UNIVERSITY OF CALIFORNIA, MERCED

Graduate Program in Biological Engineering and Small-scale Technologies (BEST), of the School of Engineering

Biomimetic Components Aiming to Improve *in vitro* Cardiac Tissue Culture Systems As Building Blocks to Reshape Failing Hearts

by

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Dissertation Presented

As a requirement for the degree of

Doctor of Philosophy

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2013

Dedication:

Dedicated with all my heart to my loved Family; my parents Isidra Carrillo Robles and Porfirio Luna Carrillo; my brothers Rogelio Manuel, Miguel Angel, and José Luis Luna Carrillo. My dear friend Llonel Nathaniel Onsurez. And To those who light up my motivation with their lives and hearts: My grandmothers Emilia Carrillo Robles†, Elidia Carrillo Rodríguez†; and to my uncles Jesús Carrillo Robles†, and Armando Carrillo Robles†.

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- Center of Integrated Nanomechanical Systems (COINS)
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The text of Chapter 3 on this dissertation is a reprint of the material as it appears in Tissue Engineering part C. The co-author listed in this publication directed and supervised research which forms the basis for Chapter 3 of this dissertation.

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EDUCATION

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Biological Engineering and Small-Scale Technologies-Emphasis in Cardiac Tissue Engineering, School of Engineering, University of California Merced, Merced, California– August 2013.

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Engineering and Biological Sciences, San Joaquin Delta College Stockton, CA– May 2006

ACADEMIC POSITIONS

2008-2011:

Teaching Assistant, School of Engineering and School of Natural Sciences, University of California, Merced

2008-2012:

Graduate Student Researcher-Biological Engineering and Small-Scale Technologies, University of California, Merced

2006-2008:

Undergraduate Student Researcher, School of Engineering and School of Natural Sciences, University of California, Merced

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Project: Doctoral Dissertation research focuses on engineering *in vitro* culture platforms containing multi-scale surface topography for the generation of diverse anisotropic cell constructs using cardiac cells including fetal, neonatal, and cardiomyocytes derived from murine and human embryonic stem cells. Additionally, investigated the cardiomyogenic effect of enriched and characterized fetal Cardiac Fibroblasts on embryonic stem cells as well mechanisms of cardiac fibrosis in *in vitro* culture systems.

Oct 2006 – May 2008

Undergraduate Research: *University of California, Merced, School of Engineering and School of Engineering, Merced, California.*
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Project: Undergraduate research involved multiple projects including engineering lab-on-a-chip-like devices using pre-stressed polystyrene sheets (Shrinky-dinks®). One of my projects involved adapting these devices, which included microfluidic channels and other microfabricated features, to study differentiation of blood stem cells into T-cells. Additionally, I developed another microfabricated device using an inkjet printer and polystyrene sheets for generating micro-wells that could be used for embryoid body formation using mESC. I also engineered an alternative cost efficient, tunable, and relatively rapid method for creating anisotropic patterned multi-scale topography that can be used for the generation of anisotropicaly and aligned confluent monolayers of neonatal cardiomyocytes as well as cardiomyocytes derived from human embryonic stem cells.

OTHER RESEARCH TRAINING

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NSF-NSC Biosensing-Bioactuation Summer Institute (BSBA) 2011. National Taiwan University, Taipei, Taiwan. Integrates advances in informatics, nanotechnology, and deeper understanding of biological systems for sensing and dynamic control of engineered systems.

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June 2008-Aug 2008

Center of Integrated Nanomechanical Systems (COINS), University of California Berkeley, and University of California Merced, Merced, California.
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Project: Fabrication of aligned microtopography for cardiac cell alignment studies using metal coated pre-stressed polystyrene sheets.

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Advisor: Juan Rafael Riesgo Escovar
Project: Characterization of *piragua,* a gene participating in the dorsal closure process in *drosophila melanogaster.*

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Center of Integrated Nanomechanical Systems (COINS) University of California Berkeley and University of California Merced, Merced, CA.
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Project: Fabrication of micro-wells for embryoid body formation using Pre-stressed polystyrene sheets and an inkjet printer.

FELLOWSHIPS:

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PUBLICATIONS

1. Hatano, R, Mercurio K, **Luna JI,** Glaser DE, Leppert V, McCloskey KE. Endothelial Cells Derived from Embryonic Stem Cells Respond to Cues from Topographical Surface Patterns. Journal of Biological Engineering , 2013;7(18):1754-1611.th

2. **Luna JI**, Ciriza J, Garcia-Ojeda M, Lieu DK, Li RA, Fowlkes CC, Khine M, McCloskey KE. Multi-scale Biomimetic Topography for the Alignment of Neonatal and Embryonic Stem Cell-derived Heart Cells. Tissue Engineering Part C: Methods, 2011;17(5):579-88. *Received the CoverHighlight*

3. Chen C, Pegan J, **Luna JI**, Xia B, McCloskey K, Chin W, Khine M. Shrinky-Dink Hanging Drops: A Simple Way to Form and Culture Embryoid Bodies. Journal of Visualized Experiments, 2008; http://www.jove.com/index/Details.stp?ID=692

4. Chen, C.S., Breslauer, D.N., **Luna, J.I.,** Grimes, A., Chin, W.C., Lee, L.P Microfluidics: 3-D Polystyrene Chips" DOI: 10.1039/b719029h

ORAL AND POSTER ABSTRACTS/ PARTICIPATION AT CONFERENCE PROCEEDINGS

Dec 15, 2011

Fetal Cardiac Fibroblasts Play a Critical Role in Cardiac Fate from Embryonic Stem Cells. Jesus Isaac Luna, Jesus Ciriza, Jennifer Manilay, Kara M. McCloskey. TERMIS-NA 2011 Continental Conference. Houston, Texas. (Oral Presentation)

July 25, 2011

Engineering of *in vitro* cardiac microenvironments for the generation of functional cardiomyocytes for tissue engineering applications. National Cheng Kung University, Tainan, Taiwan. (Oral Presentation).

July 15, 2011

Extracellular Matrix Coated Surfaces Using Fetal Cardiac Fibroblasts as Tools for Cardiac Tissue Engineering. 2011 BSBA summer training, National Taiwan University Taipei, Taiwan

June 19, 2010

Multi-scale Biomimetic Topography for the Alignment of Neonatal and Embryonic Stem Cell-derived Heart Cells. Jesus Isaac Luna, Jesus Ciriza, Kara E. McCloskey, Marcos Ojeda-Garcia, Deborah K. Lieu, Ronald A Li, Charless C. Fowlkes, Michelle Khine. XI University of California Bioengineering Symposium University of California, Davis. Davis, California.

June 20, 2009

Wrinkled microtopography to induce cell alignment and maintain contractibility of cardiac myocytes. Jesus Isaac Luna, Jesus Ciriza, Marcos E. García-Ojeda, and Michelle Khine. X University of California Bioengineering Symposium University of California Merced, Merced, California. (Oral Presentation).

Nov 3, 2007

Piragua a New Dorsal Closure Gene in *Drosophila melanogaster.* Jesus-Isaac Luna, Juan Rafael Riesgo Escovar Nestor Octavio Nazario Yepiz . Sigma Xi 2007 annual meeting. Orlando, Florida.

TEACHING EXPERIENCE

Fall 2010	Teaching Assistant: Principles of Chemistry (CHEM 002), University of California, Merced, CA.
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PROFESSIONAL MEMBERSHIPS

Emergent Behaviors in Integrated Cellular Systems- Graduate student leader Consortium 2011-2012
Tissue Engineering and Regenerative Medicine International Society (TERMIS) 2011-present
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SELECTED HONORS/AWARDS

June 2011

Best poster award- Biosensing Bioactuation Summer Institute 2011- Taipei, Taiwan. USA, NSF-Taiwan, NSC for "Extracellular Matrix Coated Surfaces using Fetal Cardiac Fibroblasts as Tools for Cardiac Tissue Engineering

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Academic Excellence Award by the California Human Development Corporation

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Research Week Poster Competition Award 2010- UC Merced for "Multi-scale Biomimetic Topography for the Alignment of Neonatal and Embryonic Stem Cell-derived Heart Cells"

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League of Latin American Citizens (LULAC) National Scholarship '05, '06, and '07

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Outstanding Student Award- San Joaquin Delta College '06

ABSTRACT:

Tissue engineering (TE) is an emergent interdisciplinary field that promises to increase our understanding of challenging issues in biomedical research that may facilitate tissue regeneration, or the replacement of dysfunctional organs and tissues. An approach of TE integrates cell sources, and materials into *in vitro* engineered substrates that may facilitate generation of tissue-like constructs, which may be used to understand pathological conditions, test pharmacological agents or elucidate pharmacokinetics, or as cell constructs for tissue replacement. Cardiac tissue, with its native *in vivo* complexity, represents a major challenge when trying to explain its physio-anatomical characteristics *in vitro*. Advances in cardiovascular tissue engineering may be improved by incorporation of materials that mimic the complex heart tissue environment, preferably those derived from primary cell sources. My findings indicate that integrating a multi-scale surface topography. along with extracellular matrix elements (ECM). facilitates the generation of confluent anisotropic cell mono-layers of fetal cardiomyocytes (FCM), neonatal cardiomyocytes NNCM, or derived from murine and human embryonic stem cells (CMdESC). In the case of primary cardiac cells, cardiac fibroblasts CF promote cell survival and phenotype. Also, my findings provide insight into the Cardiac fibroblast subpopulation including the importance of signaling derived elements to promote generation of *in vitro* cardiac microenvironments. Importantly I developed a novel simplistic, cost-efficient, tunable, multi-scale fabrication approach to produce cell culture substrates with multi-scale feature topography for the alignment of cells that improve cell connectivity while enhancing and maintaining phenotypic characteristics.

This study suggests that for promoting and enhancing functionality and phenotypic characteristics of CM cultured *in invitro* conditions, it is favorable to integrate multiple biomimetic cardiac microenvironmental factors into tissue culture systems.

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CHAPTER 1

1.1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the USA, accounting for roughly 33.6% of the total number of American deaths in 2007 (Roger et al.). This translates to about 2200 Americans dying from CVD each day, or 1 every 39 seconds. Those affected with CVD have a high propensity to experience myocardial infarction which may ultimately result in death. The lethality of this disease, coupled with its increasing annual proportion, represents the biggest challenge in finding cures to overcome this pandemic killer. The recent emergence of tissue engineering (TE) promises potential alternatives to facilitate finding cures for CVD. The aim of Cardiac tissue engineering proposes integration of materials along with biophysical and biochemical elements into cell culture platforms with the objective of generating *in vitro* cardiac tissue grafts that may be used to test pharmacological agents, to elucidate pathological conditions, or may be implanted into the human body to regenerate or partially replace damaged cardiac tissue. Biomimicry of the hearts microenvironment may potentially improve engineered *in vitro* culture systems for providing cardiac cells with an artificially created microenvironment that more closely resembles the native tissue environment. This microenvironment may significantly increase survival while enhancing phenotypic and physiologic characteristics of *in vitro* cultures cardiac cells. For instance, integration of extracellular matrix (ECM) elements, along with anisotropic

arrangement of ECM topography, and accessory cell co-cultures may also improve phenotypic characteristics of *in vitro* cultured CM. This would potentially facilitate cell-cell: integration, communication, and synchronization that could lead to cardiac tissue-like formation. Cardiac/stem cell transplantation has been proposed as a promising therapy for damaged myocardial tissue, however, this promising therapeutic approach is still under furtive investigation as it is challenged by deficiencies and limitations particularly concerning cell sources and the use of biocompatible materials. Full cardiac tissue regeneration will only become possible when two critical areas of the process are reviewed and addresses. These are: 1) the generation of a purified source of viable cardiac progenitors/cells, especially ventricular cardiomyocytes (VCMs) and 2) packaging/assembly of CM that allows further maturation and functional integration with the host tissue. ESC exhibit highly proliferative and pluripotent attributes which makes them an excellent system for studying *in vitro* cardiogenesis. Unfortunately, CMs derived from ESCs (ESC-CM) often exhibit an immature phenotype (Hudson et al.; Lieu et al.) expressing proteins specific for mature CM, while at the same time exhibiting action potential profiles more consistent with immature CMs. I propose that these ESC-dCMs fail to reach maturity *in vitro* due to the insufficient microenvironmental cues that these cells receive when compared with normal in vivo embryonic development. My proposed studies explore combinatorial signaling (i.e. soluble biochemical, cell-cell, topographical, and extracellular matrix signaling) for optimizing cardiac cell differentiation from murine ESC with an end goal of studying cardiomyogenesis *in vitro*. I integrated signaling elements derived from primary fibroblasts into a co-culture set up to study interactions of these cells leading to improve CM phenotype. Additionally, I

optimized conditions for extracting, enriching, and maintaining the phenotype of FCF with the objective to identify exclusive signaling provided by FCF when co-cultured with mESC. I present characterization studies and provide qualitative observations on CF behavior in *in vitro* culture to investigate the heterogeneity of this cardiac subpopulation. Finally, I describe the effect of topographical features contained on engineered substrates on primary and derived CM. This particular set up was assessed for identifying beneficial effects on CM phenotype enhancement as it may be adapted to *in vitro* culture system to improve conventional culture systems settings.

1.1.1. Cardiac Tissue Engineering:

Cardiac tissue engineering represent a new and emergent interdisciplinary field that integrates biophysical and biochemical elements, cells for engineering biological constructs, and devices that may facilitate the regeneration of damaged or dysfunctional tissues or organs-in my case of study, the heart. The field of cardiac tissue engineering has generated diverse methods and tools for facilitating the fabrication of complex multi-functional device in search of potential viable therapies or platforms for pharmacological studies. In general, these approaches incorporate the use of highly sophisticated pieces of equipment that may include increased cost and accessibility to conventional biological research labs. Materials or a devices biocompatibility and biomimicry, are important aspects to consider in device fabrication models for improving functionality. The materials should ideally present general quality control characteristics for producing customized pieces that are durable, with the life span of the device ideally correlating to

the remaining life span of the patient, or long enough for the organ to recover or regenerates itself in case a device is used as a temporary aid or serves as a scaffold. Moreover, the composition of the material minimizes the possibility of producing an immunological response that in many instances limited biocompatibility and can result in material rejection causing inflammation and acute attack by the immune system. Some tissue engineering therapeutic applications have produced materials or devices for partial or complete organ replacement such as certain bone sections including mechanic knee replacements devices made of inert metals and/or polymeric materials. However, this instance is not feasible for most organs, especially those with limited regenerative capabilities such as the brain or the heart where additional cell sources are needed for replacing injures tissues or supporting degenerating populations. No less important, cell sources represent a major hurdle in CTE due to limited viable sources of cells to be used as therapeutic applications. For instance, an ideal cell source should exhibit similar or improved characteristics as the materials to be used, they should present a level of biocompatibity, and integrate to the target organ and stay viable to regenerate or replace the damaged or injured site. Existing studies for developing and/or optimizing therapeutic applications for biomedical research devices include: cardiac patches, artificial cardiac valves, anisotropicaly aligned cell monolayers and cell strips, among others. The myocardial tissue is a complex organ with limited regenerative capabilities for which therapeutic applications for tissue injury repair are limited and challenged by the physiological and morphological characteristics of the organ. CTE products do not only would function as therapeutic devices, but also these are being broadly used for pharmaceutical applications such as drug discovery and testing. These devices integrate

biomedical cutting edge technology including lab-on-a-chip like devices or bioreactors with a strategic designs that may include surface topography, electric field stimulation built in settings, media perfusion and/ or mechanical stretching systems. These miniaturized devices are facilitating the understanding of the cellular behavior in *in vitro* culture systems and may soon facilitate the generation of improved devices for generating automated cell constructs that may potentially be used for therapeutic applications or to be used in pharmaceutical studies that will provide further information regarding pharmakinetic drug behavior.

The future of this field seems to be promising but not yet close to producing a functional therapeutic application that will cure infracted hearts. I consider that the most promising approach that will probably produce the most successful devices, will rely on developing *in vitro* culture systems that integrate multiple micro-environmental factors found within the native tissue that may successfully replicate the environment created during a myocardial infarction. In addition to these elements, being able to find the key factors involved in this phenomena while controlling and amending this condition will lead to the identification of important factors for creating a successful therapy.

1.1.2. Microfabrication Methods:

Studying cells outside their native environment is a routine task in many biomedical research labs. Recently it has been discovered that cells tend to lose their original characteristics when cultured in vitro using tissue culture dishes. Hence, engineering approaches have been developed for generating complex structures that biomimic native

microenvironments for improving cell viability and phenotypic characteristics. Some of these engineering approaches may require intensive operational training and may ultimately prove to be quite expensive and inaccessible to many bioresearch. Other fabrication approaches may be attained using simple custom made techniques with variables results that are more accessible to most bioresearch. In CTE, microtopography fabrication represents an important feature for inducing anisotropic alignment, which is essential to produce *in vitro* culture systems. *In vitro* culture systems have been engineered robustly in order to create complex artificial microenvironments that provide more suitable environments to cells when cultured in conventional tissue culture dishes. A core part of these systems relies on a substrate fabricated by strategic integration of one or multiple features such as surface topography or cell perfusion channels. Some of these commonly used methods are described here:

Photolithography: This method is commonly used in many labs and consists of creating a master mold that is replicated multiple times to create complex arrays containing micropatterns. This technique uses silicon wafers coated with a photo-resisting material, which is then exposed to UV source using a mask aligner containing the desired pattern to be revealed. The polymerized resin is washed and dried out by the action of organic solvents. This technique allows the generation of quite small features ranging from a couple of nanometers to few millimeters as desired. Importantly, this technique facilitates the generation of highly symmetrical and well defined features and the master mold can be usually used several times before a replacement is necessary. A trade off of this technique is an elevated fabrication cost in addition to being quite labor intensive. This

method also requires professional training and skill development before researchers can adequately utilize the equipment required for this technique.

Chemical Etching: This method consists of removing layers from a silicon wafer using a chemical etchant. This process requires skill development, includes increased cost, and the involvement of the disposal of large amounts of toxic waste. This technique has been used to generate microfluidic channels or structures containing multiple layers of different materials.

Hot embossing: This method consists of imprinting features on polymeric surfaces by raising the temperature of thermoplastic materials to reach the glass transition point and pressing a prefabricated master mold that contains the pattern to be imprinted. The next step is to cool down the thermoplastic for assuring the highest quality possible of the imprinted features.

Electro-spinning: This method consists of electro-spinning polymeric materials into a substrate that are rapidly polymerized creating complex inter-linked networks of fibrous materials with the aid of an electric-spinning device with a fine needle point from which the polymer is deposited in anisotropic or isotropic fashion.

Mechanical abrasion: Anisotropic topographical features have been created previously using this method by applying uniform pressure on a rough surface material such as sandpaper over a flat surface or using a polystyrene tissue culture dish or a glass slide. Even though this includes a low fabrication cost, the technique involves custom made instrumentation and similar results are hardly replicable.

Other microstructures commonly used for engineering CTE platforms or simple devices may include: micro-fluidic channels, micro-topographical features, micro-columns,

micro-wells, micro-pillars, or combination of these. Selecting the microstructures will depend on the purpose of the study to be performed and instrumentation and facility availability.

1.1.3. Engineering of *in vitro* Cell Culture Systems:

The environment found in conventional polystyrene tissue culture dishes does not efficiently provide culture conditions to cells as those found within native tissues. These conditions have rather adverse effect on cells, which can lead to cell dedifferentiation, phenotypic loss, and physiologic dysfunction in many cases. Integration of microfabricated features into cell culture platforms facilitates cell survival and promotes proper cell characteristics important for culturing diverse cell sources including primary cells. In CTE, incorporation of microtopographical features into cell culture platforms have provided a state of the art culture environment that has shown enhancing effects on cells as improved phenotype and physiological behavior of diverse cardiac cells from different sources. Besides substrates with microtopography, other features that can be built into devices include microcantilibers or micropilars used to measure cell's mechanical functions and study cellular behaviors when mechanical and morphological activity of cardiac cells is assessed (Thery; Baker; Coutinho et al.; Deutsch et al.; Ghibaudo et al.; Hoffman-Kim, Mitchel and Bellamkonda; Jakab et al.; H. N. Kim et al.; Ovsianikov et al.; Polonchuk et al.; Ross et al.; Zong et al.) . Notably, more sophisticated devices integrate more that one microfabrication feature in the form of bioreactors or lab-

on-a-chip devices, which creates a platform providing a more complex environment biomimicking the cardiac microenvironment increasing survival and physiology of cardiac cells. For instance, Rasidic et al. 2009, describes the advances in CTE and other subfields of TE for the development of different approaches to engineer bioreactors that incorporate diverse accessory features generating complex artificial environments that lead to improvements in cell culture systems (Heidi Au et al.). Predominant accessory elements include electric field stimulation, mechanical stretching, controlled substrate stiffness, etc. In addition to microfabricated features, naturally derived cell factors have also been used to create microenvironments that provide enhanced cell signaling elements into *in vitro* cell culture systems (Alamein et al.; LaNasa and Bryant; Yuval Eitan et al.; DeQuach et al.; Clark et al.). Some of the naturally derived elements include extracellular matrix proteins such as collagens, laminin, and fibronectin, that may come from genetically engineered sources or harvested from cell cultures known to produce such elements. Signaling factors such as cytokines, growth factors, or other phenotype enhancing proteins have also been used as part of these microenvironments (Huh et al.; Chung and King; Iyer, Chiu, Reis et al.; Nenad Bursac et al.; Rustad et al.; Place, Evans and Stevens; Radisic et al.). Elements derived from cardiac cell populations cultured in *in vitro* conditions include a mixture of ECM proteins produced by CF. These cells are kept in culture for producing a complex mixture of ECM; which is then extracted and collected by removing the CF from dishes without disrupting the deposited fibers. The harvested ECM is frequently used as coating material for improving biocompatibility of cells with the culture substrate (DeQuach et al.; Macfelda et al.; VanWinkle, Snuggs and Buja). In other instance, ECM derived from different animal sources is commercially

available and is used as coating material as well. The efficiency of coating will depend on many factors such as concentration, purity, animal source and in many cases more than one protein is used for optimal results and would depend on the characteristics of the material used to fabricate the culture substrate and the cell type to be used for the study (Macfelda et al.; Akhyari et al.). In general, CM are very active cells and may require a combination of more than one type of coating ECM protein for optimal results since the beating activity would rapidly deteriorate the culture substrate (Spahr et al.; VanWinkle, Snuggs and Buja).

Biomimicking the heart environment seems to play a critical role when trying to maintain or enhance the phenotype for studying physiological behavior of cells cultured *in vitro* in an attempt to mirror those found in the native tissue. Important aspects to be assessed include cell-cell contact and communication enhancement, cell morphology maintenance, degree of tissue reconstruction, etc. For assessing the functionality of artificially created cell culture platforms, cellular constructs are evaluated by morphological or physiological assays such as immunohistochemical assays to evaluate cell to cell connectivity using antibodies to identify protein or protein complexes expressed as cells establish connections. For instance, connexin-43 and N-cadherin have been extensively used as markers to assess cell-cell connectivity. In the case of cell-substrate connectivity, vinculin represent a common marker to measure the degree of this type of connection. Calcium signaling assays is another study that has been previously used to measure cell-cell communication analysis, which consists of using a Calcium dye such as Fluo-4AM to identify calcium waves in cultured cardiac cells. For this particular assay, cells are cultured with the dye in buffer saline solution and evaluated using a fluorescent or

confocal microscope to identify calcium activity within interconnected cells. Functionality of each device is evaluated by means of the degree to which complex interconnection between cells is established. Previous studies have provided insight of important elements to be incorporated in our CTE designs in order to obtain significant results and/or information as trying to elucidate the functionality of the heart in *in vitro* culture systems (Huh et al.; Lenas, Moos and Luyten; Radisic et al.; Ross et al.; Chung and King; Lieber et al.) .

1.1.4. Cell Sources Used in CTE:

Selecting a cell source represents critical step for establishing a cell culture that will successfully proliferate and adapt to *in vitro* culture conditions and to integrate to new devices that are designed and created to investigate particular cellular behaviors relevant to biomedical research applications. There are many variables needed to take in consideration in order to obtain a healthy and functional cell culture. These include: species from which cells are harvested, developmental stage at which cells at harvested, media content in which cells would be culture in, culture conditions, method of extraction used to harvest the cells, etc. For instance, each species would generally differ from each other as well as the culture condition in which cells would be kept in for optimal results. It is also important to define the number of cells needed for particular studies since this would vary between species or between different stages of development. For example fetal, neonatal and adult CM exhibit different morphological and physiological

characteristics and the culture conditions needed for each case are different as well as the viability of each cell type (Norris et al.; P. D. Nguyen et al.; Sreejit, Kumar and Verma; Piper et al.; Piper, Jacobson and Schwartz). Importantly, cell from different cell sources will present slightly different morphological and physiological characteristics and may also behave differently when cultured in *in vitro* conditions. Chick, quail, mice, rat, rabbit, pig, and human are some of the common cell sources used in CTE applications. The developmental stage from where cells are harvested, also presents important implications concerning cell numbers, viability, morphology, etc. It is important to keep in consideration the purpose for which the study is performed along with the information that is planned hoped to be gathered, when selecting for the best possible cell source candidate available. For instance some of the identifies developmental stages viable to harvest cells includes fetal, neonatal, adult and those derived from embryonic stem cells (ESC) or from other pluripotent cell sources such as mesenchymal or other adult pluripotent cells. Each of one of these would have variations in morphological and physiological characteristics. For example, fetal murine cells are usually more resistant, viable and adapt easily to in vitro culture conditions when compared to those from neonatal origin. Similarly, CM from neonatal sources present similar characteristics as fetal when compared to adult cell sources. The CM from fetal stage are often defined from the early developing heart to previous to the postpartum; while neonatal cells may be consider from around day 0 to 4 or 5 postpartum, and adult stage would be from the end of the neonatal stage and beyond and would be represented by the development of t-tubules that generally will occur from few days to a couple of weeks after postpartum. Maintaining the phenotypic characteristics of cells *in vitro* represents major challenge to

be addressed with previous studies having shown that fetal CM show higher plasticity than NN or adult CM in terms of maintaining original phenotype in *in vitro* culture conditions compared to other cells from more advanced developmental stages (Alford et al.; Ottaviano and Yee). Previous studies have reported differences in the proportions and frequencies of the different cell subpopulations found in the heart at different developmental stages as well as within different species (Banerjee, Fuseler, Price et al.). This is interpreted as some species having increased number of CM compared to the number of CF, or a neonatal would differ in CM numbers compared to an adult or a fetal animal. Each one of these differences and characteristics should have to be taken into consideration for having improved results when planning and expecting particular significant experimental outcomes.

1.1.5. Cell Subpopulations of the Heart:

The heart is composed of heterogeneous subpopulations of cells with different functions and phenotypic characteristics that work in a synchronous way to maintain the homeostasis of the organ and generate the systematic function of the heart consisting in pumping and regulating the blood flow in the body. The cell subpopulations of the heart can be subclassified into two main groups; Myocytes and accessory cardiac cells (Banerjee, Yekkala et al.; Banerjee, Fuseler, Price et al.). Myocytes can additionally be subclassified into atrial, ventricular, and nodal, and each one of these subgroups into myocardial, endocardial, and pericardial according to their relative position within the

heart. On the other hand, accessory cardiac cell subpopulations include smooth muscle cells (SMC), endothelial cells (ENDC), cardiac fibroblasts (CF), and peripheral blood cells. Each subpopulation would be found in different proportions and frequencies according to different stages of development and different species. For instance in adult mice, CF represent about 25%; SMC about 10%; ENDC about 5; and CM about 60% of the total cell population reported by Benerjee et al. 2007 (Banerjee, Fuseler, Price et al.). Endothelial cells are regularly found in the vasculature of the heart and are responsible for forming and controlling vascularization and new vessel formation. CF are found all over the heart and may play critical roles through development according to previous studies (Ottaviano and Yee; Togo et al.; Narmoneva et al.). Important functions of these cells include provide signaling factors and structural support to the heart. CF are mostly responsible for depositing, organizing, and maintaining the ECM of the heart for providing mechanical and cell support of the organ. SMC are mostly found in the vasculature as structural and mechanical part of veins and vessels. Peripheral blood cells are also frequently found circulating the heart in smaller proportions compared to the other described cardiac accessory cell subpopulations and include mostly cells from the immune system or other circulating pluripotent cell populations.

The myocytes represent the most frequent cell subpopulation and are responsible for the generation of the action potentials that triggers the mechanical contraction of the heart. This subpopulation can be generally classified as ventricular, atrial, and nodal CM and the name derives from the tissue of origin accordingly. Each of these cell subtypes exhibit slightly different phenotypic and physiological differences. For instance, each of these cells has a characteristic action potential and plays a unique role in the overall

electrophysiological activity of the heart. Ventricular CM generally have an elongated cell body and express the 2v isoform of the myosin light chain (MLC) protein. On the other hand, atrial CM show a more elliptical or flat cell body and express the 2a isoform of the MLC protein (Chuva de Sousa Lopes et al.; Lee et al.; Sanchez et al.; Kubalak et al.).

1.1.6. Morphological Characteristics of the Heart and *in vitro* Cardiac Biomimicry

1.1.6.1. Cardiac Anisotropy Multi-level organization: The heart is a highly anisotropic organ composed of multilayers of CM interlinked with other accessory cells and ECM fibers predominantly collagen fibers (figure 1.4)(LeGrice et al.; Caldwell et al.).The ventricular region of the heart is composed of radially overlaid multilayers of cells that range from a couple of cells in thickness with a slightly divergence of a couple degrees in orientation. On the other hand, the atrium is composed of a more complex cytoarchitecture that is less homogeneous than the ventricles (Zhao et al.; LeGrice et al.). This intrinsic architecture is the result of a highly orchestrated process speculated to originate during heart looping (ED8.5) (Figure 1.2)(Martin-Puig, Wang and Chien). Early in heart development, the heart tube undergoes sequential cytomorphological arrangements leading to myocyte elongation and alignment(Manasek, Burnside and Waterman). This alignment is maintained throughout the organs lifetime and provides important contribution to the mechanical and physiological functioning of the heart. An important function attributed to the anisotropic properties of the cardiac tissue is

facilitating mechanical and electrical coupling between cells; which is required to synchronize and orchestrate electric and mechanical propagation of the heart (Valderrabano). This anisotropic patterning of cells also facilitates the development of cytoanatomical structures as the intercalated discs at the contacting structures formed at CM terminal ends composed of gap junctions and macula adherens proteins, which is thought to occur parallel to the anisotropic cell formation and is facilitated by cell-cell contact alignment (Gutstein et al.). Another important event occurring as a result of anisotropic cytoarrangement accounts for the intracellular forging that leads to sarcomere development and maturation required for intrinsic physiological performance within the heart (Rash, Biesele and Gey). Over all, the heart architecture exhibit multi-level organization anisotropic patterning that facilitates proper organ physiological function which is necessary to pump blood all over the body (figure1.4).

1.1.6.2 Induction of CM Anisotropy in vitro: In general, most of the methods used to induce *in vitro* cardiomyogenesis fail to provide crucial signaling environmental factors needed for proper CM development and maturation such as anisotropic patterning or other signaling elements provided by accessory cell subpopulations in the heart (i.e. cardiac fibroblast or homologous progenitor cells). Biomimicking anisotropic development *in vitro* would induce cells to acquire an anatomical morphology in accordance to *in vivo* development. This particular mechanotrasduccion response has been previously induced by providing cells with a cell culture substrate containing oriented microtopograpical configurations biomimicking those described by ECM fibers or other anisotropic aligned cells found in the native tissue. Anisotropic induction has shown to promote the guidance resulting in unidirectional mechanic and electric

propagation occurring in the cardiac syncytium (D.-H. Kim et al.; Chuva de Sousa Lopes et al.; Jakab et al.; Baudino et al.). In previous studies Lieu et al., 2009 suggests that common culture conditions lacking a mechanism to induce anisotropic alignment will interfere with the development of CM-dESC resulting in failure to develop specific cellular structures such as the t-tubules required for proper CM functionality (Lieu et al.). In this report, Lieu *et al* reported that the lack of co-expression of caveolin-3, amphiphysin-2 or dihydropyridine receptors (DHPRs) and ryanodine receptors (RyRs) in both human ESC-derived CM and mESC-CM. These abnormalities may have contributed to the failure of t-tubule formation leading to obstruction of the maturation process and generating dysfunctional cells that would be inadequate for therapeutic applications.

Previous studies have suggested that anisotropic feature integration into *in vitro* CM culture systems provides beneficial environmental elements improving *in vitro* culture conditions (Motlagh et al.; Luna et al.; Zong et al.; Heidi Au et al.). These studies investigated the effect of topographical cues and contact guidance on CM cultures;, which resulted for the most part in phenotypic enhancement and anisotropic alignment of CM harvested from different sources. The mechanism(s) by which CM respond to contact guidance is not fully understood, however, it has been proposed that focal adhesions contacting the physical environment initiate the signaling cascades leading to cytoskeleton remodeling in accordance to topographic characteristics (reviewed in(Dalby et al.)). Several groups have developed fabrication approaches to create cell culture platforms containing microtopographical features that induce cell alignment. These cell grafts exhibit increased expression and localization of connexin-43 and N-cadherin at the cell-cell junctions which may be an indication of the development of intercalated disks

along with an improved electric and mechanical coupling or at least an improved cell-cell system communication development that more closely resemble the anatomical and physiological form of the native tissue. During the last decade, different groups have additionally incorporated and integrated other multiple device functionalities into CM culture platforms (i.e thermoresponsive surfaces, electric field stimulation, ECM matrices, etc.) resulting in generation of diverse cellular constructs exhibiting improved tissue like characteristics (Entcheva et al.; Sekine et al.; Heidi Au et al.; Atala, Kasper and Mikos; Matsuura et al.; Chiu et al.; Chung and King; Dengler et al.; Phillips and Brown; Rustad et al.; Stevens et al.; Birla et al.; Blan and Birla; Ovsianikov et al.; Freed et al.; N. Bursac et al.; Alekseeva et al.; Tulloch et al.; Radisic et al.; Kubalak et al.; Jakab et al.).

1.1.7. Characteristics of CM Derived from ESC for CTE Applications:

A significant challenge of ESC-dCM lies in the derivation methods used to generate CM with phenotypes similar to native CM or with improved physiological characteristics to be used in cell replacement therapies or in pharmacological studies. In the case of tissue engineering applications for therapeutic applications, CM generated *in vitro* would ideally need adapt and integrate to host tissue while need to survive the continuous systolic/diastolic forces generated by the heart's contractions. Additionally, cells need to replace the damaged/dead tissue and function as mature adult CM. Previous studies have reported an increased number of the deficiencies in CM-dESC generated using available differentiation techniques from commonly used cell sources including ESC,

mesenchymal stem cells, and/or induced pluripotent stem cells (iPSC) (Roccio et al.; Sheridan, Surampudi and Rao; Jensen, Hyllner and Bjorquist; Gherghiceanu et al.; Gonzales and Pedrazzini; Chiu et al.). In general, these techniques generate CM-like cells exhibiting unorganized sarcomeres, deficient intracellular calcium handling capabilities, mismatched gap junction distributions, and abnormal cell size and shape. Biomimicry of the temporal cardiac microenvironment occurring through development into differentiation approaches would potentially improve the efficiency of the methods in addition to generate viable CM with improved physiological and phenotypic characteristics.(Synnergren et al.; Rajala, Pekkanen-Mattila and Aalto-Setala).

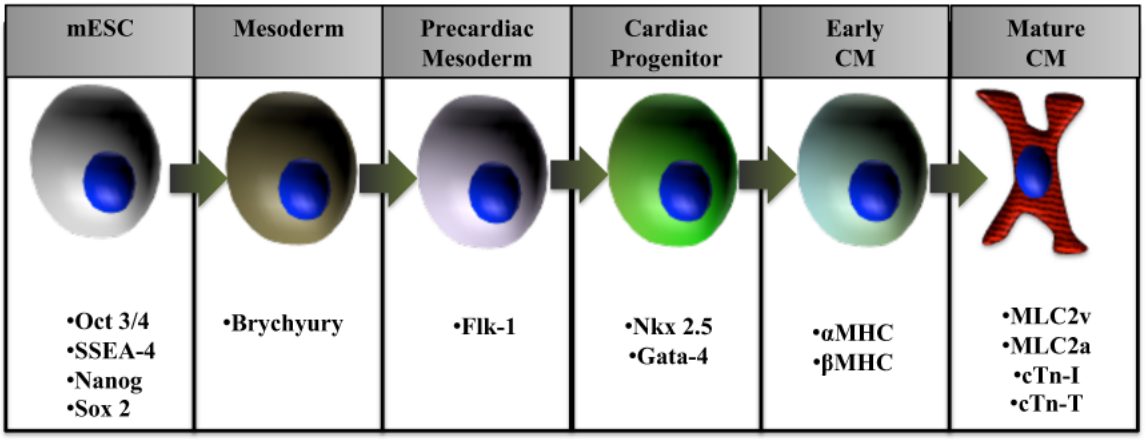


Figure 1.1: CM differentiatiation Diagram: Schematic representation depicting the process involved in cardiomyogenesis including markers commonly used to identify each corresponding developmental stage.

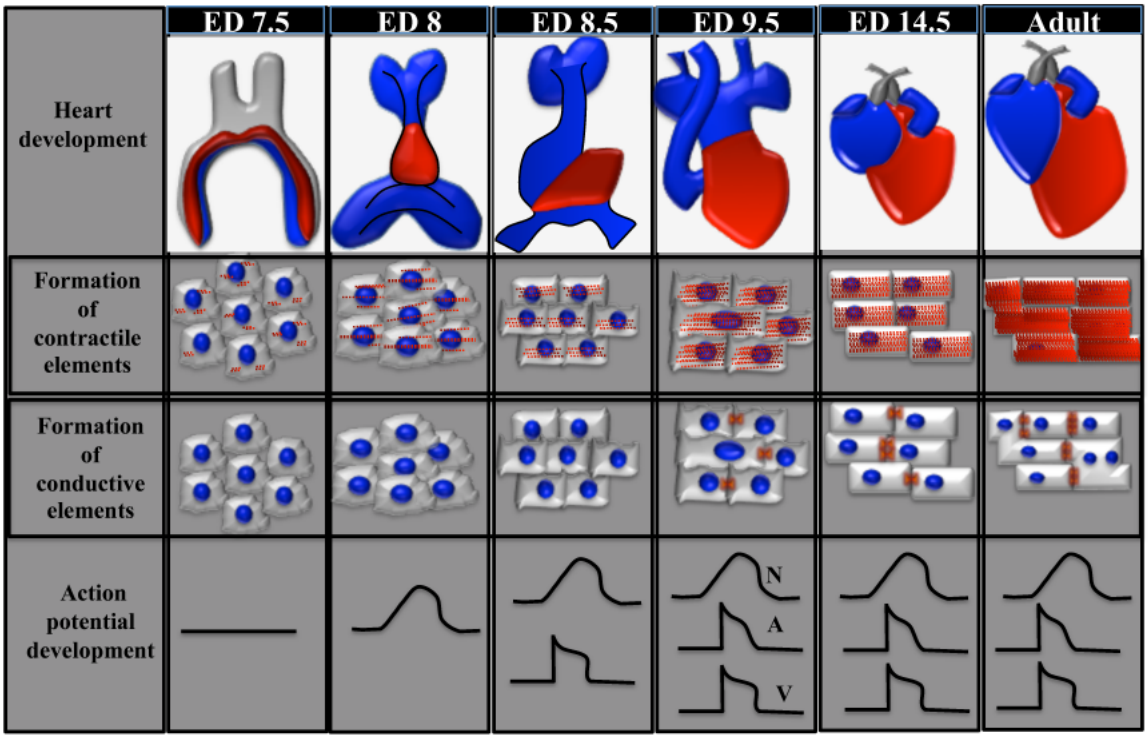


Figure 1.2: Schematic illustration of progressive contractile and conductive maturation during cardiogenesis. Schematic representation of the different stages of mouse cardiac development: cardiac crescent, cardiac tube, cardiac tube looping, embryonic, fetal, and adult heart respectively. N = nodal; A = atrial; V = ventricular.

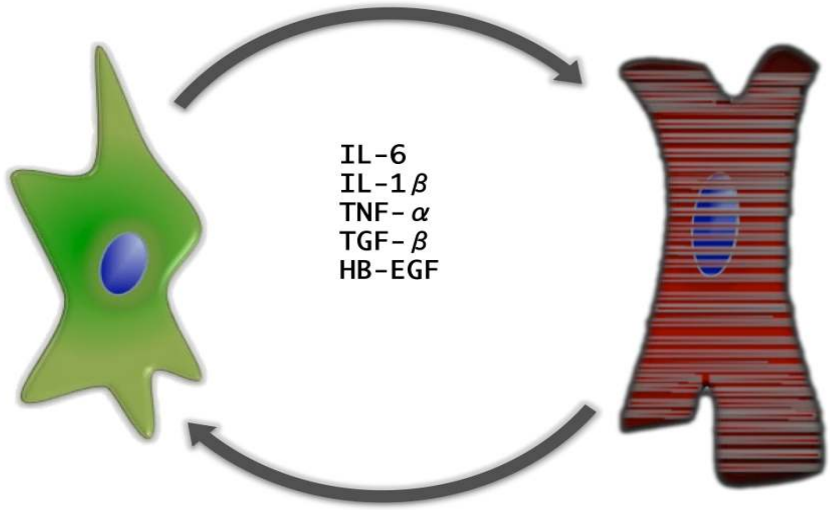


Figure 1.3: FCF-CM communication: Schematic representation depicting CF and CM signaling communication including important cytokines and growth factors thought to be involved in the crosstalk mechanisms of these cell subpopulation through development and in response to diverse stimuli.

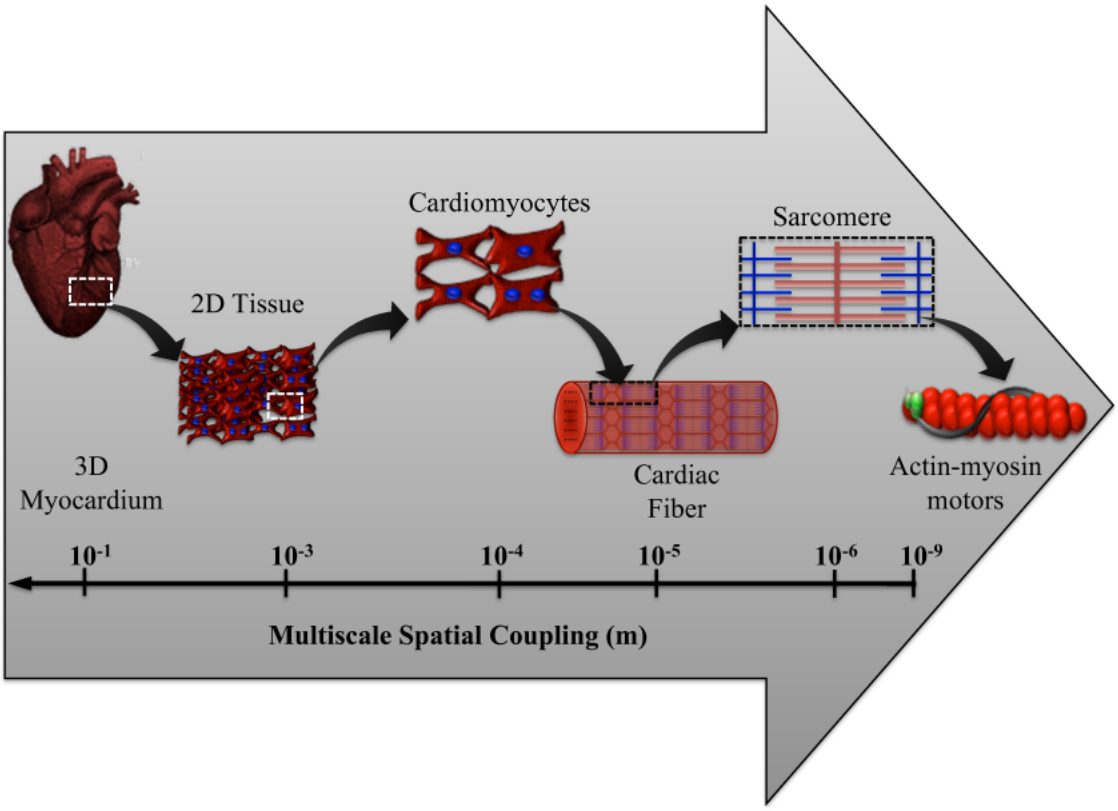


Figure 1.5: Scaling of ventricular muscle. The assembly of ventricular muscle represents a scaling problem that spans several orders of spatial magnitude from the alignment of actin-myosin complexes within a sarcomere, their alignment in myofibrils, the organization of myofibrils in a myocyte, and the coupling between myocytes in anisotropic, laminar muscle. 2D and 3D indicate two- and three-dimensional, respectively.

CHAPTER 2

MATERIALS AND METHODS:

2.1. Multi-scale topography:

Metal wrinkles were fabricated as previously reported and described in detail (Luna). Briefly, gold–palladium was deposited by sputter coating (SEM Sputter Coater; Polaron) at various thicknesses. I showed that I was able to achieve controllable heterogeneous wrinkle length-scales based on varying thickness of metal coating on pre-stressed polystyrene (PS) sheets (Grafix); coating thicknesses ranged from 15 to 90 nm, with all thicknesses generating wrinkles ranging from 20 nm to 10 mm and average wrinkles thicknesses ranging from 800 nm to 1 mm and increasing proportionally with coating thickness.31 Due to the heterogeneous range of thicknesses, all wrinkles tested in preliminary cell alignment studies using stromal cells were able to successfully induce alignment of the cells (data not shown). I chose a 60-nm coating thickness for our studies. After deposition, PS sheets were induced to thermally shrink uniaxially by constraining them from opposite sides (2 inch binder clips; OfficeMax) and heating to 150°C–160°C. The deposited metal layer on top of the PS sheet generated aligned wrinkles serve as a soft lithography mold for generating PDMS microchips for culturing the cells (Fig. 3.1). The anisotropy and length scale of the wrinkles were determined by performing a Fast Fourier transform of the scanning electron micrograph (Fig. 3.1); the critical length scale

was plotted as a probability function and range in critical dimension from the 100s of nm to several microns. Similarly, the depth of the wrinkles, as determined by profilometry (Tencor Alpha-Step 200), was on the same range with self similar features from the 100s of nm to *3 mm. A mixture of 10:1 ratio of PDMS and curing agent (Sylgard 184 Silicon Elastomer Kit; Dow Corning) was poured on the metal mold and set to cure at 75°C. It was then peeled off and cut into to a circle with diameter 15 mm to fit into 24-well plates. Controls with flat PDMS were performed following the same procedure. The chips were sterilized following standard procedures and then coated with 25 mL of 1:1 1 mg/mL laminin (Invitrogen) and 1 mg/mL bovine fibronectin (Sigma Aldrich). As apparent from Figure 1C, the features are highly anisotropic and the self-consistent structures span from the 400 nm to the 50 mm mimicking the fibrillar network of the heart's ECM. Because the metal integrates into the plastic when the plastic retracts, the metal wrinkles are robust and can withstand ultra-sonic Fatigue testing as well as multiple moldings without delamination. Moreover, the ease and simplicity of this microfabricaiton design for generating the wrinkle template molds can easily be replicated in any biological laboratory with only minimal equipment; a sputter coating system and an oven to thermally shrink the PS sheets.align upon attachment (within 2 days). This work demonstrates that I can align both murine neonatal CM (NNCM) and CM derived from human embryonic stem cells (hESC) in vitro using our biomimetic surface topography. Importantly, the CM derived from hESC *in vitro* are capable of sensing and responding to appropriate nano- to microscale mechanical cues.

2.2. Murine NNCM isolation:

All animal procedures were reviewed and approved by the UC, Merced Institutional Animal Care and Use Committee (IACUC). C57BL/6 (B6) mice were purchased from Jackson Laboratories or bred in house and housed in sterile microisolator cages. Neonatal mice were euthanized after birth by hypothermal shock. Cell extraction was performed as previously described with some modifications. Briefly, hearts were extracted from neonatal mice and placed in chilled 1 · PBS (calcium magnesium free). Blood was removed by squeezing the ventricles, rinsed with PBS, and placed in HBSS for 10 min. Auricles were removed, ventricles quarter minced, and incubated with 0.05% trypsin–EDTA (Gibco) in HBSS for additional 10 min at 37°C. The trypsin solution was removed and the predigested tissue transferred to 0.2% collagenase-type 2 (Worthington, USA 220 units/ mL) in HBSS, vortexed for 1 min, incubated in water bath at 37°C for 3 min, vortexed for 1 min again, and set to sediment for 1 min. Liquid solution was transferred to a new falcon tube containing DMEM/F:-12 (Hyclone Thermoscientific) enriched with 20% fetal bovine serum (Atlanta Biologicals) to stop enzymatic digestion. Two cycles were repeated to completely digest the tissues. Cell suspension were filtered using 70-micrometer nylon mesh (BD Bioscience) followed by centrifugation for 15 min at 2500 rpm and 4°C and then resuspended in the culture medium. CM were enriched by the following differential attachment technique. Cells were incubated in a T75 flask for 1 h. Then, nonadherent cells were collected and centrifuged for 10 min at 2500 rpm at 4°C.

Cells were counted by trypan blue exclusion test. The enriched CM were then seeded onto PDMS substrates at a concentration of 7.5 · 10cells per chip, about 1.80 cmin 1 mL of media containing 20% fetal bovine serum, 5% horse serum (Invitrogen), 100 U/mL penicillin–streptomycin (Gib- co), 2 nM L-Glutamine (Gibco), 3 mM sodium pyruvate (Cellgro Mediatech), 1 mg/mL bovine insulin (Sigma), and 0.1 mM nonessential amino acids (Gibco) at standard conditions 95% air and 5% CO2 at 37°C.

2.3 Enrichment of Cardiac Fibroblasts :

Additionally, the neonatal mouse cardiac fibroblasts (CF) were extracted and purified to analyze their behavior on the wrinkled substrate and their potential role in guiding the alignment of the NNCMs. For isolation of CF, the same protocol for cardiomyocyte extraction was performed except that the adherent cells were collected and cultured rather than the non-adherent cells. After 24 h in media containing DMEM (Gibco), 20% FBS, and 100 U/mL penicillin– streptomycin, the CF were enriched further by the selective adhesion method described above. Selective adhesion was then repeated three times to obtain highly enriched fibroblast cultures. The FBS in the medium was reduced to 10% to prevent overgrowth of cardiac fibroblast or endothelial cells. protocol for cardiomyocyte extraction was performed except that the adherent cells were collected and cultured rather than the non-adherent cells. After 24 h in media containing DMEM (Gibco), 20% FBS, and 100 U/mL penicillin–streptomycin, the CF were enriched further by the selective adhesion method described above. Selective adhesion was then repeated three times to

obtain highly enriched fibroblast cultures.

2.4 Immunostaining:

Cells were washed with PBS, fixed with 4% paraformal- dehyde, and permeabilized with 0.1% Triton X-100 with 1% BSA. Cells were then washed again and blocked with 10% BSA and normal goat serum (Jackson Immunoresearch Laboratories). Cells were incubated for 30 min with anticardiac troponin I (CTN-I; Santa Cruz Biotechnology) to identify the cardiac cells followed by anti-rabbit Alexa 488 (Invitrogen). Cells were then rinsed and costained with rhodamine- phalloidin (Invitrogen). CD31-FITC (eBioscience) was used for the identification of endothelial cells, and CD 90.2-FITC (Thy 1.2; eBioscience) was used for staining the fibroblasts.33,34 Although CD90.2 is not a specific marker of cardiac fibro- blasts, it is expressed by these cells in the heart and used to identify fibroblasts by flow cytometry and immunofluorescence analysis.35 The nuclear staining was performed using DAPI for all immunofluorescence assays. The CF were characterized by immunofluorescence analysis using antibodies against CD90.2-FITC (eBioscience) and DDR-2 (Santa Cruz Biotechnologies) followed by anti-rabbit Alexa-488 (Invitrogen), a marker specific for cardiac fibroblasts, followed by actin staining using rhodamine-phalloidin (Sigma) to analyze fibroblast morphology as previously described.36 Anticardiac troponin I (CTN-I; Santa Cruz Biotechnology) and anti-VE-cadherin-PE (eBioscience) were used to confirm the absence of cardiomyocytes and endothelial cells in the CF cultures. For gap junction and fascia adherens protein localization, the cells were fixed with 4% paraformaldehyde, blocked with 1% BSA, and

incubated with either connexin-43 (Santa Cruz Biotechnology) or N-cadherin (Abcam), respectively, followed by anti-rabbit Alexa-488 (Invitrogen). For visual localization of the focal adhesions, an antibody against vinculin (Santa Cruz Technology) was used followed by Alexa-488. Samples were mounted on a cover glass using mounting medium (Vector Laboratories) and imaged with an inverted fluorescent microscope (Nikon Eclipse TE2000- U) and digital camera (Photometrics Coolsnap). To characterize myofilament alignment in the hESC-CM, the cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.2% Triton. After blocking with 10% goat serum and 1% BSA, the cells were labeled with mouse monoclonal tropomyosin antibody (T9283; Sigma) and then Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen). Hoechst 33342 was used as the nuclear counterstain.

2.5 Flow cytometry analysis:

For CM population analysis, the cells were washed with PBS, fixed with 4% paraformadehyde for 15 min, and per- meabilized with 0.1% Triton X for 10 min. The cells were then rinsed with PBS, centrifuged at 2500 rpm for 5 min at 4°C, and blocked with 10% BSA and normal goat serum. The cells were stained with anti-CD31-FITC (eBioscience) for endothelial analysis, anti-CD90.2-FITC (eBioscience) for fibroblast analysis, and anti-Cardiac Troponin I (CTN-I; Santa Cruz Biotechnology) followed by FITC-conjugated donkey anti- rabbit (Biolegend) for quantification of the cardiac cells. Analysis was performed with a flow cytometer BD-LSRII (Biosciences) by acquiring 1 million cells per run, and analyzed using FlowJo 7.5 software. The CM, endothelial, and

fibroblasts compositions were measured for three separate neonatal heart isolations (n = 3) consisting of 4–7 neonatal hearts per isolation.

2.6 hESC culture and cardiac differentiation:

The hESC (WiCells) HES2 line (NIH code: ES02) was cultured under feeder-free conditions on hESC-qualified Matrigel (Becton Dickinson) in mTeSR (Stem Cell Technolo- gies) and differentiated as described by Yang et al.37 Briefly, hESCs were detached using trypsin (Invitrogen) and transferred to low-attachment plates to allow aggregate forma- tion. The aggregates were cultured in suspension for 12 days in StemPro-34 medium (Invitrogen) with 2 mM L-glutamine (Invitrogen), 150 mg/mL Holo-Transferrin (Sigma), 0.45 mM monothioglyerol (Sigma), and 50 mg/mL ascorbic acid (Sig- ma) with addition of 10 ng/mL BMP4 (R&D Systems), 3 ng/mL activin A (R&D Systems), and 5 ng/mL bFGF (Invitrogen) from day 1–4, 10 ng/mL VEGF (R&D Systems), and 150 ng/mL DKK1 (R&D Systems) from day 4–8, followed by 10 ng/mL VEGF, 150 ng/mL DKK1, and 5 ng/mL bFGF from day 8–12. This directed differentiation protocol yielded > 50% of spontaneously beating cardiospheres. To isolate the hESC-CM, the spontaneously contracting cardiospheres were picked and digested into single cells with collagenase II (1 mg/mL) at 37°C for 30 min, followed by 0.05% trypsin–EDTA (Invitrogen) at 37°C for 5 min. The isolated cells were then cultured in KB solution containing (mM): 85 KCl, 30 K_2HPO_4, 5 $MgSO_4$, 1 EGTA, 2 Na_2-ATP, 5 pyruvic acid, 5 creatine, 20 taurine, and 20 D-glucose, at room temperature for 30 min, followed by plating onto the wrinkles. Because these methods generated low numbers of

purified CMs, I also proceeded to transduce these hESCs with the MLC2v promoter that drives both GFP fluorescence and zeocin antibiotic resistance to more easily indentify and purify the ventricular CMs. To obtain GFP-labeled ventricular cardiomyocytes (GFP-VCM), single cells isolated from cardiospheres were plated and cultured with the medium containing 80% DMEM, 20% FBS defined (HyClone), 1 mmol/l L-glutamine, 1% NEAA, and subsequently transduced with recombinant LV-MLC2v-GFP particles at multiplicity of infection (MOI) of 5. The MLC2v promoter simultaneously drove the expression of GFP as well as zeocin for antibiotic resistance in the trans- duced hESC-VCM. The GFP-VCM were then purified with a 5-day treatment of zeocin (300 mg/mL).

2.6. Image Analysis:

The images were processed further to quantify the distribution of orientations using custom scripts written in MATLAB (MathWorks, Inc.). Images of labeled actin, CTN-I, Cx43, and N-cadherin were filtered to estimate the image gradient at each pixel location using a Guassian derivative (s = 2). The distributions of the orientations were estimated by computing a histogram of gradient orientations, where the contribution of each pixel is weighted by the gradient magnitude. This weighting limits the contribution of pixels in low contrast regions of the image where gradient orien- tation estimates are uncertain. The orientation histogram was computed for n = 12 disjointed 300 · 300 pixel subwindows in each image, and the standard deviation was computed across the subwindows. An additional measure of cell alignment was also com- puted by detecting DAPI labeled nuclei and estimating the orientation of the major axis of each nucleus.

Nuclei were segmented by thresholding followed by morphological processing and watershed segmentation to separate overlapping nuclei. The orientation of each segmented nucleus was estimated by computing the moment matrix of the segment and identifying the major and minor axes. Potential elongation of the nuclei was computed as the ratio of the lengths of the major and minor axes.

2.7. FCF isolation:

C57BL/6 (B6) mice were purchased from Jackson Laboratories and bred in-house. Pregnant females were euthanized at D15.5, fetuses extracted, and whole hearts removed and placed in chilled PBS (calcium magnesium free). Blood was removed by squeezing the hearts, rinsing with PBS, and placing in HBSS for 10 min. Hearts were then quarter minced, and incubated with 0.05% trypsin–EDTA in HBSS for additional 10 min at 37°C. The trypsin solution removed and the predigested tissue transferred to 0.2% collagenase-type 2 (220 U/mL) in HBSS, vortexed, incubated at 37°C for 3 min, vortexed again, and set to sediment for 1 min. The liquid solution was transferred to a new falcon tube containing DMEM (Gibco) enriched with 20% fetal bovine serum (Atlanta Biologicals) to stop enzymatic digestion. Two cycles are repeated to completely digest the tissues. Cell suspensions were filtered using a 70-micrometer nylon mesh (BD Bioscience) and resuspended in medium containing advanced DMEM, 10% FBS to stop enzymatic digestion. Thereafter, cells were centrifugated at 1500 rpm for 5 minutes, supernatant removed and resuspended in advanced DMEM containing 10% FBS.

2.8. FCF enrichment and phenotype maintenance:

Cells were enriched by selective adhesion method as previously described (Sreejit, Kumar and Verma). In brief, cell suspensions were incubated in a 100mm culture dishes for 1 h and non-adherent cells (CM) were removed. Adherent cells were grown to confluency for about 7 days in media containing advanced DMEM, 10% Knock-out serum replacement (KSR, GIBCO), 100 U/mL penicillin– streptomycin (Gibco), and 2000 U of Leukemia Inhibitor Factor (LIF, Esgro) and incubated in standard culture conditions replacing media twice a week. Once cells reached confluency, a second selective adhesion treatment was performed to further enrich for CF only.

2.8.1 FCF phenotype maintenance studies: FCF were extracted from C57BL/6-Tg(alpha SMA-RFP) and cultured in media containing LIF and withouth LIF as control as described. RFP expression was used to identify the differentiation of FCF into myofibroblasts after enrichment.

2.8.2. mESC maintenance: mESC line E14-Myh7-GFP were donated by Dr. Bruce Conklin, UCSF. The mESCs were maintained in mESC medium containing 80% DMEM medium (Gibco), 20% KSR (Gibco), 1 mM L-glutamine (Gibco), 0.1 mM –mercaptoethanol (Gibco), 1% nonessential amino acids (Gibco), 100 U/mL penicillin–streptomycin (Gibco), and 10ng/ml Bone Morphogenic Protein 4 (BMP4) (R&D systems), and 2000 U of LIF. E14 cells were passed about once a week by incubation in 0.05% of Trypsin-EDTA (Gibco) for 5 min at 37°C. Then, cell were replated in 60mm 0.5% gelatin coated dishes and media was replaced daily.

2.9. mESC Cell Culture:

mESC culture and differentiation assay: mESC were culture in 0.5% gelatin coated 12-well tissue culture plates at a $7.5X10^4$ cells/ml in maintenance media for 2 days. Then, media was replaced by differentiation media 1 containing 80% DMEM medium (Gibco), 15% KSR (Gibco), 1 mM L-glutamine (Gibco), 0.1 mM –mercaptoethanol (Gibco), 1% nonessential amino acids (Gibco), 100 U/mL penicillin–streptomycin (Gibco), and 0.6 ng/ml Activin A (R&D systems) for 2 additional days. Thereafter, cell were cultured in differentiation media 2 containing the same ingredients as media 1 but replacing Activin-A with 0.5ng/ml of BMP-4, under the following conditions : i) mESC in direct contact with FCF ($2.5X10^3$ cells/ml); ii) mESC cultured in indirect contact with FCF ($2.5X10^3$ cells/ml) using a transwell insert (Corning); and iii) mESC alone.

2.10. Microfabrication method using crazed polystyrene:

2.10.1. Fabrication Methods:

Chamber mold fabrication: This process includes fabrication of a master mold that contains the predetermined fabrication elements defining the culture chamber characteristics. This mold was custom made according to determined dimensions as well as the selected surface topography. The selected processed PSPS containing the features

was fixed to a base made out of polystyrene.

Structural Substrate Fabrication: This process includes the fabrication of the platform's structural base using cell-culture compatible materials or those materials commonly used in device or tool fabrication. These include materials such as: PDMS, gelatin, agarose, etc. This structure was built into a container, which is generally any commercially available cell culture dish of any size. The process for using PDMS is described as follows: A layer of PDMS/cross-linker mixture (10:1 PDMS to cross linker ration was commonly used) is added to the container uniformly covering the surface and removing air bubbles formed. Then, selected chamber-mold was placed into the unpolymerized PDSM and set to cast until completely polymerized. Thereafter, mold was removed by cutting the PDMS around the edges and by cautiously pealing off the bottom surface to minimize damage to the surface topography.

2.10.2. Cell culture platform preparation and use:

Cleaning and conditioning:

Cleaning and surface modification: For improving biocompatibility of polymers such as PDMS, the devices were plasma treated using a surface treatment instrument such as SPI Plasma-Prep II, others. Surface was air-blowed followed by tape cleaning previous to plasma treatment. Cleaning and sterilization was performed by exposing the devices to a UV-light source for about 10-30 minutes followed by phosphate buffer saline (PBS) rinse inside biohood.

Surface coating: Material determined for surface coating was applied to the cell-culture surface area according to optimized conditions for each particular cell type. Then, coated

devices were incubated with cell culture medium (similar to the medium to be used for selected cell culture) for at least 30 minutes (ideally over night) before using it.

2.10.3. Cell seeding:

Cell concentrate (solution containing cells of interest suspended in few hundred micro liters of culture medium) was placed onto the area of the culture chamber for about 30-60 minutes allowing cells to adhere to the surface. Thereafter, culture medium was increased to a volume that was enough to maintain cell viability.

2.10.5. General Fabrication Methods for Fabrication of T1 and T2:

Method T1:

i.) Metal Coating: Treated pieces were metal coated facilitated by coating systems commonly used in SEM sample preparation (i.e. Hammer VI metal sputter system with metal/alloy targets such as: Au, Au-Pd, Ag, etc.)). Optimal metal coatings with thicknesses of about 10-100 nm were commonly used and were selected based on deposition uniformity. The coating was cyclically applied in intervals of 15-30 sec and by letting samples to cool down for about 1-2 minutes to avoid material deformation caused by over heating.

ii.) Thermal Deformation: This process includes experimental settings adapted for producing anisotropic or isotropic topography features (Figure 2.4).

a. Anisotropic feature generation: Settings for anisotropic feature generation involve material deformation in a single axis. Coated pieces were mounted onto a cardboard piece and a glass slide (both 2.5-inch by 5–inch) by clamping them from the width–side using

2 pairs of 1-inch binder clips as shown in figure 2.4A. Thereafter, mounted pieces were placed on top of a hot plate or a glass-window oven pre-heated at or about 160 ° (±20°) for about 5-10 minutes. For generating features with more or less consistent characteristics, the sheets are shrunk at similar proportions as described in figure 2.4B To stop the deformation process, pieces were removed from the heat source and set to cool down at room temperature for about 10-15. Then, once the deformation process has stabilized, pieces are demounted. This fabrication process generates multi-scale, continuous anisotropic and periodic topographical features produced as a result of the mechanical unidirectional strain during the shrinking process causing a deforming retraction of the metal layer. For this process the area containing uniform anisotropic features is generally formed in the central region of the sheets and may be found in or about 0.25 cm from the length-sides and 2 cm from the width-sides (Figure 2.4Bc). This area is cut off using a laser cutter or other instrument minimazing damage to the selected surface.

b. Isotropic feature generation: Settings for this process included placing coated PSPS onto a glass slide followed by thermal shrinking similar to unidirectional shrinking, but without clamping. The pieces were completely shrunk and set to cool down at room temperature. Topography generated through this process is described by isotropic multi-scale picks.

Method II:

i.) Surface Degradation: Treated pieces were briefly immersed in an acetone bath followed by immediate transfer to an isopropanol bath. Thereafter, pieces were rinsed with distilled water and 70% ethanol; and air-dried. This process produced surface partial

crazing in thermoplastic materials forming diverse topographical features exhibiting multiple dimensions, morphologies, and frequencies. For protecting crazed pieces from further degradation and/or deformation, a light metal coating of about 10-50 nm was applied as described before or represented in figure 2.3B.

Method IV

i.) Thermoplastic surface Crazing: For this particular process, identification of fiber orientation on the PSPS previous to processing was required. Fiber orientation was performed by exposing one of the PSPS corners to acetone followed by isopropanol rinse. Pieces were cut according to the direction of the fibers and to the intended surface topography.

ii.) Surface selection: The crazing process generated an extensive surface with variable features. A detailed selection was required to identify areas of interest according to the intended application and to quality and/or uniformity of the surface topography.

iii). Supplementary surface Processing: After surface selection, the following additional process were strategically implemented to further modify thermoplastic's surface for increasing complexity and/ or functionality of surface topography(Figure 2.5B):

a.) Thermal deformation: This treatment modified surface morphology complexity, directionality, and/or dimensions.

b.) Metal coating followed by thermal deformation: This treatment generated hybrid topographical features with combined characteristics of c.) Micro-contact printing in combination with thermal deformation and/or metal coating: This process generated hybrid features with combined characteristics of features generated in methods II and/or

I. Methods described in II, and I.

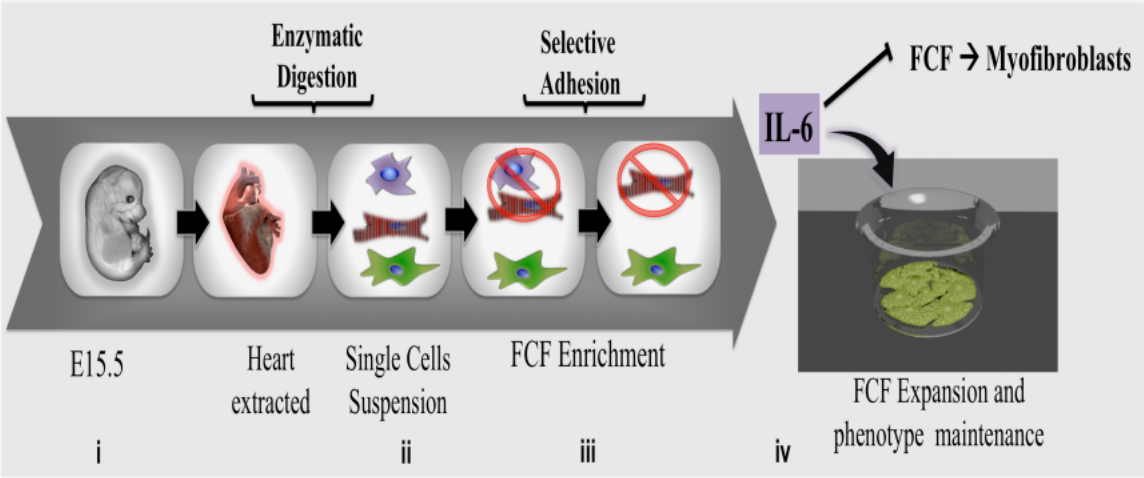


Figure 2.1: FCF extraction and enrichment. i.Hearts are extracted from ED15.5 fetal mice. ii. Enzymatic digestion (0.05%Trypsin-EDTA and Collagen type 2) to obtain single cell suspension. iii. Selective adhesion method to eliminate cardiomyocytes (CM) and endothelial (EC) cells to enrich for FCF. iv. Enriched FCF culture after 3 performance of selective adhesion cycles.

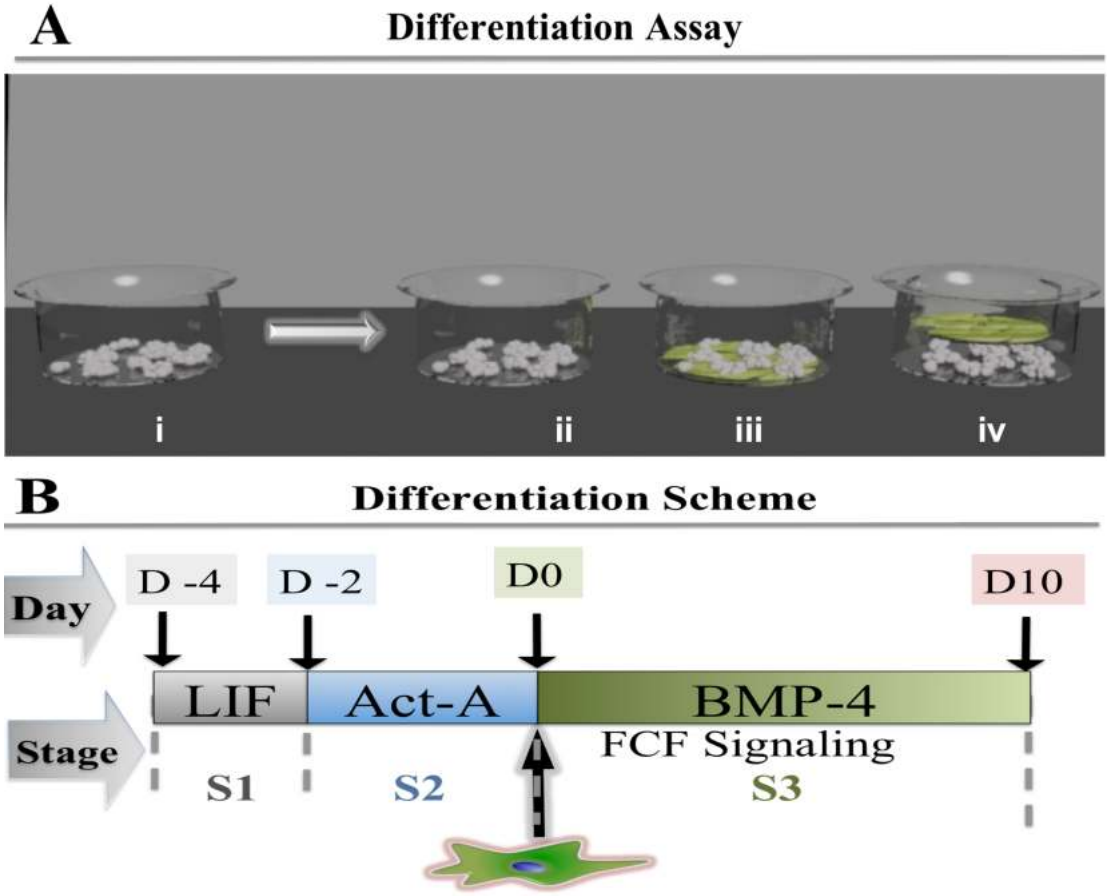

A
Differentiation Assay
i
ii
iii
iv
B
Differentiation Scheme
Day
D -4
D -2
D0
D10
LIF
Act-A
BMP-4
FCF Signaling
Stage
S1
S2
S3

Figure 2.2: Experimental design for testing FCF and its derivatives effect on mESC. **A.** Graphical representation depicting differentiation set up: i. mESC. ii. Control: mESC Only cultured in differentiation medium iii. Cell-cell signaling: Co-culture mESC with CF. iv. Biochemical signaling: Culture mESC in trans-well assay. B. Differentiation scheme depicting time line of our differentiation method used.

Antibody or Stain	Host	Source
cTn-I	Rabbit	Santa Cruz Biotechnology
Alexa 488 anti-rabbit	Donkey	Invitrogen
Rhodamine Phalloidin	---------	Invitrogen
CD 90.2-FITC	Rabbit	eBioscience
DDR-2	Rabbit	Santa Cruz Biotechnology
Rhodamine Phalloidin	---------	Sigma
VE-Cadherin-PE	Rat	eBioscience
Vinculin	Rabbit	Santa Cruz Biotechnology
Collagen I	Rabbit	Abcam
Collagen III	Rabbit	Abcam
Collagen IV	Rabbit	Abcam
Laminin	Rabbit	Abcam
Fibronectin	Rabbit	Abcam
Alexa-488-Phalloidin	--------	Invitrogen
Periostin	Rabbit	Abcam
αSMA	Donkey	Abcam
HSP-27	Rabbit	Santa Cruz Biotechnology
GATA-4	Rabbit	Santa Cruz Biotechnology
SSEA-1-FITC	Donkey	Abcam
Oct3/4	Rabbit	Abcam
Nanog	Rabbit	Abcam
Sox-2	Rabbit	Abcam
DAPI	-----	Sigma
Alexa Flour 594 anti-rabbit	Chicken	Invitrogen
Fluo-4AM-Alexa Fluor 488	---------	Invitrogen

Table 2.1: Table of antibodies or stains used for this study including name, host and vendor.

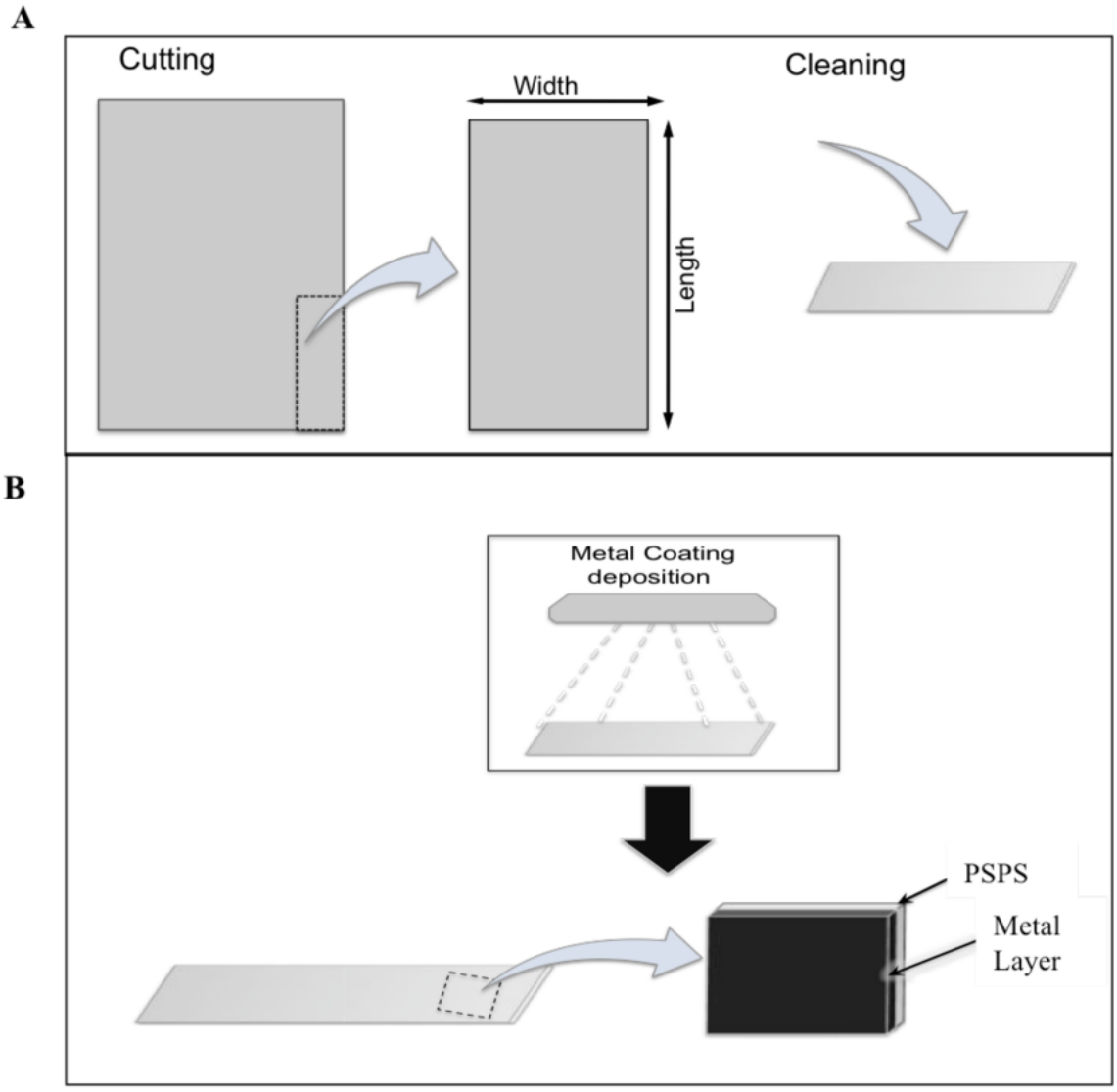


Figure 2.3: PS Cutting and Coating: Schematic representation showing the process for **A)** cutting and **B)** coating for topography fabrication.

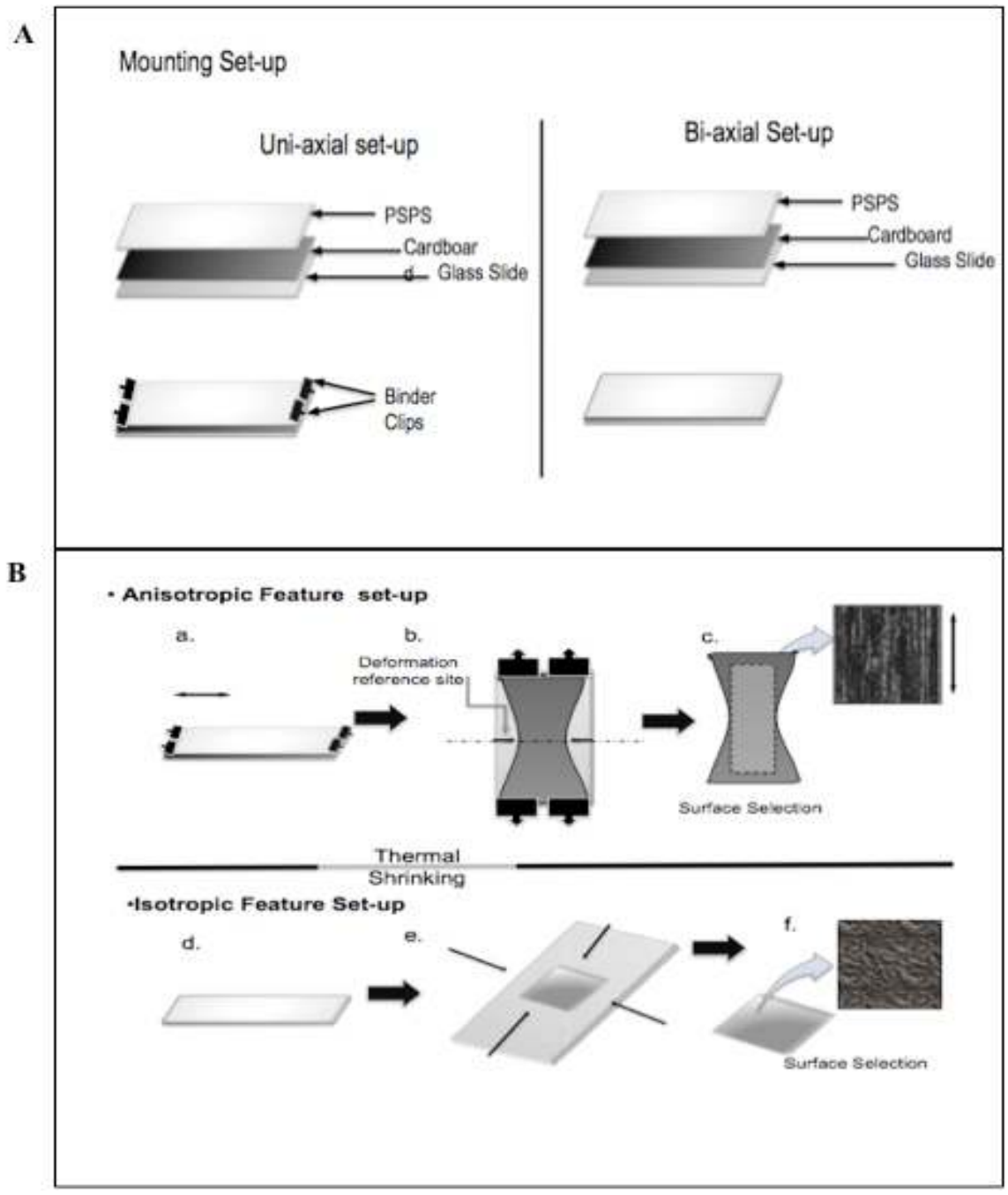


Figure 2.4: Shrinking Set up and Master Mold Fabrication: Schematic representation showing the process for: **A)** mounting PSPS for thermal shrinking; and **B)** Master mold fabrication for T1, T2 and T3.

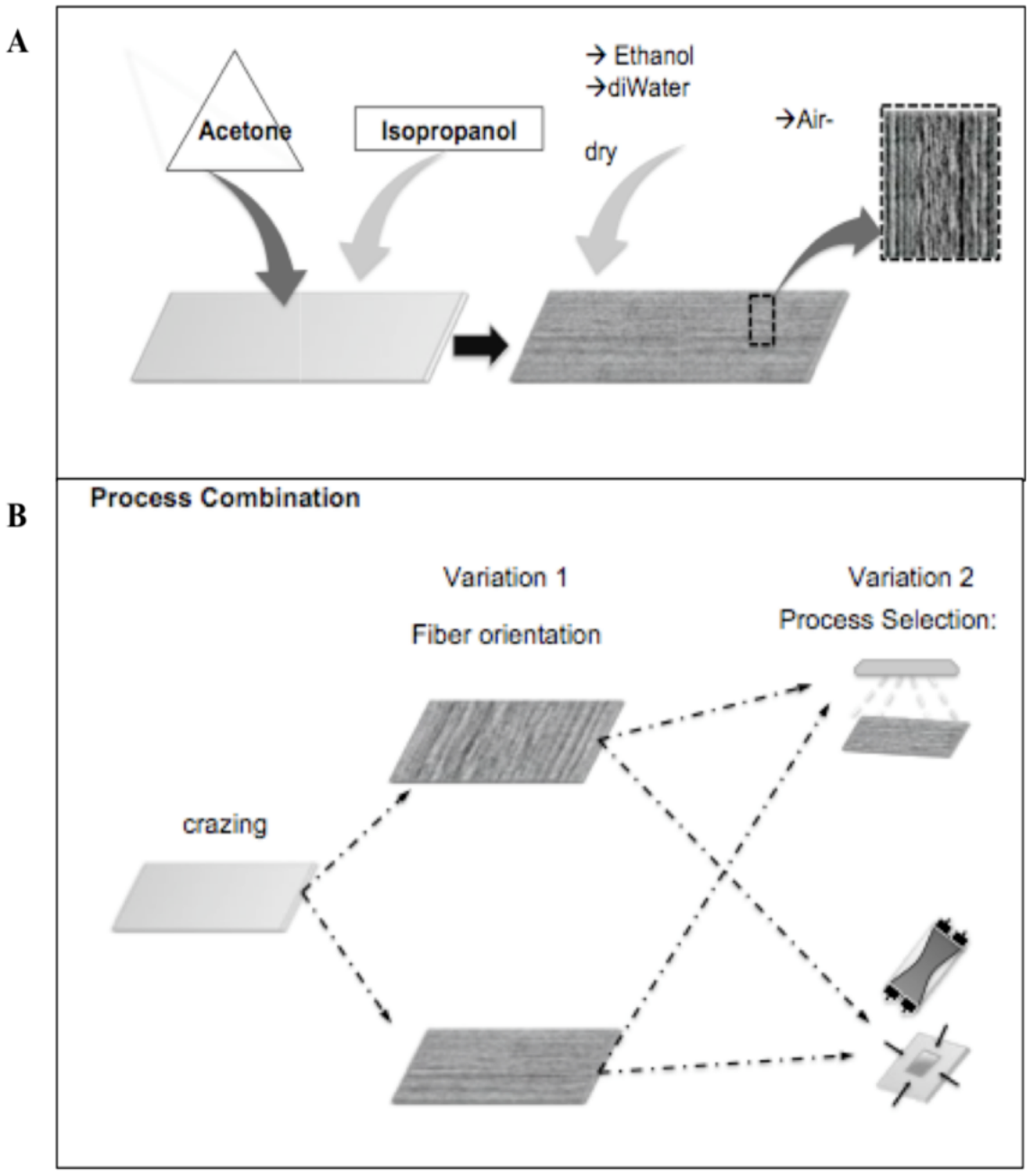


Figure 2.5: Fabrication Scheme for T1 and T2: Schematic representation showing the process for: **A)** Topography generation through PSPS acetone crazing; and **B)** Topography fabrication using methods variations.

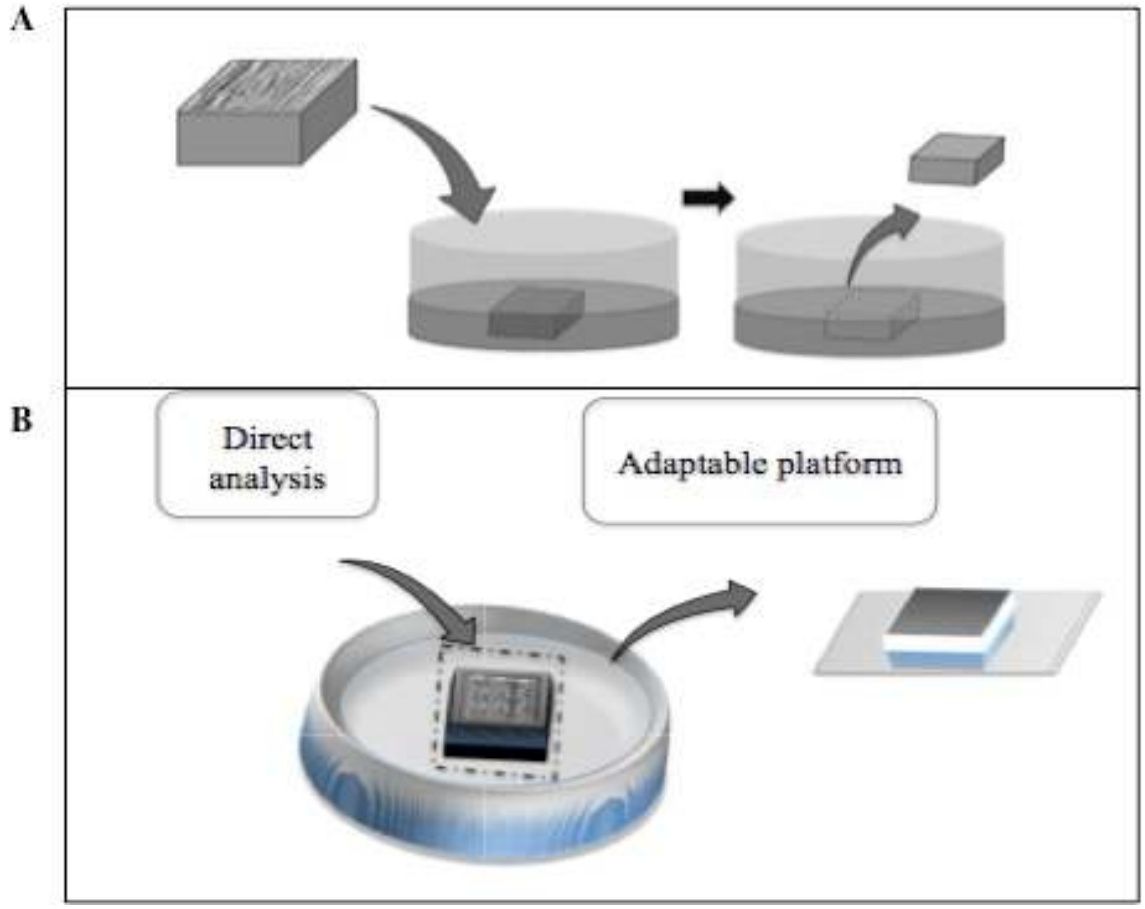


Figure 2.6: Cell culture substrate fabrication scheme: Schematic representation showing the process for: **A)** Cell culture substrate fabrication; and **B)** Chamber functionality and adaptability to different assays.

CHAPTER 3

Multiscale Biomimetic Topography for the Alignment of Neonatal and Embryonic Stem Cell-Derived Heart Cells

3.1.ABSTRACT:

Nano- and microscale topographical cues play critical roles in the induction and maintenance of various cellular functions, including morphology, adhesion, gene regulation, and communication. Recent studies indicate that structure and function at the heart tissue level is exquisitely sensitive to mechanical cues at the nano-scale as well as at the microscale level. Although fabrication methods exist for generating topographical features for cell culture, current techniques, especially those with nanoscale resolution, are typically complex, prohibitively expensive, and not accessible to most biology laboratories. Here, we present a tunable culture platform com- prised of biomimetic wrinkles that simulate the heart's complex anisotropic and multiscale architecture for facile and robust cardiac cell alignment. We demonstrate the cellular and subcellular alignment of both neonatal mouse cardiomyocytes as well as those derived from human embryonic stem cells. By mimicking the fibrillar network of the extracellular matrix, this system enables monitoring of protein localization in real time and therefore the high-resolution study of phenotypic and physiologic responses to in-vivo like topographical cues.

3.2. INTRODUCTION

Anisotropic arrangement of cells is critical in many tissues for maintaining proper physiological function (N. Bursac et al.; Biehl et al.). The role of substrate topography in controlling cell behavior is of interest for a wide variety of cell types, including neurons, skeletal, smooth muscle cells, and even corneal and lens epithelial cells (Biehl et al.; Clark et al.; Deutsch et al.; Meredith et al.; Shimizu, Fujita and Nagamori; Rajnicek, Foubister and McCaig; Recknor et al.; Sniadecki et al.; Sorensen et al.; Teixeira et al.; Webb et al.). The addition of a robust, scalable, and tunable biomimetic surface topography to in vitro cell culture systems not only allows the study of individual cells and tissue constructs in a more accurate anatomical state, but also provides important cues for inducing proper phenotypic and physiologic responses. While extensive prior studies have addressed microscale topography, recent studies have also demonstrated that topography at the nanoscale provides critical cues for cell alignment (Dalby et al.; H. N. Kim et al.; Pot et al.; Zong et al.; Yim et al.) . Submicron cues have been implicated in such critical cellular functionsas morphology, adhesion, gene regulation, and cell-to-cell communication (Dalby et al.; Yim et al.; Koo et al.). The heart in particular has a complex architecture that spans multiple length scales, from the nano- to the microscale. Comprised of layers of myocardial and matrix fibers, its anisotropic architecture helps coordinate mechanical contraction as well as electrical propagation (H. N. Kim et al.; Costa, Lee and Holmes). However, commonly used cardiomyocyte (CM) culture and tissue engineering methods do not provide the proper environmental factors to allow CM to respond morphologically, mechanically, or physiologically as they do in the native

tissue (H. N. Kim et al.; Engelmayr and Sacks). Fabrication of cell culture substrates that mimic the native environment found in the heart may improve the culture conditions of cells for the development of functional cardiac tissue (Badie and Bursac; Feinberg et al.; Hansen et al.; Thomas et al.). The alignment of CM has been studied for the last decade using different microfabrication (e.i., micro-contact printing, abrasion, photolithography, hot embossing, electrospinning, and laser ablation) approaches (Heidi Au et al.; Geisse, Sheehy and Parker; Motlagh et al.; Yeong et al.). Studies have also shown that these microtopographic cues have a greater effect on cell alignment than electrical cues (Heidi Au et al.). However, while most previous studies examined the effects of mechanical cues at the 10s to 100s of microns, recent studies indicate that structure and function at the heart tissue level is exquisitely sensitive to mechanical cues at the nano-scale level as well. Cues at the nano-scale stipulate cell mechanics, protein expression, and anisotropic action potential propagation (N. Bursac et al.). Indeed, the supporting myocardial extracellular matrix (ECM) is composed of aligned fibrils on the order of 100 nm in diameter with considerable variations in distances between the fibrals (H. N. Kim et al.). However, most current nano-fabrication approaches are complex and expensive (e-beam lithography and nanoimprint lithography) and therefore inaccessible to most biological studies. While less expensive alternative approaches are available, there is a significant trade-off; methods such as colloidal lithography have randomly ordered and oriented features and are therefore inappropriate for anisotropic alignment studies (Dalby et al.). Moreover, these systems do not span the inherent multi-scale structures of the native heart. To address this chasm, we are introducing a tunable, ultra-rapid, robust, and inexpensive non-photolithographic fabrication method to create cell culture substrates

with controllable nano- and microscale cues. The aligned grooves were created by leveraging the mismatch in stiffness between a pre-stressed polystyrene sheet and an overlying thin metal film (D. Nguyen et al.). When the plastic sheet retracts upon heating, the stiffer metal film buckles in a controllable manner causing wrinkles. Using this unique self-assembled multiscale topographical substrate, we then studied CM alignment. We created a bio-mimetic fibrillar ECM for the CM culture by first coating our polydimethylsiloxane (PDMS) substrate with fibronectin and laminin. We then studied the response of the subcellular architectural rearrangement by fluorescently staining nuclei, actin, cardiac troponin (CTN-I), gap junction protein connexin-43 (Cx43), as well as the fascia adheren protein, N-cadherin, and the focal adhesion protein, vinculin. We quantified the alignment and cell-tissue morphology from 2 to 7 days and show that the cells robustly align upon attachment (within 2 days). This work demonstrates that we can align both murine neonatal CM (NNCM) and CM derived from human embryonic stem cells (hESC) in vitro using our biomimetic multiscaled surface topography. Importantly, the CM derived from hESC *in vitro* are capable of sensing and responding to appropriate nano- to microscale mechanical cues.

3.3. RESULTS AND DISCUSSION

3.3.1. Cell population assessment

We initially used NNCM for our studies because they are relatively immature cells and can be cultured more easily in vitro, compared to adult CM that undergo profound morphological and physiological changes in long term culture (Zhang et al.).

Unfortunately, neonatal mouse hearts are limited by their size and thus in the number of cells available for experiments. Isolation of neonatal ventricular CM is challenging, in part because these cells are fully differentiated and cannot be expanded in vitro (Zhang et al.). We hypothesized that the inclusion of noncardiac cell populations would benefit our CM cultures by inducing them to physiologically respond more like native tissue. We consistently isolated *1 million cells per neonatal mouse heart by following the general protocol described by Heidi et al. (Heidi Au et al.). The heterogeneous cellular composition of the heart includes CM, endothelial cells, fibroblasts, and smooth muscle cells (Banerjee, Fuseler et al.; Brutsaert). In a previous study, it was also shown that the lack of cardiac fibroblasts in CM culture systems negatively affect the alignment, elongation, and network formation of CM (Nichol et al.). It has also been shown that the non-CM accessory cell populations are advantageous in establishing viable in vitro cultures of CM by aiding in the secretion and degradation of ECM elements that promote CM survival (Banerjee, Yekkala et al.). Further, increased apoptosis of CM was observed in cultures with only CM. Additionally, endothelial cells promote CM survival and enhance spatial organization in a 3-dimensional configuration by establishing CM-endothelium interactions (Narmoneva et al.). These previous studies reveal a value in including some accessory cells in the CM cultures; therefore, we did not attempt to completely purify the CM in our cultures. However, because fibroblasts and endothelial cells are highly proliferative, especially compared with CM, it is also helpful to maintain these accessory populations at lower levels in the cultures (Gospodarowicz et al.). We used a differential attachment plating technique to enrich our CM populations to reduce the number of fibroblasts and endothelial cells in our cultures. We then quantified the

percentage of the enriched cardiac cell populations using CTN-I, a specific marker for CM, by flow cytometry analysis (Yang et al.). Based on this marker we were able to identify an average (n = 3) of at least 70% CM purity in our sorted population with < 5% fibroblast and < 10% endothelial cells (Fig. 3.2A). To maintain these accessory cell populations at a low proliferative rate, we reduced the concentration of serum in our media from 20% to 10%.

We also examined the purified cardiac fibroblast (CF) from our mixed populations of cells isolated from the neonatal mouse heart to analyze the response of these cells on the wrinkled substrate and potential role in guiding the alignment of the NNCMs. The purity of the CF was verified based on CD90.2 (Fig. 3.2B) and DDR-2 (Fig. 3.2C) staining with no endothelial cells observed based on VE-cadherin (Fig. 3.2D) staining. Interestingly, the purified CF do not align with the wrinkles (Fig. 3.2F), and appear to fill into the spaces between the cardiac cells (Fig. 3.2G). This analysis additionally indicates that the CF are not inducing alignment of the CM cells on the wrinkled topology.

3.2 Fibrillar ECM

The incorporation of ECM proteins in the CM culture environment is thought to be important for facilitating appropriate CM-ECM adhesions (Ahumada and Saffitz; Yuval Eitan et al.). Therefore, we performed experiments to determine CM attachments to different ECM proteins by coating our substrates with fibronectin, collagen I, and laminin and combinations of these ECM proteins. We observed that the combination of laminin and fibronectin induced more cells to attach to the substrate (data not shown). Hence,

corona treatment before ECM coating and combination of fibronectin and laminin facilitate cell attachment and the generation of confluent CM monolayers. These findings are similar to previous studies where it has been shown that laminin and fibronectin induced more CM to attach compared with collagen I or IV, and maintained cell attachment from day 1 through day 8 of culture (Macfelda et al.). Using immunofluorescent analysis, we observed the distribution of laminin and fibronectin coatings on the wrinkled surface (Data not shown). We see that the ECM coats the wrinkle topography uniformly, thereby forming a fibrillar-like matrix as a guide for the cells. In the controls, coated proteins form anisotropic and more globular layer.

3.3 Cell alignment

We examined CM alignment at cellular and subcellular resolution induced by our multiscale guidance topography over a period of 7 days. For alignment experiments, the cells were cultured in three different chips and repeated three times to ensure consistent results. The actin filaments were stained to observe the internal architecture of cells. To distinguish CM from the other cell types, CM were also stained for CTN-I. We observed that the cells began plating down on both substrates within the first 24 h (Fig. 3.3A). After 48 h most of the cells attached and covered most of the surface (Fig. 3.3C). The cells also remained robustly aligned and a confluent connected layer is observed by day 7 (Fig. 3.3G). In controls, cardiac cell cultures were also more confluent, but not aligned (Fig. 3.3H). We expect that the saturated cell population at day 7 is due to the proliferation of noncardiac cells. Image analysis (Fig. 3.3I) shows the distinct alignment

condition versus the control. However, because most of the cells robustly align upon attachment, temporal information could not be obtained from these time points. The beating of the aligned cells was more coherent and synchronous compared with the cells cultured on the control flat substrate (Data not shown). In agreement with previous studies, the cells attached more robustly to the nano-topography and did not detach as in the case of the flat surface (Khademhosseini et al.). Through actin/CTN-I staining, we also observed the interaction between CM and non-cardiomyocyte cells. Recent studies indicate that heart cell elongation and alignment was enhanced by co-cultures with cardiac fibroblasts (CF) via matrix metalloprotease (MMP)-dependent mechanisms (Nichol et al.). Our biomimetic cell culture platform captures both the effects from the indigenous fibroblasts and endothelial cells as well as native topography. CNT-I positive cells appeared to connect and form a defined and consistent network with each other, whereas negative CTN-I accessory cells fill the spaces (Fig. 3.2G). To further quantify alignment of cells with the substrate, we examined nuclei alignment. Nuclei tend to be elongated with the direction of the alignment of the actin filaments as previously described (Bray, Sheehy and Parker). Using image analysis, we automatically segmented the nuclei shapes (Fig. 3.4A). We measured that the nuclei elongated by a factor of 1.43 (ratio of major to minor principal axis). A two-sampled t-test shows that the elongations of the nuclei were the same in the control and wrinkled substrates ($p > 0.12$). However, the distribution of orientations of the principal axis was substantially different in the aligned cells. Figure 3.4B shows the distribution of nuclei orientations in control and wrinkled substrates. On the wrinkled substrate, 54% of cell nuclei were aligned within –20° of the substrate. To confirm our hypothesis that the aligned cells connect and form a

defined and consistent network with each other, we stained the cells for gap junction protein Cx 43 (Fig. 3.5A, C), the fascia adheren protein N-cadherin (Fig. 3.5B, D), as well as the focal adhesion protein vinculin (data not shown) (Clark et al.). As expected, the proteins were better localized at the cell–cell junctions on the aligned cells as compared to the peripherally distributed expression on the isotropic cells. We also analyzed the localization and the anisotropic orientation distribution of Cx-43 and N-cadherin (Fig. 3.5E) and quantitatively show alignment based on the expression patterns of these proteins on the wrinkles compared with the flat substrate. We next attempted to align hESC-CMs. The control hESC- CMs cultured on flat substrates were randomly oriented cells with no obvious organization (Fig. 3.6A–C, top images).

Conversely, after attaching to the wrinkled substrate (Fig. 3.6A–C, bottom images), the hESC-CMs aligned and also displayed the typical banding pattern consistent with organized sarcomeric structure patterns. We also analyzed the orientation based on nuclei shape (Fig. 3.6D) and actin organization (Fig. 3.6E) and quantitatively show that the hESC-CMs exhibit increased alignment on the wrinkled substrates compared with the controls. In conclusion, here we have presented an easy, inexpensive, and rapid method for the fabrication of nano- to microtopographies to induce and study the cell alignment process by contact guidance, offering insight into the cell alignment process as well as in the formation of cardiac tissue-like constructs. Integration of robust and reproducible biomimetic surfaces into CM culture systems that controllably induce cell orientation may be useful for electrophysiological and pharmacological studies. Moreover, it may lead to the development of tools for tissue engineering such as patterned substrates to generate aligned tissue grafts for heart repair. The incorporation of cardiac cell

populations such as CM, cardiac fibroblasts, and endothelial cells enables the reformation of aligned cardiac tissue monolayers. We demonstrated that this platform can also align CM derived from hESC, and that this alignment aids in the development of appropriate internal organization needed for cellular contraction. Our substrate could therefore be used as a tissue engineering tool for the generation of robustly aligned cardiac cell sheets using cells derived from hESC for heart injury repair.

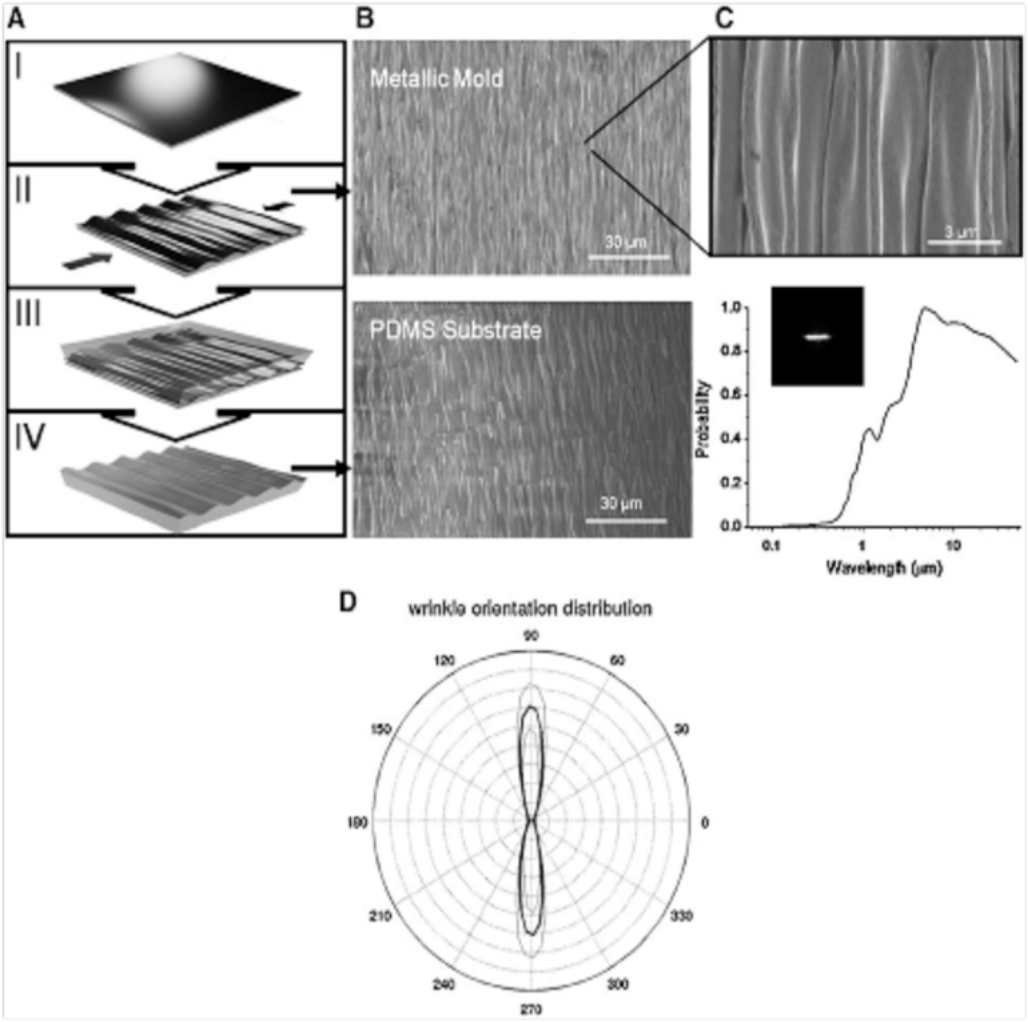


Figure 3.1: Fabrication and characterization of multiscale wrinkle substrate. (A) (I) Metallic layer is deposited on PS prestressed sheet. (II) PS is induced to thermally shrink while constrained from opposite sides to generate aligned anisoptropic wrinkles. (III) The metal wrinkles are used as a soft lithography mold to generate a PDMS substrate (IV), which is used to culture CMs. (B) Scanning electron micrographs (SEMs) of metal wrinkles (with high-resolution inset) and PDMS substrate. (C) The length scale distribution from Fast Fourier transform of SEM images. Inset shows high degree of anisotropy, as quantified by (D) computing a histogram of gradient orientations (thick lines) and standard deviation (thin lines).

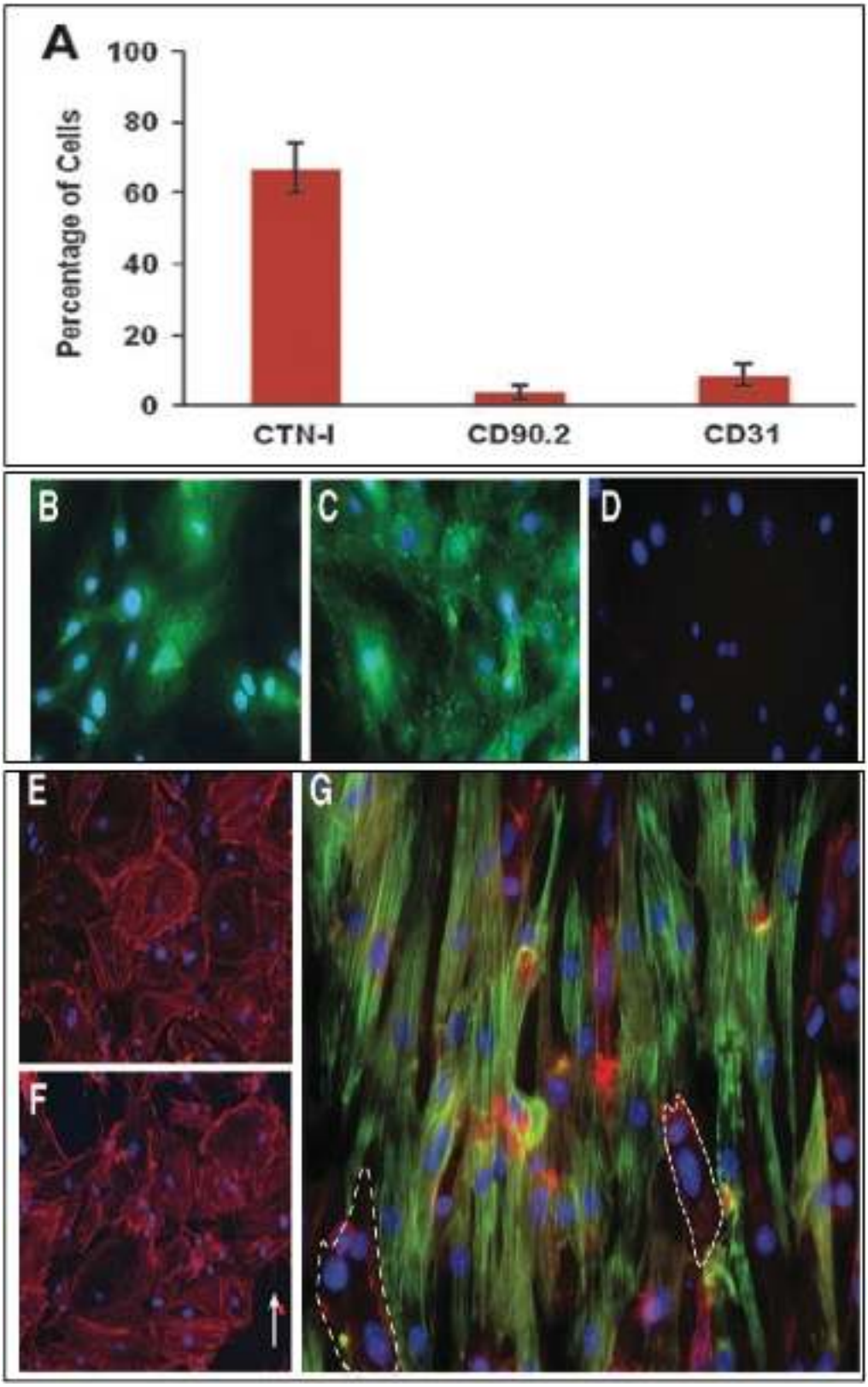
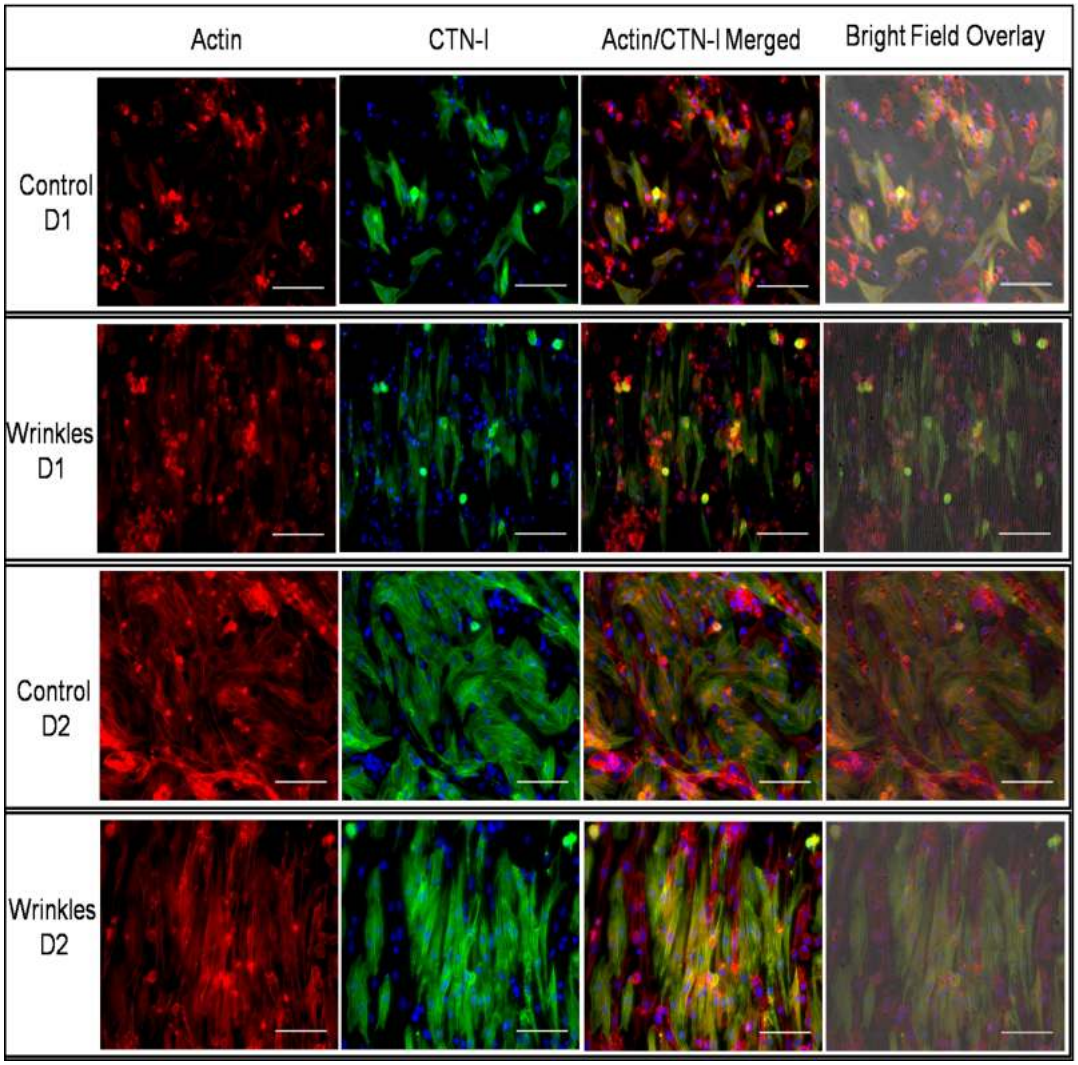
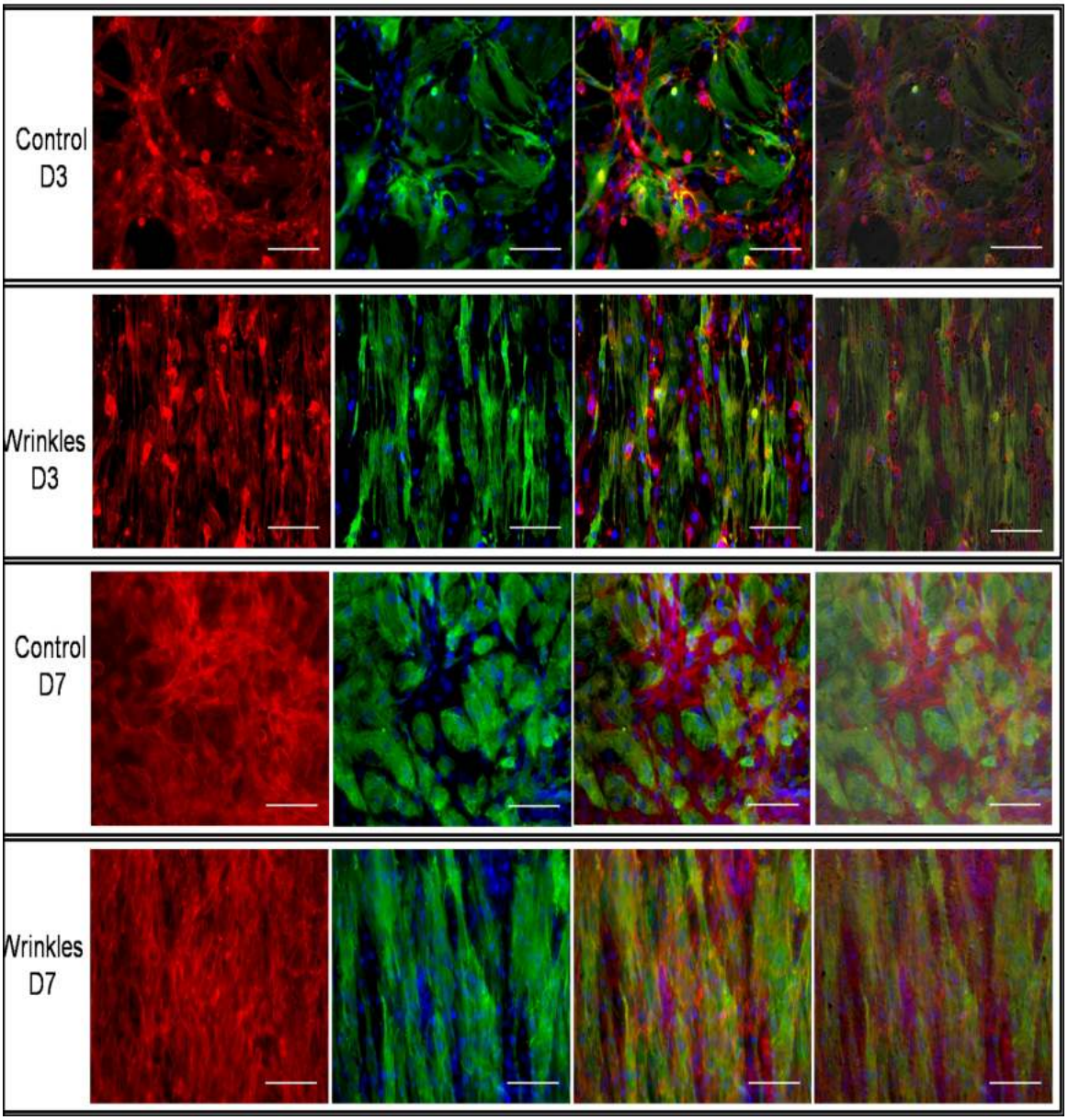

A
100
80
60
40
20
0
Percentage of Cells
CTN-I
CD90.2
CD31
B
C
D
E
F
G

Figure 3.2: Characterization of the purified neonatal cardiac cells. (A) The purified cardiac cells were stained for cardiac troponin-1 (CTN-I; cardiac cells), CD90.2 (cardiac fibroblasts), and CD31 (endothelial cells) and analyzed by flow cytometry to quantify the percentage of each population in our cultures. Separately, the CFs were also purified (NNCMs removed) and stained for fibroblasts markers (B) CD90.2 (green) and (C) DDR-2 (green), and (D) endothelial marker; VE-cadherin red). The cells were then cultured on controls and wrinkled chips for 4 days and stained with actin (red). Neither the (E) CFs on flat substrates nor the (F) CFs cultured on wrinkles substrate aligned with the wrinkled topography when cultured without CM. (G) CM cultures stained with CTN-I (green) and actin (red) show alignment on the wrinkles is by the CM cells with CF filling in the spaces. Blue indicates DAPI nuclear staining. Arrow indicates wrinkle orientation.


Actin
CTN-I
Actin/CTN-I Merged
Bright Field Overlay
Control D1
Wrinkles D1
Control D2
Wrinkles D2


Control
D3
Wrinkles
D3
Control
D7
Wrinkles
D7

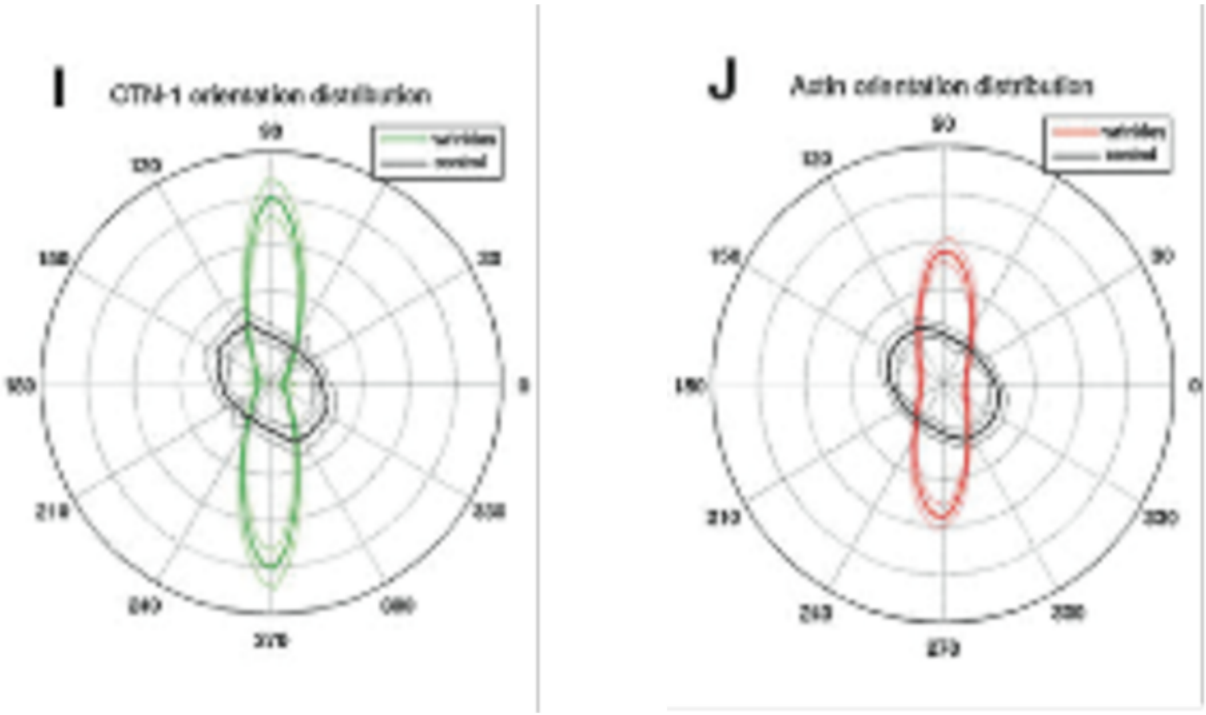


Figure 3.3: Alignment of neonatal cardiac cells on wrinkles. (A–H) Fluorescent micrographs of the cardiac cells unaligned on the flat substrates (controls) and aligning on the wrinkle substrates and at days 1, 2, 3, and 7. First column: actin (red). Second column: Cardiac troponin I (CTN-I; green). Third column: Merged images of CTN-I and actin staining. Blue is nuclear staining DAPI. Scale bars = 100 mm. (I, J) Anisotropy analysis of control (black) versus red or green (on wrinkles) by computing a histogram of gradient orientations where the contribution of each pixel was weighted by the gradient mag- nitude averaged over all 4 days. 90° is direction of wrinkles. Thinner lines indicate standard deviation.

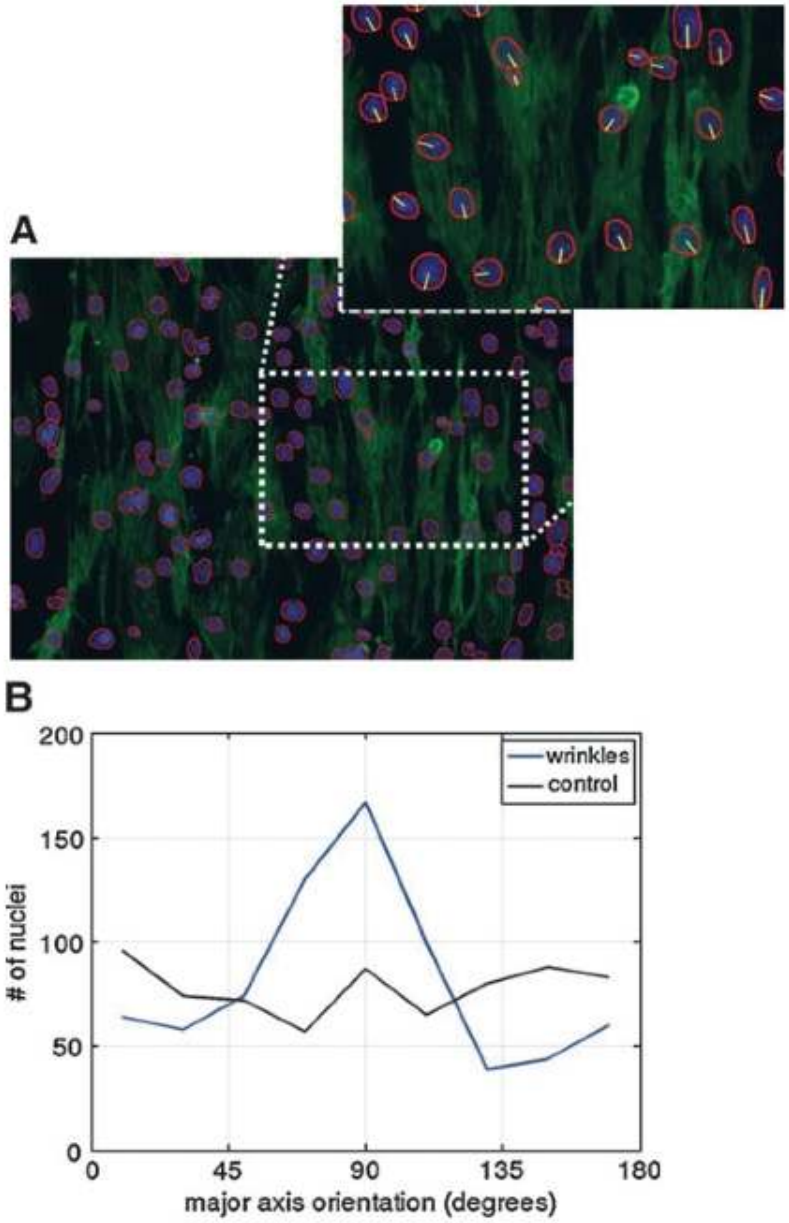
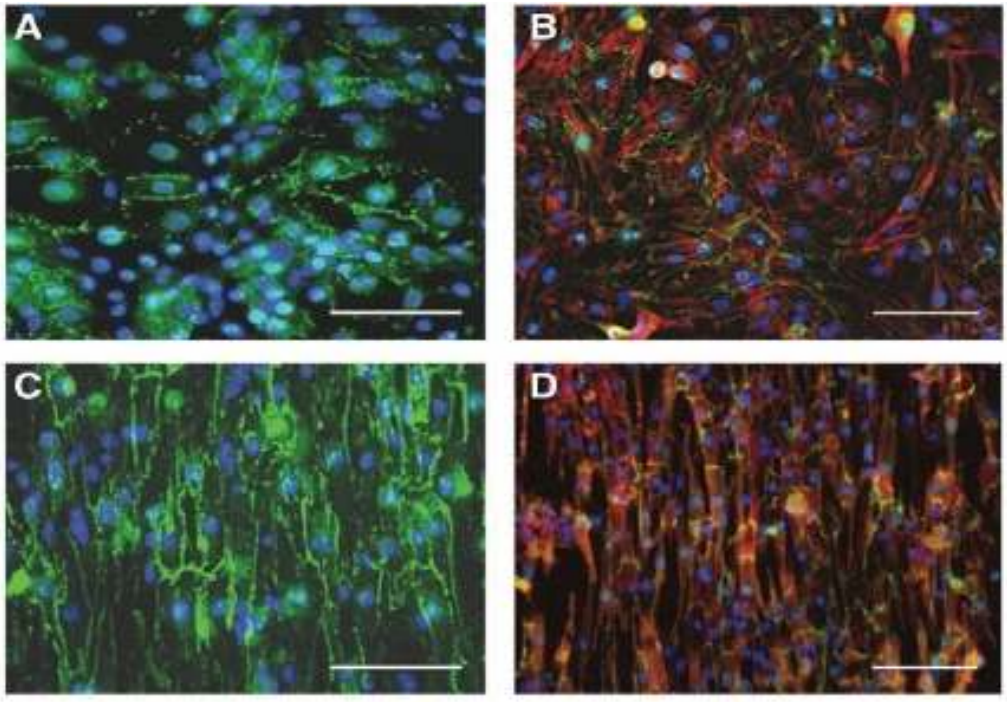


Figure 3.4. Nuclei alignment on wrinkles. Image processing was used to detect the DAPI-labeled nuclei. (A) Inset shows a high resolution image with the orientation with segmented nuclei for computing the moment matrix of the segment and identifying the major and minor axes. (B) Distribution of nuclei orientations indicating 54% of cell nuclei were aligned within – 20°.


A
B
C
D

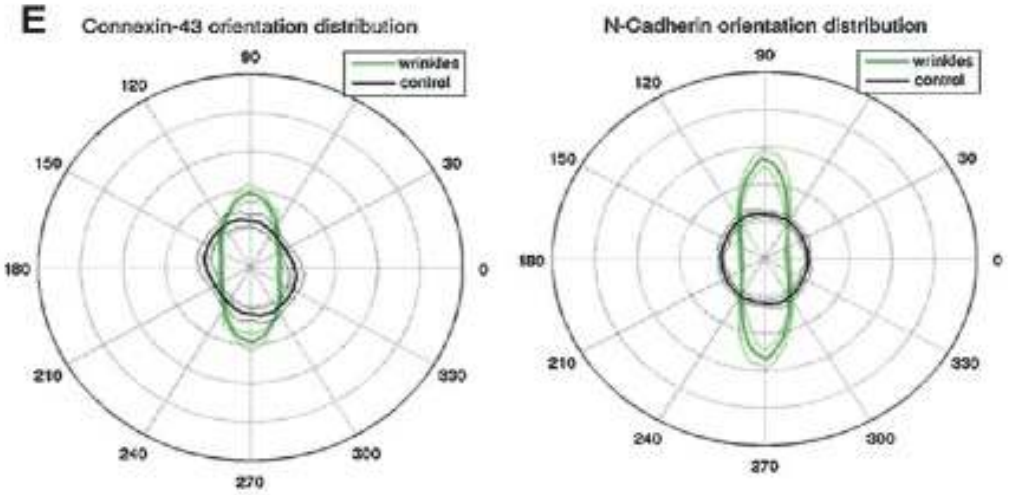

E
Connexin-43 orientation distribution
N-Cadherin orientation distribution
wrinkles
control
90
120
150
30
180
0
210
330
240
300
270

Figure 3.5. Cardiac-like tissue from aligned CMs. Fluorescent micrographs of cardiac cells cultured on (A, B) control (flat) and (C, D) wrin- kled and substrates. (A, C) Connexin-43 (green) and (B, D) N-Cadherin (green) and actin (red) expression by neonatal mouse cardiomyocytes. Blue indicates nuclear staining DAPI. Scale bars = 100 mm. (E) Anisotropy analysis of control (black) versus green (on wrinkles) was accomplished by computing a histogram of gradient orientations where the contribution of each pixel was weighted by the gradient magnitude. 90° is direction of wrinkles. Thinner lines indicate standard deviation.

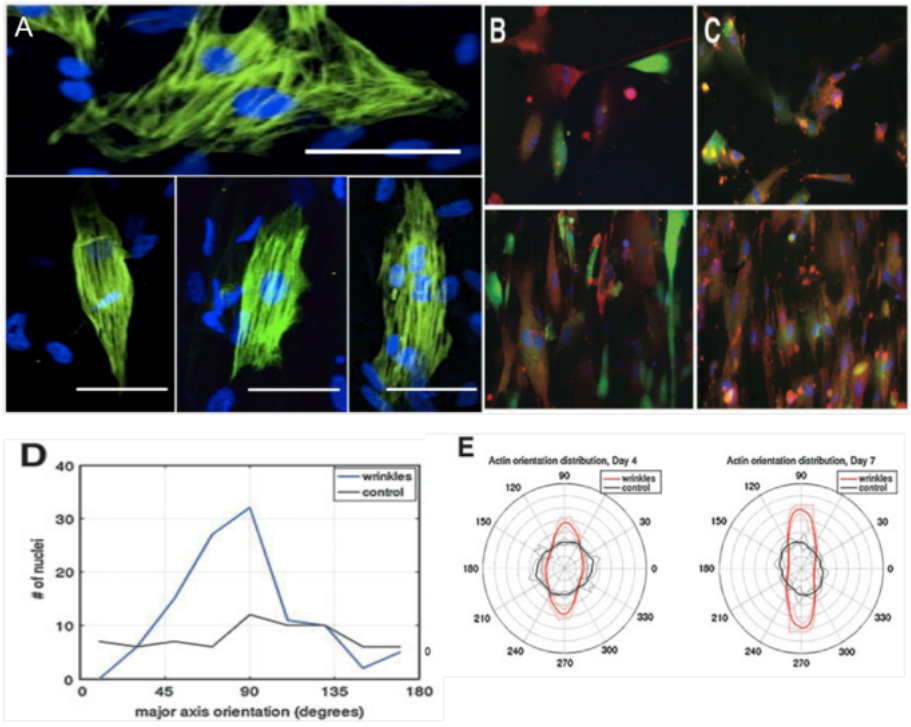


Figure 3.6: Confocal micrographs of hESC-derived CMs alignment on wrinkles. (A) Human ESC-derived CMs were isolated and cultured on flat substrate (top) and wrinkle substrates (bottom) for 8 days. Green indicates tropomyosin staining, blue nuclear staining DAPI. (B, C) Human ESC-derived CMs were also generated using an MLC2v-GFP cell line (green) and stained with actin (red) and cultured for (B) 4 days and (C) 7 days on flat (top) and wrinkled substrates (bottom). (D) Image processing was used to detect the orientation of the DAPI-labeled nuclei. (E) Anisotropy analysis of control (black) versus green (on wrinkles) showing that 90° is direction of wrinkles. The thinner lines indicate the standard deviations.

CHAPTER 4

Fetal Cardiac Fibroblasts Play a Critical Role in Cardiac Fate from Embryonic Stem Cells

4.1. ABSTRACT:

Cardiac Fibroblasts (CF) represent the most abundant non-myocyte cell subpopulation of the heart and the various functions of this cell type are thought to be critical throughout organ development. Part of the many functions attributed to CF's includes the production of regulatory signaling factors involved with cardiomyogenesis, maturation, protection, and the proper function of the heart. More specifically, CF's produce and maintain most of the signaling and structural components found within the heart's environment. The plasticity of CF's, coupled with their characteristic complex phenotype, has created challenges in defining its source of origin and level of involvement of these cells during early cardiogenesis. Here, I evaluate the effect of murine fetal CF (FCF) on the fate of embryonic stem cells (ESC), specifically the effect on cardiomyogenic induction. The mESC line E14-Myh-7-GFP is characterized by the expression of Oct-3-4, Nanog, Sox2, and SSEA-1 for evaluating pluripotency characteristics of the cells. After, the cell line was characterized, the mESC are cultured in direct or indirect contact with FCF. Early cardiomyogenesis is assessed by GFP expression associated to MHC-2β showing increased GFP+ populations with improved phenotypes in cells co-cultured directly and indirectly with FCF. GATA-4 and cTn-I expression patterns are also evaluated to identify

early and late cardiogenic events respectively. Our findings suggest that CM-like phenotypes are observed in all established culture conditions but an enhanced phenotype is found in cells influenced by FCF. Improved engraftment of GFP+ colony exhibiting healthier morphology and increased GATA-4+ populations is detected in cells exposed to the signaling effects of FCF as well. This study provides insight in the role of the complex signaling elements provided by CF and its implication in cardiomyogenic events.

4.2. INTRODUCTION:

FCF contribute a significant fraction of the total cell population of the heart and its frequency varies across species or with different stages of development (Banerjee, Fuseler et al.). This particular cardiac cell subpopulation it is thought to be involved in important cardiogenic events that are critical for proper development and homeostasis of tissues. Important contributions of these cells includes deposition and remodeling of ECM elements; secretion of multiple signaling factors, regulation and modulation of electrical activity, etc. (Vasquez, Benamer and Morley; S. L. Bowers, T. K. Borg and T. A. Baudino; Kakkar and Lee; Ieda et al.; Noseda and Schneider; Porter and Turner; Xie et al.; LaFramboise et al.; Banerjee, Yekkala et al.; Kohl et al.). Cardiogenesis development requires highly synchronized and coordinated signaling cascades that involve increased numbers of signaling molecules, which may be regulated by a cell type characteristic of a highly defined spatio-temporal genetic program; similar to CF (Noseda

and Schneider; Ottaviano and Yee). These cells may have become adapted, evolved and designed for providing myocytes with critical microenvironment factors required for proper development, function, maturation, and phenotype maintenance. For instance, these cells may be reprogrammed for differentiating into CM-like cells with the aid of the activation of a few genes previously identified to promote this behavior (DeWitt and Trounson; Jayawardena et al.; Hansson and Chien). Previous studies have aimed to understand the complex interactions between the different cell subpopulations of the heart including CF and CM for a better understanding the role of these cellular interaction in cardiac tissue development, homeostasis, and pathology (Defer et al.; McDowell et al.; J. Baum and H. S. Duffy; Porter and Turner; Dobaczewski and Frangogiannis; Fredj et al.; Manabe, Shindo and Nagai; MacKenna, Summerour and Villarreal).

Previous studies have shown that CF signaling is indeed developmental stage dependent as during the course of fetal mouse development, they provide signaling for regulating and directing hyperplacia or CM proliferation (Zhang et al.; Ottaviano and Yee; Noseda and Schneider). Subsequently, FCM acquire a more mature phenotype that includes the development of a complex anisotropic internal architecture, along with other cellular structures important for facilitating cellular communication that will result in improving tissue integration and maturation. After birth, CM continue to increase in complexity without substantial proliferation through a complex process generated by synergistic signaling interactions between CM with other cardiac subpopulation that are also involved with homeostasis regulation as well as in other physiological activities of the heart (Snider et al.; Noseda and Schneider).

As ambiguities on defining the source(s) of origin of CF persist, the lack of specific markers for these cells have limited the possibilities to identify CF progenitor(s) as well as the involvement of these cells or its progenitor in early cardiogenic events.

mESC have been extensively used to elucidate in vivo developmental events in *in vitro* culture systems (Robbins et al.; Sheridan, Surampudi and Rao; Matsuura et al.; Vidarsson, Hyllner and Sartipy). Here we evaluated the effect of FCF on the fate of pluripotent cells towards a cardiomyogenic phenotype. mESC cells were cultured directly, indirectly, or deprived of FCF to investigate if cardiomyogenic is enhanced in any of the established culture condition. Cardiomyogenic is assessed by detecting expression of CM markers in accordance to respective stage of development in progress such as GATA-4, MHC-2β, and cTn-I.

4.2.1. ESC for Cardiomyogenesis Induction:

Pluripotent stem cells represent a potential cell source to generate CM *in vitro* for CTE applications. These cells have been previously shown to differentiate into CM-like cells exhibiting phenotypes with some similarities to native CM (Ou et al.; Hudson et al.; Lee et al.; Gherghiceanu et al.; Asai et al.; DeQuach et al.; Zhu et al.). For instance, these cells exhibit spontaneous contractions and action potentials that may have close similarities to either atrial, ventricular, or nodal phenotypes. For instance, these cells may also present expression of sarcomeres with close similarities to the patterns found in CM(Gherghiceanu et al.; Ou et al.). ESC, have been previously used for cardiomyogenesis induction showing significant results leading to advances in the field

of CTE.

The ideal cell source for CTE should include the following characteristics: the source should be highly proliferative, have the ability to self renew, the ability to maintain an undifferentiated phenotype, be non immunogenic, and be able to differentiate into functional CM. ESC represent a promising candidate as they exhibit optimal characteristics when compared to other cells sources having cardiomyogenic potential (reviewed in (Gonzales and Pedrazzini)). CM can be generated *in vitro* using different approaches such as biochemical induction using growth factors such as BMP-4, Act-A,DKK1, bFBF, etc.(rev in(Lu et al.)). Another method previously used to induce Cardiomyogenesis consist of utilizing co-culture systems by culturing ESC with other cell types known to enhance cardiomyogenesis (Tulloch et al.; Janardhanan, Wang and Fisher; Rosenberg et al.; Siegel et al.). In a previous study Kattman *et al* 2011 showed that enhancement of cardiomyogenic events are achievable by the controlled temporal exposures to Activin/Nodal/TGF-b, Wnt, and BMP(Kattman, Huber and Keller; Kattman, Adler and Keller). Additionally, selecting the most efficient culture technique (i.e monolayer, embryiod body (EB), co-culture, etc) is important as the ultimate goal of tissue generation requires substantial increase of viable CM yields. Similarly, selecting an efficient cell line is important as each line has different cardiogenic capacities (Habib, Caspi and Gepstein; Habib et al.; Allegrucci and Young; Priddle et al.; Snir et al.).

Characterization and definition of pluripotent characteristics of cell lines is a critical step that generally defines the experimental outcomes involved with tissue specific induction studies. Characterization of ESC can be achieved by assessment of expression profiles facilitated by specific markers associated with pluripotent characteristics, which would

provide information about the degree of the stage of undifferentiation or pluripotency of cells. Markers for assessing pluripotent characteristics includes the surface stem-cell antigen-1(SSEA-1), SSEA-4, OCT3/4, SOX-2, NANOG, etc. Importantly, different expression patterns may differ according to cell lines or with different species. For example, expression of SSEA-1 and lack of expression of SSEA-4 may be used to assess pluripotency of murine cell lines while the reciprocal expression pattern is used to assess pluripotency on human cell lines. Induction of cardiomyogenesis *in vitro* involves the sequential progression of pluripotent cells into defined developmental stages that are allusive to in vivo homologous events (figure 1.1)(Vidarsson, Hyllner and Sartipy; Martin-Puig, Wang and Chien). An initial step for cardiomyogenic differentiation includes the cell transition from a pluripotent or embryonic stage into mesodermal like cells defined by the expression of Bry+ cells. Previous studies have described that further commitment to the cardiac fate results in the formation of the pre-cardiac-mesoderm identified by the expression of Flk-1. The development of cardiac mesoderm and the hemangioblast mesoderm occurs simultaneously and are both representative of Bry+/Flk-1+ populations(Kattman, Huber and Keller; Yang et al.). Both of these markers have been commonly used to identify the mesoderm formation process. Following this process, further commitment to the cardiac progenitor population is defined by the presence of cells expressing Mef-2c, Gata-4, Nkx-2.5, and Hand 1-2. At this particular stage of development, the cardiac progenitors will enter a critical point of the cardiomyogenic process, defined as specification where cardiac progenitors will enter a predetermined phenotype of one of the cardiomyocyte subpopulations. Afterwards, CM-like phenotypes will express cardiac troponin- I and –T (cTn-I, cTn-T); cardiac actinin-α;

MHC-α and MHC-β; and MLC-2a and/or MLC-2v according to their predetermined phenotype defined as ventricular, nodal, or atrial. Notably, in the *in vitro* cardiomyogenic induction system, the presence of the cardiac related markers is not necessarily representative of the formation of fully differentiated or functional CM. Current methods used to induce *in vitro* cardiomyogenesis have deficiencies and have produced for the most part immature or unstable CM-like cell that may undergo dedifferentiation or may fail to advance or commit to further fates. A possible cause for occurance of this process may result from depriving the CM from receiving stimuli thought to be critical for normal development (Hudson et al.; Gherghiceanu et al.; Rajala, Pekkanen-Mattila and Aalto-Setala; Vidarsson, Hyllner and Sartipy) . This phenomenon may be identified by the presence of cell subpopulations that express some of the cardiac markers but fail to physiologically behave or differentiate into fully mature CM. These dysfunctional characteristics may include the lack of formation of important protein complexes needed for proper physiological function such as the ionic handling mechanisms or the formation of a functional contracting apparatus (sarcomeres). For instance, dysfunctional or incomplete sarcomeres are a major concern observed in many of the methods used for cardiomyogenesis induction. *In vitro* studies of the cardiomyogenic process, using ESC, have provided critical information to help understand this highly complex mechanism;, which have shown homologies when compared to the in vivo cardiomyogenesis. For instance, the myosin heavy chain α (MHC-α), MHC-β, myosin light chain-2a (MLC-2a), and MLC-2v expression profiles have been strategically used as a system for studying the cardiac differentiation and specification process in vivo as well as *in vitro* (Lopez-Sanchez and Garcia-Martinez; Sanchez et al.; Robbins et al.; Lyons, Ontell et al.; Lyons,

Schiaffino et al.). It has been reported that MHC-α and MHC-β exhibit irregular and oscillatory expression patterns during murine embryonic development with similar behavior observed in ESC and with similar behavior among different species. Interestingly, after the CM have progressed into a final fate following the specification process, the MHC-α restricts its expression to atrial CM while MHC-β appears highly expresses in ventricular CM. Additionally, MLC-2a is specific to atrial CM, while MLC-2v is restricted for ventricular CM. This tracking mechanism is usually facilitated by genetic engineered cell lines that integrate detection tags such as cells expressing fluorescent proteins or antibiotic resistant genes under particular pre-specified promoters associated with one of more markers. Previous *in vitro* studies aiming to elucidate mechanisms involved in cardiomyogesis have reported homologous expression profiles compared to the in vivo process, but with slight different time-frames observed (Wobus et al.; Kubalak et al.; Baba et al.; Robbins et al.).

4.2.2. Challenges and Limitations in CM Differentiation Methods*:*

CM have been generated *in vitro* from ESC and other pluripotent stem cell sources (Roccio et al.; Chiu et al.; Gu et al.) in combination with the appropriate signaling factors known to be involved in cardiomyogenesis induction during embryonic development (Xu, Yi and Chien; Huber et al.; Gupta et al.; Doss et al.; Kolossov et al.; Yang et al.). These methods have been developed and/or improved by different groups with varying results and efficiencies. Yang et. al. 2008 describes important aspects of some of the most significant studies performed for cardiomyogenesis induction from

ESC. In general, it has been reported that different *in vitro* cardiomyogenesis induction methods produces a pool of divergent heterogeneous subpopulations of cells that may include CM-like subpopulation exhibiting phenotypes with some similarities to immature CM-like, ventricular-like, atrial-like, and nodal-like cells (figure 1.1)(Habib, Caspi and Gepstein; Doss et al.). The generate heterogeneous pool of CM-like cells is for the most part generalized in many of the previous performed studies in which a detailed classification and quantitative or qualitative assessment is absent. Also, in most cases the cardiomygenesis induction efficiency is usually relatively low and results are hardly replicated by different groups or even by the same group. For CTE applications, it would be practical to use a viable CM subpopulation that includes enriched CM-like cells selected from the overall pool that exhibit similar physiological and morphological characteristics, as differences in physiology would create an arrhytmogenic behavior limiting the functionality or usefulness of these cells. Enrichment of cell subpopulations with similar phenotypes is still a major hurdle in tissue engineering; especially in CTE as the majority of known CM specific markers found in specialized cells are expressed internally limiting the possibilities for improving enrichment techniques. For instance flow cytometry has been broadly used to enrich subpopulations of cells from heterogeneous cell pools (Kattman, Adler and Keller; Kattman, Huber and Keller). Sorting CM according to similar phenotypes would substantially improve *in vitro* development of cardiac cell constructs or tissues with anatomical and physiological characteristics resembling those found in native tissues as these are required for physiologic synchronization of cell constructs (i.e. atrial or ventricular tissue constructs) (Goh et al.). Besides improving efficiency of cardiomyogenesis induction methods, it

would be preferred to investigate cardiac cell fate decision mechanisms for advancing the generation of CM presenting defined phenotypic characteristics with improved similarities to the target cardiac tissue. However, the mechanisms involved in cardiac cell specification in vivo or *in vitro* remain for the most part unclear. *In vitro* studies using ESC for cardiomyogenesis induction may provide further understanding of the specification process having potential homologies to the *in vivo* mechanism. Detailed interpretation of the signaling mechanism or signaling factors that favor fate decisions would facilitate the generation of CM with an improved particular phenotype (Lee et al.). Cardiac accessory cells, such as fibroblasts and endothelial cells, are thought to produce a variety of complex signaling factors known to be directly involved in critical morphological events during heart development (i.e ECM, and growth factors) (Zhang et al.; Banerjee, Fuseler, Price et al.). These accessory cardiac cell subpopulations appear during early heart formation and have important contributions through development and many signaling functions along cardiogenesis that are not well identified yet. For instance, contributions of these cells may be involved in signaling mechanisms leading to the CM specification process.

4.2.3. Possible involvement of Fibroblasts in the Cardiac Differentiation and Specification Process

Previous studies aiming to gain a broader understanding of the mechanisms that leads to heart chamber morphogenesis in mice suggest that myocyte specification may occur between embryonic day 8 (ED8) and ED9.5 . The developmental stage that takes effect at

or about ED8 is represented by the formation of the heart tube followed by subsequent looping; this contributes to the assembly of the primitive heart chambers. Notably, during these developmental processes, the origination and evolution of a primitive action potential (AP) is observed, eventually progressing into a more defined AP characteristic of nodal, atrial and ventricular cell in the myocardium. These phenomena may suggest the incidence of specification events taking place around ED8 and ED9.5 (Franco et al.) (Figure 1.2). However, the intricate signaling mechanism and the key players involved in cardiac specification are for the most part undefined. Previous *in vivo* studies attempting to block the specification process by obstructing proposed signaling mechanism pathways involved in cardiac specification, resulted in the disruption of chamber morphogenesis rather than the blocking cell specification (Grego-Bessa et al.; Stankunas et al.). ECM seems to have critical affects in the mechanisms involved during cardiac development. The myocardium in the heart tube is surrounded by ECM layers that is deposited by mesenchymal cells residing at the epicardial and pericardial layers (Drake and Little). During this particular developmental stage, the developing myocytes are exposed to an environment where cells are differentially exposed to ECM elements according to its relative location within the heart tube. This environment surrounding the developing myocytes may produce significant effects with the process leading to myocyte specification or with posterior events such as those resulting in myocyte proliferation and maturation.

4.3. RESULTS AND DISCUSSION:

4.3.1: Characterization of the mESC:

Characterizing mESC is considered essential in order to generate consistent results between inductions; I cultured our mESC stock on feeder FB followed by the elimination of the FB using SAT (Figure 4.2A). Enriched populations were then expanded and evaluated to identify pluripotent characteristics of the cell populations. My characterization studies were performed by immunofluorescent analysis using the pluripotent markers SSEA-1, OCT3/4, SOX-2, and NANOG (figure 4.1). My enriched mESC popululations showed general uniform expression of all of these markers suggesting improved pluripotency of the initial population. Characterization of the population was performed not only based in a single marker but in combination of multiple in order to improve the quality of the initial mESC population according to pluripotent characteristics as well as well as to improve consistency in our results. In general, pluripotent assessment was performed regularly to the cells on stock for quality control purposes and only cells from passages of up to 10 were used for all of my experiments. The first row in figure 4.2C, the first image shows cells cultured on FB and under the presence of LIF to maintain pluripotent characteristics.

4.3.2: Preliminary Assessment of CF Cardiomyogenic Induction on mESC:

Initial cell differentiation and cardiomyogenic inductions was performed by culturing enriched mESC in monolayers and cultured in media containing BMP-4 to induce mesodermal formation. Image 4.2C is representative of the differentiation timeline used.

Image 4.2D shows undifferentiated colonies of mESC in the form of 3D colonies, which is a morphological characteristic generally exhibited by undifferentiated cells. After LIF removal, cells started to acquire a flat morphology and flat islets characteristic of pluripotent colonies in the differentiation stage (figure 4.2E). Due to the size, availability, and increased viability of CF from neonatal mice, a set of preliminary studies was conducted after previous isolation, enrichment and characterization of these cells. The stain αSMA-RFP was used to facilitate the tracking of CF in co-culture with the E14-MYH7-GFP mESC, which contains a GFP reporter under the MHCβ. These preliminary studies showed the increased GFP induction in mESC when cocultured with the RFP NNCM (Figure 4.3A-D). Notably, cells around the proximity of the RFP NNCM showed increased expression of βMHC (figure4.3A-C). On the other hand, mESC cultured without NNCF were found in lower frequencies, compared with those cultured under the presence of NNCF (figure 4.3E-F).

4.3.3. FCF Effect on mESC: Cardiogenesis Detection Using a GFP Tracking System

For assessing cardiogenic induction induced by the presence of FCF, the following conditions were tested: 1) direct FCF co-culture in direct contact with mESC; 2) FCF were co-culture using transwell assay to test the effect of soluble factors; 3) mESC were cultured without FCF as controls. This experimental set up was tracked over time using the GFP tracking system which is representative to the expression of the CM marker β-MHC which is expressed during early cardiogenic development in vivo. mESC cultured in the presence of FCF showed better engraftment and healthier morphology compared to controls from day 3 to 10 (figure 4.4). Additionally, GATA-4 immunostaining analysis indicates increased Gata-4+ cells on mESC cultured in the presence of FCF (Figure 4.5D-

H). Interestingly, mESC cultured in indirect contact showed higher frequency (figure 4.5G-H) compared to direct contact (figure 4.5 D-F).

Similarly, expression of cTn-I was observed in different patterns in each condition (figure 4.6). cTn-I+ populations were more abundant in direct contact (figure 4.6C-D) compared to control or indirect contact(figure 4.6A-B and 4.6E-H respectively). Notably, cTn-I expression exhibiting a more defined striated pattern typical of mature CM, and was only observed in indirect contact (figure 4.4E-H).

4.4. DISCUSSION:

Previous studies have provided evidence describing the signaling interactions between CF and CM through development with similar effects in CM derived from pluripotent stem cells(Pfannkuche et al.; Takeda and Manabe; Segers and Lee; Norris et al.; Martin and Blaxall; VanWinkle, Snuggs and Buja; Sopel et al.; Ottaviano and Yee; Noseda and Schneider; Banerjee, Yekkala et al.; Snider et al.; Visconti and Markwald). However, the lack of characterization to define the phenotype of the CF used for the mentioned studies may suggest the possibility of cross-signaling from other contaminant cell populations mixed with uncharacterized "CF" *in vitro*. For instance, in a report by Pfannkuche, K. et al 2010, the favorable effect of uncharacterized populations of CF extracted from ED.14.5 fetal mice on CM derived from ESC was describes, as these facilitated the engraftment to collagen matrices an enhanced the phenotypic characteristics of the cardiomyocytes. The report mentioned the appearance of improved morphology such as

organized sarcomeres and elongated oval-shape cells only when FCF were added to either hanging drop set ups for differentiation assays or mixed with CM-dESC when seeded onto collagen matrices. However, this particular study fails to provide any characterization to define the phenotypical characteristics or the purity of the fibroblasts-like population used for their studies. On another report, Xi, J. et al 2011, described a favorable effect of fetal fibroblast on promoting and improving CM derived from murine ESC engrafment ot vital and avital slices of ventricular fetal hearts. This study also fails to provide a characterization study to determine the phenotypic characteristics of the fibroblasts cell subpopulation, which were extracted from a diverse internal organs from the ED.14.5 fetal mice(Xi et al.). For our study, I carefully enriched our FCF from ED.15.5 and provided a detailed characterization previous to use my FCF cultures (presented in Chapter 6). For instance, I only selected populations presenting minor frequencies of myofibroblasts and I also used only those showing healthy morphology. In addition to providing a detail characterization assessment for the FCF used in my studies, I also performed characterizations of my stem cells populations using Nanog, Oct-3/4, Sox-2, SSEA-1 which were uniformly expressed and suggest the improved pluripotent properties of the cells. Also, I provided a live study suggesting cardiomyogenesis enhancement around the peripheries of RFP-αSMA neonatal fibroblasts, which is depicted by the appearance of GFP+ cells corresponding to cells expressing the MHC-β.

The family of the transcription factors GATA has been involved in regulating differentiation, growth and survival of different cell types(Pikkarainen et al.); In particular, GATA-4,-5,-6 which are expressed in various endodermal and mesodermal derived tissues. In order to further study temporal cardiomyogenic induction effects, I

targeted the zinc finger domain transcription factor GATA-4 which has been extensively used as a critical determinant of early cardiogenesis in vivo and *in vitro*, and which mRNA expression has been detected as early as ED. 7-7.5 and the respective protein being expressed at ED.8 during the formation of the heart tube (Pikkarainen et al.; Charron and Nemer; Charron et al.; Shiojima et al.; Lien et al.; Kelley et al.). In addition to this, GATA-4 has been strongly associated as an early marker involved in CM differentiation from ESC. It has also been reported that GATA-4 plays an important role in regulating cTn-I gene, and whose developmental pattern of expression temporally precedes that of cTn-I expression, which could be an indication that GATA-4 may be involved in mediating developmental up regulation of cTn-I(Murphy et al.). I reported the increased expression GATA 4+ populations in the presence of FCF. Interestingly, in indirect contact condition, increased expression was observed, suggesting high proliferation of early CM is enhanced by the effect of soluble factors released from FCF. Additionally, I reported an improved engraftment of mESC in the presence of FCF as reported recently(Lie-Venema et al.). The significance of the GATA-4 expression patterns could potentially be an indication not only of a potential cardiomyogensis induction, but also a latent indication that derived CM may be undergoing a process of further maturation as this gene has been previously been involved in such process (reviewed in (Pikkarainen et al.). Another important reasoning derived from our findings relies on the observation that an increased number of GATA-4 expressing cells were observed on indirect co-culture conditions. Also, in indirect co-culture conditions cells showed striation and more defined sarcomere organization. This may imply that cells in this particular environment generated improved conditions for early cardiomyogenic

events as well as for the increased frequency of cells undergoing further maturation suggested by the increased expression of cTn-I forming more defined sarcomeres.

Most important, the enhanced expression of GATA-4 by CM-like subpopulations could also be associated with the cell subpopulation expressing HSP-27 (described in Chapter 6) which may be inducing a cardioprotective mechanotrasduction response to hypertrophic signaling mechanisms involved somewhere in the process in accordance to Pikkarainen, et al. 2004 report. However, further genetic activation assays need to be performed in order to identify GATA-4 activation response.

cTn-I is part of the contractile apparatus (sarcomers) of CM and its expression has been associated with late cardiogenic events (Weeke-Klimp et al.). During heart development, the formation of sarcomeres may result from the process of increased intercellular architecture complexity, which is known as cellular hyperthrophy. Here, I provided evidence describing the possible effect of FCF involvement in hypertrophic induction; a process that is thought to be necessary for the formation of a proper internal and external morphology promoting CM functionality. Frequently, a sign of CM maturation can be identified as the appearance of organized sarcomeres showing as striation along the CM. Our findings showed that striated cells developed on FCF soluble signals, but not in direct contact, hence, these phenomenon can be attributed to soluble signals produced by FCF that are only produced when cardiac progenitor cells are not in direct contact with FCF or its respective progenitor cell. Another possible reason for the lack of sarcomere formation on direct contact co-culture conditions could be attributed to a blockage of the mechanism that leads to CM maturation by means of sarcomere formation that may have been caused by an exaggerated deposition of ECM by FCF. Observed increased

deposition of ECM elements by FCF could potentially have created the formation of multiple focal adhesion complexes, thereby increasing internal constriction forces leading to increased internal stress that may cause inhibition of sarcomere assembly. This reasoning is supported by previous reports describing the mechanotransduction response to particular ratio concentrations of collagens type I and type III which increase after birth causing CM hypertrophic and increased passive elastic modulus hence altering myofibrillar composition and myocyte function (Fomovsky, Thomopoulos and Holmes). In previous studies, Chopra A. et al. 2012 reported the mechanobiological responses of neonatal CM induced to pattern in a "Y" form exposed to fibronectin a member of the cardiac ECM and to α-catenin a member protein of the cadherin adhesion complex (Chopra, Tabdanov et al.). In this study, Chopra reported the development of well-organized sarcomeres on CM when cells were patterned on fibronecting. The same group also reported that CM showed disrupted sarcomeres when plated on patterned N-cadherin coated substrates. Similar studies reported the significance of the mechanosensing environment in accordance with the corresponding developmental stage, since disruption of the cell-cell/cell-ECM or integrin cadhering adhesion system respectively, may potentially cause severe mechanotrsaduction responses observed in the form of morphological abnormalities on cellular internal architecture and affects to overall cell physiological functioning (Chopra, Patel et al.; Chopra, Tabdanov et al.; Kresh and Chopra).

On the other hand, increased expression of GATA-4 and cTn-I in direct contact suggests a possible proliferation enhancement of early CM. Interestingly, my results obtained from co-cultures testing soluble factors suggests possible pro-hypertropic effect based on the

development of sarcomere organization. Finally, I concluded that multiple signals from FCF play a critical role in cardiomyocyte enhancement from mESC with possible temporal implications which need to be further investigated. The importance of elucidating the spatio-temporal signaling provided by FCF on *in vitro* cardiomyogenesis may result in the improvement of current methods for producing functional CM for clinical applications.

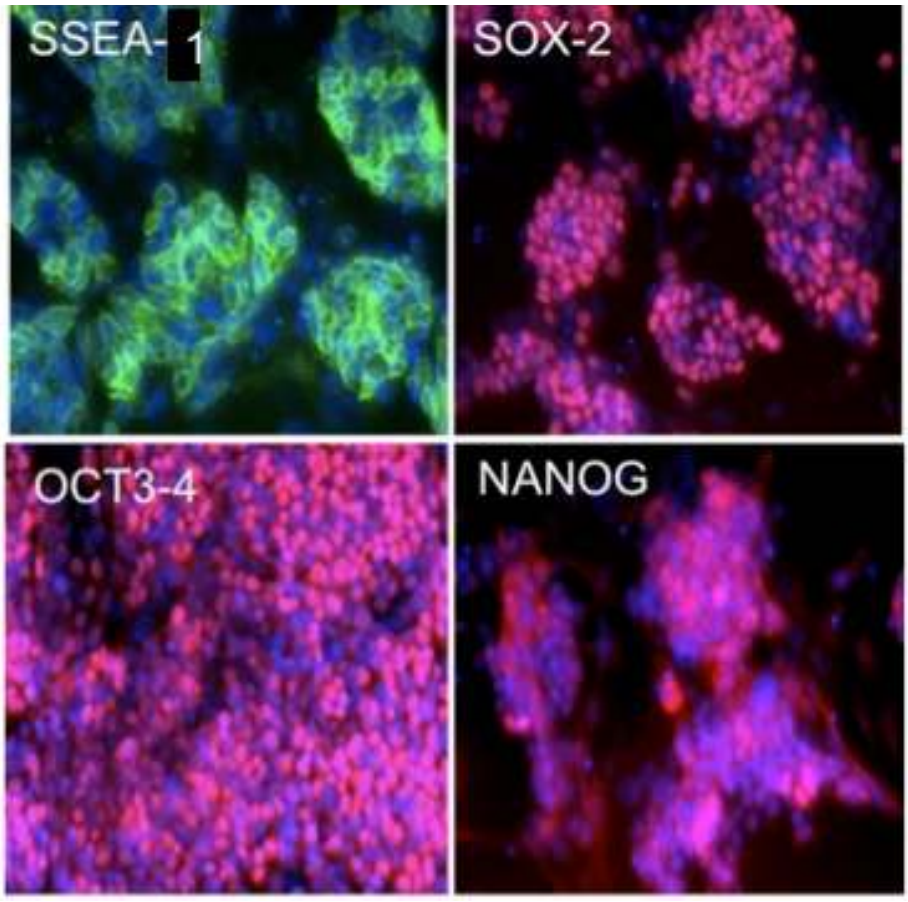


Figure 4.1: Characterization of mESC. Immunofluorecent micrographs of mESC E14-MYH7 evaluated for pluripotent characteristics using the stem cell markers SSEA-1, Sox2, Oct3/4, and Nanog.

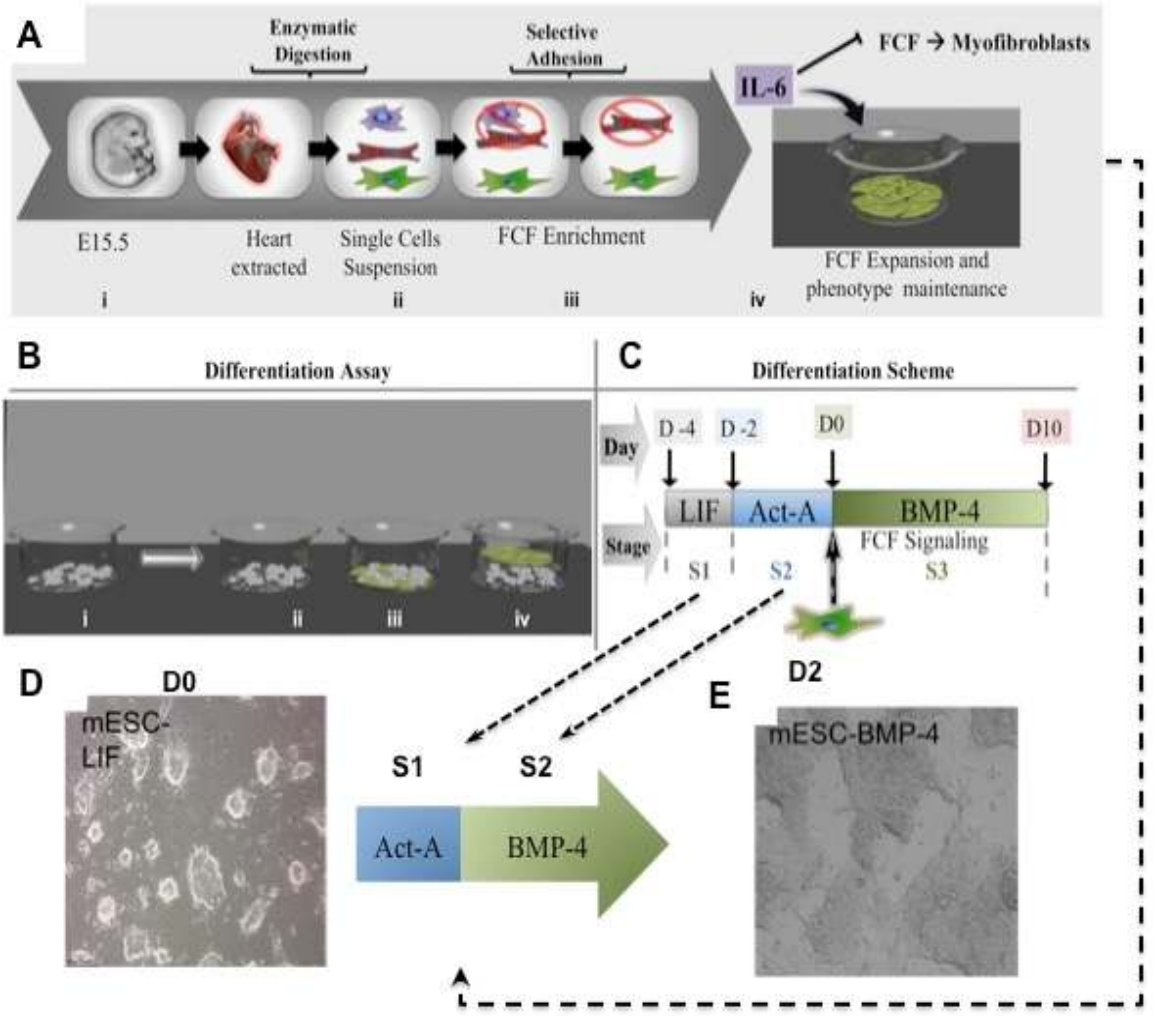


Figure 4.2: Selective adhesion and Differentiation Method: **A:** diagram representing the selective adhesion method used to enrich FCF populations. **B**: Representation of the differentiation assay used to test cardiomyogenesis of mESC: ***i)*** Undifferentiated cells*;* ***ii)*** control containing mESC only; ***iii)*** direct contact co-culture; ***iv)*** co-culture in in direct contact using trasn-well assay. **C**: Time line representing the differentiation scheme from for days previous to FCF addition to day 10 post addition . **D**: Micrograph of mESC cultured on feeders and under LIF containing media. **E**: differentiating mESC after 2 days in media containing BMP-4.

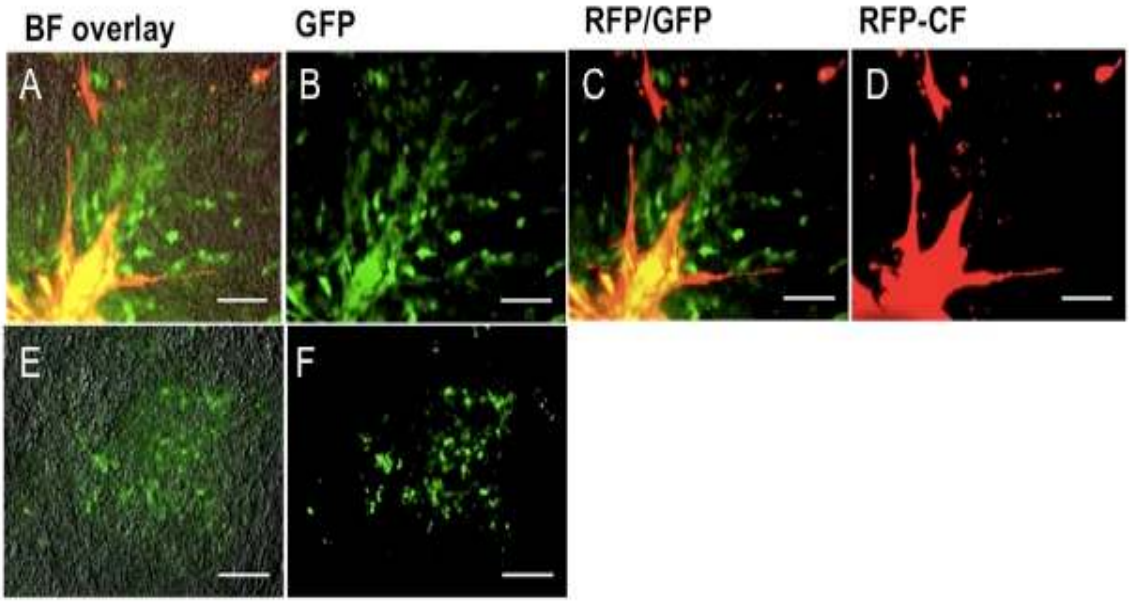


Figure 4.3: Preliminary study for cardiomyogenesis using neonatal RFP-α-SMA. A-D. Fluorescent micrographs showing enhanced cardiomyogenesis from mESC-E14-MYH7-GFP near RFP-NNCF. E-F: controls mESC E14-MYH7-GFP only **A**: overlay image of GFP, RFP and bright field. **B**: GFP only. **C:** overlay image of RFP and GFP; **D**: RFP only; **E:** overlay image of GFP and bright field; **F**: GFP only. Scale bars = 100 μm.

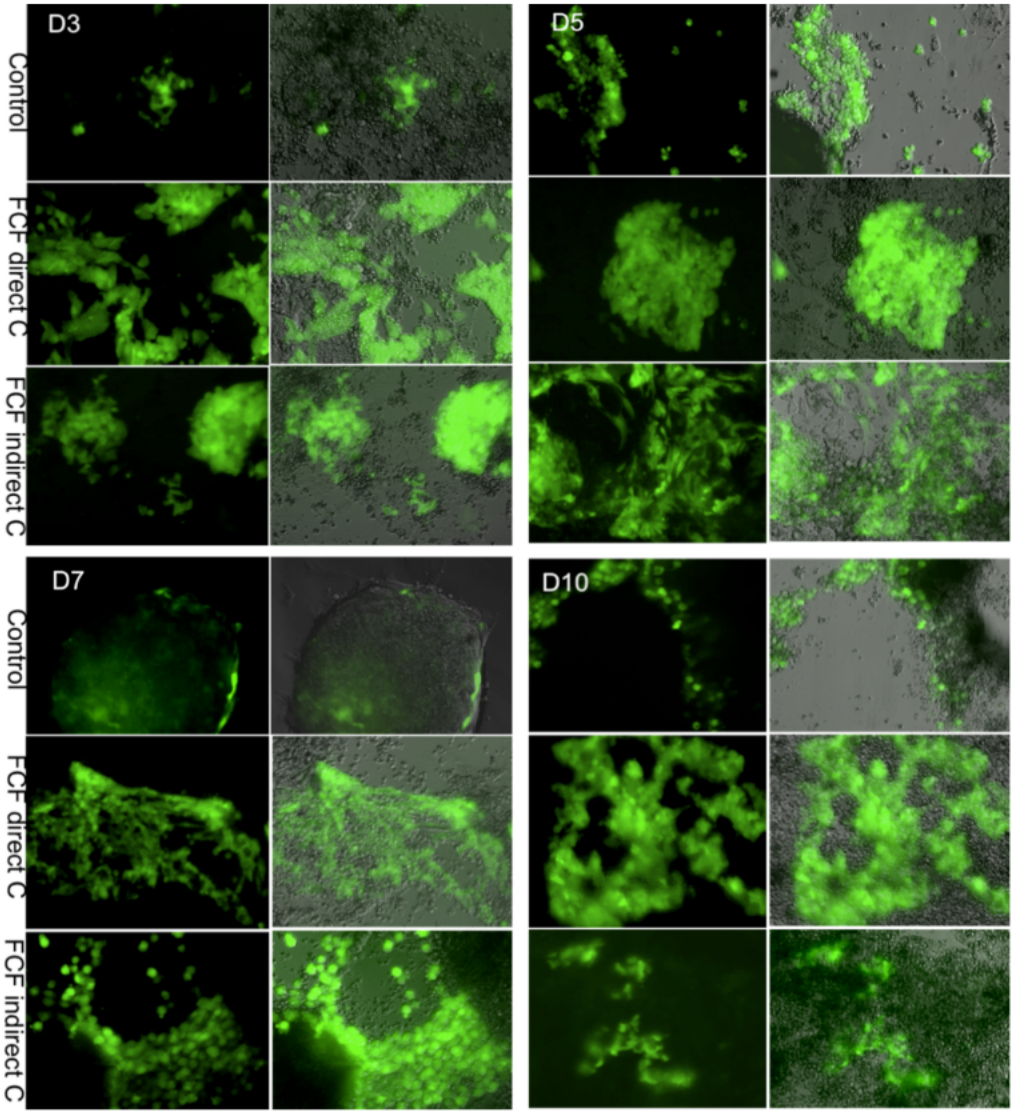


Figure 4.4: Cardiomyogenesis detected by GFP-βMHC at days 3,5,7 and 10. Micrographs of mESC- Myh7-GFP differentiation set up at days 3,5,7 and 10 respectively. Cells were culture with or without FCF. FCF were cultured in direcT or indirect contact with mESC. First row = mESC only. Second row= mESC co-cultured with FCF. Third row=mESC culture in tran-swell assay with FCF. First column= GFP micrographs. Second column= overlay of GFP with bright field images.

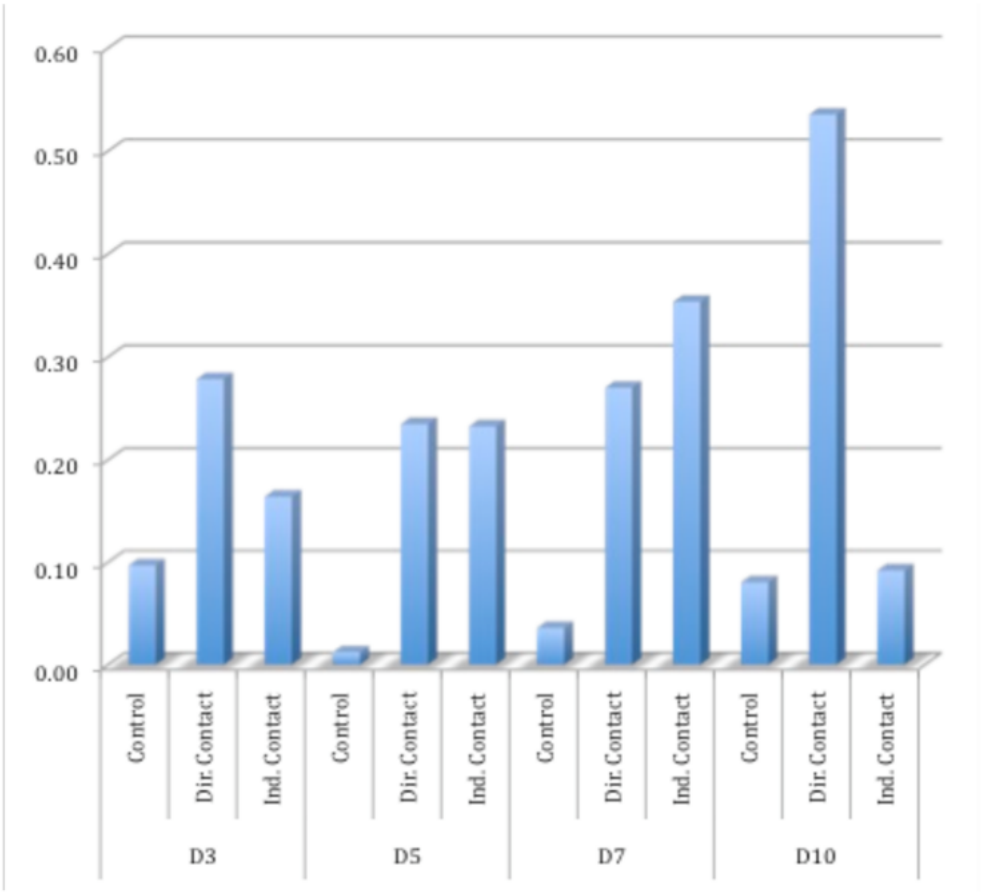


Figure 4.4B: Relative quantification from GFP+ cells. GFP+ cells area was converted to binary image and pixels under GFP expressing cells was weighted against the total image area.

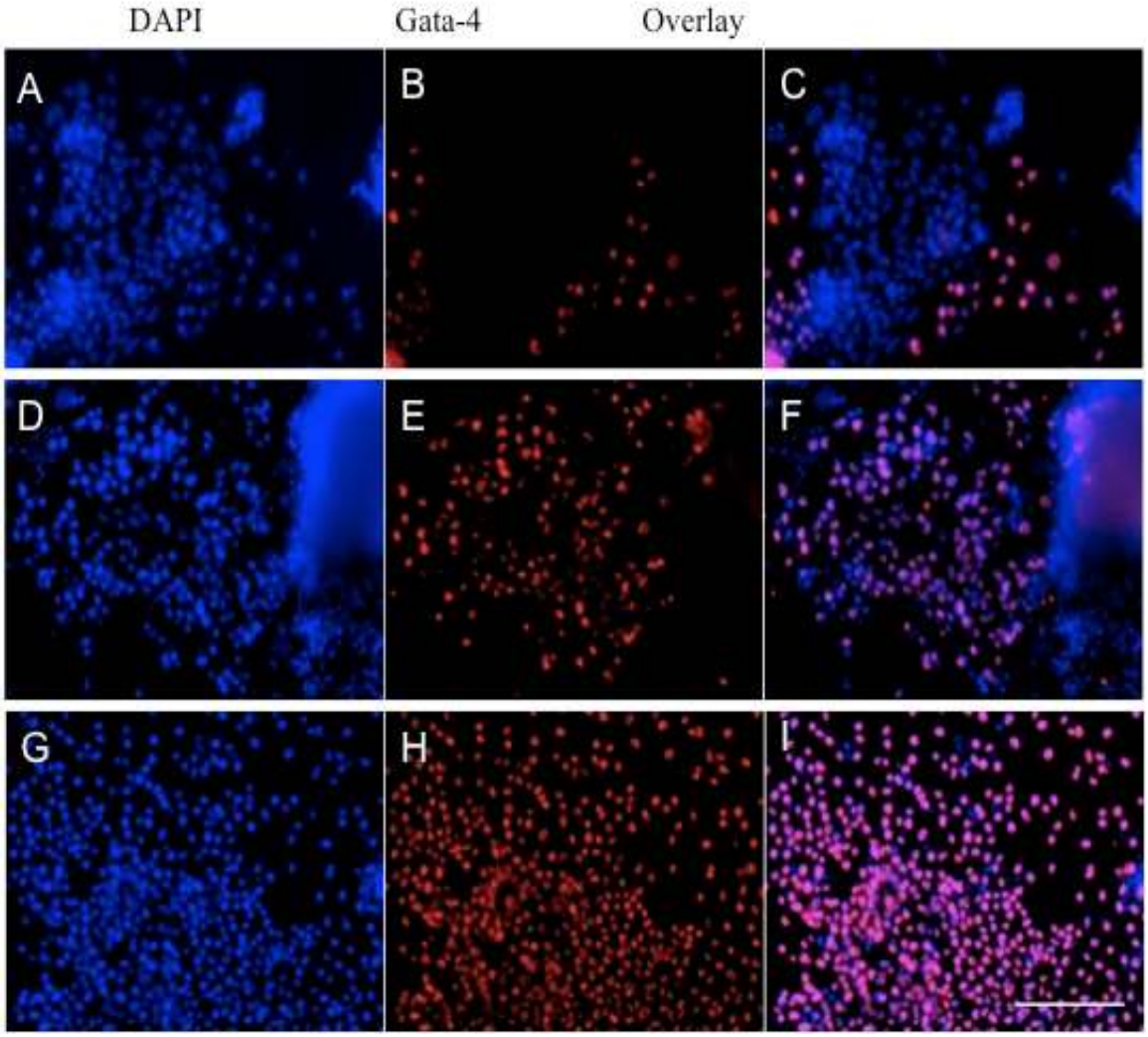


Figure 4.5: GATA-4 Expression Analysis at D10. Immunofluorescent micrographs showing the expression of GATA-4 in each different conditions indicating enhanced expression under the presence of FCF. **A-C** control. **D-F:** direct contact; **G-I:** Indirect contact in trans-well assay Red=Gata-4, Blue= nuclear staining. Scale bar =100 μm.

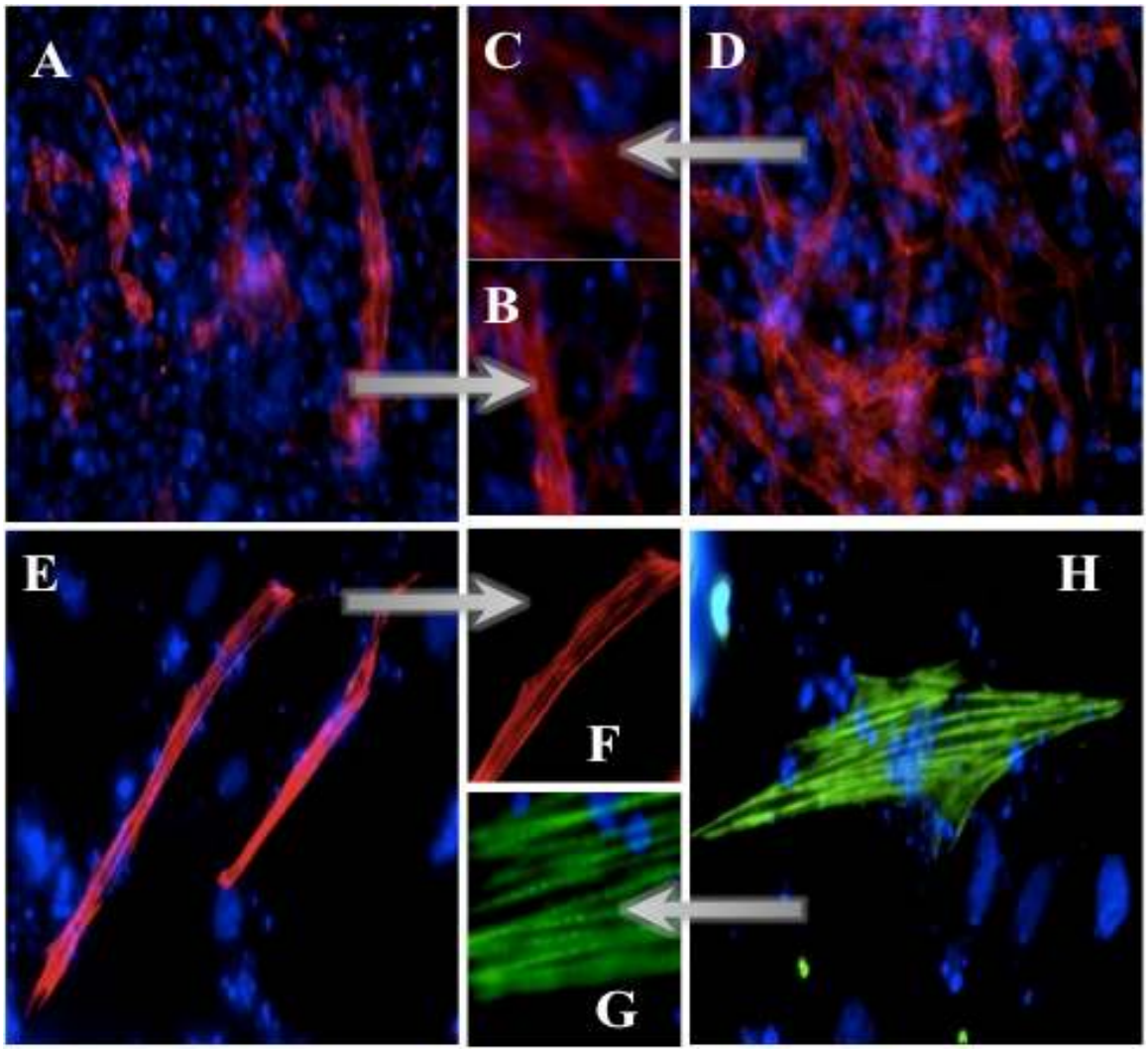


Figure 4.6: cTn-I Expression at D10 of differentiation. Immunofluorescent micrographs showing the expression of cTn-I in different conditions. **A**: Control, **D**: Direct contact, **E and H**: Soluble factors. **B,C, F** and **G** enlarged area of **A, D, E** and **H** respectively showing details of cell morphologies. Enhanced expression was detected in direct contact. Only Striated CM were found in Soluble factor condition. the presence of FCF. Red=cTn-I, Blue= nuclear staining. Green=cTn=I.

CHAPTER 5

A Characterization Study for Identifying the Morphological Diversity of Fetal Cardiac Fibroblast of the Fetal Heart

5.1. ABSTRACT:

Cardiac Fibroblasts are a cell subpopulation that is for the most part poorly characterized, and their complete role in the heart through development has not been completely defined. Here, I present a quantitative and qualitative study for enriching and identifying morphological characteristics of FCF. I report that FCF in cultured were highly enriched after 2 selective adhesion cycles and cells exhibited uniformly expression of collagens type I and III, DDR-2, fibronectin, and perisotin, but differential expression of CD90.2(Thy-1.2). I also identified important morphological feature differences which facilitated the detection of the fibroblast-to-myofibroblast differentiation process; such as expression levels and patterns of α-SMA along with cell size and appearance of stress fiber formation. Such process seemed to slow down as FCF were cultured in media containing the leukemia inhibitor factor (LIF). Additionally, ECM deposited by FCF was found to include a mixture of collagens type I and III, periostin, fibronectin, and laminin, which upon cell removal facilitated by SDS solution, remained intact as original structure was maintained and detected by immunofluorescence intensity analysis. I also detected a small cell subpopulation with morphological similarities to fibrocytes expressing HSP27

at increased proportions and which seemed to establish strong cellular communications with fibroblast and myofibroblasts. Over all this study provides insights to the complexity and heterogeneity of CF, which may contribute to the understanding of mechanisms involved with cardiac fibrosis as well as the derivation of ECM that may be used for CTE engineering applications.

5.2. INTRODUCTION:

The cardiac cell population comprises a set of cellular subpopulations that have been extensively studied, but are still poorly characterized, and the complete role of these cells through development has yet to be defined. There are several uncertainties about the entire physiological functions of the complete cardiac subpopulations, which includes cardiac ca myocytes, fibroblasts, endothelial cells, smooth muscle cells and circulating pericytes and fibrocytes derived from the bone marrow (Banerjee, Fuseler et al.). The principal cardiac subpopulation includes the cardiac myocytes that are additionally subclassified as atrial, ventricular, and nodal CM. CF is a more complex group which has not been properly classified nor characterized into more specific subgroups compared with CM (Snider et al.). The ambiguities concerning the origin and functions of CF may have been significantly challenged due to the poor characterization and proper classification of these cells. They may also have been additionally challenged by the lack of highly conspicuous morphological traits for establishing a proper classification system. Importantly, this particular subpopulation present a broad spectrum of phenotypic characteristics that differ from cell to cell, increasing the complexity of a standard group

of markers for its proper classification (Snider et al.). CD90 (Thy-1), collagen I, collagen III, perisotin, fibronectin, DDR-2, and αSMA, are some of the markers that have been used in previous studies which intended to provide characterization or classification system for FCF in their respective studies (J. Baum and H. S. Duffy; Souders, Bowers and Baudino; Francois Hudon-David et al.; S. Rohr). Even though the origin of this cell subpopulation is for the most part undefined, several progenitor candidates have been proposed (R. A. Reilkoff, R. Bucala and E. L. Herzog; Keeley, Mehrad and Strieter "Fibrocytes: Bringing New Insights into Mechanisms of Inflammation and Fibrosis; Martin and Blaxall; Snider et al.; Lie-Venema et al.; Gittenberger-de Groot et al.; DeWitt and Trounson; Grigore et al.; Lin, Wang and Zhang; J. Baum and H. S. Duffy; Ottaviano and Yee; J. J. Santiago et al.; Watsky et al.). CF's Potential cell sources include: 1) cells from epithelial to mesenchymal-transition (MET); 2) pro-epicardial cells; or 3) primary epicardial cells migrating to the myocardium followed by progressive differentiation into CF (Snider et al.; MacKenna, Summerour and Villarreal; Medici and Kalluri). CF appear in the heart on ED12.5 and continue to proliferate to postnatal day 1(Snider et al.). This particular cell subtype is thought to play critical roles through development from synthesis and deposition of ECM, participants in the electrical conductivity of the heart (Zlochiver et al.; Kohl et al.; Baum et al.; Zhang et al.; Vasquez, Benamer and Morley; Xie et al.), are involved in myocyte physiological activity and development (Ieda et al.; LaFramboise et al.; Ottaviano and Yee; Snider et al.), they deposit critical growth factors and cytokines (Dobaczewski and Frangogiannis; Fredj et al.), modulate ECM composition in response to physiological activity (hypertrophy, mechanical stress, and dystrophy) (Jourdan-Lesaux, Zhang and Lindsey; Borg et al.), etc. Cardiac fibroblasts

have been cultured *in vitro* for performing different studies aiming to identify the physiological activity and morphological characteristics of these cells (Nichol et al.; Ieda et al.; S. L. Bowers, T. K. Borg and T. A. Baudino; Bowers et al.; Golden et al.; Li et al.; F. Hudon-David et al.; LaFramboise et al.). Notably, many of the studies using CF fail to provide a detailed characterization of the cell population profiles in culture for the most part, including the likelihood of obtaining ambiguous results due to the increased plasticity of CF which are frequently observed in *in vitro* studies to differentiating into myofibroblast(S. Rohr; J. Baum and H. S. Duffy; Hinz et al.; F. Hudon-David et al.). The factors that promote the transition of cardiac fibroblasts to differentiate into myofibroblasts includes cell responses to mechanical and physiological stress that in many cases are caused by hypoxic conditions, and mechanical forces such as shear stress or stress-strain action; etc. (J. Baum and H. S. Duffy; J. J. Santiago et al.; Wipff and Hinz). In the native heart, CF migrate to the site of injury where they differentiate into myofibroblasts; these will secrete cytokines, responsible for promoting and starting the pro-inflammatory process (Martin and Blaxall; J. Baum and H. S. Duffy; Bucala; R. A. Reilkoff, R. Bucala and E. L. Herzog). As this process continues at the site of injury, a cardiac fibrosis develops which is characteristic of increased number of myofibroblasts secreting uncontrolled amounts of ECM materials causing alterations to cardiac tissue mechanical properties, as well as interference of the normal cardiac electric conduction system. A similar process seems to be observed *in vitro* conditions as CF are set to culture in conventional tissue culture dishes.

Here, I present a characterization study where cardiac cell cultures were enriched and quantified for assessing the purity of the FCF in culture while providing the culture

conditions containing highly enriched cultures of FCF. FCF were observed at high frequency based on delimiting myocyte populations using cTn-I marker after a second cycle using selective adhesion treatment (SAT). The characterization process also includes a morphocytometic analysis using nuclear measurements, such as nuclear roundness and area. This study suggests that cTn-I+ cells are characteristic of round and small size nuclei. Enriched FCF are additionally characterized using the extracellular non-myocyte markers periostin, collagen type I and III, fibronectin, and Thy-1.2(CD90.2). The enriched FCF exhibit a uniform general expression of all these markers simultaneously. In this study a set of different concentrations of sodium dodecyl sulfate (SDS) solution is used to remove cellular materials without disrupting ECM elements deposited by enriched FCF on cell culture dishes. This is additionally characterized by immunostaining analysis based on the general identification of Periostin, collagens type I and III, fibronectin and lamin. I also present a study elucidating the process of FCF-myofibroblasts transition and the effect of the leukemia inhibitor factor (LIF) on slowing down this process. I include a cell morphological analysis using combination of markers predominantly CD90.2(Thy1.2), along with fibronectin, DDR-2,and collagen type I, for identifying potential differences on FCF subopulations in culture. Finally, I present additional morphological analysis based on immunofluorescent analysis targeting HSP27. This study provides insights into the diversity of FCF based on multiple morphological and phenotypic characteristics as well as a method including quantitative and extended qualitative analysis for isolating and enriching for FCF and a method to generate cardiac ECM that may be used as coating material for *in vitro* CM culture systems or CTE applications.

5.2.1. Fibroblasts Functions in the heart:

The fibroblast is a cell type constituting several tissues in mammals and other organisms. These cells are a heterotypic cell type and for the most part have been given a vague classification based on generalized morphological characteristics that are still not well-defined. Fibroblasts in general, exhibit unique phenotypic features accompanied by a specialized genetic program that seems to be designed to perform specific functions within tissues (Baum et al.; Stephan Rohr; Ottaviano and Yee). This heterotypic cell type may diversify according to marker expression and ECM deposition/remodeling characteristics. For instance, each subtype exhibits different physiological functions according to its tissue of origin. A representative example of this occurs in the heart where fibroblasts are involved in producing and remodeling ECM, releasing growth factors, directing cell proliferation, and participating with the heart's electric activity(Ottaviano and Yee; Baum et al.; Jennifer Baum and Heather S. Duffy; Kresh and Chopra; Vasquez and Morley; Yue, Xie and Nattel). In previous studies suggest that CF may enhance survival, development and maturation of CM during cardiogenesis as well as other important functions essential to maintaining homeostasis in the heart(Vasquez and Morley; Kakkar and Lee; Noseda and Schneider; Thery). The distribution and numbers of fibroblasts differ according to several factors and developmental stages and across species(Banerjee, Yekkala et al.). Cardiac fibroblasts (CFs) maintain a defined spatio-temporal genetic program designed to provide CM with essential requirements necessary for proper function and development(Noseda and Schneider; Iyer, Chiu, Vunjak-Novakovic et al.; Vasquez and Morley; Goldsmith et al.; Bhavsar et al.; J.-J.

Santiago et al.; Banerjee, Fuseler, Intwala et al.; Ieda et al.) (Figure 1.3). During embryonic development, CFs secrete a profuse amount of ECM proteins in addition to paracrine factors, ultimately having an effect on CM proliferation and heart structural support reinforcement. However, similar signaling events produced in the adult cardiac tissue were observed, partially producing a hypertrophic rather than proliferative effect in CM. These observations on the differences of signaling transduction effect on CM provide insights on the importance CF's temporal predetermined genetic program with respect to specific developmental stages (Ieda et al.; Noseda and Schneider; Banerjee, Fuseler, Price et al.).

The origin of CF is for the most part unknown, but previous reports have associated fibroblasts with Epicardial-derived cells (EPDCs). which have been described as specialized fibroblasts with a potential common progenitor for smooth muscle cells (SMC) and fibroblasts (Lie-Venema et al.; Gittenberger-de Groot et al.). Other proposed progenitors include epithelial cells or bone marrow circulating progenitor cells also known as fibrocytes. For instance, in the lungs the condition known as interstitial lung disease, is a pathological condition where fibroblast accumulation is observed followed by the appearance of myofibroblasts leading to excessive deposition of ECM causing lung fibrosis and eventually organ dysfunction. Studies aiming to obtain insights that may lead to understanding the mechanisms involved in the course of this particular disease have suggested that the fibroblast or fibroblast-like population, which is the major contributor to the condition, may originate from sources such as circulating blood-born bone marrow cells or pulmonary epithelial cells (Fujiwara et al.). Hence, conditions described in here may suggest the possibility of the presence of heterogeneous

populations of CF as well as the possibility that these cells may come from multiple sources. Fibroblast are a very interesting cell type, exhibiting improved plastic behavior that seems to generate confusion among researchers who are attempting to create an ambiguous classification and characterization these cells. The ambiguities found while trying the classify CF have created confusion leading to the identification of the descendants and progenitors of CF as well as the role and contribution of these cells during early cardiogenic events. The significant contributions of CF to the heart during development may suggest the possibility that CF play a critical role in directing CM specification.

CF as well as other homologous fibroblast from different tissues have been cultured *in vitro* with minimal difficulty (Togo et al.; L. Chang et al.; Kaneko, Nomura and Yasuda; Iyer, Chiu, Vunjak-Novakovic et al.; Stephan Rohr; Farran et al.; S. L. K. Bowers, T. K. Borg and T. A. Baudino). A commonly used method for the extraction and isolation of these cells includes selective adhesion in which suspended cells are set to settle down for couple of minutes allowing the CF population to adhere to the surface of the culture dish in greater proportions compared to the CM or the other cardiac cell subpopulations. The technique is repeated to achieve higher purity (Golden et al.; Louch, Sheehan and Wolska; Boerma et al.). Another technique used to isolate these cells consists of cutting the extracted hearts in small pieces and placing them in normal culture conditions. The pieces of tissue slowly sediments to the bottom of the culture dish and CF slowly migrate out of the tissue to populate the surface of the dish.

CF were considered for a long time to be a contaminant cell in CM cultures. These cells have the ability to proliferate at increased rates compared to other cell types including

CM, which have limited proliferative capabilities especially *in vitro* conditions. Previous studies have reported a phenomenon that has been ignored for the most part; CF tend to differentiate into myofibroblasts which have completely different physiological characteristics than fibroblasts(Medici and Kalluri; Otranto et al.; Jennifer Baum and Heather S. Duffy; Kornelia Kis, Xiaoqiu Liu and James S. Hagood; Stephan Rohr; Wipff and Hinz; Porter and Turner).

A significant challenge in classifying CF or fibroblast in general, includes the lack of specific markers or defined functions associated with this cell type. For instance there is not a unique marker that can be used to identify these cells from other cells specially from those that present close similarities to fibroblasts such as endothelia, or smooth muscle cells. Some of the strategies used to identify these cells are based on intracellular or extracellular staining of ECM generated by these cells such as the sirus red stain. Another strategy that has been used for the identification of these cells includes finding expression of certain proteins that are not expressed by fibroblast. For instance, to identify fibroblast from CM, if the cells do not express a specific marker for CM such as cTn-I, then it is implied that there is an increased chance that the cell subpopulation under analysis could potentially be fibroblasts. The method then continues to delimit the number of possibilities until there are low chances that our cell population could not be a fibroblast population.

5.2.2. Fibrocytes, Fibroblasts, and Fibrosis:

Fibrocytes have been more recently described as a subpopulation of mesenchymal stem

cell subpopulation derived from a hematopoietic progenitor. These cells were also previously described as a circulating bone marrow cell or fibroblast-like cells (Bucala). Markers associated with this particular cell type has been frequently associated with expression profiles of CD34, CD45, and collagen-I (reviewed in(Keeley, Mehrad and Strieter "Fibrocytes: Bringing New Insights into Mechanisms of Inflammation and Fibrosis")). These cells have been frequently associated with fibroblasts and fibrosis development leading to pathological conditions of many organs all over the body such as the heart, lungs, brain, skin, kidney, arteries, eyes, etc.(Stenmark, Frid and Yeager; Jensen, Hyllner and Bjorquist; Keeley, Mehrad and Strieter "The Role of Circulating Mesenchymal Progenitor Cells (Fibrocytes) in the Pathogenesis of Fibrotic Disorders; Keeley, Mehrad and Strieter "Fibrocytes: Bringing New Insights into Mechanisms of Inflammation and Fibrosis; Keeley et al.; Sheridan, Surampudi and Rao; Synnergren et al.; Donnelly; Bucala; Kanasaki, Taduri and Koya; Fujiwara et al.; Sopel et al.; Aldrich and Kielian; Baker et al.; Kisseleva et al.; Maharjan, Pilling and Gomer; Ronald A. Reilkoff, Richard Bucala and Erica L. Herzog). Fibrocytes represent about 0.5% of the leukocyte population in the peripheral blood deriving from the CD14+ cells as result of stimulation by IL-4, IL-13 and platelet derived growth factor while being inhibited by IL-1β, IL-12, IFN-γ, and SAP immune complexes. The utilization of TGF-β1 and endothelin-1 (ET-1) is suggested for promoting differentiation of fibrocytes into connective tissue cells such as fibroblasts (Keeley, Mehrad and Strieter "Fibrocytes: Bringing New Insights into Mechanisms of Inflammation and Fibrosis"). Previous studies have identified the expression of the secondary linphoid chemokine, which is the lignad CCR7 in addition to the expression profiling of CCR2, CCR3, CCR5, CCR7,

CXCR4, αSMA, CD80, CD84, and CXCL12; Most of these are expressed as a migrating response to injury and wound healing mechanism (Bucala).

5.2.3. Mechanisms of Cardiac Fibrosis:

Fibrosis may be generally defined as the uncontrolled deposition of collagens and/or other ECM elements by fibroblast. In many cases, such as the presence of this condition in the heart after a cardiac injury, this condition may result in permanent injury and in many cases will end in death. A number of factors have been identified that can eventually lead to the development of this condition(Medici and Kalluri; Elnakish, Kuppusamy and Khan; Medina and Ghahary; Obana et al.; Bucala; Keeley, Mehrad and Strieter "The Role of Fibrocytes in Fibrotic Diseases of the Lungs and Heart; Keeley, Mehrad and Strieter "Fibrocytes: Bringing New Insights into Mechanisms of Inflammation and Fibrosis; Aldrich and Kielian; Kornelia Kis, Xiaoqiu Liu and James S. Hagood). For instance, in the case of the heart when an injury occurs as a result of a hypoxic condition, such as those caused by blockage of the coronary artery, CM will secrete cytokines, like IL-6, calling into action the cells of the immune system. Immediately following this emergency call, inflammation will occur followed by the migration of CF to the site of injury where they will differentiate into myofibroblast through the phenomenon known as the fibroblast-myofibrobalst transition. The myofibroblast have the ability to deposit increased amounts of ECM, which are used by undifferentiated fibroblast as building materials for repairing and reinforcing the hearts structure. In many cases, this reaping mechanism is hardly controlled creating an

increased number of fibroblast to differentiate into myofibroblast, and eventually leading to excessive deposition of ECM fibers, which will cause a fibrotic plug or scar tissue altering the mechanical conformation of the cardiac tissue. This change in conformation is reflected in the form of an increase of the stiffness of the cardiac tissue; which causes a hypertrophic response, by the neighboring CM. This hypertrophic response may potentially lead to CM apoptosis, which is transmitted to neighboring CM in a chain reaction effect. In many cases, propagation of the injury may result in organ failure followed by death. Previous studies aiming to obtain a deeper understanding of the mechanisms and causes produced during cardiac fibrosis have identified important key factors. Xu et al. 2007 investigated involvement of Transforming growth factor β1 (TGF-β1) in an *in vitro* set up by establishing cultures of fibroblast like cells known as mesangial cells harvested from an adult kidney(Xu, Yi and Chien). This studies suggest that TGF-β1 is shown to have significant implications on the development of fibrosis based on the observations that these cells tend to disrupt the monolayer conformation, inducing multicellular aggregation, and then producing increased amounts of collagens and other ECM proteins that are typically observed during kidney fibrosis formation. In the lungs, a similar condition has been identified with interstitial lung disease (ILD) being described as a condition characteristic of accumulation of fibroblast-like cells causing fibrosis leading to lung dysfunction(Fujiwara et al.; Keeley et al.). In another study attempting to find the fibrosis mechanisms involved during brain abscess formation fibrotic capsule, it was suggested a similar pattern involved during fibrosis formation, which includes fibroblasts or fibroblast-like cells differentiating into myofibroblasts accompanied by fibrocyte migration and the presence of microphages at the core-cite of

fibrosis formation(Aldrich and Kielian).

In the heart, significant life-threatening pathological conditions have been associated with the development of fibrosis. These include intimal hyperplasia, atherosclerosis, ischemic cardiomyopathy, and mixomatous mitral valve degeneration (Keeley, Mehrad and Strieter "Fibrocytes: Bringing New Insights into Mechanisms of Inflammation and Fibrosis; Bucala; Herzog and Bucala; Ronald A. Reilkoff, Richard Bucala and Erica L. Herzog).

5.3. RESULTS:

5.3.1 Cardiac Fibroblasts Enrichment by SAT:

The cell populations extracted from hearts and treated with a single SAT are found to include CM in high frequencies (about 48.91±12.83%, N = 3); this assessment is computed based on a myocyte exclusion analysis based on expression of cTn-I (figure 5.1A-C). The cTn-I- population was assumed to be FCF based on the absence of expression of CD144 and αSMA; markers used to identify the presence of endothelial and smooth muscle cells respectively (analysis performed after cardiac cell extraction previous to SAT; data not shown and no cells expressing these markers were substantially found). Notably, CM are found beating with their internal contractile phenotypic characteristics preserved, shown by well defined sarcomeres (Figure 5.1F).

After a second SAT cycle (Figure 5.1D-E), the frequencies for CF are computed to be at 98.03±2.42% while cTn-I+ cells represented 1.93±2.42% of the total cell population (N=3). Interestingly, the remaining cTn-I+ cells showed a decaying phenotype with undefined sarcomeres and an unhealthy appearance suggesting severe cardiomyocyte hypertrophy (Figure5.1H). For quantitative assessment N=3 data represented in graph represented on figure 5.1I.

5.3.2. Cytometric analysis using nuclei morphometry:

For identifying and characterizing FCM, I used the described image analysis method along with manual quantification using ImageJ software analysis. The parameters used to perform the study included the quantification of the area occupied by each individual nuclei along with the roundness of each nuclei which is computed using equation 5.1:

Equation 5.1……. Roundness = 4*area/(π*major_axis^2)

This is the inverse of the aspect ratio calculation and provides a measure based on fitting an ellipse. My data from after first SAT (N =3) shows how the CM nuclei morphometric measurements tend to group on the area of the graph delimited at 0.65, 325 corresponding to roundness and area in pixels respectively (Figure 5.2A). This mathematical quantification of the nuclei morphology indicates that CM generally show smaller and rounder nuclei compared to FCF populations. Cell populations from P1 showed similar patterns, but with a significant decrease in frequency compared to those from P0. Also, it was observed that cells from

second SAT show, in general, decreased area, as well as increased roundness. All cells were found contained within the quadrant 0.75, 325 corresponding to roundness and area in pixels respectively (N=3) (Figure 5.2B). From this data we also can identify the broad spectrum of nuclear morphology shown by FCF as these cells exhibit highly proliferative capabilities compared with CM that rarely divide. This morphological assessment can be used to identify the decreasing frequency of FCM as more than a single SAT is performed.

5.3.3. FCF characterization:

The data indicates that cells exhibit diverse expression patterns of the markers periostin collagen I, fibronectin, collagen III, and CD90.2 (figure 5.3). The markers periostin (figure 5.3A-C), collagen I (figure 5.3D-F), fibronectin (figure 5.3G-I), and Collagen III (figure 5.3J-L) are expressed uniformly by enriched FCF populations. In order to provide a further characterization study, a combination of more than one marker is used; in specific CD90.2 with fibronectin (figure 5.4A-C), DDR-2 (figure 5.4D-F), collagen type I (Figure 5.4G-I), or periostin(Figure5.5). In general, cells express uniformly fibronectin, DDR-2, collagen type I and periotin; but not CD90.2. This last marker is exclusively

expressed by only some cells and does not seem to have well defined morphological characteristics such as size (figure 5.5D-F).

5.3.4. Decellularization and ECM characterization:

Before to decellularizing the culture dishes, enriched FCF are grown to confluency (figure 5.6AC) and stained with sirus red (figure 5.6AD), which indicates the presence of ECM elements as red fibers. According to the results from actin/DAPI staining, any of the tested concentrations were capable of removing cellular elements with no significant differences observed between the used SDS concentrations ranging from 0.125 to 1% (Figure 5.6A-X). Images and videos (data not shown) were taken in intervals of 1s to asses the decellularization process facilitated by the staining of actin filaments and the nuclei of cells. Images were taken before and after the washing process of each concentration used in each of the decellularization process. Immunofluorescent analysis targeting Collagen type III were used to generate 3D plots for analyzing the morphological structure of the ECM left on culture dishes. According to these plots (figure 5.6Y-AB), the structure in general seems to retain a complex morphology without significant differences between any of the SDS concentrations used for this study. Immunofluorescent analysis performed on decellularized culture dishes indicate the presence of collagens type I (figure 5.7A), III (figure 5.7D) and IV (figure 5.7 G), periostin (figure 5.7J), and laminin (figure 5.7M). Each deposited ECM shows a characteristic morphological structure with noticeable differences between the collagens (images shown in middle column on binary modified micrographs of image 5.7). Further

morphological differences are observed on the skeletonized image analysis shown on third column on figure 5.7. This study suggest that the ECM deposited by FCF is composed of a complex mixture with a complex morphological structure that remained after the detergent washing process used to remove cellular materials from the surface of the culture dishes.

5.3.5. Assessing FCF-myofibroblast transition:

For maintaining the phenotypic characteristics of FCF, I cultured extracted cardiac cells with a RFP reporter under αSMA in order to identify the transition of FCF to Myofibroblast. Additionally, after a cycle of SAT, I fixed and stained the cells with cTn-I (shown in green) for identifying CM from FCF. My data suggests that cardiac cells cultured in media containing LIF show lower frequency of RFP CF (figure 5.8A-C and G-I) compared to cardiac cells cultured without LIF(figure 5.8D-E and J-L). In Cells cultured under the presence of LIF most of the RFP+ cells corresponds to cardiomyocyte populations, which are the cTn-I+RFP+ population. Interestingly, not all cTn-I+ cells express αSMA-RFP. Also, cells cultured under the presence of LIF seem to have a healthier and conserved internal morphology, such as more compact and defined sarcomeres (figure 5.8G) when compared to cells cultured without LIF (figure 5.8J). In order to further detect the process for which CF undergo differentiation I performed an immunofluorescent study targeting αSMA(figure 5.9) and a general actin stain. For this study I was able to identify conditions where cells have increased frequency of myofibroblasts, by indentifying cells with increased expression levels of

αSMA(figure5.9A-D) as well as conditions where cell cultures contained only a few cells with increased expression of αSMA(figure5.9EH). Additionally, through this study, I was able to identify important morphological features only observed on myofibroblasts, but not in FCF. The most important and noticeable morphological features include increased cell size compared to fibroblasts, as wells as increased expression of stress fibers (figure5.9I-L).

5.3.6. Cell population expressing HSP27:

To obtain a better understanding of the process involved wit the CF-myofibroblast transition, I performed an immunostaining analysis targeting the small heat shock protein (HSP)27 shown in figures 5.10 and 5.11. This study revealed the presence of a cell subpopulation that has morphological and phenotypic characteristics different to common cell subpopulation found within the cardiac cell population. Figure 5.10A-C shows how these cells seem to establish strong network interactions (figure 5.11D) leaving a trail of secreted HSP27 along its way. Interestingly I was able to identify how this cell type established complex interactions with fibroblasts (figure 5.10D-F and figure 5.11A), and how these cells camouflage themselves by mimicking morphological features similar to CF; these cells were only detected when stained for HSP27 (figure 5.10G-I). Figure 5.10J-L shows the complex anatomies of these cells, which barely show expression of actin filaments, and the elevated expression of HSP27 on cell surface. Similarly, HSP27 seemed to have been secreted along the pathway followed by these cells.

Figure 5.11 shows the diversity of the structural morphological features expressed by the small cell subpopulation, and by other cell subpopulation(s) that do not seem to exhibit morphological characteristics of CF. For instance in figure 5.11B and 5.11C, the arrows point to thin tentacle-like structures that are not usually found on fibroblasts, but are found in macrophages.

5.4. Discussion:

Several reports appoint fibroblasts as a major player in tissue homeostasis, remodeling and pathology. These cells seem to be responsible for more contributions than it is commonly thought, and which are for the most part poorly understood and need to be further explored (Porter and Turner; J. Baum and H. S. Duffy; R. A. Reilkoff, R. Bucala and E. L. Herzog; S. Rohr; Souders, Bowers and Baudino). Assigning a definition to these cell is not an easy task and further classification is required as more functionalities are discovered concerning the actual role of these cells in heart physiology (Snider et al.). Currently, CF remain for the most part a cell subtype that has been extensively study, but poorly understood and characterized. A previous study performed on adult hypertense DOCA-rats, suggested Thy1 can be used as a marker to identify CF from non myocyte cell populations (F. Hudon-David et al.). This study also used DDR-2 as a general marker for fibroblasts. In general, most groups have failed to provide detailed quantitative analysis to show the purity of the FCF used for their studies, and to confirm that the results reported on FCF are not ambiguous due to the existence of contaminant cells in their cultures such as CM or other accessory cardiac cell subpopulations found in

the heart. I have demonstrated that a single cycle of SAT fails to enrich exclusively for FCF, but included FCF as well, which appeared in increased amounts when only one SAT was performed but had a substantial decrease to a minimal amount after a second SAT cycle(figure 5.1). Identifying, characterizing, and/or classifying cell populations is a critical task in cell tissue engineering and other related fields or applications. Impurities present in cell cultures, are some of the biggest hurdles in important cellular studies or applications.

For further identifying and characterizing FCM morphological characteristics, I used the described image analysis method, along with manual quantification using ImageJ software analysis. The parameters used to perform the study included the quantification of the area occupied by each individual nuclei along with the roundness of each nuclei which is computed using equation 5.1:

Equation 5.1……. Roundness = 4*area/(π*major_axis^2)

This is the inverse of the aspect ratio calculation and provides a measure based on fitting an ellipse. The data from P0 (N =3) shows how the CM from nuclei tends to group on the area of the graph delimited at 0.65, 325, corresponding to roundness and area in pixels respectively. This mathematical quantification of the nuclei morphology indicates that CM generally show smaller and rounder nuclei compared to FCF populations. Cell populations from P1 showed similar patterns, but with a significant decrease in frequency compared to those from P0. Also, it is observed that cells from P1 in general showed a decrease in area as well as increased roundness. All cells were found contained within the quadrant 0.75, 325,

corresponding to roundness and area in pixels respectively (N=3). The significance of this study relies on selecting particular parameters that will allow improved efficiencies when trying to select for specific cell subpopulation that are highly enriched. In this particular case, when trying to enrich for CM using flow cytometry analysis, a parameter that will significantly increase the probabilities to obtain highly enriched populations would be favorable to include or take into account the size and round morphology of the cell nuclei.

In order to validate my quantitative analysis, I included an extended qualitative characterization analysis that covers significantly higher amount of markers compared to other studies where the expression of these markers are used to assess FCF subpopulations. I provided a characterization study that shown the general uniform expression of collagens type I and III, fibronectin, periostin, Thy1.2(CD90.2) (shown in figure 5.3) and DDR-2(figure 5.4). Collagens are secreted ECM and represent some of the most abundant ECM proteins deposited and synthesized by CF through development. For instance, collagens type I and III are the most deposited collagen types and form part of the interstitial ECM deposited by CF (VanWinkle, Snuggs and Buja). Periostin is also an ECM that is secreted by CF (Otranto et al.), but not by other cardiac cell subpopulations, and could potentially be used as a positive selection marker with the only limitation being that *in vitro* cell culture conditions may also be expressed by fibrocytes differentiating into fibrobalst or myofibroblast-like cell subpopulations. Periostin is considered a matricellular protein which is also expressed by cells undergoing the EMT, cells from the periosteum and periodontal ligaments, and injured vessels and metastatic

cancer cells; Periositin also has direct interactions with fibronectin and collagen I (Snider et al.). The discoidin domain receptor 2 (DDR-2) is a collagen specific receptor tyrosine kinase, that has been broadly used to partially identify fibroblast subpopulations in the heart due to the lack of expression on CM, endothelial cells, or smooth muscle cells (Camelliti, Borg and Kohl). In order to identify morphological and phenotypical differences within FCF subpopulations, I performed a combined immunosfuorescent study targeting CD90.2 with either DDR-2, collagen type III, and fibronectin (figures 5.4 and 5.5). Thorough this study I was able to identify a diversity of morphological differences between FCF. These included differential expression of CD90.2 and significant size differences among the ECM+CD90.2+ population. CD90.2 represented the point of divergence among the FCF subpopulation, and helped to improve characterization profiles of this subpopulation, suggesting the presence of more than one particular subpopulation within the subpopulation.

ECM represents an important structural and signaling element involved with most natural microenvironment within organs. Here I generated ECM derived from FCF with the purpose of identifying the ECM proteins deposited by FCF in *in vitro* culture conditions, and to study possible environmental remodeling and adaptation when these cells perceive an austere environment such as the one found on bare surfaces where cells are typically cultured on. I characterized this particular ECM based on the expression of collagen type I, collagen type III, laminin, fibronectin, and periostin deposited on decellulirized cultured dishes (figure 5.7). Each extracellular matrix element exhibits diverse morphological patterns, even in

ECM, with similar characteristics and functions with the collagen families. To further identify these morphological variants, individual images were converted into binary mode, and skeletonized using ImageJ software (figure 5.7 2nd and 3rd row respectively). These particular techniques facilitate the observation of the complex architecture of the ECM fibers as well as the interconnectivity of the same. Importantly, these study provided an insight into understand some of the functions of CF, such as depositing and arranging the ECM to create or adapt environments like the bare environment found in a tissue culture dish, into a more suitable and viable environment to house and protect CM. Previous groups have also performed decellularization studies either directly using cardiac native tissues, nor similar to my studies, by using *in vitro* tissue culture set ups collecting and/or processing the ECM to be used as a cell culture plate coating (Bick et al.; Parker and Ingber; Y. Eitan et al.). In general these studies reported favorable effect on CM culture systems when using naturally derived ECM coatings.

Culturing CF *in vitro* has been carried out extensively by many groups, however maintaining the phenotypic characteristics of these cells (keeping them from differentiating into myofibroblasts) has represented a challenge in general. In my study I investigated the FCF differentiation process into myofibrobast and reported the decreased incidence of these process when using LIF in our culture media (figure 5.8 and 5.9). Other groups have proposed that LIF, as a family member of the IL-6 cytokine sharing a common signal transducer the 130-kDA glycoprotein

(gp130), has cardio protective effects on CM as well as CF (Souders, Bowers and Baudino; Banerjee, Fuseler et al.). In my study I identified both a better preserved sarcomere, as well as noted an increase in sarcomere complexity on CM cultured under the presence of LIF. These morphological characteristics suggest the effect of LIF as a cardioprotective molecule as well as a cytokine , may be involved in enhancing CM hypertrophy as proposed by these studies. Even though LIF did not completely block the differentiation process, it helped to maintain the phenotype of FCF for a few more passages, facilitating the usage of these cells for longer periods of time than other studies. This proved quite useful in situations where maintaining the phenotype of these cells is essential in order to study the effect of FCF on cardiomyogenesis induction. I found important morphological feature differences between FCF and myofibroblasts, that were established by my group as a reference point of the CF-Myofibroblast differentiation process, and which included increased cellular size as well as the notorious presence of stress fibers detected by immunofluorescent analysis targeting αSMA shown in figure 5.9. The importance of identifying key elements in the process of CF differentiation into myofibroblast (Dobaczewski and Frangogiannis; Bucala; Kelley et al.; Keeley, Mehrad and Strieter "The Role of Circulating Mesenchymal Progenitor Cells (Fibrocytes) in the Pathogenesis of Fibrotic Disorders; Keeley, Mehrad and Strieter "Fibrocytes: Bringing New Insights into Mechanisms of Inflammation and Fibrosis; Sopel et al.; K. Kis, X. Liu and J. S. Hagood; Phillips and Brown;

Kapoun et al.), includes investigation of the mechanisms leading to cardiac fibrosis which represents a major problem concerning cardiovascular disease.

CF origin has been linked to different progenitor candidates, and when trying to understand the lineage of these cells, various differentiation models have been proposed with respect to the origin and/or appearance of these cells during development, remodeling, and pathological conditions such as fibrosis (Keeley, Mehrad and Strieter "Fibrocytes: Bringing New Insights into Mechanisms of Inflammation and Fibrosis; Bucala; Souders, Bowers and Baudino). For instance, Souders et al. 2009 proposed a model to explain the multiple site of origin of CF in the heart; this model proposes that CF are generated from the EMT and may differentiate into additional myofibroblasts in response to injury or other pathological conditions. In addition, CF may also arise from fibrocytes or pericytes, which have been associated with hematopoietic progenitors from the bone marrow. Furthermore, this model proposes the idea that pericytes may differentiate into fibroblasts that may be originated from myofibroblasts, which may also differentiate back into some fibroblast-like subpopulation. In my study I found a cell subpopulation that may correspond to pericytes exhibiting complex interactions with myofibroblasts and fibroblast (figure5.10 and 5.11). In order to identify the role of these cells, or its possible role in the CF-myofibroblasts differentiation, I performed imunofluorescent analysis targeting the small heat shock protein (HSP)27. This protein has been reported to constitutively express in many tissues at different stages of development and differentiation. HSP27 binds to cytochrome-c released from mitochondria preventing its interactions with Apaf-1, and it may be expressed in response to a variety of stressful stimuli such as hyperthermia, oxidative stress, staurosporine, etc

(Arrigo). Additionally HSP27 has been proposed to protect against apoptotic cell death that may be triggered by multiple factors. This protein complex can prevent activation of procaspase-3 and procaspase-9 and has the ability to refold polypeptides in the assembly mechanism or newly made polypeptide complexes (Arrigo; Bruey et al.; Mehlen, Schulze-Osthoff and Arrigo). Hence my study suggest that the over-expression of HSP27 on the surface and as wells as secreted by the mall cell subpopulation, may be caused in response to the accelerated process that includes the increased synthesis of protein complexes or cellular structures involved during the fibroblasts-to-myofibroblasts differentiation; including the increased expression of αSMA and the increased production of ECM proteins that is also characteristic of these differentiation process. The small cell subpopulation may also have been generated as part of the same process in accordance to the model proposed by Souders et al. 2009.

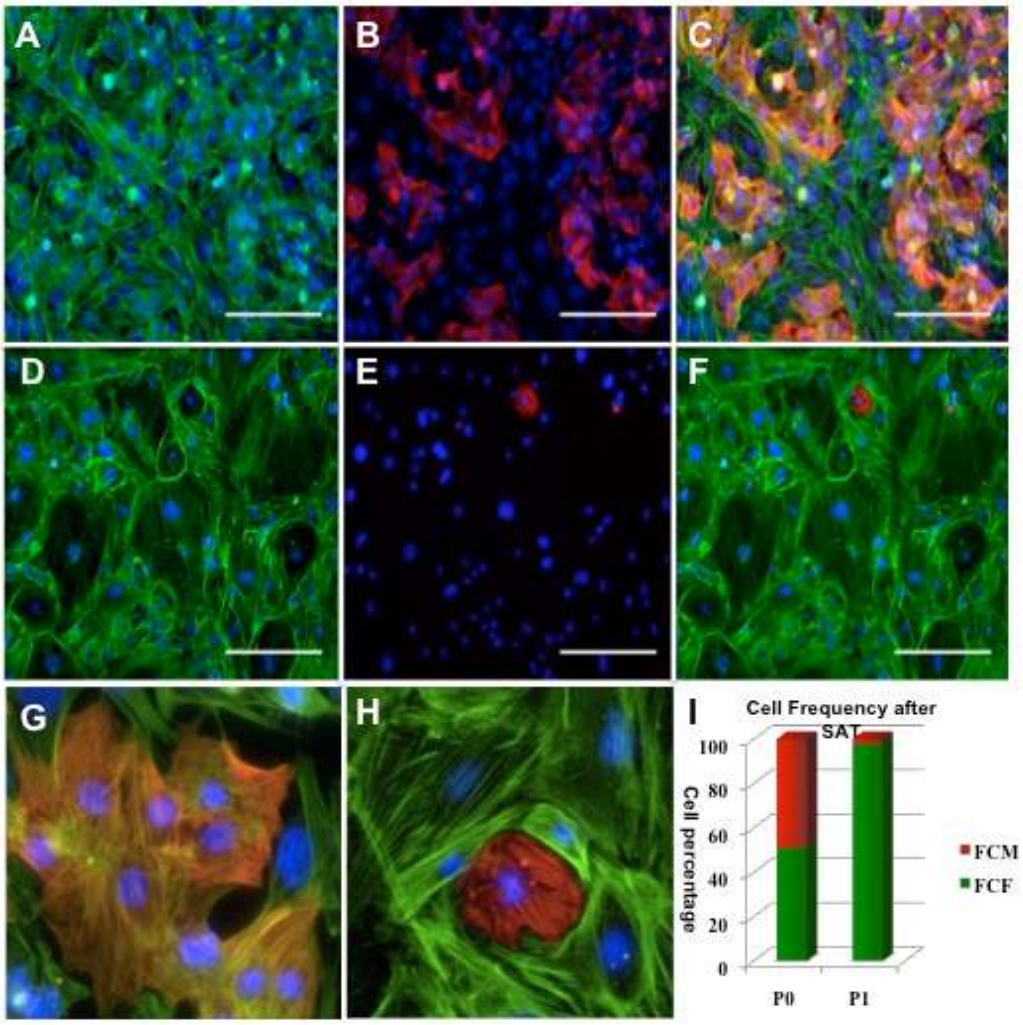


Figure 5.1: Quantification analysis of enrichment process based on cTn-I expression including characterization of enriched Population. Immunofluorescent micrographs of FCF cultures after initial SAT (**A-C**) and after a second SAT (**D-E**) CM (cTn-I+ cells) showing in Red; **G-H)** amplified image showing cTn-I+ cells morphology after 1 and 2 SAT respectively. Red= cTn-I, green= actin, blue= nuclear staining. **I**. Graph representing quantitative analysis based on immunofluorescent analysis indicating enriched populations after 2 SAT (n=3); P0 and P 1= pass number after SAT. Scale bars = 100µm

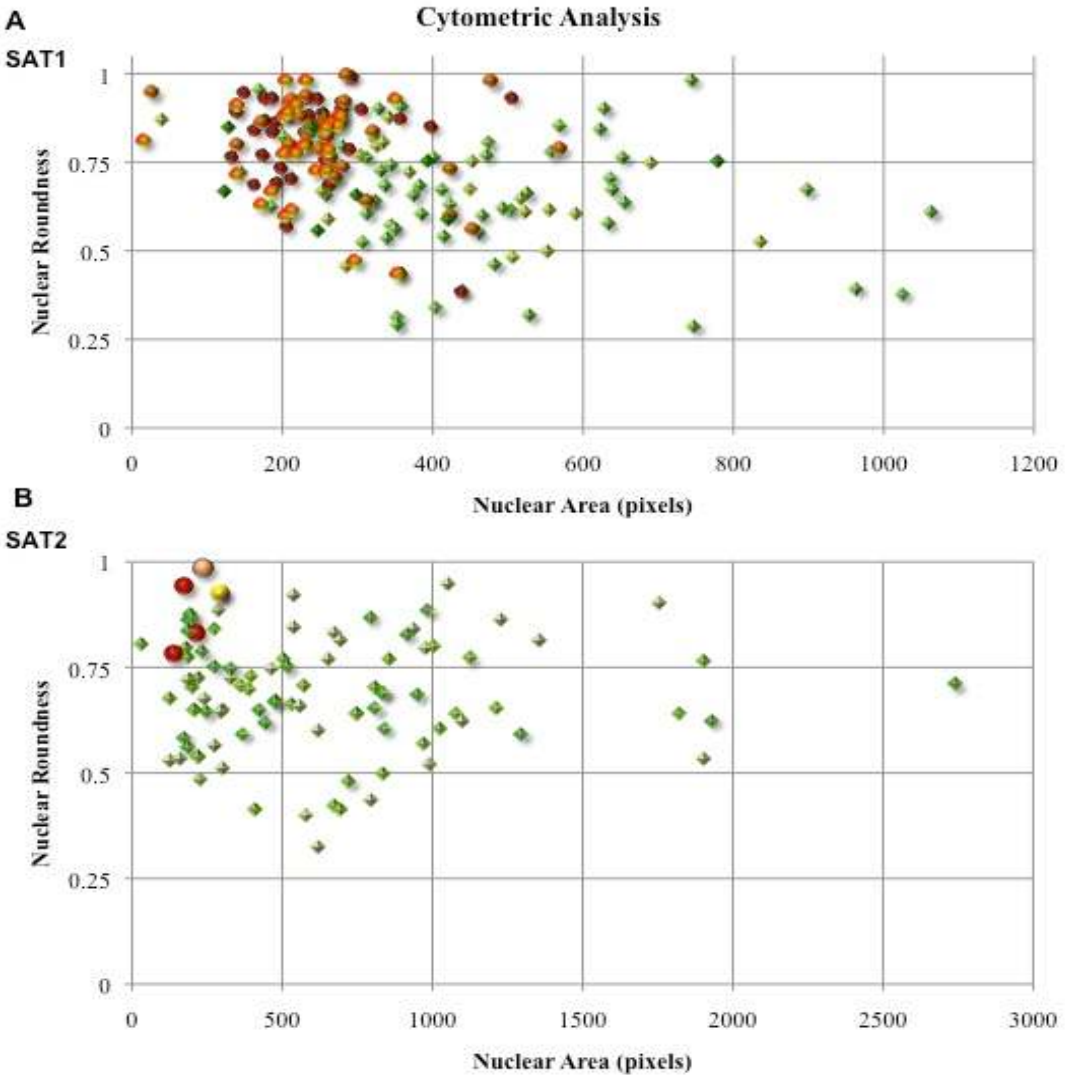


Figure 5.2: Cytometric analysis based on nuclear dimensional morphometry: (A-B) Graphical representation of nuclear morphometric analysis of cardiac cell populations after 1 and 2 SAT respectively. Each set of data from N=3 represented in a different shade. • = cTn-I+ cells and ◆ = cTn-I- cells. Nuclear roundness was computed using eq. 5.1

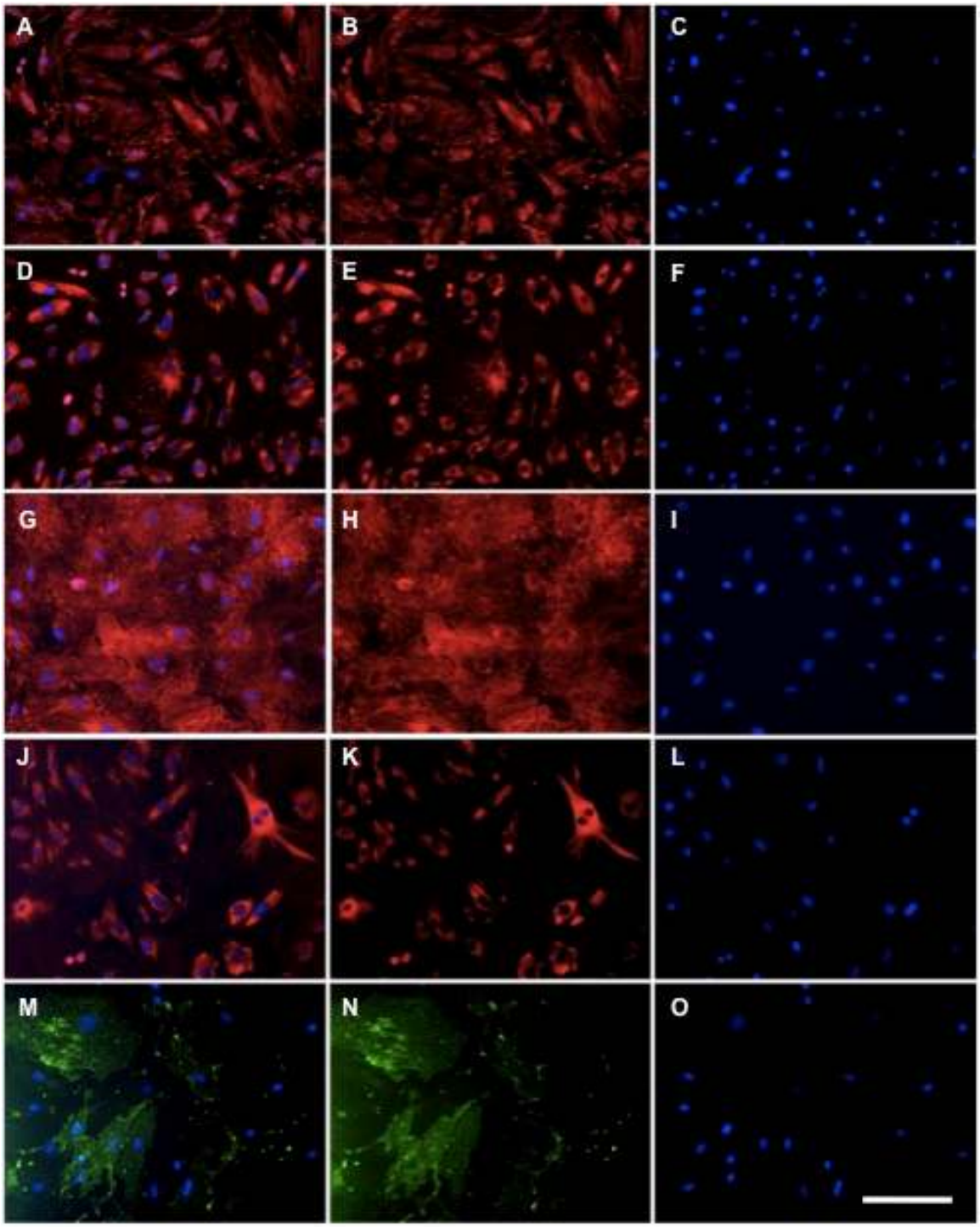

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Figure 5.3: Immunostaining Analysis for FCF Qualitative assessment and cell characterization. Immunofluorescent micrographs representative for FCF characterization indicating the expression of **A-C**) Periostin; **D-F**) Collagen type 1; **G-I**) Fibronectin; **J-L**) Collagen type III; and **M-O**) Thy1.2 (CD-90.2;green). First column = overlay image of nuclear staining and respective ECM; second column = ECM stain only; and third column nuclear stain DAPI. Scale bar = 100 μm.

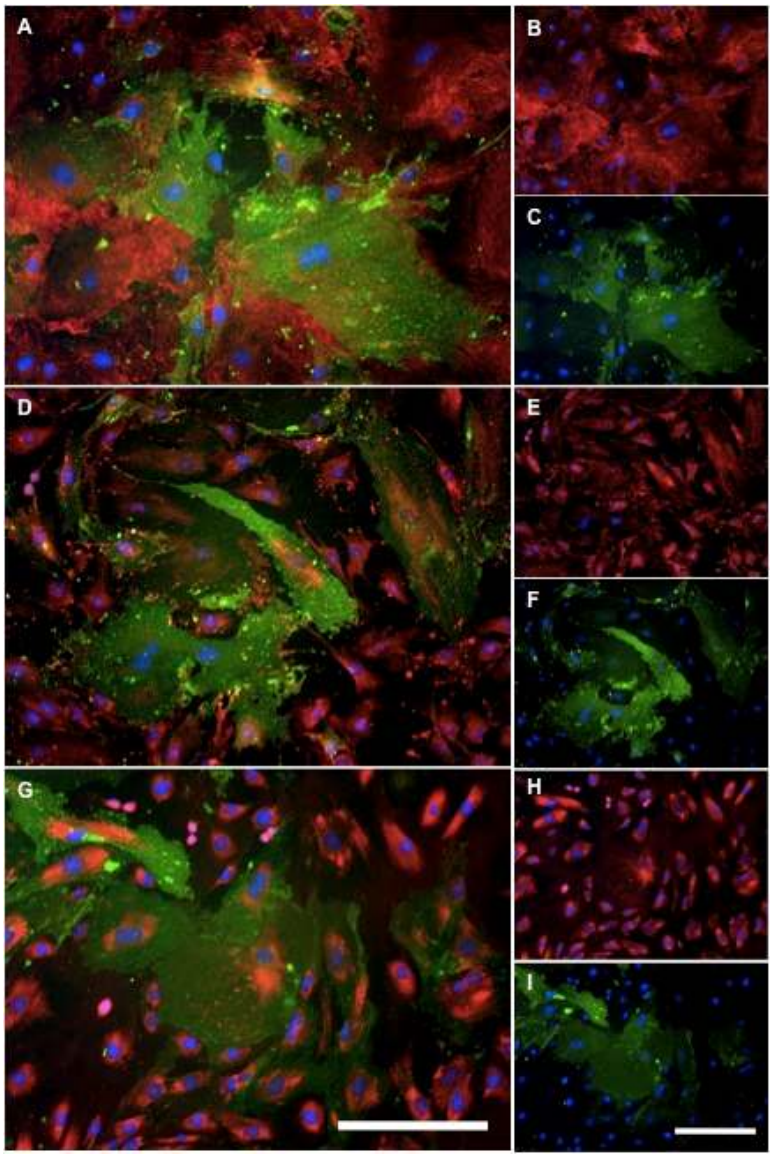

Figure 5.4: Expression diversity of FCF populations. Immunofluorescent micrograph of enriched FCF showing **A-C)** CD90.2 (green), fibronectin (Red), and nuclear staining (blue). **D-F)** CD90.2 (green), DDR-2(red), DAPI (blue). **G-I)** CD90.2(green), Collagen type I (red), and DAPI (blue)**.** Scale bar =100 μm.

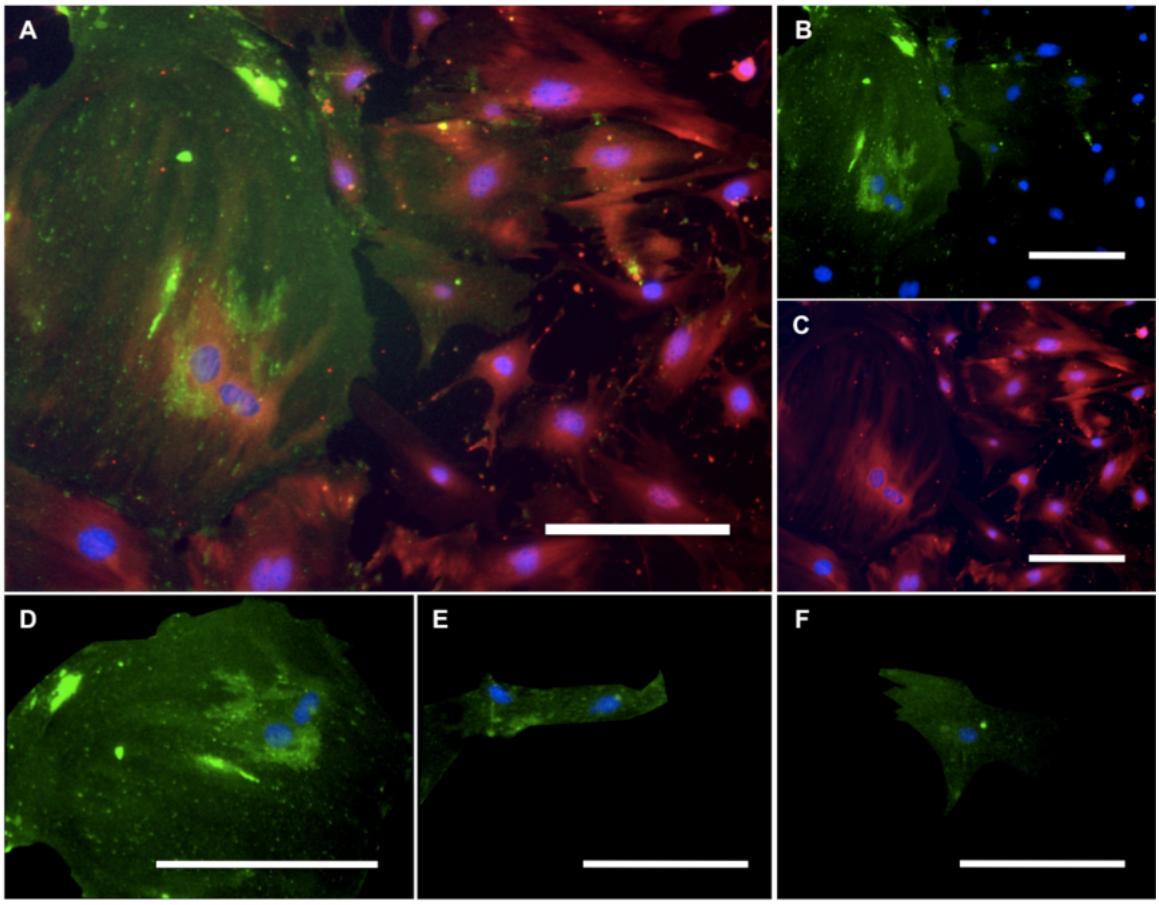

Figure 5.5: Morphological diversity of the CD90.2+/periostin+ population. A) Immunofluorescent micrograph of enriched FCF showing CD90.2 (green), periostin (Red), and nuclear staining (blue). **B)** CD90.2/DAPI. **C)** Periostin/DAPI**. D-F)** Individual CD90.2+ cells selected from image A**.** Scale bar =100 μm

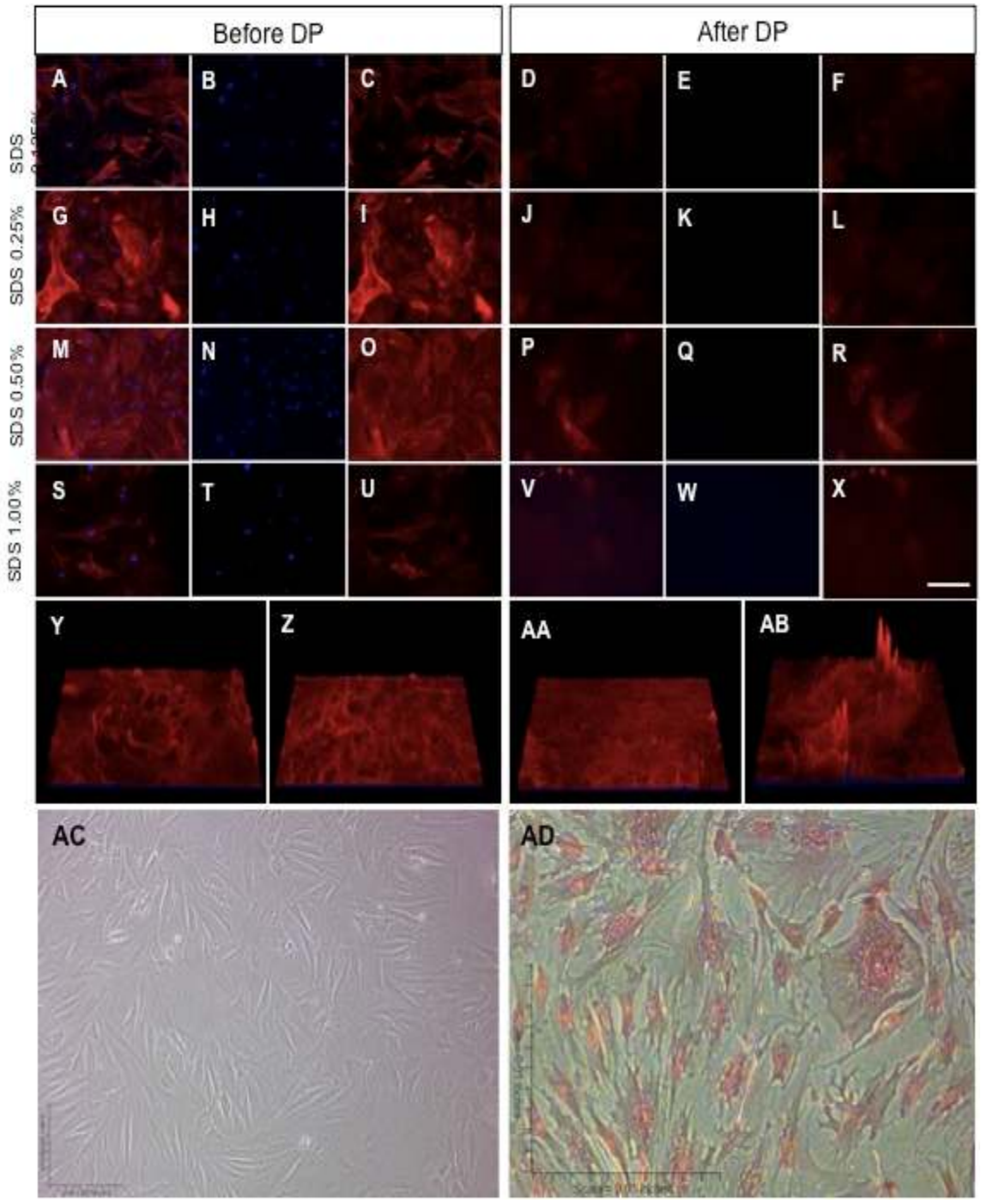
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Figure 5.6 Decellularization Study. Fluorescent micrographs showing the effect of the detergent SDS used to remove cellular materials from tissue culture dishes without removing ECM materials deposited by FCF. Images are representative of cells before and after the decellularization process. **A-F)** 0.125% SDS solution; **G-L)** 0.25% SDS solution; **M-R)** 0.5% SDS solution; and **S-X)** 1% SDS solution. Red= actin staining, blue= nuclear staining DAPI; first and fourth columns= overlay images of actin/DAPI staining; second and fifth column actin stain only; third and sixth column= DAPI staining only; Scale bar = 100μm. **Y-AB)** 3D plots from immunofluorescent micrographs using collagen type III/Alexa 594 antibodies showing the structural morphology of the ECM left on cell culture dishes after SDS treatment; plots representative to each SDS solution tested respectively. **AC)** Micrograph of FCF on dishes before the decellularization process; scale bar = 200μm**. AD)** FCF stained with Sirus red stain ; scale bar = 254 μm.

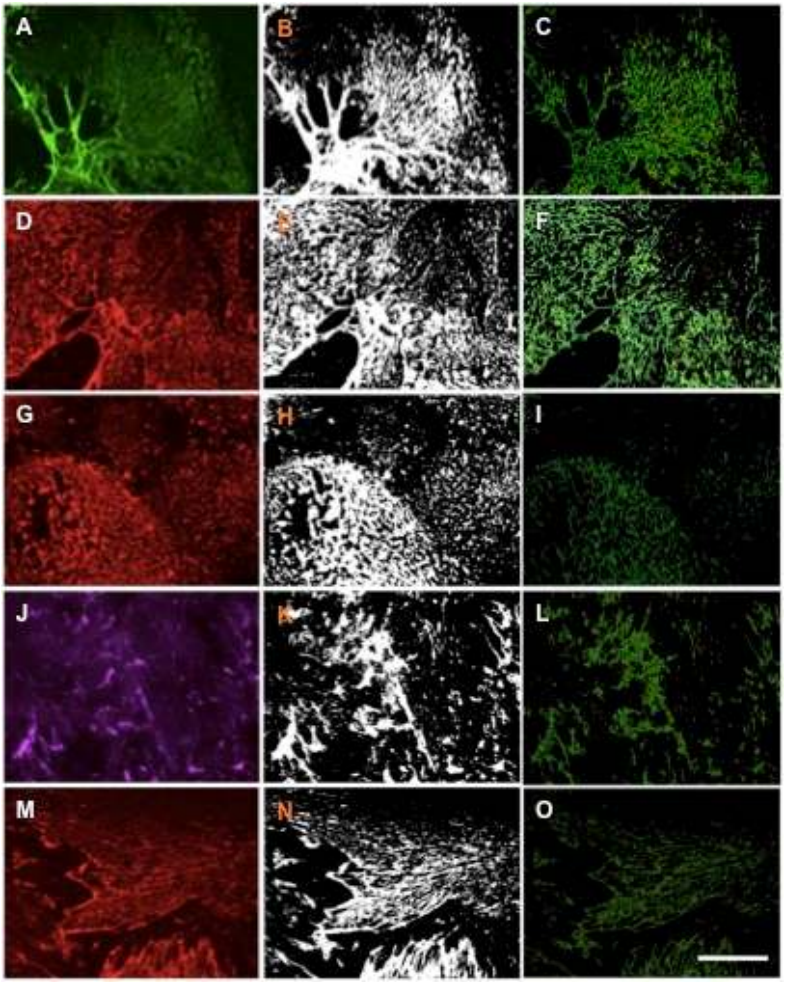

Figure 5.7: ECM Characterization Assessment. Pseudo colored fluorescent micrographs and ECM fiber analysis of decellularized culture dishes representative for **(A-C)** Collagen-I; **(D-F)** collagen-III; **(G-I)** collagen-IV;**(J-L)** periostin; and **(M-O)** laminin respectively. Column 1 = ECM micrographs; Column 2 (**(B,E,H,K,N)**= image converted to binary showing fiber morphology; and column 3 **(C,F,I,L,O)**= skeletonized image analysis. Scale bar = 100 μm.

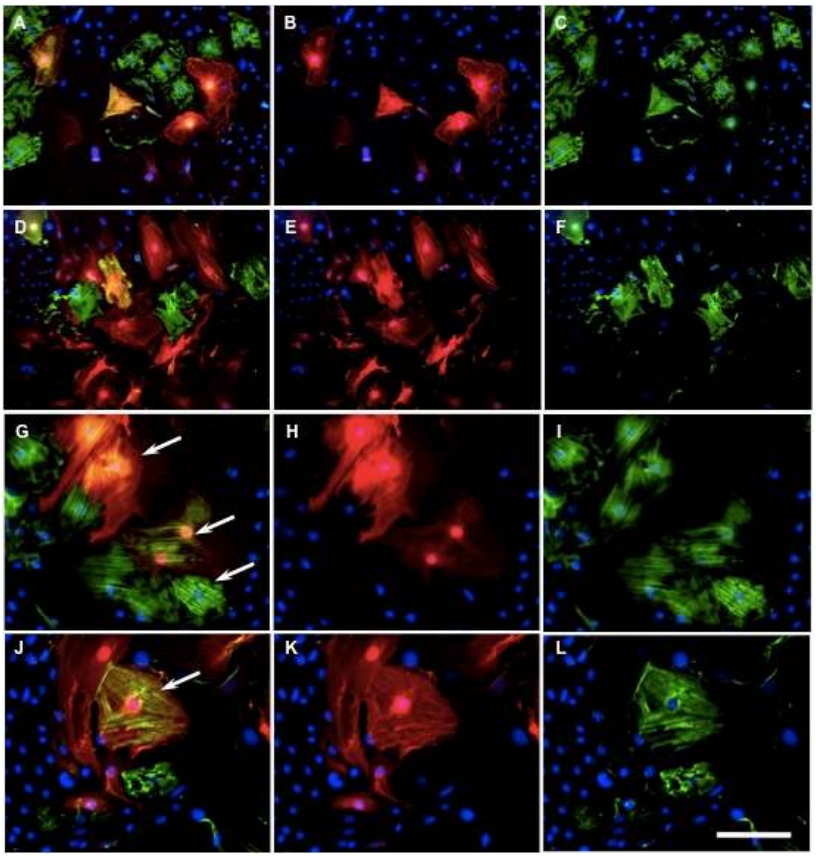

Figure 5.8: FCF Phenotype maintenance by the effect of LIF. Immunofluorescent micrographs of RFP-αSMA cardiac cells cultured either on culture media containing LIF (A-C and G-I) or without LIF (D-F and J-L). First column = overlay image of cTn-I (green)/RFP-αSMA(red)/, DAPI(blue); second column = overlay image of RFP-αSMA/DAPI; third column = overlay image of cTn-I/DAPI. G-L) Micrographs showing the morphology of cTn-I+RFP+ cells when culture with or without LIF respectively. Scale bar = 100μm.

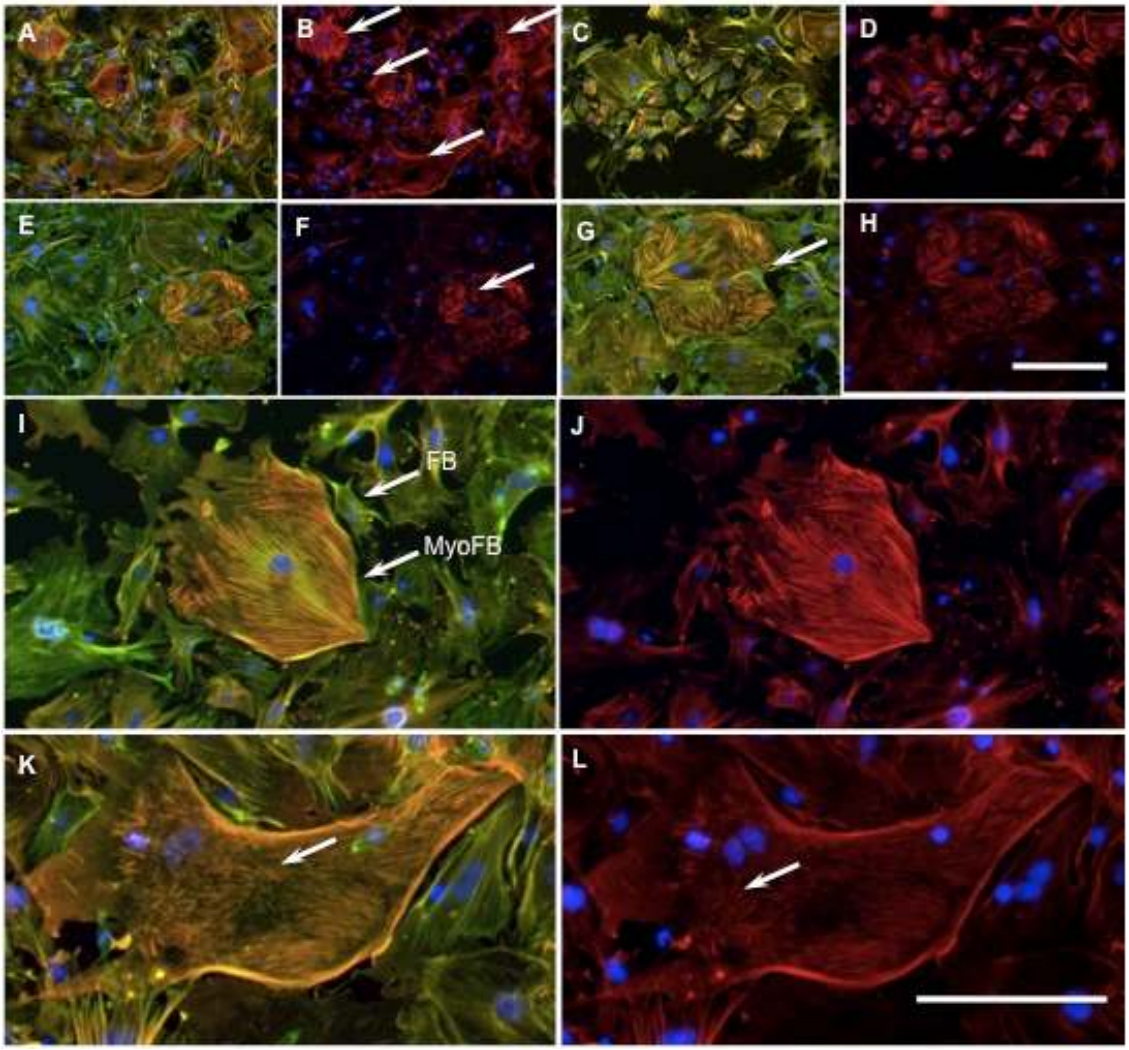
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Figure 5.9: Cardiac fibroblast-to-Myofibroblast transition assessment. Immunofluorescent micrographs showing the expression of αSMA as a marker for fibroblast differentiation assessment. **A-D)** Micrograph representing cultures with increased number of myofibrobalsts, arrow on **B** showing myofibroblasts; A,C,E,G = overlay images of actin/αSMA/DAPI; B,D,F,H = overlay images of αSMA/DAPI. E-F) Micrograph showing FCF with fewer numbers of myofibroblasts; **G-H)** Amplified image of **E-F** respectively showing the proliferation of myofobroblasts. **I-L**) Micrographs showing the morphology and increased size of Myofibroblasts compared to undifferentiated fibroblasts, arrows in **I** pointing to a fibroblast and a myofibroblast for size comparison purposes; arrow in **K** and **L** showing the formation of stress fibers on myofibroblasts. Red=αSMA, Blue= nuclear staining, green = actin staining. Scale bar =100 μm.

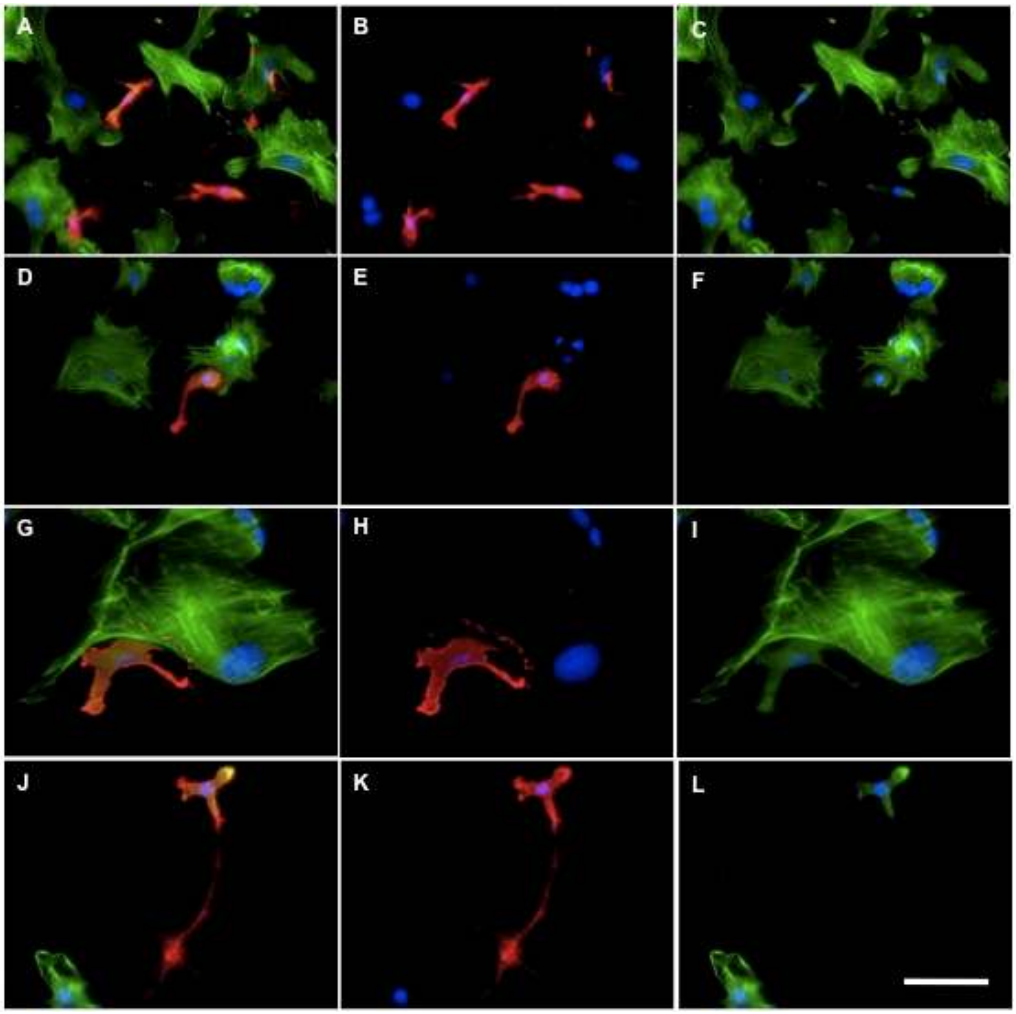


Figure 5.10: Expression of HSP27 by small fibrocyte-like subpopulation. Immunofluorescent micrograph of enriched FCF showing the presence of small elongated cells expressing HSP27 **(A-L)**. HSP27= red, actin stain =green; DAPI = blue. Scale bar =100 μm.

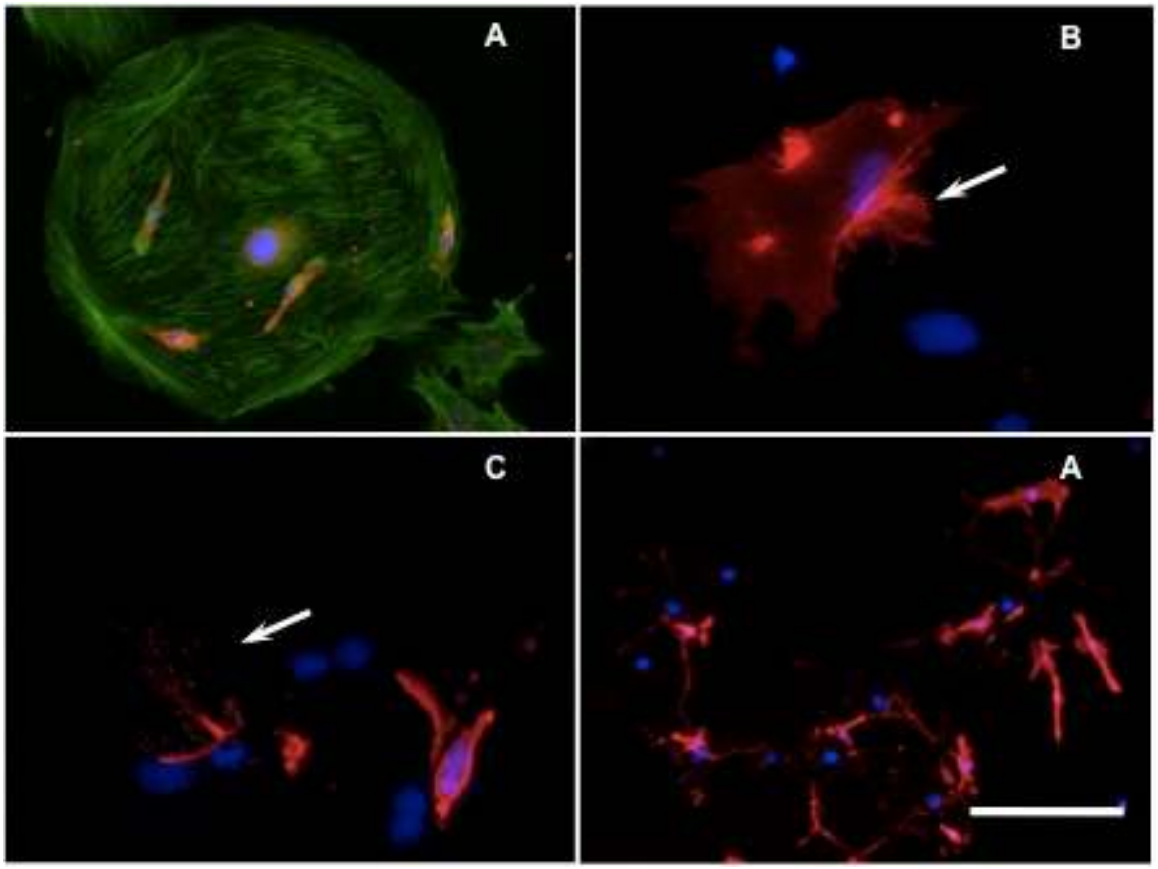


Figure 5.11: Morphological diversity of HSP27 expressing cells. Immunofluorescent micrographs showing HSP expressing cells emphasizing morphological features exhibited by these cells. HSP27= red, actin stain =green; DAPI = blue. Scale bar =100 μm

CHAPTER 6

A Simple Method to Create Complex Topography for the Generation of Diverse Cell Constructs using Fetal Cardiomyocytes

6.1. ABSTRACT:

The heart is compex organ, integrated by diverse mechanobiological anisotropic systems that span multiple organization levels ranging from the protein assembly level to the tissue organization level. This complex anisotropic organization facilitates proper organ physiological functioning to pump blood to the whole body. Biomimicry of this important characteristic in *in vitro* culture system would be favorable for promoting cell internal morphological patterning, enhancing cell-cell communications, and inducing mechanical and electrical coupling of CM. Here I describe a simple method for the fabrication of complex topographical features to induce anisotropic patterning of CM. My fabrication method includes significant cost efficiency, adaptability for producing diverse topographical features, and accessibility for most bioresearch labs. By using this method, I facilitated anisotropic alignment of single CM as well as the generation of diverse cellular constructs such as 2D and 3D cell strips and anisotropic monolayers of cells using fetal CM. Notably, individual aligned cells showed striated cells with nicely

anisotropicaly patterned sarcomeres with similar results shown on the other cell constructs. More importantly, cell constructs using multiple cells showed improved electrical connections suggested by the expression patterns of connexin-43 around the peripheries of CM, and more complex expression patterns on 3D cell constructs were also observed. Additionally, Ca^{2+} dye assay performed of confluent monolayers of aligned CM, suggested functional connectivity between cells. Finally, this study could potentially benefit the generation of biomimetic cell constructs that can be used for pharmaceutical and developmental studies or for tissue engineering applications.

6.2. INTRODUCTION:

Tissue culture systems represent a significant tool for biomedical research and pharmaceutical studies. For instance, cell culture systems may facilitate the study of the development of pathological conditions *in vitro* or can be used as drug testing platforms. However, there exist some limitations with these systems that include the lack of incorporation of signaling elements such as ECM matrices, cytokines or signaling molecules, or other biophysical and biochemical components that may be favorable or required for proper physiological functioning of cells. It has been previously shown that strategic integration of biophysical and biochemical signaling elements into cell culture platforms have significantly improved and enhanced phenotypic and physiological functioning of cells in culture while promoting tissue-like formation in *in vitro* conditions (Luna et al.; F. Wang et al.; Alekseeva et al.; Gauvin, Chen et al.; Gauvin, Parenteau-Bareil et al.; H. N. Kim et al.; Kulangara et al.; Kumar et al.; Ross et al.; Chung and

King; Greene et al.; Ho, Wang and Wang; Kang et al.; Kresh and Chopra; Mitchel and Hoffman-Kim; Barr, Hill and Bayat; Hoffman-Kim, Mitchel and Bellamkonda; Ghibaudo et al.).

Recently TE has integrated engineering tools for generating platforms that biomimic tissues and organs. Some of these elements are created by implementation of microfabrication techniques for the generation of cell culture platforms containing: 1) microtopographical features for inducing anisotropic cell patterning; 2) microelectrodes for electrophysiological recordings or application of electric field stimulation of cells; 3) microfluidic channels for perfusing media or growth factors, etc. Common microfabrication approaches used for TE applications include e-beam lithography, chemical etching, hot embossing, LBL deposition, self assembly techniques, etc (Reviewed in (H. N. Kim et al.)). In general these fabrication approaches require expensive equipment which operation requires highly skilled training making these technology hardly accessible to most bioresearch labs. I have previously reported a microfabrication technique that is cost and time efficient, tunable, and relatively easy to use for the creation of anisotropic monolayers of neonatal and CM derived from embryonic stem cells (Luna et al.) (Described in Chapter 3). For this study I engineered a cell culture platform containing multi-scale topographical features, fabricated using metal coated polystyrene sheets that were additionally constrained from opposite sides and induced to thermally shrink, creating complex anisotropic features. Here I present a continuation of these previous reported work were I extended and improved the technique by applying further material processing steps, such as organic solvent crazing for the generation of multiple diverse complex topographical features. These new topography

may be used for creating cell culture substrates for multiple applications. Here, I present a study investigating the behavior of FCM in response to different substrate topography including the substrate presented previously by our lab (T3 on figure 6.1K) or to newly created topography generated by crazing PE sheets(T1 and T2 on figure 6.1K). I aligned single FCF as wells as induced 2D and 3D anisotropic cell constructs. Individual aligned cells showed nicely aligned sarcomeres and elongated cell bodies which pattern resemble native CM. Additionally, 2D and 3D anisotropic aligned cardiac strips exhibit organized sarcomes while establishing complex gap junctions suggested by immunofluorescent analysis targeting connexin-43. I also performed a Ca^{2+} signaling study on monolayers of FCF generated using our T3 topography and using the Ca^{2+} dye Fluo-4AM, which indicates an active ionic communication between aligned cells even when the monolayers are not electrically stimulated by an external device.

This study provides insights to the diversity of cellular constructs that can be achieved using my substrate topography that ranges from the single cells to small clusters of cells. The significance of this study relies on the possibility to work with small numbers of cells, which is favorable when cell numbers are limited as when using CM-dESC or other primary cell studies that required the use of minimal amount of cells as in pharmacological studies.

6.1.1. CTE and pharmacological studies:

Research and Development in CTE has produced devices and inventions that have favored the discovery and testing of new drugs in the pharmaceutical industry (Tandon

and Jyoti; Caspi et al.; Grayson et al.; Lorts et al.; Fumimasa Nomura et al.). Novel CTE approaches have generated complex cellular constructs such as anisotropic cardiac strips or cardiac cell monolayers exhibit physiological and morphological characteristics that resemble those from native tissues with the advantage that these are grown *in vitro*. The results or effects observed on these constructs when used for pharmaceutical testing may indicate higher similarities to in vivo effects produced by the drug being tested. In addition, these constructs may facilitate assessment for drug effects in a real-time-scenario, like using cell visualization technology such as light or confocal microscopy. For instance, using cell constructs generated from primary cell sources have a more anatomical and physiological characteristics when compared to cell constructs generated from immortalized cell lines in which phenotypic and/or physiologic characteristics and behavior does not closely resemble to primary cell sources or to native tissues.

As previously described (H. N. Kim et al.; Viventi et al.), integration of multiple technologies in the form of bioreactors or lab-in-a-chip platforms, has significantly improved *in vitro* and real time pharmacological studies facilitating the physiological interrogation of small numbers of cells or even single cells. For instance, Rogers et al. 2012, integrated imprinted microelectrodes onto a flexible biodegradable substrate made out of a naturally derived polymeric material derived from silk (D.-H. Kim et al.). These platforms would facilitate recording of electrophysiological parameter at individual selected points from single cells in a monolayer of *in vitro* cultured cells. These platforms may also be used as an implantable recording patch to obtain electrophysiological recordings from whole organs such as the heart or the brain. For instance the platform would be implanted into animal models and will eventually degrade

with minimal possibilities of producing an immunological reaction due to the low reactivity of the materials involved in these devices. A similar but more rudimentary application was previously used to electrically stimulate ESC while obtaining electrophysiological readings with the same platform. Such platform were used to test the effect of different drugs on cardiogenesis induction on ESC (Serena et al.; Sauer et al.).

6.3. RESULTS:

6.3.1. Characteristics of Substrates' Topography:

I present a time efficient and a relatively simple method for the fabrication of complex topography (Figure 6.1) that may be semi-anisotropic (figure 6.1C-D), or anisotropic (figure 6.1G-H). These topographies, can be molded into polymeric materials, such as PDMS or acrylamide gels (Figure6.1E and I), and used for diverse cell mechanotrasduction responses to complex topography studies, or for TE applications. The Molded topography presents diverse peaks and valleys (Figure 6.1F and J) and as shown in the previous study (chapter 3) it contains multi-scale topographical features. Through acetone crazing PE sheet I obtained an even more complex topography (figure 6.1A) that can be further processed for increasing topography complexity or tunning the microtopographical features and that is for the most part described by the internal fibers of the polystyrene sheets. Overall, these new approach increase the diversity of topographical features that can be obtained using our microfabrication technique (figure 6.1K).

6.3.2. Alignment of FCF on Anisotropic Aligned Topographical Features:

I aligned FCF extracted from D15.5 embryos and cultured for 2 to 3 days for inducing anisotropically aligned monolayers (figure 6.2). In this study I demonstrated that FCF can be aligned using my substrate T3; for investigating internal cell morphology, I performed immunofluorescent analysis using general actin staining with phalloidin (Figure 6.2B), the CM specific marker cTn-I (Figure 6.2C), and the nuclear staining DAPI (shown in blue in Figure6.2A-C) for identifying individual cells on seeded substrates. The cells showed improved sarcomeric patterning (Figure6.2D-G) identified by the striations on cTn-I images as well as merged images of actin/cTn-I. I also observed improved cell-cell connectivity that resulted in cells expressing the fascia adherens protein N-cadherin (data not shown) and the gap junction protein Connexin-43. As cells established stronger connections, it is possible that the contraction forces generated by the beating of CM created and increased force that resulted in rupture of the monolayers and detachment from the PDMS containing the topographical features (figure 6.2H-N). Figure 6.2H-J shows the monolayers starting to rupture until the contracting forces end up detaching the aligned cells from the substrate forming a complex 3D strip of cells that did not lose their anisotropic arrangement even after detachment from PDMS (Figure 6.2K-N).

6.3.3. Cellular Connectivity and Ca2+ Signaling:

For these simple studies performed as proof of concept, I induce the alignment of FCF for 2 days and performed Ca^{2+} staining assay using Fluo4-AM in order to investigate if cardiac monolayers were able to carry on a calcium influx on the cell culture substrate set up. The videos taken while having the cells under Ca^{2+} dye, indicate fluctuations between cells according to measurements taken from vertical and horizontal groups of aligned cells based on the fluorescent intensity variation (Figure 6.3). Figures 6.3 B and C indicates the differences in values of each of the selected picks on the plot from both horizontal and vertical measurements respectively, indicated by the values at each tip of the pick. To validate and obtain better semi-quantitative measurements of the differences in fluorescent intensities with respect to time(s), I performed measurements from individual cells over time and plotted the data obtained from such measurements. My data indicates Ca^{2+} fluctuations shown in the graphical representation on figure 6.3D indicated by the curves described by the standard deviation corresponding to the computed data.

6.3.4. Alignment and Connectivity of Single Cells and Small Clusters of Cells:

For this particular study, cells were seeded into substrate containing topography, at small amounts, looking to induce the alignment of isolated events and its respective behavior in response to my topography. I used substrates created by solvent crazing, which contained ridges with complex aligned anisotropic topography at the valleys. I was able to align

individual cells, which showed nicely aligned sarcomeres and elongated shape (figure6.4A-L). I also cultured FCF on plain cell culture dishes (figure 6.4M-O) that served as controls to compare the morphological differences between cells cultured on substrates containing topographical features. The controls showed lack of alignment and unorganized sarcomeres which had also started to rupture (figure 6.4O). Additionally my data indicates that my substrate enhanced cell to cell connectivity when two or more FCM came in contact to each other as they expressed connexin-43 right at the contact sides(Figure6.5 E-F). Also, by using my substrate, I was able to induce the formation of 3D clusters of cells (Figure6.5A-D) that seem to establish electrical connections by means of connexing-43 in a complex expression patterns(Figure 6.5C-D). Figure 6.5D shows the different cells based on the nuclear staining which is shown blurred on the micrograph due to the relative position along the strip. Figure 6.5 G-J shows a 2D cell cluster with anisotropic aligned cells that also established connections at the boundaries of each cell in contact. Figure 6.5K-M shows an anisotropic aligned cardiac strip including the PDMS substrate that was used to induce alignment.

6.4. DISCUSSION:

Biomimicry of internal organ morphology has become a target of study for many groups that aim to improve and create new tissue culture systems (Freed et al.; LaNasa and Bryant; Phillips and Brown; Z.-y. Wang et al.; Coutinho et al.; Huh et al.; Maidhof et al.; Mooney et al.; H. Y. Chang et al.; Chen et al.; Luna et al.; Prabhakaran et al.; Barr, Hill and Bayat; Jakab et al.; Biela et al.; Grayson et al.; Lenas, Moos and Luyten; F. Wang et

al.; Radisic et al.; da Silva, Mano and Reis). In cardiac tissue engineering anisotropic patterning of cells represent a major morphological feature that allows cells to properly communicate and perform mechanically or electrophysiologicaly (Camelliti, Borg and Kohl; N. Bursac et al.; Sondergaard et al.; F. Nomura et al.; Heidi Au et al.; Lieu et al.). Recently, different groups have engineered different microfabrication techniques for creating substrates with anisotropic topography for the alignment of CM and other cells types that alignment represent a significant morphological characteristic for proper physiology and development such as neurons (Hoffman-Kim, Mitchel and Bellamkonda; Clark et al.; Fricke et al.), endothelial (Biela et al.; Jakab et al.), and skeletal muscle cells (Lam, Clem and Takayama; Charest, Garcia and King; Bian and Bursac; Biela et al.; Z.-y. Wang et al.).

This work represent the continuation of the method that I previously presented and facilitated the generation of complex topographical features that may be potentially used for diverse studies involving mechanotrasductio-response to topography or for the generating cellular constructs with particular anisotropic patterning for TE applications. This method uses minimal amount of costly equipment making it accessible to many bioresearch labsm as well as developing countries where costly technology is in general hardly accessible. I showed some characteristics (figure 6.1) representative to some selected topography including molded materials such as acrylamyde gels for providing information concerning the physical description of my created master molds. The advantage of replicating the master mold into different types of materials exhibiting properties to create substrates with different elasticity relies on the possibility to create substrates with tunable elastic properties for diverse studies where stiffness of the

material is favorable (Ross et al.; Farran et al.; Engler et al.; Gopalan et al.; Long and Tranquillo). Other groups have described additional methods for fabricating substrate topography that in general includes limitations such as cost efficiency or feature morphology characteristics (Review in (H. N. Kim et al.). As I reported before (Luna et al.), my lab described a method for the generation of anisotropic and confluent monolayers of neonatal cardiac cells and CM derived from hESC. Limitations of the substrates generated through this method included increased number of cells needed for alignment induction studies; additionally it was observed that as cell established cell-cell connections with neighboring cells, the monolayer confluent morphology was usually disrupted by the increased contraction forces generated by the newly created monolayer. In general, differentiation methods for generating CM derived from pluripotent cell sources produce limited amounts of cells that are available for alignment induction studies and they are additionally challenged by the available enrichment methods such as flow cytometry cell sorting that will create stressful conditions to the cells, potentially decreasing cell viability. Hence, ending up with a very limited amount of CM derived from ESC is common, and as these are seeded on substrates, they became difficult to track and many of them will die during seeding due mostly to the stress generated by the sorting process. However, my new method of creating topography T2 (Shown in figure 6.1I) provides lateral support to the cells while the smaller ridges found at the valleys induced anisotropic alignment (Figure 6.5).

T3 substrate facilitated the generation of confluent cell monolayers that may be used for Ca^{2+} signaling studies. For instance, here I present a simple Ca^{2+} staining analysis as a proof of concept using Fluo-4AM where cells were stained with the calcium die at 37°C

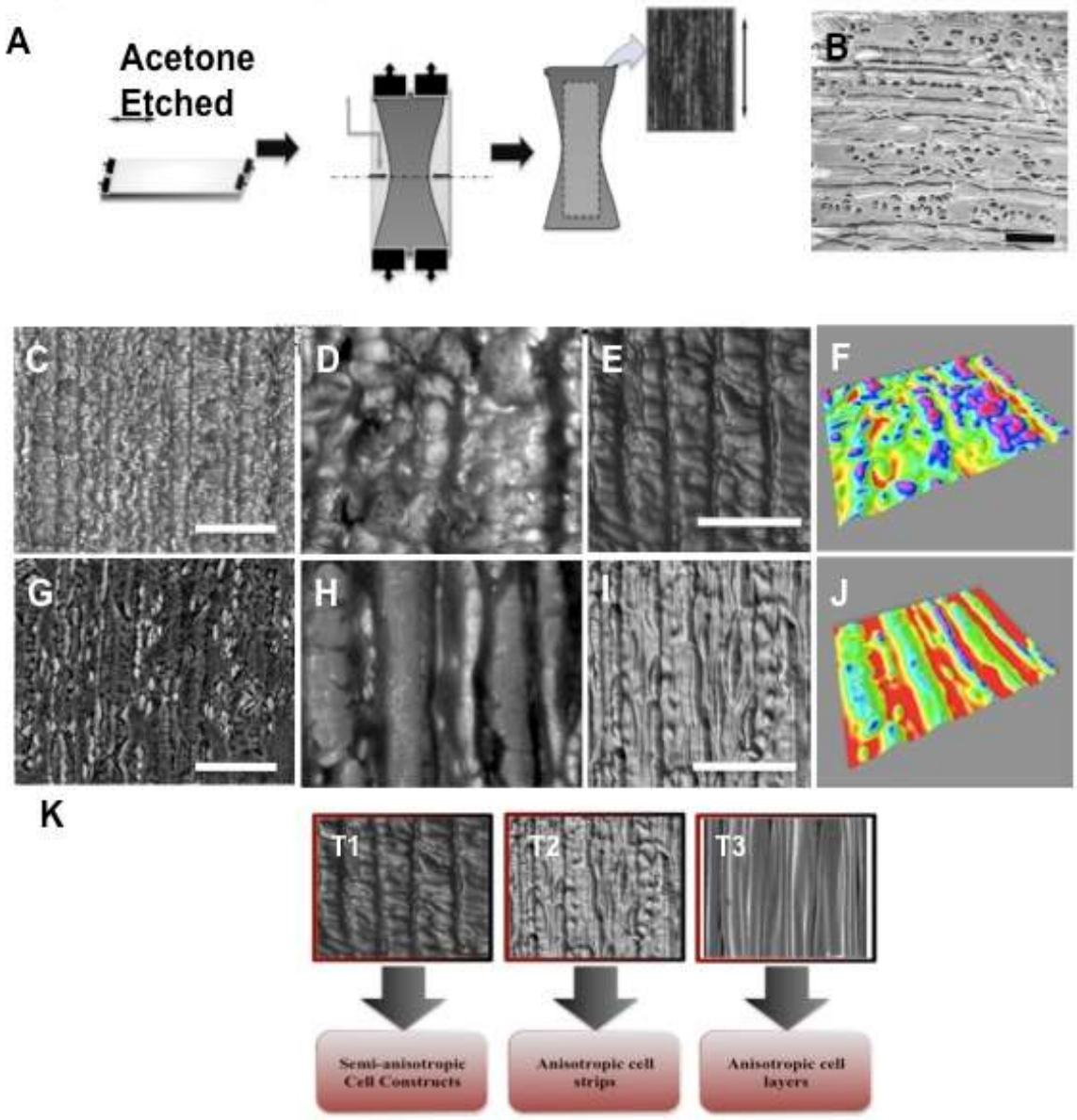
while videos were taken for identifying Ca^{2+} Influx between interconnected cells (Figure 6.3). This study showed that cells established functional connection suggested by the fluctuating values obtained from computing the fluorescent intensity generated by the Ca^{2+} influx wave generated by the cells forming syncytium-like cellular constructs. In this study, I compared relative pick values from: 1) single, and 2) a group vertical and horizontal Ca^{2+} influx measurements. Based on this relative quantification, the values suggested differences in the relative fluorescent intensity. Also, this data suggests a fluctuating behavior of the influx propagation of the Ca^{2+} wave based on the plot that shows the standard deviation curves. Previous studies that are in general more in depth, and that include sophisticated electrophysiological recordings and more detailed mathematical computation and computer simulation, reported results that are in accordance with ours (Chung and King; N. Bursac et al.).

The anatomy and physiology of the cardiac tissue is anisotropic and for proper functioning and homeostasis of CM, the cell architecture needs to be preserved, as cells are culture in *in vitro* conditions. Previous studies reported that a larger coupling resistance is observed in the transverse rather than longitudinal direction in myocytes induced to anisotropically align (N. Bursac et al.). Inducing and maintaining anisotropic characteristics of CM in *in vitro* culture conditions may result in improved results in studies performed for biomedical research and pharmacological studies. These studies additionally may provide insights into understanding cell signaling and mechanotrasnduction responses involved in microtissue architecture and development. Particular studies may be favored by the use of specific cellular constructs such as anisotropic 2D or 3D cardiac strips, or anisotropic single cells; specially when small

numbers of cells are required or preferred, or when cell numbers are limited. Here I described a method to induce the alignment of single cells and provide information on the mechanostrasduction response of FCM to the T2 topography in regards to the observed internal architecture of these cell that include sarcomere alignment that more closely resembles native tissues and that is not observed on cell cultured on common tissue culture plates. Previous studies have reported the importance of maintaining the anisotropic patterning of myocytes, highlighting this important characteristic as a mechanostructural anisotropic continuum (Kresh and Chopra; Chopra, Tabdanov et al.). I also reported the generation of more complex cellular constructs such as 2D and 3D anisotropic cardiac strips, which also exhibited striated cells described by anisotropic sarcomere patterning. The cell constructs were also created using the T2 topography and showed improved anisotropic alignment and cell-cell gap junction connectivity observed by immunofluorescent analysis targeting connexin-43. This protein complex is a major determinant of cell-cell electrical coupling in the myocardium (Motlagh et al.; J. Baum and H. S. Duffy; Lasher et al.; McCain et al.; Thomas et al.; Huang, Horackova and Pressler). For instance, connexin-43 was not detected in single isolated CM but only when two or more came in close contact (Figure 6.5 E-F). In 2D cell constructs, connexin-43 is expressed around the peripheries for cells with increased expression in those cell surrounded by other CM(Figure 6.5G-J). On the other hand, 3D cardiac strips showed an increased and more complex connexin-43 expression with particular concentration at the center of the cluster where cells seem to be more compact and concentrated. This particular expression patterning observed in multicellular constructs, seems to be in accordance to the developmental stage to where these cells belong.

Previous reports have suggested the expression of these gap-junction protein around the peripheries of cells during early development, such as fetal and neonatal development, and eventually is mostly expressed at the intercalated disc of CM which corresponds to the end sides of CM where they establish contact with the proximal cells at each end terminal (Lasher et al.).

In general my method facilitated the generation of diverse cellular constructs that may be used or adapted for biomedical research, pharmacological studies or even for tissue engineering applications.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Semi-anisotropic Cell Constructs
Anisotropic cell strips
Anisotropic cell layers

Figure 6.1: Surface topography. A) Scheme representing the fabrication approach used to generate anisotropic surface topography; PE sheets are first acetone etched and may be further induced to thermal shrinking to create more complex topographical features. **B)** Electron micrograph showing a PE etched with acetone; **C)** Micrographs of surface topography generated using acetone etched PE followed by thermal shrinking against PE fibers **D)** Amplified image view of C ;**E)** Topography imprinted into 5% Agarose substrates **F)** 3D plot showing heat map of surface topography of image D. **G)** Surface topography generated from acetone etched PE sheets without thermal shrinking **H)** Amplified view of G. **I) Topography** imprinted on 5% agarose gels using mold generated from similar method as in G. **J)** 3D plot showing heat map of surface topography of image H. **K)** Schematic representation showing topographical features and functionalities for creating cells constructs. Scale bars = 100μm.

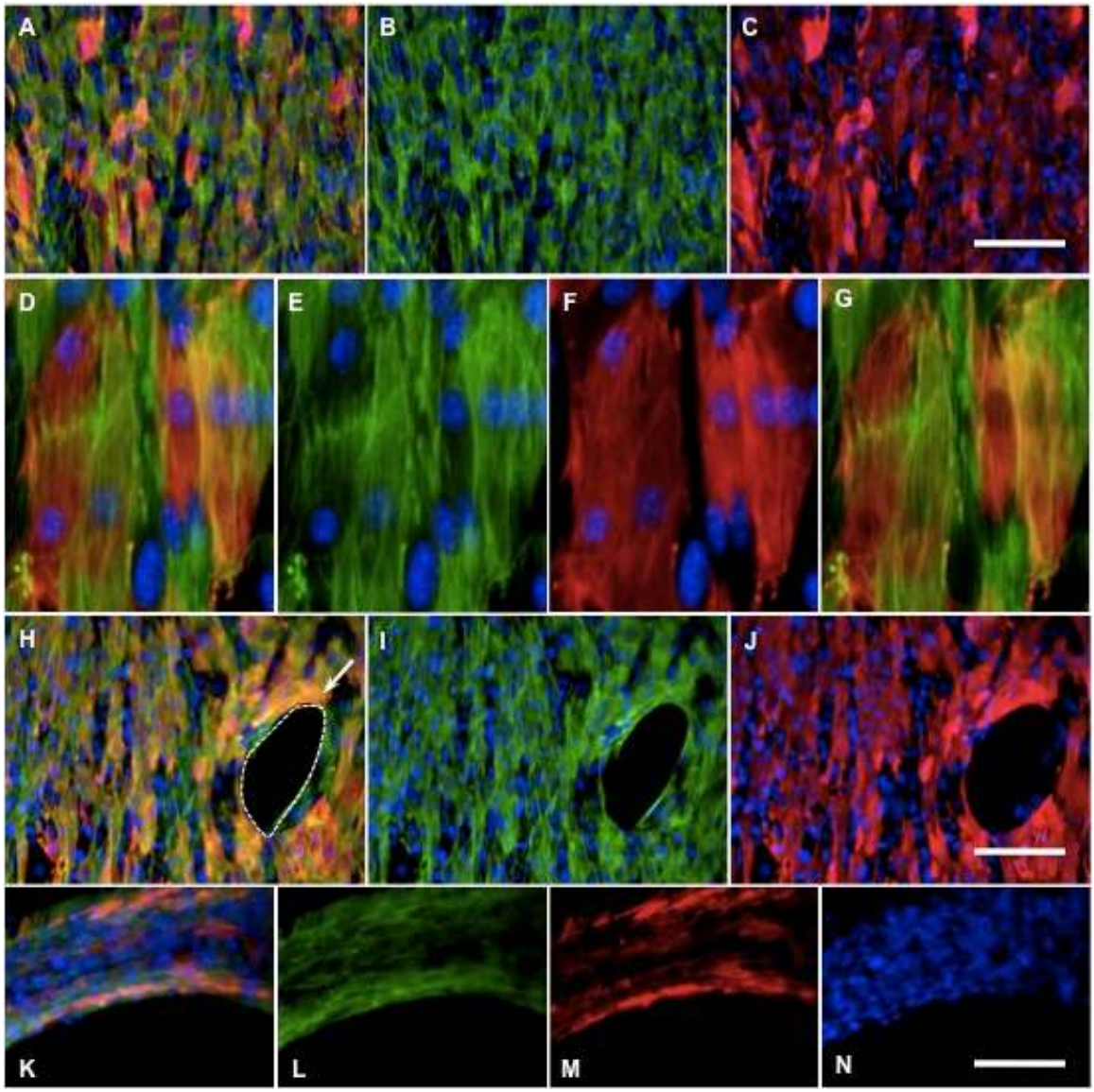
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Figure 6.2: Generation of Anisotropic monolayer of FCM and intense beating activity effects**. (A-G)** Fluorescent micrographs showing anisotropic monolayers of FCM after 3 days in culture. **D-G) Amplified** view showing sarcomeric morphology of aligned cells. **H-J)** Ruptured Anisotropic monolayer with arrow indicating whole formation due to beating generated shear forces. **K-L)** Monolayer detached from PDMS substrate forming anisotropic cell strip. red = cTn-I, green = actin, blue = DAPI. Scale bar = 100 μm.

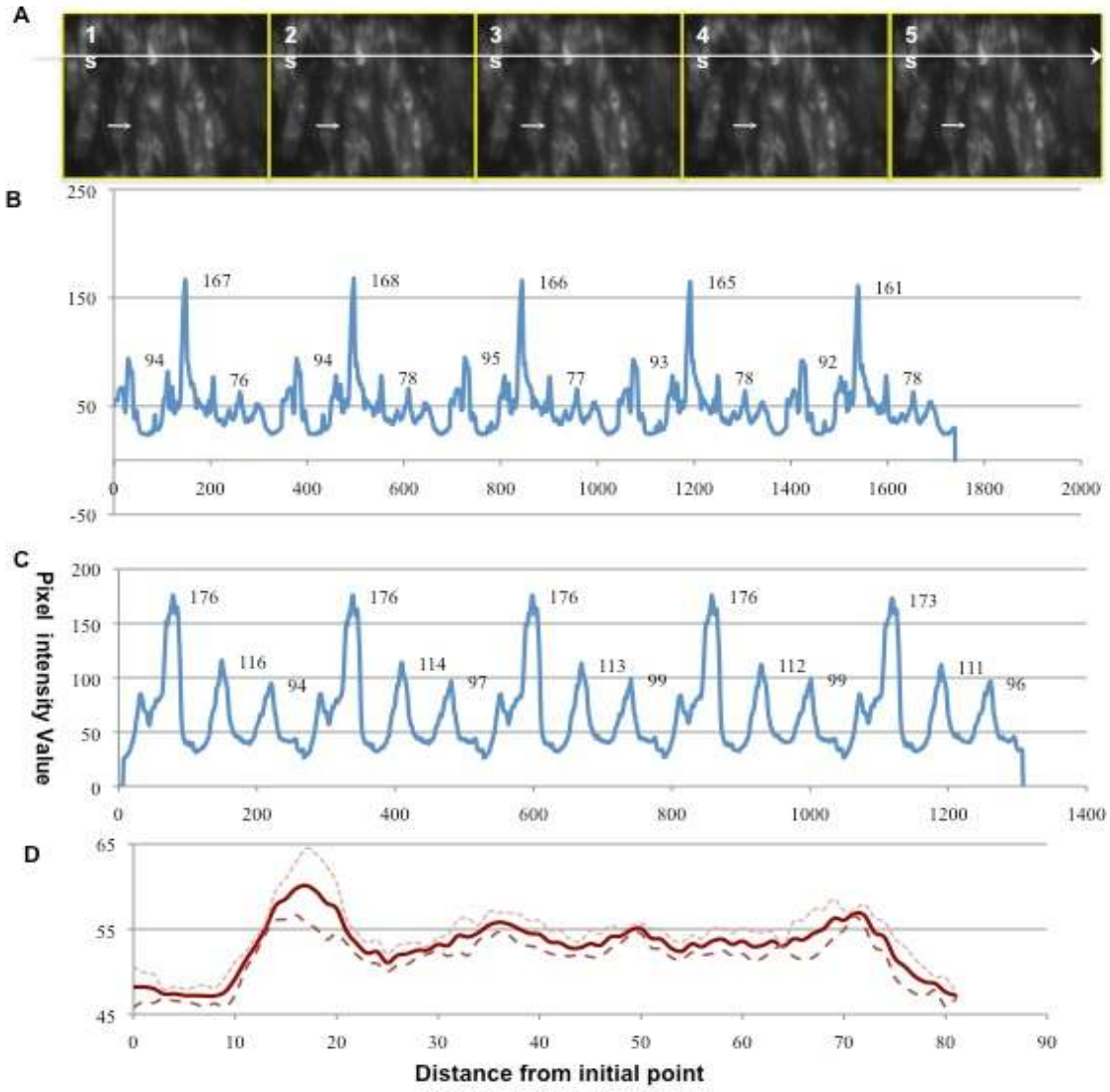

Figure 6.3: Calcium die assessment. Immunofluorescent micrographs representative of monolayer a of cardiac cells anisotropically aligned stained with Fluo-4AM. **A)** Defragmented video in 1 sec time lapses with arrows indicating group of cell measurement and individual cell measurement. **B)** scatter plot showing Ca+ influx on group of cells**. C)** scatter plot from group of cells from vertical line measurement. **D)** scatter plot from single cell measurements along with standard deviation computed from measurements.

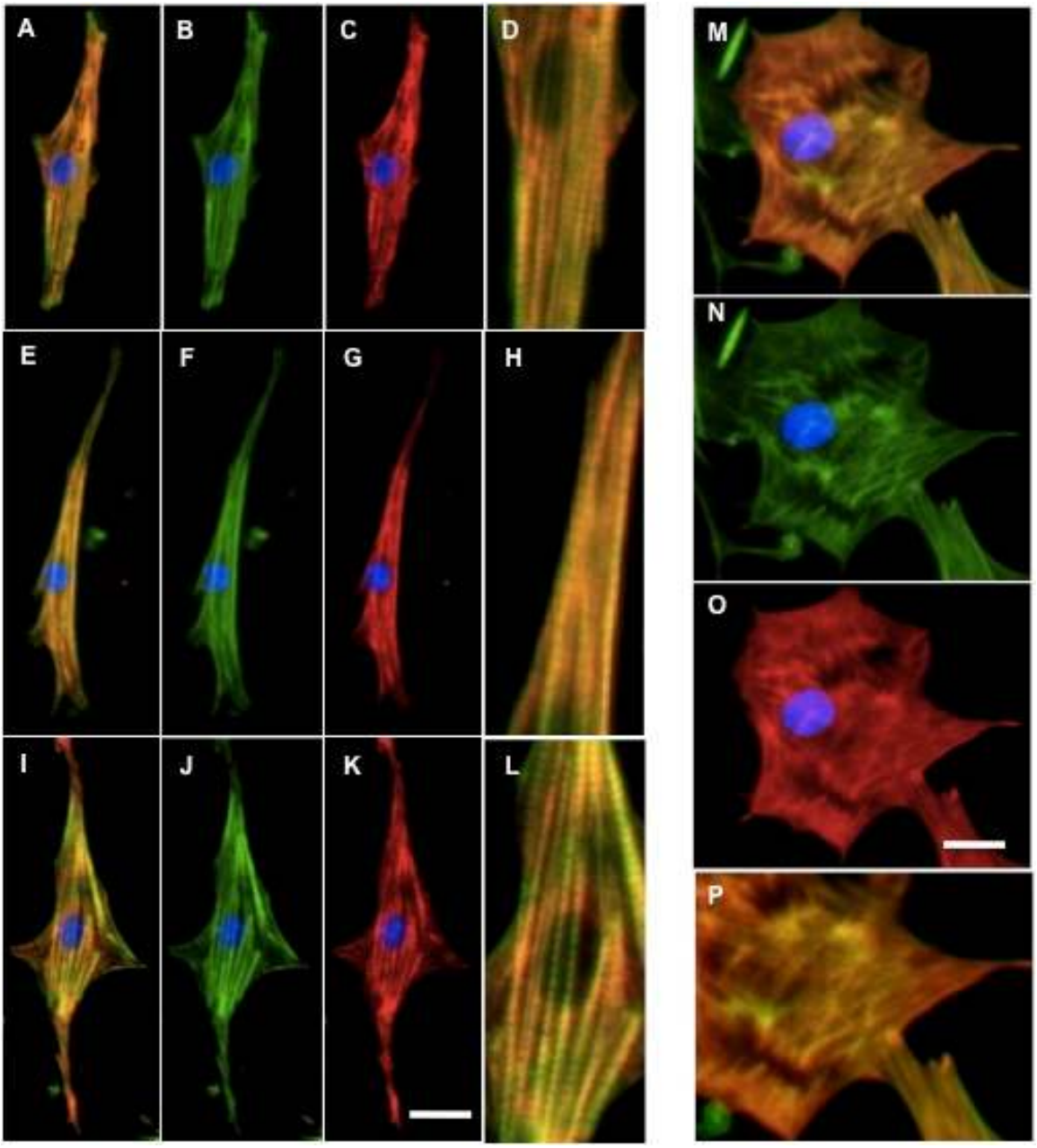

Figure 6.4. Single cell alignment**.** Fluorescent Micrographs of FCM aligned on PDMS substrates generated from crazed PE sheets; **D,H,L)** Augmented views of each respective cells showing sarcomere arrangement. **M-P) FCM** cultured on conventional culture dishes as controls. Red = cTn-I, green = actin, blue = DAPI. scale bar = 25μm.

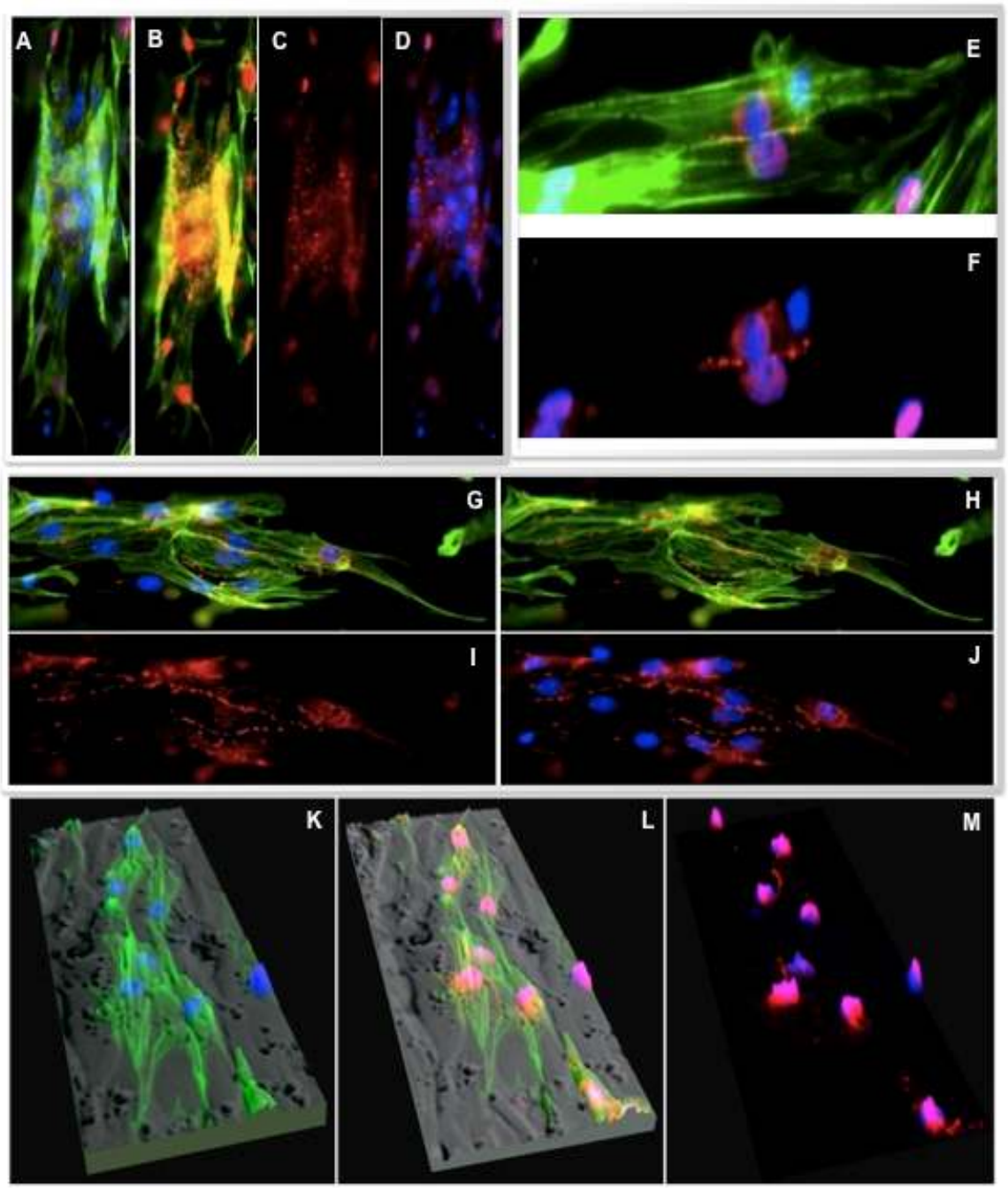
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Figure 6.5: Diverse anisotropic cell constructs. Fluorescent Micrographs of FCM aligned on PDMS substrates generated from crazed PE sheets showing diversity of generated cell constructs; **A-D)** 3D cell strips expressing connexing-43: **A**: overlay image of actin (green), connexin-43(red) and DAPI (blue); **B**: actin/connexin-43; **C:** connexin-43 only; **D:** connexin-43 and DAPI. **E-F)** A pair of FCF anisotropicaly aligned showing expression of connexin-43 at the cell-cell contact areas: **E)** Overlay image of actin, connexin-43, and DAPI; **F**: connexin-3 and DAPI only; **G-J)** 2D small FCF strip showing expression of connexin-43 at cell-cell contact areas. K-M) 3D plots from immunofluorescent micrographs showing a FCF strip and connexin-43 expression on PDMS substrate.

CHAPTER 7

CONCLUSSIONS:

Here I presented a study investigating different aspects important in cardiac tissue engineering, as well as in cardiac biomedical research applicable to pharmaceutical applications and developmental studies. In this report I included descriptions for: 1) Methods for engineering diverse topographical features for the fabrication of cell culture platforms that facilitate the alignment of neonatal, fetal, and cardiomyocytes derived from human ESC; 2) An extended study to enrich for FCF providing elements to characterize important morphological qualities, cellular behavior and responses to different stimuli, and generation ECM of these particular cell subpopulation; and 3) Provided a study showing the effect of FCF and its derivatives on murine ESC to induce cardiomyogenesis. I successfully engineered a substrate containing multi-scale topography for the alignment of neonatal and CM derived from hESC(described in chapter 3). This particular method facilitated the generation of confluent anisotropicaly aligned neonatal CM that showed improved cell-cell connections, detected by the expression patterns of connexin-43 and N-cadherin that resemble the expression patterns of native tissues at the corresponding developmental stage. Additionally, the same platform was used to align FCM; which showed similar responses as the neonatal CM. In the other study that describes the alignment of FCF(included on chapter 6), I showed that

aligned FCM were able to conduct Ca^{2+} influx waves suggested by my simple Ca^{2+} stain. In this study I presented an improvement to my previous fabrication method described in chapter 3; which facilitated the fabrication of more complex topographical features that can potentially be used for the generation of anisotropic cell constructs such as 2D or 3D cell strips or even used to align individual cardiac cells. The significance of my fabrication methods relies on the diversity of cell constructs that can be achieved as well as the cost and time efficiency, accessibility to most bioresearch labs and to the designs simplicity and functionality. From an engineering perspective I have develop a method that is capable of producing comparable results to commonly used methods that usually require high cost, expensive equipment, and that require extensive operational training. Furthermore, some of my topographical feature morphology biomimics complex topographical morphology found in native tissues such as the surface topography of the found in the heart. By using my topography, I was able to align cells from different sources such as fetal, neonatal and even CM-like cells derived from hESC.

In this report, I also provided evidence to support the idea concerning the diverse functionality of the CF in the heart, as well as demonstrated the phenotypic diversity of FCF in the heart. First I demonstrated that neonatal CF does not completely align as the CM, but rather these cells were observed to fill out the spaces found in between CM. Also I reported that FC respond to topographical features differently than CM by reorganizing the internal cytoarchitecture without necessary exhibiting a regular elongated external morphology as shown by CM (shown in chapter 3). Concerning the characterization of these cells, I demonstrated that FCF were highly enriched after a 2 cycle of SAT and that these cells have a tendency to differentiate quickly into

myofibroblasts (shown in Chapter 5). I provided an extended characterization study to identify the purity of CF in my cultures in order to avoid the presence of other contaminant cells such as smooth muscle cells or endothelial cells. Based on my characterization study, I demonstrated the presence of about 98% FCF based on the expression of collagens type I and III, fibronectin, periostin, DDR-2, and Thy-1.2(CD90.2); and the rest of the cells corresponded to apoptotic CM detected based on the expression of cTn-I. I also demonstrated that FCF subpopulations were composed of a heterogeneous subpopulation of cells that uniformly expressed collagens type I and III, DDR-2, fibronectin and periostin but with a differential expression of CD90.2. Also these cells exhibited different morphological characteristics such as size and expression levels of CD90.2. Additionally, I found a cell subpopulation that expressed high levels of HSP27 within my FCF cultures. These fibrocyte-like cells are morphologically different to CF or myofibroblasts and were found to establish strong connections with both fibroblasts and myofibroblasts, but its actual role needs to be further studied.

I also provided a method to decellularize FCF from cell tissue culture dishes using SDS and demonstrated that after removal of the cellular materials the ECM deposited by FCF remained for the most part intact. Additionally, I showed that the ECM left on dishes was composed of a complex mixture of proteins including Collagens type I and III, fibronectin, laminin, and periostin. I provided evidence concerning the process of fibroblasts-myofibroblast transition; which I identified using marked morphological treats such as the cell size differences as well as the increased expression of stress fibers and expression levels of α-SMA that are only observed in myofibroblasts but not fibroblasts. In particular, I used these differences as standards to detect the presence of

myofibroblast in my cultures. I also found that the differentiation process of CF undergoing into myofibroblasts can be slowed down by culturing enriched FCF in media containing LIF.

Lastly I demonstrated cardiomyogenesis was notably enhanced on mESC in direct or indirect co-culture with enriched FCF. For these studies, I proved that early and late cardiomyogenic markers; GATA-4 and cTn-I respectively; were expressed in higher proportions in mESC culture under the presence of FCF. I also proved that mESC co-cultured with FCF showed healthier morphology and better engraftment behavior to tissue culture plate's surface when compared to controls lacking FCF.

Over all, I presented a study that spans engineering applications as well as important aspects of cellular biology research that were strategically integrated in order to gain cardiac tissue engineering significance. In particular, I engineered a substrate containing topographical features for the generation of diverse cellular constructs that can to be potentially used to study mechanotrasnduction responses of CM or other cells types. For instance, my method for creating cardiac cellular constructs can be used for pharmaceutical studies such drug testing or to study cytotoxicity effects in *in vitro* culture conditions. Also, I provided evidence of the involvement of CF during early cardiogenic events as they enhanced cardiomyogenesis on our studies performed on mESC as well as improved the morphology of the generated CM. Another significant contribution of this work relies on my studies performed on FCF, in particular the characterization studies and the morphological traits reported to be involved on the CF-myofibroblasts process; which provides insights to elucidate the mechanisms involved in fibrogenesis as a major cause leading to cardiovascular disease. Hence, the work reported here would

significantly contribute to the generation of cardiac organoids exhibiting confluent cell-cell connectivity and anisotropic patterning that biomimics the morphological characteristics found in the native cardiac tissue. Furthermore, I presented a method for the alignment of single cells or small groups of cells that is favorable to perform studies using CM derived from ESC as the yields for generating these cells are for the most part low or limited. My method may also be used for studying developmental progression of CM maturation *in vitro* as cells start to reform the cardiac tissue to some extent. And importantly, we have created a simple method for the fabrication of diverse topographical features that can be integrated into cell culture platforms or bioreactors; I reported a dicelullarization and ECM generation method that may additionally be incorporated into cell culture platforms as coating material; investigated the cardiomyogenic enhancing effect of FCF that may be used to improved commonly used CM differentiation methods, and provided an method to generate highly enriched FCF cultures which phenotype can be additionally maintained from differentiating into myofibroblasts. All together, this pieces of work represent biomimetic components aiming to improve *in vitro* cardiac tissue culture systems: as building blocks to reshape the failing hearts.

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