Engineering 3D scaffolds with iPSCs towards regeneration of cardiac tissues

Krishnamoorthi, Muthu Kumar

2019

Krishnamoorthi, M. K. (2019). Engineering 3D scaffolds with iPSCs towards regeneration of cardiac tissues. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/106675>

<https://doi.org/10.32657/10220/48933>

ENGINEERING 3D SCAFFOLDS WITH iPSCs TOWARDS REGENERATION OF CARDIAC TISSUES

KRISHNAMOORTHY MUTHU KUMAR

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2018

ENGINEERING 3D SCAFFOLDS WITH iPSCs TOWARDS REGENERATION OF CARDIAC TISSUES

KRISHNAMOORTHY MUTHU KUMAR

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of Doctor of
Philosophy

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

22 March 2019

.....

Date

.....

Krishnamoorthi Muthu Kumar

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

22 March 2019

.....

Date

.....

Assoc. Prof. Tan Lay Poh

Abstract

This work focuses on engineering 3 dimensional (3D) fibrous hybrid scaffolds with human induced pluripotent stem cells (hiPSC) for cardiac tissue engineering (CTE). Natural materials are highly bioactive, yet they are limited by their high batch-to-batch variability, and their poorly understood bioactivity mechanisms, particularly, in affecting stem cell fate. Conversely, commonly used synthetic materials, while offering good control over different parameters, generally lack the suitable bioactivity for cellular interactions. In this work, we propose comparing and combining natural and synthetic scaffolds to enjoy the advantages of both platforms while circumventing their inherent limitations. The resulting 3D scaffolds will improve understanding of the cell material interactions and might produce a potential treatment for cardiac regeneration.

One ideal group of natural biomaterials can be obtained by decellularization, yielding tissue specific bioactive, and cell supportive scaffolds of either solid, or liquid forms. Our lab has isolated a porcine cardiac extra cellular matrix (pcECM), in both solid and liquid forms, which preserves the 3D architecture of the heart ECM, while maintaining the bioactivity for cellular interaction. Nevertheless, the exact contribution of such pcECM to stem cell and tissue specific lineage commitment, and the possible mechanisms governing such bioactivity remain largely unknown. The complexity of the pcECM composition and 3D architecture hinder our ability to discriminate between different effectors and their resulting individual or combined effects. Hence, it is necessary to generate synthetic 3D biomimetic scaffolds with controllable architecture and bioactivity profiles that would enable the study of various components in a modular way.

Electrospinning is an accessible and inexpensive means to fabricate fibrous matrices but the fundamental limitation with traditional electrospinning is that the scaffold produced is usually two dimensional (2D) dense mats rather than 3D porous structures. Here, we improvised the liquid-collector of electrospinning to fabricate 3D fibrous scaffolds with high porosity. Though this ECM mimicking synthetic polymer scaffolds offer high reproducibility, they generally lack the bioactivity inherent to natural ECM biomaterials. Thus, different approaches have been used to confer bioactivity to synthetic materials, such as natural materials (e.g., short bioactive peptides) either on the surface or within the bulk,

pre-culturing the scaffold with ECM producing cells (e.g., fibroblasts, and mesenchymal stem cells), and physical and/or chemical surface modifications.

Our work aims to obtain 3D composite scaffolds (3DCS) with ECM mimicking synthetic ultra-structures, and tissue specific biochemical cues by fabricating 3D electrospun polymeric scaffolds and functionalizing them with liquidized pcECM. The 3DCS produced were studied in comparison to the natural pcECM highlighting the roles of architecture, biochemical composition, and various combinations thereof, in affecting the function and fate of seeded human multi and pluri-potent stem cells. hiPSCs were used in this study as an ideal model cell with clinical relevancy, given their possible autologous sourcing, and their ability to differentiate into all cardiac cell types, in particular, beating cardiomyocytes (hiPSC-CM). We hypothesized that, bioactive 3D scaffolds (pcECM and/or 3DCS) that maintain a balance and cooperation between architectural and biochemical signals, are needed to initialize differentiation of hiPSC towards cardiac lineages.

Our results show that the pcECM can be mimicked by wet electrospinning of poly lactide-co-glycolide (PLGA), and poly lactide-co- ϵ -caprolactone (PLCL). However, based on the evaluated properties, and reproducibility of the 3D synthetic scaffolds, only 3D PLGA exhibited adequate profile and was therefore used for further studies. After modification with pcECM gel, the 3DCS displayed similarities with pcECM in terms of morphology, chemistry, biochemical composition. The 3DCS also displayed cardiac relevant mechanical properties and did not elicit any immunogenicity *in vitro*. 3DCS also displayed the ability for cellular attachment and growth under static conditions when human mesenchymal stem cells (hMSCs) were used as model cells. hiPSC-CM seeded 3DCS maintained CM viability, beating functionality and phenotypic identity for two weeks as evaluated by protein expression. Moreover, the scaffolds' microenvironment supported the calcium handling ability. Finally, hiPSCs seeded on these scaffolds, differentiated into cardiac lineage cells spontaneously without the addition of any external factors or molecules, asserting the role and importance of a tissue specific biochemical microenvironment for cardiac applications. Taken together, our results here contribute to the understanding of how the biology and architecture of the pcECM can affect and determine the fate of the seeded hiPSCs. This knowledge is relevant not only for basic research but also for possible CTE applications.

Lay Summary

Imagine getting a bruise on your hand or leg. The injury on the skin will be initially painful and swollen, but with time the pain reduces, the skin at the injury site acquires a scar and reverts to its original state and perform its normal function. Heart attack is an injury that happens in the heart and is caused by blocks in blood vessels that supply the heart. During a heart attack, millions of heart muscle cells die, causing an irreversible damage and a significant scar. But unlike the skin, which even after scarring performs its function without any significant loss in efficiency, the heart's function will keep decreasing if left untreated after an injury. This is because the heart lacks the potential to heal itself over time or 'regenerate'.

Currently, doctors use drugs, surgeries etc. as treatments for heart attack but these treatments do not make the patient's heart regenerate, as the treatments only keep the injury from declining further. When the situation deteriorates, the doctors recommend heart transplantation. However, it is difficult to obtain a working heart due to shortage of heart donors. Also, the wait for a donor heart can take probably months to years, which most of the times a patient cannot really afford. Hence, exploring methods to grow artificial heart or heart tissue in labs becomes an interesting prospect, which can address the problem of treating heart attack in the future.

In the 1980s, scientists discovered that they can use either natural or synthetic materials to make sheet like structures called 'scaffolds' to grow skin cells and engineer skin tissue to treat burn victims. They also successfully implanted this lab-grown tissue in humans and observed good recovery. Thus, the field of 'Tissue engineering' was born. Therefore, to build a functional tissue, two important components are required. Materials to make scaffolds that will act as the supporting skeleton of the target tissue and cells that will provide appropriate function of the target tissue.

Different natural and synthetic material choices are available for producing the scaffolds. Yet a suitable scaffold should imitate the characteristics of the target tissue, like structural design and cell function promotive properties. Both natural and synthetic materials have their own advantages and limitations. But their combination results in a product which will have the advantages of both the platforms and avoid their fundamental limitations. The

type of cells to populate the scaffolds to obtain suitable function is very critical. From heart's functional perspective, the cells to be used should be able to contract in/with the scaffold. These beating heart cells can only be obtained from 'pluripotent stem cells' (PSC), the kind of cells that can modify into any cells in our body. These cells are highly suited for this application as they can be obtained from a patient's own cells, thus eliminating the chances of immune rejection after implantation.

In this research, a 'heart construct' was developed using different cell-material combinations. The scaffold was fabricated using a natural material derived from a pig's heart combined with synthetic polymeric materials. The scaffolds were then populated with PSC and PSC modified into heart cells, resulting in a heart construct. The fabricated scaffold was observed to possess structural and biological properties suitable for heart tissue development. The scaffold, when populated with pluripotent stem cells, modified the seeded cells into heart like cells without the addition of any external factors to induce this modification. This is due to the interdependent effects of the scaffolds' heart like structural and biological characteristics. This shows that the properties of the developed scaffold were suitable for the cells to feel 'home' like in a heart environment and modify themselves into a heart like cell.

Alternatively, when the scaffolds were seeded with functional heart cells obtained from the pluripotent stem cells, the heart construct was tested for various parameters to check if the cells are communicating with the scaffold sufficiently to imitate the heart tissue. During the testing, it was observed that the heart construct displayed properties comparable to a heart environment. The construct, not only kept alive the heart cells, but also maintained the beating function of the cells.

Overall, the use of pluripotent stem cells and the option of studying their communication with materials, will facilitate the understanding of the complex heart development process. The research also opens further possibilities of growing personalized heart tissue, custom made for each patient for the treatment of heart attack, a crucial step in heart tissue engineering and regeneration.

Acknowledgements

This dissertation would not have been made possible without the support, guidance and encouragement of the following people and institutions.

I gratefully acknowledge the following funding sources that have made this PhD possible - NTU Research scholarship, NRF Funding for START program, Israeli Science Foundation and NTU MSE

I would like to express my sincere gratitude and acknowledgement to Assoc. Prof. Tan Lay Poh and Prof. Marcelle Machluf for their constant supervision, guidance, wisdom, valuable advises throughout my PhD, without which this thesis would not have been possible.

I would like to thank Prof. Subbu Venkatraman for his support and guidance to the START program.

I cannot thank Dr. Udi Sarig enough for being a great mentor and friend throughout my PhD journey. His constant encouragement and belief in my abilities have helped me mold myself as a better researcher and person.

I would like to express my sincere appreciation to Asst Prof. Dalton and Asst Prof. Wang Xiaomeng for being my thesis advisory members and sharing their scientific expertise.

I would like to thank the following NTU MSE Staff members for their help and support during my PhD - Zi Li, Patrick, Nelson, Alan, Derrik, Mastura, Victor, Fiona, Jaqueline, Siew Ling, Serika, Serena, Shazana.

I would like to thank Dr. Steve Oh, Dr. Shaul Reuveny, and Mr. Sherwin Ting, Bioprocessing Technology Institute (BTI), A-STAR, Singapore for sharing their expertise.

My PhD journey would not have been as enjoyable if not for the following friends and colleagues.

START lab friends Dr. Hadar, Dr. Priya, Dr. Susan, Dr. Scott, Jean Marc, Ramya, Dr. Evelyne, Dr. Wang Yao, Dr. Gigi, Dr. Hui Ying, Elio, Su Yin, Animesh, Kumar, Sakthi, Dr. Gopal, Dr. Maedeh, Dr. Rufaihah, Suganya, Dr. Ling Ling, Ting Xiao, Sandy and Chen Nuan.

Prof. Tan Lay Poh's lab friends Dr. Lee, Dr. Ivan, Dr. Mintu, Dr. Kirthana, Dr. Ajay, Dr. Hui Zhi, Dr. Monir, Xue Feng, Feng Lin, Amit, Prativa, Miao, Paulina, and Pen Ling.

I would like to extend my special thanks and gratitude to my friends (The Machlufia) Dr. Limor, Dr. Tomer, Inna, Dr. Yael, Noa, Tom, Tzila, Natali, Nitzan, Lior, Jacopo, Mimmo, Stasia, Sharbel and Maskit, at Prof. Marcelle's lab, who made my trip to Israel and Technion a pleasant, fruitful and enjoyable learning experience.

I also would like to thank the following faculty and their lab members from Technion for their help and support towards my PhD journey - Prof. Ester Segal, Prof. Ester Meron Holtz, Prof. Ayelet Fishman, Prof. Amram Mor, Prof. Boaz Mizrahi and Dr Nitsan Dahan.

I would also like to extend my sincere appreciation to the following Technion staff - Anat, Gabriela, Rinat, Yael, for helping me with all the bureaucracy related to my Technion trip.

Finally, I would like to take this opportunity to express my gratitude to all my previous Teachers for educating me, my Mother, Father, Wife and Son for their endless support and understanding in letting me pursue my dreams and my Friends, for always being there to provide fun and care. Thank you everyone.

Table of Contents

Abstract.....	i
Lay Summary	iii
Acknowledgements	v
Table Captions	xii
Figure Captions.....	xiii
Abbreviations	xvii
Chapter 1 Introduction	1
1.1 Background	2
1.2 Hypothesis	2
1.3 Objectives	3
1.4 Scope	4
1.5 Dissertation Overview	5
1.6 Findings and Outcomes/Originality	6
References.....	6
Chapter 2 Literature Review	9
2.1 Heart Anatomy and the Myocardium	10
2.2 Cardiovascular Diseases & Current Treatments	11
2.3 Cardiac Tissue Engineering (CTE) and Scaffold Properties	12
2.4 Natural & Synthetic Scaffold Biomaterials	14
2.5 Generating 3D Bioactive Scaffolds for CTE.....	15
2.6 Cells in Cardiac Regeneration	18
2.7 Current Strategies and Challenges in CTE.....	20
2.8 Support of PSC and PSC Derived Cells by Biomaterials	23

2.9	Effect of Cardiac ECM Based Scaffolds on Cells.....	24
2.10	Conferring Bioactivity to Synthetic Scaffolds in CTE.....	25
2.11	Effect of Architecture vs. Bioactivity on Cell Behavior and Fate.....	26
	References.....	27
Chapter 3	Experimental Methodology.....	45
3.1	Overall Experimental Design	46
3.2	General Materials and Kits.....	47
3.2.1	Antibodies	48
3.2.2	Enzymes	49
3.2.3	Cell Culture Reagents and Media	49
3.2.4	Cells, Culture Medium and Maintenance.....	50
3.2.5	Buffers and Solutions	51
3.2.6	Software	52
3.3	Decellularization and Liquid pcECM Preparation	52
3.4	Electrospinning.....	54
3.5	Morphological Analysis of the Scaffolds by Scanning Electron Microscopy	55
3.6	Scaffold Chemistry Analysis using Fourier-Transform Infrared Spectroscopy ..	56
3.7	Scaffold Mechanical Characterization using Uniaxial Testing	57
3.8	Scaffold Biochemical Composition using LC-MS/MS.....	59
3.9	Cell Characterization using Flow Cytometry	60
3.10	Immunofluorescence and Microscopy	61
3.11	Cell Viability using Alamar Blue Assay	63
3.12	Statistical Analysis	64
	References.....	64
Chapter 4	Fabrication and characterization of a 3D composite scaffold	67

4.1	Experimental Methods	68
4.1.1	<i>In vitro</i> Immunogenicity Study	68
4.1.2	Scaffold Disinfection	68
4.1.3	Cell Culture on Scaffolds	68
4.2	Results & Discussion.....	69
4.2.1	pcECM Characterization to Identify Parameters to Mimic in 3DCS.....	69
4.2.2	Wet Electrospinning of PLCL and PLGA – Optimization and Characterization	71
4.2.3	3D Synthetic Scaffolds’ Chemistry and Mechanical Characterization..	77
4.2.4	Optimization of EDC-NHS Procedure for 3DCS Fabrication	80
4.2.5	3DCS Exhibits Comparable Characteristics as the pcECM.....	84
4.2.6	3DCS is non-immunogenic <i>In vitro</i>	92
4.2.7	3DCS Supports Attachment and Proliferation of hMSC	93
4.3	Conclusion.....	98
	References.....	98
Chapter 5	Cardiac regeneration suitability of the 3DCS	103
5.1	Experimental Methods	104
5.1.1	hiPSC-CM Culture on the Scaffolds	104
5.1.2	Ca ²⁺ Signaling study using Fluo-4 AM.....	104
5.2	Results and Discussion.....	105
5.2.1	FACS Study for Cardiac Marker Expression of hiPSC-CM.....	105
5.2.2	3DCS Maintains the Viability and Functionality of Seeded hiPSC-CM	106
5.2.3	Cardiac Protein Expression of Seeded hiPSC-CM on the Scaffold.....	107
5.2.4	3DCS Exhibits Better Calcium Handling Abilities.....	114
5.3	Conclusion.....	117

References.....	118
Chapter 6 Influence of 3DCS bioactivity on pluripotent stem cells	121
6.1 Materials & Methods.....	122
6.1.1 hiPSC Attachment to the Scaffolds.....	122
6.1.2 hiPSC Culture on Scaffolds	122
6.2 Results & Discussion.....	123
6.2.1 FACS Study of Pluripotency Markers by hiPSCs.....	123
6.2.2 hiPSCs Attachment to the Scaffolds	124
6.2.3 3DCS Supports Long Term Viability and Proliferation of hiPSCs	127
6.2.4 3DCS Induces Spontaneous Differentiation of hiPSCs Towards Cardiac Lineage.....	129
6.2.5 Conditioned Medium from 3DCS-hiPSC Construct Induces Cardiac Differentiation of hiPSCs Seeded on Matrigel™.....	134
6.3 Conclusion.....	141
References.....	141
Chapter 7 Conclusion and Future Recommendations	143
7.1 Summary	144
7.2 Future recommendations	147
7.3 Conclusion.....	148
References.....	149
Chapter 8 Appendix.....	153
8.1 Supplementary Results	153
8.1.1 Analysis of hiPSC-CMs Beating on Scaffolds.....	153
8.2 Preliminary Reconnaissance Studies.....	155
8.2.1 hMSC Attachment Time and Cell Density Study	155
8.2.2 3D Scaffold and hMSC Interaction.....	156

8.2.3	hiPSC Culture on Microcarriers	157
8.2.4	hiPSC Attachment and Interaction with pcECM	158
8.2.5	hMSC Proliferation on the Scaffolds	160

Table Captions

Table 2-1 Advantages and limitations of natural and synthetic materials.....	15
Table 4-1 Poly (L-Lactide-co- ϵ -caprolactone) (70:30) electrospinning conditions.	71
Table 4-2 Poly (L-Lactide-co-glycolide) (80:20) electrospinning conditions.....	75
Table 4-3 Morphology of 3D composite scaffold's exterior and cross section at different conditions during the EDC-NHS chemistry-based modification.	83
Table 4-4 Optimized concentrations of solutions and conditions used for the fabrication of 3DCS.	84
Table 4-5 Protein Composition of pcECM gel were preserved in the 3DCS as detected using LC MS-MS.	89

Figure Captions

Figure 1-1: Scheme of aims and research questions.....	4
Figure 2-1: Anatomy of the heart left ventricular wall.....	10
Figure 2-2 Tissue engineering triad [31]	13
Figure 2-3: Influence of various factors on stem cell fate	27
Figure 3-1 Overall experimental design	47
Figure 3-2 Decellularization and liquid ECM preparation reaction scheme [3, 4]	53
Figure 3-3 Electrospinning setup A) Traditional & B) Wet electrospinning	54
Figure 3-4 Scanning electron microscopy working schematic [5]	56
Figure 3-5 FTIR working schematic [7].....	57
Figure 3-6 Uniaxial testing machine working schematic (From warwick.ac.uk)	58
Figure 3-7 LC-MS based proteomics working schematic [8]	59
Figure 3-8 FACS working schematic	60
Figure 3-9 Schematics of Immunofluorescence (A) [10] and Confocal microscopy (B) .	62
Figure 3-10 Alamar Blue TM working principle	64
Figure 4-1 Decellularization process, macroscopic appearance and characterization of architecture of decellularized pcECM.	70
Figure 4-2 SEM images of PLCL Fibers displaying defective and dense morphology.	73
Figure 4-3 SEM images of PLCL fibers obtained from a polymer solution with high DMF content showing good morphology.	73
Figure 4-4 SEM images of PLCL Fibers and the macroscopic appearance of the scaffold with dimensions.....	74
Figure 4-5 SEM images of PLGA Fibers and the macroscopic appearance pf the scaffold with dimensions.....	75
Figure 4-6 Comparison of fiber diameter distribution of 3D PLCL and PLGA scaffolds with the pcECM myocardium.....	76
Figure 4-7 FTIR spectra of PLCL (A) and PLGA (B).	78
Figure 4-8 Mechanical property of 3D electrospun scaffolds in comparison to pcECM..	79
Figure 4-9 Reproducibility of the PLGA scaffolds when compared to a batch of 3D PLCL scaffolds.....	80

Figure 4-10 Modified protocol for surface activation and functionalization of 3D electrospun PLGA using EDC-NHS chemistry.....	81
Figure 4-11 Surface morphology of PLGA scaffold does not change in the initial modification steps of the EDC-NHS modification protocol.	82
Figure 4-12 3DCS scaffold cross sectional tile exhibiting both fibrous and inter-fibrillary pcECM-like morphology on the surface and bulk of the scaffold.	86
Figure 4-13 3DCS cross sectional morphology exhibiting characteristic morphology of both electrospun and pcECM scaffolds.	87
Figure 4-14 3DCS fiber diameter and pore size as compared to the pcECM.	87
Figure 4-15 3DCS surface characterization exhibiting characteristic chemistry of synthetic PLGA polymer and natural pcECM.	88
Figure 4-16 Immunofluorescence of 3DCS exhibiting staining for Collagen I, a key component of the cardiac ECM.	91
Figure 4-17 Mechanical properties of 3DCS compared to pcECM.	92
Figure 4-18 <i>In vitro</i> immunogenicity of the scaffolds evaluated using Griess Reagent. ..	93
Figure 4-19 hMSCs Characterization using FACS.	94
Figure 4-20 hMSCs viability on the scaffolds as measured using AlamarBlue™.....	96
Figure 4-21 Phalloidin staining of hMSCs seeded on the scaffolds.....	97
Figure 5-1 hiPSC-CMs Characterization using FACS.....	105
Figure 5-2 hiPSC-CM viability on the scaffolds as measured using AlamarBlue™.....	107
Figure 5-3 Immunofluorescence of hiPSC-CMs seeded scaffolds expressing Cardiac Troponin I.	109
Figure 5-4 Isotype control for Cardiac Troponin I staining of hiPSC-CMs seeded scaffolds.	110
Figure 5-5 Immunofluorescence of hiPSC-CMs seeded UPLGA expressing Sarcomeric Alpha Actinin.	111
Figure 5-6 Immunofluorescence of hiPSC-CMs seeded 3DCS expressing Sarcomeric Alpha Actinin.	112
Figure 5-7 Immunofluorescence of hiPSC-CMs seeded pcECM expressing Sarcomeric Alpha Actinin.	113

Figure 5-8 Isotype control for Sarcomeric Alpha Actinin staining of hiPSC-CMs seeded scaffolds.....	114
Figure 5-9 Calcium handling ability of the hiPSC-CMs seeded on the scaffolds measured with Fluorescent calcium indicator, Fluo 4 - AM.	116
Figure 6-1 hiPSCs Characterization using FACS.....	124
Figure 6-2 hiPSCs attachment to the scaffolds as measured by AlamarBlue™ assay....	126
Figure 6-3 hiPSCs attachment to the scaffolds as evaluated by Hoechst staining of the cell seeded scaffolds.....	127
Figure 6-4 Long term viability and proliferation of hiPSCs on the scaffolds.	129
Figure 6-5 Immunofluorescence of hiPSCs seeded scaffolds expressing Cardiac Troponin I.....	131
Figure 6-6 Isotype control for Cardiac Troponin I staining of hiPSCs seeded scaffolds.....	132
Figure 6-7 Immunofluorescence of hiPSCs seeded scaffolds expressing Sarcomeric Alpha Actinin (SAA).....	133
Figure 6-8 Isotype control for Sarcomeric Alpha Actinin staining of hiPSCs seeded scaffolds.....	134
Figure 6-9 Immunofluorescence of hiPSCs seeded scaffolds expressing Cardiac Troponin I following addition of conditioned medium.....	137
Figure 6-10 Isotype control for Cardiac Troponin I staining of hiPSCs seeded scaffolds cultured with conditioned medium.	138
Figure 6-11 Immunofluorescence of hiPSCs seeded scaffolds expressing Sarcomeric Alpha Actinin following addition of conditioned medium.	139
Figure 6-12 Isotype control for Sarcomeric Alpha Actinin staining of hiPSCs seeded scaffolds cultured with conditioned medium.....	140
Figure 8-1 Analysis of hiPSC-CMs beating on scaffolds.....	154
Figure 8-2 3D PLGA and hMSC-RFP interaction.	155
Figure 8-3 hMSC proliferation on 3D electrospun PLGA.	156
Figure 8-4 Histological section of 3D PLGA scaffolds exhibiting interaction with hMSC-RFP.	157
Figure 8-5 Immunohistochemistry of hiPSC on microcarrier.....	158
Figure 8-6 hiPSC attachment to pcECM over different attachment time.....	159

Figure 8-7 hMSCs viability on the scaffolds as measured using AlamarBlue™.....	160
--	-----

Abbreviations

% v/v	Volumetric percentage
% w/v	Weight to Volume percentage
% w/w	Weight percentage
2D	Two dimensional
3D	Three dimensional
3DCS	3D Composite Scaffold
α MEM	Minimal Essential Medium alfa version
ANOVA	Analysis of Variance
bFGF	Basic Fibroblast Growth Factor
bmMNC	Bone marrow mono nuclear cells
bmMSC	Bone Marrow derived Mesenchymal Stem Cells
BSA	Bovine Serum Albumin
CID	Collision Induced Dissociation
cm	Centimetre
CO ₂	Carbon dioxide
CF	Cardiac Fibroblasts
CM	Cardiomyocytes
CSC	Cardiac Stem Cells
CTE	Cardiac Tissue Engineering
cTnI	Cardiac Troponin I
CVD	Cardiovascular Diseases
dl	Decilitre
DAPI	4'6- diamodino-2-phenylindole dihydrochloride Hydrate
DDW	Double Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
e.g.,	Exempli gratia
EC	Endothelial Cell
ECM	Extra Cellular Matrix

EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
NHS	N-Hydroxysuccinimide
EDTA	Ethylenediamine Tetra Acetic Acid
EGF	Epidermal Growth Factor
ESC	Embryonic Stem Cells
<i>Et. al.,</i>	Et alia
etc.	Etcetera
ETOH	Ethanol
FACS	Fluorescence Activated Cell Sorting
FB	Fibroblasts
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FTIR	Fourier Transform Infrared Spectroscopy
g	Gram
GF	Growth Factors
GAG	Glycosaminoglycan
HA	Hyaluronic Acid
HCl	Hydrochloric acid
hMSC	Human Mesenchymal Stem Cells
hr	Hour
i.e.,	Id est
hiPSC	Human Induced Pluripotent Stem Cells
kPa	Kilo Pascal
kV	Kilo Volt
L	Litre
LC-MS/MS	Liquid Chromatography coupled with Tandem Mass spectroscopy
LPS	Lipopolysaccharide
LVEF	Left ventricle ejection fraction
m	Meter
mm	Millimetre
µm	Micrometre

μL	Microlitre
mL	Millilitre
MI	Myocardial Infarction
min	Minute
mg	Milligram
MPa	Mega Pascal
MS	Mass Spectroscopy
MSC	Mesenchymal Stem Cells
N	Newton
ng	Nanogram
NO	Nitric Oxide
OCT	Optimal cutting Temperature compound
P/S	Penicillin/Streptomycin solution
PBS	Phosphate Buffered Saline
pcECM	Porcine Cardiac Extra-Cellular Matrix
PCL	Poly(ε-caprolactone)
PFA	Paraformaldehyde
PGA	Poly (glycolic acid)
PGS	Poly(glycerol-sebacate)
PLA	Poly (lactic acid)
PLCL	poly(lactide-co-ε-caprolactone)
PLGA	Poly (lactic co-glycolic acid)
PU	Polyurethane
RT	Room Temperature
s	second
SAA	Sarcomeric alpha actinin
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SMC	Smooth muscle cells
UPLGA	Unmodified PLGA

VAD	Ventricular assist devices
-----	----------------------------

Chapter 1

Introduction

This chapter presents a brief background and rationale for the design of this thesis. The current materials used in cardiac tissue engineering are discussed and outlined. Once the limitations of the outlined materials are elucidated, the hypothesis for this thesis is established. This is followed by enumerating the list of objectives used to evaluate the hypothesis of this thesis. The chapter then progresses to provide an overview of the thesis on a chapter by chapter basis and summarizing the content of each chapter. This chapter finally concludes by highlighting the novelties of this research.

1.1 Background

Decellularized scaffolds, like pcECM are used extensively for tissue engineering applications [1-6]. Even though pcECM provides preserved 3D architecture and excellent bioactivity for cellular interaction, the exact contribution of the architecture or the tissue specific bioactivity over stem cell lineage commitment remain largely unknown [7]. Another limitation of the pcECM is the batch-to-batch variability.

On the other hand, synthetic scaffolds offer tailorability such as mechanical properties, porosity, biodegradability and chemical properties. Additionally, they are also highly reproducible. The drawback of synthetic scaffold is the lack of ability to simulate the biochemistry of the natural ECM. Hence, in order to complement and compensate the inherent limitations of the natural and synthetic platforms, it is necessary to generate synthetic 3DCS with reproducible and tunable architecture and bioactivity. This will be achieved by modifying the synthetic reproducible electrospun platform with the liquid form of the bioactive pcECM.

The interaction study of pcECM and 3DCS with hiPSCs, hiPSC – CM and their comparison, we believe, will facilitate the understanding of various effectors in the cardiac ECM (pcECM). Moreover, our results may improve the knowledge of how the biology and architecture of the pcECM can affect and determine the fate of the seeded hiPSCs. The findings of the proposed study are relevant not only for basic research but also for possible CTE applications.

1.2 Hypothesis

Bioactive 3D scaffolds that maintain a balance and cooperation between architectural and biochemical signals are needed to initialize differentiation of induced pluripotent stem cells (iPSC) towards cardiac lineage.

1.3 Objectives

The overall goal of this thesis is to engineer a 3D construct based on acellular pcECM and/or 3DCS for CTE. These pcECM based 3DCS will be evaluated in terms of their efficacy and functional benefits towards improving the biological understanding of the bio-active cardiac elements in natural pcECM.

To achieve this goal the following three aims have been identified (Figure 1-1) and will be pursued.

1. To fabricate a 3D composite scaffold (3DCS) which can behave as an equivalent to the pcECM; characterize its morphology, chemistry, biochemical composition, mechanical properties and evaluate its human cell support under static conditions.
2. To study the pcECM and 3DCS bioactivity in supporting and maintaining hiPSC-CM viability, and functionality.
3. To study the pcECM and 3DCS bioactivity in supporting hiPSC attachment, viability, proliferation and differentiation under static conditions.

Figure 1-1: Scheme of aims and research questions

1.4 Scope

In this work, a synthetic biodegradable polymer is used to fabricate a 3D scaffold via wet electrospinning, a modified form of traditional electrospinning, where the solid collector was replaced with a liquid bath. The prepared 3D synthetic scaffold was modified with the liquid cardiac ECM gel using traditional EDC-NHS chemistry, resulting in a 3DCS. This scaffold was characterized for its morphology, surface chemistry, biochemical composition and mechanical properties. The scaffold further evaluated for its *in vitro* immunogenicity and cellular compatibility using RAW macrophage cells and human mesenchymal stem cells respectively.

The cardiac application suitability of the scaffold was studied using hiPSC-CMs. The ability of the scaffold to maintain the viability and beating functionality of the cells was followed for a period of two weeks. Another critical component of cardiac functionality,

Ca²⁺ handling, was also studied using fluorescent marker Fluo-4 AM under normal and external electric stimuli conditions. Finally, the scaffolds were evaluated to assess if the tissue specific bioactivity incorporated in the form of liquid cardiac gel could influence the proliferation and differentiation of hiPSCs.

1.5 Dissertation Overview

The thesis addresses the issue of a tissue specific scaffold microenvironment and their influence on hiPSC and hiPSC-CM.

Chapter 1 provides a brief background and rationale to understand the perspective of the thesis. It further presents the hypothesis, outlines the objectives, scope and novelties of the thesis.

Chapter 2 provides a review of the literature concerning the heart diseases, ECM of the heart, and recapitulating an ECM environment by fabricating a scaffold. It then discusses the components and challenges involved in scaffold fabrication, the evaluation of the scaffold's interaction with different cell types, and the importance of employing a tissue specific microenvironment for CTE.

Chapter 3 discusses the list of materials and equipment used in the studies. The choice of equipment and their working principle are also explained.

Chapter 4,5 & 6 elaborates the results of the studies and discusses the results in correlation to each other and the literature. The first part of the results focusses on the fabrication and characterization of a 3D scaffold using wet electrospinning and modifying this 3D scaffold with liquid pcECM gel to form a 3DCS. This chapter also presents the similarities of the 3DCS to the natural pcECM in terms of architecture and biochemical composition. The second part of the results chapter elucidates about the cell material interaction studies done with hiPSC-CM. The viability and functionality on these cells in the scaffold environment is discussed here. The final part of the results chapter focusses on the scaffolds' interaction

with hiPSC to elicit the influence of the tissue specific bioactivity on the pluripotent stem cells.

Chapter 5 presents the conclusions drawn from the results of the thesis. Further, this section also discusses the results obtained in relation to the objectives stated at the beginning of the thesis. To conclude, the section will present recommendations and directions for future work that can be built upon the current work.

1.6 Findings and Outcomes/Originality

The research in this thesis has led to the following novel findings.

1. Ability to successfully fabricate a modular and tunable 3DCS encompassing a tissue specific ECM and displaying morphology and chemistry similar to its source tissue ECM (in this case pcECM). While the findings and discoveries made herein were focused on the cardiac ECM, similar technology can be easily tuned for other tissue types, and so in that respect the technology presented herein is but a platform demonstrating a proof of concept with implications that are far reaching theoretically to other soft tissue types as well.
2. 3DCS can support the attachment, survival, and proliferation of stem cells (hMSCs & hiPSCs)
3. 3DCS supports and maintains the functionality of differentiated CM (hiPSC-CMs) as compared to the unmodified PLGA, showcasing the importance and role of a bioactive tissue specific molecule in maintaining cell functionality
4. 3DCS induces spontaneous commitment of the seeded hiPSCs towards cardiac lineage, which shows the influence of the tissue specific bioactivity over hiPSC differentiation

References

[1] H.C. Ott, T.S. Matthiesen, S.-K. Goh, L.D. Black, S.M. Kren, T.I. Netoff, D.A. Taylor, Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart, *Nature medicine* 14(2) (2008) 213-221.

- [2] S.F. Badylak, Xenogeneic extracellular matrix as a scaffold for tissue reconstruction, *Transplant immunology* 12(3-4) (2004) 367-77.
- [3] Y. Eitan, U. Sarig, N. Dahan, M. Machluf, Acellular cardiac extracellular matrix as a scaffold for tissue engineering: in vitro cell support, remodeling, and biocompatibility, *Tissue engineering. Part C, Methods* 16(4) (2010) 671-83.
- [4] J.P. Guyette, J.M. Charest, R.W. Mills, B.J. Jank, P.T. Moser, S.E. Gilpin, J.R. Gershlak, T. Okamoto, G. Gonzalez, D.J. Milan, G.R. Gaudette, H.C. Ott, Bioengineering Human Myocardium on Native Extracellular Matrix, *Circ Res* 118(1) (2016) 56-72.
- [5] U. Sarig, G.C. Au-Yeung, Y. Wang, T. Bronshtein, N. Dahan, F.Y. Boey, S.S. Venkatraman, M. Machluf, Thick acellular heart extracellular matrix with inherent vasculature: a potential platform for myocardial tissue regeneration, *Tissue Eng Part A* 18(19-20) (2012) 2125-37.
- [6] U. Sarig, E.B. Nguyen, Y. Wang, S. Ting, T. Bronshtein, H. Sarig, N. Dahan, M. Gvirtz, S. Reuveny, S.K. Oh, T. Scheper, Y.C. Boey, S.S. Venkatraman, M. Machluf, Pushing the envelope in tissue engineering: ex vivo production of thick vascularized cardiac extracellular matrix constructs, *Tissue Eng Part A* 21(9-10) (2015) 1507-19.
- [7] B. Oberwallner, A. Brodarac, P. Anic, T. Saric, K. Wassilew, K. Neef, Y.H. Choi, C. Stamm, Human cardiac extracellular matrix supports myocardial lineage commitment of pluripotent stem cells, *Eur J Cardiothorac Surg* 47(3) (2015) 416-25; discussion 425.

Chapter 2

Literature Review

This chapter reviews the literature providing the relevant background on heart functioning, various diseases, treatment strategies and challenges and the importance of CTE. Further, this chapter then focuses on the interaction of pluripotent stem cells (PSCs) with biomaterials of both natural and synthetic origin, which includes reviewing the literature for i) PSC and PSC derived cells support by biomaterials, ii) cardiac ECM based materials' interaction in affecting stem cells, iii) incorporation of bioactivity to synthetic scaffolds and iv) identification of stem cell fate effectors. Although current literature presents research in these topics in assorted contexts, this review concentrates on bioactivity of materials and their role in cell fate

.

2.1 Heart Anatomy and the Myocardium

Human heart is a muscular pump located in the thoracic cavity. It comprises of four chambers—two atria (right and left) on top and two ventricles (right and left) in the bottom. The left ventricle pumps oxygenated blood to all part of the body, supplying oxygen and nutrition to all living cells [1]. The left ventricular wall (Figure 2-1) has a thickness of ~1-1.5 cm [2] and consists of three tissue layers – epicardium, myocardium and endocardium.

Figure 2-1: Anatomy of the heart left ventricular wall.

The heart muscle is known as myocardium [3]. The myocardium is a network of cells in an extracellular matrix (ECM). The cellular components of the myocardium include CM, cardiac fibroblasts, endothelial cells and vascular smooth muscle cells [4]. The cells that are responsible for the contraction and relaxation of the heart are known as the cardiac muscle cells or CM [5]. These CM comprise only 30-40 % of the total number of cells, but occupy around 75 % of the volume [6]. The next major cell type in the myocardium are the cardiac fibroblasts and their primary role in the myocardium is ECM turnover and remodeling, by degrading the existing ECM and producing new one [7]. These cells' interaction with the CM aid in the electrical and mechanical signal transport and coordination across the length of the myocardium [8]. Endothelial cells and vascular smooth muscle cells comprise the blood vessels, which are responsible for oxygen and nutrient supply [4].

The cardiac ECM is a complex network of structural and adhesive proteins, growth factors (GF), and proteoglycans (glycosaminoglycan (GAG) with core protein) offering structural and biochemical cues to the cells residing within the matrix [9]. The ECM serves as a dynamic platform for the cells to adhere, proliferate, and interact with, and can in turn be modified by resident cells that secrete and remodel the existing ECM according to their requirements [9]. The major structural protein—collagen—provides the mechanical and structural support for the cells. The abundant types of collagen in the cardiac ECM are collagen I and III [10] providing the necessary strength, and visco-elastic properties which can differ depending on the location within myocardium [11]. The adhesive proteins – laminin and fibronectin aids in cell adhesion and cell – cell interaction [9, 12]. The GAG network being very hydrophilic aids in hydration of the ECM and serves as reservoir for GFs [13]. The major GAG in the heart ECM is hyaluronan [9]. The intricate organization of the cardiac ECM plays an important role in maintaining normal cardiac function. The cross talk between the cells and the ECM in the myocardium is a constantly occurring dynamic process leading to secretion of different ECM components and remodeling of existing ECM during normal physiology as well as pathology [9].

2.2 Cardiovascular Diseases & Current Treatments

Cardiovascular disease (CVD) is a worldwide health issue. CVD comprises diseases of the heart and the blood vessels. Millions die annually due to CVD, killing 1 in every 3 persons in the United States alone [1-3]. CVD comprises diseases of the heart and the vascular system. A major CVD is myocardial infarction (MI). Pathologically, ‘myocardial infarction’ is defined as the myocardial cell death due to prolonged ischemia i.e., prolonged deprivation of oxygen’ [14]. MI is caused by unstable atherosclerosis, which results in cardiac muscle ischemia—decreased oxygen and nutrient supply to the heart muscles through the coronary arteries. MI leads to severe necrotic myocardial cell death resulting in deterioration of hemodynamics and overall cardiac function [4]. Because of the limited or no regenerative ability of the heart contracting CMs, the inflammatory reaction and the irreversible event of cardiomyocyte death results in the generation of a scar tissue in the heart wall [6, 15]. The scar tissue formation following CM death is the buildup of a stiff

collagen in the infarcted site impairing the contractility of the myocardium [16]. The scar region is inactive, less resilient and deteriorates in strength over time may eventually result in heart failure—an inability of the heart to pump enough blood to meet the body's metabolic demands [7, 17].

Several treatment modalities are available for the management of MI and end stage heart failure. Lifestyle (diet) changes [18] and pharmacological (Nitroglycerine, β blockers) intervention constitutes the first level of treatment [19]. However pharmacological treatments side effects can be caused by drug dosage and unspecific drug target interactions [20-22]. Surgical management for heart failure is widely employed [23] using ventricular assist devices [24], pace makers [25], and bypass surgery [26]. Nevertheless, existing pharmacological and surgical interventions can only prevent the rapid progress of the condition, but not reverse the circumstances [27] or regenerate the infarcted scar tissue. Currently, heart transplantation remains the gold standard [23] and is faced with major challenges like limited donor organ availability, long waiting time, and requirement of immunosuppression therapy [28]. Hence development of an alternative treatment strategy for myocardial infarction and end stage heart failure is paramount, serving as the motivation for further research in the field of CTE.

2.3 Cardiac Tissue Engineering (CTE) and Scaffold Properties

Tissue Engineering is defined as “an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function” [29].

The field of CTE is vastly interdisciplinary which draws expertise from both material and biomedical engineering as well as biology and medicine. This domain entails the use of 3D scaffolds, which act as a template for tissue regeneration. Biomaterial scaffolds provide conducive environment for the cell proliferation whether cells were pre- seeded on the engineered construct *in vitro* or recruited from the host following *in vivo* implantation. Beyond scaffolds and cells, a third component often considered is the environment of the

engineered construct, which can be controlled by application of various bioactive growth factors and dedicated bioreactor technologies providing physical, mechanical or electrical stimuli to the cells [30]. This combination of scaffold, cells and growth factor/bioreactor is referred to as the tissue engineering triad (Figure 2-2) [31].

Figure 2-2 Tissue engineering triad [31]

Scaffolds can be regarded as extracellular matrix (ECM) substitutes which help in temporarily supporting cell growth and functionality. A typical ECM material consists of i) fibrous structural proteins and adhesion proteins like laminin and fibronectin; ii) specific growth factors, and iii) Glycosaminoglycans (GAG) forming interfibrillary gel like hydrated morphology [32]. Scaffolds in general should possess the following important characteristics [31]:

1. Biocompatibility — ‘Biocompatibility refers to the ability of a material to perform with an appropriate host response in a specific situation’ [33].
2. Biodegradability—ability of the seeded cells or the *in vivo* system to degrade the material without releasing nontoxic byproducts. Ideally, the scaffold should degrade in parallel to the secretion of the natural replacing ECM by the populating cells.
3. Bioactivity—presence of biomolecular cues or ligands necessary for proper cell adhesion, proliferation and differentiation.
4. Porosity—scaffolds should possess interconnected pores, thus enabling cellular migration within the scaffold; transport of nutrients and removal of degradation products.

5. Mechanical Properties—matching mechanical properties to the site or organ of interest as the integration of cells with the tissue engineered scaffold depends on the target mechanical properties [34]. This is particularly important to functional load bearing organs such as the heart.

The morphological, structural and bioactive parameters of the scaffold depend on the choice of scaffold material, and the fabrication method. The scaffold material can be predominantly sourced from either natural and/or synthetic origin.

2.4 Natural & Synthetic Scaffold Biomaterials

Various natural biomaterials have been suggested for CTE. These include collagen [35], elastin [36], fibrin [37], alginate [38], chitosan [39], silk fibroin [40], and gelatin [41]. Generally, natural biomaterials offer biological signals necessary for cellular attachment, viability, proliferation and differentiation (for stem/progenitor cells). However, the disadvantages are their high batch to batch variability, and the unknown mechanism by which these materials determine the fate of the seeded and/or the recruited cells. One common type of natural biomaterials is that produced by tissue or organ decellularization. Decellularization is a process whereby the native tissue is biochemically treated to completely remove the source tissue immunogenic cellular components, leaving behind the preserved and natural acellular ECM of that tissue. Some limitations of the natural materials are their variability and inadequate understanding of their bioactivity.

Conversely, synthetic biomaterials provide high control-ability over the chemistry, physical and mechanical properties of the scaffold, which fabrication (e.g. electrospinning, solvent casting etc.) can yield a highly reproducible product. Different synthetic polymeric materials like Poly lactic acid (PLA) [42], Poly glycolic acid (PGA) [43], Poly lactide-co-glycolide (PLGA) [42], Poly ϵ -caprolactone (PCL), Poly (L-lactide-co- ϵ -caprolactone) (PLCL) [44], Polyurethanes (PU) [45], Poly glycerol sebacate (PGS) [46], have been used alone or in combination to make CTE grafts [45, 47-59]. The notable disadvantage of the synthetic materials is the absence of any surface-active molecules to enable cell adhesion,

proliferation, and differentiation and possibly local cell-toxicity due to some polymer degradation byproducts.

The relative advantages and limitations of natural and synthetic scaffold biomaterials are summarized in Table 2-1. In summary, achieving a well-balanced inter-related properties (i.e. surface chemistry, architecture, porosity, and degradation kinetics) is a crucial requirement in the design and development of any scaffold biomaterial, and for MI treatment, in particular.

Table 2-1 Advantages and limitations of natural and synthetic materials

Material Origin	Advantages	Limitations
Natural	<ul style="list-style-type: none"> • Bioactivity • Biocompatibility • Preserved 3D architecture • Possible preservation of inherent vasculature 	<ul style="list-style-type: none"> • Batch to Batch variability • Lack of sufficient understanding of bioactivity mechanisms • Limited control-ability of product properties
Synthetic	<ul style="list-style-type: none"> • Tunable morphology and mechanical property • Minimal variability 	<ul style="list-style-type: none"> • Lack of bioactivity • Possible local toxicity due to degradation

2.5 Generating 3D Bioactive Scaffolds for CTE

Scaffold dimensions are an important parameter in tissue engineering. With the exception of thin squamous epithelium on basement membranes, 2D scaffolds fabricated by different methods do not recapitulate the usual native conditions, even if they do support cell attachment and function to some extent. The native physiological condition usually comprises a 3D environment [60], and therefore the development and evaluation of such similar 3D scaffold structures for CTE is important and suitable. 3D environments provide highly complex signals necessary for cell adhesion, proliferation, migration and differentiation in the scaffold environment [61, 62]. A 3D porous morphology not just offers structural cues, but also a downstream affected complex signaling cascade, which the scaffold architecture triggers in the interacting cells [61]. For instance, the scaffold 3D

morphology affects cell spreading [63], nutrient and oxygen diffusion gradients [60], and even the scaffold mechanical properties—all of which, were previously shown to differentially alter cellular gene expression.

In addition to possessing a 3D environment, the scaffold also needs to be bioactive to aid in appropriate cellular functions. Biomaterials are the common class of material used as scaffolds in tissue engineering applications. A biomaterial, according to European Society of Biomaterials is defined as “material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body” [31]. Scaffolds can be made bioactive by the introduction of natural biomaterials like collagen, gelatin, alginate etc. When cells are seeded on the scaffold, they impart additional biological elements to the scaffold by secreting their own ECM material, which ideally would eventually replace the scaffold material.

A promising strategy to obtain a natural 3D scaffold is decellularization of a native tissue. This has been demonstrated in both human and animal tissues of various organs (e.g., including cardiac tissue [64-66]. In this method the native tissue is subjected to enzymatic and chemical treatments to get rid of the cellular content, thus resulting in a network of conserved extracellular matrix. Tissue-specific ECM offers the advantage of preserved unique architecture and biochemical signals required for cell regulation and differentiation. Indeed, some recent studies demonstrated that acellular ECM seeded with stem cells have displayed an inclination to differentiate towards the target tissue lineage cells [64, 67].

Our group has successfully decellularized human appropriate sized pig-heart left ventricle resulting in pcECM (pcECM) [11, 68]. This material offers an ideal 3D cardiac environment with the preserved biochemical, architectural cues and the inherent vasculature [2, 11]. The material has preserved the unique cardiac architectural structures and when seeded with hMSC and endothelial cells displayed angiogenic like sprouting [2]. However, the mechanism by which the bioactive scaffold environment affects the cell fate remains unclear. Another limitation of this material is the batch to batch variability arising due to the decellularization process. A more robust fabrication method or a synthetic bio

hybrid scaffold which can exhibit similar characteristics of the pcECM would be an interesting platform to study the effectors of the pcECM architecture, bioactivity on cell fate.

Electrospinning is a popular fabrication technique to produce tissue engineered fibers (from natural and synthetic polymers materials) that are porous and having diameters in nano and microscale range [69]. Electrospinning involves the application of high voltage to a polymer solution droplet dispensed through a capillary or needle from a syringe by a pump. The polymer droplet, in the presence of the electric field will be distorted into a conical shape known as Taylor's cone [70]. When the intensity of the electric field increases, charge repulsion on the droplet surface exceeds the surface tension of the liquid. This causes elongation of the polymer droplet and evaporation of the solvent, resulting in the deposition of the polymer fiber in the counter electrode [71]. Although different studies show that electrospun fibers can be used in CTE to interact with the cells and express cardiac markers [52, 72, 73], they do not have a 3D environment comparable to the native environment. Numerous methods have been suggested for the fabrication of electrospun 3D scaffolds, such as increasing electrospinning time to get thicker fiber meshes, stacking thin scaffolds on top of each other, ultra-sonication of thin scaffolds and wet electrospinning [74, 75]. Wet electrospinning of synthetic polymers yield 3D scaffolds with controllable thickness. In wet-electrospinning the traditional solid collector is replaced with a liquid bath. This technique can be used to enhance the thickness of electrospun scaffolds producing more adequate 3D environments that can be tailored to structurally mimic the pcECM [76]. However, in order for the 3D electrospun scaffold to mimic the native ECM in more than just the fibrous architecture, further modification is necessary with bioactive molecules. A wet electrospun platform developed in our group, resulted in a fibrous tunable scaffold platform, that was successfully employed for animal model wound healing application [77], when the polymeric scaffold was modified using ECM molecules, collagen and fibronectin. This wet electrospun 3D scaffold platform has also exhibited support for hepatocyte viability and function [78].

Compositional analysis of the acellular ECM developed by our group and by other investigators has exhibited the presence of these significant molecules in the solid and

liquid form of the decellularized ECM [11, 79-83]. Hence, we believe modifying the electrospun 3D scaffold with the liquid pcECM developed in our lab will add not only a tissue specific bioactive environment but also an ECM scaffold structure that can mimic the native ECM composition.

We therefore suggest that a wet electrospinning can be used to fabricate polymer scaffolds, which—when modified with the bioactive liquidized pcECM and/or ECM producing cells—will display a similar bioactivity, structural, and morphological profile as the native pcECM. Comparing the produced 3DCS to their respective individual components and to the pcECM (positive control), will enable improving our understanding of the pcECM bioactivity mechanisms at the cell-biomaterial interface.

2.6 Cells in Cardiac Regeneration

Cell types like skeletal myoblasts (SM) [84], bone marrow mono nuclear cells (bmMNC) [85], mesenchymal stem cells (MSC) [2], Cardiac stem cells (CSC) [86-88], embryonic stem cells (ESC) [89] and induced pluripotent stem cells (iPSC) [90, 91] have been used in cardiac regenerative medicine research. When SMs were transplanted into cryoinfarcted rabbit hearts, the transplanted cells displayed cardiac like morphology but these cells in the infarcted region did not appear as healthy as the myocytes in the normal region of the heart. Also, the engrafted cells showed skeletal muscle characteristics, preventing them from integrating with the host tissue [84]. Ramshorst *et. al.* performed an intramyocardial injection of bmMNCs in patients with chronic ischemia. Although improvement in left ventricle ejection fraction (LVEF) was observed compared to placebo, limitations like absence of information on cell homing, retention and cellular mechanism were reported [85]. Autologous bone marrow derived MSCs have been used in CTE, and many clinical trials, with suggested safety and very limited efficacy profiles. MSCs generally enable limited functional salvage compared to controls, which is attributed to paracrine signaling, with the limitation being the inability of the cells to induce myogenesis and electrical coupling (to enable synchronous beating) between the cells [92]. Following the discovery of cardiac progenitor cells in the adult human myocardium that are capable of replenishing

the cardiomyocyte population [87, 88], CSC became an important cell candidate in CTE. Antonio *et. al.*, isolated CSC from adult rats and attempted to regenerate functional myocardium in an infarcted rat model. Though, the infarcted rat heart showed signs of regeneration [86], usage of these cells poses a practical difficulty in the form of isolation, characterization and clinically significant expansion *in vitro*. CSC therapy in pre-clinical and clinical setting has also exhibited improved ejection fraction of the left ventricle and their role in cardiac regeneration is being investigated [93]

An important factor in CTE is the necessity to have all the cardiac cell types—CM, fibroblasts and vascular endothelial cells—in the engineered tissue. One way of achieving this complexity of cell population within the engineered construct is to mix and match various terminally differentiated or multipotent progenitor cells to get a desired cell type ratio, at least during construct seeding. Alternatively, pluripotent cell sources—i.e., stem cells which are capable of differentiating to all three germ layers comprising the human organism—can be used and pushed towards specific relevant lineages such as CM, CF, smooth muscle cells or endothelial cells. Early research into embryonic stem cells (ESCs), demonstrated their ability to differentiate into all cardiac cells [89]. Still, the ethical issues involved in the use of ESCs stemmed research on the identification of alternative pluripotent cell sources.

In 2006, Takahashi and Yamanaka, in a breakthrough research, reported on the production of induced pluripotent stem cells (iPSC), an ESC like cells derived from somatic cells (mouse embryonic and adult fibroblast). When the somatic cells are made to ectopically express four factors—OCT3/4, Sox2, KLF, and c-Myc—the cells exhibited characteristics similar to ESC i.e. colony morphology, self-renewal [94] and the ability to differentiate into cells of all three germ layers, including CM [95]. iPSC discovery has since provided an alternative cell source for cardiac regeneration, one which is potentially autologous—as iPSCs can be derived from reprogramming of the patients own adult somatic cells—thus necessitating the need for ethically questionable ESCs [96-98]. Indeed, similar to ESCs, iPSC as well were shown to differentiate into all the major cardiac lineage cells [99-103].

Evidence to the impact of the discovery of iPSCs is the vast ongoing research in this relatively young field. Since the first report in 2006, iPSCs have been obtained, albeit with sometime different genetic combinations, from mice [94], human [104], rhesus [105], pig [106], rat [107], rabbit [108], canine [109], sheep [110] and bovine [111]. Furthermore, many different types of iPSC lines have been established using the same or different factors for inducing pluripotency [112]. Similarly, multiple protocols have been developed for the mass expansion of iPSCs *in vitro*, which makes translation of iPSC related studies towards clinical studies possible [101, 113, 114]. In the field of CTE, a few studies have been done using iPSC as a candidate cell for cardiac regeneration. Intra-myocardial injection of undifferentiated iPSCs in adult mouse models ceased the progression of remodeling in infarcted hearts and exhibited low expression of cardiac, smooth muscle and endothelial markers [90]. Human iPSCs injected in long term surviving porcine models exhibited endothelial like phenotype [91], though the actual mechanism of the differentiation is unclear. Taken together, the advantages of iPSCs make them an interesting and most promising candidate for CTE applications, and yet the interactions between this pluripotent cell source and complex bioactive environments remains largely uncharted.

2.7 Current Strategies and Challenges in CTE

Current tissue engineering strategies, to aid in regeneration of the infarcted myocardium, can be broadly categorized into either patch deposition or injectable delivery approaches [115]. In both cases, either cells alone, biomaterials alone or various combinations thereof are delivered aiming to augment the myocardium, prevent further deterioration and even restore cardiac function. Traditional cell injection therapies were first demonstrated in the 1990s, by direct injection of C2C12 myoblasts and AT-1 cardiomyocyte in the myocardium of animal models [116, 117]. Menasche *et al.* have demonstrated injecting skeletal myoblasts in post infarcted scars in human patients but did not show any significant improvement in heart function [118]. In a study by Hoffman *et al.* they found that the transplanted cells were mostly found in liver and spleen [119]. Even to-date after many clinical trials with various cell sources [120], cell-only therapy approaches produce but

dismal results due to poor cell retention in the injected area and low survival of the few engrafted cells [121].

Another cell-only strategy is the scaffold free cell patch where cells are cultured and allowed to form their own ECM [122], the limitation being that these scaffold free cell patches did not form a successful graft post implantation. Cell sheet engineering is another approach which uses poly (N-isopropylacrylamide), a temperature responsive polymer culture surface. Layers of cells and their secreted ECM grown on these surfaces can be easily detached and assembled into a 3D tissue structure [123]. It has also been demonstrated that these cell sheets can be directly implanted effectively without cell loss as the cells maintain their inherently secreted ECM [124]. However, to achieve human equivalent tissue thickness, several cell sheets need to be stacked together limiting the applicability of this approach due to insufficient oxygen and nutrition supply to the center of the construct [125].

Tissue engineered biomaterial scaffolds have exhibited the ability to support cell seeding, survival and engraftment following implantation. For instance, cardiac like tissue constructs were reported using rat neonatal CM, and/or pluripotent stem cell derived CM that are pre-seeded on collagen or gelatin matrix *in vitro* prior to implantation. Following *in vivo* implantation these grafts displayed improved cell retention and survival at the infarcted area [35, 81, 126-133]. In addition to cell support and retention, biomaterials (patches or injectables) provides also mechanical support to the infarcted region (law of Laplace [134]). Thus, by reducing the stress on the infarcted myocardium, the adverse remodeling process slows down, and cardiac function is maintained. Though this approach helps in alleviating the existing condition, it does not successfully regenerate the infarct site [56].

Various naturally obtained materials induce different levels of response, dependent on the origin and specific characteristics of the material itself. For instance, patches made from Collagen, an important constituent of the cardiac extracellular matrix (cECM) have been demonstrated to survive *in vivo* in immunosuppressed rats [135]. Another study involving

gelatin, a hydrolyzed form of collagen was used to showcase the ability of this material to perform as cardiac graft [136]. Alginate, a negatively charged polysaccharide graft was shown to encourage neovascularization in rat models [49]. Maintenance of cardiac function after MI has been demonstrated using fibrin glue alone [37]. Small intestine submucosa has been used as a scaffold material with seeded mesenchymal stem cells. Though this seeded scaffold showed significant heart function improvement in short term (4 weeks) when compared to the control scaffold without cells, a chronic inflammatory reaction in the graft area was observed. Also, there was no electromechanical communication between the implant and the host tissue [137]. Another appropriate candidate for use in cardiac patch for MI treatment would be ECM derived from cardiac origin. Our group has successfully fabricated an acellular cardiac ECM from porcine heart ventricle. This ECM preserves the distinctive cardiac architecture, porosity and the inherent biochemical signals [68]. Thus, the type of architecture and biochemical signals that comprise each natural biomaterial, differentially affects the interaction with the seeded cells as well as recruited cells *in vivo*.

Synthetic biomaterials also have been used in CTE. PLCL patch has been used in post MI rat models, but no significant difference was observed in improvement of left ventricular repair when compared to control animals [51]. Poly glycolide-co-caprolactone with bone marrow mononuclear cells have been studied in rat models. This displayed attenuation of left ventricle function, but the cardiac differentiation of the seeded cells in the graft site was not uniform [138]. Sepideh *et. al.* fabricated an electrospun gelatin/PCL hybrid scaffold. This hybrid scaffold exhibited improved adhesion and migration of human adipose derived stem cells (hASCs) when compared to control electrospun PCL scaffold [139].

Nevertheless, despite many years of research, countless biomaterials and cell sources tested, different infarction animal models and clinical trials, and under various immunological conditions, the dynamic interactions between the cells and the biomaterials remain complex and are not sufficiently elucidated. Furthermore, various biomaterials induce different levels of response, dependent on the origin and specific characteristics of the material itself. Moreover, this variable property of the materials and the existing lack of

understanding of their vague interaction mechanism with cells, causing spontaneous differentiation of stem cells or dedifferentiation of differentiated cells, makes additional research in CTE necessary.

2.8 Support of PSC and PSC Derived Cells by Biomaterials

Though different natural, synthetic and hybrid scaffold platforms have been studied for iPSC or iPSC-CM interaction, to the best of our knowledge there has been no investigation of these cells' interaction in a 3D bioactive environment (pcECM and 3DCS) under conditions without any external differentiation factors. Hence, we intend to work on bridging this knowledge gap, by seeding hiPSC on pcECM and 3DCS under normal culture condition and investigate if the inherent physical and tissue specific biochemical cues are sufficient to stimulate proliferation and spontaneous differentiation of the seeded cells.

Previous works have demonstrated the ability of several biomaterials to support PSC. Zhang *et. al.*, developed a matrix sandwich method using MatrigelTM for differentiation of iPSC into CM in the presence of additional growth factors. This work has monitored the ability of this matrix system to support the viability of CM over a period of 30 days by examining cardiac specific markers (Troponin and myosin light chain 2) [140]. However, MatrigelTM is a non-tissue specific ECM material, thereby requiring a combination of small molecules and growth factors to induce differentiation. However, to study the ability of a cardiac specific microenvironment in affecting cell fate, a tissue-based ECM like the pcECM or the 3DCS can be used to evaluate this microenvironment's ability to induce spontaneous differentiation of PSC towards cardiac lineage in the absence of external growth factors or small molecules. Perfusion assisted recellularization of an entire mouse heart with human iPSC derived multipotent cardiac progenitor cells showed regeneration of the muscle as well as the vasculature in the mouse heart. The recellularized heart showed spontaneous contractions [141, 142]. Though this is an interesting result exhibiting the concept of iPSC usage, the production of a whole heart decellularization strategy is effortful and the resulting decellularized mouse hearts are much smaller than clinically required. Also, the scale up of this technology involves challenges such as nutrient

diffusion, recellularization and the variability of the natural material which could hamper the commercial application.

Synthetic material platforms have also been studied for the support of PSC. Aligned PLGA nanofibers have exhibited alignment of hiPSC-CM compared to normal tissue culture plates (TCPs). But this was demonstrated in a scaffold of only 50 μm thickness yielding a monolayer of aligned hiPSC-CM [143]. An electrospun PCL scaffold with a murine embryonic fibroblast feeder layer exhibited spontaneous differentiation of murine iPSCs seeded in the presence of a differentiation medium [144]. This result though very interestingly displaying spontaneous differentiation, was carried out in an environment which is not comparable to native 3D conditions.

2.9 Effect of Cardiac ECM Based Scaffolds on Cells

Even though the bioactivity of cardiac ECM scaffolds, such as our pcECM, influences the seeded cells by altering the attachment, proliferation and differentiation, the underlying factors responsible for these effects remain unknown.

Williams *et. al.* fabricated a rat heart ventricle ECM gel-fibrin scaffold crosslinked with transglutaminase. Cardiac progenitor cells seeded on this scaffold showed expression of cardiac, smooth muscle cell and endothelial marker expression. A limitation in this work as mentioned by the authors is the gelling ability of the rat ECM (to fibrin) at high ratios and the differences of a rat ECM model for human application [145]. Stoppel *et. al.* fabricated an aligned silk-porcine cardiac ECM based hybrid scaffold [146]. Though this scaffold displays the ability to support human embryonic stem cell derived CM, this scaffold might not be optimal for *in vivo* cardiac applications as long term *in vitro* study is required (> 7 days). Research by our group with thick (~1.5 cm) pcECM and hMSC-HUVEC co culture exhibited *in vitro* angiogenesis and vessel sprouting by a time dependent addition of vascular endothelial growth factor in a dynamic bioreactor cultivation system [2]. This process of angiogenesis was attributed to the preserved biochemical cues in the bulk as well the preserved vascular infrastructure of the pcECM

[2]. Our group has also developed the liquid form of the pcECM and it exhibited excellent potential in its cell supportability and cardiac regeneration in rat models [147]. But the tunability of this material in terms of mechanical properties is challenging. Also, our group has also fabricated electrospun scaffolds with the pcECM and this electrospun scaffold is highly cell supportive [148]. Though both the liquid pcECM and the electrospun scaffold are very interesting materials, the origin of their bioactivity still remains uncovered. In an interesting work, Oberwallner et al, demonstrated the interaction between human cardiac ECM and murine iPSCs. In this study, the iPSCs were shown to differentiate towards cardiac lineage by expressing cardiac specific markers. This study demonstrated the ability of organ specific ECM in pushing the seeded PSCs towards the cells of that target organ. The limitation in this work is that the availability of the cardiac ECM that is being derived from human patients with serious cardiovascular conditions. Also, though the use of murine cells resulted in interesting results, to get clinically relevant understanding, human cells' interaction with these type of bioactive environments is necessary [64]. A recent study by Guyette *et. al.* has used perfusion recellularization of an entire human decellularized heart with iPSC derived CM [149]. Although it is reported that the bioactivity of the human cardiac ECM contributed to the viability and functionality of the seeded iPSC-CM, the mechanism by which the native ECM has affects the seeded iPSC-CM to maintain functionality remains unclear.

Hence a biomimetic scaffold with a modular architecture and bioactivity profile would enable improved understanding of the pcECM mechanism of action.

2.10 Conferring Bioactivity to Synthetic Scaffolds in CTE

Although different hybrid scaffold platforms were studied for CTE applications, a knowledge gap remains in deducing an ideal scaffold with appropriate architecture and biochemical cues in comparison to an existing natural bioactive scaffold such as our pcECM.

The scaffold materials' bioactivity plays an important role for a dynamic interaction between the seeded cell and the scaffold. Various modifications can be done to render the

materials more bioactive if they are inherently not one [150]. The easiest way to make a material bioactive is to modify the surface by coating with biomolecules like collagen [151], gelatin [41], fibronectin [152], vitronectin [153], laminin [45], and synthetic peptide [154] molecules. The surface modification with these molecules have been shown to improve the cell adhesion and proliferation of the cells cultured on synthetic surfaces [155, 156]. Incorporation of bioactive molecules in the bulk material, release of loaded growth factors and scaffold surface peptides has enabled cellular functions like proliferation, migration and differentiation [157-162].

A PCL-collagen-elastin-gelatin hybrid system was fabricated using electrospinning for CTE. Though this scaffold displays adhesion, migration and proliferation of the seeded human adipose derived stem cells, the thickness of the scaffold used was only 100-200 μm , which is not comparable to the thickness of the native cardiac 3D environment [139]. Another composite scaffold was fabricated by electrospinning of poly (glycerol sebacate)-carbon nanotubes (CNTs) dispersed in gelatin methacrylate. This construct exhibited improved ability of rat CM to interact and spontaneously beat when compared to the control PGS scaffold without CNTs [163]. Though this CNT incorporated hybrid exhibits good electrophysiological environment, *in vivo* investigation is necessary to get information on the immunological effects this scaffold triggers and the effect of the scaffold degradation products. You *et. al.* fabricated a 3D conductive scaffold using hydroxyethyl methacrylate and gold nanoparticles. Though this platform displayed adhesion and beating of rat neonatal CM without any natural bioactive molecules, the duration of study was limited to only 8 days, requiring longer period of studies to investigate if the scaffold can maintain the beating of the CM over extended experimental durations [164].

Hence, bioactivity of a scaffold material, either inherent or introduced by modification, is a guiding factor in maintaining an active communicating environment between the scaffold material and the cells seeded therein.

2.11 Effect of Architecture vs. Bioactivity on Cell Behavior and Fate

Many studies have reported on the importance of cell-biomaterial scaffold interactions for tissue engineering and regenerative medicine applications. These studies, often are based on the ability of these interactions to influence the cellular fate. In particular, both inherent and external signals, in a cell scaffold environment can influence and determine the fate of the seeded stem cell [165-174]. These works point to the fact that factors such as scaffold architecture, biochemical cues and external stimuli has an important role in determining the seeded cellular fate. However, with respect to the effect of biomaterial scaffolds on the interacting pluripotent stem cell's lineage commitment - the literature is quite scarce. Also, the influence of cardiac ECM mimicking microenvironments (in terms of architecture and biochemical composition) on human PSC differentiation and lineage commitment remains unexplored. A schematic of various factors that can affect stem cell fate is represented in Figure 2-3.

Figure 2-3: Influence of various factors on stem cell fate

References

- [1] P.A. Iaizzo, Handbook of cardiac anatomy, physiology, and devices, Springer Science & Business Media 2009.
- [2] U. Sarig, E.B. Nguyen, Y. Wang, S. Ting, T. Bronshtein, H. Sarig, N. Dahan, M. Gvartz, S. Reuveny, S.K. Oh, T. Scheper, Y.C. Boey, S.S. Venkatraman, M. Machluf, Pushing the envelope in tissue engineering: ex vivo production of thick vascularized cardiac extracellular matrix constructs, *Tissue Eng Part A* 21(9-10) (2015) 1507-19.
- [3] R.H. Anderson, R. Razavi, A.M. Taylor, Cardiac anatomy revisited, *Journal of anatomy* 205(3) (2004) 159-77.
- [4] R. Lakshmanan, U.M. Krishnan, S. Sethuraman, Living cardiac patch: the elixir for cardiac regeneration, *Expert Opin Biol Ther* 12(12) (2012) 1623-40.
- [5] N.J. Severs, The cardiac muscle cell, *BioEssays : news and reviews in molecular, cellular and developmental biology* 22(2) (2000) 188-99.
- [6] H.W. Vliegen, A. van der Laarse, C.J. Cornelisse, F. Eulerink, Myocardial changes in pressure overload-induced left ventricular hypertrophy. A study on tissue composition, polyploidization and multinucleation, *European heart journal* 12(4) (1991) 488-94.
- [7] D. MacKenna, S.R. Summerour, F.J. Villarreal, Role of mechanical factors in modulating cardiac fibroblast function and extracellular matrix synthesis, *Cardiovascular research* 46(2) (2000) 257-63.
- [8] P. Camelliti, T.K. Borg, P. Kohl, Structural and functional characterisation of cardiac fibroblasts, *Cardiovascular research* 65(1) (2005) 40-51.
- [9] M. Rienks, A.P. Papageorgiou, N.G. Frangogiannis, S. Heymans, Myocardial extracellular matrix: an ever-changing and diverse entity, *Circ Res* 114(5) (2014) 872-88.
- [10] S.L. Bowers, I. Banerjee, T.A. Baudino, The extracellular matrix: at the center of it all, *Journal of molecular and cellular cardiology* 48(3) (2010) 474-82.
- [11] U. Sarig, G.C. Au-Yeung, Y. Wang, T. Bronshtein, N. Dahan, F.Y. Boey, S.S. Venkatraman, M. Machluf, Thick acellular heart extracellular matrix with inherent vasculature: a potential platform for myocardial tissue regeneration, *Tissue Eng Part A* 18(19-20) (2012) 2125-37.
- [12] Z. Kassiri, R. Khokha, Myocardial extra-cellular matrix and its regulation by metalloproteinases and their inhibitors, *Thrombosis and haemostasis* 93(2) (2005) 212-9.

- [13] C. Frantz, K.M. Stewart, V.M. Weaver, The extracellular matrix at a glance, *J Cell Sci* 123(Pt 24) (2010) 4195-200.
- [14] K. Thygesen, J.S. Alpert, H.D. White, E.S.C.A.A.H.A.W.H.F.T.F.f.t.R.o.M.I. Joint, Universal definition of myocardial infarction, *J Am Coll Cardiol* 50(22) (2007) 2173-95.
- [15] S.E. Senyo, R.T. Lee, B. Kuhn, Cardiac regeneration based on mechanisms of cardiomyocyte proliferation and differentiation, *Stem cell research* 13(3 Pt B) (2014) 532-41.
- [16] K.T. Weber, From inflammation to fibrosis: a stiff stretch of highway, *Hypertension* 43(4) (2004) 716-9.
- [17] W.J. Remme, K. Swedberg, D. Task Force for the, E.S.o.C. Treatment of Chronic Heart Failure, Guidelines for the diagnosis and treatment of chronic heart failure, *European heart journal* 22(17) (2001) 1527-60.
- [18] S.L. Cobb, D.J. Brown, L.L. Davis, Effective interventions for lifestyle change after myocardial infarction or coronary artery revascularization, *Journal of the American Academy of Nurse Practitioners* 18(1) (2006) 31-9.
- [19] M.G. Sutton, N. Sharpe, Left ventricular remodeling after myocardial infarction: pathophysiology and therapy, *Circulation* 101(25) (2000) 2981-8.
- [20] F.J. Azuaje, L. Zhang, Y. Devaux, D.R. Wagner, Drug-target network in myocardial infarction reveals multiple side effects of unrelated drugs, *Scientific reports* 1 (2011) 52.
- [21] L. Gullestad, P. Aukrust, T. Ueland, T. Espevik, G. Yee, R. Vagelos, S.S. Froland, M. Fowler, Effect of high- versus low-dose angiotensin converting enzyme inhibition on cytokine levels in chronic heart failure, *J Am Coll Cardiol* 34(7) (1999) 2061-7.
- [22] M. Packer, P.A. Poole-Wilson, P.W. Armstrong, J.G. Cleland, J.D. Horowitz, B.M. Massie, L. Ryden, K. Thygesen, B.F. Uretsky, Comparative effects of low and high doses of the angiotensin-converting enzyme inhibitor, lisinopril, on morbidity and mortality in chronic heart failure. ATLAS Study Group, *Circulation* 100(23) (1999) 2312-8.
- [23] E.M. Delmo Walter, R. Hetzer, Surgical treatment concepts for heart failure, *HSR proceedings in intensive care & cardiovascular anesthesia* 5(2) (2013) 69-75.
- [24] S.R. Wilson, M.M. Givertz, G.C. Stewart, G.H. Mudge, Jr., Ventricular assist devices the challenges of outpatient management, *J Am Coll Cardiol* 54(18) (2009) 1647-59.

- [25] E.B. Friedrich, M. Bohm, Management of end stage heart failure, *Heart* 93(5) (2007) 626-31.
- [26] S.S. McCoy, C.S. Crowson, H. Maradit-Kremers, T.M. Thorneau, V.L. Roger, E.L. Matteson, S.E. Gabriel, Longterm outcomes and treatment after myocardial infarction in patients with rheumatoid arthritis, *The Journal of rheumatology* 40(5) (2013) 605-10.
- [27] H. Jawad, A.R. Lyon, S.E. Harding, N.N. Ali, A.R. Boccaccini, Myocardial tissue engineering, *British medical bulletin* 87 (2008) 31-47.
- [28] C. Söderlund, G. Rådegran, Immunosuppressive therapies after heart transplantation — The balance between under- and over-immunosuppression, *Transplantation Reviews* 29(3) (2015) 181-189.
- [29] R. Langer, J.P. Vacanti, Tissue engineering, *Science* 260(5110) (1993) 920-6.
- [30] I. Martin, D. Wendt, M. Heberer, The role of bioreactors in tissue engineering, *Trends in biotechnology* 22(2) (2004) 80-6.
- [31] F.J. O'brien, Biomaterials & scaffolds for tissue engineering, *Materials Today* 14(3) (2011) 88-95.
- [32] S.F. Badylak, Xenogeneic extracellular matrix as a scaffold for tissue reconstruction, *Transplant immunology* 12(3-4) (2004) 367-77.
- [33] D.F. Williams, On the mechanisms of biocompatibility, *Biomaterials* 29(20) (2008) 2941-53.
- [34] U. Sarig, H. Sarig, A. Gora, M.K. Krishnamoorthi, G.C.T. Au-Yeung, E. de-Berardinis, S.Y. Chaw, P. Mhaisalkar, H. Bogireddi, S. Ramakrishna, F.Y.C. Boey, S.S. Venkatraman, M. Machluf, Biological and mechanical interplay at the Macro- and Microscales Modulates the Cell-Niche Fate, *Scientific reports* 8(1) (2018) 3937.
- [35] T. Eschenhagen, C. Fink, U. Remmers, H. Scholz, J. Wattchow, J. Weil, W. Zimmermann, H.H. Dohmen, H. Schafer, N. Bishopric, T. Wakatsuki, E.L. Elson, Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system, *FASEB J* 11(8) (1997) 683-94.
- [36] T. Mizuno, T.M. Yau, R.D. Weisel, C.G. Kiani, R.K. Li, Elastin stabilizes an infarct and preserves ventricular function, *Circulation* 112(9 Suppl) (2005) I81-8.

- [37] K.L. Christman, H.H. Fok, R.E. Sievers, Q. Fang, R.J. Lee, Fibrin glue alone and skeletal myoblasts in a fibrin scaffold preserve cardiac function after myocardial infarction, *Tissue Eng* 10(3-4) (2004) 403-9.
- [38] J. Leor, S. Tuvia, V. Guetta, F. Manczur, D. Castel, U. Willenz, O. Petnehazy, N. Landa, M.S. Feinberg, E. Konen, O. Goitein, O. Tsur-Gang, M. Shaul, L. Klapper, S. Cohen, Intracoronary injection of in situ forming alginate hydrogel reverses left ventricular remodeling after myocardial infarction in Swine, *J Am Coll Cardiol* 54(11) (2009) 1014-23.
- [39] S. Lu, H. Wang, W. Lu, S. Liu, Q. Lin, D. Li, C. Duan, T. Hao, J. Zhou, Y. Wang, S. Gao, C. Wang, Both the transplantation of somatic cell nuclear transfer- and fertilization-derived mouse embryonic stem cells with temperature-responsive chitosan hydrogel improve myocardial performance in infarcted rat hearts, *Tissue Eng Part A* 16(4) (2010) 1303-15.
- [40] C. Patra, S. Talukdar, T. Novoyatleva, S.R. Velagala, C. Muhlfeld, B. Kundu, S.C. Kundu, F.B. Engel, Silk protein fibroin from *Antheraea mylitta* for cardiac tissue engineering, *Biomaterials* 33(9) (2012) 2673-80.
- [41] M. Kharaziha, M. Nikkhah, S.R. Shin, N. Annabi, N. Masoumi, A.K. Gaharwar, G. Camci-Unal, A. Khademhosseini, PGS:Gelatin nanofibrous scaffolds with tunable mechanical and structural properties for engineering cardiac tissues, *Biomaterials* 34(27) (2013) 6355-66.
- [42] A. Lesman, M. Habib, O. Caspi, A. Gepstein, G. Arbel, S. Levenberg, L. Gepstein, Transplantation of a tissue-engineered human vascularized cardiac muscle, *Tissue Eng Part A* 16(1) (2010) 115-25.
- [43] U.A. Stock, J.E. Mayer, Jr., Tissue engineering of cardiac valves on the basis of PGA/PLA Co-polymers, *J Long Term Eff Med Implants* 11(3-4) (2001) 249-60.
- [44] J. Jin, S.I. Jeong, Y.M. Shin, K.S. Lim, Y.M. Lee, H.C. Koh, K.S. Kim, Transplantation of mesenchymal stem cells within a poly (lactide - co - ϵ - caprolactone) scaffold improves cardiac function in a rat myocardial infarction model, *European journal of heart failure* 11(2) (2009) 147-153.

- [45] T.C. McDevitt, K.A. Woodhouse, S.D. Hauschka, C.E. Murry, P.S. Stayton, Spatially organized layers of cardiomyocytes on biodegradable polyurethane films for myocardial repair, *J Biomed Mater Res A* 66(3) (2003) 586-95.
- [46] Y. Wang, G.A. Ameer, B.J. Sheppard, R. Langer, A tough biodegradable elastomer, *Nat Biotechnol* 20(6) (2002) 602-6.
- [47] Q.Z. Chen, A. Bismarck, U. Hansen, S. Junaid, M.Q. Tran, S.E. Harding, N.N. Ali, A.R. Boccaccini, Characterisation of a soft elastomer poly(glycerol sebacate) designed to match the mechanical properties of myocardial tissue, *Biomaterials* 29(1) (2008) 47-57.
- [48] E.L. Dvorin, J. Wylie-Sears, S. Kaushal, D.P. Martin, J. Bischoff, Quantitative Evaluation of Endothelial Progenitors and Cardiac Valve Endothelial Cells: Proliferation and Differentiation on Poly-glycolic acid/Poly-4-hydroxybutyrate Scaffold in Response to Vascular Endothelial Growth Factor and Transforming Growth Factor β 1, *Tissue engineering* 9(3) (2003) 487-493.
- [49] J. Leor, S. Aboulafia-Etzion, A. Dar, L. Shapiro, I.M. Barbash, A. Battler, Y. Granot, S. Cohen, Bioengineered cardiac grafts: A new approach to repair the infarcted myocardium?, *Circulation* 102(19 Suppl 3) (2000) III56-61.
- [50] J. Leor, S. Cohen, Myocardial tissue engineering: creating a muscle patch for a wounded heart, *Annals of the New York Academy of Sciences* 1015 (2004) 312-9.
- [51] K. Matsubayashi, P.W. Fedak, D.A. Mickle, R.D. Weisel, T. Ozawa, R.K. Li, Improved left ventricular aneurysm repair with bioengineered vascular smooth muscle grafts, *Circulation* 108 Suppl 1 (2003) II219-25.
- [52] Y. Orlova, N. Magome, L. Liu, Y. Chen, K. Agladze, Electrospun nanofibers as a tool for architecture control in engineered cardiac tissue, *Biomaterials* 32(24) (2011) 5615-5624.
- [53] T. Ozawa, D.A. Mickle, R.D. Weisel, N. Koyama, S. Ozawa, R.K. Li, Optimal biomaterial for creation of autologous cardiac grafts, *Circulation* 106(12 Suppl 1) (2002) II76-82.
- [54] A.P. Pego, A.A. Poot, D.W. Grijpma, J. Feijen, Biodegradable elastomeric scaffolds for soft tissue engineering, *Journal of controlled release : official journal of the Controlled Release Society* 87(1-3) (2003) 69-79.
- [55] A.P. Pego, B. Siebum, M.J. Van Luyn, X.J. Gallego y Van Seijen, A.A. Poot, D.W. Grijpma, J. Feijen, Preparation of degradable porous structures based on 1,3-trimethylene

carbonate and D,L-lactide (co)polymers for heart tissue engineering, *Tissue Eng* 9(5) (2003) 981-94.

[56] U. Sarig, M. Machluf, Engineering cell platforms for myocardial regeneration, *Expert Opin Biol Ther* 11(8) (2011) 1055-77.

[57] W.K. Wan, G. Campbell, Z.F. Zhang, A.J. Hui, D.R. Boughner, Optimizing the tensile properties of polyvinyl alcohol hydrogel for the construction of a bioprosthetic heart valve stent, *Journal of biomedical materials research* 63(6) (2002) 854-61.

[58] W.Y. Yeong, N. Sudarmadji, H.Y. Yu, C.K. Chua, K.F. Leong, S.S. Venkatraman, Y.C. Boey, L.P. Tan, Porous polycaprolactone scaffold for cardiac tissue engineering fabricated by selective laser sintering, *Acta biomaterialia* 6(6) (2010) 2028-34.

[59] G. Zund, C.K. Breuer, T. Shinoka, P.X. Ma, R. Langer, J.E. Mayer, J.P. Vacanti, The in vitro construction of a tissue engineered bioprosthetic heart valve, *Eur J Cardiothorac Surg* 11(3) (1997) 493-7.

[60] Q.L. Loh, C. Choong, Three-dimensional scaffolds for tissue engineering applications: role of porosity and pore size, *Tissue engineering. Part B, Reviews* 19(6) (2013) 485-502.

[61] T.G. Deluzio, D.G. Seifu, K. Mequanint, 3D scaffolds in tissue engineering and regenerative medicine: beyond structural templates?, *Pharmaceutical Bioprocessing* 1(3) (2013) 267-281.

[62] C.M. Murphy, M.G. Haugh, F.J. O'Brien, The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering, *Biomaterials* 31(3) (2010) 461-6.

[63] B.J. Lawrence, S.V. Madhally, Cell colonization in degradable 3D porous matrices, *Cell adhesion & migration* 2(1) (2008) 9-16.

[64] B. Oberwallner, A. Brodarac, P. Anic, T. Saric, K. Wassilew, K. Neef, Y.H. Choi, C. Stamm, Human cardiac extracellular matrix supports myocardial lineage commitment of pluripotent stem cells, *Eur J Cardiothorac Surg* 47(3) (2015) 416-25; discussion 425.

[65] H.C. Ott, T.S. Matthiesen, S.-K. Goh, L.D. Black, S.M. Kren, T.I. Netoff, D.A. Taylor, Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart, *Nature medicine* 14(2) (2008) 213-221.

- [66] J.M. Wainwright, C.A. Czajka, U.B. Patel, D.O. Freytes, K. Tobita, T.W. Gilbert, S.F. Badylak, Preparation of cardiac extracellular matrix from an intact porcine heart, *Tissue Engineering Part C: Methods* 16(3) (2009) 525-532.
- [67] P.M. Crapo, S. Tottey, P.F. Slivka, S.F. Badylak, Effects of biologic scaffolds on human stem cells and implications for CNS tissue engineering, *Tissue Eng Part A* 20(1-2) (2014) 313-23.
- [68] Y. Eitan, U. Sarig, N. Dahan, M. Machluf, Acellular cardiac extracellular matrix as a scaffold for tissue engineering: in vitro cell support, remodeling, and biocompatibility, *Tissue engineering. Part C, Methods* 16(4) (2010) 671-83.
- [69] D.H. Reneker, I. Chun, Nanometre diameter fibres of polymer, produced by electrospinning, *Nanotechnology* 7(3) (1996) 216.
- [70] T. Lu, Y. Li, T. Chen, Techniques for fabrication and construction of three-dimensional scaffolds for tissue engineering, *International journal of nanomedicine* 8 (2013) 337-50.
- [71] G.C. Rutledge, S.V. Fridrikh, Formation of fibers by electrospinning, *Advanced drug delivery reviews* 59(14) (2007) 1384-91.
- [72] M.P. Prabhakaran, D. Kai, L. Ghasemi-Mobarakeh, S. Ramakrishna, Electrospun biocomposite nanofibrous patch for cardiac tissue engineering, *Biomedical materials* 6(5) (2011) 055001.
- [73] K.S. Rho, L. Jeong, G. Lee, B.M. Seo, Y.J. Park, S.D. Hong, S. Roh, J.J. Cho, W.H. Park, B.M. Min, Electrospinning of collagen nanofibers: effects on the behavior of normal human keratinocytes and early-stage wound healing, *Biomaterials* 27(8) (2006) 1452-61.
- [74] J.B. Lee, S.I. Jeong, M.S. Bae, D.H. Yang, D.N. Heo, C.H. Kim, E. Alsberg, I.K. Kwon, Highly porous electrospun nanofibers enhanced by ultrasonication for improved cellular infiltration, *Tissue Eng Part A* 17(21-22) (2011) 2695-702.
- [75] B. Sun, Y.Z. Long, F. Yu, M.M. Li, H.D. Zhang, W.J. Li, T.X. Xu, Self-assembly of a three-dimensional fibrous polymer sponge by electrospinning, *Nanoscale* 4(6) (2012) 2134-7.
- [76] Y. Yokoyama, S. Hattori, C. Yoshikawa, Y. Yasuda, H. Koyama, T. Takato, H. Kobayashi, Novel wet electrospinning system for fabrication of spongiform nanofiber 3-dimensional fabric, *Materials letters* 63(9) (2009) 754-756.

- [77] H. Chen, Y. Peng, S. Wu, L.P. Tan, Electrospun 3D Fibrous Scaffolds for Chronic Wound Repair, *Materials* (Basel) 9(4) (2016).
- [78] J.H. Brown, P. Das, M.D. DiVito, D. Ivancic, L.P. Tan, J.A. Wertheim, Nanofibrous PLGA electrospun scaffolds modified with type I collagen influence hepatocyte function and support viability in vitro, *Acta biomaterialia* 73 (2018) 217-227.
- [79] J. Barallobre-Barreiro, A. Didangelos, F.A. Schoendube, I. Drozdov, X. Yin, M. Fernandez-Caggiano, P. Willeit, V.O. Puntmann, G. Aldama-Lopez, A.M. Shah, N. Domenech, M. Mayr, Proteomics analysis of cardiac extracellular matrix remodeling in a porcine model of ischemia/reperfusion injury, *Circulation* 125(6) (2012) 789-802.
- [80] K. Methe, H. Backdahl, B.R. Johansson, N. Nayakawde, G. Dellgren, S. Sumitran-Holgersson, An alternative approach to decellularize whole porcine heart, *BioResearch open access* 3(6) (2014) 327-38.
- [81] J.M. Singelyn, J.A. DeQuach, S.B. Seif-Naraghi, R.B. Littlefield, P.J. Schup-Magoffin, K.L. Christman, Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering, *Biomaterials* 30(29) (2009) 5409-16.
- [82] J.M. Wainwright, C.A. Czajka, U.B. Patel, D.O. Freytes, K. Tobita, T.W. Gilbert, S.F. Badylak, Preparation of cardiac extracellular matrix from an intact porcine heart, *Tissue engineering. Part C, Methods* 16(3) (2010) 525-32.
- [83] B. Wang, M.E. Tedder, C.E. Perez, G. Wang, A.L. de Jongh Curry, F. To, S.H. Elder, L.N. Williams, D.T. Simionescu, J. Liao, Structural and biomechanical characterizations of porcine myocardial extracellular matrix, *Journal of materials science. Materials in medicine* 23(8) (2012) 1835-47.
- [84] D.A. Taylor, B.Z. Atkins, P. Hungspreugs, T.R. Jones, M.C. Reedy, K.A. Hutcheson, D.D. Glower, W.E. Kraus, Regenerating functional myocardium: improved performance after skeletal myoblast transplantation, *Nature medicine* 4(8) (1998) 929-33.
- [85] J. van Ramshorst, J.J. Bax, S.L. Beeres, P. Dibbets-Schneider, S.D. Roes, M.P. Stokkel, A. de Roos, W.E. Fibbe, J.J. Zwaginga, E. Boersma, M.J. Schalij, D.E. Atsma, Intramyocardial bone marrow cell injection for chronic myocardial ischemia: a randomized controlled trial, *Jama* 301(19) (2009) 1997-2004.
- [86] A.P. Beltrami, L. Barlucchi, D. Torella, M. Baker, F. Limana, S. Chimenti, H. Kasahara, M. Rota, E. Musso, K. Urbanek, A. Leri, J. Kajstura, B. Nadal-Ginard, P.

Anversa, Adult cardiac stem cells are multipotent and support myocardial regeneration, *Cell* 114(6) (2003) 763-76.

[87] B. Nadal-Ginard, P. Anversa, J. Kajstura, A. Leri, Cardiac stem cells and myocardial regeneration, *Novartis Foundation symposium* 265 (2005) 142-54; discussion 155-7, 204-11.

[88] D. Torella, G.M. Ellison, S. Mendez-Ferrer, B. Ibanez, B. Nadal-Ginard, Resident human cardiac stem cells: role in cardiac cellular homeostasis and potential for myocardial regeneration, *Nature clinical practice. Cardiovascular medicine* 3 Suppl 1 (2006) S8-13.

[89] O. Caspi, A. Lesman, Y. Basevitch, A. Gepstein, G. Arbel, I.H. Habib, L. Gepstein, S. Levenberg, Tissue engineering of vascularized cardiac muscle from human embryonic stem cells, *Circ Res* 100(2) (2007) 263-72.

[90] T.J. Nelson, A. Martinez-Fernandez, S. Yamada, C. Perez-Terzic, Y. Ikeda, A. Terzic, Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells, *Circulation* 120(5) (2009) 408-16.

[91] C. Templin, R. Zweigerdt, K. Schwanke, R. Olmer, J.R. Ghadri, M.Y. Emmert, E. Muller, S.M. Kuest, S. Cohrs, R. Schibli, P. Kronen, M. Hilbe, A. Reinisch, D. Strunk, A. Haverich, S. Hoerstrup, T.F. Luscher, P.A. Kaufmann, U. Landmesser, U. Martin, Transplantation and tracking of human-induced pluripotent stem cells in a pig model of myocardial infarction: assessment of cell survival, engraftment, and distribution by hybrid single photon emission computed tomography/computed tomography of sodium iodide symporter transgene expression, *Circulation* 126(4) (2012) 430-9.

[92] G. Vunjak-Novakovic, N. Tandon, A. Godier, R. Maidhof, A. Marsano, T.P. Martens, M. Radisic, Challenges in cardiac tissue engineering, *Tissue engineering. Part B, Reviews* 16(2) (2010) 169-87.

[93] E. Cambria, F.S. Pasqualini, P. Wolint, J. Gunter, J. Steiger, A. Bopp, S.P. Hoerstrup, M.Y. Emmert, Translational cardiac stem cell therapy: advancing from first-generation to next-generation cell types, *NPJ Regen Med* 2 (2017) 17.

[94] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, *Cell* 126(4) (2006) 663-76.

- [95] R. Josowitz, X. Carvajal-Vergara, I.R. Lemischka, B.D. Gelb, Induced pluripotent stem cell-derived cardiomyocytes as models for genetic cardiovascular disorders, *Current opinion in cardiology* 26(3) (2011) 223-229.
- [96] Z. Liu, J. Zhou, H. Wang, M. Zhao, C. Wang, Current status of induced pluripotent stem cells in cardiac tissue regeneration and engineering, *Regenerative medicine research* 1(1) (2013) 6.
- [97] M.C. Puri, A. Nagy, Concise review: Embryonic stem cells versus induced pluripotent stem cells: the game is on, *Stem Cells* 30(1) (2012) 10-4.
- [98] J.J. Savla, B.C. Nelson, C.N. Perry, E.D. Adler, Induced pluripotent stem cells for the study of cardiovascular disease, *J Am Coll Cardiol* 64(5) (2014) 512-9.
- [99] W.J. Adams, Y. Zhang, J. Cloutier, P. Kuchimanchi, G. Newton, S. Sehrawat, W.C. Aird, T.N. Mayadas, F.W. Lusinskas, G. Garcia-Cardena, Functional vascular endothelium derived from human induced pluripotent stem cells, *Stem Cell Reports* 1(2) (2013) 105-13.
- [100] D. Iyer, L. Gambardella, W.G. Bernard, F. Serrano, V.L. Mascetti, R.A. Pedersen, A. Talasila, S. Sinha, Robust derivation of epicardium and its differentiated smooth muscle cell progeny from human pluripotent stem cells, *Development* 142(8) (2015) 1528-41.
- [101] S. Ting, A. Chen, S. Reuveny, S. Oh, An intermittent rocking platform for integrated expansion and differentiation of human pluripotent stem cells to cardiomyocytes in suspended microcarrier cultures, *Stem cell research* 13(2) (2014) 202-13.
- [102] Y. Wang, J. Hu, J. Jiao, Z. Liu, Z. Zhou, C. Zhao, L.J. Chang, Y.E. Chen, P.X. Ma, B. Yang, Engineering vascular tissue with functional smooth muscle cells derived from human iPS cells and nanofibrous scaffolds, *Biomaterials* 35(32) (2014) 8960-9.
- [103] A.D. Witty, A. Mihic, R.Y. Tam, S.A. Fisher, A. Mikryukov, M.S. Shoichet, R.K. Li, S.J. Kattman, G. Keller, Generation of the epicardial lineage from human pluripotent stem cells, *Nat Biotechnol* 32(10) (2014) 1026-35.
- [104] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, *Cell* 131(5) (2007) 861-72.
- [105] H. Liu, F. Zhu, J. Yong, P. Zhang, P. Hou, H. Li, W. Jiang, J. Cai, M. Liu, K. Cui, X. Qu, T. Xiang, D. Lu, X. Chi, G. Gao, W. Ji, M. Ding, H. Deng, Generation of induced

pluripotent stem cells from adult rhesus monkey fibroblasts, *Cell stem cell* 3(6) (2008) 587-90.

[106] T. Ezashi, B.P. Telugu, A.P. Alexenko, S. Sachdev, S. Sinha, R.M. Roberts, Derivation of induced pluripotent stem cells from pig somatic cells, *Proc Natl Acad Sci U S A* 106(27) (2009) 10993-8.

[107] W. Li, W. Wei, S. Zhu, J. Zhu, Y. Shi, T. Lin, E. Hao, A. Hayek, H. Deng, S. Ding, Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors, *Cell stem cell* 4(1) (2009) 16-9.

[108] A. Honda, M. Hirose, M. Hatori, S. Matoba, H. Miyoshi, K. Inoue, A. Ogura, Generation of induced pluripotent stem cells in rabbits: potential experimental models for human regenerative medicine, *The Journal of biological chemistry* 285(41) (2010) 31362-9.

[109] H. Shimada, A. Nakada, Y. Hashimoto, K. Shigeno, Y. Shionoya, T. Nakamura, Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors, *Molecular reproduction and development* 77(1) (2010) 2.

[110] Y. Li, M. Cang, A.S. Lee, K. Zhang, D. Liu, D. Kaufman, Reprogramming of sheep fibroblasts into pluripotency under a drug-inducible expression of mouse-derived defined factors, *PloS one* 6(1) (2011) e15947.

[111] B. Huang, T. Li, L. Alonso-Gonzalez, R. Gorre, S. Keatley, A. Green, P. Turner, P.K. Kallingappa, V. Verma, B. Oback, A virus-free poly-promoter vector induces pluripotency in quiescent bovine cells under chemically defined conditions of dual kinase inhibition, *PloS one* 6(9) (2011) e24501.

[112] J. Yu, M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, Slukvin, II, J.A. Thomson, Induced pluripotent stem cell lines derived from human somatic cells, *Science* 318(5858) (2007) 1917-20.

[113] R. Olmer, A. Haase, S. Merkert, W. Cui, J. Paleček, C. Ran, A. Kirschning, T. Scheper, S. Glage, K. Miller, Long term expansion of undifferentiated human iPS and ES cells in suspension culture using a defined medium, *Stem cell research* 5(1) (2010) 51-64.

[114] R. Zweigerdt, R. Olmer, H. Singh, A. Haverich, U. Martin, Scalable expansion of human pluripotent stem cells in suspension culture, *Nature protocols* 6(5) (2011) 689-700.

- [115] R. Tee, Z. Lokmic, W.A. Morrison, R.J. Dilley, Strategies in cardiac tissue engineering, *ANZ J Surg* 80(10) (2010) 683-93.
- [116] G.Y. Koh, M.G. Klug, M.H. Soonpaa, L.J. Field, Differentiation and long-term survival of C2C12 myoblast grafts in heart, *The Journal of clinical investigation* 92(3) (1993) 1548-54.
- [117] G.Y. Koh, M.H. Soonpaa, M.G. Klug, L.J. Field, Long-term survival of AT-1 cardiomyocyte grafts in syngeneic myocardium, *The American journal of physiology* 264(5 Pt 2) (1993) H1727-33.
- [118] P. Menasche, O. Alfieri, S. Janssens, W. McKenna, H. Reichenspurner, L. Trinquart, J.T. Vilquin, J.P. Marolleau, B. Seymour, J. Larghero, S. Lake, G. Chatellier, S. Solomon, M. Desnos, A.A. Hagege, The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation, *Circulation* 117(9) (2008) 1189-200.
- [119] M. Hofmann, K.C. Wollert, G.P. Meyer, A. Menke, L. Arseniev, B. Hertenstein, A. Ganser, W.H. Knapp, H. Drexler, Monitoring of bone marrow cell homing into the infarcted human myocardium, *Circulation* 111(17) (2005) 2198-2202.
- [120] A. Behfar, R. Crespo-Diaz, A. Terzic, B.J. Gersh, Cell therapy for cardiac repair--lessons from clinical trials, *Nat Rev Cardiol* 11(4) (2014) 232-46.
- [121] F. Wang, J. Guan, Cellular cardiomyoplasty and cardiac tissue engineering for myocardial therapy, *Advanced drug delivery reviews* 62(7-8) (2010) 784-97.
- [122] K.R. Stevens, K.L. Kreutziger, S.K. Dupras, F.S. Korte, M. Regnier, V. Muskheli, M.B. Nourse, K. Bendixen, H. Reinecke, C.E. Murry, Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue, *Proc Natl Acad Sci U S A* 106(39) (2009) 16568-73.
- [123] S. Masuda, T. Shimizu, M. Yamato, T. Okano, Cell sheet engineering for heart tissue repair, *Advanced drug delivery reviews* 60(2) (2008) 277-285.
- [124] N. Matsuda, T. Shimizu, M. Yamato, T. Okano, Tissue engineering based on cell sheet technology, *Advanced Materials* 19(20) (2007) 3089-3099.
- [125] N. Sekiya, G. Matsumiya, S. Miyagawa, A. Saito, T. Shimizu, T. Okano, N. Kawaguchi, N. Matsuura, Y. Sawa, Layered implantation of myoblast sheets attenuates

adverse cardiac remodeling of the infarcted heart, *The Journal of thoracic and cardiovascular surgery* 138(4) (2009) 985-93.

[126] R.E. Akins, R.A. Boyce, M.L. Madonna, N.A. Schroedl, S.R. Gonda, T.A. McLaughlin, C.R. Hartzell, Cardiac organogenesis in vitro: reestablishment of three-dimensional tissue architecture by dissociated neonatal rat ventricular cells, *Tissue Eng* 5(2) (1999) 103-18.

[127] R.L. Carrier, M. Papadaki, M. Rupnick, F.J. Schoen, N. Bursac, R. Langer, L.E. Freed, G. Vunjak-Novakovic, Cardiac tissue engineering: cell seeding, cultivation parameters, and tissue construct characterization, *Biotechnology and bioengineering* 64(5) (1999) 580-9.

[128] T. Chandy, G.H. Rao, R.F. Wilson, G.S. Das, The development of porous alginate/elastin/PEG composite matrix for cardiovascular engineering, *Journal of biomaterials applications* 17(4) (2003) 287-301.

[129] A. Dar, M. Shachar, J. Leor, S. Cohen, Optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds, *Biotechnology and bioengineering* 80(3) (2002) 305-12.

[130] T. Eschenhagen, M. Didie, F. Munzel, P. Schubert, K. Schneiderbanger, W.H. Zimmermann, 3D engineered heart tissue for replacement therapy, *Basic research in cardiology* 97 Suppl 1 (2002) I146-52.

[131] T. Kofidis, P. Akhyari, J. Boublik, P. Theodorou, U. Martin, A. Ruhparwar, S. Fischer, T. Eschenhagen, H.P. Kubis, T. Kraft, R. Leyh, A. Haverich, In vitro engineering of heart muscle: artificial myocardial tissue, *The Journal of thoracic and cardiovascular surgery* 124(1) (2002) 63-9.

[132] R.K. Li, Z.Q. Jia, R.D. Weisel, D.A. Mickle, A. Choi, T.M. Yau, Survival and function of bioengineered cardiac grafts, *Circulation* 100(19 Suppl) (1999) II63-9.

[133] W.H. Zimmermann, C. Fink, D. Kralisch, U. Remmers, J. Weil, T. Eschenhagen, Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes, *Biotechnology and bioengineering* 68(1) (2000) 106-14.

[134] R.J. Lee, A. Hinson, S. Helgersson, R. Bauernschmitt, H.N. Sabbah, Polymer-based restoration of left ventricular mechanics, *Cell transplantation* 22(3) (2013) 529-33.

- [135] W.H. Zimmermann, I. Melnychenko, T. Eschenhagen, Engineered heart tissue for regeneration of diseased hearts, *Biomaterials* 25(9) (2004) 1639-47.
- [136] P. Akhyari, P.W. Fedak, R.D. Weisel, T.Y. Lee, S. Verma, D.A. Mickle, R.K. Li, Mechanical stretch regimen enhances the formation of bioengineered autologous cardiac muscle grafts, *Circulation* 106(12 Suppl 1) (2002) I137-42.
- [137] M.Y. Tan, W. Zhi, R.Q. Wei, Y.C. Huang, K.P. Zhou, B. Tan, L. Deng, J.C. Luo, X.Q. Li, H.Q. Xie, Repair of infarcted myocardium using mesenchymal stem cell seeded small intestinal submucosa in rabbits, *Biomaterials* 30(19) (2009) 3234-3240.
- [138] H. Piao, J.S. Kwon, S. Piao, J.H. Sohn, Y.S. Lee, J.W. Bae, K.K. Hwang, D.W. Kim, O. Jeon, B.S. Kim, Y.B. Park, M.C. Cho, Effects of cardiac patches engineered with bone marrow-derived mononuclear cells and PGCL scaffolds in a rat myocardial infarction model, *Biomaterials* 28(4) (2007) 641-9.
- [139] S. Heydarkhan-Hagvall, K. Schenke-Layland, A.P. Dhanasopon, F. Rofail, H. Smith, B.M. Wu, R. Shemin, R.E. Beygui, W.R. MacLellan, Three-dimensional electrospun ECM-based hybrid scaffolds for cardiovascular tissue engineering, *Biomaterials* 29(19) (2008) 2907-14.
- [140] J. Zhang, M. Klos, G.F. Wilson, A.M. Herman, X. Lian, K.K. Raval, M.R. Barron, L. Hou, A.G. Soerens, J. Yu, S.P. Palecek, G.E. Lyons, J.A. Thomson, T.J. Herron, J. Jalife, T.J. Kamp, Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method, *Circ Res* 111(9) (2012) 1125-36.
- [141] B. Lin, T.Y. Lu, L. Yang, Hear the beat: decellularized mouse heart regenerated with human induced pluripotent stem cells, *Expert Rev Cardiovasc Ther* 12(2) (2014) 135-7.
- [142] T.Y. Lu, B. Lin, J. Kim, M. Sullivan, K. Tobita, G. Salama, L. Yang, Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells, *Nat Commun* 4 (2013) 2307.
- [143] M. Khan, Y. Xu, S. Hua, J. Johnson, A. Belevych, P.M. Janssen, S. Gyorke, J. Guan, M.G. Angelos, Evaluation of Changes in Morphology and Function of Human Induced Pluripotent Stem Cell Derived Cardiomyocytes (hiPSC-CMs) Cultured on an Aligned-Nanofiber Cardiac Patch, *PloS one* 10(5) (2015) e0126338.
- [144] Y. Chen, D. Zeng, L. Ding, X.-L. Li, X.-T. Liu, W.-J. Li, T. Wei, S. Yan, J.-H. Xie, L. Wei, Three-dimensional poly-(ϵ -caprolactone) nanofibrous scaffolds directly promote

the cardiomyocyte differentiation of murine-induced pluripotent stem cells through Wnt/ β -catenin signaling, *BMC cell biology* 16(1) (2015) 22.

[145] C. Williams, E. Budina, W.L. Stoppel, K.E. Sullivan, S. Emani, S.M. Emani, L.D. Black, 3rd, Cardiac extracellular matrix-fibrin hybrid scaffolds with tunable properties for cardiovascular tissue engineering, *Acta biomaterialia* 14 (2015) 84-95.

[146] W.L. Stoppel, D. Hu, I.J. Domian, D.L. Kaplan, L.D. Black, 3rd, Anisotropic silk biomaterials containing cardiac extracellular matrix for cardiac tissue engineering, *Biomedical materials* 10(3) (2015) 034105.

[147] Y. Efraim, H. Sarig, N. Cohen Anavy, U. Sarig, E. de Berardinis, S.Y. Chaw, M. Krishnamoorthi, J. Kalifa, H. Bogireddi, T.V. Duc, T. Kofidis, L. Baruch, F.Y.C. Boey, S.S. Venkatraman, M. Machluf, Biohybrid cardiac ECM-based hydrogels improve long term cardiac function post myocardial infarction, *Acta biomaterialia* 50 (2017) 220-233.

[148] S. Beth, A. Ron, B. Limor, E. Yael, G. Idit, E. Ofek, D. Tzila, G. Lior, Z. Eyal, M. Marcelle, Electrospun Extracellular Matrix: Paving the Way to Tailor-Made Natural Scaffolds for Cardiac Tissue Regeneration, *Advanced Functional Materials* 27(34) (2017) 1700427.

[149] J.P. Guyette, J.M. Charest, R.W. Mills, B.J. Jank, P.T. Moser, S.E. Gilpin, J.R. Gershlak, T. Okamoto, G. Gonzalez, D.J. Milan, G.R. Gaudette, H.C. Ott, Bioengineering Human Myocardium on Native Extracellular Matrix, *Circ Res* 118(1) (2016) 56-72.

[150] H. Shin, S. Jo, A.G. Mikos, Biomimetic materials for tissue engineering, *Biomaterials* 24(24) (2003) 4353-64.

[151] S. Iwai, Y. Sawa, H. Ichikawa, S. Taketani, E. Uchimura, G. Chen, M. Hara, J. Miyake, H. Matsuda, Biodegradable polymer with collagen microsphere serves as a new bioengineered cardiovascular prosthesis, *The Journal of thoracic and cardiovascular surgery* 128(3) (2004) 472-9.

[152] L.A. Hidalgo-Bastida, J.J. Barry, N.M. Everitt, F.R. Rose, L.D. Buttery, I.P. Hall, W.C. Claycomb, K.M. Shakesheff, Cell adhesion and mechanical properties of a flexible scaffold for cardiac tissue engineering, *Acta biomaterialia* 3(4) (2007) 457-62.

[153] D.A. Stout, J. Yoo, A.N. Santiago-Miranda, T.J. Webster, Mechanisms of greater cardiomyocyte functions on conductive nanoengineered composites for cardiovascular application, *International journal of nanomedicine* 7 (2012) 5653-69.

- [154] E. Rosellini, C. Cristallini, G.D. Guerra, N. Barbani, Surface chemical immobilization of bioactive peptides on synthetic polymers for cardiac tissue engineering, *Journal of biomaterials science. Polymer edition* 26(9) (2015) 515-33.
- [155] M.J. Humphries, S.K. Akiyama, A. Komoriya, K. Olden, K.M. Yamada, Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion, *The Journal of Cell Biology* 103(6) (1986) 2637-2647.
- [156] M. Tallawi, E. Rosellini, N. Barbani, M.G. Cascone, R. Rai, G. Saint-Pierre, A.R. Boccaccini, Strategies for the chemical and biological functionalization of scaffolds for cardiac tissue engineering: a review, *J R Soc Interface* 12(108) (2015) 20150254.
- [157] M. Borkenhagen, J.F. Clemence, H. Sigrist, P. Aebischer, Three-dimensional extracellular matrix engineering in the nervous system, *Journal of biomedical materials research* 40(3) (1998) 392-400.
- [158] D.L. Elbert, J.A. Hubbell, Conjugate addition reactions combined with free-radical cross-linking for the design of materials for tissue engineering, *Biomacromolecules* 2(2) (2001) 430-41.
- [159] J.A. Hubbell, Bioactive biomaterials, *Current opinion in biotechnology* 10(2) (1999) 123-9.
- [160] B.K. Mann, R.H. Schmedlen, J.L. West, Tethered-TGF- β increases extracellular matrix production of vascular smooth muscle cells, *Biomaterials* 22(5) (2001) 439-444.
- [161] R.A. Stile, K.E. Healy, Thermo-responsive peptide-modified hydrogels for tissue regeneration, *Biomacromolecules* 2(1) (2001) 185-94.
- [162] K.P. Walluscheck, G. Steinhoff, S. Kelm, A. Haverich, Improved endothelial cell attachment on ePTFE vascular grafts pretreated with synthetic RGD-containing peptides, *European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery* 12(3) (1996) 321-30.
- [163] M. Kharaziha, S.R. Shin, M. Nikkhah, S.N. Topkaya, N. Masoumi, N. Annabi, M.R. Dokmeci, A. Khademhosseini, Tough and Flexible CNT-Polymeric Hybrid Scaffolds for Engineering Cardiac Constructs, *Biomaterials* 35(26) (2014) 7346-54.
- [164] J.O. You, M. Rafat, G.J. Ye, D.T. Auguste, Nanoengineering the heart: conductive scaffolds enhance connexin 43 expression, *Nano letters* 11(9) (2011) 3643-8.

- [165] E. Alsberg, H.A. von Recum, M.J. Mahoney, Environmental cues to guide stem cell fate decision for tissue engineering applications, *Expert Opin Biol Ther* 6(9) (2006) 847-66.
- [166] F.P. Barry, J.M. Murphy, Mesenchymal stem cells: clinical applications and biological characterization, *Int J Biochem Cell Biol* 36(4) (2004) 568-84.
- [167] A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher, Matrix elasticity directs stem cell lineage specification, *Cell* 126(4) (2006) 677-89.
- [168] F. Gelain, D. Bottai, A. Vescovi, S. Zhang, Designer self-assembling peptide nanofiber scaffolds for adult mouse neural stem cell 3-dimensional cultures, *PloS one* 1 (2006) e119.
- [169] A. Kumar, A. Patel, L. Duvalsaint, M. Desai, E.D. Marks, Thymosin β 4 coated nanofiber scaffolds for the repair of damaged cardiac tissue, *J Nanobiotechnol* 12(10) (2014).
- [170] G. Kumar, C.K. Tison, K. Chatterjee, P.S. Pine, J.H. McDaniel, M.L. Salit, M.F. Young, C.G. Simon, Jr., The determination of stem cell fate by 3D scaffold structures through the control of cell shape, *Biomaterials* 32(35) (2011) 9188-96.
- [171] X. Lian, J. Zhang, S.M. Azarin, K. Zhu, L.B. Hazeltine, X. Bao, C. Hsiao, T.J. Kamp, S.P. Palecek, Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ β -catenin signaling under fully defined conditions, *Nature protocols* 8(1) (2013) 162-175.
- [172] M.M. Nava, M.T. Raimondi, R. Pietrabissa, Controlling self-renewal and differentiation of stem cells via mechanical cues, *BioMed Research International* 2012 (2012).
- [173] M. Radisic, H. Park, H. Shing, T. Consi, F.J. Schoen, R. Langer, L.E. Freed, G. Vunjak-Novakovic, Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds, *Proc Natl Acad Sci U S A* 101(52) (2004) 18129-34.
- [174] H.G. Senel Ayaz, A. Perets, H. Ayaz, K.D. Gilroy, M. Govindaraj, D. Brookstein, P.I. Leikes, Textile-templated electrospun anisotropic scaffolds for regenerative cardiac tissue engineering, *Biomaterials* 35(30) (2014) 8540-52.

Chapter 3

Experimental Methodology

This chapter summarizes the materials, methods and instruments used in this thesis. The first section illustrates the list of materials used in the thesis. The following sections describe the methods used of the scaffold fabrication followed by the characterization techniques used to study the scaffolds' morphology, chemistry, composition, mechanical properties, cell interactions, and statistics.

3.1 Overall Experimental Design

Figure 3-1 represents the overall experimental design of the work. Aim 1 focuses on additional characterization of the pcECM to identify the parameters which can be mimicked in the electrospinning process. Based on the pcECM characterization, wet electrospinning was used to fabricate a 3D synthetic scaffold. This scaffold was characterized for morphology, pore size and mechanical properties. The scaffold was modified with liquid pcECM to incorporate tissue specific bioactivity. Finally, the ability of this 3D composite scaffold (3DCS) to support cells was studied. Aim 2 and Aim 3 focused on the interaction of hiPSC-CM and hiPSC, respectively, with the pcECM and their equivalent 3DCS. Protein expression using immunofluorescence was carried out to investigate and compare the interaction between cells of various potency levels and the pcECM and its equivalent 3DCS.

OVERALL EXPERIMENT DESIGN

Figure 3-1 Overall experimental design

3.2 General Materials and Kits

Alamar Blue TM Bio-Rad (BUF012B)

Albumin from bovine serum (BSA) Sigma (#A2153)

Chloroform Bio-Lab (#03080521)

DAPI Sigma (#D-8417)

DMSO Merck (#2324948)

DNase Deoxyribonuclease (For RT-PCR) Promega (#M610A)

EDC Sigma (#39391)

Ethanol absolute Frutarom LTD (#5551640)

Fetal bovine serum (FBS) Biological industries (#04-001-1A)
Fluoromount-G (+DAPI) Southern Biotech (#0100-20)
Formaldehyde (Formalin, 37% in DDW) Aldrich (#533998)
Griess reagent system Promega (#G2930)
Hoechst 33258 Life sciences (#H21491)
Hydrochloric Acid (HCl) Merck (#109057)
Isopropyl alcohol Gadot (#5551640)
Lipopolysaccharide (LPS) Sigma (#L3129)
Methanol Frutarom LTD (#5558520)
Microscope slides superfrost plus Menzel Glaser (#J1800AMNZ)
Paraformaldehyde (PFA) EMS (#15710)
Phalloidin -FITC
Potassium Chloride (KCl) Merck (#12257)
Potassium hydroxide (KOH) Frutarom (#2355528900)
Potassium Phosphate (KH₂PO₄) Sigma (#P5655)
Sodium azide Acros organics (#26628-22)
Sodium acetate Sigma (#S2889)
Sodium chloride Frutarom (#5550950)
Sodium dodecyl sulfate (SDS) Sigma (#L3771)
Sodium hydroxide Merck (#1.06498.1000)
Sodium Phosphate Carlo Erba (#480087)
Tissue-Tek OCT compound Sakura (#4583)
Tri Reagent TM Sigma (#T9424)
Triton X-100 Merck (#11869)
Trypan Blue Solution 0.4% Sigma (#T8154)

3.2.1 **Antibodies**

PE/Cy5 anti-human CD90 (Thy1) Antibody, Biolegend (#328112)
APC anti-human CD44 Antibody, Biolegend (#338806)
APC anti-human CD29 Antibody, Biolegend (#303008)
APC anti-human CD105 Antibody, Biolegend (#323208)

APC anti-human CD73 Antibody, Biolegend (#344003)

Human Embryonic marker panel (Oct4, Nanog, Tra-1-60, SOX2 and SSEA4), Abcam, (ab109884)

Mouse anti human Sarcomeric α -actinin, Sigma (#A7811)

Mouse anti human Cardiac troponin I, Millipore (#MAB1691)

Goat anti mouse. Alexa Flour 647 Molecular probes (#A21235)

3.2.2 **Enzymes**

- Pepsin- Sigma (#P7000)
- Trypsin from porcine pancreas (type IX-S, 13,000-20,000 BAEE units/mg protein) (#T0303, Sigma-Aldrich)
- Trypsin from porcine pancreas for tissue culture (#T4799, Sigma-Aldrich)

3.2.3 **Cell Culture Reagents and Media**

Unless otherwise stated, all cell culture and reagents were purchased from Biological Industries, Beit Haemek, Israel.

FCS - Fetal Calf Serum- Biological Industries, (#04-001-1A)

FBS – Defined fetal bovine serum, Hyclone (#3007003)

CaCl₂ - Baker (#0070)

Non-essential amino acid- (#01-340-1)

L-glutamine- s (#41-218)

Medium M-199 (Earle's) with L-glutamine (#01-080-1A)

Medium DMEM with D-glucose 4500 mg/lit, with L-glutamine (#11-050-1M)

Medium DMEM (low) with D-glucose 1000mg/lit (#11-050-1G)

Medium α -MEM (#01-042-1G)

F-12 supplement (Gibco, #21765029)

2-mercaptoethanol (Sigma, M3148)

bFGF (Sigma, #F0291)

Penicillin & Streptomycin (P/S) x10, 10000unit/per ml (#03-031-5B)

Trypsin EDTA- solution, EDTA 0.02% (#03-050-1A)

Aquagard-1 (for disinfecting water in CO₂ incubators), 10ml per L (#01-867-1B)

Aquagard-2 solution (for disinfecting water bath), 2ml per L (#01-867-1B)

BioMYC-3 antibiotic solution 100x (#03-038-1C).

Amphotericin B (Fungizone, #15290026, Life sciences, Gibco)

3.2.4 Cells, Culture Medium and Maintenance

All cultures were maintained in 37°C and humidified atmosphere of 5% CO₂.

RAW 264.7 – Murine macrophage cell line, (#TIB-71, ATCC). Were used for In-Vitro biocompatibility assays. The base medium for this cell line is DMEM supplemented with 10% FCS, 1% Pen-Strep® and 0.4% Fungizone.

Human bone marrow mesenchymal stem cells (hMSCs) (Lonza, Switzerland) were cultured using α MEM, supplemented with 10% FCS, 1% Pen-Strep, 0.4% Fungizone, and 5ng/mL basic fibroblast growth factor (Biological Industries, Israel). Media was replaced every 2 days. Cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Two HiPSC (hiPSCs) were used in this thesis.

HiPSC (hiPSC), IMR-90 (WiCell bank, WI, with signed MOU), were cultured in mTeSR™1 (STEMCELL Technologies, Canada) medium on tissue culture plates coated with Geltrex (Life technologies, Singapore) at 37 °C in 5% CO₂ incubator. The training for maintenance, expansion and differentiation of the hiPSC was performed at the Bioprocessing Technology institute, Singapore (BTI) under a signed non-disclosure agreement (NDA) and is based on a micro carrier (MC) platform as previously published [1]. Briefly, hiPSCs were cultured for 7 days on Geltrex coated micro carriers with daily media changes for expansion. The differentiation was performed in a spinner flask with small molecules CHIR99021 and IWR 1 endo. Following 12 days of differentiation, the were harvested with TrypLE dissociation.

Another hiPSC cell line (UT) was kindly provided by Prof. Lior Gepstein's lab (Faculty of Medicine, Technion, Israel), and cultured according to their protocol [2]. The hiPSC

colonies were plated on Matrigel-coated plates and cultured in complete mTeSR1 medium. When cells reach ~85% confluency, they were split at 1:6 using 0.5mM EDTA and re-plated in mTeSR medium supplemented with 2 μ M Thiazovivin (ROCK inhibitor). After 4 days (day 0), if cells are 80- 90% confluent, the differentiation process was initiated by changing the culture medium to the differentiation medium. The differentiation medium used was RPMI-B27 and containing RPMI supplemented with B27 minus insulin. On day 0 the mTeSR was replaced with RPMI-B27supplemented with 6 μ M CHIR99021 (mimicking activation of the canonical Wnt pathway). On day 2 the medium was exchanged with RPMI-B27 supplemented with 2 μ M Wnt-C59 (a potent Wnt signaling pathway inhibitor). From day 4 onward, the cells were cultured with RPMI-B27 medium. Contracting areas usually appear from day 8 to day 12 of differentiation.

3.2.5 **Buffers and Solutions**

PBS

8.1mM Na₂HPO₄

1.47mM KH₂PO₄

0.137M NaCl

2.68mM KCl

pH= 7.4

Saline

NaCl 0.9%

pH = 7.4

Trypsin-EDTA

0.05% Trypsin

0.02% EDTA

Triton X-100- Ammonium hydroxide

1% triton X-100 and 1% NH₄OH in PBS

Formalin

10% formalin in PBS

4% PFA

4% PFA in PBS (diluted from purchased ampule of 16% PFA in DDW).

Natural Buffered Formalin 10%:

3.2.6 Software

NIS Elements – Nikon, Japan. Used for quantification of fluorescence and histology images.

ZEN light edition, 2011 – blue and black versions, Carl Zeiss, Germany. Used for image acquisition and analyses for confocal microscopy (LSM710 and LSM510meta) results.

ImageJ, NIH free ware. Used for image post processing.

FCS Express, De Novo, Used for FACS data analysis

OriginPro 8, OriginLab, Used for all statistical analysis

3.3 Decellularization and Liquid pcECM Preparation

Decellularization is a technique in which the extracellular matrix (ECM) of a tissue is isolated by the removal or stripping of the cellular components of the tissue through physical, chemical and/or enzymatic treatments. The process results in a decellularized ECM which can be used

as a scaffold material in artificial organ development or tissue engineering. Post isolation, this decellularized ECM materials can be seeded with different cells to achieve a tissue construct.

For decellularization, hearts from healthy commercial slaughter-weight female pigs were harvested and immersed in ice-cold sterile PBS, with antibiotics (Penicillin/Streptomycin and Fungizone). The atria, right ventricle and any excess fat were removed, and the left ventricle was excised, and 3 mm slices were prepared.

Figure 3-2 Decellularization and liquid ECM preparation reaction scheme [3, 4].

The decellularization procedure (Figure 3-2) consisted of three steps: alternating hyper/hypo tonic solutions (1.1% and 0.7% (w/v) NaCl in DDW, respectively); enzymatic treatment using 0.05% (w/v) trypsin (Sigma, St. Louis, MO) supplemented with 0.02% (w/v) EDTA (Sigma, St. Louis, MO) calibrated to pH 8.2 and maintained at 37°C for optimal trypsin activity; and detergent washes with 1% (v/v) Triton-X-100 (BioLab Ltd., Israel) in PBS supplemented with 1% (v/v) ammonium hydroxide (BioLab Ltd., Israel).

The decellularized pcECM was then frozen in liquid nitrogen, crushed and lyophilized to a dry fine powder. The powder was solubilized (10 mg/ml) in 0.01M HCl using 1 min sonication, followed by enzymatic digestion using 1 mg/ml pepsin. The solution was then adjusted to pH 5 with NaOH (0.1M) and kept at 4°C.

3.4 Electrospinning

Electrospinning is a widely used technique for the fabrication of polymeric fibers from polymer solutions in the presence of a high voltage (Figure 3-3). The fiber formation process in electrospinning is the consequence of competition between two forces; electrostatic repulsion and surface tension. The electrospinning spinning setup consists of a syringe pump, high voltage supply and a collector. Polymer solution loaded in a syringe is pumped to form a polymer droplet in the needle connected to the syringe and a high external voltage is applied to the needle and the collector is grounded. When the charges on the surface of the droplet (electrostatic repulsion) overcomes the surface tension of the liquid droplet, the macromolecular polymers in the droplet results in fiber formation on the surface of the collector. Various parameters influence the success of fiber formation like polymer solution concentration and viscosity, polymer solvent volatility, syringe pump flow rate, needle diameter, applied voltage, distance between the needle and the collector.

Figure 3-3 Electrospinning setup A) Traditional & B) Wet electrospinning.

Copolymer of L-lactide and ϵ -caprolactone (PLCL) (PURASORB PLC 7015, Corbion, Netherlands) in 70/30 molar ratio (Inherent Viscosity 1.5 dl/g) was used. PLCL solutions with different solvent combinations were prepared and electrospun. Similarly, copolymer of DL-lactide and glycolide (PLGA) (RESOMER LG 824 S, Germany) in 80/20 molar ratio (Inherent Viscosity 1.7-2.6 dl/g) was dissolved in a solvent mixture containing 70 %

Chloroform and 30 % Dimethyl formamide. The obtained solution was electrospun at a voltage of 18 kV, tip to target distance of 4 cm and at a flow rate of 0.5 mL/h using a 23 G (inner diameter – 0.337 mm) needle in electrospinning machine (NANON, Japan). For wet electrospinning, a metal mold and a liquid collector was used containing a solution of isopropyl alcohol and distilled water in the ratio of 7:3, with poloxamer F108 used as surfactant. Post wet electrospinning, the collected scaffold was frozen, washed 2-3 times in distilled water and refrozen. The frozen samples were freeze dried for 24-48 h. To obtain stable electrospinning conditions various parameters were altered—concentration of the polymer solution (10-15 %), solvent mixtures (DCM: DMF, TFE: CF, CF: DMF), applied voltage (15-25 kV), flow rate (0.1 – 0.5 mL/h), needle gauge (23 G, 25 G) and tip to collector distance (4-12 cm). Fibers collected on aluminum foil (solid collector) were used as reference for wet (liquid collector) electrospinning conditions.

3.5 Morphological Analysis of the Scaffolds by Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a surface characterization technique which uses focused electron beams to scan and reveal their morphological structures under vacuum (Figure 3-4). SEM was used to study the morphology of our scaffolds, as the SEM can resolve structures in the order of micro and nanometers, which are the expected scale of structures in our scaffolds. Typically, an electron microscope consists of an electron source, which upon application of voltage emits electrons. These emitted electrons are focused using electromagnetic lenses and used for scanning the surface of the sample in a raster pattern. Based on the type of interaction and the interaction volume of the electron beam with the sample surface the type of emitted signals varies from backscattered, secondary, and X-rays. These signals can be detected using an array of detectors present in the SEM.

Figure 3-4 Scanning electron microscopy working schematic [5]

The morphology of the obtained fibrous scaffolds was characterized by scanning electron microscopy (SEM) (JEOL 6360, Japan). Scaffolds were sputter-coated with a thin layer of gold for 30 sec using SPI-Module Sputter Coater System and observed under SEM using an accelerating voltage of 5-10 kV. Diameter J [6], an ImageJ plugin, was used to quantify the fiber diameter of randomly collected SEM images ($n=5$) obtained at 2000x magnification of the 3D electrospun and pcECM scaffolds. Pore size of the scaffolds were calculated manually using Image J.

3.6 Scaffold Chemistry Analysis using Fourier-Transform Infrared Spectroscopy

Fourier-Transform Infrared (FTIR) spectroscopy is an absorption spectroscopy technique which uses an infrared radiation's interaction with materials to study the chemistry of the material (Figure 3-5). Attenuated total reflection -FTIR (ATR-FTIR), is a method to study the surface of the materials, as it is simple and straightforward to use without the need for extensive sample preparation. When IR radiation interacts with materials, the materials absorb the energy and the chemical bonds in the material undergo different kinds of vibration characteristic of the bond type. The detector in the FTIR instrument recognizes

this absorption corresponding to different wavelength or wave numbers and records it as a spectrum with transmittance in the y-axis and wave number in the x-axis. This spectrum can be used to identify characteristic chemical groups present in the materials and identify them qualitatively.

Figure 3-5 FTIR working schematic [7]

Perkin Elmer® FTIR- Frontier [Perkin Elmer Inc., Shelton, USA] was used in attenuated total reflection mode was used to study the surface chemistry of the electrospun scaffolds with the as received polymers acting as controls and the pcECM. The samples were analyzed in the range of 600-4000 cm^{-1} at 32 scans per sample.

3.7 Scaffold Mechanical Characterization using Uniaxial Testing

Uniaxial testing offers a simple method to study a material's response to loading. By administering a controlled displacement along an axis, we can obtain the change in dimensions and the resulting parameters from a stress-strain curve in a strain to break test (Figure 3-6).

Figure 3-6 Uniaxial testing machine working schematic (From warwick.ac.uk)

Uniaxial tensile testing machine uses a load cell to measure the load at constant extension rate. The geometry and dimensions of the sample are used here to compute the stress and strain from the load applied and the corresponding strain. The stress, strain and Young's modulus are calculated as follows.

$$\sigma = F/A_0$$

Where σ is the stress in Pa, F is the force or load applied, A_0 is the initial cross-sectional area in the direction perpendicular to extension.

$$\varepsilon = \Delta L/L_0$$

Where ε is the strain, ΔL is the change in length of the sample and L is the original length of the sample.

$$E = \sigma/\varepsilon$$

Where E is the Young's modulus calculated as the slope from the stress strain curve in the linear region.

Instron 5943 Universal Testing Instrument [Instron, Norwood, MA] with 50N load cell was used. Device control and data acquisition were performed with BlueHill® Software. All samples were lyophilized before mechanical testing. Specimens (n=3 for each group) of 5 mm width, 8 mm thickness and 10 mm length were mounted with a pneumatic grip and were stretched at a rate of 0.05 mm/s until sample failure. Linear region of the stress strain curves was used to calculate Young's modulus. The peak value of the stress strain curve was used to calculate the ultimate tensile stress.

3.8 Scaffold Biochemical Composition using LC-MS/MS

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is a technique used for the separation and identification of molecules from a mixture (Figure 3-7). To characterize the scaffold biochemical composition, we used LC-MS/MS. Prepared samples are loaded to the instrument, where LC separates the multiple components of the mixture. This is followed by the first MS, where molecules are ionized and separated based on mass to charge ratio (m/z) and in the second MS further ionization takes place to form fragments and molecules are detected with higher sensitivity.

Figure 3-7 LC-MS based proteomics working schematic [8]

Proteomic analysis was performed at the Smoler Protein Research Center, Technion – Israel Institute of Technology, as per previously published protocol [9]. Briefly, samples were digested with trypsin and the resulting peptides were analyzed by liquid

chromatography tandem mass spectrometry (LC-MS/MS). Peptide fractionation was performed by high-pressure liquid chromatography (HPLC) and electrospray ionization. Collision induced dissociation was used for further fractionation of the peptides, for additional analysis and identification. Proteome discoverer software (Thermo-Scientific) was used for peptide analysis and identification, against the porcine part of the UniProt database. Uncharacterized protein in the database was further analyzed using a basic local alignment search tool (BLAST) on the UniProt database to find similar sequences to identify members of the same gene family.

3.9 Cell Characterization using Flow Cytometry

Flow cytometry is a technique that can be used for characterization of cell fluorescence with a laser. This technique can be used to characterize the cellular properties based on their surface or intracellular protein expression and followed by immunostaining.

Figure 3-8 FACS working schematic

Flow cytometry uses a combination of microfluidics and laser to quantify the cells (Figure 3-8) based on the presence or absence of fluorescence in cell population, which is a direct manifestation of the expression of the target protein or its absence. The main components of a flow cytometer are a flow cell, laser, and detector. The flow cell contains a liquid called as sheath fluid which helps in the flow of the cells in an order to form droplets of

single cells. Laser irradiation on these cells if resulting in a fluorescence will be recorded by the detector and further processing is done using a computer-based software.

To determine cell marker expression, isolated cells were fluorescently labeled and analyzed using FACS (Calibur, BD, USA). Cell labeling was performed with the antibodies listed in Table 5. Briefly, cells were washed once with PBS and fixated with 0.5% formaldehyde for 10 min at 37°C, washed with PBS and then permeabilized with 0.25% Triton for 5 min on ice. Cells were then washed with PBS, resuspended in a wash buffer (0.5% BSA in PBS), and aliquoted to 500,000 cells in 100 µl according to the no. of antibodies used for staining + isotype (per secondary antibody) and cell only controls. Aliquots were incubated with primary antibodies diluted according to their optimal dilution factor in wash buffer for 30min at room temperature. This was followed with couple of washes in wash buffer (2x5min each) and incubation with a secondary antibody (diluted in wash buffer) for 30 min at room temperature (covered with aluminum foil). Stained cells were washed twice with wash buffer prior to FACS analyses.

3.10 Immunofluorescence and Microscopy

Immunofluorescence is the study of antigen antibody interaction in biological specimens (Figure 3-9 A). This can be used to characterize cellular components and/or structures based on protein expression in the cell. Immunofluorescence was used here as a tool to qualitatively characterize the cell-scaffold interaction and characterize for expression for cardiac markers. Traditionally, immunofluorescence uses a sequence of processes to prepare the sample with primary and secondary antibody to render the target protein fluorescent and then are imaged using fluorescent microscopes and/or confocal laser scanning microscopes. An external light source is used to excite the fluorescent dye and the emission is observed as fluorescence. Multiple cellular components can be imaged using different fluorescent molecules.

Figure 3-9 Schematics of Immunofluorescence (A) [10] and Confocal microscopy (B)

The fluorescent microscope consists of a mercury vapor lamp light source, filter cube set for excitation and emission, objective lens, mirrors, detectors/camera and eye piece. These fluorescent microscopes are easy to use and can be used for initial assessment of the samples. However, the limitation in terms of using thicker samples, resulting in blurred images requires better imaging systems which can aid in imaging thicker specimens with better resolution. Hence confocal microscopy is used. The major differences between the normal fluorescence microscope and a confocal microscope are the light source and additional components to avoid blurred images. In confocal microscope (Figure 3-9 B), lasers are used instead of mercury vapor lamps and a pin hole in front of the detector to remove out of focus signal. Other advantages of the confocal microscope include the option of controlling depth of field, focal plane and focal plane stacking (z-stacking) for thicker samples.

For immunofluorescence the samples were processed as described below.

The specimens were placed in plastic molds and embedded in OCT for 1hr at RT. OCT was replaced with fresh one and molds were frozen over dry ice. The molds were kept at -80°C until use. Sections, $10\mu\text{m}$ -thick (unless otherwise stated), were sliced from the blocks

using a cryostat (Leica) and mounted onto positively charged glass slides (Menzel glaser, Germany). The slides were kept at -20°C until staining was performed. OCT-embedded sections were allowed to reach RT equilibrium for 1hr in the darkness prior to fixation. Slides were carefully placed in -20°C cold methanol containing dish placed inside a 4°C refrigerator for 20min. To remove OCT from the slides, fixated slides were carefully transferred into PBS solution for 20min (RT). For histochemistry, OCT free slides were washed with DDW 2x to get rid of excess salts and remaining OCT, followed by air-drying inside a chemical hood for 5-10min prior to staining.

Each specimen was carefully circled using a PAP pen. If permeabilization needed, samples were incubated with 1% Triton X-100 for no more than 10 min at RT. Blocking unspecific protein binding sites was performed with 5% FBS for 1 hr at RT. Specimen were incubated with primary antibodies diluted in 3% FBS (+0.1% Triton X-100 in PBS if using intracellular antibody) at 4°C overnight. After 3x washes in PBS, the samples were subsequently incubated with secondary antibodies diluted in 3% FBS in PBS for 1 hr at RT (protected with aluminum foil). Samples were then washed 3x in PBS (DAPI or Hoechst staining in PBS for 10 min can be performed at that stage) and finally mounted with fluoromount for bleaching prevention. The stained slides were imaged with a confocal laser scanning microscope (LSM 710, Zeiss).

3.11 Cell Viability using Alamar Blue Assay

Alamar Blue is a bioassay which uses a redox indicator to evaluate cellular health and metabolism *in vitro*. Alamar Blue assay is used to monitor cellular viability of cell seeded scaffolds as this method offers an option for continuous monitoring over longer culture periods. Alamar Blue works on the principle of using the cells reducing environment to reduce the active ingredient, resazurin (Figure 3-10). The reduced molecule is stable in the culture environment and does not interfere with the general metabolism of the cells and reduced product can be detected by their fluorescence (Excitation – 540 nm; Emission – 590 nm).

Figure 3-10 Alamar Blue™ working principle

Following different cell interaction studies, AlamarBlue™ assay is performed as follows. To the culture plate wells containing the cell-scaffold construct, 1 ml of complete culture medium with 10% AlamarBlue™ was added to each well. After 2.5 hours (for hMSCs) and 4-5 h (for hiPSCs) incubation with AlamarBlue™, 100 μ L of each well in three replicates were transferred in a black 96-well plate for fluorescence reading on the Varioskan flash reader (excitation 540 nm, emission 595 nm) to measure cell viability.

3.12 Statistical Analysis

All data are presented in the form of mean $\pm$ standard deviation. ANOVA (One way/Two way) followed by post-hoc Tukey analysis and student t-test were employed to compute statistical significance between experimental groups. Origin 8 (OriginLab, USA), was used to conduct all the statistical analysis. Unless otherwise stated, $p < 0.05$ is considered statistically significant.

References

- [1] S. Ting, A. Chen, S. Reuveny, S. Oh, An intermittent rocking platform for integrated expansion and differentiation of human pluripotent stem cells to cardiomyocytes in suspended microcarrier cultures, *Stem cell research* 13(2) (2014) 202-13.
- [2] R. Shinnawi, I. Huber, L. Maizels, N. Shaheen, A. Gepstein, G. Arbel, A.J. Tijssen, L. Gepstein, Monitoring Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes

with Genetically Encoded Calcium and Voltage Fluorescent Reporters, *Stem Cell Reports* 5(4) (2015) 582-96.

[3] Y. Efraim, H. Sarig, N. Cohen Anavy, U. Sarig, E. de Berardinis, S.Y. Chaw, M. Krishnamoorthi, J. Kalifa, H. Bogireddi, T.V. Duc, T. Kofidis, L. Baruch, F.Y.C. Boey, S.S. Venkatraman, M. Machluf, Biohybrid cardiac ECM-based hydrogels improve long term cardiac function post myocardial infarction, *Acta biomaterialia* 50 (2017) 220-233.

[4] T.Y. Lu, B. Lin, J. Kim, M. Sullivan, K. Tobita, G. Salama, L. Yang, Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells, *Nat Commun* 4 (2013) 2307.

[5] F.Y. Zhu, Q.Q. Wang, X.S. Zhang, W. Hu, X. Zhao, H.X. Zhang, 3D nanostructure reconstruction based on the SEM imaging principle, and applications, *Nanotechnology* 25(18) (2014) 185705.

[6] N.A. Hotaling, K. Bharti, H. Kriel, C.G. Simon, Jr., DiameterJ: A validated open source nanofiber diameter measurement tool, *Biomaterials* 61 (2015) 327-38.

[7] F. Faghihzadeh, N.M. Anaya, L.A. Schiffman, V. Oyanedel-Craver, Fourier transform infrared spectroscopy to assess molecular-level changes in microorganisms exposed to nanoparticles, *Nanotechnology for Environmental Engineering* 1(1) (2016) 1.

[8] A.C. Gucinski, E.D. Dodds, W. Li, V.H. Wsocki, Understanding and Exploiting Peptide Fragment Ion Intensities Using Experimental and Informatic Approaches, in: S.J. Hubbard, A.R. Jones (Eds.), *Proteome Bioinformatics*, Humana Press, Totowa, NJ, 2010, pp. 73-94.

[9] S. Beth, A. Ron, B. Limor, E. Yael, G. Idit, E. Ofek, D. Tzila, G. Lior, Z. Eyal, M. Marcelle, Electrospun Extracellular Matrix: Paving the Way to Tailor-Made Natural Scaffolds for Cardiac Tissue Regeneration, *Advanced Functional Materials* 27(34) (2017) 1700427.

[10] C. Has, Y. He, Research Techniques Made Simple: Immunofluorescence Antigen Mapping in Epidermolysis Bullosa, *Journal of Investigative Dermatology* 136(7) (2016) e65-e71.

Chapter 4

Fabrication and characterization of a 3D composite scaffold

This chapter illustrates the results of the fabrication of the composite scaffold obtained from the combination of the 3D synthetic polymeric scaffold and the natural liquid pcECM gel. The chapter begins with the characterization of the pcECM for different parameters that the synthetic scaffolds can mimic. Wet electrospinning was employed to fabricate 3D scaffolds of PLCL and PLGA. These synthetic scaffolds were characterized for their morphology and pore size and mechanical property. Based on this assessment and the ease of fabrication, PLGA scaffold was chosen for further investigations. This scaffold was further modified with the liquid pcECM using traditional EDC-NHS chemistry with few modifications to obtain a 3D composite scaffold (3DCS). The 3DCS mimicked the morphology, chemistry, biochemical composition of the pcECM scaffold and had suitable mechanical properties for cardiac applications. Furthermore, the 3DCS did not display any immunogenicity in vitro and supported human stem cells' attachment and proliferation up to 14 days.

4.1 Experimental Methods

4.1.1 In vitro Immunogenicity Study

RAW 264.7 cells (ATCC, TIB-71™) were cultured with high glucose DMEM (DMEM-HIGH, Sigma, St. Louis, MO) supplemented with 10% FCS, 1% Pen-Strep® and 0.4% Fungizone® for one day, after which the medium was changed to a low-serum medium (3% serum). Once 70% confluence was reached; the cells were exposed to unmodified PLGA, 3DCS, pcECM scaffolds. Lipo polysaccharide (LPS) was used as the positive control and cell without treatment were used as baseline controls. Levels of secreted nitric oxide (NO) were measured as an indication of inflammatory response and immunogenicity. After 16 h incubation with the samples, secreted NO levels were measured and determined against a standard calibration curve (0-100µM sodium nitrite in low-serum medium) as the free stable nitrite form (NO²⁻) using the Griess reagent system (Promega, Madison, WI) according to the manufacturer's instructions.

4.1.2 Scaffold Disinfection

Electrospun PLGA and 3DCS scaffolds were sterilized by UV irradiation for 30 min, and washed by DI water and sterile filtered phosphate-buffered saline (PBS) for three times. Before seeding the cells, the scaffolds were aseptically transferred to a fresh tissue culture plate for further experiments. pcECM scaffolds were disinfected by washing with 70 (v/v) % ethanol in distilled water, three times for 1 hr each, followed by three washes in sterile PBS for 1 hr each. Following sterilization/disinfection, both scaffold types were immersed in complete cell culture media without antibiotics and kept over-night at 37°C, 5% CO₂ incubation to verify lack of microbial cell growth by microscopy examination of the supernatant at 40x magnification.

4.1.3 Cell Culture on Scaffolds

Electrospun PLGA, 3DCS and pcECM 3D scaffolds were prepared to have a dimension of 10 mm diameter. Scaffolds were aseptically transferred into the wells of 48 well plates (flat bottom) and allowed to occupy the entire available surface area of each well. hMSCs were harvested using trypsin and the obtained cells were diluted in culture medium to reach 200,000 cells per 100 μ L. The cell suspension was added to the scaffolds' surface gently and making sure to cover majority of the scaffolds surface. The cell seeded scaffolds were incubated in a CO₂ incubator at 37°C for initial attachment. Each scaffold was subsequently washed with PBS to remove the non-attached cells and were transferred into a well of a 12-well plate with 2 mL of media. The cell viability was performed on day 3, 7 and 14 as described in section 3.11.

4.2 Results & Discussion

4.2.1 pcECM Characterization to Identify Parameters to Mimic in 3DCS

Decellularized pcECMs were obtained (as mentioned in section 3.3) from Professor Marcelle Machluf's laboratory at the Technion-Israel Institute of Technology. The decellularization involves a three-step process as previously published [1, 2]. pcECM characterization in this work was carried out to investigate the architecture, pore size, and mechanical property to identify and mimic these parameters during the fabrication of a synthetic 3D electrospun scaffold. The pcECM developed by our group was characterized extensively and exhibited promising *in vitro* cell support ability, differentiation ability and angiogenesis in the preserved vasculature [2, 3]. Further characterization of the pcECM for its morphological properties were carried out to determine parameters that can be mimicked using a synthetic scaffold. To mimic the fibrous morphology of the pcECM, wet electrospinning of PLCL and PLGA was carried out, as it is widely accepted that electrospinning results in ECM mimicking fibrous morphologies [4-6]. Figure 4-1 represents the process of decellularization, the macroscopic appearance, architecture, and pore size distribution of the pcECM.

Figure 4-1 Decellularization process, macroscopic appearance and characterization of architecture of decellularized pcECM.

Reaction scheme for decellularization of pcECM (A); Decellularized pcECM (B); Freeze dried cross-section view of pcECM (C); SEM image of the endocardial layer (D); Myocardial layer (E); Epicardial layer (F) of the pcECM; Fiber diameter distribution (G); Pore size distribution (H); Representative strain to break assay curve (out of n=3 repetitions) (I).

Fiber diameter distribution was observed from nano to micro range in the pcECM due to the inherent strut and fiber morphology. The pcECM myocardial fibers have a mean diameter of $0.431 \pm 0.058 \mu\text{m}$. Due to the native fiber and strut morphology of the pcECM,

the diameter exhibited here is not absolute and is an overall estimate obtained through SEM image analysis. The pcECM has an overall mean pore size of $11.33 \pm 4.97 \mu\text{m}$. The pcECM myocardial layer had a Young's modulus of $193.9 \pm 99.8 \text{ kPa}$. These are the parameters of interest that we would try mimicking in the 3D electrospun scaffold.

4.2.2 Wet Electrospinning of PLCL and PLGA – Optimization and Characterization

Electrospinning was performed using methods mentioned in section 3.4. Different experiments were carried out to optimize various solvent combinations, polymer concentrations, flow rates, applied voltages (Table 4-1) for the stable fabrication of 3D PLCL scaffolds. During electrospinning the polymer jet emerging from the needle was inspected to assure continuous and stable flow. PLCL concentration of 10, 12 and 15 % (w/v) was used for the optimization. The tip to target distance was not modified during the solid electrospinning. However, for wet electrospinning, the distance was modified to obtain reproducible spinning conditions. The effect of solvent and solvent combinations were also studied as this affects the polymer solution properties and spinning process [7-10].

Table 4-1 Poly (L-Lactide-co- ϵ -caprolactone) (70:30) electrospinning conditions.

Electrospinning conditions of PLCL (7030) polymer solution of inherent viscosity – 1.5 dL/g

Collector#	Concentration (w/v) %	Solvents*	Applied Voltage (kV)	Flow rate (mL/h)	Tip to collector distance (cm)	Solution flow and spinning ability
Solid	10	DCM: DMF (8:2)	15	0.5	12	Resulted in solution flow suitable for spinning
	12	DCM: DMF (8:2)	15	0.5	12	
	12	DCM: DMF (7:3)	15	0.4	12	
	15	TFE:CF (7:3)	15	0.5	12	
	15	TFE:CF (7:3)	25	0.5	12	
Liquid	10	DCM: DMF (8:2)	15	0.1	4	Did not result in

12	DCM: DMF (8:2)	16.5	0.4	5	proper solution flow
12	DCM: DMF (7:3)	17	0.2	3.5	
15	TFE:CF (7:3)	15	0.5	4	Unstable flow over longer periods of spinning
15	TFE:CF (7:3)	25	0.5	4	Proper solution flow but not reproducible scaffold structure

* DCM – Dichloromethane, DMF – Dimethylformamide, TFE – trifluoroethanol, CF – Chloroform; # - solid - aluminum foil, liquid – liquid bath collector. 23 G needle was used for this experiment.

In all the conditions, the electrospun fibers obtained by using a 2D solid collector (aluminum foil, internal control), exhibited a defect free homogeneous fiber formation (Figure 4-2 A). However, when the same solution conditions were used for the fabrication of 3D fibers using the liquid collector, there were defects in the fiber formation resulting in fiber merging and/or pockets of densely crowded fibers (Figure 4-2 B&C, respectively). The beaded structures were probably due to insufficient solution viscosity and surface tension effects on the polymer jet [11]. Though the fiber morphology in Figure 4.2 B&C, can be compared to the anisotropic morphology present in the pcECM, the visual inspection of the solution flow from the needle tip of the electrospinning setup was found to be irregular. Hence the conditions that led to these morphologies were not used further.

Figure 4-2 SEM images of PLCL Fibers displaying defective and dense morphology.

Fibers collected using aluminum foil, 2D spinning conditions (A); PLCL fibers collected in liquid medium showing defects (B) and dense morphology (C); 10 % PLCL in DCM:DMF 8:2, 15 kV, 4 cm, 0.1 mL/h. Representative images are shown out of n=3 repetition in each set of electrospinning conditions.

To solve the issue of beaded fibers, the polymer concentration and DMF ratio were modified. Increasing PLCL concentration and DMF content which altered the polymer solution viscosity and conductivity respectively, led to better 3D fiber structures (Figure 4-3). However, the condition was not reproducible as the polymer solution flow was not consistent during repeated attempts.

Figure 4-3 SEM images of PLCL fibers obtained from a polymer solution with high DMF content showing good morphology.

12 % PLCL DCM: DMF 8:2, 15 kV, 4 cm, 0.1 mL/h (A); 12 % PLCL DCM: DMF 7:3, 15 kV, 4 cm, 0.1 mL/h (B). The fibers were collected in a liquid medium and the SEM fibers show defect free morphology, but the condition was not reproducible.

A fluorinated solvent, TFE in combination with CF was used to check for the formation of defect free fibers in the wet electrospinning conditions. This modified solvent combination for PLCL resulted in defect free fiber morphology (Figure 4-4 A, B &C). Increasing the

voltage aided in better solution behavior over extended periods of spinning, but the reproducibility of the process was poor nonetheless. Macroscopic images of the scaffold displayed a diameter of 1.8 cm and a thickness of 0.8 cm (Figure 4-4 D, E, & F). The PLCL 3D fibers had a mean diameter of $1.06 \pm 0.69 \mu\text{m}$ as measured by Diameter J, an image J plugin.

Figure 4-4 SEM images of PLCL Fibers and the macroscopic appearance of the scaffold with dimensions.

Fibers collected using aluminum foil (A); PLCL fibers collected in liquid medium at 15 kV (B) & 25 kV (C); 15 % PLCL in TFE:CF 7:3, 4 cm, 0.5 mL/h; (D), (E) & (F) show the scaffold in the mold, from top and side views, respectively. Representative images are shown out of $n=3$ repetition in each set of electrospinning conditions.

PLGA 8020 electrospun fibers collected both using the solid aluminum foil collector and liquid medium (Table 4-2) showed defect free fiber morphology. Macroscopic images of the scaffold displayed a diameter of 1.8 cm and a thickness of 1.1 cm (Figure 4-5). The PLGA 3D fibers had a mean diameter of $0.75 \pm 0.23 \mu\text{m}$ as measured by Diameter J, an image J plugin.

Table 4-2 Poly (L-Lactide-co-glycolide) (80:20) electrospinning conditions.

Electrospinning conditions of PLGA (8020) polymer solution of inherent viscosity – 1.7-2.6 dL/g

Collector [#]	Concentration (w/v) %	Solvents*	Applied Voltage (kV)	Flow rate (mL/h)	Tip to collector distance (cm)	Solution flow and spinning ability
Solid	8	CF: DMF (7:3)	18	0.5	12	Proper solution flow and
Liquid					4	reproducible scaffolds

* DMF – Dimethylformamide, CF – Chloroform; # - solid - aluminum foil, liquid – liquid bath as described above. 25 G needle was used for this experiment.

Figure 4-5 SEM images of PLGA Fibers and the macroscopic appearance of the scaffold with dimensions.

Fibers collected using aluminum foil (A); PLGA fibers collected in liquid medium using 8 % PLGA in CF: DMF 7:3, 4 cm, 0.5 mL/h (B, C shows different magnifications of the same region); (D), (E) & (F) shows the scaffold in the mold, the top view and the side view respectively. Representative images are shown out of n=3 repetition in each set of electrospinning conditions.

Digital analysis of the SEM images for fiber diameter distribution of the 3D electrospun scaffold and pECM originating from the myocardium showed nano to micro in all scaffold

fiber diameters Figure 4-6. Only the myocardial layer of the pcECM could be compared to the 3D scaffold fiber diameter distribution.

Figure 4-6 Comparison of fiber diameter distribution of 3D PLCL and PLGA scaffolds with the pcECM myocardium.

This is due to two factors i) the thickness of the myocardial layer in the left ventricle and ii) the presence of fibrous morphology in the myocardial layer. The myocardial layer is the thickest of the left ventricle compared to the epicardium and endocardium. Also, both the other two surfaces, epicardial and endocardial layer, did not display any visible fibers (Figure 4-1 D & F respectively) at the chosen magnification. Moreover, the cellular composition of the myocardium is predominantly made up of CM, which is responsible for the beating functionality of the cardiac muscle [12]. Hence, fabricating a construct to mimic the myocardial layer that is mainly responsible for cardiac functionality seems to be the logical way to go for cardiac patch development. Along with the above-mentioned reasons, the fact that wet electrospinning results in a fibrous morphology and the tunable nature of the eventual scaffold shape and thickness can be matched to the thickness of the target tissue also resulted in drawing the comparison of the fibers of the synthetic scaffolds with the myocardium.

4.2.3 3D Synthetic Scaffolds' Chemistry and Mechanical Characterization

FTIR was performed using the method mentioned in section 3.6. The idea of characterizing the electrospun scaffolds with FTIR (Figure 4-7A&B) is to provide reference spectra for future biological functionalization of the synthetic electrospun scaffold. The functional groups on the electrospun fibers were compared to the commercial polymer granules used as the control. Characteristic ester C=O stretching were found in both PLCL and PLGA 3D fibers (1748 and 1752 cm^{-1} respectively). 3D PLCL [13, 14] fibers showed characteristic peak at 2944 and 2860 cm^{-1} corresponding to CH stretching of alkyl groups in copolymers of PLCL. For 3D PLGA [15, 16] peaks at 2995 cm^{-1} (CH bending), 1454 and 1424 cm^{-1} (CH₃ stretching), 1045 cm^{-1} (C-O stretching), and 845 cm^{-1} (rocking CH₂) were found. There was no visible difference observed between the FTIR spectra of the pure polymer and the fabricated scaffold.

Figure 4-7 FTIR spectra of PLCL (A) and PLGA (B).

The FTIR spectra of the polymer electrospun 3D scaffolds were compared to that of pure polymer granules. Representative graphs are presented out of $n=3$ repetitions. These graphs serve as baseline and reference for the subsequent characterization of successful 3DCS functionalization with the liquidized pcECM.

Mechanical properties (measured using methods in section 3.7) of the 3D scaffolds were measured using uniaxial tensile testing machine. 3D PLCL and 3D PLGA scaffolds displayed a tensile modulus of 12.3 ± 8.7 and 13.7 ± 4.3 kPa respectively as shown in Figure 4-8. This tensile modulus was calculated based on the linear region of the stress–strain curves. Both the synthetic 3D scaffolds showed lower tensile modulus when compared with the pcECM, which displays a modulus of 193.9 ± 99.8 kPa. The lower Young's moduli of the synthetic scaffolds is most probably due to the structure of the fibrous scaffold fabricated using the wet electrospinning method. In wet electrospinning, fibers were solidified rapidly when they come into contact with the liquid collector. These fibers are entangled due to the fast speed that they were deposited but no crosslinking takes place between fibers. Therefore, when these fibers were stretched during the tensile testing, sliding/gliding and disentangling takes place. There were no chemical crosslinks to resist the stretching leading low modulus measured. Nevertheless, when comparing the modulus, the standard deviation of the 3D scaffolds' modulus is much smaller compared to that of the pcECM which exhibits that the synthetic platform is more reproducible in terms of their properties. Moreover, considering the elastic modulus for normal cardiac tissue, our

fabricated 3D scaffolds are within the required range for CTE applications — 10 and 15 kPa [17, 18].

Figure 4-8 Mechanical property of 3D electrospun scaffolds in comparison to pcECM.

Stress strain curves of 3D PLCL (A); 3D PLGA (B); pcECM (C). Modulus calculated from the linear region of the stress-strain curves (D). All samples were of similar dimensions as specified in 3.7. Results represent mean $\pm$ SD of n=3 repetitions per group.

When the scaffolds are fabricated using wet electrospinning, the polymeric fibers are collected in the liquid bath and then formed into cylindrical shapes using customized molds. For PLCL, the process of scaffold shape and size was not consistent and less reproducible during multiple attempts as opposed to PLGA, the wet electrospinning of which displayed satisfying reproducibility over repeated attempts. This reproducibility is an important factor, as the resulting scaffold's property is reliant on the scaffold dimensions and it is more prudent to use a thoroughly reproducible and controllable fabrication method for scaffold preparation. Though, further modifications to the PLCL electrospinning conditions, may result in a better reproducible scaffold, our aim here was to manufacture a working polymer scaffold model to proceed with the fabrication of the 3D composite scaffolds. Hence, further investigation of PLCL was not carried out and based on the different characterization methods and the reproducibility of the scaffold's macroscopic form and dimensions, during wet electrospinning process, (Figure 4-9), PLGA scaffold was used for further cell material interaction studies.

Figure 4-9 Reproducibility of the PLGA scaffolds when compared to a batch of 3D PLCL scaffolds.

3D PLGA fabrication exhibited better reproducibility compared to 3D PLCL.

4.2.4 Optimization of EDC-NHS Procedure for 3DCS Fabrication

The wet electrospun 3D PLGA scaffold, displaying a porous and fibrous morphology remain inert in terms of bioactivity required for cellular interaction. Polymeric scaffolds can be conferred bioactivity using natural materials such as collagen, gelatin, and alginate [19-25]. However, for the scaffolds to be suitable for a target tissue application, the bioactivity of the scaffold also needs to be tissue specific. This tissue specific bioactivity can only be obtained from decellularized ECM materials, as they would preserve the native microenvironment's complex biochemical composition. Thus, we used a liquified porcine cardiac ECM to modify the bio-inert 3D PLGA scaffold to form a 3D composite scaffold (3DCS). This modification of the 3D PLGA scaffold with liquid pcECM gel was achieved using EDC-NHS chemistry with few modifications.

Modification of the 3D electrospun PLGA with the pcECM gel was carried based on EDC-NHS chemistry. Some parameters of the protocol were optimized to homogenously incorporate the unique natural material, the pcECM gel onto the 3D electrospun porous polymeric scaffolds.

Figure 4-10 Modified protocol for surface activation and functionalization of 3D electrospun PLGA using EDC-NHS chemistry.

Two factors were considered here for successful modification of the 3D PLGA scaffold: i) The protein material used here is the liquid pcECM gel which can form a gel at higher temperatures ($\sim 37^{\circ}\text{C}$). ii) The thickness of the 3D scaffold, where the aim was to obtain a completely modified 3D scaffold and not just limiting the modification to the scaffold surface. When the scaffolds were modified as per the standard protocol which does not include a gelation (Table 4-3 A) step for the protein used, there was no pcECM (Table 4-3 D) like morphology observed either on the surface or in the cross section of the scaffold. The fiber morphology of the scaffolds was also monitored and found to be unchanged during each step of the EDC-NHS modification as shown in Figure 4-11. Although, SEM images exhibit that there are no visible changes in the scaffold morphology post EDC-NHS treatment, any possible change in molecular weight or degradation during NaOH treatment should also be taken into consideration.

Figure 4-11 Surface morphology of PLGA scaffold does not change in the initial modification steps of the EDC-NHS modification protocol.

Scaffolds were characterized using SEM to evaluate for morphological changes during the EDC-NHS treatment. Representative images are shown out of n=3 repetition in each condition.

This gelation step was added not only to aid surface modification of the 3D PLGA, but also to obtain pcECM mimicking morphologies. When a 12 h gelation (Table 4-3 B) step was included to assist in the gelation of the pcECM gel, pcECM (Table 4-3 D) like structures were observed on the surface of the scaffold but not in the cross section of the scaffold. To address the challenge of the scaffold thickness, the incubation temperature of the liquid pcECM solution following EDC-NHS surface activation was modified. When the traditional temperature of 4°C was used, only the surface of the scaffold exhibited pcECM like morphology, while the cross section of the scaffold displayed only normal fibrous morphology of the 3D PLGA (Table 4-3 B). This was attributed to the combination of the low temperature and the thickness playing a role in limiting the diffusion of the liquid pcECM through the entirety of the 3D scaffold. Hence the incubation temperature was increased to room temperature (~20°C). This condition as presented in Table 4-3 C, exhibited pcECM mimicking morphology in both the scaffold surface as well as the cross section of the 3D PLGA scaffold, which resulted in the formation of a 3D composite scaffold (3DCS). The optimized scaffold modification conditions, the concentrations of the solutions used, the incubation times are listed in Table 4-4.

Table 4-3 Morphology of 3D composite scaffold’s exterior and cross section at different conditions during the EDC-NHS chemistry-based modification.

Treatment	Scaffold Exterior	Scaffold Cross Section
A. No Gelation
B. Incubation at 4°C followed by 12 h Gelation Time
C. Incubation at Room Temperature (~20°C) followed by 12 h Gelation Time
D. pcECM scaffold Comparison

Table 4-4 Optimized concentrations of solutions and conditions used for the fabrication of 3DCS.

EDC-NHS Protocol
0.05 (w/v) % NaOH for 30 min at RT
3X D.H ₂ O wash
40 mM EDC + 80 mM NHS in 50 mM MES buffer (pH 6.0), Incubated for 4 h at 4°C
3X D.H ₂ O wash
500 µg/mL pcECM gel in 1X PBS (pH 7.2), overnight RT (~20°C)
37°C for 12 h (to induce gelation)
3X PBS Wash
Freeze dry
Characterize

4.2.5 **3DCS Exhibits Comparable Characteristics as the pcECM**

The scaffold exterior and cross-sectional morphology of the 3DCS are shown in Figure 4-12 and Figure 4-13 respectively. The morphology of the scaffold post wet electrospinning and after modification with pcECM gel, was characterized using scanning electron microscopy (SEM). The 3DCS displayed a combination of morphologies; a fibrous morphology as expected from the wet electrospinning and an inter fibrillary matrix like morphology due to the pcECM functionalization. The SEM images were used to further characterize the scaffold parameters. The fiber diameter varied between 0.1 to 1.5 µm with an average diameter of 1.01 ± 0.50 µm (Figure 4-14). The pore size of the 3DSC ranged from 8 to 25 µm with an average pore size of 14.05 ± 3.99 µm (Figure 4-14). These scaffold characteristics were comparable to the natural pcECM material (Figure 4-13). The large distribution in fiber diameter in the pcECM can be attributed to the fiber and strut morphology found on decellularized cardiac ECM [26]. The same nano to micro dimension fiber diameter present in the 3DCS can be attributed to the wet electrospinning process. Similar trend was observed by other groups for the wet electrospinning of PCL using a liquid bath [27]. Another factor that contributes to this fiber diameter distribution is the presence of the pcECM gel in the 3DCS and the resulting combination of morphologies from the electrospun fiber and the natural material. The characterization of the pore size of

the 3D scaffold was necessary to establish if the scaffold can support the cell growth and nutrient transfer in the scaffold structure. Though there are various porosimetry techniques available viz mercury porosimetry, micro computed tomography etc., we employed SEM image analysis as it a simple and easy method that does not require specialized equipment and training [28]. Also, methods using pressure for liquid intrusion like mercury porosimetry also poses limitations in terms of high pressure, cost and environmental toxicity. Hence, ImageJ based analysis of SEM images was used to characterize an estimation of the scaffolds' pore size.

Figure 4-12 3DCS scaffold cross sectional tile exhibiting both fibrous and inter-fibrillary pcECM-like morphology on the surface and bulk of the scaffold.

Functionalized 3DCS scaffold surface morphology as fabricated with the optimized protocol mentioned in Table 4-4. The image is a manually stitched image of multiple SEM images of the surface of the 3DCS.

Figure 4-13 3DCS cross sectional morphology exhibiting characteristic morphology of both electrospun and pcECM scaffolds.

3DCS exhibited fibrous morphology characteristic of the wet electrospun polymer and an interfibrillary matrix like structure of the pcECM. Representative images are shown out of $n=3$ repetition in each sample.

Figure 4-14 3DCS fiber diameter and pore size as compared to the pcECM.

3DCS displayed comparable fiber diameter and pore size distributions similar to the pcECM. The mean fiber diameter and pore sizes of 3DCS was significantly higher as computed using Image J software. Results represent mean $\pm$ SD of $n=3$ repetitions per group.

ATR-FTIR was employed to characterize the chemistry of the different scaffolds after the unmodified PLGA was functionalized using the pcECM gel through EDC-NHS chemistry. The spectra of the different scaffolds are shown in Figure 4-15. Both unmodified PLGA (UPLGA) and 3DCS displayed a common intense peak at 1740 cm^{-1} characteristic of the C=O stretching of the PLGA backbone, which was not observed in the pcECM sample. Three distinct peaks were found in the 3DCS scaffolds sample at 3320 , 1640 and 1540 cm^{-1} . The peak detected at 3320 cm^{-1} is associated with the N-H stretching from amines. Also, the peaks at 1640 and 1540 cm^{-1} correspond to N-H bending from primary amines [6]. The presence of these characteristic amine peaks in the ECM and the ECM modified PLGA scaffold compared to the UPLGA scaffolds indicate that the UPLGA scaffolds were successfully functionalized with the pcECM gel.

Figure 4-15 3DCS surface characterization exhibiting characteristic chemistry of synthetic PLGA polymer and natural pcECM.

Pink highlight represents the region corresponding the polymeric carbonyl backbone and yellow highlight represents the region corresponding the protein amide group and natural pcECM

To assess the similarity of the protein composition (performed using method as mentioned in section 3.8) between the starting ECM gel and the 3DCS (i.e., pcECM gel modified

UPLGA scaffold), both these materials were subjected to proteomic analysis. The list of proteins maintained in both the scaffold types are illustrated in Table 4-5. The proteomic analysis exhibit that the cardiac ECM is primarily made up of collagens. Collagen types I and III, the predominant types of collagen in the cardiac ECM was present in the both the scaffolds. Other types of collagen found among the composition are collagen II, IV, V, VI and XI. Additionally, sequences possessing ~60 % identity to proteoglycan sequences were also found, which could contribute towards the ECM environment by providing a hydration to the tissue construct [29]. The proteomics results reveal the presence of important cardiac environment proteins, Collagen I and III. This preserved fibrous collagen composition grants the scaffold, the necessary structural and biochemical properties as well as contributes to the overall cardiac microenvironment in terms of maintaining the tissue structure and supporting cardiac cells [30, 31]. The rest of the proteins deduced by proteomics play a role in forming network like structures and interfibrillary matrix structures, which is characteristic of a native ECM environment [32]. Additionally, sequences possessing ~60 % identity to proteoglycan sequences were also detected. Ratio of areas of unique peptides of the 3DCS compared to the pcECM gel based on Discoverer software (Thermo Fisher Scientific, UK) revealed no significant difference between the two samples.

Table 4-5 Protein Composition of pcECM gel were preserved in the 3DCS as detected using LC MS-MS.

Accession No.	GENE Name	3DCS/pcECM gel Ratio #
F1RT61	COL1A1 (Inferred) ^a	1.69
F1SFA7	COL1A2	0.88
I3LJX2	COL1A1 (Inferred) ^b	1.31
F1RYI8	COL3A1	1.98
F1RXW0	COL5A2	1.59
I3LV61	Proteoglycan 3 (Inferred) ^c	2.29
F1S021	COL5A1	1.87
I3LSV6	COL2A1	0.66
I3LEF9	Proteoglycan 3 (Inferred) ^d	2.24

F1S3G7	COL5A3	1.00
Q9TV36	FBN1	1.39
I3LQ84	COL6A2	0.64
F1RLL9	COL4A2	0.46
F1S571	COL11A1	1.02
I3LUR7	COL6A3	0.54
F1S2C1	COL11A2	0.52

3DCS – 3D electrospun PLGA scaffold modified with pcECM gel
pcECM gel – Porcine cardiac ECM gel
3DCS/pcECM gel Ratio – Relative variability in protein composition between the 3DCS and pcECM gel. A ratio of ≥ 4 indicates a difference from the control
^a 100 % identical to Collagen 1 Alpha 1 (*Sus scrofa domestica*) in a Uniprot BLAST
^b 81.9 % identical to Collagen 1 Alpha 1 (*Sus scrofa domestica*) in a Uniprot BLAST
^c 62.5 % identical to Proteoglycan 3 (*Homo sapiens*) in a Uniprot BLAST
^d 59.4 % identical to Proteoglycan 3 (*Homo sapiens*) in a Uniprot BLAST

To determine the homogenous functionalization of the 3DCS with the ECM gel, we performed immunostaining (performed using method as mentioned in 3.10) for the presence of collagen I, a major protein in cardiac ECM as found in our proteomics results. As shown in Figure 4-16, 3DCS showed a similar collagen I staining as compared to the pcECM, thus further confirming the modification of the scaffold with the ECM gel. The uniform collagen I fluorescence observed in the cross section of the 3DCS, that is comparable to the pcECM, represents the homogenous distribution and modification across the 3D UPLGA by the liquid pcECM. This uniform presence of the ECM molecule in the 3DCS scaffold structure is key in addressing the position or location-based bioactivity that was referred as a limitation in the natural pcECM material. By using a synthetic scaffold as a tunable platform and modifying this with the liquid form of the pcECM, this limitation of varied bioactivity profile is normalized considerably. Other methods like elemental analysis can also be used to measure the amount of amine or sulphur containing groups to obtain similar information.

Figure 4-16 Immunofluorescence of 3DCS exhibiting staining for Collagen I, a key component of the cardiac ECM.

To study the mechanical properties of the scaffolds, tensile testing was performed (Figure 4-17). Both the Young's modulus and the ultimate tensile stress of the 3DCS scaffolds were higher when compared the UPLGA scaffold. UPLGA had a mean modulus of 13.7 ± 4.3 kPa whereas the 3DCS had almost three times the value at 38.6 ± 4.3 kPa. pcECM had the highest modulus among the three scaffolds at 193.9 ± 99.8 kPa. The ultimate stress values also followed the same trend where pcECM was the highest followed by 3DCS and then UPLGA at 296.3 ± 111.8 , 25.8 ± 0.4 and 10.8 ± 1.2 kPa respectively. The value for pcECM is consistent with published results showing high modulus values for decellularized ECM scaffolds due to the presence of predominantly collagenous composition [1, 2, 33]. In the case of 3DCS, the modulus while lower compared to the pcECM, is significantly higher than that of UPLGA. This is due to the addition of the pcECM gel to the PLGA scaffold through EDC-NHS modification which results not only in surface functionalization but also the formation of interfibrillary structures which mimics the native pcECM. The modulus of UPLGA was the lowest among the three scaffolds which can be due to the highly porous nature of the scaffold, a result of the wet

electrospinning process. The low modulus can also be beneficial for eventual cellular integration and interaction as it has been shown to aid in better initial cellular integration [34].

Figure 4-17 Mechanical properties of 3DCS compared to pcECM.

Uniaxial testing of the scaffolds was performed to calculate the modulus (A) and ultimate stress (B) of the scaffold materials. Samples were fixed to the instrument as depicted in (C) and data was recorded for the samples where the failure location was in the center of the sample structure and not near the grips. All samples were of similar dimensions as specified in 3.7. Results represent mean $\pm$ SD of $n=3$ repetitions per group. * represents significant increase of 3DCS with PLGA ($p < 0.05$); # represents significant increase of pcECM when compared to PLGA & 3DCS ($p < 0.05$).

4.2.6 3DCS is non-immunogenic *In vitro*

As the 3DCS was fabricated through different chemical processes and modifications, we studied their immunogenicity through *in vitro* means. RAW macrophage cell stimulation was used performed (using method as mentioned in section 4.1.1) with the different scaffold groups, as the macrophage stimulation involves triggering pro-inflammatory factors such as NO [35]. To characterize this, the macrophage cells were allowed to react with the materials. After the macrophages were exposed to different scaffolds, the level of pro-inflammatory factor NO was analyzed using Griess reagent. From this result (Figure

4-18) it was evident that the scaffolds did not elicit any immunogenic response in terms of NO release as measured by Griess reagent compared to the positive LPS control. This confirms that our scaffold fabrication process and the resulting scaffold are not immunogenic as demonstrated in previous publications for similar materials [36].

Figure 4-18 *In vitro* immunogenicity of the scaffolds evaluated using Griess Reagent.

RAW macrophage cells were exposed to the scaffold materials for 16 h and the immunogenicity was recorded as the amount of NO released using Griess Reagent. Results represent mean $\pm$ SD of n=3 repetitions per group. * represents statistical significance of LPS with PLGA, 3DCS, pcECM and cells only ($p < 0.05$).

4.2.7 3DCS Supports Attachment and Proliferation of hMSC

The hMSCs used in the study were characterized for MSC markers to validate the stemness of the starting cell population. Flow cytometry was performed (using protocol mentioned in section 3.9) to evaluate expression of CD29, CD44, CD73, CD90 and CD105. Isotype controls were used to calculate the percentage of positive cells. The FACS results exhibited that the hMSCs were of high quality as they were more than 90% positive for all the markers analyzed as shown in Figure 4-19.

Figure 4-19 hMSCs Characterization using FACS.

Detection by FACS demonstrates positive expression for various MSC markers. Dot plot of the representative population (A). Populations are more than 95 % positive for CD29 (C), CD73 (D), CD44 (E), CD90 (F) and CD105 (G). Black histogram is the antibody only control (B).

hMSCs were seeded on different scaffolds (UPLGA, 3DCS and pcECM) for up to two weeks; the viability and proliferation of the cells were compared between scaffolds using AlamarBlue™ (performed using protocol mentioned in section 3.11). As shown in Figure 4-20 the seeded hMSCs maintained their viability and proliferated in number from day 3 till day 14. Phalloidin and DAPI staining of the scaffolds demonstrated the presence of cells in the scaffolds as shown in Figure 4-21.

Though hMSCs are not cardiac cells, they are a widely accepted cell model for CTE [37] as they can be cultured and multiplied easily, could be derived from autologous sources and are less immunogenic. 3DCS showed initial high densities and reached the original seeding density in two weeks. Here, the combination of UPLGA substrate and the cell binding moieties from the pcECM gel, contribute to the initial cellular attachment and the eventual proliferation. Similar studies have shown that the presence of bioactive molecules increases cellular attachment and proliferation on different substrates [38]. UPLGA showed a lower cell density than the 3DCS. This is due to the lack of bioactivity [5, 6] in the pristine 3D UPLGA polymer where the cells need time to secrete their own ECM materials. pcECM had the lowest starting cell densities, but they showed the highest increase in cell number during the culture period of two weeks indicating higher proliferation rate (Figure 4-20). This could be due to the higher stiffness of pcECM as similar behaviour for hMSCs have been observed by other groups, where hMSCs exhibit higher proliferation rate in stiffer substrates compared to the soft ones [39].

Figure 4-20 hMSCs viability on the scaffolds as measured using AlamarBlue™.

hMSC interaction and proliferation on 3DCS compared to the unmodified PLGA and pcECM scaffold. All three scaffolds exhibited ability to support the viability and proliferation of the seeded cells. 3DCS has significantly higher cell densities than PLGA at days 3 and 7; also, 3DCS has significantly higher cell densities than pcECM during all time points (3,7&14); PLGA is not significant to pcECM at all time points ($p < 0.05$, ANOVA). Cell densities were calculated based on the initial seeding density and the surface of the scaffold used. hMSCs calibration curve was used to convert AlamarBlue™ fluorescent intensity into cell numbers and eventually cell densities for each time point. Same graph in terms of cell number is presented in the supplementary section 8.2.5.

Figure 4-21 Phalloidin staining of hMSCs seeded on the scaffolds.

Representative images of histological section of 3DCS, unmodified PLGA and pcECM stained (Day 14) with Phalloidin and DAPI; Scale bar: 50 μ m.

4.3 Conclusion

Collectively, we have demonstrated the fabrication of a 3D composite scaffold (3DCS) by combining an optimized synthetic 3D wet electrospinning protocol and modification of the resulting scaffold with porcine cardiac ECM gel using a slightly modified EDC-NHS chemistry. Morphological analysis of the 3DCS exhibited similarity in terms of fiber diameter and porosity to the pcECM scaffold. Also, the 3DCS mimicked the surface chemistry of the pcECM and maintained the biochemical composition of the pcECM gel. The mechanical properties of the synthetic 3D scaffold were improved by the addition of the pcECM gel. The scaffold was found to be non-immunogenic *in vitro* and was supportive of viability and proliferation of seeded hMSCs. The specific suitability of these scaffolds for CTE applications is addressed in the subsequent chapters.

References

- [1] Y. Eitan, U. Sarig, N. Dahan, M. Machluf, Acellular cardiac extracellular matrix as a scaffold for tissue engineering: in vitro cell support, remodeling, and biocompatibility, *Tissue engineering. Part C, Methods* 16(4) (2010) 671-83.
- [2] U. Sarig, G.C. Au-Yeung, Y. Wang, T. Bronshtein, N. Dahan, F.Y. Boey, S.S. Venkatraman, M. Machluf, Thick acellular heart extracellular matrix with inherent vasculature: a potential platform for myocardial tissue regeneration, *Tissue Eng Part A* 18(19-20) (2012) 2125-37.
- [3] U. Sarig, E.B. Nguyen, Y. Wang, S. Ting, T. Bronshtein, H. Sarig, N. Dahan, M. Gvirtz, S. Reuveny, S.K. Oh, T. Scheper, Y.C. Boey, S.S. Venkatraman, M. Machluf, Pushing the envelope in tissue engineering: ex vivo production of thick vascularized cardiac extracellular matrix constructs, *Tissue Eng Part A* 21(9-10) (2015) 1507-19.
- [4] D.H. Reneker, I. Chun, Nanometre diameter fibres of polymer, produced by electrospinning, *Nanotechnology* 7(3) (1996) 216.
- [5] J.H. Brown, P. Das, M.D. DiVito, D. Ivancic, L.P. Tan, J.A. Wertheim, Nanofibrous PLGA electrospun scaffolds modified with type I collagen influence hepatocyte function and support viability in vitro, *Acta biomaterialia* 73 (2018) 217-227.

- [6] H. Chen, Y. Peng, S. Wu, L.P. Tan, Electrospun 3D Fibrous Scaffolds for Chronic Wound Repair, *Materials* (Basel) 9(4) (2016).
- [7] S.E. Aniagyei, L.B. Sims, D.A. Malik, K.M. Tyo, K.C. Curry, W. Kim, D.A. Hodge, J. Duan, J.M. Steinbach-Rankins, Evaluation of poly(lactic-co-glycolic acid) and poly(dl-lactide-co-epsilon-caprolactone) electrospun fibers for the treatment of HSV-2 infection, *Mater Sci Eng C Mater Biol Appl* 72 (2017) 238-251.
- [8] S.Q. Huan, G.X. Liu, G.P. Han, W.L. Cheng, Z.Y. Fu, Q.L. Wu, Q.W. Wang, Effect of Experimental Parameters on Morphological, Mechanical and Hydrophobic Properties of Electrospun Polystyrene Fibers, *Materials* 8(5) (2015) 2718-2734.
- [9] A. Haider, S. Haider, I.K. Kang, A comprehensive review summarizing the effect of electrospinning parameters and potential applications of nanofibers in biomedical and biotechnology, *Arab J Chem* 11(8) (2018) 1165-1188.
- [10] H.Z. Chen, Y.S. Lui, J.H. Zhao, L. Xu, L.P. Tan, Effect of solvent composition of electrospun PLGA fibers on paclitaxel release, *Mater Technol* 33(11) (2018) 716-722.
- [11] H. Fong, I. Chun, D.H. Reneker, Beaded nanofibers formed during electrospinning, *Polymer* 40(16) (1999) 4585-4592.
- [12] M. Ieda, T. Tsuchihashi, K.N. Ivey, R.S. Ross, T.-T. Hong, R.M. Shaw, D. Srivastava, Cardiac Fibroblasts Regulate Myocardial Proliferation through $\beta 1$ Integrin Signaling, *Developmental cell* 16(2) (2009) 233-244.
- [13] T. Elzein, M. Nasser-Eddine, C. Delaite, S. Bistac, P. Dumas, FTIR study of polycaprolactone chain organization at interfaces, *Journal of colloid and interface science* 273(2) (2004) 381-387.
- [14] V. Shalini, S. Jonnalagadda, N. Kumar, Fast degradable poly (L-lactide-co-epsilon-caprolactone) microspheres for tissue engineering: synthesis, characterization, and degradation behavior, *J Polym Sci A1* 45 (2007) 2755-2764.
- [15] C.D.A.C. Erbetta, R.J. Alves, J.M. Resende, R.F. de Souza Freitas, R.G. de Sousa, Synthesis and Characterization of Poly (D, L-lactide-co-glycolide) Copolymer, *Journal of Biomaterials and Nanobiotechnology* 3(02) (2012) 208.
- [16] M.M. Stevanović, B. Jordović, D.P. Uskoković, Preparation and characterization of poly (D, L-lactide-co-glycolide) nanoparticles containing ascorbic acid, *BioMed Research International* 2007 (2007).

- [17] R. Akhtar, M.J. Sherratt, J.K. Cruickshank, B. Derby, Characterizing the elastic properties of tissues, *Materials today* 14(3) (2011) 96-105.
- [18] P.A. Janmey, R.T. Miller, Mechanisms of mechanical signaling in development and disease, *J Cell Sci* 124(Pt 1) (2011) 9-18.
- [19] L.A. Hidalgo-Bastida, J.J. Barry, N.M. Everitt, F.R. Rose, L.D. Buttery, I.P. Hall, W.C. Claycomb, K.M. Shakesheff, Cell adhesion and mechanical properties of a flexible scaffold for cardiac tissue engineering, *Acta biomaterialia* 3(4) (2007) 457-62.
- [20] S. Iwai, Y. Sawa, H. Ichikawa, S. Taketani, E. Uchimura, G. Chen, M. Hara, J. Miyake, H. Matsuda, Biodegradable polymer with collagen microsphere serves as a new bioengineered cardiovascular prosthesis, *The Journal of thoracic and cardiovascular surgery* 128(3) (2004) 472-9.
- [21] M. Kharaziha, M. Nikkhah, S.R. Shin, N. Annabi, N. Masoumi, A.K. Gaharwar, G. Camci-Unal, A. Khademhosseini, PGS:Gelatin nanofibrous scaffolds with tunable mechanical and structural properties for engineering cardiac tissues, *Biomaterials* 34(27) (2013) 6355-66.
- [22] T.C. McDevitt, K.A. Woodhouse, S.D. Hauschka, C.E. Murry, P.S. Stayton, Spatially organized layers of cardiomyocytes on biodegradable polyurethane films for myocardial repair, *J Biomed Mater Res A* 66(3) (2003) 586-95.
- [23] E. Rosellini, C. Cristallini, G.D. Guerra, N. Barbani, Surface chemical immobilization of bioactive peptides on synthetic polymers for cardiac tissue engineering, *Journal of biomaterials science. Polymer edition* 26(9) (2015) 515-33.
- [24] H. Shin, S. Jo, A.G. Mikos, Biomimetic materials for tissue engineering, *Biomaterials* 24(24) (2003) 4353-64.
- [25] D.A. Stout, J. Yoo, A.N. Santiago-Miranda, T.J. Webster, Mechanisms of greater cardiomyocyte functions on conductive nanoengineered composites for cardiovascular application, *International journal of nanomedicine* 7 (2012) 5653-69.
- [26] K.K. Parker, D.E. Ingber, Extracellular matrix, mechanotransduction and structural hierarchies in heart tissue engineering, *Philos Trans R Soc Lond B Biol Sci* 362(1484) (2007) 1267-79.
- [27] E. Kostakova, M. Seps, P. Pokorny, D. Lukas, Study of polycaprolactone wet electrospinning process, *Express Polymer Letters* 8(8) (2014) 554-564.

- [28] Q.L. Loh, C. Choong, Three-dimensional scaffolds for tissue engineering applications: role of porosity and pore size, *Tissue engineering. Part B, Reviews* 19(6) (2013) 485-502.
- [29] M. Yanagishita, Function of proteoglycans in the extracellular matrix, *Acta Pathol Jpn* 43(6) (1993) 283-93.
- [30] H.K. Graham, M. Horn, A.W. Trafford, Extracellular matrix profiles in the progression to heart failure. European Young Physiologists Symposium Keynote Lecture-Bratislava 2007, *Acta Physiol (Oxf)* 194(1) (2008) 3-21.
- [31] N. Nagaya, T. Fujii, T. Iwase, H. Ohgushi, T. Itoh, M. Uematsu, M. Yamagishi, H. Mori, K. Kangawa, S. Kitamura, Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis, *American Journal of Physiology-Heart and Circulatory Physiology* 287(6) (2004) H2670-H2676.
- [32] H.B. Kwak, Aging, exercise, and extracellular matrix in the heart, *J Exerc Rehabil* 9(3) (2013) 338-47.
- [33] G. Chi Ting Au-Yeung, U. Sarig, H. Sarig, H. Bogireddi, T. Bronshtein, L. Baruch, A. Spizzichino, J. Bortman, B.Y.C. Freddy, M. Machluf, S.S. Venkatraman, Restoring the biophysical properties of decellularized patches through recellularization, *Biomaterials Science* 5(6) (2017) 1183-1194.
- [34] U. Sarig, H. Sarig, A. Gora, M.K. Krishnamoorthi, G.C.T. Au-Yeung, E. de-Berardinis, S.Y. Chaw, P. Mhaisalkar, H. Bogireddi, S. Ramakrishna, F.Y.C. Boey, S.S. Venkatraman, M. Machluf, Biological and mechanical interplay at the Macro- and Microscales Modulates the Cell-Niche Fate, *Scientific reports* 8(1) (2018) 3937.
- [35] S.F. Badylak, T.W. Gilbert, Immune response to biologic scaffold materials, *Semin Immunol* 20(2) (2008) 109-16.
- [36] S. Beth, A. Ron, B. Limor, E. Yael, G. Idit, E. Ofek, D. Tzila, G. Lior, Z. Eyal, M. Marcelle, Electrospun Extracellular Matrix: Paving the Way to Tailor-Made Natural Scaffolds for Cardiac Tissue Regeneration, *Advanced Functional Materials* 27(34) (2017) 1700427.
- [37] U. Sarig, M. Machluf, Engineering cell platforms for myocardial regeneration, *Expert Opin Biol Ther* 11(8) (2011) 1055-77.

- [38] J. Yu, A.R. Lee, W.H. Lin, C.W. Lin, Y.K. Wu, W.B. Tsai, Electrospun PLGA fibers incorporated with functionalized biomolecules for cardiac tissue engineering, *Tissue Eng Part A* 20(13-14) (2014) 1896-907.
- [39] M. Sun, G. Chi, P. Li, S. Lv, J. Xu, Z. Xu, Y. Xia, Y. Tan, J. Xu, L. Li, Y. Li, Effects of Matrix Stiffness on the Morphology, Adhesion, Proliferation and Osteogenic Differentiation of Mesenchymal Stem Cells, *Int J Med Sci* 15(3) (2018) 257-268.

Chapter 5

Cardiac regeneration suitability of the 3DCS

This chapter explains the results of the interaction between the 3DCS and hiPSC-CM. CM were obtained by directed differentiation of hiPSC using small molecules. The cells were characterized for cardiac markers using flow cytometry and were seeded on the scaffolds to evaluate the scaffolds' hiPSC-CMs support-ability. Post seeding, the scaffolds demonstrated capacity to not just support the viability of the seeded cells but also support their beating functionality. The cells seeded on the scaffolds also expressed cardiac proteins and exhibited calcium handling capabilities, a crucial characteristic for CM functionality.

5.1 Experimental Methods

5.1.1 hiPSC-CM Culture on the Scaffolds

HiPSC-CM (hiPSC-CM) were obtained from hiPSCs (UT) as mentioned in section 3.2.4 and were seeded on the different scaffolds (~500,000 cells per scaffold) and cultured for 14 days. The hiPSC-CM seeded scaffolds were cultured in RPMI Medium and supplemented with 2% B-27 minus insulin. Culture medium was replaced every second day. Cell viability was evaluated using the AlamarBlue™ according to the manufacturer protocol. The beating of the scaffolds was monitored and recorded with a light microscope.

5.1.2 Ca²⁺ Signaling study using Fluo-4 AM

The ability of the CM to process Ca²⁺ is an important functionality in the cardiac environment. Characterization of cardiac tissue engineered constructs for this functionality provides information on the cardiac suitability of the construct and the extent to which the scaffold provides a cardiac microenvironment for the seeded CMs to function as they are intended to. An easy method to characterize the Ca²⁺ handling ability is to use a fluorescent calcium indicator Fluo 4 AM, which is cell permeable. The change in intracellular Ca²⁺ can be recorded using a confocal microscope.

Human pluripotent stem cells derived CM were obtained and cultured on the scaffold as mentioned in section 5.1.1. Ca²⁺ imaging was performed per previously published protocol [1]. Briefly, cell seeded scaffolds were loaded with 5 mM fluo-4 (Molecular Probes) fluorescent Ca²⁺ indicator with Pluronic F-127 (Molecular Probes) at 2:1 dilution to aid in recording intracellular Ca²⁺ transients. An indicator-free Tyrode solution (in mM: NaCl-140, KCl-5.4, CaCl₂-1.8, MgCl₂-1, HEPES-10, glucose-10) at pH 7.4 is used for sample incubation at 37°C. For pacing of the scaffolds, they were plated in a 35 mm optical bottom plate (Matek) with field stimulation electrodes (RC-37FS; Warner Instruments) and paced using a stimulus isolation unit (SIU-102; Warner instruments), by applying 5 ms suprathreshold bipolar stimulation pulses up to 50 mA. Intracellular Ca²⁺ transients were

recorded using a Zeiss LSM 710 laser scanning confocal imaging system equipped with a 60X water objective. Data were analyzed using Matlab-based custom written software.

5.2 Results and Discussion

5.2.1 FACS Study for Cardiac Marker Expression of hiPSC-CM

The hiPSC-CMs derived using small molecules based directed differentiation used in the study were characterized for their cardiac marker expression to verify the quality of the cell population.

Figure 5-1 hiPSC-CMs Characterization using FACS.

Detection by FACS demonstrates positive expression for cardiac markers. Dot plot of the representative population (A). Populations are more than 90 % positive for cTnI (C) and 80 % positive for SAA (D). Black histogram is the antibody only control (B).

Flow cytometry was performed (using protocol mentioned in section 3.9) to evaluate the pluripotent markers' expression. Cells were stained for Cardiac Troponin I (cTnI) and Sarcomeric Alpha Actinin (SAA), two prominent markers for CM. Isotype controls were used to calculate the percentage of positive cells. The FACS results exhibited that the

hiPSC-CMs were of high quality as they were more than 90% positive for cTnI and more than 80% for SAA as shown in Figure 5-1.

5.2.2 **3DCS Maintains the Viability and Functionality of Seeded hiPSC-CM**

It is suggested that a cardiac tissue construct engineered *in vitro* should possess tissue mimicking characteristics such as ECM mimicking microenvironment, non-immunogenicity, mechanical properties, cardiac cellular compatibility, contractility, and calcium handling [2]. The results of the first objective exhibited suitable scaffold properties. To check the cardiac suitability and supportability of the engineered 3DCS scaffold hiPSC-CM were seeded on these scaffolds and their health and functionality was monitored. Beating of the scaffolds were also monitored as a parameter for cellular functionality, as it is a widely used method to monitor the functional characteristic of the differentiated hiPSC-CMs [3].

To demonstrate the ability of the scaffolds to maintain the functionality of seeded cardiac cells, hiPSC-CMs were seeded on the scaffolds for two weeks and the cell viability was measured using AlamarBlue™ (performed with the protocol mentioned in section 3.11) during days 3, 7 and 14. As shown in Figure 5-2, at all the time points, 3DCS had significantly higher cell viability compared to UPLGA and pcECM. CM are not proliferating cells and all the scaffolds demonstrated the ability to maintain the viability of the seeded CM throughout the culture period. Yet, only 3DCS and pcECM could maintain the beating functionality (as shown in supplementary Figure 8-1) of the seeded hiPSC-CM cells while the UPLGA failed to maintain the crucial cardiomyocyte functionality, which could be due to the absence of a bioactive cardiac microenvironment. Beating functionality, a simple parameter to measure the cells' viability and functionality in the scaffold microenvironment. The quantity of cells active on a scaffold and the resulting beating, is therefore determined by both seeding density (which should be high enough to enable cell-scaffold and cell-cell contacts) and by the scaffold provision of supporting microenvironment.

Figure 5-2 hiPSC-CM viability on the scaffolds as measured using AlamarBlue™.

hiPSC-CMs interaction on 3DCS compared to the unmodified PLGA and pcECM scaffold.

All three scaffolds supported the viability of the seeded cells.

5.2.3 Cardiac Protein Expression of Seeded hiPSC-CM on the Scaffold

Apart from just viability, supporting the functionality of the seeded cells is an important criterion for a cardiac patch. Therefore, the hiPSC-CM seeded scaffolds were further studied for their cardiac marker expression (performed using protocol mentioned in section 3.10).

Cardiac Troponin I expression was observed in all three scaffolds (Figure 5-3) as expected as the seeded cells were characterized previously using FACS. The same trend was observed for Sarcomeric alpha actinin (SAA) as well, but with an absolute difference in their expression pattern between the cardiac ECM containing scaffolds 3DCS and pcECM and the UPLGA scaffolds. The SAA striation and organization was prominent (as shown by the green arrows in the inset) in the 3DCS (Figure 5-6) and pcECM (Figure 5-7) and was completely absent in the UPLGA scaffold as shown in Figure 5-5. The isotype controls for both cTnI and SAA were negatively stained as shown in Figure 5-4 and Figure 5-8 respectively. SAA is a protein located in the sarcomeric assembly and it was selected to

explain the contractile characteristic of the CM. The stark difference in the SAA pattern can be attributed to the role of the cardiac microenvironment present only in the 3DCS and pcECM scaffolds. Yu et al showed that SAA staining of neonatal rat CM seeded on unmodified and protein modified electrospun PLGA mats followed similar expression patterns [4]. The lack of SAA expression in UPLGA could also be attributed to dedifferentiation of hiPSC-CMs [5, 6]. The presence of SAA organization in the 3DCS and pcECM scaffolds and not in UPLGA scaffold further explains the lack of beating of the seeded cells in the UPLGA scaffolds. This, further endorses the criticality of a tissue specific bioactive microenvironment for cardiac regeneration.

Figure 5-3 Immunofluorescence of hiPSC-CMs seeded scaffolds expressing Cardiac Troponin I.

Representative images of hiPSC-CMs seeded scaffolds stained (14 days) for cTnI with primary cardiac troponin I antibody, Alexa Fluor 647 secondary antibody and DAPI for nuclear counterstaining. Scale: 20 µm.

Figure 5-4 Isotype control for Cardiac Troponin I staining of hiPSC-CMs seeded scaffolds. Representative images of isotype control for cTnI staining stained (14 days) only with the secondary antibody-Alexa Fluor 647 and DAPI for nuclear counterstaining. Scale: 20 μ m.

Figure 5-5 Immunofluorescence of hiPSC-CMs seeded UPLGA expressing Sarcomeric Alpha Actinin.

Representative images of hiPSC-CMs seeded scaffolds stained (14 days) for SAA with primary sarcomeric alpha actinin antibody, Alexa Fluor 647 secondary antibody and DAPI for nuclear counterstaining. Scale: 20 µm. No sarcomeric pattern was observed in this scaffold.

Figure 5-6 Immunofluorescence of hiPSC-CMs seeded 3DCS expressing Sarcomeric Alpha Actinin.

Representative images of hiPSC-CMs seeded 3DCS stained (14 days) for SAA with primary sarcomeric alpha actinin antibody, Alexa Fluor 647 secondary antibody and DAPI for nuclear counterstaining. Insets panel show zoomed (Scale: 5 μm) in images. Green arrow points to the striation pattern. Scale: 20 μm.

Figure 5-7 Immunofluorescence of hiPSC-CMs seeded pcECM expressing Sarcomeric Alpha Actinin.

Representative images of hiPSC-CMs seeded pcECM stained (14 days) for SAA with primary sarcomeric alpha actinin antibody, Alexa Fluor 647 secondary antibody and DAPI for nuclear counterstaining. Insets panel show zoomed (Scale: 5 μm) in images. Green arrow points to the striation pattern. Scale: 20 μm.

Figure 5-8 Isotype control for Sarcomeric Alpha Actinin staining of hiPSC-CMs seeded scaffolds.

Representative images of isotype control for SAA staining stained (14 days) only with the secondary antibody-Alexa Fluor 647 and DAPI for nuclear counterstaining. Scale: 20 μm .

5.2.4 3DCS Exhibits Better Calcium Handling Abilities

Ca^{2+} is a key modulator in cardiomyocyte functioning. It aids in regulating the electrical signaling and the contractile function of the resident CM in the heart myocardium [7]. We investigated the hiPSC-CMs seeded scaffolds' calcium handling ability using a qualitative fluorescent Fluo-4 AM molecule and confocal microscopy (using protocol mentioned in section 5.1.2), a characterization required to evaluate the suitability of these cell scaffold

constructs for cardiac regeneration. Calcium fluorescence under normal conditions and external electric field stimulation was tested [8].

In (Figure 5-9), for each scaffold, the upper panel represents the fluorescent distance time plots and the lower panel represents the same data processed using a MATLAB program to result in a line scan plot. At normal/spontaneous conditions without any external stimuli, the 3DCS exhibited higher number of calcium fluorescence pulse in the measured time (18 s) as compared to the pcECM and UPLGA. When the scaffolds were exposed to external electrical stimuli of 1 Hz, all the scaffolds showed similar performance. At 1 Hz, UPLA, 3DCS and pcECM were able to maintain the calcium fluorescent pulses based on the external frequency (1 pulse per second). Whereas at 1.5 Hz, the UPLGA did not reciprocate the effect of increased calcium handling at higher external stimuli as opposed to the 3DCS and the pcECM. Here, the UPLGA at 1.5 Hz was not able to produce calcium fluorescent pulses concurrently with the external electrical stimulation as opposed to 3DCS and pcECM.

Figure 5-9 Calcium handling ability of the hiPSC-CMs seeded on the scaffolds measured with fluorescent calcium indicator, Fluo 4 - AM.

Representative fluorescent distance time plots (upper panel) of the scaffolds loaded with Fluo 4 AM and images under a confocal laser scanning microscope using line scanning mode. The images were processed using a MATLAB program to result in a line scan tracing (lower panel). Blue scale bar indicates 1 s.

Most cardiac disorders are characterized by the impaired ability of the CM to perform perfectly in terms of their contractile property, for which Ca^{2+} is a crucial regulator [9]. Hence, the cell scaffold constructs' characterization to support this functionality is crucial in evaluating the suitability of this construct as a cardiac patch. The calcium handling abilities of CM have been related to the ECM stiffness they are being exposed to [10]. Various results have reported the relationship between the substrate stiffness and cellular calcium handling dynamics. Neonatal rat ventricular myocytes (NRVMs) seeded on softer

collagen coated polyacrylamide gel substrates in the range of 10 kPa (elastic modulus) have been shown to exhibit better calcium handling when compared to stiffer (50 kPa) substrates [11]. Another group used NRVMs on fibronectin coated structures and reported cells on 15 kPa substrate showed better calcium handling compared to 3 kPa [11]. Chicken embryo CM seeded on hyaluronic acid and poly acrylamide gels suggest that improper stiffness and increasing matrix stiffness drastically, could negatively affect the calcium handling of seeded CM [10]. The cell material interaction of the fabricated scaffolds in terms of cardiac handling displays a similar logic, that an intermediate stiffness combined with the biochemical composition could have a positive effect. The low stiffness UPLGA shows issues in efficient calcium handling while the higher stiffness pcECM showed better calcium handling in external field stimulus conditions. The 3DCS, with the intermediate stiffness, shows a better overall calcium handling by striking a balance in terms of cell material interaction. Still, factors that are different from the cited results should also be taken in consideration in terms of the complex relationship between architecture and biochemical composition of the ECM environment used in our study, when drawing the above conclusions.

Ca^{2+} signaling ability of the PSC derived CM is also related to the maturation state of the hiPSC-CMs, where the presence or improved Ca^{2+} indicates the presence of a mature cardiomyocyte [9, 12]. Here as shown in Figure 5-9, the 3DCS exhibited superior support to this crucial functionality in the normal/spontaneous conditions and also under external field stimulation. This indicates that the 3DCS scaffold system could also aid in the maturation of the seeded hiPSC-CMs.

5.3 Conclusion

In this chapter, the suitability of the fabricated 3DCS for cardiac application for cardiomyocyte viability and functionality was studied. hiPSC was differentiated into CM using small molecule-based protocols. These CM were seeded on the different scaffolds – UPLGA, 3DCS and pcECM. All three scaffolds maintained the viability of the seeded cells but only the 3DCS and the pcECM maintained the beating functionality and exhibited

patterned SAA expression. Also, the 3DCS and pcECM scaffolds seeded with hiPSC-CMs displayed better calcium handling compared to the UPLGA scaffold. In summary, the results here show that a cardiac ECM containing microenvironment supports better cardiomyocyte functionality and suitability for cardiac regenerative applications.

References

- [1] S. Beth, A. Ron, B. Limor, E. Yael, G. Idit, E. Ofek, D. Tzila, G. Lior, Z. Eyal, M. Marcelle, Electrospun Extracellular Matrix: Paving the Way to Tailor-Made Natural Scaffolds for Cardiac Tissue Regeneration, *Advanced Functional Materials* 27(34) (2017) 1700427.
- [2] N.J. Kaiser, K.L. Coulombe, Physiologically inspired cardiac scaffolds for tailored in vivo function and heart regeneration, *Biomedical materials* 10(3) (2015) 034003.
- [3] E. Laurila, A. Ahola, J. Hyttinen, K. Aalto-Setälä, Methods for in vitro functional analysis of iPSC derived cardiomyocytes - Special focus on analyzing the mechanical beating behavior, *Biochim Biophys Acta* 1863(7 Pt B) (2016) 1864-72.
- [4] J. Yu, A.R. Lee, W.H. Lin, C.W. Lin, Y.K. Wu, W.B. Tsai, Electrospun PLGA fibers incorporated with functionalized biomolecules for cardiac tissue engineering, *Tissue Eng Part A* 20(13-14) (2014) 1896-907.
- [5] A. Leri, M. Rota, F.S. Pasqualini, P. Goichberg, P. Anversa, Origin of cardiomyocytes in the adult heart, *Circ Res* 116(1) (2015) 150-66.
- [6] Y. Yahalom-Ronen, D. Rajchman, R. Sarig, B. Geiger, E. Tzahor, Reduced matrix rigidity promotes neonatal cardiomyocyte dedifferentiation, proliferation and clonal expansion, *Elife* 4 (2015).
- [7] C.J. Fearnley, H.L. Roderick, M.D. Bootman, Calcium signaling in cardiac myocytes, *Cold Spring Harb Perspect Biol* 3(11) (2011) a004242.
- [8] I. Itzhaki, S. Rapoport, I. Huber, I. Mizrahi, L. Zwi-Dantsis, G. Arbel, J. Schiller, L. Gepstein, Calcium handling in human induced pluripotent stem cell derived cardiomyocytes, *PloS one* 6(4) (2011) e18037.
- [9] T.J. Kolanowski, C.L. Antos, K. Guan, Making human cardiomyocytes up to date: Derivation, maturation state and perspectives, *Int J Cardiol* 241 (2017) 379-386.

- [10] J.L. Young, K. Kretschmer, M.G. Oudeck, A.C. Zambon, A.J. Engler, Mechanosensitive kinases regulate stiffness-induced cardiomyocyte maturation, *Scientific reports* 4 (2014) 6425.
- [11] M. Tallawi, R. Rai, A.R. Boccaccini, K.E. Aifantis, Effect of substrate mechanics on cardiomyocyte maturation and growth, *Tissue engineering. Part B, Reviews* 21(1) (2015) 157-65.
- [12] A.H. Fong, M. Romero-Lopez, C.M. Heylman, M. Keating, D. Tran, A. Sobrino, A.Q. Tran, H.H. Pham, C. Fimbres, P.D. Gershon, E.L. Botvinick, S.C. George, C.C. Hughes, Three-Dimensional Adult Cardiac Extracellular Matrix Promotes Maturation of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes, *Tissue Eng Part A* 22(15-16) (2016) 1016-25.

Chapter 6

Influence of 3DCS bioactivity on pluripotent stem cells

This chapter describes the results of the interaction of hiPSC (hiPSCs) with the 3D composite scaffold and the influence of the modified 3DCS' bioactivity profile on the stemness and commitment of hiPSCs towards ECM source-tissue specific lineages. The hiPSCs were characterized for their pluripotency using flow cytometry. The cells were seeded on the scaffolds and were cultured in a spontaneous differentiation medium to evaluate if the scaffolds' bioactivity can influence the differentiation of the seeded cells preferably towards cardiac lineage. All scaffolds exhibited hiPSCs attachment, viability and proliferation. After 4 weeks of culture, only the cells seeded on the 3DCS expressed cardiac markers spontaneously without the addition of any external factors. Conditioned medium from this experiment was obtained and administered to hiPSCs seeded on Matrigel (a non-differentiating control as observed in our experiments) to evaluate for any soluble factors in the conditioned medium. Surprisingly, the hiPSCs seeded on Matrigel, in the presence of this conditioned medium expressed cardiac lineage markers.

6.1 Materials & Methods

6.1.1 hiPSC Attachment to the Scaffolds

hiPSCs obtained from Professor Gepstein's lab (hiPSCs-UT) were cultured as mentioned in section 3.2.4. Scaffolds were prepared with a dimension of 6 mm diameter and disinfected as mentioned in section 4.1.2. hiPSCs were seeded into each sterilized scaffold by pipetting at different positions on the surface of the scaffold. The seeded scaffolds in 96-well plate were placed at 37°C to allow for cell attachment. Following different incubation periods (0 min, 45 min, 90 min, and 150 min) scaffolds were quickly washed with sterile PBS to remove non-attached cells and were subsequently transferred into a 24-well plate for culturing. Two ml of complete culture medium with 10% AlamarBlue™ was added to each well. After 5 h incubation with AlamarBlue™, 100 µL of each well in three replicates were transferred in a black 96-well plate for fluorescence reading on the Varioskan flash reader (excitation 540 nm, emission 595 nm) to measure cell viability. Also, the cell seeded scaffolds were stained with Hoechst and imaged using a fluorescent microscope (Nikon, TE2000).

6.1.2 hiPSC Culture on Scaffolds

hiPSCs - UT were cultured as mentioned in section 3.2.4 and were seeded on the different scaffolds and cultured for 28 days. To study the effect of preserved cardiac bioactivity of the different scaffolds on the spontaneous differentiation of hiPSCs, the hiPSCs were harvested and seeded on different scaffolds (5×10^5 cells per scaffold) for 24 hr in complete mTeSR medium. One-day post seeding, the medium was replaced with a spontaneous differentiation medium (a nutrient medium previously reported and used for PSC cultivation, which has less stringent biochemical control of PSC purity maintenance compared to mTeSR [1]) containing 79% DMEM, 20% defined Fetal Bovine Serum (HyClone SH30070.03), 1% nonessential amino acids, 0.1 mM β -mercaptoethanol, and 100 U/mL penicillin–100 mg/mL streptomycin (Biological Industries). The media was

replaced every three days and at the end of four weeks, the scaffolds were processed for immunofluorescence.

6.2 Results & Discussion

6.2.1 FACS Study of Pluripotency Markers by hiPSCs

The hiPSCs used in the study were characterized for their pluripotency to validate the stemness of the starting cell population. Flow cytometry was performed (using protocol mentioned in section 3.9) to evaluate the pluripotent markers' expression. Cells were stained for OCT4, Nanog and SSEA4 three widely used markers for pluripotency. Isotype controls were used to calculate the percentage of positive cells. The FACS results exhibited that the hiPSCs were of high quality as they were more than 90% positive for all the markers analyzed as shown in Figure 6-1.

Figure 6-1 hiPSCs Characterization using FACS.

Detection by FACS demonstrates positive expression for pluripotent markers. Dot plot of the representative population (A). Populations are more than 95 % positive for OCT4 (C) & SSEA4 (D) and 90 % positive for NANOG (E). Black histogram is the antibody only control (B).

6.2.2 hiPSCs Attachment to the Scaffolds

The common assumption is that a suitable ECM microenvironment contributes crucial cues towards organ specific differentiation of stem cells. However, such interactions have not been demonstrated with human pluripotent stem cells to study the influence of an organ specific bioactivity on the pluripotency of the cells. Also, the specific interaction in this cell scaffold interaction, evoking cell viability, proliferation and differentiation remains unclear. To study these interactions of the hiPSCs with the scaffold platforms, suitable culture conditions need to be established for favorable initial attachment, proliferation and possibly differentiation.

Evaluation of optimal cell attachment time of hiPSCs to the scaffolds was studied by culturing cells on the scaffolds for different initial attachment times (0, 45, 90 and 150 min) at 37°C and 5% CO₂. Subsequently, AlamarBlue™ assay (performed using protocol

mentioned in section 6.1.1) was performed to determine the viable cells attached on the scaffold. Based on different attachment times, all the scaffolds displayed high attachment at 90 min (Figure 6-2).

Both UPLGA and 3DCS showed relatively higher attachment than the pcECM scaffold at all the attachment times. Given that there was no significant difference between 90 and 150 min, subsequent experiments used an attachment time of 90 min which also corresponds to the standard used in our lab for other cell types for pcECM. The attachment time results revealed that an initial attachment time of 90 min is required for the cells to attach to the scaffolds efficiently, a trend observed in all scaffold platforms. An ideal initial incubation time for attachment is required as a shorter incubation time results in an insufficient attachment and longer times could lead to compromising cell viability due to nutrient depletion resulting in cell death [2].

Figure 6-2 hiPSCs attachment to the scaffolds as measured by AlamarBlue™ assay. iPSC was incubated with pcECM for 0, 45, 90, 135 and 180 min to attach to the surface. Fluorescent intensity (FI) is represented mean $\pm$ SD from n=3. * represents statistical significance of PLGA, 3DCS & pcECM at 90 min when compared to 0 and 45 min ($p<0.05$); ns represents statistical non-significance of PLGA, 3DCS & pcECM at 150 min when compared to 90 min ($p<0.05$).

The effect of attachment time of hiPSCs to the scaffolds was also observed by staining the cell seeded scaffolds with Hoechst nuclear stain and imaged under a fluorescent microscope. The same trend as in the AlamarBlue™ was also observed here as shown in Figure 6-3. The pcECM at 0 min had very few cells and it gradually increases as the attachment time increased. Also, the nuclear staining images reveal the colony like structures, characteristic of the hiPSCs.

Figure 6-3 hiPSCs attachment to the scaffolds as evaluated by Hoechst staining of the cell seeded scaffolds.

iPSC was incubated with the scaffolds for 0, 45, 90, and 150 min to attach to the surface.

Representative Hoechst stained images of the seeded scaffolds Scale: 200 µm.

6.2.3 3DCS Supports Long Term Viability and Proliferation of hiPSCs

The long-term viability of 28 days and proliferation of hiPSCs on the scaffolds was measured using AlamarBlue™ (performed using protocol mentioned in section 3.11). All three scaffold platforms supported the long-term culture of hiPSCs (Figure 6-4) with an observed difference during the initial time points of day 1 and 7. During these time points, the UPLGA and pcECM had significantly lower number of cells compared to the 3DCS.

However, more cells were observed at later time points as observed from the increased fluorescent intensities (FI). All three scaffolds exhibited significant increase in cells from day 1 to 28. At day 28, the 3DCS had significantly higher FI than pcECM. Day 21 showed the highest FI for both UPLGA and pcECM, not significantly different to the 3DCS, corresponding to cellular proliferation during the culture period. The initial lower FI corresponding to lower cells in the UPLGA can be attributed to the absence of cell binding moieties. However surprisingly, the pcECM scaffold with its known bioactivity and cell adhesion abilities, did not exhibit the highest attachment which was also observed in our attachment time characterization. Though there were only very low number of cells in the pcECM during initial time points, the cells proliferated much better at higher time points. Maricela *et al* have reported that when hiPSCs were seeded on collagen coated PCL electrospun scaffold with various stiffness, it was reported that at a stiffness of 193 kPa (a value very similar to the Young's modulus of pcECM), the iPSCs proliferated faster up to 12 days [3]. Moreover, such effects of stiffness and nanotopographical cues have been reported to have effect the cellular proliferation, pluripotency and differentiation potential of seeded hiPSCs [4]. Also, the lower initial cellular attachment of the hiPSCs to the pcECM (as observed in Figure 6-2 and Figure 6-3), could be responsible for the initial low FI of the hiPSCs seeded on the pcECM. Another reason could be the architecture of the pcECM scaffold which can support cellular infiltration only up to 100 μm at static conditions [5]. After the initial attachment of the seeded cells, the cells compete for scaffold surface to proliferate and infiltrate and this combined with the initial lower cell number could be responsible for the delayed proliferation that is observed for hiPSCs seeded on the pcECM.

Figure 6-4 Long term viability and proliferation of hiPSCs on the scaffolds.

hiPSCs interaction and proliferation on 3DCS compared to the unmodified PLGA and pcECM scaffold. All three scaffolds exhibited ability to support the viability and proliferation of the seeded cells.

6.2.4 **3DCS Induces Spontaneous Differentiation of hiPSCs Towards Cardiac Lineage**

To study the influence of the scaffolds' bioactivity on the pluripotency of the hiPSCs, the cells were seeded on the scaffolds in a spontaneous differentiation medium for 28 days. Immunofluorescence for cardiac markers were performed (using protocol mentioned in section 3.10) 4 weeks post seeding to inspect for cardiac specific protein expression. Figure 6-5 shows the scaffold stained for cardiac troponin I, a key cardiac marker. Both the 3DCS and the pcECM displayed cTnI marker expression. In comparison, the UPLGA scaffold seeded with hiPSCs did not display any cTnI expression. Further, the scaffolds were stained for SAA and the expression of these markers; similarly as cTnI; were positive in the 3DCS and the pcECM but not in the UPLGA samples as shown in Figure 6-7. Isotype controls for both cTnI and SAA exhibited negative staining as shown in Figure 6-6 and Figure 6-8.

The differentiation of hiPSCs on the pcECM, is in acceptance with previously published results where mouse ESC and iPSC cells were observed to differentiate in the presence of a 300 μm thick decellularized human cardiac ECM slices [6]. The differentiation observed

in 3DCS could therefore be attributed to the complex cardiac ECM biochemistry that was used to functionalize the UPLGA. Even though other ECM formulations like MatrigelTM were shown to support cardiac differentiation of pluripotent stem cells, they were achieved with the addition of external factors and molecules [7]. Here we show that the scaffolds' bioactivity and physical cues are solely responsible for the effect without external induction mediums. In addition, this tissue specific bioactivity takes leverage of the pluripotency of the seeded cells and aids in their 'embryonic tissue' development and differentiation, by pushing their differentiation towards a lineage that is dictated by the surrounding tissue ECM microenvironment.

Figure 6-5 Immunofluorescence of hiPSCs seeded scaffolds expressing Cardiac Troponin I.

Representative images of hiPSCs seeded scaffolds stained (Day 28) for cTnI with primary cardiac troponin I antibody, Alexa Fluor 647 secondary antibody and DAPI for nuclear counterstaining. Scale: 20 µm.

Figure 6-6 Isotype control for Cardiac Troponin I staining of hiPSCs seeded scaffolds. Representative images of isotype control for cTnI staining stained (Day 28) only with the secondary antibody-Alexa Fluor 647 and DAPI for nuclear counterstaining. Scale: 20 μm .

Figure 6-7 Immunofluorescence of hiPSCs seeded scaffolds expressing Sarcomeric Alpha Actinin (SAA).

Representative images of hiPSCs seeded scaffolds stained (Day 28) for SAA with primary sarcomeric alpha actinin antibody, Alexa Fluor 647 secondary antibody and DAPI for nuclear counterstaining. Scale: 20 µm.

Figure 6-8 Isotype control for Sarcomeric Alpha Actinin staining of hiPSCs seeded scaffolds.

Representative images of isotype control for SAA staining stained (Day 28) only with the secondary antibody-Alexa Fluor 647 and DAPI for nuclear counterstaining. Scale: 20 μm.

6.2.5 Conditioned Medium from 3DCS-hiPSC Construct Induces Cardiac Differentiation of hiPSCs Seeded on Matrigel™

One of the major hurdles that this thesis tries to decouple, in studying pcECM bioactivity mechanisms, relates to the effect of direct structural cues vs. that of pcECM biochemical composition on human stem cell commitment. While elucidating the exact molecular mechanisms behind the remarkable spontaneous differentiation of hiPSC towards ECM source-tissue specific lineages is beyond the scope of this thesis, an initial assessment of

the possible involvement of soluble factors in the process was performed. We hypothesized that if soluble factors, regardless of their source, are involved in the apparent differentiation process, then a conditioned media produced from spontaneous differentiation of hiPSC seeded on 3DCS or pcECM, might elicit a similar effect on pluripotency in a non-differentiating control. MatrigelTM (a commercial secreted and soluble ECM source commonly used for hiPSC culture and maintenance) coated plates were used as the non-differentiating control, as it does not induce directed spontaneous differentiation with the regular non-conditioned medium (Figure 6-9 and Figure 6-11, MatrigelTM Ctrl panel). Moreover, pcECM gel was obtained from solid pcECM and 3DCS were fabricated using pcECM gel. Hence, the hypothesis that the conditioned medium from these cardiac scaffolds' interaction with hiPSCs could induce cardiogenic differentiation was studied using MatrigelTM. Other members of our laboratory, have previously compared the effect of soluble pcECM (data not shown) to that of MatrigelTM—on hiPSC spontaneous differentiation. It turned out that unlike the tissue specific pcECM, commercial growth factor rich MatrigelTM does not induce spontaneous differentiation. Therefore, MatrigelTM coated tissue culture plates were seeded with hiPSCs and cultured in either plain spontaneous differentiation media or in pcECM/ standard 3DCS-hiPSC conditioned media for 28 days. Immunofluorescence performed (using protocol mentioned in section 3.10) at the end of 4 weeks exhibited, that only the conditioned medium has induced a spontaneous differentiation towards cardiac lineage, as apparent by both cTnI and SAA staining (Figure 6-9, Figure 6-11) and isotype control (Figure 6-10, Figure 6-12). The fact that only the hiPSCs seeded on cardiac ECM containing scaffold differentiated towards cardiac lineage can be due to two scenarios: i) There could be factors being secreted and released into the culture medium by the cells themselves as a response to the interaction with the scaffold. ii) There could be molecules being released into the medium from the cardiac ECM as a result of degradation during the culture period. In both cases, the assumption is that the released factors could be present in the culture medium and that they are acting on the cells and influencing their differentiation fate—as opposed to insoluble factors such as direct cell-cell or cell-ECM contact induced differentiation. This hypothesis was confirmed when the conditioned medium was able to induce cardiac lineage differentiation of hiPSCs seeded on MatrigelTM. This is significant as the hiPSCs seeded MatrigelTM cultured with

regular medium instead of the conditioned medium did not result in any cardiac lineage differentiation. Hence these set of results exhibits the importance of a dynamic cell material interaction and the crucial presence of a cardiac ECM microenvironment, which might have been recognized by the cells as an initiator of their differentiation towards the target tissue lineage. Also, even though the differentiation of the seeded cells only exhibited lineage commitment and did not actually result in completely functional CM, these results reiterate our hypothesis and the long-held assumption that an organ specific ECM microenvironment; both in biochemical and physical environments; could influence the seeded stem cell's fate in terms of lineage commitment and differentiation.

Figure 6-9 Immunofluorescence of hiPSCs seeded scaffolds expressing Cardiac Troponin I following addition of conditioned medium.

Representative images of hiPSCs seeded MatrigelTM samples stained (Day 28) for cTnI with primary cardiac troponin I antibody, Alexa Fluor 647 secondary antibody and DAPI for nuclear counterstaining. MatrigelTM Ctrl – hiPSCs cultured on Matrigel coated plates and cultured with non-conditioned medium; 3DCS to MatrigelTM - hiPSCs cultured on MatrigelTM coated plates and cultured with conditioned medium from 3DCS-hiPSC interaction; pcECM to MatrigelTM - hiPSCs cultured on MatrigelTM coated plates and cultured with conditioned medium from pcECM-hiPSC interaction; Scale: 20 μm.

Figure 6-10 Isotype control for Cardiac Troponin I staining of hiPSCs seeded scaffolds cultured with conditioned medium.

Representative images of isotype control for cTnI staining stained (Day 28) only with the secondary antibody-Alexa Fluor 647 and DAPI for nuclear counterstaining. Matrigel™ Ctrl – hiPSCs cultured on Matrigel™ coated plates and cultured with non-conditioned medium; 3DCS to Matrigel™ - hiPSCs cultured on Matrigel™ coated plates and cultured with conditioned medium from 3DCS-hiPSC interaction; pcECM to Matrigel™ - hiPSCs cultured on Matrigel™ coated plates and cultured with conditioned medium from pcECM-hiPSC interaction; Scale: 20 μm.

Figure 6-11 Immunofluorescence of hiPSCs seeded scaffolds expressing Sarcomeric Alpha Actinin following addition of conditioned medium.

Representative images of hiPSCs seeded MatrigelTM samples stained (Day 28) for SAA with primary sarcomeric alpha actinin antibody, Alexa Fluor 647 secondary antibody and DAPI for nuclear counterstaining. MatrigelTM Ctrl – hiPSCs cultured on MatrigelTM coated plates and cultured with non-conditioned medium; 3DCS to MatrigelTM - hiPSCs cultured on MatrigelTM coated plates and cultured with conditioned medium from 3DCS-hiPSC interaction; pcECM to MatrigelTM - hiPSCs cultured on MatrigelTM coated plates and cultured with conditioned medium from pcECM-hiPSC interaction; Scale: 20 µm.

Figure 6-12 Isotype control for Sarcomeric Alpha Actinin staining of hiPSCs seeded scaffolds cultured with conditioned medium.

Representative images of isotype control for SAA staining stained (Day 28) only with the secondary antibody-Alexa Fluor 647 and DAPI for nuclear counterstaining. Matrigel Ctrl – hiPSCs cultured on Matrigel coated plates and cultured with non-conditioned medium; 3DCS to Matrigel - hiPSCs cultured on Matrigel coated plates and cultured with conditioned medium from 3DCS-hiPSC interaction; pcECM to Matrigel - hiPSCs cultured on Matrigel coated plates and cultured with conditioned medium from pcECM-hiPSC interaction; Scale: 20 μ m.

6.3 Conclusion

This chapter illustrates the influence of the scaffolds' bioactivity on the differentiation ability of the pluripotent cells. hiPSCs were seeded on the scaffolds and their attachment and proliferation were studied using AlamarBlueTM. All the scaffolds displayed ability to support the attachment and proliferation of the seeded cells for a period of 28 days. Immunofluorescence evaluation of the cell seeded scaffolds exhibited spontaneous differentiation of the seeded cells towards cardiac lineage in 3DCS and pcECM scaffolds. Conditioned medium from hiPSC and cardiac ECM containing scaffolds (3DCS and pcECM), when added to hiPSC culture on MatrigelTM, induced similar spontaneous differentiation of the cells towards cardiac lineage, denoting the importance of the soluble factors secreted by cells cultured on 3DCS and/or pcECM in modulating hiPSC differentiation. The presence of the soluble factors sheds light on the cardiac lineage commitment of the seeded hiPSCs on the cardiac ECM containing scaffolds (3DCS & pcECM) and opens up the possibility of further identifying the bioactive molecules responsible for this phenomenon.

References

- [1] G. Arbel, O. Caspi, I. Huber, A. Gepstein, M. Weiler-Sagie, L. Gepstein, Methods for Human Embryonic Stem Cells Derived Cardiomyocytes Cultivation, Genetic Manipulation, and Transplantation, in: R.J. Lee (Ed.), Stem Cells for Myocardial Regeneration: Methods and Protocols, Humana Press, Totowa, NJ, 2010, pp. 85-95.
- [2] Y. Wang, T. Bronshtein, U. Sarig, F.Y.C. Boey, S.S. Venkatraman, Endothelialization of acellular porcine ECM with chemical modification, International Journal of Bioscience, Biochemistry and Bioinformatics 2(5) (2012) 363.
- [3] M. Maldonado, L.Y. Wong, C. Echeverria, G. Ico, K. Low, T. Fujimoto, J.K. Johnson, J. Nam, The effects of electrospun substrate-mediated cell colony morphology on the self-renewal of human induced pluripotent stem cells, Biomaterials 50 (2015) 10-9.

- [4] R.G. Ireland, C.A. Simmons, Human Pluripotent Stem Cell Mechanobiology: Manipulating the Biophysical Microenvironment for Regenerative Medicine and Tissue Engineering Applications, *Stem Cells* 33(11) (2015) 3187-96.
- [5] U. Sarig, E.B. Nguyen, Y. Wang, S. Ting, T. Bronshtein, H. Sarig, N. Dahan, M. Gvirts, S. Reuveny, S.K. Oh, T. Scheper, Y.C. Boey, S.S. Venkatraman, M. Machluf, Pushing the envelope in tissue engineering: ex vivo production of thick vascularized cardiac extracellular matrix constructs, *Tissue Eng Part A* 21(9-10) (2015) 1507-19.
- [6] B. Oberwallner, A. Brodarac, P. Anic, T. Saric, K. Wassilew, K. Neef, Y.H. Choi, C. Stamm, Human cardiac extracellular matrix supports myocardial lineage commitment of pluripotent stem cells, *Eur J Cardiothorac Surg* 47(3) (2015) 416-25; discussion 425.
- [7] J. Zhang, M. Klos, G.F. Wilson, A.M. Herman, X. Lian, K.K. Raval, M.R. Barron, L. Hou, A.G. Soerens, J. Yu, S.P. Palecek, G.E. Lyons, J.A. Thomson, T.J. Herron, J. Jalife, T.J. Kamp, Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method, *Circ Res* 111(9) (2012) 1125-36.

Chapter 7

Conclusion and Future Recommendations

This chapter explains and discusses the results in Chapter 4, 5 & 6 in relation to the existing literature and elicits the extent to which the hypothesis was tested. Finally, the chapter concludes by presenting future recommendations and opportunities that can be pursued based on this thesis.

7.1 Summary

Cell material interactions play a crucial role in tissue engineering and regeneration, as a suitable native ECM mimicking microenvironment can result in a favorable interaction regime, tissue regeneration and homeostasis, as opposed to inflammatory response, fibrosis and scarring [1, 2].

Factors that formulate a native ECM can be broadly classified into architectural and biochemical components which in turn act as cues for seeded cells *in vitro* or recruited cell *in vivo* [3]. Decellularized ECM materials from different species and tissue sources are considered excellent candidates for tissue regeneration applications, as they possess a preserved native ECM architecture and all the inherent tissue specific bioactive molecules [4]. Though decellularized pcECM has demonstrated tissue source specificity in improving cardiac function following MI, while being delivered either as an insoluble (patch) or as a soluble injectable form [5, 6], its exact mechanisms of action remains unclear. One major impediment remains, our ability to decouple the architecture of insoluble ECM scaffolds from their soluble biochemical composition, be it naturally soluble or solubilized following interaction with various ECM-remodeling human cells.

Many 3D passive ECM structures can be mimicked using synthetic polymeric materials and modern scaffold fabrication techniques, however, they usually lack an appropriate bioactive profile [7]. pcECM on the other hand may be overly bioactive, and is subject to high batch to batch variability, which may limit both reproducibility in basic research studies, as well as large scale production strategies. We provide a proof-of-concept for conferring tissue specific bioactivity to completely synthetic 3D ECM mimicking scaffolds, while offering better control and higher ‘tune-ability’ over architectural and soluble cues—with clear implications to both basic as well as applied research.

While electrospinning has been widely recognized for the fabrication of ECM mimicking architecture at nano and micro scales [8], traditional electrospinning results only in a 2D matrix structure that is far from the native 3D environment [9-11]. Wet electrospinning is

a modified method which uses a liquid bath in place of the solid collector of the traditional electrospinning, resulting in a highly three-dimensional scaffold [12-14]. Though the wet electrospinning process results in a native mimicking 3D structure and morphology, they lack the necessary bioactivity required for cellular interactions. Hence suitable chemical modifications are necessary to transform these scaffolds bioactive [12-14].

Hence, we hypothesized that combining the wet electrospun 3D scaffold with the liquid form of the cardiac ECM will result in a 3D composite scaffold (3DCS) that can recapitulate a native ECM microenvironment and can be compared to the pcECM, with better tunability. This would enable us in exploiting the advantages of both the natural ECM and the synthetic polymeric scaffold, while circumventing their inherent limitations. Therefore, we fabricated a 3D composite scaffold and characterized this scaffold in terms of morphology, chemistry, biochemical composition, mechanical properties, *in vitro* immunogenicity and cellular compatibility with hMSCs (hMSCs). The cardiac suitability of the scaffold was studied with human induced pluripotent derived CM (hiPSC-CMs). Additionally, the scaffolds' cardiac specific bioactivity was studied with hiPSC (hiPSCs).

In Aim 1, the key objective was to study the feasibility for the fabrication of a synthetic hybrid scaffold, that can compensate for the limitations of the pcECM and the synthetic scaffold systems individually. To achieve this, various parameters, particularly the overall morphology and chemistry of the pcECM were characterized to identify and recapitulate them using a more tunable synthetic system. The fabrication of the synthetic 3D scaffold to mimic the morphological characteristics of the pcECM was performed using wet electrospinning of PLGA polymer, a technique which results in a fibrous construct with tunability over the scaffold dimensions. To equip the 3D synthetic scaffold with the bioactivity essential for further cellular interaction, the 3D PLGA scaffold was functionalized using the liquid form for the pcECM using EDC-NHS chemistry, to for the 3DCS. This 3DCS when characterized for different scaffold parameters, were found to mimic the pcECM in terms of morphology as well as chemistry. The 3DCS were also characterized to be non-immunogenic *in vitro* and supported the growth and proliferation

of seeded hMSCs, an accepted model cell type for CTE. All these results, answered our research questions established for Aim 1 as shown in Figure 1-1.

Building on Aim 1 and to further characterize the 3DCS cardiac compatibility (Aim 2 research questions, Figure 1-1), 3DCS were further seeded with hiPSC-CM, exhibiting pro-longed cell viability and support-ability for over two weeks, a pre-requisite for CTE applicability. Although all the scaffolds, i.e., UPLGA, 3DCS and pcECM maintained viability of the seeded hiPSC-CMs, only the scaffolds with cardiac bioactivity (3DCS and pcECM) could maintain CM beating functionality. To further understand this interaction, immunofluorescence for cardiac markers were performed. All the scaffolds exhibited positive staining for cardiac troponin I, a key marker for cardiomyocyte characterization. The expression pattern of sarcomeric alpha actinin explained the presence and absence of beating functionality in the hiPSC-CMs seeded on cardiac ECM containing scaffolds and UPLGA respectively, as organized actinin structure, a key component for cardiac beating, was detected only in 3DCS and pcECM scaffolds. Another key parameter for cardiac functionality, calcium handling of the seeded hiPSC-CMs was also studied using fluorescent calcium marker Fluo-4 AM. In normal conditions, the calcium handling of the seeded hiPSC-CMs were higher in the hybrid 3DCS followed by natural pcECM and finally the UPLGA, a trend that could be attributed to the differences in their mechanical property and the availability of cardiac bioactivity. These results reiterated the importance of a cardiac specific bioactive microenvironment for cardiomyocyte viability, protein expression and the influence of a dynamic balance between scaffold morphological properties and chemistry for cardiomyocyte calcium handling.

After having established that the hybrid 3DCS can support the viability and functionality of hiPSC-CMs, the extent of support by the 3DCS for hiPSCs attachment, proliferation and differentiation was studied in Aim 3 (Figure 1-1). hiPSCs when seeded on the three scaffold platforms (3DCS, pcECM and UPLGA), were able to attach to the scaffolds after an incubation time of 90 min and proliferation was observed for four weeks. To characterize the influence of the scaffolds on the differentiation of the seeded hiPSCs, immunostaining was performed for cardiac markers cardiac troponin I and sarcomeric alpha actinin. The

immunostaining results revealed that the scaffolds containing cardiac ECM i.e. 3DCS and pcECM influenced the seeded hiPSCs and induced their differentiation specifically to cardiogenic lineage without the addition of any external factors, an important finding towards our research question. To explore how the scaffold bioactivity supports the observed differentiation pattern, conditioned medium from hiPSCs seeded cardiac ECM containing scaffolds (3DCS and pcECM) were added to hiPSCs seeded on Matrigel coated tissue culture plates. The hypothesis that the cardiac ECM bioactivity is responsible for the cardiogenic differentiation was confirmed here, as only the hiPSCs on Matrigel with conditioned medium expressed cardiac markers and the hiPSCs on Matrigel with regular medium did not express cardiac markers. This result, though promising, does not pin point the major components or combination of molecules responsible for this observed effect and further investigation in this regard is necessary to understand the interactions occurring in this backdrop.

Taken together, it is evident that there is a dynamic crosstalk occurring in the overall cell scaffold interaction regime resulting in varied viability, proliferation and differentiation responses. The results obtained from the interaction of the hiPSCs and hiPSC-CMs with the scaffold platforms, manifests the importance of a favorable ECM microenvironment and the required balance between the architectural and biochemical cues necessary for cellular viability and functionality.

7.2 Future recommendations

In this work, a 3D composite scaffold was fabricated from the combination of a natural and synthetic material platform to enjoy the advantages of both the material platforms. The scaffolds, though characterized extensively on various parameters, needs to be studied in other conditions too.

The cell interaction work presented in this thesis is completely performed at static conditions. The same cell interactions could be very much different if they were to be performed under dynamic conditions with a bioreactor setup. This is possible as the

fabrication of the 3D electrospun scaffold produced by wet electrospinning is extremely tunable in their resulting dimensions. Larger scaffolds can be designed and functionalized using the pcECM gel and the scaffold can be studied under bioreactor environment with external electrical and mechanical stimuli. This dynamic environment could improve the overall functionality of the scaffold than that is presented in the current work. Also, the performance of this scaffold in an *in vivo* model could be an interesting avenue to pursue as a cardiac patch material in managing MI and cardiac regeneration.

The results of the scaffold bioactivity influence, though exciting and supports the overall hypothesis of this thesis, does not really pin point the molecule(s) responsible for the actual effect. Our results here exhibit that the conditioned medium contains factors that could be responsible for the observed effect. Hence the future work would be to analyze this conditioned medium using proteomic analysis to characterize for the presence of any or a combination of ECM based matrikines or cell secretome or cytokines that can be related to cardiac differentiation. Once a set of molecules are identified, these could be compared to existing targets of cardiac differentiation pathways using bioinformatics tools to identify possible peptide sequences. This would not only answer the question of the origin of the bioactivity in the cardiac ECM, but also can be considered as a product. Though the pcECM presented in this thesis as cardiac ECM has exhibited excellent properties, there could be reservations when translating this material into clinical trials as they are of ‘xeno’ origin. If a set of molecules are found using the combination of proteomics and bioinformatics, then the identified sequences can be synthesized *in vitro* chemically with more control and reproducibility, thus alleviating the ‘xeno’ concern. These synthesized molecules can be used in clinical settings for cardiac regeneration or can also be used *in vitro* to study cardiac regeneration or tissue development, information which could be useful for overall cardiac regeneration understanding.

7.3 Conclusion

This thesis reports the fabrication of a 3D composite scaffold obtained using wet electrospinning and liquid pcECM gel. The 3DCS mimicked the natural pcECM, in many

parameters like architecture, surface chemistry, protein composition and non-immunogenic nature. There was a slight difference observed in terms of mechanical properties between these two scaffolds, but this does not seem to be an issue, as the cells seeded on the 3DCS were able to maintain their growth and functionality.

The hypothesis of the thesis that a ‘balance and cooperation of architectural and biochemical signals’ are necessary for a scaffold was exhibited when we seeded hiPSC CMs and hiPSCs on the 3DCS.

The 3DCS when seeded with human iPSC derived CM, not only maintained cellular viability, but also the functionality in terms of protein expression and calcium handling. In addition, the same trend was observed in the study of the influence of cardiac specific bioactivity of the scaffold on hiPSC differentiation. This shows the essential role of the cardiac specific biochemical composition in hiPSCs differentiation. Coupled with the highly porous, tailorable and reproducible synthetic 3D fibrous scaffold, this hybrid structure of 3DCS has great potential to be developed into a cardiac patch with the right mechanical support and functionality. Further, while this thesis focused on the fabrication of a cardiac suitable scaffold, these results theoretically can also be extrapolated as a proof of concept study for the fabrication of other tissue type specific 3D scaffold platforms. This can be achieved by producing different 3D tunable synthetic scaffolds and functionalizing them with tissue specific ECM combinations, resulting in an array of scaffolds expressing characteristics of the respective tissue types, which could have potential applications in soft tissue regeneration.

References

- [1] C.M. Nelson, M.J. Bissell, Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer, *Annu Rev Cell Dev Biol* 22 (2006) 287-309.
- [2] S.W. Lane, D.A. Williams, F.M. Watt, Modulating the stem cell niche for tissue regeneration, *Nat Biotechnol* 32(8) (2014) 795-803.

- [3] K.H. Nakayama, L. Hou, N.F. Huang, Role of extracellular matrix signaling cues in modulating cell fate commitment for cardiovascular tissue engineering, *Adv Healthc Mater* 3(5) (2014) 628-41.
- [4] U. Sarig, M. Machluf, Engineering cell platforms for myocardial regeneration, *Expert Opin Biol Ther* 11(8) (2011) 1055-77.
- [5] Y. Efraim, H. Sarig, N. Cohen Anavy, U. Sarig, E. de Berardinis, S.Y. Chaw, M. Krishnamoorthi, J. Kalifa, H. Bogireddi, T.V. Duc, T. Kofidis, L. Baruch, F.Y.C. Boey, S.S. Venkatraman, M. Machluf, Biohybrid cardiac ECM-based hydrogels improve long term cardiac function post myocardial infarction, *Acta biomaterialia* 50 (2017) 220-233.
- [6] Y. Eitan, U. Sarig, N. Dahan, M. Machluf, Acellular cardiac extracellular matrix as a scaffold for tissue engineering: in vitro cell support, remodeling, and biocompatibility, *Tissue engineering. Part C, Methods* 16(4) (2010) 671-83.
- [7] S. Stratton, N.B. Shelke, K. Hoshino, S. Rudraiah, S.G. Kumbar, Bioactive polymeric scaffolds for tissue engineering, *Bioact Mater* 1(2) (2016) 93-108.
- [8] D.H. Reneker, I. Chun, Nanometre diameter fibres of polymer, produced by electrospinning, *Nanotechnology* 7(3) (1996) 216.
- [9] Y. Orlova, N. Magome, L. Liu, Y. Chen, K. Agladze, Electrospun nanofibers as a tool for architecture control in engineered cardiac tissue, *Biomaterials* 32(24) (2011) 5615-5624.
- [10] M.P. Prabhakaran, D. Kai, L. Ghasemi-Mobarakeh, S. Ramakrishna, Electrospun biocomposite nanofibrous patch for cardiac tissue engineering, *Biomedical materials* 6(5) (2011) 055001.
- [11] K.S. Rho, L. Jeong, G. Lee, B.M. Seo, Y.J. Park, S.D. Hong, S. Roh, J.J. Cho, W.H. Park, B.M. Min, Electrospinning of collagen nanofibers: effects on the behavior of normal human keratinocytes and early-stage wound healing, *Biomaterials* 27(8) (2006) 1452-61.
- [12] J.H. Brown, P. Das, M.D. DiVito, D. Ivancic, L.P. Tan, J.A. Wertheim, Nanofibrous PLGA electrospun scaffolds modified with type I collagen influence hepatocyte function and support viability in vitro, *Acta biomaterialia* 73 (2018) 217-227.
- [13] H. Chen, Y. Peng, S. Wu, L.P. Tan, Electrospun 3D Fibrous Scaffolds for Chronic Wound Repair, *Materials (Basel)* 9(4) (2016).

- [14] Y. Yokoyama, S. Hattori, C. Yoshikawa, Y. Yasuda, H. Koyama, T. Takato, H. Kobayashi, Novel wet electrospinning system for fabrication of spongiform nanofiber 3-dimensional fabric, *Materials letters* 63(9) (2009) 754-756.

Chapter 8

Appendix

8.1 Supplementary Results

8.1.1 Analysis of hiPSC-CMs Beating on Scaffolds

The beating video obtained was converted into sequence of images and two images were chosen at certain intervals to account for rest state (diastole) or maximum movement or displacement (systole) in the scaffold edges. Arrows are drawn from the border of the systole image to the edge of the scaffold. Then the arrows were copy pasted on to the diastole images to maintain the dimensions of the arrow. The scaffold edges were inspected for displacement as shown in Figure 8-1. hiPSC-CMs seeded on 3DCS and pcECM exhibited displacement in the scaffold edges, whereas there was no displacement observed in UPLGA scaffolds.

Figure 8-1 Analysis of hiPSC-CMs beating on scaffolds.

Representative images obtained from beating videos. Inset represents the zoomed in images of the scaffold edges shown in the black square region.

8.2 Preliminary Reconnaissance Studies

8.2.1 hMSC Attachment Time and Cell Density Study

Cell attachment and cell density studies (Figure 8-2) were performed on the 3D electrospun PLGA scaffolds by varying the attachment time (0, 45, 90, 135 & 180 min) and the initial cell seeding density (100K, 500K, 1M, 2M, and 6M cells/cm²).

Figure 8-2 3D PLGA and hMSC-RFP interaction.

Percentage of hMSCs attached to the 3D electrospun PLGA scaffold after different incubation times (A); hMSC cell density on 3D PLGA scaffold under different initial seeding density after 24 h (B).

hMSC-RFP was used as a model cell to study the initial cell attachment and cell density studies. Optimal parameters balancing the cell attachment time and initial cell density is important in order to obtain a constant number of attached cells at the beginning of each assay. Based on these preliminary results, an attachment time of 90 min was found to be suitable. The low attachment percentage (compared to the previous work by our lab showing approximately 20 % of hMSC attachment to pcECM) of cells to the scaffold may be due to the lack of cell binding moieties and surface hydrophobicity of the scaffold. To evaluate the effect of various initial cell seeding density on the 3D PLGA scaffold, different initial cell numbers were seeded. As the initial seeding density increased, the attached cell density increased. Yet there is no significant increase in the attached cell density for initial seeding density of 2 and 6 million cells/cm² indicating a saturation maximal scaffold

carrying capacity of 1.3×10^6 cells/cm² for hMSCs. This can be attributed to the lack of available surface area on the scaffold to accommodate the incremental increase in seeding density beyond the saturation value.

8.2.2 3D Scaffold and hMSC Interaction

To determine the interaction of the hMSC with the 3D electrospun scaffold and the ability of the scaffold to support cell viability and proliferation, hMSCs were seeded on the 3D PLGA scaffolds and cell proliferation was measured on day 3, 7 & 14 using AlamarBlueTM. As seen in Figure 8-3, the scaffold supports the viability of the cells over a period of 14 days. There is no significant decrease in cell numbers between day 3 & 7. However, a significant increase in the cell number is observed from day 7 to day 14.

Figure 8-3 hMSC proliferation on 3D electrospun PLGA.

hMSC-RFP interaction and proliferation on unmodified 3D electrospun PLGA scaffold. A significant difference in terms of cell number was observed between day 14 when compared to both day 3 and day 7.

The delayed proliferation of hMSC on the 3D PLGA scaffold can be attributed to cell acclimation to the scaffold conditions and possible modification / secretion of their own ECM which can possibly facilitate their proliferating after 7 days of culture. Others have shown similar results where MSCs significant proliferation after 14 days of seeding on a

electrospun PLGA mat [180]. Histological sections (Figure 8-4) of the scaffolds on day 3 and day 14 show the interaction of the hMSC-RFP cells with the 3D PLGA scaffold.

Figure 8-4 Histological section of 3D PLGA scaffolds exhibiting interaction with hMSC-RFP.

Representative images of histological section of 3D PLGA stained with H & E on day 3 (A) and 14 (B); hMSC-RFP fluorescence of day 3 (C) and 14 (D); Scale: 500 µm.

8.2.3 hiPSC Culture on Microcarriers

IMR-90 hiPSCs were used for the preliminary studies mentioned here and all hiPSC cells used for the attachment and the interaction studies with the pcECM expressed the pluripotent markers OCT 4 and TRA 1-60 as visualized from the immunohistochemistry (IHC) staining of the hiPSC cells present in the microcarriers used for their mass expansion (Figure 8-5).

Figure 8-5 Immunohistochemistry of hiPSC on microcarrier.

Pluripotency of the hiPSC on microcarrier was evaluated using IHC stain of pluripotent markers OCT 4 (A) & TRA 1-60 (B). Cell nuclei are counter stained with hematoxylin. Scale: 100 μ m.

8.2.4 hiPSC Attachment and Interaction with pcECM

The optimal attachment time of hiPSC on pcECM was determined after seeding 500,000 cells on top of thin (3 mm) pcECM slices that were allowed to attach in static conditions for 0, 45, 90, 135 & 180 min at 37°C and 5 % CO₂. Subsequently, AlamarBlue™ assay was performed to determine the number of cells attached on the scaffold.

Figure 8-6 hiPSC attachment to pcECM over different attachment time.

hiPSC was incubated with pcECM for 0, 45, 90, 135 and 180 min to attach to the surface (A). B, C, D, E, & F show the H&E staining images of hiPSC seeded pcECM after 0, 45, 90, 135 and 180 min of incubation respectively. Representative images and mean $\pm$ SD values are shown out of 3 repetitions where black arrow indicates cell clusters at different time points. (*) denotes statistical significance ($p < 0.05$) as calculated using ANOVA. Scale bar: 50 μ m.

Based on different attachment times (Figure 8-6 A), there was no significant difference between 45 to 180 min in the attachment of iPSCs to the pcECM surface. A significant difference between 0 and 90, 135, 180 min was observed. Therefore, a minimum incubation time of 45 min is required for the iPSC attachment to the pcECM. Given that there was no significant difference between 45 and 90 min of attachment time, subsequent experiments used an attachment time of 90 min which also corresponds to the standard used in our lab for other cell types for pcECM. The effect of the attachment time was also observed by the H&E staining images (Figure 8-6 B-F)). There were no observable cells at 0 min, but cells were observed at all the other incubation times.

8.2.5 hMSC Proliferation on the Scaffolds

Figure 8-7 hMSCs viability on the scaffolds as measured using AlamarBlue™.

hMSC interaction and proliferation on 3DCS compared to the unmodified PLGA and pcECM scaffold in terms of cell numbers.