation compared to collagen hydrogel.[25] However, there has not been any study that investigates the direct comparison of traditional substrates, such as Matrigel, and the newly developed tissue specific ECM on iPSC differentiation into CMs. In this study, the effects of the coating substrates Matrigel and cardiac ECM were compared during the CM differentiation from iPSC, and we determined that cardiac ECM enhances CM development.

RESULTS

Characterization of decellularized bovine cardiac tissue and Matrigel

In order to determine if native cardiac ECM affects CM differentiation of iPSC, we decellularized bovine adult heart tissue with the detergents sodium dodecyl sulfate (SDS)

and TritonX-100 to isolate the cardiac ECM. The removal of cells during the decellularization process was confirmed by the absence of nuclei in the H&E stained tissues (Fig. 2.1A,B). We used nanoLC MS/MS to identify the matrix protein composition of cardiac ECM and Matrigel and found that the cardiac ECM is primarily composed of collagen I, collagen III, collagen IV, fibrillin-1, fibrinogen, elastin, fibronectin, periostin and other matrix proteins (Table 2.1). Conversely, Matrigel was primarily composed of laminin, entactin, proteoglycan, fibrinogen, and collagen IV (Table 2.1). The ECM composition of cardiac ECM and Matrigel varies greatly in protein composition and these differences may affect CM development. In addition to studying the composition of cardiac ECM, we also investigated if there were any differences in CM differentiation on cardiac ECM when compared to Matrigel, the most commonly used substrate coating for CM differentiation.

Cardiac ECM enhances gene expression during CM differentiation

To study the effect of cardiac ECM on CM development, iPSC were differentiated into CM using the recombinant proteins, activin A and bone morphogenetic protein 4 (BMP4), and seeded onto cardiac ECM or Matrigel coated plates. After 21 days of differentiation on ECM from bovine or porcine heart or on Matrigel the iPSC-derived CMs expressed the sarcomeric protein, alpha-actinin, and ~10% of the cells were positive for cardiac troponin T (cTNT) (Fig. 2.2A,B). In order to determine the effect of cardiac ECM, compared to Matrigel, on CM development, gene expression was measured by qPCR and evaluated every 7 days during the 21 day differentiation. The CM differentiation on cardiac ECM resulted in significant increased expression of Gata4,

MEF2C, Isl1, cTNT, and Tbx20 (10-300 fold) compared to cells differentiated on Matrigel (Fig. 2.3). As expected, the expression of the developmental genes, Gata4 and MEF2C decreases after day 14 because cardiac fate has already been determined. Expression of the adult cardiac genes, cTNT and Tbx20, continues to increase with time as expected. We conclude that cardiac ECM, in contrast to Matrigel, greatly enhances the gene expression of cTNT and transcription factors involved with both cardiac development and function during CM differentiation.

DISCUSSION

The *de novo* generation of adult cardiomyocytes is critical if we are to develop new cellular treatments for cardiovascular disease. Since there is no known treatment that can regenerate the damaged myocardium, the use of iPSC to derive CMs has become ideal to replace the diseased muscle because they are an unlimited cell source and have the ability to create autologous tissue-specific cells for transplantation. Many studies have demonstrated how the transplantation of hESC and iPSC-derived CMs into an infarcted heart resulted in many beneficial therapeutic outcomes including reduced scar formation, improved ejection fraction, and increased left ventricle wall thickening.[1-8] Because of the clinical utility of using PSC-derived CM for transplantation into diseased hearts, many protocols have been created to generate CMs from PSC. [9-13] The majority of these CM differentiation protocols utilize Matrigel, mouse tumor ECM, as the substrate coating. Since there is evidence that tissue-specific ECM greatly affects CM differentiation,[17, 22-24] we investigated cardiac ECM—extracted from decellularized heart tissue—and Matrigel's impact on CM development and discovered that cardiac ECM enhances the differentiation of iPSC-derived CM compared to Matrigel.

The protein composition of Matrigel and cardiac ECM was assessed by nano-LC MS/MS and we determined that their compositions are very different. The proteins unique for Matrigel are laminin, proteoglycans, and entactin, and those unique for bovine cardiac ECM are collagen I, collagen III, fibrillin-1, fibronectin, elastin, fibulin-1/5, lumican and periostin. The only similarities in the matrix composition are fibrinogen and collagen IV. Periostin is known to induce proliferation of differentiated rat neonatal CMs,[26] and

therefore the presence of periostin within a substrate scaffold may increase the ability for our differentiated CMs to re-enter cell cycle. However our data demonstrate that we have equal numbers of cTNT positive cells after 21 day differentiation when seeded on to both cardiac ECM and Matrigel, thereby demonstrating that 21 days may be too early to be able to see a difference in CM proliferation, since the CMs are still developing, and the benefits of periostin within the substrate scaffold.

The cardiac ECM contains significantly more ECM proteins than Matrigel, demonstrating the increased complexity of native cardiac ECM. Besides the increased number of ECM proteins, the presence of specific unique proteins may be providing the correct microenvironment that is needed to support CM development. Specifically, fibronectin promotes hESC differentiation into CMs[17] and therefore the lack of fibronectin in the Matrigel may be detrimental during CM differentiation. This suggests that using mouse tumor ECM may, not surprisingly, be inappropriate to recapitulate the cardiac environment, and therefore Matrigel is not able to induce the enhanced gene expression of key transcription factors during CM differentiation.

The tissue-specific ECM from the heart has a positive influence in CM development. Duan and colleagues determined that cardiac ECM, compared to collagen I, induced increased expression of cTNT similar to our data. In addition, when cardiac progenitors are seeded onto porcine cardiac ECM, compared to collagen I, ECM results in increased expression of Gata4, Nkx2.5, tnnC and alpha-myosin heavy chain,[22] which is again similar to the increases in gene expression we found during CM differentiation on cardiac

ECM. In addition, hESC-CMs on cardiac ECM, compared to gelatin, promoted increased expression of desmoplakin, suggesting increased CM maturation.[23] These findings support our data that cardiac ECM enhances gene expression of cardiac markers and that the enhanced expression suggests the iPSC-derived CMs are more developed and functional. However, further studies are needed to support this hypothesis.

Many CM differentiation protocols have been created because of the beneficial impact PSC-derived CMs have on creating new therapies to treat heart disease. These therapies include cellular transplantation, tissue engineering, and discovering new pharmaceutical drugs. In addition to developing new therapies, the use of PSC-derived CMs allows scientists the opportunity to study CM development as well as being able to model diseases *in vitro*. The majority of these protocols utilize Matrigel as the coating substrate for CM differentiation. However there has not been a study investigating if there are other ECMs that may be more appropriate to use instead of the mouse tumor ECM, Matrigel. Our research suggests that using cardiac ECM, which better mimics the *in vivo* cardiac microenvironment, as the substrate for iPSC differentiation into CMs led to enhanced expression of cardiac genes when compared to Matrigel. By using ECM native to cardiac tissues, we have created a better platform for CM differentiation and now provide potentially more functional CMs that can be used to develop new therapies for cardiovascular disease.

ACKNOWLEDGEMENTS

I would like to thank Dr. Charles Murry for extensive training in cardiac differentiation of pluripotent stem cells and Dr. Leslie Lock for contributing the CiF1.4 iPSC line. Lastly, I would like to thank the funding agencies National Science Foundation and California Institute for Regenerative Medicine.

METHODS AND MATERIALS

iPSC maintenance

All experiments were done with approval from UCI's Human Stem Cell Research Oversight Committee. The human CiF1.4 iPSCs, a gift from Dr. Leslie Lock (UC Irvine), were maintained on irradiated CF-1 mouse embryonic fibroblasts (MEF) and fed daily with KO DMEM/F12 (LifeTechnologies), 20% Knock Out Serum Replacement (LifeTechnologies), Non-Essential Amino Acids, Glutamax, β-mercaptoethanol, and 10 ng/ml bFGF (Pepprotech). Before cardiac differentiation, iPSCs were subcultured for at least 3 passages on Matrigel-coated plates and fed MEF conditioned medium.

CM differentiation

The iPSCs were seeded 2 days before cardiac induction at 215,000 cells/cm^2 onto Matrigel Growth Factor Reduced (BD) or cardiac ECM-coated plates. The tissue culture plates were coated with 0.166 mg/ml ECM for 2 hours at room temperature. The directed differentiation protocol created by Laflamme and colleagues was used for the monolayer cardiac differentiation.[10] On Day 0 cells were fed 100 ng/ml Activin A in RPMI/B27 (minus insulin) (Life Technologies), and 18 hours later exchanged with 10 ng/ml BMP4 (PepproTech) in RPMI/B27 (minus insulin). Fresh medium was replaced on Day 5 and fed with RPMI/B27 (plus insulin) every 2-3 days.

Cardiac tissue decellularization and ECM generation

Bovine and porcine hearts were purchased from Sierra Medical. Prior to decellularization the tissue was processed by removing all excess fat, rinsed with PBS with heparin to

remove blood clots, and stored in -80°C for at least 16 hours. The tissue was cut into $3mm^3$ sized pieces and subjected to continuous stirring at 350rpm in the following solutions: ddH_20 for 30 minutes, 2x PBS for 15 minutes, 1% SDS for 72 hours, 1% Triton for 30 minutes, and followed by 7 washes in ddH_20 for 30 minutes. Decellularized tissues were lypholized and then milled with a cheese grater (amazon.com) into powder. The ECM powder was digested with 1 mg/ml pepsin diluted in 0.01M HCl and continuously stirred for 48-72 hours or until fully digested. Approximately 10mg of cardiac ECM is added to 1mg of pepsin and batches were generated using 100 mg of cardiac ECM.

Mass Spectrometry

Solubilized cardiac ECM and Matrigel samples were analyzed using nano-LC MS/MS. Protein levels were measured using BCA assay. Equivalent masses were treated with iodacetamide and trypsinized as described.[27-29] The peptides were desalted with stacked C18/SCX tips and subjected to nanoLC- MS/MS using an LTQ Orbitrap Velos Pro mass analyzer (Thermo Fisher Scientific) connected to a nanoLC-Easy1000. Fourier transform mass spectrometry precursor spectra were acquired at 60,000 resolution and subjected to rapid CID fragmentation and ion trap analysis.

Resulting data was calculated using Mascot Distiller 2.5.0 (Matrix Science) and peaklists were searched using SwissProt with *Bos Tuarus* and *Mus musculus.* False discovery rates were thresholded at 5% or below. Mascot protein score were compared using in-house software.

Quantitative PCR

Total RNA was harvested from differentiated iPSC using Trizol (LifeTechnologies) according to the manufacture's recommended protocol. Three micrograms of RNA was used to reverse transcribe cDNA using iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed using SYBR green chemistry and analyzed on iQ5 iCycler (Bio-Rad). Primers were synthesized by Integrated DNA Technologies, and their sequences can be found in supplemental methods (Table 2.S1). All mRNA levels were normalized to 18S expression.

Flow cytometry

Cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes. Samples were incubated with 0.75% saponin and 1:100 anti-cTNT antibody (Sigma) or isotype control mouse IgG followed by goat anti-mouse FITC secondary antibody. Samples were analyzed with BD FACSCalibur flow cytometer and data were assessed with FlowJo software.

Immunocytochemistry

Cells were fixed with 4% PFA for 30 mins. Samples were permeabilized with 0.05% TritonX-100 in PBS and incubated in blocking buffer (4% bovine serum albium in PBS and 0.01% TritonX-100). The cells were then incubated in blocking buffer with antibodies against alpha-actinin and myosin light chain 2V and then goat anti-mouse FITC and goat anti-rabbit Alexa Fluor 568 secondary antibodies. Samples were stained

with DAPI for visualization of nuclei and imaged with confocal microscope (Olympus FV1000).

FIGURES

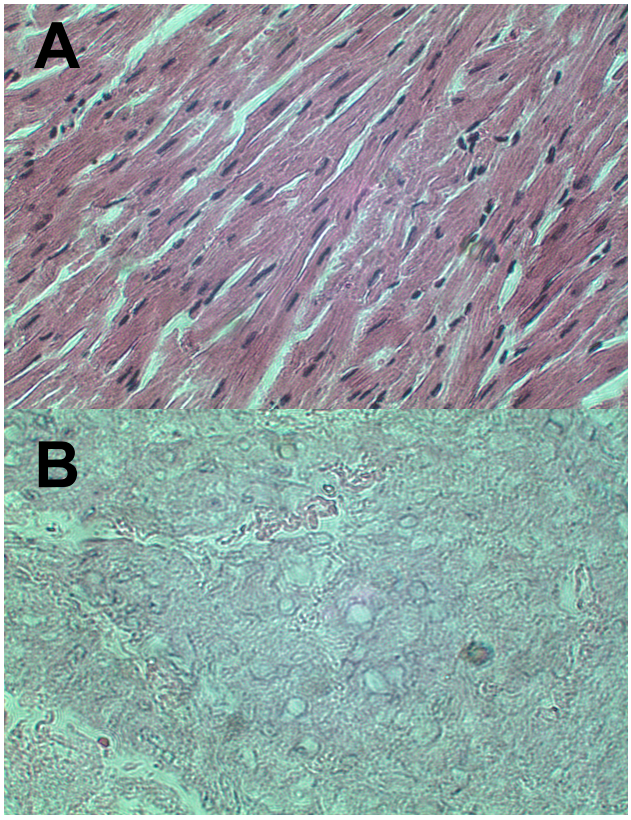

Figure 2.1. Characterization of bovine cardiac ECM. H&E stain of normal (A) and decellularized (B).

Cardiac ECM	Matrigel
Collagen I	Collagen IV
Collagen III	Fibrinogen
Collagen IV	Proteoglycan
Elastin	Laminin
Fibrinogen	Nidogen1/Entactin
Fibrillin-1	
Fibronectin	
Fibulin-1	
Fibulin-5	
Periostin	
Lumincan	

Table 2.1. Key matrix proteins of bovine cardiac ECM and Matrigel identified using nano-LC MS/MS.

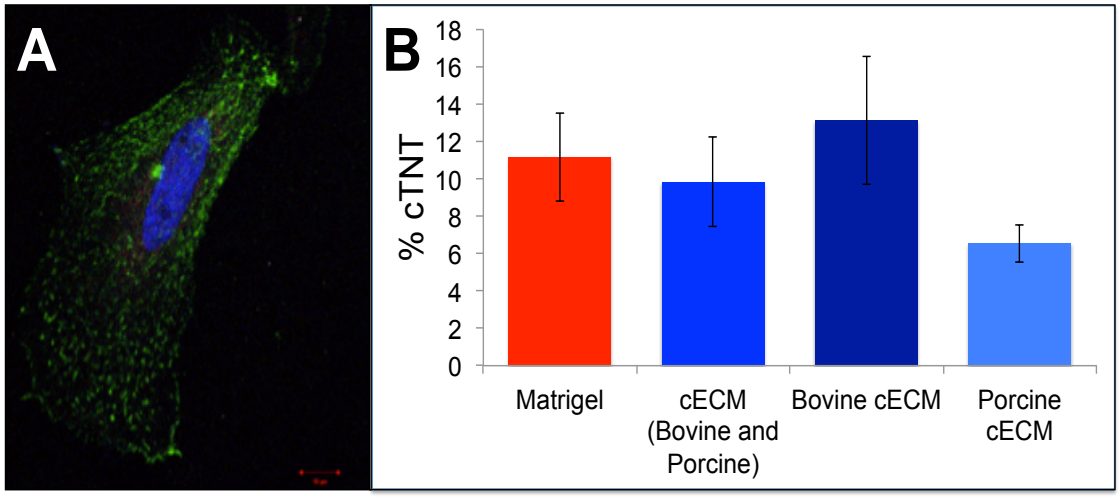

Figure 2.2. **The number of cTNT (+) cells is not altered by cECM.** A. Immunofluorescence image of iPSC-derived CM: nucleus (Dapi, blue), myosin light chain 2V (red) and α-actinin (green). B. Flow cytometry analysis for cTNT (+) cells comparing substrates of Matrigel and cardiac ECM (cECM).

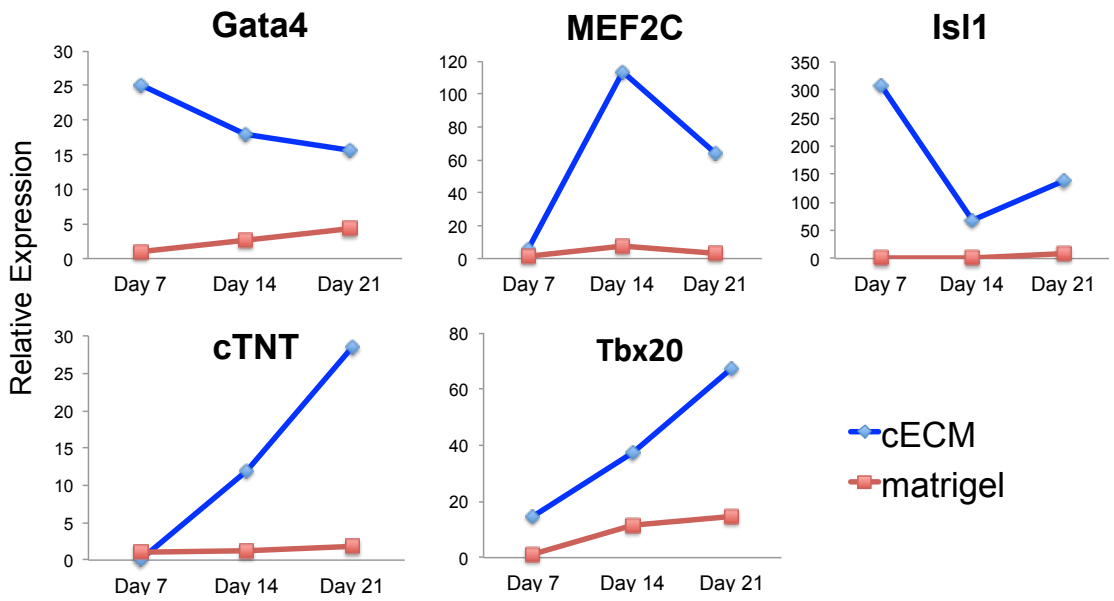

Figure 2.3. Cardiac ECM promotes increased expression of CM development and structural genes. Overall expression level of Gata4, MEF2C, ISL1, cTNT and TBX20 are increased by cardiac ECM (cECM). The iPSCs were differentiated on Matrigel and cECM. The RNA was harvested at Day 7, 14, 21 and the gene expression was measured using qRT-PCR.

Gene Name	Sequences 5'-> 3'
18S FWD	CCC CGG CCG TCC CTC TTA
18S REV	CGC CCC CTC GAT GCT CTT AG
cTNT FWD	GAC CCG CGG GAA GGC TAA AG
cTNT REV	GTG GGG GCA GGC AGG AGT G
ISL1 FWD	CGC GTG CGG ACT GTG CTG AAC
ISL1 REV	TTG GGC TGC TGC TGC TGG AGT
GATA4 FWD	CGG GGA CAT AAT CAC TGC GTA ATC
GATA4 REV	GGG CCT CCT TCT TTG CTA TCC TC
MEF2C FWD	TAACTTCTTTTCACTGTTGTGCTCCTT
MEF2C REV	GCC GCT TTT GGC AAA TGT T
TBX20 FWD	CCC TGG CCC CGC TGT GA
TBX20 REV	CGT TGG CCC GAG AGG AGA GTT

Supplemental Table 2.1. Real time qPCR primer sequences.

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CHAPTER 3

Three-dimensional adult cardiac extracellular matrix promotes maturation of human induced pluripotent stem cell-derived cardiomyocytes

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ABSTRACT

Pluripotent stem cell-derived cardiomyocytes (CM) have great potential in the development of new therapies for cardiovascular disease. In particular, human induced pluripotent stem cells (iPSC) may prove especially advantageous due to their pluripotency, their self-renewal potential, and their ability to create patient-specific cell lines. Unfortunately, pluripotent stem cell-derived CM are immature, with characteristics more closely resembling fetal CM than adult CM, and this immaturity has limited their use in drug screening and cell-based therapies. Extracellular matrix (ECM) influences cellular behavior and maturation, and so we tested the hypothesis that native cardiac ECM might enhance the maturation of iPSC-derived CMs *in vitro*. We demonstrate that compared to fetal cardiac ECM, matrix from adult heart tissue drives iPSC-derived CM maturation, as indicated by enhanced expression of Junctin. Further, maturation of iPSC-derived CM was also enhanced when cells were seeded into a three-dimensional (3D)

cardiac ECM scaffold, compared to two-dimensional (2D) culture. 3D cardiac ECM promoted increased expression of calcium handling genes Junctin, CaV1.2, NCX1, and CASQ2. Consistent with this, we find that iPSC-derived CM in 3D adult cardiac ECM show increased calcium signaling (amplitude) and kinetics (maximum upstroke and downstroke), compared to cells in 2D. Cells in 3D culture were also more responsive to caffeine, likely reflecting an increased availability of calcium in the sarcoplasmic reticulum. Taken together, these studies provide novel strategies to mature iPSC-derived CMs that may have applications in drug screening and transplantation therapies to treat heart disease.

Key Words: 2D cell culture, 3D cell culture, ECM, Heart (tissue engineering applications), iPSC

INTRODUCTION

Cardiovascular disease is the number one cause of death in the US and most of the developed world. It most commonly manifests as a myocardial infarction, an ischemic event in the heart that results in the death of millions to billions of cardiomyocytes (CM). The loss of CMs can impair heart function, and repair is limited due to the extremely low proliferation rate of endogenous CMs. Compounding the insult, damaged tissue undergoes fibrosis which increases the risk of arrhythmia and subsequent heart failure. There is no available drug treatment that can regenerate the lost myocardium, resulting in a critical need to find new treatment strategies.

Recent studies have focused on the potential regenerative capacity of exogenous CMs as a novel treatment following myocardial infarction, and in particular, on the derivation of these from induced pluripotent stem cells (iPSCs) generated from the patient's own cells. Several studies report transplantation of human embryonic stem cell (hESC) and iPSC-derived CMs into ischemic hearts, largely in mouse and rat, where improvements in heart function have been observed. [1-8]

A limitation to this approach is that pluripotent stem cell-derived CMs generated through current methods are immature, displaying characteristics of fetal CM. Specifically, the cells show disorganized sarcomere structures, weak force contraction, automaticity (spontaneous beating), improper calcium handling and electrophysiological signaling, low expression of critical cardiac proteins, and altered responses to drugs when compared to mature CMs.[9] Not surprisingly then, there is growing evidence that mature CMs

outperform immature CM for tissue replacement strategies and screening of novel pharmacologic compounds – they produce stronger force contraction, and show appropriate calcium handling and electrophysiology.[10-12] In addition, the loss of automaticity seen in mature CM (which require pacemaker cells to trigger beating) will greatly improve safety by eliminating potential arrhythmic events. Lastly, the use of mature CMs for drug screening has clear advantages over the use of immature cells for predicting efficacy and potential side effects.[13] It is clear, therefore, that strategies are needed that promote more efficient generation of mature CM from iPSC.

Several methods have been developed to induce maturation of pluripotent stem cell derived-CMs, including exposure to triiodothyronine (T3 thyroid hormone),[11] exposure to non-CM cells,[14] mechanical stress,[15] or microRNA,[16] as well as overexpression of potassium channels[17] and long-term culture.[10] In addition, there is also evidence that extracellular matrix (ECM) can affect both cell and organ maturation.[18-25] Importantly, 2D culturing on porcine cardiac ECM affects rat and human CM development and maturation,[26-28] and cardiac ECM can have therapeutic effects when injected into the heart after a myocardial infarction.[29] Studies have shown that fetal and adult cardiac ECM have different effects on rat neonatal CM proliferation,[30] however, whether this is true for human iPSC-derived CM is not known. It is also unclear how a 2D versus a 3D growth environment might influence CM maturation, or whether matrix from fetal versus adult heart might make a difference. Here we compare the effects of 2D versus 3D, and fetal versus adult matrix on the maturation of iPSC-derived CMs, and find that both source and geometry of the matrix are important.

RESULTS

Characterization of extracellular matrix from decellularized adult and fetal bovine cardiac tissue

To investigate the potential differences between fetal and adult cardiac ECM and their respective contributions to human iPSC-derived CM maturation, we isolated cardiac ECM through sodium dodecyl sulfate (SDS)- and Triton-X100 detergent-mediated decellularization of fetal and adult bovine heart muscle. We found that similar polymerization characteristics could be obtained using 1% SDS for the adult tissue and 0.5% SDS for the fetal tissue (Table S1). Importantly, the fetal cardiac ECM decellularized with either 0.5% or 1% SDS induced a similar gene expression profile compared to adult cardiac ECM when used in 2D assays (Fig. S1). H&E staining confirmed the removal of cells during the decellularization process (Fig. 1A-D).

The protein compositions of fetal and adult cardiac ECM were characterized by nano-LC MS/MS. At the score thresholds employed (Table S2 legend), the prominent proteins were fibrinogen, collagen, periostin, fibrillin, fibulin, fibronectin and other matrix proteins (Table S2). The fetal cardiac ECM samples contained two unique proteins not found in the adult samples, mimecan and versican (Table S2). To examine the structure and distribution of collagen and elastin within the ECM, we used second harmonic generation (SHG) to detect collagen fibers and autofluorescence to detect elastin fibers. The elastin fibers of the fetal ECM, prior to pepsin digestion, were more evenly distributed, while there were more defined, organized bundles in the adult ECM (Fig. 1E,F). In contrast to fetal ECM, the decellularized adult cardiac ECM displayed a higher

collagen fiber signal intensity and larger bundles evident by increased fiber width and number (Fig. 1G-J). Hydrogels were formed with the adult and fetal cardiac ECMs, and storage modulus (G') and loss modulus (G'') were measured by parallel plate rheology. The adult cardiac ECM (67.5 ± 12.6 Pa) was ~10-fold stiffer than fetal cardiac ECM (7.2 ± 3.2 Pa) (Table 1, Fig. S2)).

3D adult cardiac ECM promotes expression of CM maturation genes

In order to determine if cardiac ECM affects iPSC-derived CM maturation we examined gene expression (by qPCR) in cells cultured in 2D and 3D fetal and adult cardiac ECM. After 7 days in culture, iPSC-derived CMs seeded into 3D cardiac ECM (from fetal or adult tissue) expressed higher levels of numerous maturation-related genes compared to cells in 2D gels. These included genes related to calcium handling, such as Junctin (JCN), L-type voltage-dependent calcium channel (CACNA1C/CaV1.2), calsequestrin 2 (CASQ2), and sodium-calcium exchanger 1 (NCX1), which were augmented between 2 and 120-fold (Fig. 2A,B). We also saw similar increases in expression of several other genes related to cardiac maturation, including Triadin (88-fold; n=2), sarcoplasmic reticulum Ca2+ ATPase (SERCA2a) (30-fold; n=1), and potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) (7-fold; n=1) in 3D versus 2D culture (data not shown). In addition, we also noted strong induction of these same genes, such as JCN, when comparing adult to fetal matrix, with the augmentation being particularly marked in 3D cultures (Fig. 2C,D). We next examined the expression of key structural and calcium-handling genes at the protein level. After 21 days of cardiac induction, 50-90% of differentiated cells expressed cardiac troponin T

(cTNT), which is a marker of CM differentiation (Fig. 3A). Confirming our qPCR data, we found a strong induction of CaV1.2 and MYL2 protein in iPSC-derived CMs cultured in 3D adult cardiac ECM, compared to 2D (Fig. 3B). In addition, the iPSC-derived CMs cultured on adult cardiac ECM exhibited increased expression of MYL2 compared to cells on fetal ECM (Fig. S3). Thus, both the source (fetal vs adult) and the geometry (2D vs 3D) of ECM strongly influences maturation of iPSC-derived CM.

Cardiac ECM geometry affects iPSC-derived CM calcium signaling

The genetically-encoded fluorescent calcium flux reporter GCaMP6[31] was utilized to assess the functional effects of ECM geometry on iPSC-derived CM calcium handling. We first compared single cells in 2D culture with single cells embedded in 3D gels. When cultured for 7 days in 3D cardiac ECM, the iPSC-derived CMs showed a significant decrease in beat rate compared to 2D culture (Fig. 4A). Interestingly, we also saw a decrease in calcium transient amplitude, maximum upslope, and maximum downslope, and a concomitant increase in time to 50% decay (Fig. 4B-E). These data demonstrate a clear effect of matrix geometry on CM calcium handling. *In vivo* CMs are in close association and the formation of syncitia promotes electrical coupling. To mimic this we cultured aggregates of iPSC-derived CM in 3D gels and also allowed these to settle onto 2D gels. Perhaps, not surprisingly, we found the calcium handling capabilities of the cells under these conditions to be quite different from those seen when the cells are not in close association. Representative traces of calcium influx are shown in Fig. 4F. Calcium transient amplitude, maximum upslope, and maximum downslope were all increased (they were decreased with single cells) and the time to 50% decay was

correspondingly decreased when cells were in 3D versus 2D (Fig. 4 G-J). Beat rate was not significantly altered by matrix geometry (data not shown). We also examined iPSC-derived CM behavior in a third model – cardiac spheroids embedded in 3D hydrogels composed of either adult porcine cardiac ECM, fibrin, or collagen I. Compared to either fibrin or collagen I, cells in native cardiac matrix showed a decreased beat rate (Fig. S4). These data are consistent with the promotion of CM maturation by native heart matrix.

3D adult cardiac ECM increases iPSC-derived CM response to drugs

To further assess the functionality of iPSC-derived CMs in 3D cardiac ECM, cells were exposed to isoproterenol and propranolol, drugs known to modulateβ-adrenergic signaling, and consequently, calcium handling. Consistent with the known behavior of adult CM, the iPSC-derived CMs increased their beat rate upon the addition of 1 μM isoproterenol (Fig. 5A), and this was decreased to baseline levels following the addition of 10 μM propranolol (Fig. 5A). Caffeine targets the ryanodine receptor (RyR), resulting in an increase in available calcium. Exposure of iPSC-derived CMs to caffeine resulted in a more rapid release of calcium in 3D culture than in 2D (Fig. 5B). The upslope was steeper and the time to maximum calcium concentration was quicker (Fig. 5C,D). Thus, iPSC-derived CMs are responsive to drugs with known cardiac effects and these effects are more pronounced in 3D versus 2D culture.

DISCUSSION

Myocardial infarction results in the death of millions to billions of CMs, and the lack of endogenous CM regeneration within the heart means that these cells are not replaced. A potential strategy to help these patients is to transplant CMs grown outside of the body, which will require establishment of procedures for the generation of large quantities of mature, or close to mature, CMs. iPSC-derived CMs provide an unlimited, autologous cell source that could be utilized for therapeutic transplantation, as well as drug screening, disease modeling, and cardiotoxicity studies. A drawback, however, is that current protocols generate iPSC-derived CMs that are immature and display characteristics that resemble fetal CMs, as demonstrated by a weak force contraction, improper electrophysiology, and reduced or absent expression of several critical cardiac proteins.[10, 39] Consequently, there is considerable interest in finding strategies that promote maturation of iPSC-derived CMs. In the current study, we investigated the properties and composition of native cardiac ECM from adult and fetal bovine hearts and tested their ability to promote iPSC-derived CM maturation in both 2D and 3D conformations. We found several notable differences that call into question the continued use of 2D cultures for the generation of iPSC-derived CM, notably that that the composition and stiffness of the 3D matrix conveys critical maturation signals to the developing CMs.

Multiple groups have investigated strategies to induce the maturation of pluripotent stem cell-derived CMs,[10-12, 14, 40-42] however, a majority of these studies investigated maturation of CMs in 2D. While 2D studies have certainly advanced our knowledge of

CM maturation, the 3D environment greatly impacts cell behavior and maturation,[23, 43] and so a full understanding of this process will require the use of a more physiologic (3D) environment.[44] Previous studies have shown that growing pluripotent stem cell-derived CMs in 3D benefits maturation induction,[40-42, 45] however, these studies used only single-protein ECM scaffolds, which do not recapitulate the complexity of the *in vivo* ECM. DeQuach and colleagues showed that porcine cardiac ECM affects CM maturation,[26] however, only modest changes were observed, likely due to the use of a 2D geometry.

In the current study, we found that iPSC-derived CMs grown in a complex 3D scaffold behave differently to those in single protein gels (collagen I or fibrin) and that cells in 3D differ considerably from those cultured in 2D. This was especially notable when we studied calcium handling and expression of maturation markers. Although a complete mechanistic understanding of how the geometry of the cultures impacts cell phenotype is not yet available, we hypothesize that biomechanical cues, likely mediated through integrins,[44, 46-48] are critical.

The idea of biomechanical cues driving cell maturation is supported by our finding that the fetal and adult cardiac ECM derived from decellularized heart tissue have a similar composition but differ in their mechanical properties and fiber architecture. The adult ECM has a higher density of collagen compared to the fetal ECM, which is similar to what has been shown in rat adult cardiac ECM.[30] In addition, the stiffness of the adult ECM hydrogel is 10-fold greater than the fetal hydrogel. These data are in agreement with findings that adult hearts are much stiffer than fetal hearts (10-50KPa versus

6KPa),[44] and previous studies have shown that substrate mechanics, particularly stiffness, influence CM growth and maturation.[44, 49] We think it is likely that the induction of maturation genes, such as JCN, by 3D culture and by the presence of adult versus fetal matrix, is largely the result of gel stiffness, which is a consequence of gel composition. It is also possible that specific matrix proteins engage with specific integrins to mediate maturation signals, however we do not have any data to directly support this hypothesis.

Our investigation into iPSC-derived CM maturation concentrated on examining calcium handling. We found that iPSC-derived CMs cultured in 3D cardiac ECM showed increased expression of calcium handling genes compared to 2D cultures, which is consistent with previous studies that primarily focused on the expression of ion channels and contractile machinery.[10, 11, 14] Our data showing an increase in calcium handling genes supports the hypothesis that a 3D geometry significantly influences CM maturation. *In vivo,* adult CMs express higher levels of calcium handling proteins than do fetal cells,[39] which again is consistent with the idea that expression of calcium-handling genes can be used as a measure of CM maturity. Interestingly, CMs of the adult heart express NCX1 at a lower level than CMs of the fetal heart,[39] however, we see increased NCX1 expression in our 3D cultured CMs, which is similar to results described by Rog-Zielinska et al 2015.[50] We speculate that the 3D cardiac ECM is only one component involved in promoting CM maturation and that additional maturation stimuli (hormones, growth factors, mechanical stretching, signals from non-CM) may also affect NCX1 expression. In addition, only a subset of "maturation" genes we studied reached

elevated levels indicative of maturation when cells were cultured in 3D gels, which is consistent with our CM maturation model being incomplete.

Consistent with changes in gene expression we found significant differences in the calcium-handling in CMs, as measured by the calcium flux reporter GCaMP6. Recent publications show that mature CMs display increased calcium signaling and kinetics.[10, 11] In line with this, we found that in aggregates of iPSC-derived CMs, calcium transient amplitude, maximum upslope, and maximum downslope were all increased and the time to 50% decay was correspondingly decreased when cells were in 3D versus 2D. These data are entirely consistent with our findings of increased expression of calcium handing genes/proteins such as JCN, CASQ2, CaV1.2, and SERCA2a in 3D, which would act to increase the amount of calcium within the sarcoplasmic reticulum (SR) and create CMs with a greater capacity to handle calcium during calcium-induced calcium release.

Interestingly, in contrast to this, when we seeded CMs as single cells into 3D gels (to allow a more direct comparison of matrix effects in 2D versus 3D) we found that calcium handling kinetics were different – calcium transient amplitude, maximum upslope, and maximum downslope were all decreased and the time to 50% decay was correspondingly increased when cells were in 3D versus 2D. This strongly implies that gene expression is not enough to determine calcium-handling characteristics. Rather, it is the association of cells and the coupling of these calcium-handling systems that determines the kinetics of calcium handling. Clearly, future studies in 3D should focus on aggregated, rather than single, cells.

In the current study, CMs are cultured for 7 days before measuring calcium signaling, similar to Yang and colleagues;[11] however others have cultured CMs for over 100 days before assaying for maturation.[10] Interestingly, our unpublished data demonstrate that iPSC-derived CMs cultured long term (comparing a 5 month to a 2 month culture) exhibit increased calcium signaling and kinetics suggesting that maturation continues over an extended period of time. Since endogenous CMs take several years before maturation is complete, it is not entirely surprising that maturation of CM in culture may take considerable time.

An important characteristic of CMs, and one that needs to be recapitulated by iPSC-derived CMs, is their response to drugs. We found that iPSC-derived CMs cultured in 3D were more responsive to various stimuli compared to cells cultured in 2D. The addition of the β-adrenergic agonist, isoproterenol, to the iPSC-derived CMs cultured in 3D induced an increased beat rate compared to cells in 2D culture. Furthermore, exposure to caffeine induced a steeper slope and a shorter time to maximum calcium concentration in the CMs cultured in the 3D cardiac ECM compared to 2D. Caffeine is known to increase RyR sensitivity by lowering the luminal calcium threshold for calcium release from the SR.[51] We hypothesize that the 3D culturing of iPSC-derived CMs increases the slope and decreases the time to maximum calcium signaling due to an increase in absolute calcium and/or faster release of calcium from the SR caused by the increased expression of JCN, CASQ2, and triadin, which are all proteins that handle calcium within the SR and modulate its release through the RyR.

In summary, we have used 3D adult cardiac ECM as a strategy to promote iPSC-derived CM maturation. We determined that the age (fetal vs. adult) of the cardiac ECM modulates its mechanical properties and fiber architecture, and these aspects affect CM gene and protein expression. After 7 days of culture within the 3D adult cardiac ECM hydrogels, the iPSC-derived CMs showed increased expression of many calcium-handling genes, consistent with the increase in these genes seen in adult versus fetal CMs. We also report that calcium signaling in the iPSC-derived CMs is enhanced in 3D compared to 2D, consistent with the changes in gene expression. The utilization of 3D adult cardiac ECM to culture pluripotent stem cell-derived CMs better recapitulates the *in vivo* environment than traditional 2D cultures or the use of non-native matrix, thereby promoting CM maturation. Our studies help advance strategies for maturing pluripotent stem cell-derived CMs, so that they can be safely and effectively used for therapeutic applications to treat heart disease.

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DISCLOSURE STATEMENT

No competing financial interests exist.

METHODS AND MATERIALS

iPSC maintenance and cardiac differentiation

All experiments were performed with approval from UC Irvine's Human Stem Cell Research Oversight Committee and used the human WTC-11 GCaMP iPSC line, which was a gift from Dr. Bruce Conklin (UCSF).[31] The WTC-11 iPSC line was derived from a healthy male volunteer with a normal electrocardiogram and no known family history of cardiac disease. The GCaMP iPSC line was generated using nuclease-mediated (TALEN) introduction of GCaMP6f, [32] and the resulting cell line reports calcium fluxes through GFP fluorescence. The iPSC were maintained on Growth Factor Reduced Matrigel (Corning, Salt Lake City, Utah) and fed daily with mTeSR1 medium (StemCell Technologies, Vancouver, Canada). To induce CM differentiation, iPSC were seeded as single cells into 12 well plates coated with 1 mg Matrigel Growth Factor Reduced (BD #354230), in the presence of 10 nM Y-27632 (Ascent, Cambridge, Massachusetts). Cardiac induction was initiated 2-4 days later (designated as Day 0), when cells were 75-90% confluent, by the addition of glycogen synthase kinase 3 inhibitor (CHIR99021, 6 μM, Tocris Inc, Bristol, United Kingdom) in RPMI/B27 (without insulin, Life Technologies, Carlsbad, California).[33] Twenty-four hours later (Day 1) the medium was changed to fresh RPMI/B27 (without insulin). On Day 3, Wnt inhibitor (IWP2, 5 μM, Tocris) was added in RMPI/B27 (without insulin), and at Day 5 cells were fed fresh

RMPI/B27 (without insulin). The cells were subsequently fed RMPI/B27 (with insulin) (Life Technologies) every 3 days starting at Day 7. Cells began to spontaneously beat by Day 12.

Cardiac tissue decellularization and ECM generation

Bovine adult and fetal hearts were purchased from Sierra Medical. Prior to decellularization the tissue (100-200 g) was processed by removing all excess fat, and stored at -80°C for at least 16 hours. Next the tissue was cut into 3 mm^3 sized pieces and subjected to continuous stirring at 330 RPM during incubation in the following solutions at room temperature: ddH_20 for 30 min, 2x PBS for 15 min, sodium dodecyl sulfate (SDS) (1% for adult tissue and 0.5% for fetal tissue, Bio-Rad) for 72 hours, and 1% Triton for 90 min. This was followed by 7 washes in ddH_20 for 30 min each, and 18 hours in PBS with Antibiotic-Antimycotic (Life Technologies) at 4°C.

Decellularized tissues were lyophilized and then milled using a cheese grater (Amazon.com, Seattle, Washington) to create a fine powder. The ECM powder was digested with 2 mg/ml pepsin (Sigma-Aldrich, St. Louis, Missouri) diluted in 0.01 M cell culture grade hydrochloric acid (HCl). Approximately 200 mg of cardiac ECM was added to 20 mg of pepsin and continuously stirred at 330 RPM at room temperature for 48-72 hours or until fully digested. The digested ECM was divided into 1 ml aliquots and stored at -80°C until needed.

Multiphoton Imaging and Fiber Analysis

Fibrillar collagen generates second-harmonic (SHG) signals when incident light interacts with its non-centrosymmetric structure, and SHG microscopy has been widely used to study collagen fibers *in vivo* and *in vitro*.[34-36] In addition, elastin fibers are autofluorescent due to elastin's tricarboxilic, triamino pyridinium derivatives, and are therefore visualized using multiphoton microscopy.[37]

Imaging was performed in the Laboratory for Fluorescence Dynamics (LFD) at UCI.[38] A Ti:Sapphire laser (Mai Tai, Spectra Physics, Irvine, CA) was used for two-photon fluorescence excitation with a wavelength of 740 nm (SHG) and 860 nm (autofluorescence) with an incident power of 20 mW. The signal was collected using a water objective with a long working distance (LUMPlanFl 40x/0.80 W, Olympus, Tokyo, Japan). The SHG signal was obtained using a 320nm-390nm bandpass filter. The autofluorescent signal was collected using a 500nm long pass filter. Images were captured every 5 μm through the 3D scaffolds with a field of view of 8,464 μm^2. Data acquisition was performed in SimFCS (software developed by the LFD). The images were analyzed using ctFIRE MATLAB (http://loci.wisc.edu/software/ctfire) by extracting information corresponding to the fiber width and number. The data were collected from 3 separate z-stacks for each sample.

Statistical Analysis

Split-Unit ANOVA was used to analyze qRT-PCR data using R analysis ($^{\delta}p>0.05$ $^{\delta\delta}p>0.01$). Figures show the standard error of the mean with n=3 unless otherwise stated.

Student's T-test was performed on GCaMP calcium transients and standard error of the mean is shown. (*** $p<0.001$, ** $p<0.01$, *$p<0.05$)

Additional information and explanation of methods can be found in the **Supplemental Materials.**

Supplemental Materials

iPSC-derived CM maturation

2D cultures: Prior to seeding the iPSC-derived CMs (Day 28-64 post induction), tissue culture plates were coated with 1 mg/ml of fetal or adult bovine cardiac ECM at 37°C overnight. The cells were harvested with 200 Units/ml collagenase II (LifeTechnologies) for 1 hour, followed by 3x washes with PBS (Ca/Mg free), 5 min incubation in 10X TryplE (LifeTechnologies) and then seeded at 45,000 cells/cm^2.

3D cultures: The cardiac ECM was adjusted to pH 7.4 with 0.1N NaOH and supplemented with 1/9 volume with 10x PBS (with Ca/Mg). The 3D scaffolds were composed of 5.25 mg/ml cardiac ECM and 1.75 mg/ml fibrinogen (Sigma). The cells were seeded at 1.6-3x10^6 cells/ml. The 3D gels were polymerized by the addition of 1.3 Units/ml thrombin (Sigma) and incubation at 37°C for 2 hours before media was added. Each 3D tissue construct also contained 0.15 U/mL aprotinin (Sigma) in a total scaffold volume of 75µl. Completed 3D gels were cultured in polydimethylsiloxane (PDMS, Dow Corning, Midland, Michigan) retention rings, each with a diameter of 8 mm and an approximate height of 1 mm. Cells were harvested after 7 days by digesting the tissue constructs with 500 Units/ml Collagenase II for 35-45 min.

Three independent biological experiments of the maturation studies were performed with each experiment using cardiac ECM from independent batch preparations and cardiac differentiations.

Quantitative RT-PCR

Total RNA was harvested from differentiated iPSC using Trizol (Life Technologies) according to the manufacturer's recommended protocol and was treated with RQ1 DNAse (Promega, Madison, Wisconsin) for 1 hour. Three micrograms of RNA was used to generate cDNA using iScript cDNA Synthesis Kit (BioRad, Hercules, California). qPCR was performed using SYBR green chemistry and an iQ5 BioRad icycler. mRNA levels were normalized to 18S expression and measured in triplicate. Primers were synthesized by Integrated DNA Technologies (Coralville, Iowa) and their sequences can be found in Table S1.

Calcium imaging and analysis

The iPSC-derived CMs have been engineered to express a calcium indicator (GCaMP6f reporter) that emits fluorescence in the presence of cytosolic calcium. Calcium transients were measured using an epifluorescent microscope with an inverted 10x objective (IX70 Olympus, Tokyo, Japan). Images were captured using a SPOT Idea 3.0 megapixel color fluorescent camera and SPOT acquisition software (SPOT Imaging Solutions, Sterling Heights, Michigan). The cells were placed in a 37°C heated stage during imaging. The video was captured at 33 frames/second with 8x8 binning. ImageJ was used to process and extract the temporal fluorescence intensity of the acquired video data.

Subsequent data were analyzed using MATLAB software (MathWorks, Torrance, California). A customized algorithm determined the troughs and peaks of each calcium wave and measured the amplitude, max upslope, max downslope, time to 50% decay, and beat rate. Additional measurements included the effect of caffeine on the slope of amplitude change.

Pharmacological Treatment of CMs

Baseline and drug recordings were taken before and after 10 min incubation with the drug(s) on the 37°C stage. All recordings were taken over 1 min. The order of the β-adrenergic drug treatments was baseline, 1 μM isoproterenol (Sigma #I6504), and 1 μM Isoproterenol + 10 μM Propranolol (Sigma #P0884). The effect of caffeine (Tocris #2793) on calcium signaling was assessed at a final concentration of 20 mM. The video recordings were acquired before and during the addition of caffeine.

nanoLC-MS/MS

Decellularized fetal and adult tissue pellets were fully solubilized in a buffer comprising 4% SDS, 10 mM TCEP, 100 mM TEAB through repeated cycles of heating (95°C) / ultrasonication (40°C) followed by 120 min of ultrasonication. Protein levels were subsequently quantitated using the BCA assay. Equivalent mass amounts of the resulting samples were treated with iodoacetamide and trypsinized as described[52] with modifications[53], and the resulting peptides desalted using stacked C18/SCX tips[53, 54]. The resulting peptides from each sample individually were subjected to nanoLC-MS/MS using an LTQ Orbitrap Velos Pro mass analyzer (Thermo Fisher Scientific, Waltham,

Massachusetts) connected to a nanoLC-Easy1000, with peptide separation in an in-house-packed 25 cm x 75 μm ID C18 nanospray tip. Peptides were resolved in segmented solvent gradients running from 6 to 35% CH_3CN in 0.1% formic acid over 135 min. FTMS precursor spectra were acquired at 60,000 resolution, and up to 20 of the most intense ions with charge states of +2 and higher in each precursor spectrum were subjected to rapid CID fragmentation and ion trap analysis.

Spectral data were re-calculated using Mascot Distiller 2.5.0 (Matrix Science, Boston, Massachusetts) and the resulting peaklists were searched against SwissProt (July 2014) with *Bos Taurus* taxonomy along with a database of common contaminants using Mascot Server 2.5.0 (Matrix Science), with Carbamidomethyl (C) and Oxidation (M) as fixed and variable modifications, respectively, and precursor and fragment mass tolerances of 10 ppm and 0.5 Da, respectively. Homology false discovery rates were either below, or thresholded at 5%. Mascot protein score and emPAI values in Mascot exports were compared between samples using in-house software.

Mechanical analysis of 3D cardiac ECM hydrogels

Parallel plate rheology was conducted using an MCR 302 (Anton Paar, Ashland, VA) to determine the storage and loss moduli (reported as pascals, Pa) of fetal and adult 3D cardiac ECM hydrogels composed of 5.25 mg/ml cardiac ECM and 1.75 mg/ml fibrinogen. Hydrogels were polymerized similar to the above experiments with measurements occurring after the two-hour incubation period at 37°C. Heating was conducted within a P-PTD200 Peltier-temperature-controlled hood (Anton Paar). The

storage modulus (G') and loss modulus (G'') was measured at oscillation frequencies ranging from 0.1 to 10 Hz, with a constant strain amplitude of 1%. Experiments were conducted with a gap height of 200 μm using a 25-mm parallel plate. Each of the respective samples was measured in triplicate (n=3).

Flow cytometry

Cells were fixed with 4% PFA for 10 min and then incubated in 0.75% saponin in PBS. Samples were stained with 1:100 anti-cTNT antibody (ThermoScientific) or isotype control mouse IgG and subsequently incubated with goat anti-mouse FITC secondary antibody. Samples were run on a BD FACSCalibur flow cytometer and data were analyzed using FlowJo software.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% TritonX100, and then blocked with 0.1% TritonX100 and 4% bovine serum albumin (blocking buffer). Cells were incubated in primary antibodies: 1:100 MYL2 (ProteinTech, Illinois), 1:100 CaV1.2 (ThermoFisher) and 1:800 α-actinin (Sigma-Aldrich) at 4°C overnight, followed by secondary Alexa Fluor 488/568 for 2 hours in blocking buffer. Cell nuclei were subsequently stained with DAPI for 30 min. The cells were imaged with either an epifluorescent microscope (Nikon Eclipse TE300) or a confocal laser microscope (Leica TCS SP8).

Hydrogel Encapsulation of Human iPS-CM Spheroids

iPSC-derived CM were seeded into AggreWell 400 (STEMCELL Technologies) at 720,000 cells per well. CM spheroids were allowed to aggregate and self-assembled for two days before encapsulation within ECM.

ECM hydrogels consisted of 10 mg/mL bovine fibrinogen (Sigma-Aldrich) dissolved in Dulbecco's phosphate buffered saline (Life Technologies) and combined with 9 mg/mL rat tail collagen (BD Biosciences) and 10 mg/mL cardiac ECM. All matrices denoted as a partial percentage had the remaining volume completed with fibrin. Thrombin was mixed with the cell and extracellular matrix solution to yield a final concentration of 3 Units/mL (0.3 Units/mg fibrin). Spheroids resuspended in ECM mixture were cultured in three PDMS retention rings attached to a glass bottom dish (World Precision Instruments, Sarasota, Florida). The tissues were incubated for 30 min at 37°C to allow full polymerization, and then fed RPMI 1640 supplemented with B27. The medium was replaced every 2 days.

FIGURES

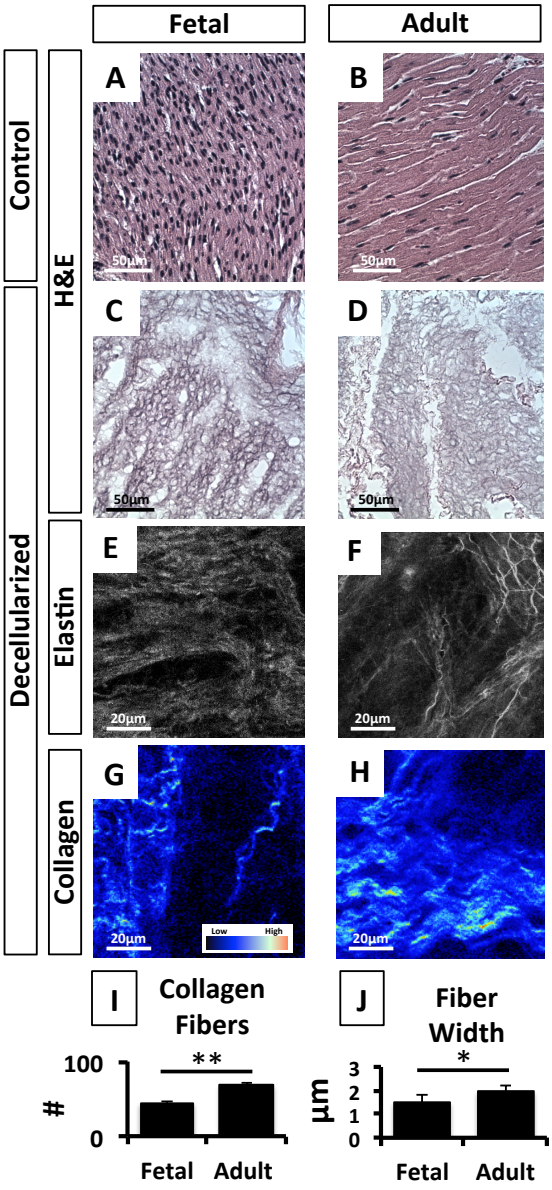


Figure 3.1. **Characterization of cardiac ECM from decellularized bovine fetal and adult heart tissue.** Decellularized adult bovine cardiac tissue has a more prominent fibrillar structure than fetal cardiac tissue. (A,C,E,G) Fetal heart tissue. (B,D,F,H) Adult heart tissue. (A,B) Control tissue. (C-H) Decellularized tissue. (E,F) Elastin fibers visualized by autofluorescence at 860 nm. (G,H) Collagen fibers visualized by second harmonic generation at 740 nm. (I) Quantification of the number of collagen fibers comparing fetal and adult decellularized cardiac tissue. (J) Collagen fiber width comparing fetal and adult decellularized cardiac tissue. Results are expressed as mean ± standard error. (n=3; *p<0.05 **p<0.01, Student's t-test)

Table 3.1. Mechanical properties of fetal and adult cardiac ECM hydrogels. Parallel plate rheology was used to determine storage and loss modulus at an oscillation rate of 1 Hz with a 1% strain amplitude. Results are expressed as mean ± standard error.

Source (3D Hydrogel)	Storage Modulus (G', Pa)	Loss Modulus (G'', Pa)
Fetal (n=3)	7.2 ± 3.2	1.3 ± 0.58
Adult (n=3)	67.5 ± 12.6	26.2 ± 14.1

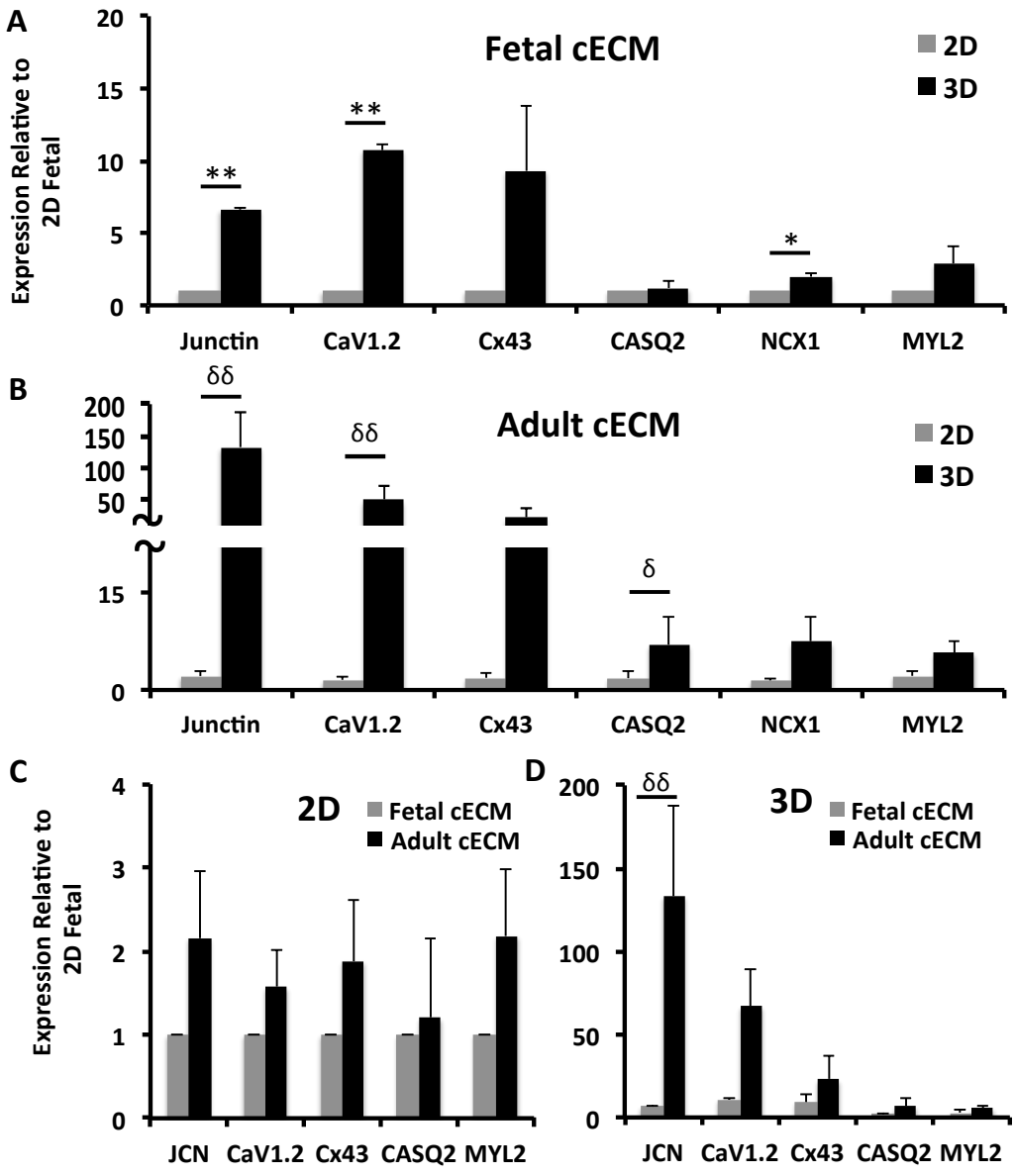


Figure 3.2. 3D adult cardiac ECM increases iPSC-derived CM expression of structural and functional cardiac genes. Gene expression was measured using qRT-PCR and expression normalized to 2D fetal cardiac ECM. Cardiac genes measured were JCN (Junctin), CACNA1C/CaV1.2 (L-type voltage-dependent calcium channel), Cx43 (connexin-43), CASQ2 (calsequestrin 2), NCX1 (sodium-calcium exchanger 1), and MYL2 (myosin light chain 2). (A) Gene expression in iPSC-derived CM cultured in (3D) or on (2D) fetal cardiac ECM. (*$p>0.01$ **$p>0.001$, Student's T-test) (B) Gene expression in iPSC-derived CM cultured in (3D) or on (2D) adult cardiac ECM. (C,D) Data from (A,B) replotted to aid direct comparison of gene expression in iPSC-derived CM cultured on adult and fetal 2D cardiac ECM (C) and 3D cardiac ECM (D). Unless otherwise indicated, results are expressed as mean ± standard error. (n=3; $^{\delta}p>0.05$ $^{\delta\delta}p>0.01$, Split-Unit ANOVA)

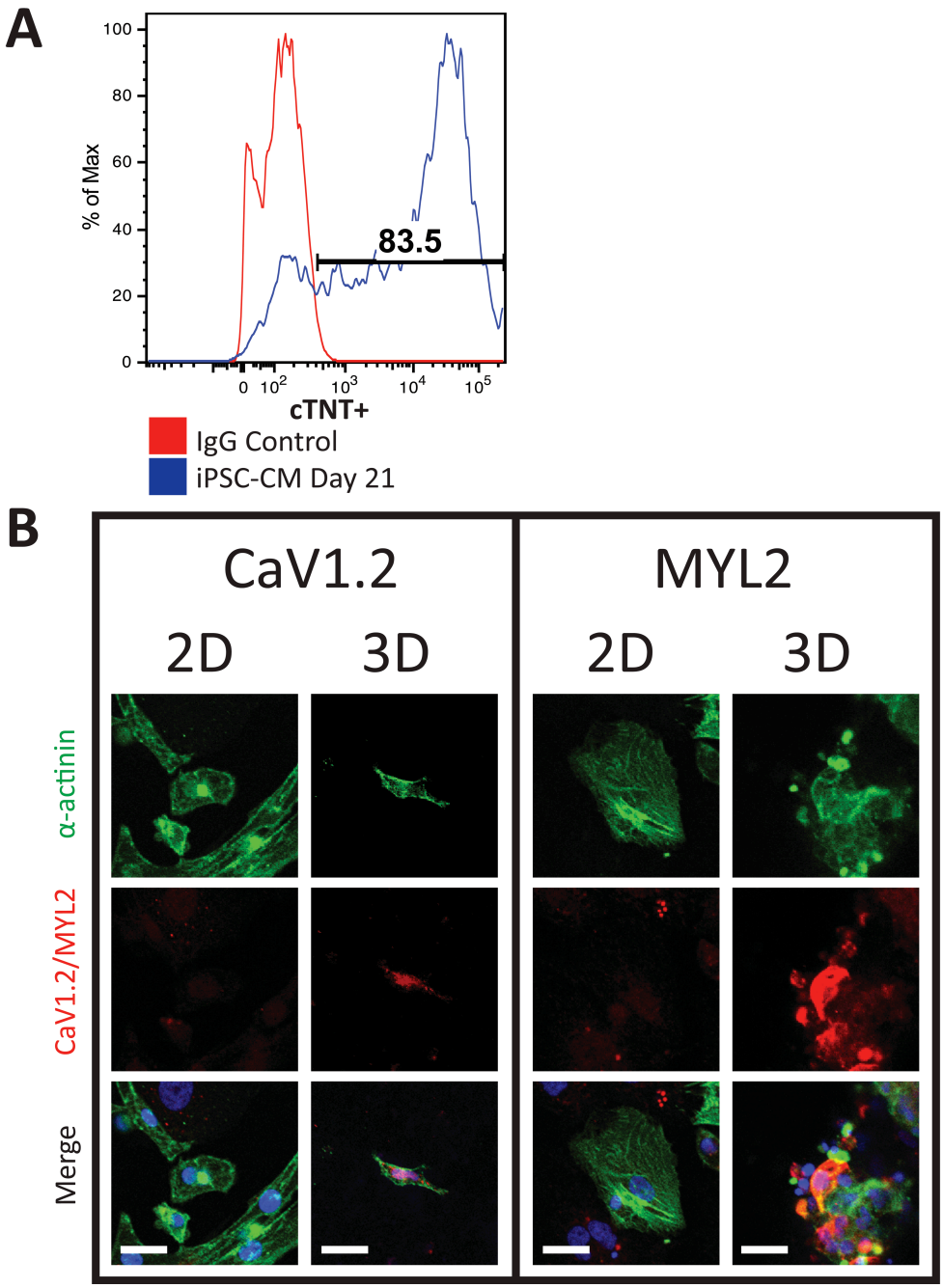


Figure 3.3. 3D adult cardiac ECM increases iPSC-derived CM protein expression of structural and calcium handling proteins. iPSC-derived CM express sarcomere and calcium handling proteins. (A) Flow cytometry analysis for cTNT(+) cells after 21 days of differentiation. (B) Immunofluorescent staining for MYL2 and CaV1.2 in iPSC-derived CMs cultured in 2D and 3D cardiac ECM. (Right panels) MYL2 staining. (Left panels) CaV1.2 staining. Scale bar is 30 μm.

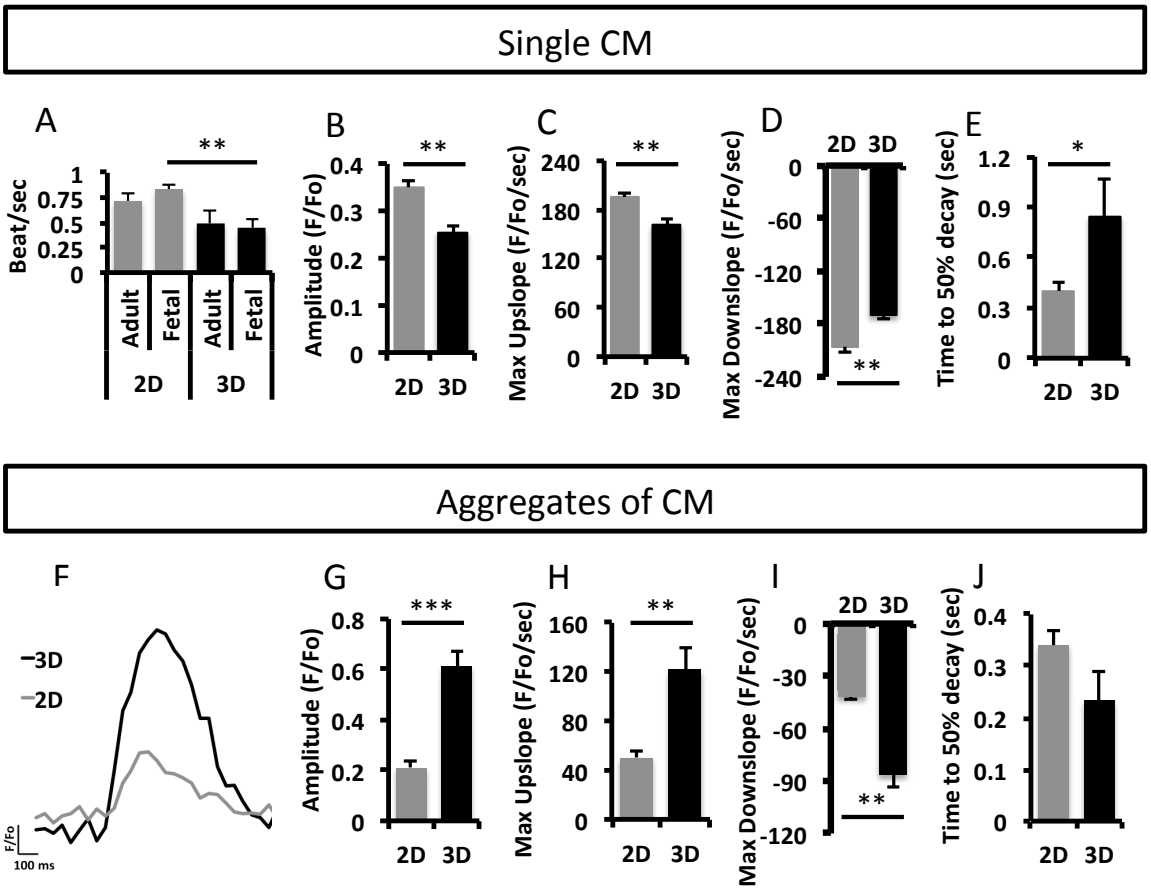


Figure 3.4. 3D adult cardiac ECM increases calcium signaling in iPSC-derived CM. A GCaMP reporter was used to visualize calcium transients in iPSC-derived CMs after 7 days in 2D and 3D adult cardiac ECM. (A-E) iPSC-derived CMs cultured in 3D as single cells displayed decreased calcium signaling and kinetics. (F-J) iPSC-derived CM cultured in 3D as aggregates displayed an increase in calcium signaling and kinetics compared to 2D. (A) Beat rate of iPSC-derived CM cultured in 2D and 3D adult/fetal cardiac ECM. (B,G) Amplitude. (C,H) Max Upslope. (D,I) Max Downslope. (E,J) Time to 50% decay. (F) Representative calcium wave transient of iPSC-derived CM culture in 2D and 3D adult cardiac ECM. Results are expressed as mean ± standard error. (n=4-12; * $p<0.05$ ** $p<0.01$ *** $p<0.001$, Student's t-test)

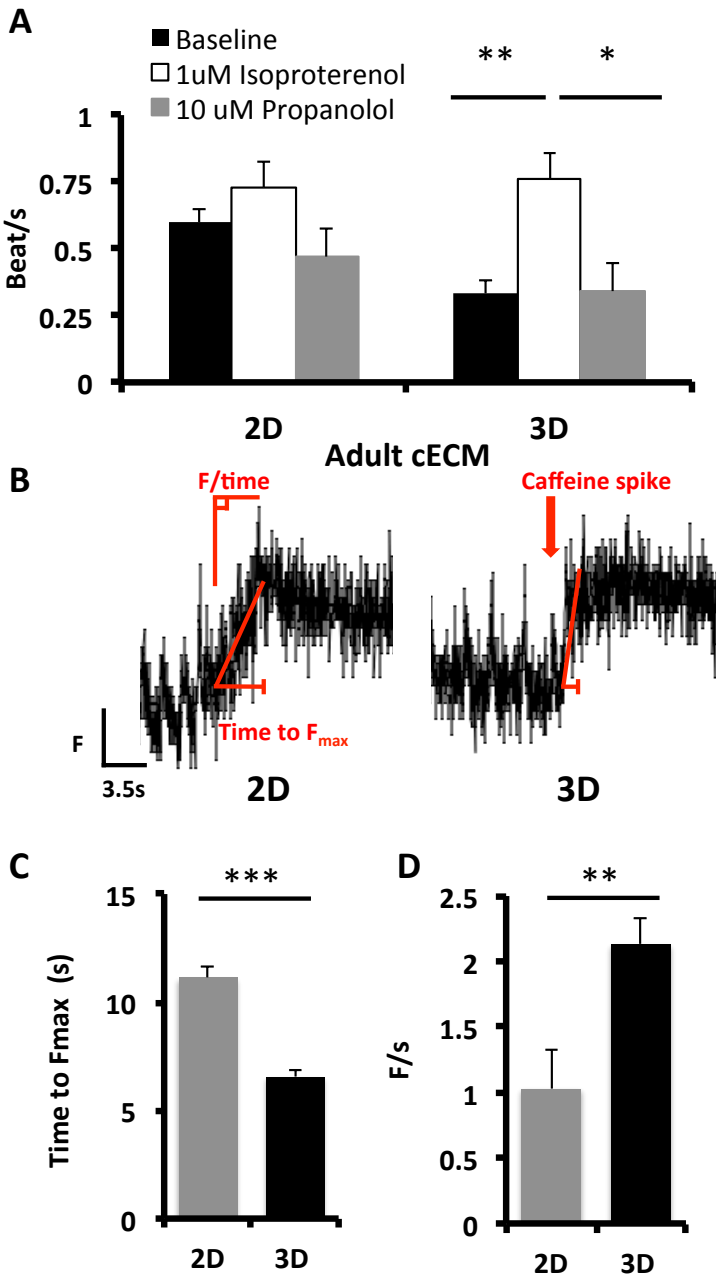


Figure 3.5. 3D adult cardiac ECM increases iPSC-derived CM response to drugs. iPSC-derived CMs cultured in 3D were more responsive to β-adrenergic stimuli and caffeine than cells in 2D cultures. (A) The effect of isoproterenol and propranolol on the beat rate of iPSC-derived CM. (B) A representative fluorescent signal of the iPSC-derived CM calcium transient in response to 20mM caffeine cultured in 2D and 3D adult cardiac ECM. (C) The time to maximum fluorescent output (F_{max}) was measured after the addition of caffeine. (D) The velocity of calcium release F/time. Results are expressed as mean ± standard error. (n=13-17; ** $p<0.01$ ***$p<0.001$, Student's t-test)

Supplemental Table 3.1. SDS concentration used to decellularized cardiac tissue affects polymerization of fetal and adult bovine cardiac ECM.

Fetal cardiac ECM hydrogel

	1% SDS				Day 3
	1 hour		2 hour		100% Decellularized
	50 ul	75 ul	50 ul	75 ul	
16 mg/ml	-	-	gel	watery	
12 mg/ml	-	-	-	-	
10 mg/ml	-	-	-	-	
7 mg/ml	-	-	-	-	

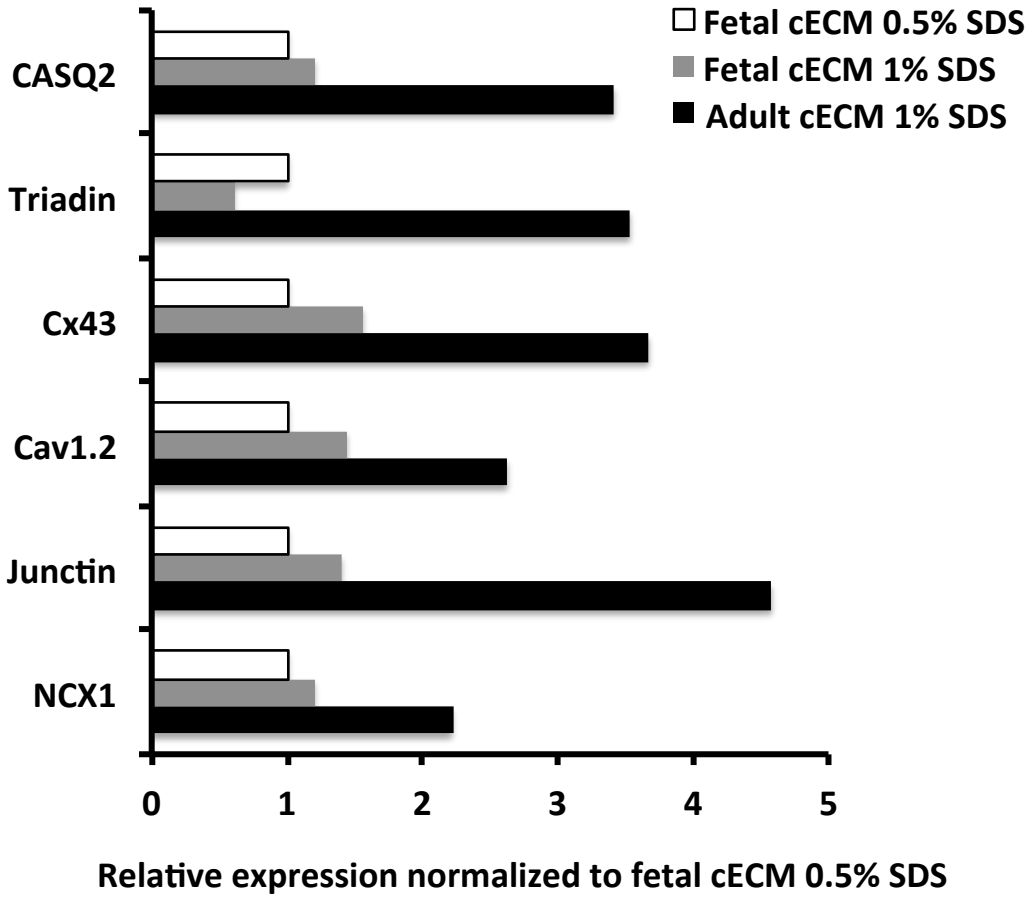
	0.5% SDS				Day 3
	1 hour		2 hour		90% Decellularized
	50 ul	75 ul	50 ul	75 ul	
16 mg/ml	gel	gel	gel	gel	
12 mg/ml	-	-	gel	gel	
10 mg/ml	-	-	watery	watery	
7 mg/ml	-	-	watery	watery	

	0.25% SDS				Day 3
	1 hour		2 hour		80% Decellularized
	50 ul	75 ul	50 ul	75 ul	
16 mg/ml	gel	gel	gel	gel	
12 mg/ml	-	-	gel	gel	
10 mg/ml	-	-	gel	watery	
7 mg/ml	-	-	watery	watery	

	0.1% and 0.05% SDS				Day 6
	1 hour		2 hour		<70% Decellularized
	50 ul	75 ul	50 ul	75 ul	
16 mg/ml	gel	gel	gel	gel	
12 mg/ml	gel	gel	gel	gel	
10 mg/ml	-	-	gel	gel	
7 mg/ml	-	-	watery	watery	

Adult cardiac ECM hydrogel

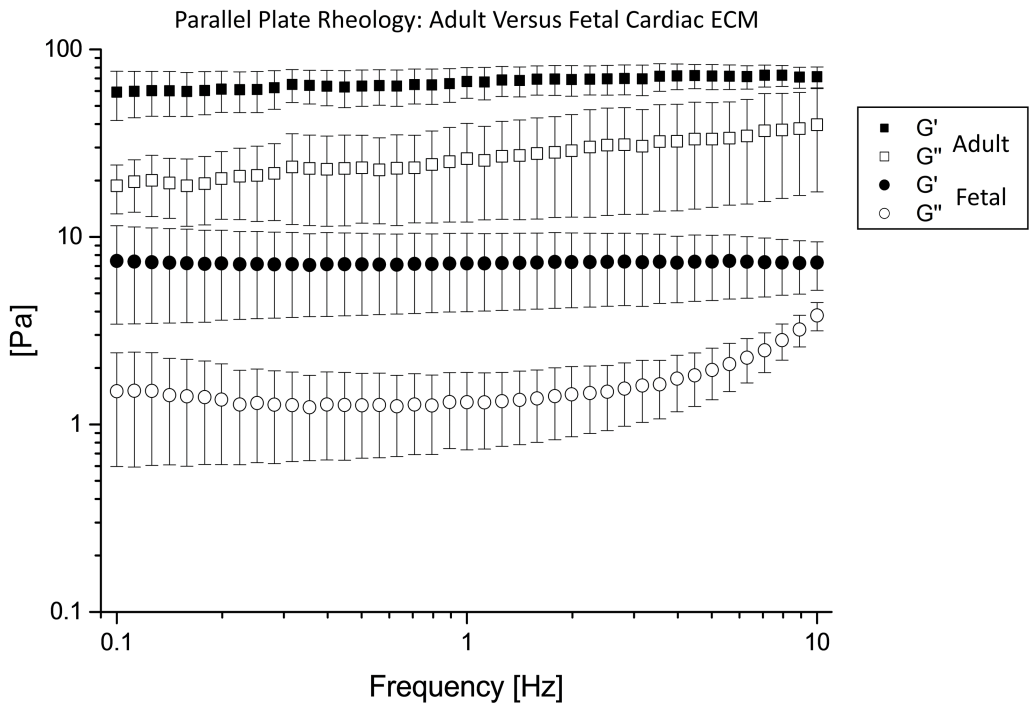
	1% SDS				2-3 Days
	1 hour		2 hour		100% Decellularized
	50 ul	75 ul	50 ul	75 ul	
16 mg/ml	gel	gel	gel	gel	
12 mg/ml	gel	gel	gel	gel	
10 mg/ml	gel	gel	gel	gel	
7 mg/ml	gel	gel	gel	gel	



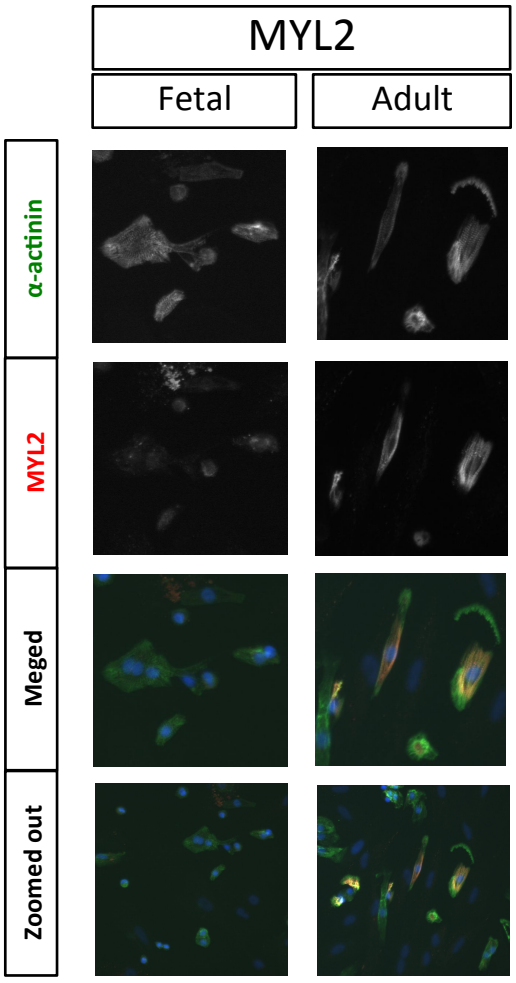
Supplemental Figure 3.1. Induction of iPSC-derived CM cardiac genes is indistinguishable on gels derived from fetal tissues decellularized with 0.5 and 1% SDS. Cardiac genes were measured using qRT-PCR and expression normalized to 2D fetal cardiac ECM decellularized with 0.5% SDS. The cardiac genes measured were Triadin, Junctin, CACNA1C/CaV1.2 (L-type voltage-dependent calcium channel), Cx43 (connexin-43), CASQ2 (calsequestrin 2), NCX1 (sodium-calcium exchanger 1). Comparing expression from iPSC-derived CM cultured for 7 days on 2D fetal cardiac ECM decellularized with 0.5% and 1% SDS to 2D adult cardiac ECM.

Supplemental Table 3.2. Key proteins identified in decellularized adult and fetal bovine heart tissue preparations by nanoLC-MS/MS. Data were thresholded at 4.4% and 4.8% FDR for adult and fetal samples, respectively.

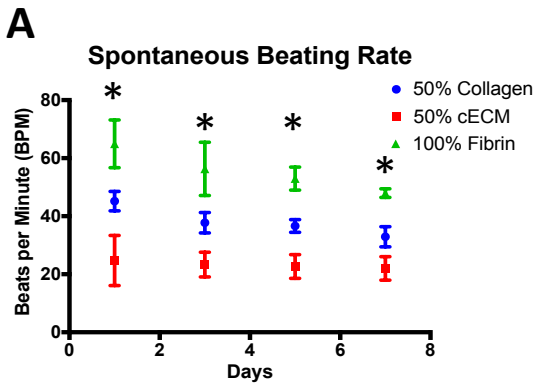
	Bovine Heart	
	Fetal	**Adult**
Fibrinogen	x	x
Collagen IV	x	x
Periostin	x	x
Fibrillin-1	x	x
Collagen III	x	x
Lumican	x	x
Fibronectin	x	x
Fibulin-5	x	x
Mimecan	x	
Collagen I	x	x
Decorin	x	x
Fibulin-1C	x	x
Elastin	x	x
Dermatopontin	x	x
Versican	x	



Supplemental Figure 3.2. Adult cardiac ECM hydrogels have higher storage and loss modulus compared to fetal hydrogels. Parallel plate rheology was used to quantify the storage and loss moduli of each sample. A frequency sweep was performed at a strain amplitude of 1% from 0.1 to 10 Hz. Results are expressed as mean ± standard error for each frequency. (n=3)



Supplemental Figure 3.3. iPSC-derived CM cultured on adult cardiac ECM compared to fetal has increased protein expression of MYL2. Immunofluorescent staining of iPSC-derived CMs cultured in 2D cardiac ECM for MYL2**.**



Supplemental Figure 3.4. 3D adult cardiac ECM decreases iPSC-derived CM beat rate. (A) Spontaneous beating rate significantly decreased with respect to time in culture and matrix composition. (*$p<0.0001$, two-way ANOVA)

Supplemental Table 3.3. Quantitative RT-PCR primer sequences.

Gene Name	Sequences 5'-> 3'
Junctin FWD	ATT GCA TTG CTG GGC GTC TG
Junctin REV	GGC ATC ATC CAC ATC AAA ATC TCC
MYL2 FWD	AGC GGA CCC TGA GGA AAC CAT T
MYL REV	GGG AAG GCG GCG AAC ATC T
NCX1 FWD	GTC CAT CGC TGC CAT CTA CCA C
NCX1 REV	TAC AGC AGC ACC CCC ACA TTG A
Cx43 FWD	TCC CCT CTC GCC TAT GTC TCC TC
Cx43 REV	CTG CCC CAT TCG ATT TTG TTC TG
CaV1.2 FWD	ACA AGG GCC CCA TCT ACA ACT ACC
CaV1.2 REV	CGA TGA CGA AGC CCA CGA AGA T
18S FWD	CCC CGG CCG TCC CTC TTA
18S REV	CGC CCC CTC GAT GCT CTT AG
Triadin FWD	AGA GCC CCC AGG TTT TGA CAC A
Triadin REV	CGG GGG ATT TGG GCA CAG
CASQ2 FWD	GTG GCC CAG GTC CTT GAA CAT AAA
CASQ2 REV	GCT GCA AAC TCG CCA TCA AAC TCT

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CHAPTER 4

Endothelial cells promote maturation of induced pluripotent stem cell-derived cardiomyocytes through endothelin-1 and notch signaling

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ABSTRACT

Induced pluripotent stem cell-derived cardiomyocytes (CM) hold great potential for the creation of new therapies to treat cardiovascular disease. However, CMs generated from pluripotent stem cells demonstrate an immature phenotype, displaying characteristics of fetal CM rather than adult CM. As a result, immature pluripotent stem cell-derived CMs are not safe for transplantation nor effective in drug discovery applications. During development the maturation of CMs is influenced by endothelial cells (EC) and so we hypothesized that EC may promote maturation of iPSC-derived CM. Here, we demonstrate that both EC/CM co-culture and EC-conditioned medium promote CM maturation, as demonstrated by increases in expression of the maturation markers Junctin, SERCA2a, CaV1.2, NCX1, and Cx43. Moreover, in line with these gene expression changes, EC-conditioned medium promotes an increase in iPSC-derived CM calcium signaling as measured using a GCaMP6 reporter. In addition, we determined that EC express endothelin 1 (ET-1) and the notch ligands, Delta-like 4 (DLL4), Jagged 1 (JAG1), and Jagged 2 (JAG2), and that blocking ET or notch signaling in CM reduced

expression of the CM maturation markers. In summary, we have generated a new strategy that utilizes EC to promote maturation of iPSC-derived CM, and suggest that this may aid in the generation of more mature CM for use in the development of safe and effective cardiovascular disease therapies.

Keyword words: endothelial cell, iPSC, cardiomyocyte, maturation, ET-1, notch signaling

INTRODUCTION

The de novo generation of adult cardiomyocytes (CM) is central to therapies that aim to replace damaged heart tissue, and induced pluripotent stem cells (iPSC) are an ideal source material. They provide an unlimited cell source, can differentiate into CMs, and are readily derived from patient cells to create autologous cell lines. Previous studies demonstrate that the transplantation of human embryonic stem cells (hESC) and iPSC-derived CMs into infarcted rodent hearts results in improved therapeutic outcomes.[1] However, CMs generated from pluripotent stem cells (PSC) are immature and display characteristics of fetal CM such as automaticity (spontaneous beating), weak contraction forces, and compromised expression of calcium handling genes.[2] As a result these immature CMs can cause arrhythmias, making them unsafe for transplantation. Thus, in order to develop safe and effective therapies for heart disease using iPSC as a source it is imperative that we develop strategies for promoting maturation of iPSC-derived CM so that the cells more closely resemble adult CMs.

Endothelial cells (EC) have been shown to influence maturation of multiple cell types, including hepatocytes,[3] hESC-derived pancreatic progenitors,[4] and hESC-derived CMs.[5] During development, autocrine and paracrine signaling between EC and CMs likely influences CM maturation.[6] In particular, endothelin-1 (ET-1), a potent vasoconstrictor produced by both CM and EC influences CM contractility and growth.[7] Specifically, ET-1 decreases CM proliferation and promotes binucleation and increases in cell size.[8] Besides ET signaling, other studies have indicated that notch signaling plays an important role in cardiac differentiation.[9] However, to date, no studies have looked at the role of EC in promoting iPSC-

derived CM maturation. Here we report that both EC co-culturing and the addition of EC-conditioned medium promote iPSC-derived CM maturation. Moreover, we determined that this maturation is dependent, in part, on both ET-1 and notch signaling.

RESULTS

EC/CM co-culture and EC-conditioned medium increase iPSC-derived CM expression of maturation-associated genes

In order to study CM maturation, iPSC were differentiated into CMs using small molecule inhibitors of glycogen synthase kinase 3 (CHIR99021) and Wnt signaling (IWP2). After 21 days, the iPSC-derived CMs were 50-80% positive for cardiac troponin T (cTNT) (Fig. 1A) and expressed the sarcomeric protein, α–actinin (Fig. 1B).

To determine the effect of EC on CM maturation, we co-cultured iPSC-derived CMs with EC for 7 days and evaluated expression of maturation markers by qRT-PCR. We also examined expression of these markers in EC using a previously-generated RNAseq dataset (unpublished data) and found that EC robustly express connexin 43 (Cx43), along with low levels of Junctin and SERCA2a. CaV1.2 and NCX1, however, were not expressed (Fig. 1C). As a further control, for comparison to CM/EC co-cultures we isolated RNA from separate cultures of EC and CM and then pooled the RNA. As shown in Fig. 1D we found that EC strongly induce expression of the gap junction and calcium handling genes, Junctin, Cx43, L-type voltage-dependent calcium channel (CaV1.2), and sodium-calcium exchanger 1 (NCX1) (Fig. 1D). SERCA2a (sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a) was also strongly induced but this was not statistically significant. In

order to determine if the ability of EC to increase maturation is due to soluble factors produced by the EC, iPSC-derived CMs were cultured with either fresh or EC-conditioned medium for 7 days. We found strong and significant upregulation of all five marker genes, including SERCA2a (Fig. 1E). These results demonstrate that EC induce maturation of iPSC-derived CM and that this can be mediated by soluble factors.

EC-conditioned medium increases iPSC-derived CM calcium signaling

To further investigate the influence of EC on CM maturation, we measured the effect of EC-conditioned medium on iPSC-derived CM calcium signaling using a GCaMP reporter to visualize the calcium transient. After 7 days, the iPSC-derived CM cultured with EC-conditioned medium resulted in a statistically significant increase in the calcium transient amplitude, and an increase in max upslope and max downslope that did not quite reach statistical significance (Fig. 1F-H). The EC-conditioned medium had no effect on iPSC-derived CM beat rate (Fig. 1I).

EC effects on iPSC-derived CM gene expression are mediated through notch and endothelin-1 (ET-1)

Based on previously published studies[7-9] we focused on possible roles for ET-1 and notch signaling in EC-induced maturation of CM, and confirmed that both ET-1 (but not ET-2 or ET-3) and several notch ligands, including Dll4, Jagged-1 and Jagged-2, are expressed by EC in vitro (Fig. 2A, B). To examine the importance of ET-1 in this setting we cultured CM in EC-conditioned medium and blocked ET-1 signaling in the CM using PD145065, an endothelin receptor inhibitor. Under these conditions we saw a strong

decrease in expression by CM of the maturation markers Junctin, SERCA2a, Cx43, CaV1.2, and NCX1 (Fig. 2C), confirming the important role that EC-derived ET-1 has in inducing maturation of iPSC-derived CM. We next tested whether cell contact-dependent signaling through notch might have a role by inhibiting notch signaling with the gamma-secretase inhibitor DAPT. Again, we saw a significant decrease in expression of CM maturation markers, including Junctin, SERCA2a, and CaV1.2 (Fig 2D). This confirms a role for notch signaling in EC-induced maturation of iPSC-derived CM.

DISCUSSION

There is a critical need to produce adult CMs in order to create novel treatments for heart disease and a promising source for these is autologous iPSC. However, iPSC-derived CMs are immature and have characteristics of fetal CMs, rather than of adult CMs. Here we report that signals from EC can significantly increase expression of several maturation markers in iPSC-derived CMs.

We found that both EC-conditioned medium and EC co-culture promote CM maturation as evidenced by significantly increased expression of Junctin, SERCA2a, Cx43, CaV1.2, and NCX1. This is consistent with an increase in maturation as *i*n vivo the calcium handling proteins, Junctin, SERCA2a, and CaV1.2 are expressed at higher levels in adult CMs compared to fetal CMs.[10] Co-culturing EC with CMs is also known to upregulate expression of Cx43,[5] which is consistent with our conditioned medium experiments.

In mammals, the ET family consists of three active ET isoforms, with ET-1 most prominently expressed within the cardiovascular system.[7] ET-1 is secreted by CMs[7] and EC (Fig. 2A), and regulates cardiac contraction, hypertrophy and calcium signaling.[11] Since ET-1 is known to promote terminal differentiation of developing CMs we hypothesized that ET-1 is in part responsible for the increase in maturation induced by EC, and this turned out to be the case. The ET receptor antagonist PD142893 strongly inhibited upregulated expression of several maturation genes, including NCX1, which was previously identified as an ET-1 target.[7, 11] While there are two known ET receptors (ETA and ETB), ETA is predominantly expressed by CMs[7] and we used a dose of

PD142893 (10 nM) that should preferentially target ETA over ETB. Thus EC-derived ET-1 is likely working through CM-expressed ETA. Further studies would be required to confirm this finding, perhaps using siRNA knockdown of the receptors.

Notch signaling plays a significant role in the myocardium by regulating CM differentiation and proliferation during development and cardiac remodeling.[9] Koyangagi and colleagues determined that notch inhibition during EC/CM co-culture resulted in a decrease in cTNT expression,[12] similar to the decrease in Junctin, SERCA2a, and CaV1.2 observed in our EC/CM co-culture. Unlike the conditioned media experiments where gene expression could be unequivocally attributed to the CM, in the co-culture experiments it is possible that some gene expression (Junctin, SERCA2a, Cx43) is being contributed by the EC, however, as shown in Fig. 1C, EC do not express CaV1.2. Our data are thus consistent with the hypothesis that notch signaling is a key regulator of iPSC-derived CM maturation.

We also tested whether these changes in expression of key calcium-handling genes would result in altered calcium transients on the CM, and this was indeed the case. In CM cultured in EC-conditioned medium we saw increases in calcium transient amplitude, max upslope and max downslope, indicative of increased calcium available for signaling and increased kinetics. In line with this, Yang and colleagues reported that CM maturation in response to T3 supplement resulted in increased calcium transient amplitude and kinetics.[2]

In summary, EC play a significant role in promoting the maturation of iPSC-derived CM *in vitro,* through both secreted and contact-dependent pathways (Fig. 2E). These findings suggest that future protocols for generation of mature iPSC-derived CM should incorporate EC and/or EC-derived factors.

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METHODS AND MATERIALS

Cardiac Differentiation

The WTC-11 GCaMP iPSC line, a gift from Dr. Bruce Conklin (UCSF), was used in accordance with UC Irvine's Human Stem Cell Research Oversight Committee. The iPSCs were maintained with mTeSR medium (StemCell Technologies) on Matrigel Growth Factor Reduced (BD) coated dishes. Cardiac differentiation was induced by glycogen synthase kinase-3 inhibition (CHIR99021, 6 μM, Tocris Inc) and Wnt inhibition (IWP2, 5 μM, Tocris), as described previously.[13]

Endothelial Cell Isolation

Endothelial progenitor cells (ECs) were isolated from human umbilical cord blood through CD31(+) cell purification from the mononuclear cell fraction.[14]

EC co-culture and condition medium

EC and iPSC-derived CMs were plated onto Matrigel Growth Factor Reduced (BD) coated dishes. Cells were fed with RPMI media supplemented with B27 (Life Technologies) and Endothelial Cell Growth Supplement (ECGS; BD Biosciences) every 2-3 days. EC-conditioned medium was collected after 1-3 days of EC growth. Endothelin inhibition was achieved by adding 10 nM PD 145065 inhibitor (EMD Millipore). Notch inhibition was achieved with 20 μM DAPT (Sigma) provided at each medium change. Cells were fed on days 1 and 4 and then harvested after 7 days.

qRT-PCR

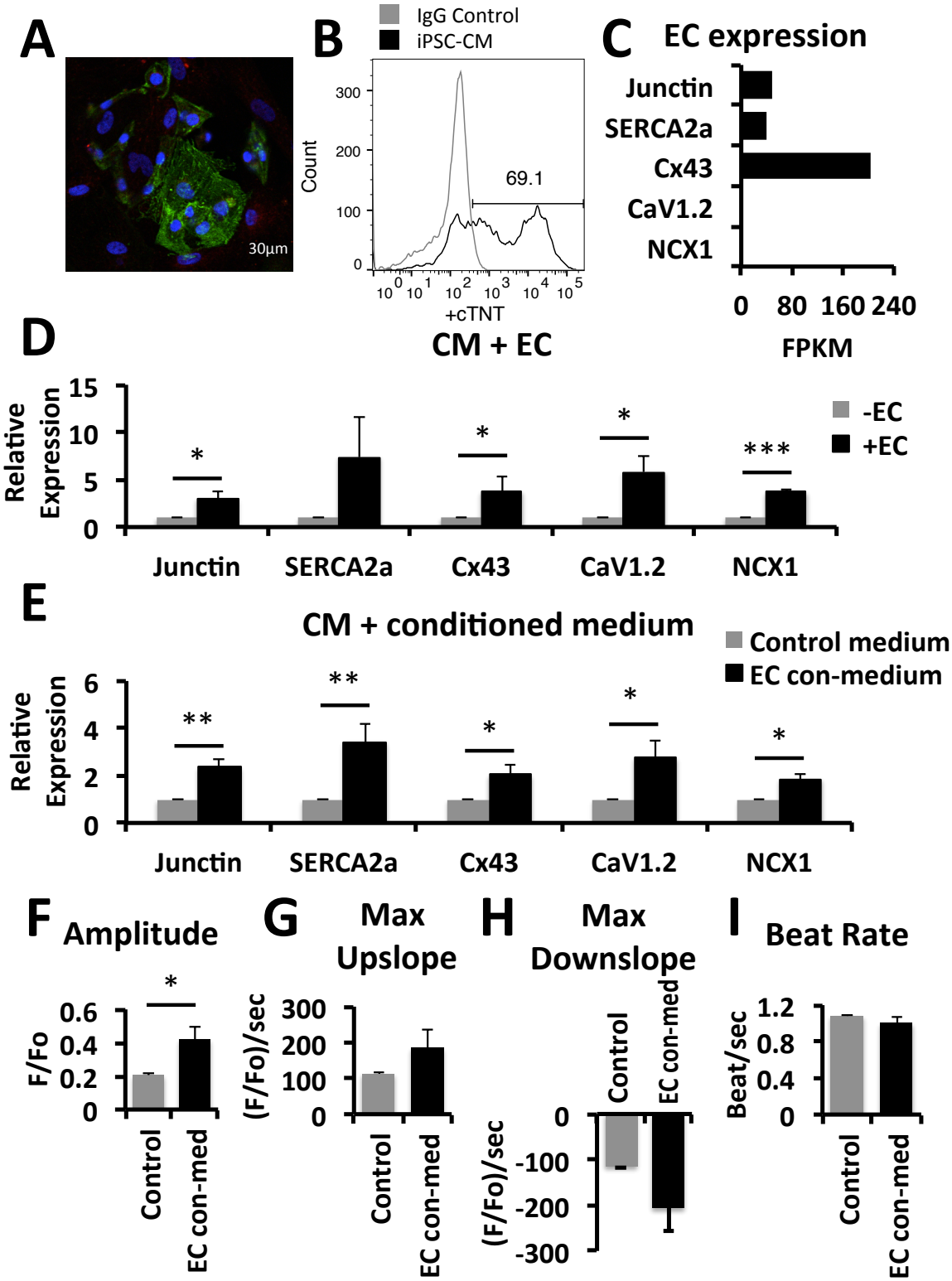
RNA was extracted using TRIzol (Life Technologies) according to the manufacturer's instructions. Three micrograms of RNA was used to generate cDNA using the iSCRIPT cDNA Synthesis Kit (Bio-Rad). The reaction mix included SYBR green chemistry and primers generated by IDT Technologies. The samples were analyzed using an iQ5 Real Time PCR Detection System (Bio-Rad). The control sample, compared to EC/iPSC-derived CM co-culture, consisted of a mix of equal parts EC and iPSC-derived CM RNA that were isolated separately. (n=3; * $p<0.05$ ** $p<0.01$ *** $p<0.001$, Student's t-test)

Calcium imaging and analysis

A GCaMP reporter [15] was used to visualize the presence of cytosolic calcium. Calcium transients were measured using an epifluorescent microscope with an inverted 10x objective (IX70 Olympus, Tokyo, Japan). Images were captured using a SPOT Idea 3.0 megapixel color fluorescent camera and SPOT acquisition software (SPOT Imaging Solutions, Sterling Heights, Michigan). ImageJ was used to process and extract the temporal fluorescence intensity of the acquired video data.

Subsequent data were analyzed using MATLAB software (MathWorks, Torrance, California). A customized algorithm determined the troughs and peaks of each calcium transient and measured the amplitude, max upslope, max downslope, and beat rate. (n=6; * $p<0.05$, Student's t-test)

FIGURES


A
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Junctin
SERCA2a
Cx43
CaV1.2
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FPKM
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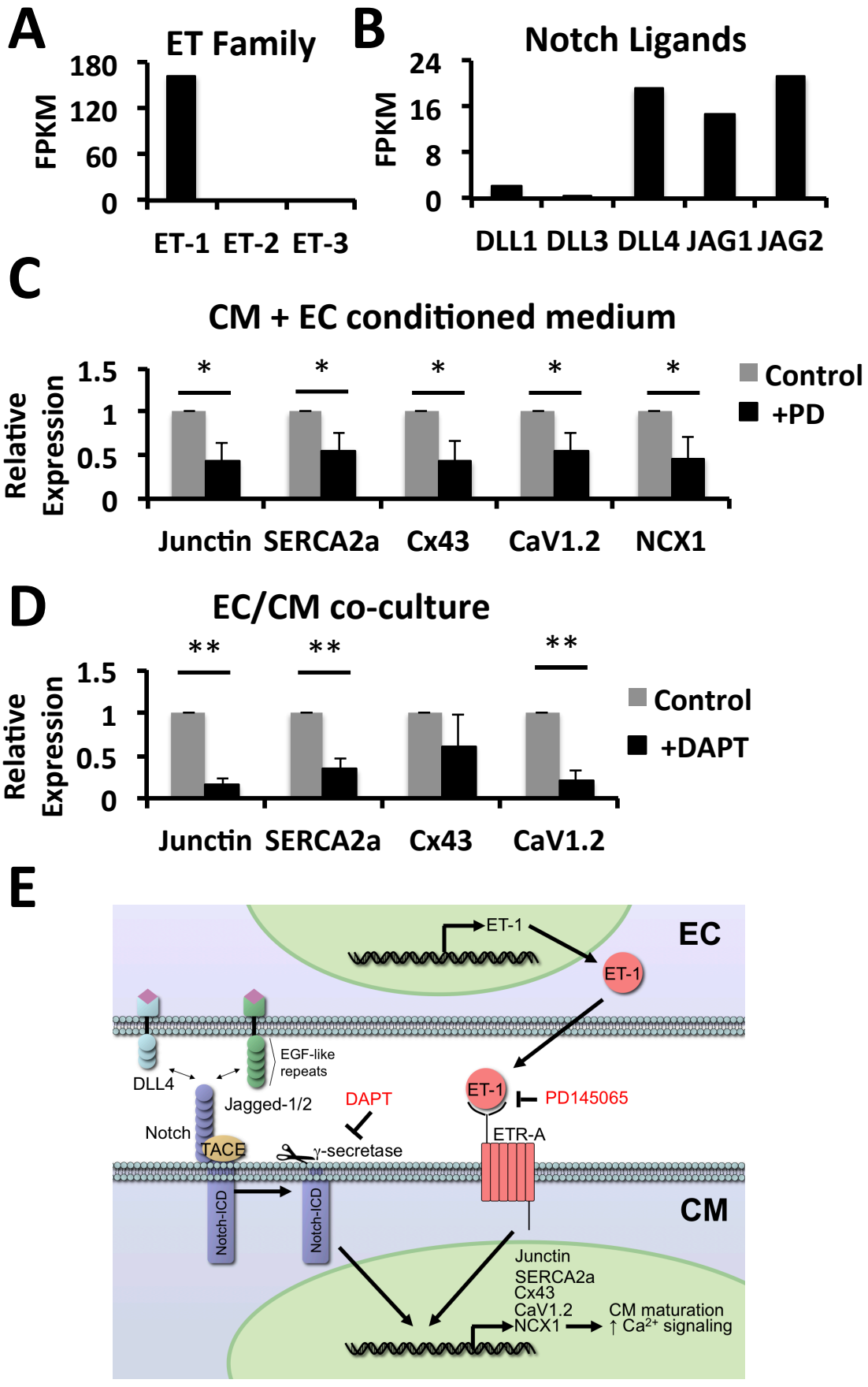
Figure 4.1. EC promote increased expression of cardiac maturation markers in iPSC-derived CM. (A) Immunofluorescent staining of iPSC-derived CM for α-actinin (green), myosin light chain 2V (red), and nuclei (blue). (B) Flow cytometry analysis for cTNT (+) cells after 21 days of differentiation. (C) RNA-sequence analysis of average expression of CM maturation genes in primary EC (mean expression in three independent lines). CM maturation genes include GJA1 (Cx43), ATP2A2 (SERCA2a), ASPH (Junctin), SLC8A1 (NCX1), and CACNA1C (CaV1.2). (D,E) EC promote iPSC-derived CM expression of CM marker genes. (D) The effect of EC/CM co-culture on iPSC-derived CM gene expression after 7 days. (E) The effect of EC-conditioned medium on iPSC-derived CM gene expression after 7 days. Genes analyzed (by qRT-PCR) included: Junctin, SERAC2a (sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a), Cx43 (connexin-43), CACNA1C/CaV1.2 (L-type voltage-dependent calcium channel), and NCX1 (sodium-calcium exchanger 1). (n=3; * $p<0.05$ **$p<0.01$, Student's t-test). (F-I) A GCaMP reporter was used to visualize calcium transients in iPSC-derived CMs after 7 days in control medium or EC-conditioned medium. (F) Amplitude. (G) Max Upslope. (H) Max Downslope. (I) Beat rate. (n=6; * $p<0.05$, Student's t-test)


A
ET Family
FPKM
180
120
60
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ET-1 ET-2 ET-3
B
Notch Ligands
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Relative Expression
1.5
1
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Junctin SERCA2a Cx43 CaV1.2 NCX1
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Relative Expression
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Junctin SERCA2a Cx43 CaV1.2
Control
+DAPT
E
EC
ET-1
ET-1
EGF-like repeats
DLL4
Jagged-1/2
DAPT
ET-1
PD145065
Notch
TACE
γ-secretase
ETR-A
Notch-ICD
Notch-ICD
CM
Junctin
SERCA2a
Cx43
CaV1.2
NCX1
CM maturation
↑ Ca2+ signaling

Figure 4.2. Inhibition of ET and notch signaling decreases iPSC-derived CM expression of calcium handling genes. (A) RNA-sequence analysis showing expression of ET family members (ET-1, ET-2, ET-3) in primary EC (mean expression in three independent lines). (B) The average expression of notch ligands (DLL1, DLL3, DLL4, JAG1, JAG2) in EC. (C-D) The small molecule inhibitors, PD142893 (ET) and DAPT (notch), were incubated with the iPSC-derived CMs (in conditioned medium or in coculture) and after 7 days gene expression of maturation markers (Junctin, SERCA2a, Cx43, CaV1.2) was measured using qRT-PCR. (C) The effect of ET inhibition on iPSC-derived CM cultured in EC-conditioned medium. (D). The effect of notch inhibition on iPSC-derived CM co-cultured with EC. (n=3; * $p<0.05$ **$p<0.01$, Student's t-test). (E) Schematic showing the contact-dependent and -independent interactions between EC and iPSC-derived CM.

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CHAPTER 5

Summary and Conclusions

DISCUSSION

In the past 15 years, very little progress has been made in developing new treatments for heart disease except for medical device intervention. The lack of new pharmacologic and biologic CVD treatments creates a critical need to discover novel therapeutic approaches that can regenerate the damaged myocardium.[1] PSC-derived CM provide a powerful tool for disease modeling, drug screening, and cell-based transplantation. Since CMs differentiated from PSC are immature and their immaturity leads to safety and efficacy concerns for transplantation and drug screening applications,[2] many groups are investigating specific strategies to induce maturation of CMs. However, researchers are primarily focused on one specific stimulus and therefore ignore the big picture – during development CMs receive not just one signal, but a myriad of signals from other cell types,[3] ECM,[4] paracrine signals,[5, 6] hormones,[7] mechanical stimulation,[8] and more that work in synchrony to instruct the CM to mature.[9] The purpose of this dissertation is to investigate stimuli that affect CM maturation and understand the underlying mechanisms responsible for maturation. In order to have a more holistic approach, we are examining the role of the cardiac microenvironment on iPSC-derived CM development and maturation *in vitro.* Since it is impossible to impose all the signals generated within the cardiac microenvironment, we sought to focus on 3 main components: cardiac ECM, 3D culturing, and endothelial cells.

Recently, ECM is gaining recognition as an integral component that modulates cellular behavior, development, and maturation rather than just a scaffold that provides architectural support.[10, 11] Due to the large influence ECM has on development, it is critical to employ the appropriate ECM when directly differentiating stem cells into desired cell types, such as CM. Furthermore, the development of decellularization techniques has provided researchers with an abundance of tissue-specific native ECM that retains the original matrix composition and mechanical properties. With this in mind, we hypothesized that cardiac ECM derived from decellularized cardiac tissue would provide a better substrate-coating for CM differentiation than the traditionally used Matrigel, and this was indeed the case. Our results reflect similar findings from the Vunjak-Novakovic group and the Christman group, thereby providing further evidence of the critical role cardiac ECM has in CM development.[12, 13]

In addition to development, we have also shown that cardiac ECM promotes iPSC-derived CM maturation *in vitro*. To investigate the developmental-stage differences within cardiac ECM and their potential impact on CM maturation, we extracted native cardiac ECM by detergent-mediated decellularization from bovine adult and fetal heart tissue. We found that iPSC-derived CM cultured in adult cardiac ECM upregulated cardiac maturation markers relative to cells grown in fetal matrix, thereby implicating cardiac ECM age as an important factor in CM maturation. During our investigation of fetal and adult cardiac ECM we determined that the matrices are similar in protein composition, however, they have very different mechanical properties and fiber architecture. Since mechanical properties are known to affect CM maturation and the

adult cardiac ECM is stiffer compared to the fetal ECM, the difference in mechanical properties potentially explains why adult cardiac ECM promotes CM maturation over fetal cardiac ECM.

Specifically, the adult cardiac ECM is 10 fold stiffer and contains more collagen fibers than the fetal cardiac ECM. The increase in collagen fibers within the adult cardiac ECM contributes to hydrogel's increased stiffness compared to fetal ECM. The differences between fetal and adult cardiac ECM's stiffness and fiber architecture may modulate specific biomechanical transduction signals within the CM that are responsible for regulating gene expression of maturation markers. Furthermore, CMs cultured in 3D compared to 2D will exhibit different biomechanical transduction signals because CM can sense their environment and respond accordingly.[14] Specifically, we hypothesize that the biomechanical transduction signals responsible for CM maturation are mediated by integrins. Integrins are transmembrane proteins that function, by non-covalently binding to ECM proteins outside the cell, to mechanically connect the outside world to the cell's cytoskeleton. The integrin bridge enables the cell to sense the outside environment and regulate tissue patterning, cell trafficking, proliferation, migration, spreading, differentiation and cell fate determination.[15-17]

Within CMs, the protein complex of ECM-integrin-costamere, also known as focal adhesion complex, is a mechanical sensor that can detect stretch-induced deformation resulting in the recruitment and activation of several signaling proteins including FAK, Src, Rho-associated coiled-coil containing protein kinase, PKCε, and mitogen-activated

protein kinases.[18-21] In addition, the focal adhesion complexes bind to cytoskeletal proteins such as α-actinin, a protein within the sarcomere.[22] These studies suggest that the CM's deformation caused by mechanical changes within the environment, such as stretching or increased stiffness, is sensed by the ECM-integrin-costamere complex, which thereby regulates cytoskeletal reorganization, downstream signaling cascades, and ultimately gene expression.

CMs express the integrin subunits α1-, α3-, α5-, α6-, α7-, α9-, and α10 and β1A and β1D.[23] During development rodent fetal CMs express the integrin subunitsβ1A and β1D, however as the CMs transition to the neonatal stage β1A is down regulated, and therefore the adult mouse CMs predominantly express β1D.[24] In conjunction, fetal and neonatal CMs express the integrin β 1 associated subunits α 3, α 6 and low levels of α7, and after development the adult CMs upregulate the expression of integrin subunit α7.[25] Specifically, the CM integrins α5β1 binds fibronectin and α3β1 binds to collagen I, fibronectin, and laminin.[26] Recent studies have demonstrated that β 1 integrin participates in hypertrophic responses in rat ventricular myocytes[27] and overexpression of β 1D within neonatal rat CMs increased promoter activity of atrial natriuretic factor and myosin regulatory light chain 2V implicating the role of β 1D in CM maturation.[27] We further speculate that the presence of increased collagen fibers within our adult cardiac ECM hydrogels and 3D culture conditions, compared to fetal ECM and 2D cultures, may lead to an upregulation of the integrin subunit β 1D, and therefore explain our observed increase in the expression of MYL2 and other maturation markers of the iPSC-derived CMs that were cultured in adult cardiac 3D ECM hydrogels. Future experiments may

include knocking down integrin subunit β 1D and determining if the loss of β 1D dampens the maturation effects the 3D adult cardiac ECM has on iPSC-derived CMs. Conversely, we can also determine if the presence of additional collagen in the fetal ECM or overlaying of additional adult cardiac ECM on the CM cultured in 2D, such as a sandwich method, induces integrin β 1D and promotes gene expression of the CM maturation markers. Ultimately, these proposed experiments may determine if integrins, specifically β1D, are responsible for the biomechanical transduction signals that induce CM maturation.

During the past 100 years, scientists have traditionally studied and cultured cells *in vitro* in 2 dimensions (2D), however, CMs within the body are not grown in 2D, but in 3D as muscle fibers. In order to determine the impact of dimension on CM maturation, iPSC-derived CMs were cultured in 3D cardiac ECM hydrogels, and compared to cells on 2D coated-surfaces. 3D culture resulted in an increase of CM maturation markers, calcium signaling, and responsiveness to drugs similar to the maturation induced by T3 hormone and long-term culturing.[7, 28] Although the determination of how 3D culturing, compared to 2D culturing, induces CM maturation was beyond the scope of our study, future studies should focus on uncovering the mechanisms responsible for maturation and the resulting cascade of events including changes in gene and protein expression, morphology, contraction forces, and electrophysiology and calcium signaling. We hypothesize that integrins may be responsible for the mechanical transduction signals leading to maturation, however we do not yet have data to support this suggestion. Overall, our results highlight the limitations of 2D culturing, and should urge scientists to

change from the traditional 2D culturing paradigm to 3D culture systems, especially when generating mature stem cell-derived cell types.

During our evaluation of iPSC-derived CM calcium signaling, we discovered that calcium handling varied greatly when the CMs were cultured as single cells versus aggregates. We determined that culturing single CMs in 3D was not enough to alter calcium-handling characteristics, but rather the close association CMs and coupling of calcium handling systems that dictate the kinetics of calcium signaling. In addition, we observed high variability in calcium signaling and beat rate when studying single CMs compared to the more uniform characteristics in CMs cultured as aggregates or spheroids. Taken together, future studies in 3D should focus on aggregated CM rather than single CM.

More recently, researchers are understanding the importance of vasculature during development because of its role in cellular maturation in several tissues. Similarly to the Hsieh group, we also found that the co-culture of EC with PSC-derived CM promotes CM maturation, as demonstrated by increases in maturation markers and calcium signaling(ref). Furthermore, iPSC-derived CMs cultured in EC-conditioned medium also induced CM maturation, thereby suggesting that EC produce soluble factor(s) that contribute to CM maturation. Upon further examination, we determined that both ET-1 and notch signaling are responsible, in part, for mediating EC's role in CM maturation.

Our studies, similar to other published work, demonstrate that our CM maturation protocol is still incomplete.[29, 30] This is not surprising since we only focused on 3 aspects of the cardiac microenvironment (Cardiac ECM, 2D vs 3D and presence of EC), and therefore are missing a number of other factors. Future studies should include additional components of the microenvironment, such as cardiac fibroblasts, cyclic stretching, and T3 hormone. And rather than study one component at a time, it would be ideal to simultaneously apply all known stimuli when inducing maturation. Potentially, the myriad of signals will be able to generate fully mature PSC-derived CM that mimic adult CM. For instance, our future studies should focus on applying all three cardiac microenvironment components simultaneously by culturing iPSC-derived CM in 3D cardiac ECM hydrogels with EC and determine if the multiple maturation stimuli results in additive benefits leading towards more complete maturation.

Since our lab has developed a high-throughput drug screening device that investigates drugs' effects on 3D microvascularized tissues, we are capable of recreating the cardiac microenvironment within the drug screening platform to include 3D culturing, cardiac ECM, vasculature (stromal cells and EC), and flow dynamics that are capable of inducing mature iPSC-derived CM. Another important aspect to using mature CM is to improve predictability of drug's efficacy and potential cardiotoxicity that immature CMs would have failed to identify. To test this hypothesis, we would evaluate specific drugs that have been pulled from the market due to cardiotoxicity such as Astemizole or Grepafloxacin and determine if mature CMs could have accurately predicted the negative side effects. Furthermore, by combining our CM maturation strategy with our drug screening device,

we have created a novel high-throughput platform that is capable of screening various compound libraries to discover potential “hits” that can be further tested in the clinic as a potential therapy to treat heart disease.

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APPENDIX I

Oligonucleotides used in this study

qRT-PCR Primers

Gene Name	Sequence 5` → 3`	
18S	CCC CGG CCG TCC CTC TTA	(forward)
	CGC CCC CTC GAT GCT CTT AG	(reverse)
CASQ2	GTG GCC CAG GTC CTT GAA CAT AA	(forward)
	GCT GCA AAC TCG CCA TCA AAC TCT	(reverse)
CaV1.2	ACA AGG GCC CCA TCT ACA ACT ACC	(forward)
	CGA TGA CGA AGC CCA CGA AGA T	(reverse)
cTNT	GAC CCG CGG GAA GGC TAA AG	(forward)
	GTG GGG GCA GGC AGG AGT G	(reverse)
Cx43	TCC CCT CTC GCC TAT GTC TCC TC	(forward)
	CTG CCC CAT TCG ATT TTG TTC TG	(reverse)
GATA4	CGG GGA CAT AAT CAC TGC GTA ATC	(forward)
	GGG CCT CCT TCT TTG CTA TCC TC	(reverse)
HCN4	CAG GAG ACC ACA GCA GAA AT	(forward)
	CCT TTC TGT CAC CTC ATT TG	(reverse)
ISL1	CGC GTG CGG ACT GTG CTG AAC	(forward)
	TTG GGC TGC TGC TGC TGG AGT	(reverse)
Junctin	ATT GCA TTG CTG GGC GTC TG	(forward)
	GGC ATC ATC CAC ATC AAA ATC TCC	(reverse)
MEF2C	TAACTTCTTTTCACTGTTGTGCTCCTT	(forward)
	GCC GCT TTT GGC AAA TGT T	(reverse)
MYL2	AGC GGA CCC TGA GGA AAC CAT T	(forward)
	GGG AAG GCG GCG AAC ATC T	(reverse)
NCX1	GTC CAT CGC TGC CAT CTA CCA C	(forward)
	TAC AGC AGC ACC CCC ACA TTG A	(reverse)
Tbx20	CCC TGG CCC CGC TGT GA	(forward)
	CGT TGG CCC GAG AGG AGA GTT	(reverse)
Triadin	AGA GCC CCC AGG TTT TGA CAC A	(forward)
	CGG GGG ATT TGG GCA CAG	(reverse)
SERCA2a	GAG GCT CTG TAA CCT TAT CT	(forward)
	GAG GGA TCT GGC TAC TTA C	(reverse)

APPENDIX II

List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AAV	Adeno-associated virus
αMHC	Alpha-myosin heavy chain
ANOVA	Analysis of variance
BMP	Bone morphogenetic protein
BPM	Beats per minute
cECM	Cardiac ECM
CaV1.2	L-type voltage-dependent calcium channel 1.2
CHIR99021	GSK3 inhibitor
CHO	Chinese hamster ovary
CM	Cardiomyocyte
CRISPR	Clustered regularly-interspaced short palindromic repeats
CSQ	Calsequestrin
cTNT	Cardiac troponin T
CTS	Cardiac tissue sheet
CVD	Cardiovascular disease
Cx43	Connexin 43
DAPT	Gamma-secreatase inhibitor
DKK1	Dickkopf homolog 1
DLL4	Delta-like protein 4

EB	Embryoid body
EC	Endothelial cell
ECGS	Endothelial growth supplement
ECM	Extracellular matrix
ESC	Embryonic stem cell
ET	Endothelin
FGF	Fibroblast growth factor
END-2	Visceral endoderm-like cells
FBS	Fetal bovine serum
HEK	Human embryonic kidney
HCl	Hydrochloric acid
HCN4	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4
H&E	Hematoxylin and eosin stain
hESC	Human embryonic stem cell
iPSC	Induced pluripotent stem cell
IWP2	Wnt inhibitor
GCaMP6f	Genetically encoded ultra-sensitive protein calcium sensors
GFP	Green fluorescent protein
GSK-3β	Glycogen synthase kinase 3β
HBSS	Hank's balanced salt solution
hERG	Human ether-a-go-go-related gene also known as Kv11.1
IL-6	Interleukin 6
ISL1	Insulin gene enhancer protein 1

JAG1	Jagged 1
JCN	Junctin
Kir	Inward-rectifier potassium ion channel
Kv	Voltage-gated potassium channel
MEF	Mouse embryonic fibroblasts
MEF2C	Myocyte-specific enhancer factor 2C
miR	MicroRNA
nanoLC MS/MS	Nanoscale liquid chromatography coupled to tandem mass spectrometry
MYL2	Myosin light chain 2
NaV1.5	Voltage-gated sodium channel
NCX1	Na+-Ca2+ exchanger 1
PBS	Phosphate buffer solution
PD 145065	Endothelin receptor inhibitor
PDMS	Polydimethylsiloxane
PSC	Pluripotent stem cells
qRT-PCR	Quantitative real time polymerase chain reaction
RyR	Ryanodine receptor
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SERCA2A	Sarcoplasmic reticulum calcium ATPase 2a
SEM	Standard error of the mean
SHG	Second Harmonic Generation
SSEA	Stage-specific embryonic antigen

SR	Sarcoplasmic Reticulum
T3	Triiodothyronine
TALEN	Transcription Activator-Like Effector Nucleases
Tbx20	T-box transcription factor 20
TGF-β	Transforming growth factor-beta
TS	Timothy Syndrome
T-tubule	Traverse tubules