Engineering Three Dimensional Cardiac Micro-Tissues Encapsulated with the Co-Culture of Cardiomyocytes and Cardiac Fibroblasts

by

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ABSTRACT

Cardiac tissue engineering has major applications in regenerative medicine, disease modeling and fundamental biological studies. Despite the significance, numerous questions still need to be explored to enhance the functionalities of the engineered tissue substitutes. In this study, three dimensional (3D) cardiac micro-tissues were developed through encapsulating co-culture of cardiomyocytes and cardiac fibroblasts, as the main cellular components of native myocardium, within photocrosslinkable gelatin-based hydrogels. Different co-culture ratios were assessed to optimize the functional properties of constructs. The geometry of the micro-tissues was precisely controlled using micro-patterning techniques in order to evaluate their role on synchronous contraction of the cells. Cardiomyocytes exhibited a native-like phenotype when co-cultured with cardiac fibroblasts as compared to the mono-culture condition. Particularly, elongated F-actin fibers with abundance of sarcomeric α-actinin and troponin-I were observed within all layers of the hydrogel constructs. Higher expressions of connexin-43 and integrin β1 indicated improved cell-cell and cell-matrix interactions. Amongst co-culture conditions, 2:1 (cardiomyocytes: cardiac fibroblasts) ratio exhibited enhanced functionalities, whereas decreasing the construct size adversely affected the synchronous contraction of the cells. Therefore, this study indicated that cell-cell ratio as well as the geometrical features of the micropatterned constructs are among crucial parameters, which need to be optimized in order to enhance the functionalities of engineered tissue substitutes and cardiac patches.

DEDICATION

I dedicate my work to my beloved parents, Mrs. & Mr. Bikramjit Singh.

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LIST OF ABBREVIATIONS:

3D	Three dimensional
2D	Two dimensional
ANOVA	Analysis of Variance
BMMNC	Bone marrow derived mononuclear cells
BSA	Bovine Serum Albumin
CMs	Cardiomyocytes
CFs	Cardiac Fibroblasts
CNT	Carbon Nanotube
Cl	Calcein
DAPI	4’,6-diamidino-2-phenylindole
DPBS	Dulbecco’s Phosphate Buffered Saline
ECM	Extracellular matrix
EthD	Ethidium Homodimer
FFT	Fast Fourier Transform
GelMA	Gelatin methacrylate
GNPs	Gold nanoparticles
GO	Graphene oxide
HA	Hyaluronic acid
HBSS	Hank’s Balanced Salt Solution
hESC	Human embryonic stem cell
IGF	Insulin like growth factor

iPSCs	Induced pluripotent stem cells
MeTro	Methacrylated tropoelastin
MMP-2	Matrix metalloprotease-2
MSC	Mesenchymal stem cell
PANI	Polyaniline
PCL	Polycaprolactone
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PEG-DA	Polyethylene glycol diacrylate
PF	Paraformaldehyde
PGCL	Poly-glycolide-co-caprolactone
PGS	Poly(glycerol-sebacate)
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PLLA	Poly(L-lactide acid)
PLN	phospholamban
PNIPAAm	Poly-N-isopropylacrylamide
RLP	Resilin-like polypeptide
TMSPMA	3- (trimethoxysilyl) propyl methacrylate
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Harpinder Saini, Feba S. Sam, Mahshid Kharaziha, Mehdi Nikkhah **"**Micropatterning Techniques to Control Cell-Biomaterial Interface for Cardiac Tissue Engineering" in "*Cell and Material Interface: Advances in Tissue Engineering, Biosensor, Implant, and Imaging Technologies*", Editors: Kris Iniewski, Nihal Engin Vrana, Taylor & Francis, (2014) *(Accepted, In press).* Reproduced with permission.

1.1. Native Cardiac Tissue

1.1.1. Major Cell Types within the Native Myocardium

There are three main cell types embedded within the native myocardium: cardiomyocytes, cardiac fibroblasts and endothelial cells (Iyer, Chiu, and Radisic 2009, Fleischer and Dvir 2013, Severs 2000). Cardiomyocytes account for 30% of the total number of cells within the myocardium (Ehler 2015, Iyer, Chiu, and Radisic 2009) . They are well distributed throughout the heart but those that beat fastest and determine the natural beating frequency of cardiac muscle are known as pacemaker cells(Mark and Strasser 1966). In human heart these cells are located at sinoatrial node (Malmivuo and Plonsey 1995). Specifically, cardiomyocytes have active machinery of myofibrils that contains thick and thin filaments of actin, myosin and titin (arranged in section known as sarcomeres) which under the effect of propagating electrical impulses lead to their contraction and relaxation (Nag 1980, Malmivuo and Plonsey 1995). These cells act coherently with each other through intracellular junctions (*e.g.* gap junctions) thus forming a three dimensional (3D) syncytium (Radisic et al. 2007a).

Among non-cardiomyocytes, cardiac fibroblasts are present in abundance (Camelliti, Borg, and Kohl 2005). They are crucial for extracellular matrix (ECM) synthesis and degradation within the myocardium (Souders, Bowers, and Baudino 2009, Castaldo et al.

2013). These cells also secrete various growth factors and cytokines in response to different stimuli via paracrine/autocrine signaling, which regulates processes such as cell growth, differentiation, migration etc (Souders, Bowers, and Baudino 2009). Cardiac fibroblast also participate in electrotonic conduction of impulses thereby leading to synchronous contraction of distant cardiomyocytes. Additionally, they play a significant role in cardiac tissue remodeling when presented with numerous biophysical/biochemical signals in various diseased states (Souders, Bowers, and Baudino 2009, Castaldo et al. 2013).

Endothelial cells, on the other hand are responsible for the formation of blood vessels and capillaries for oxygen supply, and also for waste removal throughout the tissue similar to the other organs (Radisic et al. 2007a). They are also involved in regulating the blood supply in accordance to hormonal and hemodynamic demand through vasodilation/ vasoconstriction of the capillaries (Brutsaert 2003b). The paracrine signaling between the endothelial cells and cardiomyocytes has been shown to significantly influence the functional properties such as contractility and rhythmicity of the myocardial tissue (Brutsaert 2003a, Ramaciotti et al. 1992, Narmoneva et al. 2004). Additionally, endothelial cells play a significant role in tissue remodeling by regulating infiltration of immune cells such as lymphocytes/macrophages at infarcted zone after onset of myocardial infarction (Frangogiannis, Smith, and Entman 2002). Myocardium also constitutes of various other cell types such as cardiac stem cells, pericytes cells, mast cells and macrophages; however they exist in small population and function in response to various conditions such as inflammation or presence of cytokines (Tirziu, Giordano, and Simons 2010).

1.1.2. Myocardial ECM

The myocardial ECM is a 3D architectural network with well-defined anisotropic structure, composed of major proteins such as collagen, elastin, vitronectin, fibronectin and laminin etc. (Castaldo et al. 2013). Various compositions of these proteins notably influence the characteristics of the matrix within healthy and diseased states and contribute to the contractile capacity of the heart (Castaldo et al. 2013, Marsano et al. 2010, Engler et al. 2008). For instance, collagen is the main loadbearing protein that transmits the force generated by the cardiomyocytes in systole phase while imparting the passive stiffness within the diastole. Collagen also prevents the dilation and edema of the muscle over a long period of time (Godier-Furnemont and Vunjak-Novakovic 2013, Chen et al. 2008a). Different types of collagen that have been identified in myocardial ECM consist of collagen type I, III, IV and VI. Collagen type I comprise around 85% of the fibrillar collagen affecting the overall rigidity of the heart muscle. Alternatively, collagen type III modulates matrix elasticity (Engler et al. 2008, Marsano et al. 2010, Chen et al. 2008a). Topography of the cardiac muscle can also be attributed to the folded and highly ordered structure of its components maintained by disulphide and hydrophobic bonds (Wang and Carrier 2011) . Overall, the stiffness and the architecture of the myocardial tissue provides the necessary signaling cues to support cardiac cells phenotype and functions such as cellular survival, proliferation, differentiation, migration etc. (Tandon et al. 2013, Ma, Halade, and Lindsey 2012). Furthermore, ECM also comprise of various biomolecules such as proteoglycans, glycoproteins etc.that can bind to other bioactive molecules and impact processes such as tissue remodeling, electrical transduction, mechanical transduction etc. (Ma, Halade, and Lindsey 2012).

1.1.3. Cell: Matrix Interactions

The interaction of cardiac cells with the anisotropic structure of myocardium is paramount for regulation of the tissue properties such as synchronous contractility (Feinberg et al. 2007, Au et al. 2007, Zhang et al. 2012, Au et al. 2009). In particular, cellular organization and the orientation of the actin fibers, through a process known as contact guidance, significantly influence the contractile force generated throughout the tissue (Au et al. 2007, Zhang et al. 2012, Au et al. 2009). For instance, in a study by Kim *et. al.*, it was shown that non-aligned cardiomyocytes generate 65-85% less contractile force in comparison to highly organized cells which exhibit rod-like morphologies (Kim et al. 2008). In this regard, cardiac tissue remodeling due to a diseased state (*e.g.* arrhythmia) can affect the ECM composition (*e.g.* excessive collagen deposition) and consequently lead to poor cellular organization and tissue contractility (Baig et al. 1998, Biernacka and Frangogiannis 2011). Intracellular calcium dynamics, which is a crucial factor during systole and diastole phases, gets also altered due to the changes in cellular alignment (Yin, Bien, and Entcheva 2004). Equivalently, It has been shown that junctional markers such as N-cadherin and connexin 43, which are responsible for mechanical and electrical signal propagations, are significantly influenced by cellular organization (Patel, Desai, and Kumar 2011). These markers in particular, which regulate synchronous beating and contraction of the cells will be highly expressed when cells are properly aligned within the tissue matrix (Patel, Desai, and Kumar 2011). Therefore, well ordered arrangement of cardiac cells is essential for viable structural integrity and proper functioning of the myocardium in a healthy state.

Besides biophysical cues, ECM also provides biochemical cues such as various growth factors and ligands within the myocardium. It has been established that cells interact with the matrix through a combination of proteins collectively called as focal adhesion complex (Samarel 2005a). Various transmembrane proteins such as vinculin and integrin β1 help in direct attachment of cell cytoskeleton to ECM and thus help in bidirectional transfer of biochemical/mechanical signals (Samarel 2005a). For instance, Integrin β1 help in mechanotransduction between ECM and cardiomyocytes or cardiac fibroblasts (Samarel 2005a, Ross and Borg 2001). They also assist in chemical signaling when any ligand binds to either intercellular receptor (inside-outside signaling) or to extracellular receptor (outside-in signaling); initiating a cascade of events (Ross and Borg 2001). Integrin β1 are also characterized to play a definite role in myofibrillogenesis, cellular phenotypes and cell migration (Ross and Borg 2001).

1.1.4. Cell: Cell Interaction

Cell: Cell interactions play a significant role in maintaining the intercellular communication between the cells (Zhang, Su, and Mende 2012). In myocardium, homogenous or heterogeneous cell types communicate intercellularly either through gap junctions or adherens (Zhang, Su, and Mende 2012). Gap junction are intercellular proteins that allow the direct flow of molecules, solutes and ions from one cytoplasm into another in between the adjacent cells (Souders, Bowers, and Baudino 2009). In addition, gap junctions are mainly responsible for electrical impulse propagation between the two coupled cells (Souders, Bowers, and Baudino 2009). The most commonly identified gap junctions in the heart have been connexin such as connexin 43, 45 and 40 (Souders, Bowers, and Baudino 2009, Camelliti, Borg, and Kohl 2005). Various studies have

observed that connexin 43 gets expressed at either homogenous cellular junction such as in between cardiomyocytes or and at heterogeneous cellular junction between cardiomyocyte and cardiac fibroblast cells (Camelliti, Borg, and Kohl 2005). Similarly, connexin 45 are also found to be expressed either at heterogeneous junctions between cardiomyocyte and cardiac fibroblasts or at homogeneous junctions between adjacent cardiac fibroblasts (Camelliti, Borg, and Kohl 2005). On the other hand, connexin 40 is mainly known to be expressed at homogenous cellular junction between cardiac fibroblasts (Camelliti, Borg, and Kohl 2005) . Due to the presence of gap junction molecules, an electrical syncytium is established in between the myocardial cells, which thereby ensures synchronous contraction of the tissue within the myocardium (Eckardt et al. 2004).

Cardiac cells are also known to communicate with each other intercellularly through adherens junctions which are transmembrane proteins that interconnect the actin/intermediate filaments of adjacent cells (Zhang, Su, and Mende 2012). These class of protein complexes are known to mechanically couple the cells and participate in myofibril organization along with other proteins such as Integrin β1 (Luo and Radice 2003). Some of the most common adheren molecules are N-cadherins which interconnect cardiomyocyte and cardiac fibroblast as well as cardiac fibroblast to cardiac fibroblast (Luo and Radice 2003). Cadherin-11 are expressed in between cardiac fibroblast and endothelial cells (Souders, Bowers, and Baudino 2009), while cadherin-13 play a major role in maintaining the robustness of vasculature within the heart (Souders, Bowers, and Baudino 2009).

1.2. Microengineering technologies and their application in Cardiac Tissue Engineering

The emergence of micro- and nanofabrication technologies in the past few years has enabled researchers to create platforms with precise geometrical features resembling the native *in vivo* cellular microenvironment (Chung, Kang, and Khademhosseini 2007, Murtuza, Nichol, and Khademhosseini 2009, Dvir, Timko, Kohane, et al. 2011, Nikkhah, Edalat, et al. 2012a, Zorlutuna et al. 2012, Khademhosseini et al. 2006). These technologies have been successfully adopted by biologists and bioengineers for a wide range of applications from tissue engineering to biosensors design and fundamental biological studies (Khademhosseini et al. 2006, Park and Shuler 2003, Gillette et al. 2008, Park et al. 2007, Atala, Kasper, and Mikos 2012). In particular, photolithography techniques adapted from semiconductor industry have gained significant attention to fabricate scalable topographical features using advanced biomaterials such as hydrogels (Khademhosseini et al. 2006, Nikkhah, Edalat, et al. 2012a). Using these techniques, it is possible to develop well-ordered structures (*e.g.* grooves, pillars, ridges) with precisely defined geometrical dimensions to control cell substrate interactions (Nikkhah, Edalat, et al. 2012a, Bettinger, Langer, and Borenstein 2009). Alternatively, soft lithography techniques such as microcontact printing and microfluidic patterning have been successfully employed to create two dimensional (2D) patterned features of ECM proteins to generate geometrically defined arrangement of cells (Xia and Whitesides 1998, Whitesides et al. 2001).

In the past few years, microfabrication techniques have found significant applications in numerous aspects of tissue engineering in general (Khademhosseini et al.

2006) and cardiovascular tissue engineering in particular (Chung, Kang, and Khademhosseini 2007, Camci-Unal et al. 2014, Zhang et al. 2011, Annabi et al. 2013, Iyer et al. 2012, Nikkhah, Eshak, et al. 2012). These techniques have enabled the development of *in vitro* bioengineered cardiac tissue substitutes to mimic the anisotropic architecture of native myocardium for regenerative medicine applications and fundamental biological studies (Zhang et al. 2011, Bursac et al. 2002, Kim et al. 2010). Furthermore using these technologies, it is possible to generate physiologically relevant cardiac related disease models for high throughput drug screening (Agarwal, Goss, et al. 2013, Natarajan et al. 2011). The following sections provides an overview on application of microengineering technologies to engineer *in vitro* cardiac tissues. First a brief summary has been provided on various types of biomaterials used in cardiac tissue engineering followed by recent use of micropatterning techniques to develop cardiac constructs.

1.3. Biomaterials in cardiac regeneration

The success of tissue engineering strategies for cardiac repair and regeneration is highly dependent on the selection of appropriate scaffolding biomaterials with enhanced biomechanical/biological properties (Camci-Unal et al. 2014, Vunjak-Novakovic et al. 2010). In particular, the selected biomaterial should be biocompatible and exhibit suitable mechanical robustness while supporting the cyclic contraction of the cardiac cells (Walsh 2005, Camci-Unal et al. 2014, Lanza, Langer, and Vacanti 2014). Biomaterials can be obtained naturally by either using cardiac tissue ECM (decellularized matrix) (Duan et al. 2011, Wainwright et al. 2010) or can be chemically synthesized (Chen et al. 2008a). Natural biomaterials such as collagen are considered to be suitable

candidates for cardiac regeneration since they are biocompatible in nature and they induce different signals to cells through surface receptor interactions. (Vunjak-Novakovic et al. 2010). Furthermore, these materials can be patterned using microcontact printing or micromolding techniques to provide sufficient anisotropy for generation of cardiac organoids (Kofidis et al. 2002, Chiu, Janic, and Radisic 2012, Zimmermann, Schneiderbanger, et al. 2002, Black et al. 2009). To date, numerous natural biomaterials such as collagen and matrigel (Chiu, Janic, and Radisic 2012, Zimmermann, Schneiderbanger, et al. 2002, Simpson and Dudley 2013) hyaluronic acid (Ifkovits et al. 2010, Yoon et al. 2009), gelatin (Li et al. 2000, Li et al. 1999), chitosan (Karp et al. 2006, Fujita et al. 2005), alginate (Zieber et al. 2014b, Amir et al. 2009), laminin (McDevitt et al. 2002), elastin (Annabi et al. 2013), fibrin (Black et al. 2009, Birla et al. 2005), cellulose-based scaffolds (Entcheva et al. 2004), plant origin polysaccharide (Venugopal et al. 2013), silk fibroin (Yang et al. 2009, Patra et al. 2012), as well as self-assembling peptides (Soler-Botija et al. 2014, Davis et al. 2006, Hsieh et al. 2006) have been used for cardiac regeneration. For instance in a study by Li *et. al.*, fetal rat ventricular cells were seeded on commercial gelatin-based foams (Gelfoam®), and implanted into rats onto the scarred area (Li et al. 1999). Seven days after implantation it was found that the cells on the implanted graft were beating, and the density of cardiomyocytes in the area was higher than initial seeding density (Li et al. 1999). In another study, artificial myocardial tissue was created by seeding cardiomyocytes on a collagen scaffold. The cells started beating by 36 hours of culture and maintained contractility for 12 weeks *in vitro* (Kofidis et al. 2002). Although natural biomaterials have numerous advantages, they suffer from poor mechanical properties (Kofidis, Mueller-Stahl, and Haverich 2007, Chen et al.

2008a). Furthermore, their degradation rate may not be optimal to develop engineered cardiac tissue substitutes to allow for sufficient extracellular matrix deposition (Chen et al. 2008a). Therefore, researchers have tried to overcome these limitations by developing synthetic biomaterials.

Biodegradable synthetic polymers have had extensive use in medical field for numerous applications including the development of patches and scaffolds for cellular transplantation and myocardial repair (Chen et al. 2008a). In particular, various synthetic polymers such as poly(ethylene glycol) (Jongpaiboonkit et al. 2008, Kraehenbuehl et al. 2008), polyglycolic acid (PGA) (Solan et al. 2003), poly(lactic-co-glycolic acid) (PLGA) (Ayaz et al. 2014), poly-glycolide-co-caprolactone (PGCL) (Piao et al. 2007), poly(L-lactic acid) (PLLA) (Zong et al. 2005, Caspi et al. 2007) and its copolymers with PLGA (Zong et al. 2005, Caspi et al. 2007), poly(glycerol-sebacate) (PGS) (Engelmayr et al. 2008, Radisic, Park, et al. 2008), poly-N-isopropylacrylamide (PNIPAAm) and their copolymers (Miyagawa et al. 2005, Naito et al. 2004, Wang et al. 2010), polyurethane (Rockwood et al. 2008), and polycaprolactone (PCL) (Soler-Botija et al. 2014, Shin et al. 2004) have been widely utilized in cardiac regeneration. For instance, in a study by Piao *et. al.*, PGCL scaffolds were seeded with bone marrow derived mononuclear cells (BMMNC) for treatment of myocardial infarction in animal model (Piao et al. 2007). The developed scaffolds were found to be biocompatible while promoting vascular formation as well as the migration of BMMNCs into the epicardial region (Piao et al. 2007).

With the advancements in cardiac tissue engineering, there has been an increasing demand toward the development of innovative biomaterials, which can respond to the microenvironmental cues and provide sufficient signaling to the surrounding cells for

rapid regeneration of the injured tissue (Sakiyama-Elbert and Hubbell 2001). Particularly, composite biomaterials have been proposed to address these needs (Qazi et al. 2014, Kharaziha et al. 2013, Ozawa et al. 2004, Kharaziha et al. 2014, McGann, Levenson, and Kiick 2013). For instance, the blends of natural and synthetic polymers such as copolymers made of gelatin with PCL, PLA and PGS are among the examples of composite biomaterials, which have been widely used for cardiac regeneration (Kharaziha et al. 2013, Ozawa et al. 2004, Ifkovits et al. 2009). Hybrid biomaterials incorporated with nanoparticles, such as carbon nanotubes (CNTs) (Shin et al. 2013, Kharaziha et al. 2014), gold nanoparticles (GNPs) (Dvir, Timko, Brigham, et al. 2011), and graphene oxide (GO) (Shin et al. 2014), also belong to the emerging class of innovative biomaterials for cardiac tissue engineering. For instance, Shin *et. al.* embedded CNTs within photocrosslinkable gelatin methacrylate (GelMA) hydrogel to develop functional cardiac patches. Incorporation of CNTs significantly enhanced the electrical conductivity and mechanical robustness of the hybrid hydrogel and ultimately improved electrophysiological functionalities of cardiomyocytes(Shin et al. 2013). In a similar study, an electrically conductive cardiac patch was developed via integration of gold nanowires within alginate hydrogel. The developed constructs significantly enhanced protein expression, alignment, and synchronous contraction of cardiac cells (Dvir, Timko, Brigham, et al. 2011). Oxygen-releasing materials are also considered to be suitable candidates which provide the cardiac cells with sufficient oxygen to maintain their viability and functionality (Oh et al. 2009).

1.4. Micropatterning techniques in cardiac tissue engineering

1.4.1. Microfabricated 2D in vitro models

To date, numerous studies have utilized 2D *in vitro* cardiac tissue models for various applications ranging from fundamental biological studies (*e.g.* cell-substrate interactions) to regenerative medicine, disease modeling and drug screening (Cimetta et al. 2009, Salick et al. 2014, McDevitt et al. 2002, Khademhosseini et al. 2007, Thery 2010, Grosberg et al. 2012, Feinberg et al. 2012, Alford et al. 2010, Bray et al. 2010, Kuo et al. 2012, Shim et al. 2012, Feinberg et al. 2013, Bursac et al. 2002, Serena et al. 2012, Natarajan et al. 2011, Agarwal, Farouz, et al. 2013, McDevitt et al. 2003, Yasukawa et al. 2013). The 2D models are mainly engineered through micropatterning of ECM proteins using soft lithography techniques such as microcontact printing and microfluidic patterning (Zhang et al. 2011, Folch 2012). Microcontact printing is a well-respected technique where the proteins of interest can be easily transferred from a microfabricated rubber stamp to the desired areas of the substrate, which comes in contact with the stamp (Xia and Whitesides 1998, Whitesides et al. 2001). Stamps with various geometrical features are usually fabricated in PDMS using soft lithography techniques. The transfer of the desired proteins on the substrate is mainly governed due to differences in the hydrophilicity of the surfaces (Xia and Whitesides 1998, Whitesides et al. 2001). In a recent study by Salick *et. al.*, PDMS stamps with different aspect ratios (width/length ratio) were used to pattern fibronectin and matrigel on glass slides (Salick et al. 2014). Human embryonic stem cell differentiated cardiomyocytes (hESC-CMs) were seeded on the patterned features, and the effect of constructs' aspect ratio on sarcomere alignment was investigated. Based on the findings of this study, the width of the constructs had a

pronounced effect on sarcomere alignment as compared to the aspect ratio (Salick et al. 2014). Furthermore, it was shown that that constructs with a width in the range of 30-80 μm notably enhanced sarcomere alignment **(Figure 1.1A)** (Salick et al. 2014). Similarly, this technique was successfully employed by McDevitt *et. al.*, where laminin was micropatterned on non-adhesive polystyrene surfaces to study the effects of geometrical constraint of laminin lanes (width) on synchronous beating of neonatal cardiomyocytes (McDevitt et al. 2002). According to this study, narrower laminin lanes with 15-20 μm width, resulted in aligned and bipolar cell-cell junctions similar to native myocardium **(Figure 1.1B)** (McDevitt et al. 2002). Although microcontact printing on 2D surfaces has been widely accepted as an efficient technique for the patterning of cardiac cells (Cimetta et al. 2009, Salick et al. 2014, Thery 2010, Grosberg et al. 2012, Feinberg et al. 2012, Bray et al. 2010, Kuo et al. 2012, Bursac et al. 2002, Yasukawa et al. 2013), there are several disadvantages associated with this approach. Some limitations include denaturation of patterned proteins, necessity of using multiple stamps to pattern several proteins onto same substrate, stamp deformation, etc. (Zhang et al. 2011, Folch 2012, Perl, Reinhoudt, and Huskens 2009). Another popular technique for 2D micropatterning is microfluidic patterning which addresses some of the limitations of microcontact printing (Folch 2012, Wang et al. 2014). The advantages of microfluidic patterning are delivery of proteins on selective areas of the substrate in their natural form and thus preserving them from denaturation (Folch 2012). This technique utilizes the microchannels formed when a fabricated PDMS stamp comes into contact with the substrate. The proteins to be patterned are then delivered, with a fluidic carrier, to the areas of substrate which do not come into contact with PDMS stamp (Folch 2012).

Khademhosseini *et. al.*, successfully employed microfluidic patterning technique to pattern hyaluronic acid onto glass substrates and generate contractile cardiac constructs (Khademhosseini et al. 2007). They showed successful alignment of neonatal rat cardiomyocytes along with the patterns layout. Interestingly, the patterned cells were detached from the substrate and formed contractile organoids after 3 days of culture (Khademhosseini et al. 2007). The use of electrical field in microfluidic systems has been also suggested to develop highly organized cellular constructs mimicking the anisotropy of the native cardiac tissue (Yang and Zhang 2007). For instance in a study by Yang *et. al.*, dielectrophoresis in a microfluidic device, was used to induce cellular alignment along the direction of electrical field. The results of this study demonstrated successful cellular orientation in between the electrodes (Yang and Zhang 2007). Similar to microcontact printing, microfluidic patterning also suffers from many limitations such as buckling of the PDMS stamp and leakage of ECM proteins from microfluidic channels (Folch 2012).

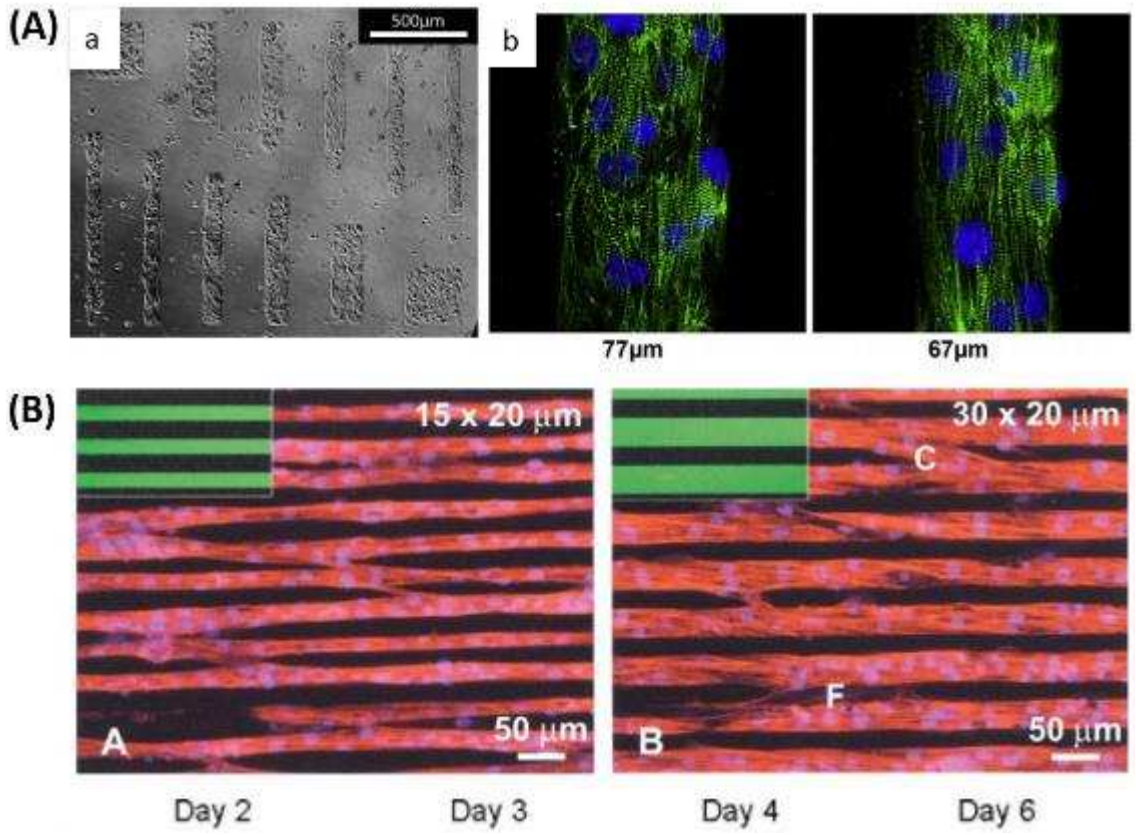

Figure 1.1: Application of 2D micropatterning techniques in cardiac tissue engineering; (A) Micropatterned matrigel-fibronectin features with different aspect ratios seeded with hESC-CMs; (a) Phase contrast images of the patterned cardiac cells, (b) Confocal images demonstrating sarcomere organization (green stain) and nuclei (blue) orientation across different widths. Adapted from Salick *et. al.* with permission from Elsevier [Biomaterials], copyright (2014) (Salick et al. 2014). (B) Microcontact printed lanes of laminin demonstrating the actin cytoskeleton (red) and nuclei (blue) organization of aligned cardiomyocytes across patterns with variable width. Inset images show laminin printed lanes. Adapted from McDevitt, *et. al.* with permission from Wiley [Journal of Biomedical Materials Research], copyright (2002) (McDevitt et al. 2002).

Overall, microcontact printing and microfluidic patterning techniques have both shown great potential as simple and efficient approaches to form highly organized cardiac constructs to study fundamental biological questions regarding cardiac cells and ECM interactions (Cimetta et al. 2009, Salick et al. 2014, Thery 2010, Grosberg et al. 2012, Feinberg et al. 2012, Bray et al. 2010, Kuo et al. 2012, Bursac et al. 2002, Yasukawa et al. 2013). However, many of the geometrical cues, which modulate native like cellular

functionalities, are missing on 2D patterned surfaces. For instance, the lack of third dimension in these approaches significantly influences cellular phenotype and function (Thery 2010). Therefore, in the past few years, there has been tremendous efforts to engineer physiologically relevant cardiac tissue models using 3D topographical surfaces or micropatterned hydrogels. These approaches are summarized in the following section.

1.4.2. Microfabricated 3D tissue constructs

3D tissue constructs, fabricated using polymeric biomaterials or hydrogels, provide a more realistic microenvironment compared to 2D models for various tissue engineering applications (Thery 2010, Camci-Unal et al. 2014, Khademhosseini et al. 2006, Zorlutuna et al. 2012). To date, 3D microfabricated models such as surface topographies, micropatterned hydrogel constructs, and microengineered polymeric biomaterials have been utilized to impart necessary biophysical cues to control cardiac cells phenotype, cytoskeletal organization and contractility (Kolewe et al. 2013, Engelmayr et al. 2008, Rodriguez et al. 2014, Guillemette et al. 2010, Bian, Jackman, and Bursac 2014, Rao et al. 2013, Entcheva and Bien 2003, Bian et al. 2014b, Motlagh et al. 2003, Chen et al. 2014, Arai et al. 2008b, Wang et al. 2013, Au et al. 2007, Luna et al. 2011, Yu et al. 2013, Bursac et al. 2002, Kajzar et al. 2008, Zhang et al. 2012, Yin, Bien, and Entcheva 2004, Patel, Desai, and Kumar 2011, Deutsch et al. 2000, Annabi et al. 2013). Different topographical features can be fashioned in polymeric or rigid biomaterials (*e.g.* silicon) using photolithography and micromolding techniques (Folch 2012, Bettinger, Langer, and Borenstein 2009, Nikkhah, Edalat, et al. 2012a). Patterned substrate can be etched to generate 3D surface topographies with the desired geometrical features to proceed with the biological studies (Bettinger, Langer, and Borenstein 2009,

Nikkhah, Edalat, et al. 2012a). On the other hand, patterned or etched substrates can be used as a master mold to replicate the features in polymeric biomaterials (*e.g.* PDMS) using micromolding techniques (Xia and Whitesides 1998). For instance, in an early study by Desai group, microtextured surfaces in silicone including two distinct designs of microposts (micropegged features) and microgrooves coated with laminin were fabricated for cardiac tissue engineering (Deutsch et al. 2000). The goal of this study was to develop a physiologically relevant cell culture substrate that can enhance cardiac cell attachment and alignment. Cardiac cells oriented, bridged the gaps and attached to the end points of the pegged features. Alternatively, the cells on microgrooved features aligned along the direction of the grooves. Overall, cellular attachment was higher on the micropegs as compared to flats surfaces (control) (Deutsch et al. 2000). In another study by Luna *et. al*., a non-photolithographic technique was used to generate parallel wrinkles in the range of nano- and microscale on a PDMS substrate to study cardiac cell organization and protein marker expression (Luna et al. 2011). In this approach, a layer of metal (gold palladium) was first deposited on a pre-stressed polystyrene sheet to generate wrinkles. Thereafter, the wrinkled substrates were used as molds to cast PDMS a. Seeding of neonatal mouse cardiomyocytes and hESC-CMs resulted in the formation of highly organized cellular constructs on the wrinkled surfaces. Further analysis confirmed the orientation of N-Cadherin as well as connexin-43 along the major axis of the wrinkles (Luna et al. 2011). Rodriguez *et. al.* fabricated an array of patterned microposts to quantify passive tension, twitch force and the frequency of spontaneous beating of human induced pluripotent stem cells derived cardiomyocytes (hiPSC-CMs) (Rodriguez et al. 2014). The elastomeric microposts were fabricated in PDMS using soft

lithography technique. The microposts were then stamped with different ECM proteins such as collagen type IV, fibronectin and laminin to enhance cellular attachment. The findings of this study demonstrated that microposts coated with laminin resulted in enhanced cellular attachment with higher spreading as compared to collagen and fibronectin coated substrates. This platform was reported to be versatile to measure the contractility of the cardiac cells in healthy, diseased, differentiated and undifferentiated states. However, a major limitation of this study was the lack of cellular alignment on the micropost array (Rodriguez et al. 2014). In a similar context, Rao *et. al.*, studied the effects of fibronectin coated PDMS microgrooves on calcium cycling of hiPSC-CMs. Comparing to flat substrates (control condition), the cells exhibited enhanced alignment and sarcomere organization on the microgrooved features. Furthermore, improved Ca^{2+} cycling, in response to caffeine, was observed on structured surfaces **(Figure 1.2A)** (Rao et al. 2013). In another interesting study by Engelmayr *et. al.*, accordion like honeycomb structures were fabricated to provide a biomimetic microenvironment for cardiac tissue engineering (Engelmayr et al. 2008). The rationale behind the development of such structures was the honeycomb architecture of collagen fibers surrounding cardiomyocytes within the native myocardial tissue. These structures were fabricated using microablation technique through orienting two square shaped pores at 45° on a PGS scaffold. The microfabricated 3D scaffolds exhibited excellent anisotropic mechanical properties matching the native cardiac tissue properties. Seeding neonatal rat cardiomyocytes on the developed scaffolds, resulted in enhanced cellular orientation and directional contractile properties **(Figure 1.2B)** (Engelmayr et al. 2008).

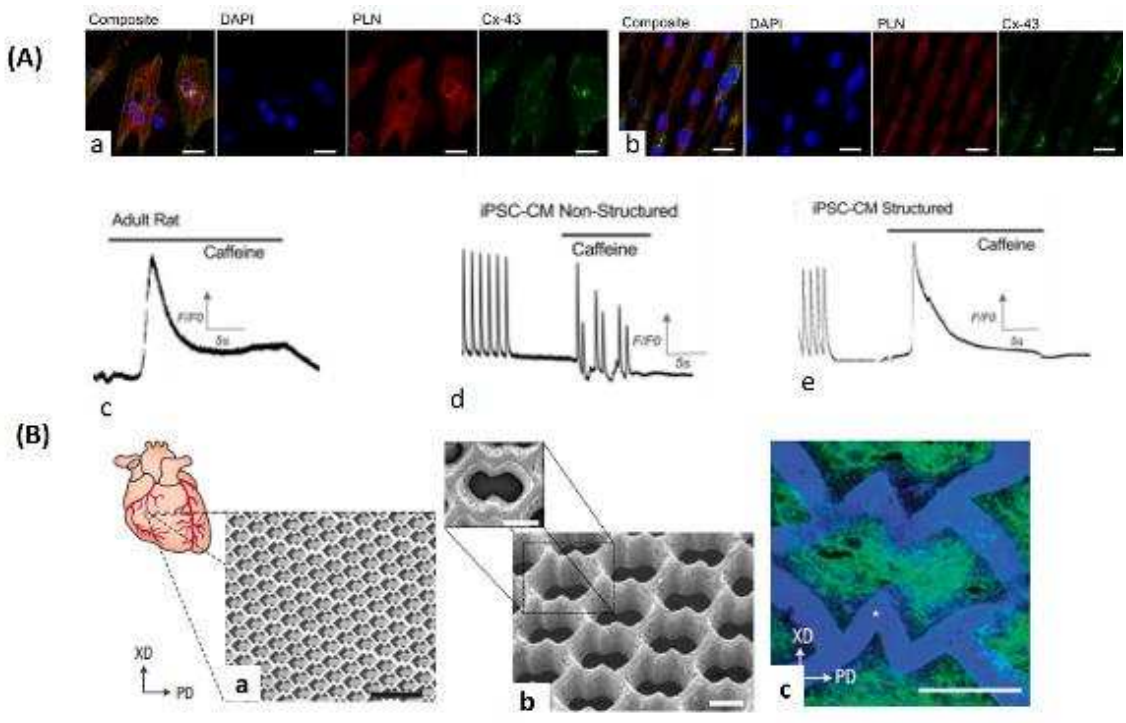

Figure 1.2: Illustrative examples for 3D scaffolds for cardiac tissue engineering. (A) Microgrooved PDMS structures seeded with hiPSC-CM ; (a) Immunostained hiPSC-CM on (a) flat and (b) Structured PDMS substrates demonstrating the expression of cardiac specific and nuclei marker, (c, d, e) Representative traces of Ca^{2+} release from sarcoplasmic reticulum in response to caffeine for (c) Adult rat heart, (d) Cells seeded on unstructured and (d) Structured PDMS substrates. Adapted from Rao *et. al.* with permission from Elsevier [Biomaterials], copyright (2013) (Rao et al. 2013). (B) Honeycomb structure fabricated in PGS; (a) Representative images showing the honeycomb structure of collagen fibers in native cardiac tissue, (b) Microfabricated accordion-like honeycomb structure, (c) Confocal images demonstrating actin cytoskeleton (green) organization and cellular alignment. Adapted from Engelmayr *et. al.* with permission from Nature publishing group [Nature Materials], copyright (2008) (Engelmayr et al. 2008).

Hydrogels are favorable biomaterials with attractive properties for cardiac tissue engineering applications (Camci-Unal et al. 2014, Zorlutuna et al. 2012, Shin et al. 2013, Dvir, Timko, Brigham, et al. 2011). Hydrogels exhibit high water content, tunable mechanical properties (*e.g.* stiffness) and structural architecture (*e.g.* porosity) while providing a 3D native microenvironment to support cellular growth and assembly (Peppas et al. 2006). To date, several studies have used micropatterned hydrogel

constructs to provide biomimetic topographical anisotropy for cardiac tissue engineering (Agarwal, Farouz, et al. 2013, Annabi et al. 2013, Zhang et al. 2013, Iyer, Chui, and Radisic 2009, Al-Haque et al. 2012, Chiu, Janic, and Radisic 2012, Karp et al. 2006, Aubin et al. 2010, Iyer, Chiu, and Radisic 2009). For instance, in a recent study by Annabi *et. al.*, methacrylated tropoelastin (MeTro) hydrogel, with suitable resilience, was used to develop micropatterned cardiac patches (Annabi et al. 2013). Patterns of 20 x 20 μm (width x spacing) and 50 x 50 μm channels were formed, using replica molding technique, and subsequently, photocrosslinked through UV exposure. Unpatterned MeTro and micropatterned GelMA hydrogel were used as control. The findings of this study demonstrated that neonatal rat cardiomyocytes exhibited higher cellular attachment, proliferation, elongation as well as cardiac marker expression (troponin I, connexin 43, sarcomeric α actinin) on micropatterned MeTro features as compared to control conditions. Furthermore, micropatterned MeTro substrates significantly promoted the spontaneous contractility of the cardiac cells for a long period of culture time (two weeks) **(Figure 1.3A)** (Annabi et al. 2013). In another study, Zhang *et. al.* studied functional and structural maturation of hESC-CMs on micropatterned fibrinogen and matrigel hydrogels (Zhang et al. 2013). The differentiated cardiac cells were encapsulated in the hydrogel solution and poured over the PDMS micromold, to polymerize at 37° C. Within 2 weeks of culture, the cardiac cells reorganized, with aligned actin fibers, along the patterned layouts. In addition, the cells expressed high levels of sarcomeric α actinin, N-cadherin, troponin-T and connexin 43 along with enhanced conduction velocity (action potential propagation) within the 3D micropatterned hydrogel patches as compared to 2D

monolayer substrates **(Figure 1.3B)** (Zhang et al. 2013).Table 1-1 provides a brief summary of some other 2D/3D studies in the field of cardiac tissue engineering.

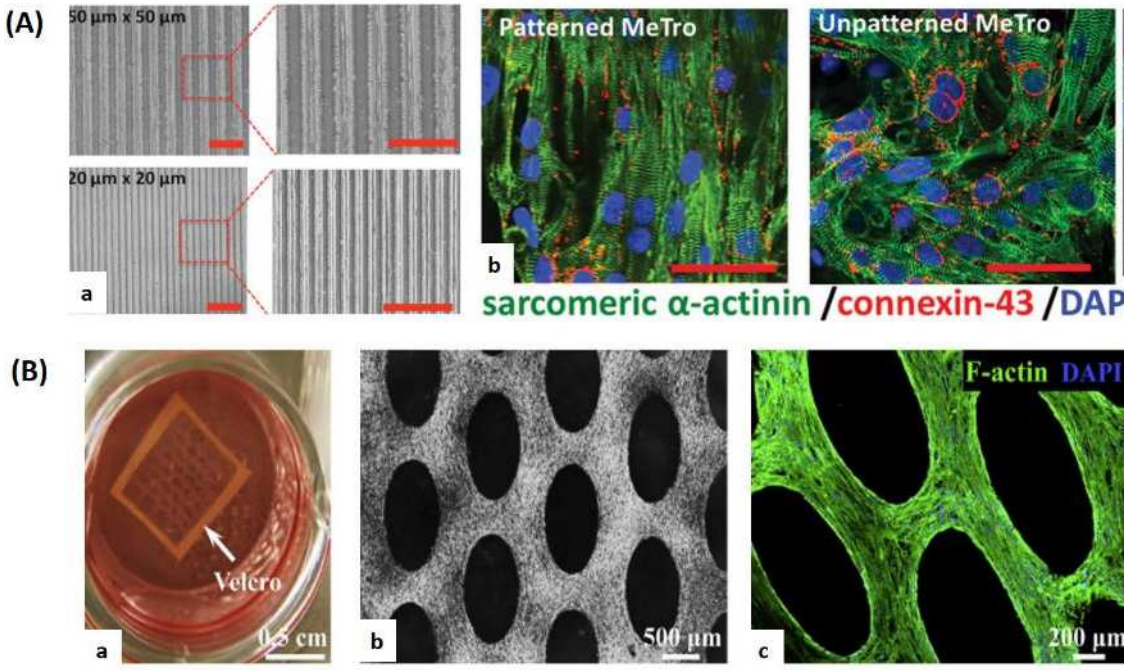

Figure 1.3: Representative examples of 3D micropatterned hydrogels for cardiac tissue engineering. (A) Honeycomb structure fabricated in PGS; (A) Micropatterned MeTro hydrogels: (a) Phase contras images showing the patterned layouts with variable dimensions, (b) Confocal images of cardiac specific markers on patterned and unpatterned substrate. Adapted from Annabi *et. al.* with permission from Wiley [Advanced Functional Materials], copyright (2013) (Annabi et al. 2013). (B) Fibrin based cardiac tissue patch; (a) Representative image of the patch, (b) Elliptical pores within the patch, (c) Densely aligned cells with highly organized actin cytoskeleton fibers embedded within the patch. Adapted from Zhang *et. al.* with permission from Elsevier [Biomaterials], copyright (2013) (Zhang et al. 2013).

Table 1. A brief summary of some other studies utilizing micropatterning techniques in Cardiac Tissue Engineering. Note: 2D and 3D describes substrate topographies.

2D/3D	Cell Type	Material	Design Feature	Purpose of Study	Ref.
2D	CM	Poly-Vinyl Chloride	Microabrasion, Micropatterning	Control anisotropy at macro scale	(Bursac et al. 2002)
2D	HESC derived CM	Poly-acrylamide	Micropatterning	In-Vitro Disease Model	(Serena et al. 2012)
2D	CM and VSMC	PNIPAAM coated PDMS	Micropatterning	Contractility Measurements	(Alford et al. 2010)
2D	CM and VSMC	Alginate	Micromolding/ Microcontact printing	Contractility Measurements	(Agarwal, Farouz, et al. 2013)
2D	CM, CF	Collagen Type I	Microfluidic patterning	In-Vitro 2D model, alignment	(Camelliti, McCulloch, and Kohl 2005)
2D	CM, CF, EC	Alginate bound with RGD/HBP	Co_2 Laser Cutting/ Engraving system	Vessel like network formation, localization of cells upon tri-culture	(Zieber et al. 2014b)
3D	CM, CF, EC	Matrigel	Microchannels of PEG Disc	Optimal timing and fraction for seeding	(Iyer, Chui, and Radisic 2009)
3D	CM , CF	Collagen Type I and Matrigel	Microwells containing Microposts	Mimic organization of myocardium under different conditions.	(van Spreeuwel et al. 2014)
3D	CM , CF	Collagen Type I	MicroTUG containing microposts	Study electro-mechanical coupling using AFM	(Galie et al. 2015)
3D	mESC-CM, mESC-CVP	Fibrin	PDMS mold with hexagonal posts	Engineer tissue patch from stem cells and CVP's, improved level of differentiation,	(Liau et al. 2011)

1.4.3 Effect of electrical and mechanical stimulation on microfabricated cardiac tissues

In addition to structural and topographical cues, electrical and mechanical stimulations enhance the maturity and functionality of engineered cardiac tissues (Hsiao et al. 2013, Hirt et al. 2014, Miklas et al. 2014, Zimmermann, Melnychenko, Wasmeier, Didie, et al. 2006). Cells grown on scaffolds have to be stimulated via either electrical signals (Radisic et al. 2004) or mechanical stimulation (Zimmermann, Schneiderbanger, et al. 2002) to achieve optimal conditions similar to those in the native heart. Particularly, electrical stimulation results in the alignment of cardiac fibers, promotes cellular differentiation, and enhances contractile properties of the tissue (Kreutziger and Murry 2011, Radisic, Marsano, et al. 2008, Radisic et al. 2004, Radisic et al. 2007a, Annabi et al. 2013, Zimmermann, Melnychenko, and Eschenhagen 2004, Park et al. 2104). In this regard, numerous studies have incorporated the effects of electrical stimulation on micropatterned scaffolds (Annabi et al. 2013, Thavandiran et al. 2013, Park et al. 2104, Boudou et al. 2012, Tandon et al. 2009, Alford et al. 2010, Chiu, Janic, and Radisic 2012). For instance, Park *et. al.*, utilized microfabricated PGS scaffold to study the individual and combined effects of insulin like growth factor (IGF-1) and electrical stimulation on maturation of engineered cardiac tissues (Park et al. 2104). PGS scaffolds, with excellent mechanical and biodegradability properties, were fabricated with rectangular shape pores using photolithography and micromolding techniques. Neonatal cardiac cells were then seeded on the scaffolds under four different conditions including: IGF-1 only, electrical stimulation only, with IGF-1 and electrical stimulation, and without electrical stimulation and IGF-1. Monophasic electrical stimulations, with 5V/cm amplitude and 1 HZ frequency for duration of 2 ms were used to induce contractility

within the engineered tissue constructs. Electrical stimulation enhanced the orientation of tissue like bundles, parallel to the electrical field, and significantly improved the expression of matrix metalloprotease-2 (MMP-2). The presence of IGF-1 reduced the excitation threshold, while the integration of IGF-1 and electrical stimulation further promoted the expression of cardiac gap junction markers (connexin 43) and sarcomere organization (Park et al. 2104). In another study, Chiu *et. al.* studied the combined effects of topographical cues and electrical stimulation on the engineered cardiac tissues fabricated in collagen-chitosan hydrogels (Chiu, Janic, and Radisic 2012). The topographical features were composed of microgrooves with the width in the range of 10, 20, and 100 μm. A custom made bioreactor system was used to induce 2.5 V/cm biphasic electrical pulses with 1 Hz frequency on the engineered tissue constructs seeded with neonatal rat cardiomyocytes. Cardiac cells reorganized along the major axis of the microgrooved features upon six days of culture, while cellular alignment significantly reduced the electrical stimulation threshold. Specifically, 10 μm width microgrooves resulted in the formation of complete contractile tissues comprised of mature gap junctions while the presence of electrical stimulation promoted cellular density (Chiu, Janic, and Radisic 2012). Au *et. al.* also investigated the combinatorial effects of electrical field stimulation and surface topography on cardiomyocytes organization on polyvinyl substrates consisting of V-shaped grooves of 13 μm width and 700 nm high. Their findings demonstrated the topographical cues and electrical field stimulation resulted in enhanced cellular elongation and alignment along the direction of microgrooves (Au et al. 2007).

Similar to electrical stimulation, a number of other studies have utilized mechanical stimulation to enhance the maturity and functionalities of engineered cardiac tissues (Shachar, Benishti, and Cohen 2012, Zimmermann, Schneiderbanger, et al. 2002, Miklas et al. 2014, Zhang et al. 2012). Zimmermann and Eschenhagen performed numerous studies on this subject using cardiac tissues fabricated in collagen and matrigel (Eschenhagen et al. 1997, Fink et al. 2000, Zimmermann, Didie, et al. 2002, Zimmermann, Schneiderbanger, et al. 2002, Zimmermann, Melnychenko, Wasmeier, Didie, et al. 2006). Their findings demonstrated that mechanical stimulation could lead to enhanced cardiomyocyte organization with increased mitochondrial density and improved length of myofilaments. They further concluded that under the effect of mechanical stimulation, highly differentiated cardiac muscle syncytium will be developed with contractile and electrophysiological characteristics similar to the native myocardium (Zimmermann, Melnychenko, Wasmeier, Didie, et al. 2006, Zimmermann, Schneiderbanger, et al. 2002, Fink et al. 2000). In another study by Miklas *et. al.*, a custom made bioreactor setup was used to simultaneously induce electrical and mechanical stimulation on patterned cardiac tissues (Miklas et al. 2014). The bioreactor design consisted of eight individual microwells fabricated in PDMS. Each microwells had two end posts acting as fixation points to the tissue along with two electrodes for electrical stimulation. Mechanical stimulation (5% cyclic stretch) was induced using a pneumatically actuated stretching setup while electrical stimulation was generated through paired carbon electrodes within each chamber. Neonatal rat cardiomyocytes were encapsulated in collagen type I hydrogel and then injected within each microwell for subsequent experimental analysis. Following cell culture for three days, electrical and

mechanical stimulation in individual and combinatorial settings were applied to the micro tissues for duration of three days. Mechanical stretch along with electrical stimulation significantly enhanced sarcomere and troponin-T expression throughout the tissues as compared to individualized stimulation conditions (Miklas et al. 2014).

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CHAPTER 2

THREE DIMENSIONAL CARDIAC MICRO-TISSUES ENCAPSULATED WITH THE CO-CULTURE OF CARDIOMYOCYTES AND CARDIAC FIBROBLASTS

Harpinder Saini, Ali Navaei, Alison Van Putten, Mehdi Nikkhah: "Three Dimensional Cardiac Micro-Tissues encapsulated with the Co-Culture of Cardiomyocytes and Cardiac Fibroblasts". Advanced Healthcare Materials. 2015. In Press.

2.1. INTRODUCTION

Heart failure and cardiovascular diseases are increasingly affecting numerous people across the globe, with the number of fatalities estimated to increase to 23.6 million by 2030 (Mozaffarian et al. 2015). Myocardial infarction alone can lead to a significant cell loss and adverse remodeling of ventricular myocardium in a short period of time (Laflamme and Murry 2011, Tous et al. 2011). Due to the limited regeneration potential of ventricular specific cardiomyocytes, myocardial infarction can ultimately lead to catastrophic heart failure (Laflamme and Murry 2011). In this regard, heart transplantation has been a partially successful approach due to inadequate number of donors and the clinical complications associated with the surgical procedures (Chen et al. 2008b).

During the past few years, tissue engineering has emerged as a powerful alternate to develop *in vitro* cardiac constructs with native like properties (Hirt, Hansen, and Eschenhagen 2014). Engineered tissue substitutes can be potentially used for regeneration medicine, fundamental biological studies, disease modeling and drug screening applications (Hirt, Hansen, and Eschenhagen 2014, Lee et al. 2015). To date, different types of synthetic (Ren et al. 2012, Kolewe et al. 2013) and natural-based

(Radisic et al. 2003, Gishto, Farrell, and Kothapalli 2015) scaffolding biomaterials have been utilized for specific applications in cardiac tissue engineering. Additionally, integration of micro- and nanoengineering technologies (Yu et al. 2013, Arai et al. 2008a, Tsang et al. 2015, McDevitt et al. 2002, Agarwal, Farouz, et al. 2013, Guillemette et al. 2010, McCain et al. 2014, Annabi et al. 2013) and advanced biomaterials (Shin et al. 2013, Dvir, Timko, Brigham, et al. 2011, Kharaziha et al. 2013) has enabled the development of cardiac tissues with well-defined architecture and cellular organization. While these studies have significantly advanced the knowledge in terms of biological behavior of cardiomyocytes, numerous aspect still needs to be optimized to induce superior tissue-level functionalities. For instance, it is well known that native myocardium is made up of only 30% of myocytes; and the rest of the tissue is populated by non-myocyte cells such as fibroblast, endothelial and smooth muscle cells (Radisic et al. 2007b). However, the majority of the previously reported studies have focused on mono-culture condition through incorporation of cardiomyocytes within their models. Therefore, a crucial criteria to generate *in vivo* like properties, is through the incorporation of non-myocyte cells along with cardiomyocytes within engineered tissue constructs (Hussain et al. 2013, Bian et al. 2014a, Iyer, Chui, and Radisic 2009).

Among the non-myocytes, cardiac fibroblasts are the largest cell population in the myocardium (Radisic et al. 2007b). Cardiac fibroblasts are primarily responsible for the synthesis or degradation of various components of the extracellular matrix (ECM) including fibrin and collagen (Camelliti, Borg, and Kohl 2005). These cells also play a significant role in mechanotransduction and electrical conduction across the heart (Radisic et al. 2007b, Chiquet et al. 2009). Furthermore, cardiac fibroblasts affect

cardiomyocyte phenotype (Kakkar and Lee 2010), in a way that the communication between the two cell types significantly influence the overall functionalities of the tissue (Radisic et al. 2007b, Camelliti, Borg, and Kohl 2005). To date, a number of studies have exploited the co-culture of cardiomyocytes and cardiac fibroblasts within engineered tissue constructs (Lancaster et al. 2014, Zimmermann, Melnychenko, Wasmeier, Didié, et al. 2006, Hussain et al. 2013, van Spreeuwel et al. 2014, Galie et al. 2015, Iyer, Chiu, and Radisic 2009, Zieber et al. 2014a, Camelliti, McCulloch, and Kohl 2005, Iyer, Chui, and Radisic 2009). For instance, Lancaster *et al.* used three dimensional (3D) scaffolds embedded with human dermal fibroblasts and then seeded the scaffolds with neonatal rat cardiomyocyte for treatment of chronic heart failure. The outcome of this study demonstrated enhanced electrophysiological properties of infracted rat heart confirmed through increased levels of ejection fraction, cardiac index and mean arterial pressure (Lancaster et al. 2014). Similarly, other studies have utilized cardiac fibroblasts to promote differentiation of stem cells towards adult like cardiomyocyte (Parrag, Zandstra, and Woodhouse 2012, Liau et al. 2011, Zhang et al. 2013). These studies suggest that co-culture of cardiomyocytes and cardiac fibroblasts is a key step in promoting the maturity of engineered tissues. However, another crucial factor to enhance tissue-level functionalities is the incorporation of the cells within 3D matrices rather than seeding on 2D scaffolds. For instance, in a recent study, more mature T-tubule development and enhanced excitation-contraction coupling were demonstrated when cardiac cells encapsulated within matrigel as opposed to seeding of the cells on 2D substrates (Bian et al. 2014a).

In this work, 3D hydrogel-based cardiac micro-tissues were developed, with variable geometrical features, comprised of the co-culture of cardiomyocytes and cardiac fibroblasts. The primary hypothesis was that the ratio of cardiac fibroblasts should be optimized in order to enhance the structural and functional properties of the *in vitro* constructs. Furthermore, it was postulated that the geometrical features of the constructs would significantly impact the synchronous contraction of the cells. The insights provided by this study can be useful in the development of the next generation of cardiac tissue substitutes for regenerative medicine and disease modeling applications.

2.2. EXPERIMENTAL SECTION

2.2.1. Synthesis of gelatin methacrylate (GelMA) hydrogel

GelMA was prepared as described in previous protocols (Nichol et al. 2010). Briefly, Gelatin (type A; sigma) was added to Dulbecco's phosphate buffered saline (DPBS; Gibco) at concentration of 10% (w/v). The resultant mixture was continuously stirred at 50° C for one hour until clear solution was observed. This was followed by drop-wise addition of 8% (v/v) methacrylic anhydride (Sigma) to synthesize high degree methacrylated GelMA. After 3 hours, the reaction was stopped by adding warm DPBS and the resultant mixture was dialyzed for one week in distilled water by using 12-14 KDa cut off dialysis tubing. Finally, the solution was lyophilized for one week and kept at -80°C for future experimental use.

2.2.2. Isolation of neonatal cardiomyocytes and cardiac fibroblast cells

Cardiomyocytes and cardiac fibroblasts were obtained from two day old neonatal rats (Annabi et al. 2013) based on well-defined protocol approved by Institution of Animal Care at Arizona State University. Briefly, the thorax of two day old neonatal rats

was opened and heart was surgically removed. Upon removing the atria, the hearts were cut into 3-4 medium sized pieces and placed in 0.05% Trypsin solution (without EDTA; Gibco) prepared in Hank's Balanced Salt Solution (HBSS; Gibco) for 14-16 hours at 4°c with continuous gentle shaking. After trypsination, the heart pieces were neutralized in cell culture media and further subjected to collagenase type II treatment to release population of cells comprised mainly of cardiomyocytes and cardiac fibroblasts. The cell suspension obtained were then preplated in T-175 flasks to enrich cardiomyocytes and cardiac fibroblasts. After an hour, the unattached cells, which were essentially cardiomyocytes, were separated and counted. Cardiac fibroblasts were either harvested from the same isolation, or by using continuous cultures with maximum passage number of three.

2.2.3. Engineering of cardiac micro-tissues

The glass slides, on top of which the constructs were micropatterned, were prepared by primarily treating them with 3-(trimethoxysilyl) propyl methacrylate (TMSPMA; Sigma) prior to the experiments. Poly (Ethylene Glycol) diacrylate (PEG-DA; Polysciences), a cell repellant hydrogel, was prepared in DPBS containing 0.5% (w/v) photoinitiator ((2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone; Sigma) at a final concentration of 20% (w/v). Furthermore, GelMA prepolymer solution was formulated by mixing 5% (W/V) into DPBS containing photoinitiator with the same concentration as defined above.

To develop cardiac micro-tissues, cardiomyocytes and cardiac fibroblasts were mixed in well-defined proportions (cardiomyocytes: cardiac fibroblasts; 2:1 and 1:1) with a final concentration of 7.5×10^6 cells/300 µl of GelMA prepolymer solution. As control,

constructs were also prepared by encapsulating only cardiomyocytes with same cell density as the co-culture conditions. The glass slides were primarily coated with PEG-DA hydrogel by putting a drop of 6.5 µl of hydrogel solution onto TMSPMA treated glass slide which was covered by putting a glass coverslip on top of it. This set up was exposed to UV for 50 seconds (800 mW, 360-480 nm). Subsequently, a drop of 17 µl of GelMA prepolymer solution suspended with the cells was pipetted onto a spacer of height 100 µm; on top of which of which the PEG-DA coated glass slide was inverted. A photomask designed using AutoCAD was placed on top of this set up and exposed to UV with optimized timing. We utilized a 1 cm × 1 cm photomask layout to engineer an array of micro-tissue of variable geometries (Length x Width; M1: 2000 µm × 500 µm, M2: 1000 µm × 250 µm, M3: 500 µm × 125µm) and constant aspect ratio. The spacing between the constructs were set to be 500 µm. Upon UV exposure, the glass slide was removed and the sample was washed with warm DPBS to remove the uncrosslinked hydrogel. The prepared samples were then placed into 24 well plates with 500 µl of media. The cell culture media utilized for these experiments was composed of DMEM (Gibco), 10% fetal bovine serum (FBS; Gibco), 1% penicillin- streptomycin (Gibco) and 1% L-glutamine (Gibco). The media was changed on Day 1 followed by a change on every alternate day to maintain consistent experimental conditions.

2.2.4. Cell viability analysis

Cell viability within the micro-tissues was analyzed after 7 days of culture using Live/Dead Assay kit (Life technologies) containing calcein AM (Cl) and ethidium homodimer (EthD). A solution for experimental use was prepared by adding 0.5 µl/ml Cl and 2 µl/ml ETD in warm DPBS. Culture media was removed from the well plates

followed by washing the samples three times in DPBS. The Live/Dead solution of about 150 µl was then added to each well followed by incubation for 30 minutes. After incubation, fluorescent tile images were taken with the 10X magnification to visualize viable (green stain) and non-viable (red stain) cells using an inverted fluorescent microscope (Zeiss Axio Observer Z1). The images were analyzed using Image J software (v 1.48, NIH) and cell survival percentage was subsequently quantified within each sample. In particular, the number of dead cells (stained with ETD) and the total number of cells were calculated using Image J within each image. The number of live cells were then obtained by subtracting number of non-viable cells from total number of cells. The cellular viability was calculated by dividing number of viable cells with total number of cells. To obtain statistically significant results, images were taken from triplicate samples across all experimental conditions for M1-M3 constructs. Further, each tile image, was divided into three equivalent images for quantification purposes and the results were reported as average± standard deviation.

2.2.5. Cell proliferation analysis

To determine cellular proliferation, Alamar Blue Assay (Invitrogen) was utilized at Day 1, 3 and 5 of the culture. Briefly, media was removed from the well plate and the samples were washed three times with DPBS. The Alamar Blue solution was prepared in warmed media at concentration of 10 % (v/v) and then added to each well plate followed by incubation for three hours. Next, 100 µl of the Alamar Blue solution from each well plate was transferred to 96 well plate. Subsequently, fluorescence intensity was measured using a fluorescent plate reader at 544-590 nm wavelength. Duplicate Alamar Blue solution from each sample were recorded. As control, we prepared GelMA

micropatterned constructs without cells with the same geometrical features. The values of the control were subtracted from the cell-encapsulated hydrogel-based constructs samples and final values at day 3 and 5 were normalized with respect to day 1 of culture. To obtain statistically significant results three samples were prepared for each condition.

2.2.6. Quantification of cells' nuclei alignment

To quantify the cells' nuclei alignment across different micro-tissues within all the conditions, samples were fixed at day 9 using 4 % (v/v) paraformaldehyde (PF; Electron Microscopy Sciences) solution in DPBS for 30 minutes. After fixing, 1:1000 ratio of DAPI (4', 6-diamidino-2-phenylindole; Life Technologies) solution in DPBS was pipetted in to the samples for 20 minutes followed by gentle wash in DPBS. Subsequently, fluorescent tile image from triplicate samples for each construct across all the conditions were taken. Each tile image was further cut into three equivalent areas and analyzed using Image J software according to previously defined method (Nikkhah, Eshak, et al. 2012). Using built-in function of Image J, an ellipse was fitted across the DAPI stained nuclei and then angle of major axis of the ellipse with respect to x axis was measured. All alignment angles were normalized with respect to average angle of each image and grouped in 10° increments.

2.2.7. Staining for F-actin cytoskeleton and FFT analysis

In order to visualize the organization of F-actin fibers of the cells encapsulated within the micro-tissues, samples were fixed at day 9 in 4% PF solution. Cells were then permeabilized using 0.1 % X-100 Triton(Sigma) for 20 minutes followed by blocking with 1% Bovine Serum Albumin (BSA) for 45 minutes. In order to stain for F-actin, Alexa fluor 488 phalloidin (Life Technologies) was prepared in 0.1% BSA with 1:40

ratio and added to the samples for 45 minutes. This was followed by staining the cells' nuclei with DAPI. The stained samples were inverted on drop of ProLong Diamond antifade solution (Life Technologies) on a glass coverslip for 3D imaging. Z-stack images of the samples were taken at 20X magnification using an inverted fluorescent microscope (Zeiss Axio Observer Z1) equipped with ApoTome.2. Upon 3D imaging, FFT analysis was performed using built-in feature of Image J software to assess the organization of F-actin fibers within specific micro-tissues. Orientation Index of the F-actin fibers was calculated to be one minus the ratio of length of minor axis to major axis as defined by previous studies (Nichol et al. 2010). Higher values of orientation index signifies better cytoskeleton alignment whereas lower values indicates random orientation. For statistical relevance, orientation index was calculated from three FFT thresholded images for each geometrical feature in mono-culture and co-culture conditions. The values were then reported as average ± standard deviation.

2.2.8. Immunostaining for cardiac specific and cell adhesion markers

Primary monoclonal antibodies specific to sarcomeric α-actinin, connexin-43 and Integrin-β1 was obtained from Abcam. The primary antibody for Troponin-I was obtained from Developmental Studies Hybridoma Bank. The secondary antibodies; goat anti mouse alexa fluor 488 for troponin-I, goat anti mouse alexa fluor 594 for sarcomeric α-actinin (pseudo-colored with green), goat anti rabbit alexa fluor 488 for connexin-43 (pseudo-colored with red) and goat anti rabbit 594 for Integrin-β1 were purchased from Abcam. For immunostaining, the samples were fixed in PF and washed with DPBS three times. For all the cardiac specific markers (sarcomeric α-actinin, connexin 43, Troponin-I), cell membrane was permeabilized using 0.5% X-100 Triton for 45 minutes. For

Integrin-β1 staining, no permeabilization was performed to prevent loss of cell adhesion markers expressed extracellularly. Upon fixation and permeabilization, the samples were washed with DPBS three times and further blocked in 10% goat serum (Life Technologies). After blocking, the primary antibodies for sarcomeric α-actinin, connexin 43 and Integrin-β1 were prepared at a dilution of 1:100 in 10% goat serum and added to the constructs for overnight incubation. The primary antibody for Troponin-I was prepared at dilution of 2μg of Ig/ ml of DPBS solution as per Developmental Studies Hybridoma Bank protocol and then added to the samples for overnight incubation. This was followed by washing the constructs with DPBS for five times and adding the respective secondary antibodies at dilution of 1:200 for six hours. The samples were then stained with DAPI and 3D Z-stack fluorescent images were taken at 20X/40X objective using Zeiss fluorescent microscope equipped with ApoTome.2.

2.2.9. Quantification of protein area coverage

For quantification of area coverage by different proteins, images of stained samples for F-actin, Integrin-β1 ,Sarcomeric α-actinin, Troponin-I were taken at 20X magnification using Zeiss fluorescent microscope equipped with ApoTome.2. Duplicate windows of about 100 x 100 μm (10000 μm^2) were selected from each image. Using ImageJ software the area fraction covered by the protein was quantified within the selected window. In order to obtain statistically relevant results, the quantification was done on three images for each construct in mono-culture and co-culture conditions. The data was presented as percentage of protein area covered (per 10^4 $\mu m^{2)}$.

2.2.10. Characterization of the beating behavior of the cardiac micro-tissues

In order to quantify the beating characteristics of the microengineered cardiac tissues, real time video microscopy was used on each day of culture from day 2 using built-in zeiss software. The beating signal patterns within each micro-tissue were obtained using a custom written MATLAB program. To obtain statistically significant results, videos were taken from at least three replicates for each sample across all the experimental conditions.

2.2.11. Statistical analysis

All statistical analysis were done using GraphPad Prism (v.6, GraphPad San Diego) software. In order to analyze the significant difference between the means of the groups, one way ANOVA (for cell's nuclei alignment and synchronous beating) and two way ANOVA (for cell viability, cell proliferation, orientation index, protein area coverage) were used. Furthermore, for multiple comparison, Tukey's test for one way ANOVA and Bonferroni's post hoc test for two way ANOVA were implemented. All the statistical difference with a $p<0.05$ was considered to be significant.

2.3. RESULTS

2.3.1. The development of 3D micro-tissues with different geometrical features

We utilized micropatterning technique to generate 3D micro-tissues embedded with the co-culture of cardiomyocytes and cardiac fibroblast using Gelatin Methacrylate (GelMA) with high degree of methacrylation (~92%)(Shin et al. 2013). GelMA was preferred for this study due to its established ease of microfabrication along with suitable cell responsive properties (Nichol et al. 2010, Dolatshahi-Pirouz et al. 2014). We utilized 5% GelMA for micropatterning the constructs since previous studies have indicated that

increasing the concentration of the hydrogel to 10 or 15% leads to lower cellular viability and spreading.(Nichol et al. 2010) As evident from **Figure 2.1B**, the constructs were successfully developed with different geometrical features (**Figure 2.1A**: M1: 2000 µm × 500 µm, M2: 1000 µm ×250 µm, M3: 500 µm ×125 µm). The height of the constructs was set to be 100 µm across all the experimental conditions. To preform preliminary assessment on the morphology of the encapsulated cells, we acquired phase contrast images from day 1 to day 9 of culture (Figure 1B). Cardiomyocytes in mono-culture condition displayed round morphology with minimum spreading during the entire culture time. On the other hand, upon co-culture with cardiac fibroblasts, the cells initiated spreading and formed networks through different layers of 3D hydrogel matrix.

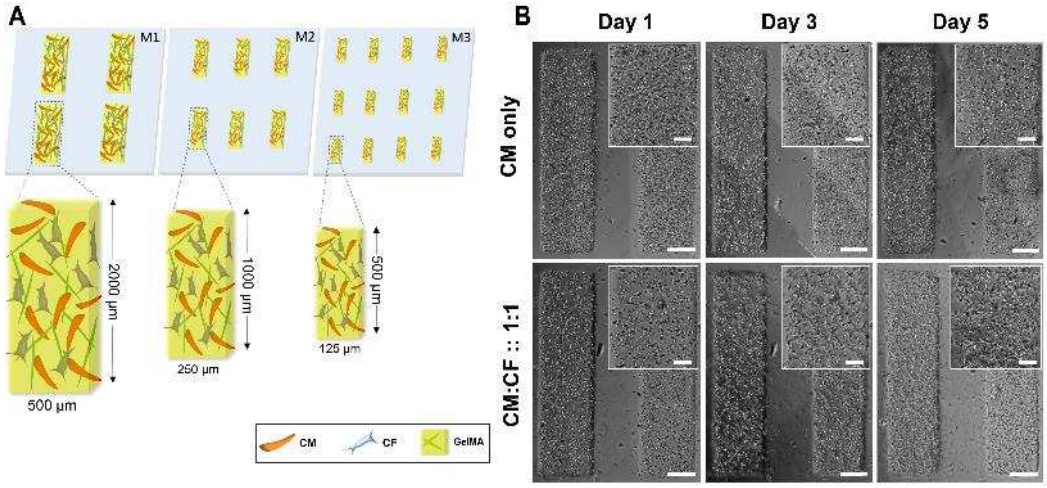

Figure 2.1: (A) Schematic drawing demonstrating engineered constructs of different geometries with their respective dimensions. (B) Representative phase constrast images depicting minimal spreading of cardiomyocytes (CM) within M1 construct across mono-culture condition and enhanced network formations of cardiomyocytes with cardiac fibroblasts (CF) in 1:1 co-culture condition. Scale bars represent 250 µm. Insets show magnified images of the constructs to closely visualize the morphology of the cells. Scale bars represents 100 µm.

2.3.2. Cellular viability and proliferation

We assessed cellular viability within engineered constructs in mono-culture and co-culture conditions after one week. This time point was selected since most of the construct in co-culture depicted cellular spreading and spontaneous beating whereas no cellular elongation or beating was evident in mono-culture even at day 7 of culture. Overall cellular viability was quantified to be about 84.6% ±2.83 (n=9) within all the constructs across different cellular ratios; consistent with previous studies (Shin et al. 2013). Additionally, no significant difference was observed between the mono-culture and the co-culture conditions **(Figure 2.2A, B)**. This further strengthens the argument that even though cardiomyocytes remained viable during all culture days, they adopted a round morphology within the 3D GelMA hydrogel.

Next, we assessed the cellular proliferation within the constructs at day 3 and 5 of the culture **(Figure 2.2 C)**. Evaluating the proliferation on the longer culture time points (day 7, 9) was technically difficult due to detachment of samples during multiple washes. Across all the micro-tissue within the mono-culture condition, no significant cellular proliferation was observed. These results are in tune with the native characteristics of the cardiomyocytes with limited proliferative capacity (Bergmann et al. 2009).However, higher fluorescent signal on day 5 as compared to day 3 in mono-culture condition was expected to be due to presence of cellular impurities, such as cardiac fibroblasts, typically contained within the enriched cardiomyocyte population. Alternatively, among the co-culture conditions, significant proliferation across all the constructs was observed between day 3 and day 5 of culture. Higher cellular proliferation in co-culture conditions could therefore be attributed to the presence of significant amount of cardiac fibroblasts

at the initial culture time. These data clearly indicated that the population of cardiac fibroblasts in the co-culture conditions increased as a function of time. The spreading and proliferation of cardiac fibroblasts was envisioned to essentially assist cardiomyocytes to establish interconnected networks leading to the formation of functional 3D micro-tissues. To test such hypothesis, we further assessed cytoskeletal organization along with cell adhesion and cardiac specific markers expression within specific micro-tissues.

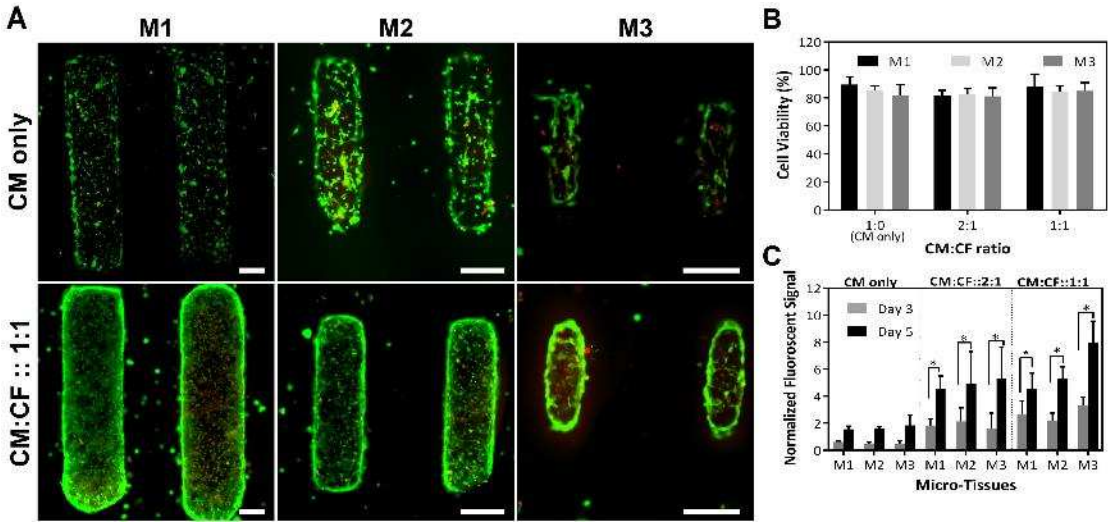

Figure 2.2: (A) Representative live/dead stained images of the engineered micro-tissues across mono-culture (CM only) and co-culture groups (CM:CF::1:1) at day 7 (Viable cells: green stain, Non-viable Cells: red stain). (B) Quantitative representation of cell survival across all the experiemental conditions at day 7 of culture. (C) Normalized fluoroscent signal from Alamar Blue assay, depicting proliferation of cell-laden micro-tisssues at day 3 and 5 normalized to day 1. Scale bars represent 100 µm.

2.3.3. Alignment of encapsulated cells within the micropatterned constructs

It has been well established that cellular alignment significantly improves within micropatterned features having width less than 100 µm (Salick et al. 2014, Nikkhah, Edalat, et al. 2012b). As we intended to study the sole impact of tissue geometry on functional properties of cardiac constructs, we speculated the absence of any significant alignment within the micropatterned features. In order to test our hypothesis, we assessed the cells' nuclei organization, as an indicator of global alignment(Nikkhah, Eshak, et al.

2012), across all the constructs at day 9 of culture **(Figure 2.3)**. As expected, there were no significant cells' nuclei alignment, across all the micro-tissues of variable geometrical features. This trend was valid regardless of mono-culture or co-culture conditions.

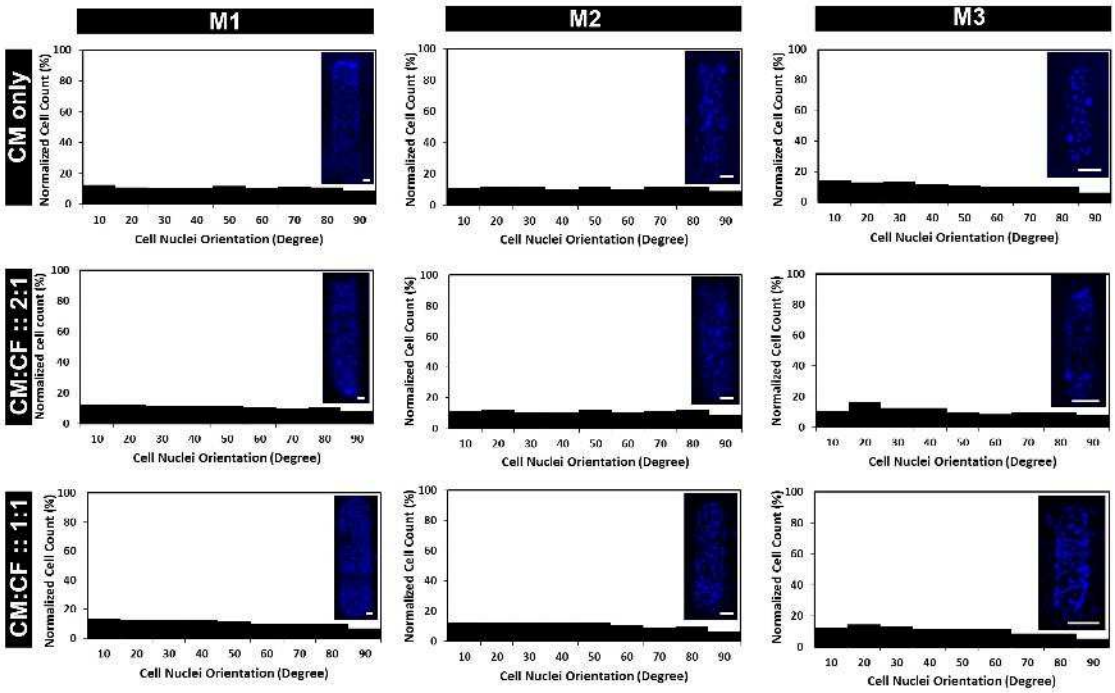

Figure 2.3: Histograms representing cells' nuclei alignment within cardiac micro-tissues in mono-culture (CM only) and co-culture conditions (CM:CF::2:1 and CM:CF::1:1) at day 9. The insets illustrate the representative DAPI stained images of individual constructs across different experiemental groups. No significant alignment observed within the micro-tissues as a function of geometrical features. Scale bars represent 125 μm.

2.3.4. Actin Cytoskeleton organization

To visualize the organization of F-actin fibers and assess the differences between mono-culture and co-culture conditions, fluorescent images were taken from the samples at day 9 of the culture (time point of highest synchronous beating). Across all the geometrical features within the mono-culture, most of the cells had limited expression of actin fibers and adopted a round morphology, consistent with phase contrast images **(Figure 2.4A)**. However, upon addition of cardiac fibroblasts, significantly higher

expression of F-actin fibers was observed throughout the constructs **(Figure 2.4B)**. This trend was consistent across all the constructs among the mono-culture and co-culture conditions. Additionally, Z-stack images of actin fibers clearly depicted the distribution of the fibers within different layers of GelMA hydrogel demonstrating the formation of 3D micro-tissues **(Figure 2.4A)**.

In order to study the local alignment of the cell cytoskeleton, we performed FFT (Fast Fourier Transform) analysis on F-actin fibers and further quantified the orientation index **(Top right insets, Figure 2.4A, 2.4C)** similar to previous studies (Nichol et al. 2008). Overall, the addition of cardiac fibroblasts enhanced the orientation index within each specific construct. Furthermore in mono-culture and 2:1 cell ratio, the orientation index enhanced through decreasing the geometrical features of the construct **(Figure 2.4C)**. Similar observations were also reported in other studies among co-culture and mono-culture conditions when cells were encapsulated within self-assembled gels(Nichol et al. 2008).

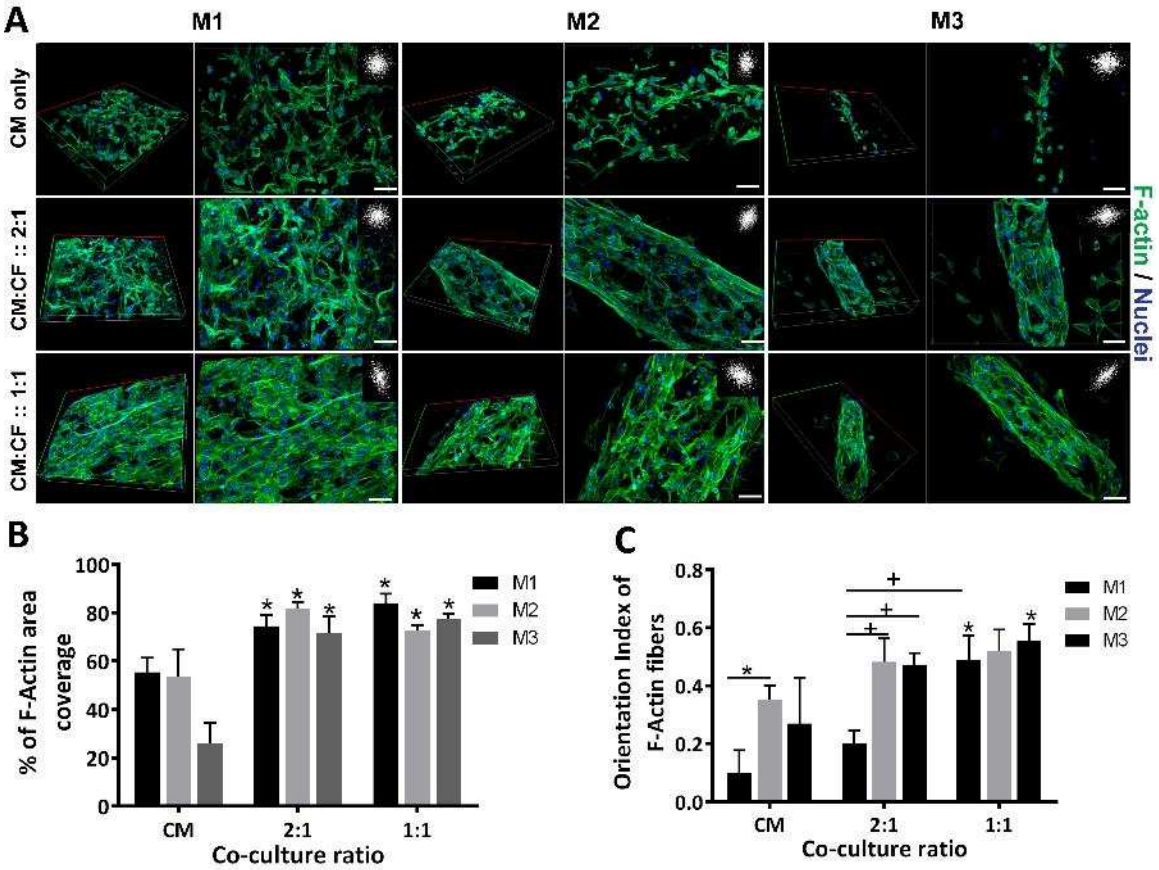

Figure 2.4: (A) Representative images depicting 3D and top view of the actin cytoskeleton organization of encapsulated cells in different micro-tissues at day 9 of culture depicting marked differences between mono-culture and co-culture conditions. FFT images (insets) representing the actin fiber alignment within individual constructs. Scale bars represent 50 μm. (B) Quantified analysis of percentage of area covered by F-actin fibers per 10^4 μm^2 across different constructs in mono-culture and co-culture conditions. "*" represents significant difference ($p<0.05$) with respect to mono-culture condition. (C) Analysis of Orientation Index of F-actin fibers within all the exprimental groups. "*"/ "+" represents significant difference ($p<0.05$) with respect to mono-culture condition and within co-culture conditions respectively.

2.3.5. Analysis of expression of transmembrane protein Integrin- β1

In order to study the effect of co-culture condition on cell-matrix interactions within different constructs, we analyzed integrin-β1 expression. Integrin-β1 is a well-established transmembrane protein responsible for cellular mechanotransduction and cell-ECM interactions (Sheehy, Grosberg, and Parker 2012). Fluorescent images of the stained samples at day 9 of culture were taken across mono-culture and co-culture

conditions **(Figure 2.5A)**. Constructs in the co-culture depicted higher area coverage by Integrin-β1, thereby suggesting enhanced cell-ECM interaction compared to mono-culture condition **(Figure 2.5B)**. Furthermore, 3D Z-stack images depicted integrin-β1 expression across different layers of hydrogel similar to F-actin distribution **(Figure 2.5A)**.

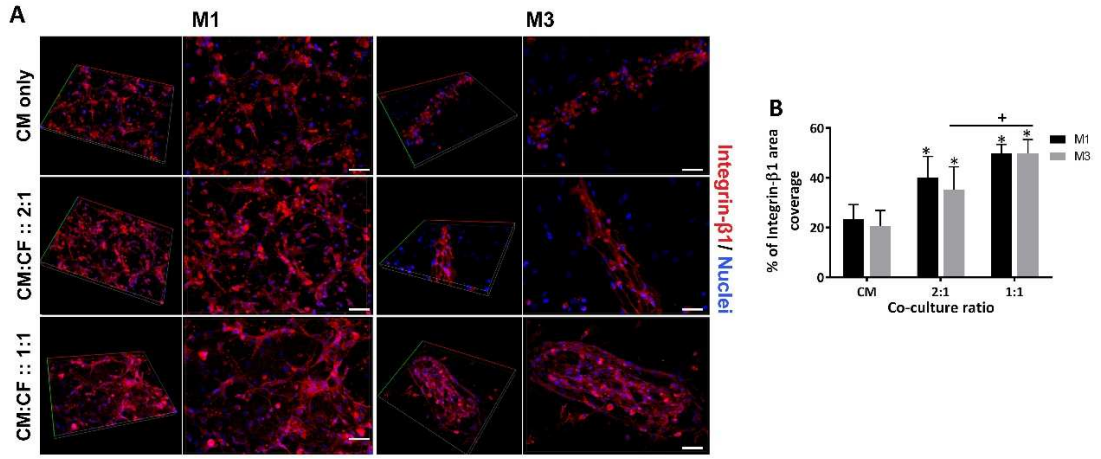

Figure 2.5: (A) Representative images depicting 3D and top view of the transmermbrane protein Integrin-β1 across M1 (2000 µm x500 µm) and M3 (500 µm x125 µm) micro-tissues in mono-culture and co-culture conditions at day 9. Scale bars represent 50 µm. (B) Quantification of percenatge of area covered by Integrin-β1 per 10^4 μm^2 across M1 to M3 constructs in mono-culture and co-culture conditions. " *"/ "+" depicts significant difference ($p<0.05$) with respect to mono-culture and within co-culture conditions.

2.3.6. Assessment of beating characteristics of cell-encapsulated micro-tissues

We further analyzed synchronized beating (BPM) of the 3D micro-tissues as a function of cell-cell ratio and the geometrical features of the constructs from day 2 to day 9 of the culture. This time frame was chosen to be consistent to previous studies which have reported high functionalities of *in vitro* cardiac construct within day 7 to day 9. (Shin et al. 2013, Annabi et al. 2013, Kharaziha et al. 2013)As expected, due to less spreading, round morphology and the lack of well-established cell-ECM interactions,

cardiomyocytes in mono-culture condition exhibited individual beating rather than synchronous tissue-level contraction across all the days of the culture.

Within co-culture conditions (1:1 and 2:1), we observed synchronous tissue level beating **(Supplementary movies S1-S6)**. We contemplate that such difference in synchronous beating was due to the presence of cardiac fibroblast cells and enhanced cellular network formation and cell-matrix interactions as confirmed by actin cytoskeletal organization and integrin-β1 expression. Amongst the co-culture conditions, higher beating frequency was specifically evident in 2:1 cellular ratio within M1 and M2 constructs **(Figure 2.6B; Supplementary movies S1-S2, S4-S5)**. For instance, M1 and M2 constructs in 2:1 ratio exhibited ~58±13 BPM and ~56±16 BPM respectively on day 9 of the culture. On the other hand, in 1:1 co-culture condition, these constructs revealed ~34±11 BPM and ~28±20.3 BPM respectively. While there was statistically significant difference within both M1 and M2 constructs between two co-culture groups, no significant differences was observed in M3 construct **(Figure 2.6B)**. Interestingly, within 2:1 co-culture condition, we observed the influence of reducing the geometrical features of the constructs on synchronous contraction of the cells. In particular, M1 (~58±13 BPM) and M2 (~56±16 BPM) exhibited significantly higher beating frequency as compared to M3 constructs (~17± 12 BPM) **(Figure 2.6A)**. These observations primarily indicated the impact of cellular ratios on synchronous beating of the micro-tissues. In addition, within the optimized cellular ratio (2:1), the decrease in geometrical features of the constructs negatively influence the synchronous contraction of the cells **(Figure 2.6A)**.

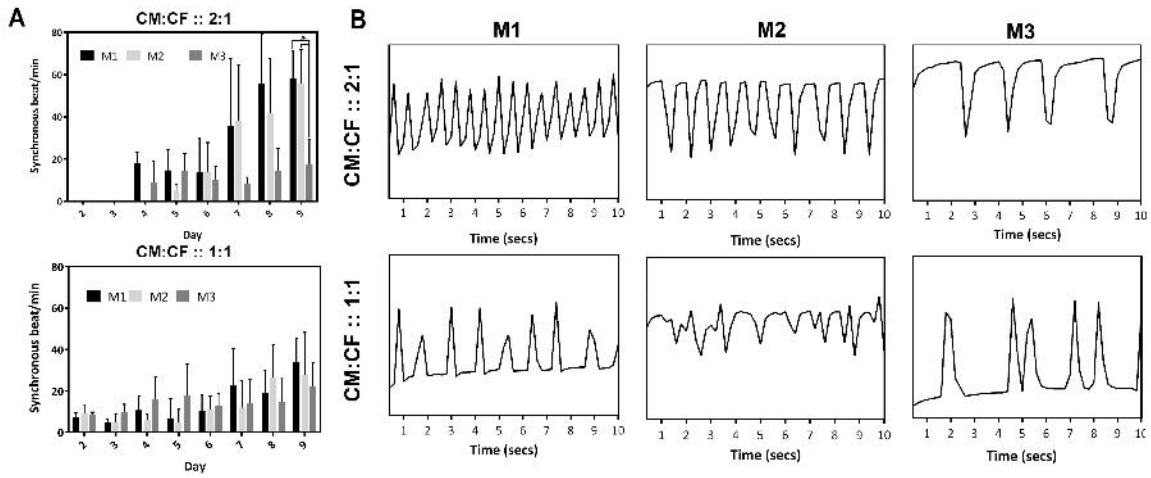

Figure 2.6: Cardiomyocyte beating characteristics in co-culture conditions. (A) Quantitative analysis of synchronous beats per min (BPM) of different micro-tissues (M1, M2, M3) from day 2 to day 9 demonstrating higher beating frequency in 2:1 comapred to 1:1 cellular reatio. "*" suggests p value<0.05 (B) Patterns within beating signals of enginnered micro-tissues in 2:1 and 1:1 co-culture conditions at day 9 of culture.

2.3.7. Expression of Sarcomeric α-actinin, Connexin-43 and Troponin-I

We evaluated the differences in cardiac marker expressions among mono-culture and co-culture conditions across all the constructs (M1-M3) on day 9 of culture. Cardiomyocytes in mono-culture minimally expressed sarcomeric α-actinin with occasional appearance of cross striations **(Figure 2.7A, B)**.On the other hand, within the co-culture conditions in M1 construct, significantly higher area was covered by sarcomeric α-actinin **(Figure 2.7B)**. In M3 construct however, no significant difference was quantified among the mono-culture and co-culture conditions **(Figure 2.7A, B)**. Furthermore, within each co-culture condition, there was notably higher area coverage by sarcomeric α-actinin in M1 construct as compared to M3 construct. These findings suggested that in the absence of cardiac fibroblasts within the 3D GelMA hydrogel, cardiomyocytes maintained a round morphology and do not exhibit a well-established native-like phenotype. However upon addition of cardiac fibroblasts, cardiomyocytes

express higher sarcomeric α-actinin with elongated and interconnected cellular morphology and well-defined cross striations.

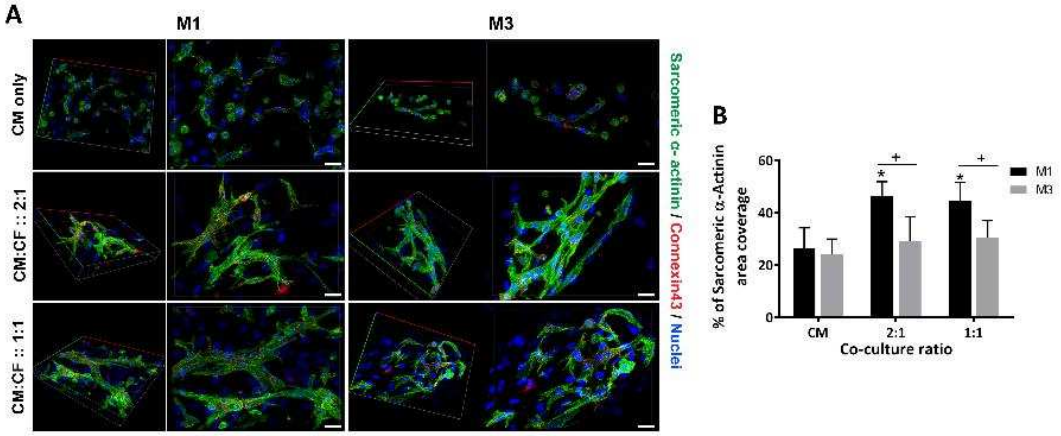

Figure 2.7: (A) Representative images deicting 3D and top view fluoroscent images of the sarcomeric α-actinin (green) and connexin-43 (red) stained cardiomyocyte in M1 and M3 micro-tissues within mono-culture and co-culture conditions at day 9. Scale bars represent 20 μm. (B) Quantification of percentage of area covered by Sarcomeric α-actinin per 10^4 μm^2 in M1-M3 constructs across mono-culture and co-culture conditions. "*"/ "+" represents significant difference ($p<0.05$) with respect to mono-culture and within co-culture conditions.

Similarly, from **Figure 2.7A**, it is evident that Connexin-43, a cell-cell gap junction protein responsible for synchronous beating of the cells (Eckardt et al. 2004), was well expressed in co-culture as compared to mono-culture. Troponin-I was also observed to be well expressed for 1:1 cellular ratio in M1 construct **(Figure 2.8A, B)** as compared to the mono-culture condition. The 3D images of Sarcomeric α-actinin, Connexin-43 and Troponin-1 clearly indicated the penetration of cellular network inside the hydrogel matrix within the co-culture conditions with well-defined cardiac markers expression **(Figure 2.7A, 2.8A)**. Such observations thus, suggested that cardiomyocytes and cardiac fibroblasts were highly interconnected across different layers of 3D GelMA hydrogel leading to superior structural and tissue-level functionalities (i.e. beating).

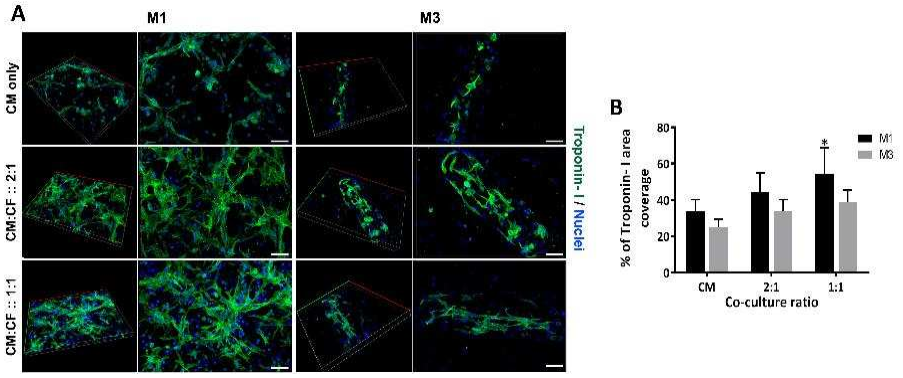

Figure 2.8: (A) Representative images depicting 3D and top view for cardiomyocytes stained for Troponin-I in different micro-tissues (M1, M3) within mono-culture and co-culture conditions at day 9. Scale bars represent 50 μm. (B) Quantified analysis of percentage of area covered by Troponin-I per 10^4 μm^2 across different constructs within mono-culture and co-culture conditions. "*" represents significant difference with respect to the mono-culture condition.

2.4. DISCUSSION

In this work, we generated 3D cardiac micro-tissues with variable geometrical features to study the effect of cardiomyocytes and cardiac fibroblasts co-culture (varying cell-cell ratio) and the geometrical features of the constructs on the functionalities of the engineered tissues. GelMA hydrogel was particularly selected due to its suitable crosslinking properties to precisely control the geometrical feature of the 3D constructs. Furthermore, gelatin, which is denatured form of collagen (Nichol et al. 2010), provided an excellent matrix for cellular adhesion and proliferation, thereby mimicking the native ECM of myocardial tissue.

A major finding of our study was the superior structural and functional properties of the engineered micro-tissues comprised of the co-culture of cardiomyocytes and cardiac fibroblast cells over the mono-culture. The majority of cardiomyocytes within the mono-culture condition maintained a round morphology, although viable, and did not spread through the 3D hydrogel matrix. On the other hand, upon co-culture with cardiac

fibroblasts, these cells elongated and displayed enhanced cell-based network formations. In fact, integrin-β1 expression, a transmembrane protein that directly links cellular cytoskeleton to ECM (Tirziu, Giordano, and Simons 2010), confirmed superior cell-matrix interactions in co-culture conditions as compared to mono-culture of cardiomyocytes.

The co-culture conditions further revealed enhanced local alignment of actin fibers as confirmed by increased orientation index **(Figure 2.4C)**. Although there was absence of global nuclear alignment, higher organization of actin fibers led to improved local anisotropy within the co-culture conditions. In fact, many studies have revealed similar local alignment within the native heart tissue and hence attempted to engineer bio-inspired constructs to enhance the overall tissue-level functionalities (Nichol et al. 2008, Streeter et al. 1969, Bian et al. 2014a). For instance, Bian *et al.,* utilized diffusion tensor magnetic resonance imagining (DTMRI) maps to design *in vitro* 3D cardiac patches with local cellular alignment. The outcome of this study demonstrated enhanced structural and functional properties of the cardiac cells encapsulated in a mixture comprised of fibrinogen and matrigel.(Bian et al. 2014a) In another study by Nichol *et al.,* local cellular alignment was demonstrated to be due to enhanced MMP-2 expression when cardiomyocytes are co-cultured with cardiac fibroblasts (Nichol et al. 2008).

Immunostaining results further showed that cardiomyocytes in co-culture with cardiac fibroblasts expressed a mature phenotype. For instance, sarcomeric α-actinin in mono-culture was elusive of any cross striated structures, whereas in co-culture conditions the cells expressed well-organized sarcomeric α-actinin. This was also evident from Troponin-I staining which was minimally expressed in mono-culture as compared

to co-culture conditions. Our results also indicated enhanced cell-cell interactions in co-culture conditions manifested through high expression of connexin-43. It is well established that communication between adjacent myocytes occur through gap junction proteins mainly by connexin-43, which allows the passage of ions and solutes in between cells (Kohl 2003).These junctions also ensures synchronous contraction of the cells and play a prominent role in generating tissue level syncytium (Kohl 2003). In fact, a recent study by Hussain *et al.,* reported a similar findings in which cardiac cells encapsulated in chitosan nanofibrous scaffolds minimally expressed connexin-43 in mono-culture condition as opposed to co-culture with cardiac fibroblasts (Hussain et al. 2013). While connexin-43 is expressed between neighboring myocytes, many studies have revealed that these gap junction proteins also express in between cardiomyocytes and cardiac fibroblasts (Gaudesius 2003, Rook et al. 1992). Additionally, studies have also demonstrated that cardiac fibroblast, when electrically coupled with cardiomyocytes, offer high membrane resistance such that they can transmit electrical signals over distances extended to 300 μm without significant loss of electrical activity (Radisic et al. 2007b, Kohl 2003, Camelliti, Borg, and Kohl 2005). High resistance along with enhanced expression of gap junctions indicates the significance of the cell-cell interactions between cardiomyocytes and cardiac fibroblasts. Therefore, it is envisioned that the co-culture of cardiomyocytes with cardiac fibroblast cells, improves the overall cell-cell communication thereby enhancing the synchronization and spontaneous activity of adjacent cardiomyocytes.

The cross- talk between cardiomyocytes and cardiac fibroblasts could also be attributed to paracrine/autocrine signaling factors. There are many soluble mediators

which are released by the cardiac cells including FGF (fibroblast growth factor), Interleukins, TGF-β (transforming growth factor) and other cytokines which interact with specific cell receptors and affect the performance of the tissue (Samarel 2005b). Thus, it is plausible to speculate that a combination of improved cell-matrix, cell-cell interaction along with paracrine/autocrine signaling leads to the overall enhanced tissue functionalities in co-culture compared to the mono-culture condition. The detailed analyses on paracrine/autocrine signaling between cardiomyocytes and cardiac fibroblast cells, within engineered micro-tissues, is the subject of our future studies.

Another major aspect of our work was the optimization of the ratio of encapsulated cardiac fibroblasts to cardiomyocytes. Although we observed higher alignment of actin fibers in 1:1 cell ratio as compared to 2:1 co-culture condition, the 2:1 ratio (two cardiomyocyte to one cardiac fibroblast) gave superior synchronous beating. We speculate that this could be due to higher number of cardiomyocytes in the 2:1 cell ratio, which promotes the formation of a more mature functional syncytium. We also envision that higher number of cardiomyocytes limited the proliferation of cardiac fibroblasts due to contact inhibition (Olson et al. 2005). Further, high number of fibroblasts in 1:1 condition can lead to low conduction velocity and therefore give rise to suboptimal beating characteristics (Vasquez, Benamer, and Morley 2011). In fact, in a study by Iyer *et al.*, it was speculated that the starting population of cardiac fibroblasts should be reduced in order to achieve enhanced electrophysiological properties of the constructs (Iyer, Chiu, and Radisic 2009). Moreover within the 2:1 co-culture condition, we noticed that as we decrease the overall geometrical features from M1 (2000 μm x 500 μm) to that of M3 (500 μm x 125 μm) constructs, despite improved local alignment of

actin fiber, there is a significant decrease in synchronous contraction. This clearly depicts that reducing the geometrical features of the construct has a notably adverse effect on the synchronous contraction of the cells. Perhaps, this is due to the overall less number of cardiomyocytes within M3 compared to M1 and M2 constructs. Such behavior highlights the need for optimization of cellular ratios as well as the geometrical features of the constructs in designing the next generation of cardiac patches.

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CHAPTER 3

CONCLUSION AND FUTURE PERSPECTIVES

3.1. Conclusion

The goal of this thesis was to engineer three dimensional (3D) cardiac micro-tissues exhibiting structural and functional properties similar to the native myocardium. While in the past, innumerous research has been done in the field of cardiac tissue engineering, there are still some key aspects which need careful thought and optimization for better functionalities of engineered cardiac tissue substitutes. The findings presented herein, can be useful to engineer the next generation of cardiac patches for regenerative medicine and disease modeling applications.

In this study, cardiac micro-tissues were engineered by encapsulating co-culture of cardiomyocytes and cardiac fibroblasts. The tissue was micropatterned into different geometrical features of constant aspect ratio to assess their role on construct functionalities. The findings on cellular morphology and expression of various proteins (F-actin fibers, Integrin β1, Sarcomeric α-actinin, Connexin 43, Troponin- I) clearly demonstrated a more native like phenotype of cardiomyocytes upon co-culture with cardiac fibroblasts as compared to mono-culture condition across all the tissue geometries. Furthermore, among the co-culture conditions, high number of cardiomyocytes led to improved tissue-level functionalities as observed by enhanced spontaneous beating in constructs from 2:1 as compared to 1:1 cellular ratios. Additionally, for an optimized cell: cell ratio, the impact of tissue geometry was clearly perceived as decreasing the size of the micro-patterned constructs adversely affected the functionality of the constructs.

3.2 Future Perspectives

While it is evident that a comprehensive study has been performed during past one year in the current MS thesis, there are various questions yet to be explored to optimize the structural and functional properties of the cardiac patches for implantation purposes. The following section thus explores few of the future novel aspects which can be further pursued in this subject.

3.2.1. Impact of External Stimulation on the Functionalities of Micro-Tissues Co-Cultured with Cardiomyocytes and Cardiac Fibroblasts within 3D Environment

In the current study, cardiac micro-tissues were not externally stimulated (electrical or mechanical stimulation). While this was crucial to understand the *in vitro* dynamics of encapsulated cells in terms of structural and functional properties, we didn't observe any significant global cellular alignment (although significant local alignment was evident in 1:1 group as compared to mono-culture condition). Thus, for future experiments one can study the impact of external stimulation on cellular alignment in these tissue geometries and the possible impact on structural and functional properties of cardiac cells. External stimulation can aid in better design of cardiac patches towards clinical applications.

3.2.2. Encapsulation of Endothelial cells along with Cardiac Fibroblasts and Cardiomyocytes within the 3D Constructs

It is well-known that the vascularization of engineered tissues is paramount for the clinical success of the transplants. While the findings from this study demonstrates the favorable effects of cardiac fibroblast on cardiomyocyte phenotype and functionalities, one can also study the impact of tri-culture with endothelial cells on

spontaneous contraction of the constructs. In this regard, the localization of different cells within the 3D micro-tissues can also be determined. As it is well known that cardiac fibroblast help in vascular formation through various growth factors and adheren junctions (such as cadherin 11), such studies can be also utilized to explore the impact of cardiac fibroblasts on endothelial morphogenesis.

3.2.3. Engineering 3D Models to analyze different cell transduction pathways between Cardiomyocytes and Cardiac Fibroblasts

The overall functionality of the engineered cardiac tissue patch is resultant due to the interplay of various signal pathway such as paracrine/autocrine signaling, cell: cell interaction and cell: matrix interaction. As the presented model is 3D in nature it can provide a better appreciation of different cell transduction pathways. For instance, by blocking cell: cell interaction and cell: matrix interaction, an understanding regarding the role of paracrine/ autocrine signaling can be developed.

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Figure 1.2 (B)

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