Developing vascularization in cardiac decellularized porcine ECM graft using bioreactor

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DEVELOPING VASCULARIZATION IN CARDIAC DECELLULARIZED PORCINE ECM GRAFT USING BIOREACTOR

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Table of Contents

<i>List of Figures</i>	viii
<i>Nomenclature</i>	x
<i>Abstract</i>	xiii
Chapter 1 Introduction and Literature Review.....	1
1.1 Introduction.....	1
1.2 Literature review	2
1.2.1 Cardiac patch.....	3
1.2.2 Cells	4
1.2.3 Biomaterials for cardiac patch engineering	7
1.2.4 Bioreactors	12
1.2.5 Vascularization of thick cardiac patch	13
1.3 Research hypothesis and objective.....	15
1.4 Research Novelty and Significance	16
Chapter 2 Materials and Methods	18
2.1 Cell culture.....	18
2.1.1 Cell types and medium.....	18
2.1.2 Cell metabolic rate and proliferation analysis.....	18
2.2 Cell growth and metabolism in various medium compositions	19
2.3 Co-culture studies	20
2.3.1 HUVEC and MSC co-culture.....	20
2.3.2 Analysis of growth dynamics of MSC and HUVEC co-culture using a differential model.....	21
2.3.3 Imaging population dynamics using DiI pre labeling	23
2.3.4 Phenotypic properties of co-cultured cells.....	23
2.3.5 Cell seeding density studies	24
2.4 Static cell cultivation on ECM surface.....	25
2.4.1 Decellularization and preparation of ECM scaffold	25
2.4.2 HUVECs attachment study	26
2.4.3 HUVEC growth study	27

2.4.4 Protein treatment for acellular ECM	28
2.4.5 Gelatin staining using CBP-TAMRA	28
2.4.6 Co-culture study	29
2.5 Dynamic cell cultivation of ECM vasculature	29
2.5.1 Perfusion and Rotation Bioreactors	29
2.5.2 Endothelialization and dynamic cultivation of ECM.....	34
2.5.3 Dynamic endothelialization with protein treatment and co-culture	35
2.6 Microscope imaging and analysis	36
2.7 Histological studies	37
2.7.1 Tissue sample fixation.....	37
2.7.2 OCT blocking preparation and cryo-sectioning	38
2.7.3 H&E staining.....	38
2.7.4 Masson's Trichrome staining	39
2.7.5 ABC DAB staining for CD31	39
2.7.6 Immunofluorescent staining for OCT embedded sections	40
2.8 Lenti-virus generation and cell transfection.....	41
Chapter 3 Results and Discussion.....	44
3.1 Effect of medium on cell growth and metabolism	44
3.2 MSC and HUVEC co-culture studies	47
3.2.1 Growth rate and metabolic indices of MSC and HUVEC in co-culture	47
3.2.2 Analysis of growth dynamics of MSC and HUVEC co-culture using a differential model.....	50
3.2.3 Phenotypic properties of MSC and HUVEC grown in co-culture	57
3.2.4 DiI staining of MSC and HUVEC co-culture	61
3.2.5 Effect of low seeding density on population dynamics.....	63
3.2.6 Effect of medium volume and seeding density on population dynamics.....	67
3.3 HUVEC attachment and growth on decellularized ECM	70
3.3.1 HUVEC attachment and growth on ECM slices.....	70
3.3.2 Cell morphology on ECM.....	76
3.4 Protein Treatment for Acellular ECM.....	81
3.4.1 Effect of treatment on attachment and growth of HUVEC and MSC.....	81

3.4.2 Gelatin staining with CBP-TAMRA	84
3.4.3 Effect of seeding sequence on co-culture.....	86
3.4.4 Effect of treatment on co-culture	88
3.5 HUVEC-GFP and MSC-mTomato expression and culture	90
3.6 Endothelialization of acellular ECM.....	95
3.6.1 Static culture of HUVEC on ECM vasculature.....	95
3.6.2 HUVEC attachment and growth using perfusion bioreactor	99
3.6.3 Dynamic co-culture of HUVEC and MSC on vasculature	106
3.6.4 Effect of protein treatment on vascularization	111
Chapter 4 Conclusions and future recommendations.....	118
Reference	121
Appendix.....	133

List of Figures

Figure 1. Acellular cardiac scaffold structure and composition.	11
Figure 2. Preservation of inherent vasculature.	12
Figure 3. Medium and air circulation layout in bioreactor system.....	32
Figure 4. Tubular and rotational bioreactor system setup.....	33
Figure 5. Effect of medium compositions	46
Figure 6. Effects of culturing time, medium and cell ratio on HUVEC and MSC growth.....	49
Figure 7. Population dynamics of MSCs and HUVECs co-cultured under different medium compositions.....	54
Figure 8. Model correlations for MSC and HUVEC co-cultures growth dynamics.....	56
Figure 9. Phenotypic analysis for MSC.....	59
Figure 10. Phenotypic analysis for HUVEC	60
Figure 11. Phenotypic analysis for MSC and HUVEC co-culture	61
Figure 12. Cell tracking using membrane labeling dye.....	63
Figure 13. MSCs and HUVECs co-cultured under low seeding densities	65
Figure 14. Simulation of population dynamics under low initial seeding densities	65
Figure 15. Validation of MSC staining by fluorescent membrane cell trackers using flow cytometry analysis.....	67
Figure 16. Seeding density effect on co-culture growth.....	68
Figure 17. H&E staining for acellular and native ECM vasculature	71
Figure 18. HUVEC percentage attachment on ECM slices.....	73
Figure 19. H&E images for HUVEC attachment to ECM utilizing various attachment times	74
Figure 20. HUVEC growth on ECM scaffold	76
Figure 21. Morphological properties of HUVEC, MSC, and their co-culture.....	77
Figure 22. SEM images for acellular and re-seeded ECM	80
Figure 23. Proliferation over 21 days on biochemically modified ECM scaffold.....	83
Figure 24. CBP-TAMRA staining for gelatin coating on ECM.....	85
Figure 25. Proliferation of single and co-cultured cells.....	88
Figure 26. Cell proliferation on ECM surface with protein treatment.....	90
Figure 27. Proliferation curve for HUVEC	92

Figure 28. Expression of HUVEC-GFP	93
Figure 29. Expression of MSC-mTomato.....	94
Figure 30. HUVEC growth on ECM vasculature under static culture conditions.....	96
Figure 31. H&E staining for static study on ECM vasculature	97
Figure 32. Masson's Trichrome staining for static study on ECM vasculature	98
Figure 33. Confocal imaging for cross-section of ECM vasculature seeded with HUVEC-GFP	99
Figure 34. HUVEC growth on ECM vasculature under dynamic culture condition.	100
Figure 35. H&E staining of dynamic study for HUVEC seeded ECM vasculature	104
Figure 36. MSC growth on ECM vasculature under dynamic culture condition	105
Figure 37. Longitudinal view with DiI pre-stained MSCs using confocal microscopy	106
Figure 38. HUVEC-MSC co-culture growth on ECM vasculature under dynamic culture condition	107
Figure 39. Masson's Trichrome staining for cross-sections of ECM vasculature sequentially re-seeded with MSCs followed by HUVECs with a 7 day interval under dynamic culture condition	110
Figure 40. Confocal imaging for cross section of ECM vasculature sequentially re-seeded with HUVECs and MSCs under dynamic culture condition.	110
Figure 41. HUVEC growth on gelatin treated ECM vasculature under dynamic culture condition	112
Figure 42. Masson's Trichrome staining for cross-section of gelatin treated ECM vasculature seeded with HUVECs under dynamic culture condition.....	112
Figure 43 Masson's Trichrome staining for cross-sections of native porcine and gelatin treated ECM vasculature.....	113
Figure 44. Confocal image for longitudinal view of vasculature. Gelatin treated ECM vasculature seeded with HUVEC under dynamic culture condition	115

Nomenclature

α MEM	Minimum Essential Medium Eagle, Alpha Modification
bFGF	basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CBP	Collagen Binding Protein
DAPI	4', 6-diamidino-2-phenylindole
DDW	Double Distilled Water
DiI	1, 1-Dioctadecyl-3, 3, 3, 3-tetramethylindocarbocyanine
DiO	3, 3'-Dioctadecyloxacarbocyanine Perchlorate
ϵ_i	Basal Metabolic Indices
EC	Endothelial Cell
ECM	Extracellular Matrix
EGM-2	Endothelial Growth Media-2
ESC	Embryonic Stem Cell
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal Bovine Serum
FI	Fluorescence Intensity
FITC	Fluorescein Isothiocyanate
GAG	Glycosaminoglycans
GFP	Green Fluorescent Protein
HCl	Hydrogen Chloride
H&E	Hematoxylin and Eosin

HUVEC	Human Umbilical Vein Endothelial Cell
IHC	Immunohistochemistry
iPSC	induced Pluripotent Stem Cell
k_i	Self-effect
l_i	Other-effect
LAD	Left Anterior Descending
MI	Myocardial Infarction
MSC	Mesenchymal Stem Cell
OCT	Optimal Cutting Temperature Compound
PBS	Phosphate-Buffered Saline
PcECM	porcine cardiac ECM
PCL	Poly Caprolactone
PE	Phycoerythrin
PFA	Paraformaldehyde
PGA	Poly Glycolic Acid
PLA	Poly Lactic Acid
PTFE	Polytetrafluoroethylene
ROI	Region of Interest
RPM	Revolutions per Minute
RSM	Response Surface Model
SEM	Scanning Electron Microscopy
SMC	Smooth Muscle Cell
TGF- β	Transforming Growth Factor beta

μ_i	Basal Growth Rate
VEGF	Vascular Endothelial Growth Factor
vWF	Von-Willebrand Factor

Abstract

The creation of thick cardiac tissue for scar replacement therapy following myocardial infarction (MI) has been hindered by the lack of functional and enduring vascular supply. Most cells do not survive more than a few hundred of micrometers diffusion barrier away from the blood vessel *in vivo*, hence tissue suffering from lack of oxygen and other nutrients experience ischemia and necrosis. Effective vascularization process requires the proper selection of cell types, scaffold containing inherent vasculature infrastructure with suitable mechanical and biochemical properties, and optimized culturing conditions.

Since the process of re-vascularization involves the interaction and collaboration of more than one cell type, it is critical to understand the factors affecting the growth dynamics of the co-culture system to direct the process toward a desirable regeneration. Mesenchymal stem cells (MSC) have been used in various engineering applications owing to their regeneration and differentiation capabilities as well as their therapeutic potentials. Efforts have been made by applying both MSC and human umbilical vein endothelial cell (HUVEC) in co-culture system for vasculature development and regeneration, however, no comprehensive studies exist to address and analyze the effects of culturing parameters on the population dynamics. In this thesis, we suggest a modified Lotka-Volterra model to quantitatively describe the population dynamics of co-cultured cells under the influence of different culturing parameters, including cell ratio, medium compositions, and culturing time. This model, commonly referred to describe the prey-and-predator behavior of subpopulations sharing the same closed ecological niche, can now be used to predict the population dynamics in cell co-culture systems under given culturing conditions. The

empirical results indicate that under most conditions, endothelial cell (EC) growth was inhibited by their own species but promoted by MSCs, which coincides perfectly with the model prediction.

Similar results were also observed when cells were cultured on more complex 3-D acellular extracellular matrix (ECM) scaffolds, derived from porcine left ventricle tissue (pcECM). The decellularized thick pcECM exhibits advantages such as comprehensive 3-D architecture with well-preserved vasculature framework, mechanical and chemical properties that are comparable to native tissue, and ECM attachment proteins that are normally missing in synthetic materials. The use of decellularized animal ECM scaffolds for tissue engineering is not new, but vascularization of cardiac derived ECM remains a critical problem for long term survival of thick and clinically relevant sized tissue constructs. In this study, we demonstrated the supportability of pcECM scaffold surface for the attachment and growth of HUVECs and MSCs, as model cells for angiogenic processes. Two approaches were carried out in parallel to improve HUVEC survival and proliferation on the pcECM scaffold: co-culture of HUVEC with MSC, and protein modification of the pcECM scaffold prior to cell seeding. Studies were carried out first on the surface of small samples (0.5cm^2) for screening and optimization purposes, under static culture conditions, i.e. in the tissue culture plate. These studies were followed *ex-vivo* by dynamic culturing of more complex and clinically relevant thick pcECM patches in a mimicking physiological-like milieu. In the co-culture approach, we demonstrated the supporting function of MSCs in HUVEC attachment and proliferation, which further strengthens the findings of our mathematical modeling performed in standard tissue

culture plates. The importance of seeding sequence for co-culture was also revealed. In the second approach, protein treatment of the pcECM was implemented with common attachment proteins, such as gelatin and fibronectin. In both methods, significant improvement of cell growth over time was observed.

Finally, we applied the knowledge gained from the latter findings in a 2-D co-culture model and simplified small 3-D ECM scaffolds, on a much more complex thick ECM patch in a dynamic environment mimicking the physiological setting. This dynamic cultivation was provided by a custom built perfusion bioreactor system, which provides efficient medium circulation and shear stress to the cells, seeded within the vasculature. Cells were seeded on the preserved vasculature inherent to the thick acellular pcECM slabs. Confluent monolayers of endothelial cells lining the vasculature lumens were obtained by both sequential co-culturing approach and protein treatment approach following dynamic culturing for up to 21 days.

These findings collectively validate the suitability of thick ECM patch for cardiovascular regeneration and replacement therapy. The population dynamics between MSCs and HUVECs were elucidated for the first time using a quantitative model, which could be extended for other co-culture cell models. The successful endothelialization of the thick cardiac tissue patch serves as a proof of concept with a promising potential for cardiac replacement therapy and other clinical applications.

Chapter 1 Introduction and Literature Review

1.1 Introduction

Cardiovascular disease is the leading cause of death in developed countries with more than 600,000 people diagnosed every year [1]. MI, commonly referred to as heart attack, is caused by insufficient blood supply to the cardiac tissue from partially or fully blocked arteries. Resulted cardiovascular tissue ischemia or damage, downstream to the blocked artery, can lead to ventricular remodeling. During the remodeling process, the fibrous scar tissue lacking the contractile and electrical conductive functions significantly compromises the ventricular function, and would eventually give rise to heart failure [2]. Clinical statistics demonstrated the high correlation of MI incidence to age, with approximately 50% of MI patients aged above 65 in United States [3]. With the aging populations in developed world, the need for effective prevention and treatment for MI is ever increasing [4]. While heart transplantation remains the definite treatment for end-stage patients, the invasive procedure combined with high cost and lack of donors are critical issues for high demand every year [5, 6]. Another drawback of heart transplantation is that patients require the use of immunosuppressant therapy following transplantation (to avoid rejection) having reported side effects such as hypertension, diabetics, and possibly leading to renal failure [7]. Pharmacological therapies were shown to help slow down the progression of MI for advanced but not end-stage patients, but rarely prevent the irreversible end-stage disease from happening [8]. Other surgical approaches such as left ventricular assist devices, restraints using biventricular pacemakers or external constraining mesh [9-11] were also proven to help preventing or

reduce the degree of cardiac wall thinning and ventricular dilatation even at end-stage, however, its complicated implantation procedure gave conflicting results from different groups in animal and clinical trials [12]. None of above mentioned strategies actually heals or regenerates the infarcted heart tissue. Hence, alternative approaches using tissue engineering techniques have been explored with the aim of providing viable replacement or augmenting solutions that may facilitate cardiac tissue regeneration.

1.2 Literature review

As a fast developing field of research, tissue engineering is interdisciplinary and aims to “apply the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function” [13]. The current cardiac tissue engineering therapies can be categorized into two approaches: cell therapy and tissue engineering [4, 14]. Compared with patch based therapy, cell based injection therapy is relatively less invasive and simple. Since cardiomyocytes have limited regeneration capability and very limited source of origin [5], other cell types including embryonic stem cells (ESC) [15, 16], induced pluripotent stem cells (iPSC) [17, 18], and mesenchymal stem cells (MSC) [19-21], which are regenerative and possess the potential to differentiate into cardiomyocytes are the popular candidates for cellular therapy. Autologous cells can be harvested from bone marrow or other organs, cultured and expanded *ex vivo*, and delivered to targeted injured sites. Similar and improved approaches, including cells encapsulation with liquidized biomaterial scaffold, have also been explored to improve cell survival and host integration [22]. However, the function of

cellular therapy in cardiac tissue regeneration is still limited without supporting scaffold materials. Tissue engineering approach, such as cardiac patch therapy, applies biomaterials scaffold with supportive biochemical and mechanical properties for grafted cells, offers mechanical support with functional vascular systems and regenerative cells to be integrated with host tissue once implanted and restore the contractile function of the heart [14, 23].

1.2.1 Cardiac patch

Comparing to full-sized regenerated heart, heart patch is definitely a more realistic goal to achieve. Different by the nature of scaffold, most commonly studied cardiac patches can be grouped into tissue based, hydrogel based, and cell sheet based patches [5, 24-26]. Regardless of their different biochemical properties, mechanical strength, and fabrication methods, they all serve the purpose of delivering regenerative cells to infarcted area on/in supporting constructs with compatible biochemical and mechanical properties that are readily integrated into host tissue post implantation and help in restoring some extent the cardiac function.

The ideal cardiac patch for tissue augmentation or scar replacement therapy should possess the following qualities: suitable selection of cell types with specialized functional roles and regenerative potential; chemical and mechanical properties that are comparable to physiological conditions; and functional vascular network to provide efficient blood supply for the long term survival and ready integration to the host surrounding.

1.2.2 Cells

The native human heart is made from a complex combination of cell types, which serve their respective functions. The major cell group making up to 70% of the entire cardiovascular population is that of cardiac fibroblasts [27]. The other 30% of cardiac cell population is cardiomyocytes [28]. Other cells types including endothelial cells (EC), SMC, pericytes and adipose tissue are also present with their special functions, though with much smaller population compared to the first two groups [27]. The critical interplay between different cell types in native tissue suggests the importance of applying co-culture or tri-culture system in directing the population of interest toward a desirable regeneration process and achieving stable and functional tissue structure [29-34].

Among the cardiac cell types, cardiomyocytes are essential for cardiac contraction. Since they have limited regeneration capability and cannot be expanded *ex vivo*, finding the right source for cardiomyocytes is critical for all cardiac tissue engineering applications [5] [35]. Autologous and allogeneic cells are often the optimal choices for tissue engineering applications due to their reduced immunogenicity, which may prevent graft rejections. However, one big challenge of using these cells is the limited resources of donor cells. As a result, one focus of cardiac tissue engineering is to find the optimal cell type or combination of cell types, which fulfill several major requirements. First the cells should be easily harvested or expanded *ex vivo*. Second, cells should be able to integrate well with the host tissue upon injection or transplantation while provoking a minimal

immune-response. Last, cells should have the potential to differentiate into desired functional cells (mainly for pluripotent/multipotent cell types) [36].

The term “Regenerative Medicine” was born with the discovery and establishment of human embryonic stem cells and embryonic germ cells in 1998, [37]. Owing to their pivotal regenerative potential, availability and expandability *in vitro*, stem cells have been widely studied for tissue engineering applications [8, 38-44]. MSCs express various markers including CD44, CD90, CD105, but none of which is specific for MSCs [45, 46]. Hence, MSCs are generally characterized by their abilities for self-regeneration and multi-lineage differentiation [47]. In cardiovascular regeneration process, autologous MSCs derived from bone marrow are shown to be genetically stable for many generations [47], yet can be differentiated into cardiomyocytes, cardiac fibroblasts, and endothelial cells under the right condition [44, 48-51]. This solves the issues for cells expansion, immune-response and differentiation potential at the same time. BM-MSCs injected into damaged heart tissue have demonstrated improved cardiac function after acute MI [41, 52-54]. Apart from MSCs, other cell types including iPSC, ESC, and cardiac stem cells also possess the ability for cardiac regeneration [5].

Endothelial cells are usually applied to achieve vascularization for regenerated cardiac tissue with the support of other cell types [55-57]. Human umbilical vein endothelial cells (HUVEC) are well studied and commonly used by researchers for their abilities to form capillary like networks *in vitro* [58-60]. HUVECs were demonstrated to have the

potentials to promote cardiomyocytes growth and stability and improve the spatial reorganization when being co-cultured with them [61].

Co-cultures of two or more cell types are commonly adopted for regenerative applications. To achieve functional vascular system with HUVECs, we applied co-culture approach with MSCs in focus here owing to their well-studied behavior and differentiation capabilities, as well as previous reported promising results when co-cultured with EC [62, 63]. Co-culture approach with progenitor cells, such as MSCs have been attempted to create functional blood vessels for various applications including bone [64, 65], and cardiovascular regeneration [66, 67], both demonstrated successful formation of 3D prevascular network. Interaction between MSC and HUVEC when co-cultured together was also studied by different groups in terms of cell-cell contact and reciprocal signaling [68, 69]. Co-cultured MSCs with ECs were found to exhibit strong pro-angiogenic and vasculogenic effects that were associated with the ability of MSCs to stabilize the formation of tubular vascular-like structures both *in vitro* [63, 66, 70] and *in vivo* [71-73]. MSCs were also shown, under certain *in vitro* conditions, to trans-differentiate into ECs, further reaffirming their *in vivo* association [44, 71, 74, 75]. Despite their popularity, the interplay between the two cell groups in co-culture system, and how it affects the overall population dynamics are still not clear [76]. The factors governing these dynamics have not been thoroughly quantified even in respect to the most fundamental effectors such as medium composition, seeding density, and cell seeding ratios, let alone in a comprehensive model that combines two or more of the above effector.

1.2.3 Biomaterials for cardiac patch engineering

A more specific definition of “Tissue Engineering” to differentiate it from cell therapy involves the usage of scaffolding materials [77], which provide mechanical and architectural support for the cellular component mimicking the natural ECM in native tissue. Several requirements for scaffold materials have to be fulfilled. Ideally the scaffold should be biodegradable and degrade in parallel to the secretion of substitute ECM by the cells. The scaffold material and its degradation by products should be biocompatible to minimize cytotoxicity and immune responses. Finally, the scaffold material should possess tissue matching mechanical properties, such as elasticity, viscosity and stiffness, according to the site of need. Other requirements are porosity, which in turn enables better mass diffusion, roughness and presence of essential adhesion proteins which are desired to better support and integrate with the grafted cells [78, 79].

Scaffolding biomaterials can be broadly classified into two groups, synthetic and natural materials. Commonly used synthetic biomaterials include Poly lactic acid (PLA), poly glycolic acid (PGA), poly- ϵ -caprolactone (PCL) and their copolymers. Using the synthetic approach, precise chemical and mechanical properties can be tailored achieving high reproducibility and low batch-to-batch variability [80-82]. Ideally, the degradation rate of the polymer should be comparable to the in-growth rate of ECM to achieve slow replacement while providing the necessary mechanical support [2]. However, synthetic materials would mostly suffer from bulk degradation leading to the sudden collapse and

loss of mechanical support [83].

In contrast to synthetic materials, natural materials have poor process-ability and less tunable mechanical strength compared to synthetic materials. Nevertheless, natural materials, such as collagen, fibrin, and chitosan based scaffolds are still often preferred due to their good biocompatibility and the presence of inherent natural adhesion proteins [5]. Thin natural scaffold incorporating ECM or its protein molecules were used in the attempt to mimic ECM [84-93], and improvement for small MI animal models were observed [94]. Though encouraging, current achievements in small animal models lack translational potentials as the implanted engineered constructs are generally too thin and small to produce a desired effect in the clinical setting, where much thicker and complex scar tissue is formed [5].

With the advance of decellularization techniques, researchers can nowadays obtain natural ECM from different tissue sources, including heart valves [24], blood vessels [22], urinary bladder [95], liver [96], and many more which are made commercially available [97].

ECM isolated from different tissue types possesses different structural properties and protein compositions [97, 98] and it is therefore speculated that myocardial ECM would be the most suitable one for myocardial tissue engineering. Myocardial ECM has an optimal heart compliance with an interlocking mesh of fibrous proteins and glycosaminoglycans (GAGs) to support the cardiomyocytes embedded in the network.

The main components of native ECM is collagen type I and type III [27, 99]. The two types of collagen exhibit distinctive mechanical properties. Type I collagen is more rigid, which is commonly found in bone and scar tissue, whereas type III collagen is more elastic, contributing to the elasticity of skin tissue and artery walls [100]. The combination of two collagen types, together with other adhesion molecules, supports the cardiomyocyte attachment, proliferation, alignment, and signaling pathways [78, 79, 101], and has significant effects on the contractile force transmission and host remodeling *in vivo* [102-104].

Cardiac ECM scaffolds isolated from animals such as rat [7, 105] and pig [14, 106-111] have been intensively studied in recent years owing to their compositional and mechanical properties [5, 29, 31, 110]. In Ott's study in 2008, cardiomyocytes and ECs were grafted on relatively thin rat whole heart cardiac ECM with complete vascular architecture and intact cardiac geometry isolated by coronary perfusion with detergent. Under mechanical and electrical stimulation generated by a modified Langendorff apparatus, rhythmic pump function was observed for up to 8 days and single layers of endothelial cells were formed within the ventricular cavities and vascular lumens of the cardiac construct [7]. Wang's group fabricated cardiac patch using isolated porcine ECM and recellularized it with differentiated bone marrow mononuclear cells. Infiltration and proliferation of cells in the ECM construct was observed and pre-differentiated cells expressed stable cardiomyocyte makers [109]. The use of porcine heart as an ECM source is advantageous compared to small animals such as rat [112] as it shares similar physiological properties with the human heart.

Our group also developed efficient decellularization processes to isolate thin [110] and thick [5] pcECM from porcine cardiac tissue. With the 3D architecture, protein molecules (Figure 1) [110], and major vasculature network well preserved (Figure 2) [14], the isolated porcine ECM exhibited desirable mechanical properties and supporting functions in enhancing the survival and proliferation of commonly used cell types, including fibroblasts, cardiomyocytes, and MSCs [5, 110]. Moreover, beating ability of cardiomyocytes seeded in thin pcECM scaffold with expression of typical functional cardiac markers was demonstrated by our group, indicating that the isolated porcine ECM is a suitable scaffold material candidate for cardiac tissue engineering [110].

Though these thin ECM constructs are promising, their potential for bulk cardiac tissue regeneration is still limited [5]. In contrast, thick (15 mm) acellular ECM constructs, which are derived from the full thickness myocardial tissue, possess fiber morphologies and ultra-structural characteristics that mimic the native tissue, and may therefore be used for trans-mural scar replacement therapy altogether [14]. An alternative approach involving whole heart decellularization has also been achieved by other groups with more aggressive decellularization processes [111, 113]. Nevertheless, the clinical applicability of this approach is hindered by the high CM quantities required for bulk recellularization and the higher complexity, arising from the attempted cultivation of an entire heart in the lab. We have therefore suggested that the usage of smaller yet clinically feasible tissue parts (which size is determined during decellularization) may be advantageous in allowing more efficient studies to be conducted by using specific cardiac parts for specific

therapeutic targets. In our thick porcine cardiac ECM, the inherent vasculature network was proven to be successfully preserved after the decellularization and can be used as a supporting platform for endothelialization process [14]. Through these vascular networks, sufficient nutrient and oxygen can be provided to the rest of the tissue construct with the assistance of bioreactor system within a dynamic culture environment, which mimics the physiological conditions of human hearts.

Figure 1. Acellular cardiac scaffold structure and composition. Sections of native (a) and decellularized (b) cardiac tissue samples stained for F-actin (green) and nuclei (bright blue). SEM images of native (c) and acellular (d) cardiac ECM scaffold surface together with deconvolved images of immunofluorescent staining for collagen I (red), fibronectin (green) (e) and collagen III (red) and fibronectin (green) (f) show the preserved structure and protein composition.

Figure 2. Preservation of inherent vasculature. Macroscopic view of a 10 mm-thick acellular perfused matrix (A). Preservation of inherent vasculature was evaluated by corrosion casting of native (B, C) and acellular pcECM (D, E). Patency and functionality of the acellular vasculature ECM is demonstrated by the perfusion of fluorescently labeled Rhodamine-Dextran at consecutive time points (F and G with a 1 min interval).

1.2.4 Bioreactors

A bioreactor for tissue engineering applications is a device that provides biological and physiological conditions *in vitro*, thus mimics the *in vivo* conditions [114]. The parameters that can be controlled by bioreactors include; temperature, humidity, pCO₂,

pH, and pO_2 . In addition, bioreactors can offer a dynamic environment when using perfusion [7, 115, 116], mechanical, and electrical stimulations [117, 118], which cannot be provided by the static culture environment. The first bioreactor was developed in 1993 by Langer's group in the application of cartilage tissue engineering to provide cells with sufficient mass transfer and shear stress at the implant surfaces [119]. After which, various types of bioreactors for different tissue engineering applications have been developed [31, 70, 120, 121]. Rotational bioreactors enhanced the mass transportation via laminar flow and provide the microgravity to the culturing environment [122, 123]. From simple mixed flasks to rotation chamber, one advanced stage in bioreactor evolution is that of perfusion bioreactors [116, 124, 125], which allow convection of mass transport both on the surface and within the tissue construct. Media perfusion is achieved through either vascular-like infrastructures or through pre-formed channels/pores [2, 126]. Another type of bioreactor, rotational bioreactor, can enhance the mass transportation via laminar flow and provide the microgravity to the culturing environment [122, 123]. For thick engineered tissue with complicated physiological structures like cardiac tissue, combination of properties from different bioreactor types is normally required [127, 128].

1.2.5 Vascularization of thick cardiac patch

As mentioned in earlier sections, effective and long term regenerated cardiac tissue requires careful selection of cell sources, compatible and supportive scaffold material, as well as functional vascular system with the assistance of dynamic culturing environment. Among the three, sufficient and functional vascularization stays the crucial hurdle

hindering the long term survival of regenerative tissue construct, especially the thick ones [129].

Without functional and efficient blood supply, the lack of oxygen and nutrient will cause tissue ischemia and necrosis. Cardiac tissue possesses a complicated and hierarchical network of blood vessels. From veins and arteries to capillaries, regardless of their sizes and locations, every single blood vessel plays a critical role in transporting nutrients and oxygen throughout the cardiac tissue. In the absence of vasculature, regenerated tissue has a critical diffusion thickness, greater than which, diffusion of oxygen and nutrients will not be sufficient to support the needs of tissue. Research by Radisic's group indicated that without functional vascularization, the mass transport can only reach as far as 100 μ m into the tissue. With the assistance of bioreactors, culture medium perfusion and electric stimulation can effectively increase the depth to about 200 μ m [130]. However, once the regenerated tissue is transplanted to the host body, the lack of vascularization remains a critical issue *in vivo*.

The formation of blood vessels *in vivo* involves two pathways: vasculogenesis and angiogenesis. In vasculogenesis the *in situ* assembly of capillaries from endothelial cells and their progenitor cells occurs, in which endothelial cells form nascent endothelial tubes that eventually would connect to form vascular network [129]. In comparison, angiogenesis involves the forming of new blood vessels from pre-existing vasculature network through vessel sprouting. This process involves stages of vasodilation, basement membrane degradation, adherent cell migration, capillary lumen formation, basement

membrane synthesis, and recruitment of pericytes and vascular smooth muscle cells (SMC) [131, 132]. Similar pathways could be exploited *ex vivo* in a tissue engineering approach. Thus a vasculogenic-like process will bring about the *de novo* formation of nascent tubes by seeded co-cultured endothelial and embryonic fibroblasts, as previously reported by the Levenberg's group [133]. In an angiogenic-like process, the use of explanted blood vessel *ex vivo* was shown to provide blood vessel like network sprouting in a tissue engineered construct, as recently reported by the Radisic's group [134]. Hence, to achieve functional vascular system, three important requirements must be met: stable endothelial in confluent monolayer lining the vasculature with assistance from supporting cells, vascular structure with necessary structural and adherent ECM proteins, and dynamic environment with perfusion functions.

1.3 Research hypothesis and objective

The ultimate goal of this research is to regenerate functional blood vessels within a thick (10-15 mm) and clinically relevant acellular cardiac ECM patch, for possible cardiac replacement therapy. Previous studies from our group have proved the suitability of decellularized thick porcine ECM scaffold in terms of its biochemical, mechanical, and structural properties. Other groups have shown that MSCs possess the potential to support and enhance the formation of blood vessels when being co-cultured with ECs. We therefore hypothesized that 1) there are certain patterns existing in which MSCs and ECs influence the proliferation and metabolism of each other when co-cultured in the same niche; 2) the attachment and proliferation of EC on 2D and even 3D scaffold can be

enhanced by co-culture with MSC in the right manner; 3) with the optimal combination of cell culture, scaffold and environment, blood vessels with full EC coverage can be regenerated within the thick cardiac construct.

The research goal was pursued by following three major aims:

1. Investigating the effect of culturing conditions (media composition, culture media ratio for MSC and EC, cell seeding densities, etc.) on population dynamics under static culture conditions as a basis for subsequent dynamic cultivation. This includes the development of a quantitative model to investigate the interaction mechanism (physical or chemical signaling based) of HUVEC and MSC in co-culture.
2. Investigating the effect of different ECM protein treatments on proliferation of singly and co-cultured cells to enhance the ECM's supportability for targeted cells.
3. Applying dynamic culture environment with customized perfusion bioreactor system for optimal cell seeding and dynamic culturing environment by applying the optimized approaches from aim 1 and 2.

1.4 Research Novelty and Significance

The long-term survival of regenerated cardiac tissue depends critically on the effective and sufficient blood supply to the surrounding tissue, and functional vascular system is the key. Numerous studies were carried out pursuing blood vessel regeneration by applying targeted cell lines, i.e. ECs and supporting cells. Nevertheless, no systematic model has been developed to investigate the co-culture dynamics of endothelial and

MSCs with different culturing conditions. For the first time, a quantitative model was applied to study the dynamic population, and a prey-and-predator behavior was discovered with substantial influence from culturing medium composition, cell combination, and culturing duration. This elucidation of cell interaction provides insightful guidance in determining the culturing parameters for co-culture approaches and can be applied to various systems in tissue engineering research.

Decellularized ECM scaffolds applied for cardiac tissue regeneration in previous studies, were mostly thin and clinically inadequate. Achieving thicker constructs requires the development of a vascular system to supply oxygen and nutrient for cell long-term survival. We demonstrated that the co-culture and chemical enhancement / modification approaches have both been proven effective for the revitalization of vascular like network within the thick pcECM scaffold. This inherent and revitalized vascular like network was shown to be efficiently covered with desired monolayers of ECs, functionally delivering media, nutrient and oxygen *ex vivo* in a custom designed bioreactor for controlled dynamic cultivation. Furthermore, this approach may provide instant blood supply *in vivo* through relatively simple anastomosis to the host vasculature. This would enable probable prevention of blood coagulation and improve the survival and integration post-implantation in future large animal pre-clinical evaluation or in any possible clinical application.

Chapter 2 Materials and Methods

2.1 Cell culture

2.1.1 Cell types and medium

Human bone marrow derived MSC and HUVEC were both purchased from Lonza (Basel, Switzerland). MSCs were maintained in Minimum Essential Medium Eagle, alpha modification (α MEM, Sigma., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Langley, OK, USA), 1% L-Glutamine (Gibco), 5 ng/mL basic fibroblast growth factor (bFGF) (Life Technologies, Carlsbad, CA, USA), and 1% Antibiotic-Antimycotic (Gibco). HUVECs were maintained in Endothelial Growth Media-2 (EGM-2) complete medium (Lonza) supplemented with 1% Antibiotic-Antimycotic (Gibco).

2.1.2 Cell metabolic rate and proliferation analysis

The cell metabolic rate and viability were determined using the AlamarBlueTM reagent (Life Technologies). Cells seeded on tissue culture plates or ECM samples were incubated and sampled at various time points in respective medium with 10% AlamarBlueTM reagent (Life Technologies), at 37°C in dark. The fluorescence intensity of each sample was measured with Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. AlamarBlueTM fluorescence intensities (FI) were determined as measures of culture viability or converted to cell numbers for proliferation analysis (when applicable) using standard calibration curves under the same conditions. The fluorescent signal was monitored at 545nm

excitation wavelength and 590nm emission wavelength.

A calibration curve for each cell type under pre-determined culturing condition, i.e. cell density, medium type, scaffold type, etc. was generated by plotting the FI reading from AlamarBlue™ assay against pre-determined cell numbers. The slope of each curve was used to calculate cell number with fluorescent intensity (an example of HUVEC calibration curve with EGM-2 medium on 24-well plate is shown in Appendix - Supplementary data). The growth rate of each cell type was obtained by plotting the cell number against culturing days (an example of HUVEC growth curve with EGM-2 medium on 24-well plate is shown in Figure 27a, with cell growth rate of 0.3624 elucidated from the equation of fitted exponential trendline). The basal cell metabolic index for each cell type can be elucidated with AlamarBlue™ fluorescent intensity reading at time zero, or the intercept of cell growth curve, and was then normalized to 1000 cells.

2.2 Cell growth and metabolism in various medium compositions

In order to investigate the effect of different medium compositions on cell growth, designated MSC and HUVEC culture media were mixed in a series of six combinations: 100% α MEM, 80% α MEM+ 20% EGM-2, 60% α MEM+ 40% EGM-2, 40% α MEM+ 60% EGM-2, 20% α MEM+ 80% EGM-2, and 100% EGM-2. Both cell types were singly cultivated and passaged at 70% to 80% confluence. Cells from passage 3 to 7 were used for experiments. Medium was replaced every 2 days. MSC and HUVEC were

counted with Countess automated cell counter (Life Technologies) and seeded separately at density of 5000 cells per well in 24-well plates (NUNC, Roskilde, Denmark). Cells were cultured for 7 days in above mentioned culture media combinations. Three replicates were prepared for each medium combination. Medium combinations with no cells served as control. AlamarBlueTM reading was taken every second day to obtain the metabolism and proliferation rate of the cells.

The growth curves for each cell type under each medium composition were elucidated from AlamarBlueTM assay over the 7 days culture period. The growth rates for each cell type were calculated as the slope of the exponential growth phase ($R^2 > 0.95$, $p < 0.001$) and were plotted against the medium composition (α -MEM: EGM2, respectively). The AlamarBlueTM fluorescence intensity at time zero was elucidated from the intercept of the growth curves ($p < 0.001$), normalized to 1000 cells and plotted as metabolic index for each cell type against medium composition.

2.3 Co-culture studies

2.3.1 HUVEC and MSC co-culture

To study the cell growth under co-culture condition with different medium combinations, MSCs and HUVECs were counted and mixed with 9 different ratios: 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, and 1:5. A total number of 3000 cells were seeded in each well of 96-well plate (NUNC) and cultured for 7 days. High initial seeding densities were used (9000 cells/cm²) to allow cultures to achieve confluence after a short duration. Three

combinations of culture medium were used for each cell mixing ratio: complete α -MEM, complete EGM-2, and a 1:1 mix (v/v) of the above media. Co-culture viability was measured daily using AlamarBlueTM. However, since the two cell types in our co-cultures have different metabolic rates, their AlamarBlueTM readings (as raw FI values) cannot be correlated directly to population sizes (cell numbers) but only presented as measures of total cell viability.

2.3.2 Analysis of growth dynamics of MSC and HUVEC co-culture using a differential model

A differential model that was shown to correspond with the Lotka-Volterra model (“prey-and-predator” in a closed ecological niche model) was fitted to explain the AlamarBlueTM fluorescence intensities’ profile as a function of time (and consequent cell number or cell population size). For the model fitting, it can be stipulated that under each medium composition the change in the population of X and Y species (i.e., MSC and HUVEC), living in a closed ecological niche (i.e., a tissue culture plate) can be expressed by the following equations (1) and (2) for populations X and Y respectively.

$$(1) \frac{dX}{dt} = X(\mu_x + k_x X + l_x Y) \quad (2) \frac{dY}{dt} = Y(\mu_Y + k_Y Y + l_Y X)$$

Where μ_i is the basal growth rate of population ‘i’ when grown separately and unlimited by space or nutrients. The coefficients k_i and l_i describe the ‘self-effect’ and the ‘other-effect’ on the growth rate of cell type ‘i’ respectively. For example, negative k value for

MSC ($k_{MSC} < 0$) will indicate that the MSC inhibit their own growth, while a positive l value for HUVEC ($l_{HUVEC} > 0$) will indicate that HUVEC growth is encouraged by the MSC in co-culture etc. Therefore, the basal growth rates (μ_X and μ_Y), under each medium composition, can be easily elucidated from the exponential growth phases of the separately cultured cells.

When $X(t)$ and $Y(t)$ are the cell numbers (or the population size) of their respective populations at time 't', the FI of the AlamarBlueTM assay for any co-culture of X and Y at time 't' can be expressed by the following equations (3) and (4).

$$(3) \text{ FI}(t) = \epsilon_X X(t) + \epsilon_Y Y(t) + C \quad (4) \text{ FI}(t) = \epsilon_X \int_0^t \frac{dX}{dt} + \epsilon_Y \int_0^t \frac{dY}{dt} + C$$

C is a constant standing for the assay blank reading without cells, if the blank is already subtracted from the FI values then $C=0$. The coefficients ϵ_X and ϵ_Y are the cells' basal metabolic indices (FI/cell) that when multiplied by the cell number (at any given time t) will result in the AlamarBlueTM fluorescence intensity readings (for time t). Having the initial cell populations at time ZERO (border conditions, $t=0 \rightarrow X_0$ and Y_0), according to the known seeding density and ratios, along with the previously elucidated basal growth rates (i.e., μ_X and μ_Y) and metabolic indices (i.e., ϵ_X and ϵ_Y) under each medium composition, it is possible to numerically estimate the value of equation (4) for any given time and a set of 4 parameters: k_X , k_Y , l_X and l_Y . By utilizing any optimization software (e.g., Microsoft-Excel-SolverTM) it is possible to optimize the values of k_X , k_Y , l_X and l_Y using the least squares method to achieve the highest fit (with the lowest sum of squares

and highest R^2) between the real measured FI values (Real FI) and the model calculated FI values (Model FI). By doing so, the k and l parameters for both MSC and HUVEC were evaluated.

2.3.3 Imaging population dynamics using DiI pre labeling

In order to trace the cells in co-culture for up to 7 days, non-specific membrane labeling dye DiI (Life Technologies) was used to stain MSCs prior to seeding. The cells were harvested and washed with phosphate-buffered saline (PBS) (Gibco). DiI dye was diluted into 12.5 μ M concentration. After 30 minutes of incubation at 37°C, the unbound dye was removed by washing with two PBS washes. The cells were then plated separately and cultured overnight to get rid of the burst release of the dye from the cell membrane. Pre-stained MSCs and unstained HUVECs were then plated into circular cell culture plate with diameter of 14cm (NUNC) with 1:1 seeding ratio. The cells were co-cultured in EGM-2 medium for 7 days, with medium replaced every second day. All the steps were carried out in dark. Flow cytometry (EMD Millipore, Billerica, MA, USA) was applied to analyze the percentage of DiI-labeled MSC in co-culture population after 7 days. Fluorescent and phase contrast light microscopic pictures were taken on day 3 and 7 using Nikon ECLIPSE Ti microscope (Nikon Instruments Inc., NY, USA).

2.3.4 Phenotypic properties of co-cultured cells

After being single or co-cultured for 7 days in EGM-2 medium, MSCs and HUVECs were trypsinized and fixed with 4% paraformaldehyde (PFA) on ice for 30 minutes. The

fixated cells were then washed with cold PBS at 4°C and permeablized with 0.1% Triton X-100 in PBS. After 10 minutes of incubation, cells were washed twice with PBS and incubated with 0.5% bovine serum albumin (BSA) in PBS for another 30 minutes followed by another two washes with PBS. Cell pellets were obtained by centrifuging at 250 g for 5 minutes and re-suspending in 0.1% BSA in PBS. Cells were then incubated with primary antibodies at 1:50 dilution against human PECAM-1(CD31) (Santa Cruz, Dallas, TX, USA), Von-Willebrand factor (vWF) (Santa Cruz), CD44 (BioLegend, San Diego, CA, USA) and CD105 (BD Biosciences, NJ, USA) for 30 minutes followed by staining with secondary antibodies conjugated with fluorescent dyes with dilution factor of 1:100 (Santa Cruz) for another 30 minutes in dark. All the incubations were carried out at 4°C. After washing with PBS, cells were suspended in 0.1% BSA. 5000 events for each sample were acquired using Guava easyCyte HT System (EMD Millipore) and analyzed using FlowJo flow cytometry analysis software (Tree Star Inc., Ashland, OR, USA). Antibodies used for immunostaining were as follows: rabbit (α -human CD31): phycoerythrin (PE), rabbit (α -human Von-Willebrand factor (vWF)): fluorescein isothiocyanate (FITC), rat (α -human CD44): PE, and mouse (α -human CD105): FITC. Cells without any antibody served as background for setting adjustment and cells stained with non-specific molecules of the same isotype as the antibodies served as negative controls.

2.3.5 Cell seeding density studies

HUVECs and MSCs mixed in a 1:1 ratio were seeded on tissue culture plates with

different culture areas (NUNC; 12, 24, and 48 wells) to achieve three seeding densities: 1000, 1500, and 3000 cells/cm². The total number of cells per well was maintained constant. Cells were then cultured for up to 7 days. 1 or 0.2mL of complete EGM-2 was used to culture the cells and was replenished daily to study the effect of total medium volume on cell proliferation; their viability was evaluated using AlamarBlueTM assay.

To study the effect of absolute population size, the population dynamics of MSC and HUVEC co-culture was studied using low seeding densities. MSCs, HUVECs, and 1:1 co-culture were seeded on 10 cm tissue culture plates at low initial densities (500 cells/cm²) and cultured in complete EGM-2 for 7 days. Before seeding, MSCs were labeled with fluorescent membrane tracker DiI (Life Technologies). To evaluate population size, 7-day-old co-cultures were followed up by flow cytometry (EMD Millipore), fluorescent microscopy, and phase contrast light microscopy using ECLIPSE Ti microscope (Nikon Instruments Inc.).

2.4 Static cell cultivation on ECM surface

2.4.1 Decellularization and preparation of ECM scaffold

Thick pcECM slabs were decellularized in Technion, Israel, by Prof. Machluf's group according to their patented decellularization technique [14, 110]. Briefly, and The left ventricular tissue slabs with thickness of about 15mm were harvested from healthy female pig hearts and excised in parallel to the left anterior descending (LAD) coronary artery and its adjacent veins. The decellularization process consists of mainly three steps:

alternating hyper/hypo NaCl tonic solutions, enzymatic treatment with Trypsin-EDTA (Sigma), and Triton-X 100 detergent wash. The decellularization cycle was repeated with replenished washing solution. The acellular ECM slabs were disinfected with intensive wash using sterile PBS, followed by 70% ethanol and DDW before shipped to Singapore and stored in sterile PBS solution supplemented with 2% Antibiotic-Antimycotic.

Two sizes of ECM scaffold were used for screening / optimization and vascularization purposes respectively. For static studies with cells seeding on ECM surface, myocardial portions of the ECM slabs were cut into small ECM samples with average surface area of about 0.5cm^2 . For static or dynamic vascularization studies with cells seeding on ECM vasculature, the whole thick (70mm (w) x 90mm (l) x 15mm (t)) tissue constructs were used (Figure 2a). Two catheters were sutured onto each end of decellularized LAD structure to allow perfusion of solution and medium through the vasculature and diffusion into the entire tissue slab. Prior to each experiment, small or large matrices were once again disinfected with 70% ethanol (1x30min, 1x2hr and 1x12hr) followed by at least 3 washes with PBS (3x30min), soaking with complete culture media for 12 hours in CO₂ incubator and air drying in a sterile hood for 2 hours immediately before seeding. Contamination test was done by sampling medium (20μL) with 12-hour incubation on agar plates made with LB broth.

2.4.2 HUVECs attachment study

This set of experiments was designed to study the compatibility of decellularized ECM

for cell attachment and proliferation, which is a prerequisite for endothelialization and its long term survival, and to determine the optimal time needed for HUVEC attachment. Prior to seeding, ECM slices (0.5cm^2) were placed individually into 96-well low binding plate (NUNC). 200,000 HUVECs suspended in 40 μL EGM-2 medium were seeded with micropipette on top of each ECM sample. The cells were allowed to attach to ECM for 5 attachment periods: 5, 45, 90, 135, and 180 minutes. After each seeding period, ECM slices were taken out from the 96-well plate, washed briefly with PBS, and transferred to 24-well low binding plate for AlamarBlueTM assay. Each re-seeded ECM sample was incubated with 10% AlamarBlueTM with total volume of 2mL for 5 hours at 37°C. After AlamarBlueTM reading, ECM samples were histologically evaluated in the histopathology laboratory of the Institute of Molecular and Cell Biology (IMCB, A*Star).

2.4.3 HUVEC growth study

To obtain the optimal seeding density of HUVECs on ECM scaffold, after washing as described in Section 2.4.2, ECM slices were seeded with HUVECs in 4 seeding densities: 100,000, 500,000, 1,000,000, and 2,000,000 cells per piece, in 96-well low binding plate. After seeding, cells were allowed to attach on ECM for 90 minutes in incubator. Seeded ECM slices were washed with PBS briefly and transferred to 24-well low binding plate to be cultured in EGM-2 medium for 7 days. AlamarBlueTM readings were taken every second day.

2.4.4 Protein treatment for acellular ECM

After disinfection, ECM slices (0.5cm^2) were coated with sterile gelatin, fibronectin, or laminin solution. 4mg/mL gelatin (Sigma), 10 $\mu\text{g/mL}$ fibronectin (Sigma), or 100 $\mu\text{g/mL}$ laminin (Sigma) coating solutions were prepared by dissolving protein powders in PBS and disinfected by autoclaving. ECM slices were immersed in 3mL of each coating solutions on shaker for 24 hours, and washed with PBS three times. Non-coated ECM served as control.

2.4.5 Gelatin staining using CBP-TAMRA

Titration was done by preparing collagen binding protein (RGDSCQDSETRTFY, CBP) conjugated to TAMRA (CBP-TAMRA, Sigma, stocking solution: 1 mg/ml in DDW) in 0.2% gelatin with 12 serial dilution factors (from 1 mg/ml to $0.4875\mu\text{g/ml}$) in 96-well plate with four replicates for each dilution factor. Signals for both bulk solution and surface coating on the well bottom after overnight incubation in 37°C , 5% CO_2 and a subsequent PBS wash, were detected using Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific) with excitation wavelength 549nm and emission wavelength as 577nm. The optimal dilution factor was identified as the last one before titration curve (FI signal against CBP-TAMRA to gelatin ratio (w/w)) reaches saturation and represents the equivalent titration point for this set of experiments. This equivalent point dilution factor was used for following gelatin staining studies.

After cutting and sterilization process as discussed in Section 2.4.1, ECM samples (0.5

cm²) were blocked with 5% FBS at 37⁰C overnight. Following a brief wash with PBS, non-conjugated CBP blocking was performed at 37⁰C overnight. CBP-TAMRA staining with the optimal dilution factor obtained in previous steps was done in 0.2% gelatin solution, followed by overnight incubation at 37⁰C in dark and PBS wash. Control samples were similarly blocked yet were stained without being gelatin treated. The stained samples were kept in PBS solution (with 5% FBS and 2x antibiotics, replaced every two days) before being fixed with 4% PFA at various time points and observed under confocal microscope (LSM 700 laser scanning confocal microscope, Gain: 677, lens: 10x, Carl Zeiss).

2.4.6 Co-culture study

For simultaneous co-culture, 150,000 MSCs and 150,000 of HUVECs were seeded together in a mixed suspension on each ECM slice (0.5cm²). For sequential co-culture, HUVECs were seeded 7 days after MSCs. For single culture, 300,000 HUVECs or 300,000 MSCs were seeded separately. Seeded ECM slices were cultured in 24-well low binding plates (NUNC) in 2mL EGM-2 for 21 days. Medium was replenished every second day.

2.5 Dynamic cell cultivation of ECM vasculature

2.5.1 Perfusion and Rotation Bioreactors

Tubular bioreactor system was designed to culture the ECM sample over long period of time within a sterile environment at culturing temperature of 37⁰C, with 5% CO₂ and

medium perfusion. The circulation layout of the entire system is shown in Figure 3. The overall bioreactor system is composed of 4 sections: a MasterFlex pump (Cole-Parmer Instrument Company, Vernon Hills, IL, USA) to circulate the medium throughout the system at a tunable rate; an oxygenator (Radnoti LLC, Monrovia, CA, USA) which allows air perfusion into medium to reach its desired CO₂ level; the tubular bioreactor which holds the ECM sample into position and allows medium to be perfused through the vasculature of ECM slab; and a medium reservoir to store excess medium for exchange (Corning, Figure 4a)

Tubular bioreactor can provide cultured tissue with optimal growing environment and adjustable perfusion rate; however, preliminary results from imaging analysis indicated that a more uniform cell distribution might be possible with rotation chamber to assist the cell attachment in the initial seeding process. A rotational tissue culture chamber was designed and customized based on commercially available cell culture system (Synthecon, Houston, TX, USA) to improve the initial attachment of cells on ECM vasculature (Figure 4b). The transparent polycarbonate chamber sits on a customized holding frame with a small rotational motor, which is attached on the side. The rotation velocity and orientation can be adjusted by a controller. A holding frame with a central cavity (Figure 4c) was customized for positioning the ECM slab (Figure 4d) tightly inside the chamber. The two catheters that are already sutured to the thick pECM on both sides (Section 2.4.1) were attached to two cannulas, which in turn were connected to the outside tubing through designated connectors for medium perfusion and circulation. The seeding frames have numerous pores on both sides to allow sufficient medium exchange

(Figure 4c).

To serve the purposes of both disinfection and dynamic culturing of thick ECM slab, the bioreactor system was installed with silicon tubing and lure connectors (Cole-Parmer) linking up the oxygenator, bioreactor chamber and medium reservoir. The entire bioreactor system consists of two circulation loops: medium and air flow (Figure 3). The medium flow was driven by the pump from medium reservoir to oxygenator, perfuse the entire ECM slab through its vasculature in bioreactor chamber, and back to medium reservoir. In the air loop, an air pump was used to constantly pump air from CO₂ incubator into oxygenator to allow air perfusion through air permeable silicon tubing into medium before entering the bioreactor chamber. The only non-air permeable tubing (Tygon® R-3603) instead of silicon in the entire system was used to connect oxygenator to bioreactor chamber inlet, where minimum air exchange was desired.

After installation, the entire bioreactor system was sterilized by washing with 70% ethanol for 30 minutes and circulating with fresh ethanol three times (2 x 2hr, 1 x overnight). Same procedure was repeated with sterile PBS containing 2 x antibiotics.

Figure 3. Medium and air circulation layout in bioreactor system. MasterFlex pump (a), oxygenator (b), bioreactor (c), medium reservoir (d). Air was pumped into oxygenator via an air pump from CO₂ incubator. Both bioreactor chamber and oxygenator were kept in the incubator (zone in read dashed line). Arrows indicate direction of medium (solid line) and air (dashed line) flow.

Figure 4. Tubular and rotational bioreactor system setup. Tubular (a) and rotational (b) bioreactor systems, sample holding frame within the rotational chamber (c) and ECM slab sample (d).

2.5.2 Endothelialization and dynamic cultivation of ECM

As mentioned earlier, vasculature system embedded inside the thick pcECM construct was successfully preserved after the decellularization process and can be used as excellent platform for endothelialization (Figure 2). After identifying the inlet and outlet of the vasculature by syringe injection, catheters were sutured at the construct's two ends to guide the medium in and out during perfusion. The thick ECM slab was then installed into the bioreactor chamber (Figure 4). Before cell seeding, the entire bioreactor system with ECM was then disinfected once more with the same procedure described in Section 2.5.1.

To assess the attachment of HUVECs on ECM vasculature, which differs physiologically from myocardial ECM surface, HUVECs were seeded on the vasculature wall by syringe injection to the ECM inlet catheter. To confine the HUVECs inside the main vasculature and minimize leaking, disinfected ECM slab was taken out of the bioreactor chamber and placed on sterile tissue culture plate to allow two clamps to close up the ends of branches along the main vasculature in a sterile hood. 3 million HUVEC (in 1mL EGM-2 medium) was injected into each ECM slab. ECM patch was then installed back into the rotational bioreactor chamber pre-filled with medium to allow uniform attachment with rotation (3.3 RPM) for 2 hours. After the initial attachment, rotation was stopped and the re-seeded ECM slab can be moved to the tubular bioreactor for continuing culture to free the rotational bioreactor chamber for parallel experiments.

For dynamic studies, same initial steps were followed. After 2 hours of initial attachment as described earlier, medium perfusion was started with 1mL/min perfusion rate and a

total volume of 200mL EGM-2 medium was used to circulate the entire system for up to 21 days. AlamarBlueTM assay was performed every 2-3 days, before and after which, medium was replenished. Contamination test was performed every 2 days by sampling 20 μ L medium from bioreactor system using a sterile needle through a heparin lock sampling port (Biometrix, Israel), and incubating on agar plate for 12 hours.

For comparison purpose, static experiment using the same system set up and initial cell seeding procedure was performed. The ECM slabs, after initial cell attachment, were then either cut into four equal cross-sectional pieces along the vasculature subsequently incubated in AlamarBlueTM reagent in 6-well low binding plate, or sacrificed for histological studies. 3 replicates were prepared for each group of these experiments. At the end of each experiment, samples were sacrificed for fluorescent imaging and histological studies.

2.5.3 Dynamic endothelialization with protein treatment and co-culture

Two different approaches were tried to improve endothelialization in terms of cell attachment, growth and morphology. For ECM vasculature with protein treatment, after disinfection procedure for bioreactor system with ECM slab installed, as described in Section 2.5.2, 80mL of 0.2 w/v% sterile gelatin solution in PBS was slowly injected into the ECM vasculature with a syringe and covered the entire ECM slab in a T75 flask to be incubated on a shaker overnight. Three washes with sterile PBS containing double antibiotics with circulation throughout the bioreactor system were performed after the

treatment to remove any unbounded gelatin from the ECM slab.

Two parallel dynamic approaches were carried out. For dynamic co-culture model, sequential seeding approach was adopted. 3 million MSCs (in 1mL EGM-2 medium) were seeded 7 days prior to another 3 million HUVECs (in 1mL EGM-2 medium) in the same way as single culture (Section 2.5.2).

For each set of experiment, 3 replicates were used. AlamarBlue™ assay was performed every 2-3 days, before and after which, medium was replenished. Contamination test was performed as described before (Section 2.5.2). At the end of each experiment, samples were sacrificed for fluorescent imaging and histological studies.

2.6 Microscope imaging and analysis

Scanning electron microscopy (SEM) was applied to study the morphological properties of cells on ECM surface. ECM samples seeded with cells were gently washed with PBS and fixed in 2% PFA overnight. Samples were then washed with PBS three times, dehydrated with ethanol in ascending concentration from 30% to 100%, and air dried. The dehydrated samples were gold sputtered and mounted on JSM-6360 SEM for imaging at voltage of 5kV (JEOL, Tokyo, Japan).

Fluorescent microscopy, and phase contrast light microscopy using ECLIPSE Ti microscope (Nikon Instruments Inc.), as well as confocal laser scanning microscopy using

LSM 700 laser scanning confocal microscope with UV (405nm), Blue (488nm), and Green (555nm) lasers and 10x or 20x objectives (Zeiss, Oberkochen Germany) were also used to observe cell density, morphology and location in 2D and 3D scaffold. Fluorescent Binocular microscope with UV (excitation: 370-390, emission: 400-460), Green (excitation: 460-480, emission: 495-540), and Red (excitation: 535-555, emission: 570-625) filters (Olympus, Tokyo, Japan) was used to offer a much larger view of interest, which allows easy observing of population dynamics and bulk samples or samples in bioreactors in a large scale without compromising the sterile environment.

2.7 Histological studies

2.7.1 Tissue sample fixation

To protect the Green Fluorescent Protein (GFP) signal of HUVEC-GFP seeded ECM during the dynamic studies,, tissue samples were fixated prior to freezing. Tissue slabs were injected with 80mL of 2% PFA solution in PBS very slowly at 10mL/min with a syringe. Tissue was incubated for 5 hours in a T75 flask before a sterile PBS dilution at 1:1 ratio to reach 1% PFA concentration, again with slow injection to the vasculature where the HUVEC-GFP were seeded. After 2 hour incubation, same procedure was carried out with 30% sucrose solution in PBS containing 0.05% sodium azide and with 24 hour incubation time. The sucrose injection and incubation was repeated one more time with fresh solution.

2.7.2 OCT blocking preparation and cryo-sectioning

Tissue-Tek OCT (Optimal cutting Temperature compound) compound (Sakura, The Netherlands) was injected into the vasculature of ECM slabs. The ECM sample was then embedded in an open plastic container with 50mL OCT for 15 minutes at room temperature. The ECM slabs were then cut into smaller samples to fit into the plastic molds and embedded with OCT with cross-section of vasculature facing downwards. The samples were left on dry ice till they are completely frozen (with OCT turning solid and opaque) and then kept at -80°C for storage.

For cryo-sectioning, frozen sample blocks were sliced with $10\mu\text{m}$ thickness at -22°C using a cryostat machine (Leica, Wetzlar, Germany) and mounted onto positively charged glass slides (Menzel glaser, Braunschweig, Germany). Sample on slides can be used for staining immediately or stored at -20°C for later use.

2.7.3 H&E staining

Hematoxylin and eosin (H&E) staining was performed to observe cell distribution and alignment on tissue cross sections. Tissue sections were fixed on glass slides with cold methanol (-20°C) for 15 minutes and washed with double distilled water (DDW) twice with 2 minutes gentle shaking to remove OCT. Sections were stained with hematoxylin solution for 10 minutes followed by washing with clean DDW until the water is clear from hematoxylin residue. Sections were then briefly dipped into 0.5% hydrogen chloride (HCl) solution prepared in 70% ethanol, followed by 0.05% ammonium hydroxide in

DDW for 30 seconds. Sections were washed with DDW after each step. Sections were then immersed in 80% ethanol for 2 minutes before transferred into eosin solution for another 2 minutes. The dehydration process was performed by immersing the stained sections into a series of ethanol and xylene solutions: 95% ethanol for 1 minute, twice of 100% ethanol for 1 minute, xylene for 1 minute, a second round of xylene for 2 minutes. Finally the sections were mounted using xylene-based mounting medium (Thermo Scientific, Waltham, MA, USA) and sealed with cover slip, and left to air dry in a chemical hood overnight before imaging. The DDW used for all the washing steps were pre-adjusted with pH value at 7.2.

2.7.4 Masson's Trichrome staining

Masson's Trichrome staining was performed to observe cell nuclei and cytoplasm in contrast to the ECM collagen in re-seeded tissue samples. Masson Trichrome staining kit with aniline blue (Bio-Optica, Milan, Italy) was used for the cryo-sliced, OCT embedded sections, according to manufacturer's protocol.

2.7.5 ABC DAB staining for CD31

Immunohistochemistry (IHC) was applied to stain specific HUVEC markers, CD31 to assess the phenotypic properties of HUVEC after being cultured on 3-D scaffold for long time with an ABC staining kit (Thermo Scientific). After cryo-sectioning, sample on glass slides were brought to 4⁰C overnight from their storage condition (-20⁰C) on the day before staining and incubated in humid chamber at room temperature for 20 minutes

immediately before staining. Samples were fixed by dipping in cold acetone (-20°C) for 10 minutes, and quenched with 3% H_2O_2 in methanol for another 10 minutes. After brief wash with PBS, blocking was performed with 10% FBS in PBS. At least 50 μl of primary antibody (rabbit anti human CD31, SC-8306, Santa Cruz) with 1:100 dilution factor in blocking solution was added to each sample and incubated for 90 minutes at room temperature. After washing the sample 3 times with PBS, secondary biotinylated antibody (1:200 in blocking solution) was added and incubated for 45 minutes at room temperature, followed by PBS wash. The ABC reagent was then prepared and added followed by DAB staining according to manufacturer's protocol. After staining, samples were dehydrated with 96% and 100% ethanol and followed with xylene before mounting with cover glass for observation under microscope. Samples with only secondary antibody served as isotype control.

2.7.6 Immunofluorescent staining for OCT embedded sections

Fixated section slides were washed three times with PBS and incubated for 5 minutes each time. To confine the liquid in staining area, circle around the sample on glass slides using a PAP pen (Abcam, England) to create a thin film like barrier. The slides were laid flat to avoid overflow of liquid. 100 μl of 5% FBS in PBS was added to each sample as blocking solution, and incubated for 1 hour at room temperature. 100 μl of primary CD31 antibody (rabbit anti human, SC-8306, Santa Cruz) with 1:50 dilution factor in PBS containing 3% FBS was added to each sample, and incubated at 4°C overnight. On the second day of staining, wash the sections three times with PBS before adding 100 μl of

secondary PE-conjugated donkey anti rabbit antibody (SC-3745, Santa Cruz) for one hour incubation in dark. To get rid of excess antibodies, the stained sections were then washed with PBS three times with 5 minutes incubation for each in dark. Sample slides without staining and stained with only secondary antibody was used as control. The entire staining experiment was carried out with section slides placed in a humid chamber created with slide box and damp paper cloth to prevent evaporation, especially for long hour incubation. For nuclei counterstaining, sections were mounted with DAPI-Fluoromount-G (SouthernBiotech, Birmingham, AL, USA).

2.8 Lenti-virus generation and cell transfection

For long term monitoring of HUVECs after seeding on tissue scaffold, and distinguishing them from other cell types in co-culture studies, HUVECs and MSCs were transfected with GFP and mTomato carrying Lenti-virus system, respectively. Both of the plasmids were given as a gift generously by Rambam hospital, Israel, and were inserted into XL-1 blue competent cells. Competent cell suspension was thawed on ice for 40 minutes. 2.5 μ g of each plasmid was added into 100 μ L (about 10^9 cells/ μ L) cell suspension, and allowed to incubate on ice for another 40 minutes. Heat shock was performed on cell plasmid mix at 42 $^{\circ}$ C for 90 seconds followed by 5 minutes incubate on ice. Cell suspension was cultured in LB (Luria-Bertani) broth (BD Biosciences) for one hour in a shaking incubator at 37 $^{\circ}$ C before 100 μ l of which was added evenly on agar plate containing ampicillin (100mg/L in LB broth). The bacterial cells on agar plates were cultured overnight in the incubator at 37 $^{\circ}$ C, and single colony was picked up using a sterile pipette

tip and cultured in 100mL LB broth in a sterile conical flask in shaking incubator for 12-18 hours.

The amplified plasmid DNA was harvested using PureLink® HiPure Plasmid Filter Maxiprep Kit according to manufacturer's protocol (Life Technologies). Briefly, cells were harvested by centrifugation overnight LB cell culture at 4000g for 10 minutes. Cell pellet was re-suspended in Resuspension Buffer with RNase A and lysed with Lysis Buffer. The lysate was clarified with negative charged DNA binding to positive resin surface when passing through the anion exchange column fitted with filtration cartridge unit. After washing the DNA adherent column with Wash Buffer to remove RNA, proteins, carbohydrates and other impurities, only plasmid DNA remained bound to the resin. The plasmid DNA was then eluted with Elution Buffer, desalted and concentrated by washing with isopropanol and 70% ethanol with centrifugation at >12000g for 30 minutes at 4⁰C after each wash. DNA pellet was air dried for 10 minutes, resuspended in TE buffer, and stored at -20⁰C if not used immediately. All the materials and solutions used were included in the kit (Life Technologies). The DNA concentration was checked using NanoDrop (N100, Thermo Scientific).

To generate the Lenti-virus with target plasmid DNA, a commercially available ViraPower™ Lenti-viral Expression System (Life Technologies) was applied, and manufacturer's protocol was followed. Briefly, 5M 293FT cells (Life Technologies) were plated on 10cm tissue culture plate one day before transfection, and cultured overnight in DMEM medium with high glucose (Sigma) to reach 90-95% confluence. On the day of

transfection, culture medium was replaced with Opti-MEM I medium containing serum (Life Technologies). In a sterile centrifuge tube, 9µg ViraPower™ Packaging Mix was mixed with 3µg of Lenti expression plasmid DNA prepared earlier in 1.5mL Opti-MEM I medium without serum. In a separate tube, 36µL Lipofectamine™ 2000 was diluted in 1.5mL Opti-MEM medium with gentle mixing, and incubated at room temperature for 5 minutes. DNA-Lipofectamine™ 2000 complexes were prepared by mixing the DNA and Lipofectamine™ 2000 gently and incubating at room temperature for 20 minutes. The complexes (3mL) were added drop wise to each plate of cells and cultured in CO₂ incubator overnight. The complex containing medium was replaced with 10mL complete DMEM medium without antibiotics on the next day, and plates were left for 48 hour incubation for virus production. Virus containing supernatant was centrifuged at 500g for 15 minutes to get rid of cell debris, and stored at -80⁰C if not used immediately. For primary cell transfection, 1mL of virus containing medium harvested in the previous stage was added to each 10cm plate with pre-plated cells. 6µg/mL polybrene (Sigma) was added to enhance transfection efficiency. Cells were incubated with virus for 24 hours before the medium was replaced with fresh growth medium. The transfection efficiency was assessed using an ECLIPSE Ti microscope (Nikon Instruments Inc.)

Chapter 3 Results and Discussion

3.1 Effect of medium on cell growth and metabolism

Several cell types were previously reported to be involved in the process of vascularization [62]. Out of various cell-type combinations, HUVEC and MSC co-culture system are among the most popular options for cardiovascular tissue regeneration [66-69, 75]. In the context of cardiac tissue engineering, even though MSCs do not possess the beating ability as CMs do, they are repeatedly used as CM model cells [5] owing to their easy expansion *in vitro*, self-regeneration capabilities, and their proven supporting function for vascular tubular arrays [62, 63, 66, 135]. Hence MSC interaction with pcECM under both static and dynamic conditions have been extensively studied by our group with promising results [5, 14, 110, 136]. Endothelial cells, such as HUVECs, are most commonly used for both vasculogenesis and angiogenesis in tissue regeneration applications [56-58]. And the feasibility of using HUVECs to recellularize and form a coating monolayer within the lumens of acellular arterial ECM biograft has been verified in our previous studies as well [31]. In this thesis, the application of HUVECs and MSCs for regenerating vascular structure within thick cardiac patch was firstly investigated.

However, prior to co-culturing both cell types, the culture parameters need to be optimized for each cell type individually, particularly since the designated medium and metabolic indices of each type are distinctly different. Surprisingly, to the best of our knowledge, no comprehensive study was performed to date on the effects of different media compositions and seeding density ratios on the co-culture population dynamics and

on each individual cell type metabolism, survival and proliferation. Therefore, six culture media mixing ratios were used to singly culture HUVEC and MSC as detailed in the methodology section.

The growth rate and metabolic indices obtained for MSC and HUVEC are shown in Figure 5. Cell numbers were determined using the AlamarBlueTM assay against each cell type's respective calibration curve ($R^2 > 0.99$, $p < 0.0001$). Various assays have been developed for cell viability studies over time, based on nucleotide incorporation, protein binding, and cell metabolic activity, most of which, such as MTT assay, are cell destructive, and are not suitable for continuous monitoring of the same samples [137, 138]. AlamarBlueTM assay measures the metabolism and proliferation of cells in a fast, accurate, non-radioactive, and non-toxic manner that allows continuous study on the same batch of samples without sacrificing the cells or the need of detaching the cells from scaffold [139]. As AlamarBlueTM dye is reduced by the cellular metabolic activity, this assay provides an indirect indicator for cell number based on the overall metabolic activity of viable cells [140]. It has been compared with other viability assays with good correlations, and has been widely applied in tissue engineering applications [55, 141-144].

When MSC and EC were applied in co-culture system for tissue regeneration applications, most of the literature publications report the use of either MSC [55] or HUVEC [64, 138] designated medium to culture both cell types in the same niche without much explanation and reasoning. The most commonly used medium for either of the cell

type might not be the optimal for the co-culture system, hence, effort must be made to optimize the medium composition to support the growth and metabolism of both cell types. It is evident that the medium composition has no effect on the growth rate of MSCs. However, HUVECs did not grow in 100% α MEM medium and had significantly higher growth rate with 40% or higher EGM-2 present in medium combo (Figure 5a). The metabolic indices of both MSC and HUVEC inclined with increasing proportion of EGM-2 medium, and reached the highest with complete EGM-2 medium (Figure 5b). These findings suggest that EGM-2 serves as the optimal culture medium for MSC and HUVEC co-culture when compared to α MEM or even combination of the two.

Figure 5. Effect of medium compositions. Growth rates (a) and metabolic indices (b) of MSC and HUVEC under various medium compositions (n=3).

For the first time, the effect of medium compositions on cell growth and metabolism was addressed and comprehensively studied. Results showing significant influence on both cell types using different medium combinations verify the importance of this preliminary evaluation before more complicated models can be applied. The methodology employed in this study enables the proper identification of the right medium to be used for

subsequent studies, and can also be applied to other co-culture applications in the future.

3.2 MSC and HUVEC co-culture studies

3.2.1 Growth rate and metabolic indices of MSC and HUVEC in co-culture

To study the growth dynamics of cells under co-culture conditions, the effects of medium type and cell mixing ratio were studied. Three types of medium were used for cell culture, 100% EGM-2, 50% EGM-2 and 50% α MEM, and 100% α MEM. Nine cell mixing ratios were studied for co-culture of MSC and HUVEC, from 5:1 to 1:5. Also, time of culture is an important factor as AlamarBlueTM reading was taken daily during the 7 days. The viability of the cells in co-culture was measured by fluorescence intensities (FI) of AlamarBlueTM assay to represent the sum of the two co-cultured cell numbers weighted according to their specific metabolic indices (FI/cell). As the two cell types in the co-culture system each possess different metabolic rates, their AlamarBlueTM readings (as raw FI values) cannot be resolved to reflect on the cell number of each component separately [145], but only presented as measures of viability of the entire co-culture system [137]. The term “cellular viability” is commonly used instead of the cell number here and in most applied cell models to replace “cell number” [146], when the size of overall and each subpopulation were estimated [29, 139]. We found that culturing time, medium compositions, and cell seeding ratios, significantly affected these viability values, most likely affecting the population growth and consequent cell numbers. Thus, these factors were considered and included in an elaborate model to explain the co-culture population dynamics.

In Figure 6, the average FIs of the overall co-culture system were analyzed using one-way ANOVA with Tukey's HSD post-hoc correction, and plotted against culturing time (a), medium compositions (b) and MSC to HUVEC ratios (c) showing a significant effect of each of the parameters (time, medium and ratio) over the fluorescence intensity. As expected, an increase in FI was observed with increasing culturing time ($p < 0.0001$), despite a slight and insignificant drop in FI values from day 1 to day 2 (Figure 6a); the effect of medium composition on total FI was small yet significant ($p < 0.05$), with a small advantage of mixed medium over pure α MEM and EGM-2 (Figure 6b); A significant increase was observed with increasing proportion of MSC over HUVEC in the co-culture system ($p < 0.001$, Figure 6c), which could be attributed to the MSC's intrinsic higher growth rate compared to HUVEC.

A simple response surface model (RSM) was fitted to summarize the effects of the three factors (culturing time, medium composition, and MSC to HUVEC ratio) together on the natural logarithm of the observed FI values, assuming all three as continuous variables ($R^2 > 0.70$, $p < 0.0001$):

$$\ln(FI) = \begin{cases} \alpha\text{MEM} \Rightarrow -0.78 + 0.16t + 1.88R \\ 1 : 1\text{mix} \Rightarrow -0.15 + 0.16t + 1.88R \\ \text{EGM} - 2 \Rightarrow -0.52 + 0.16t + 1.88R \end{cases}$$

$t \equiv$ time (day)

$R \equiv$ MSC ratio, 0 = only HUVEC ... 1 = only MSC

As shown in Figure 6d, depicting the natural logarithm of the real FI versus the natural logarithm of the predicted FI (by the RSM model), a significant fit was found ($R^2 = 0.70$, $p < 0.0001$) along with the perfect fit line ($Y=X$, bold line) and the $\pm 95\%$ confidence intervals for the fit (dashed lines), suggesting that these factors can be used later in a more complex and “biologically correct” model. After validating the significance of the single factors, a differential model, which was shown to correspond to the Lotka-Volterra model (Section 3.2.2), was fitted to explain the variance in the FIs of the AlamarBlue™ assay as a function of time and seeding ratios under each medium composition.

Figure 6. Effects of culturing time, medium and cell ratio on HUVEC and MSC growth. Average FI of MSC and HUVEC co-culture under different culturing time (a), medium compositions (b), and MSC to HUVEC ratio (c). The natural logarithm of the observed FIs are plotted against the RSM predicted values with a fitted statistical RSM to explain the variance in Ln(FI) versus culturing time, medium composition, and MSC to HUVEC ratio, assuming all three as continuous variables ($R^2 > 0.70$, $p < 0.0001$, d, $n=3$).

The monitoring and analyzing cell growth metabolism over time can be a challenge, especially in co-culture systems each cell type exhibit different growth profile, and the overall measurement cannot be easily resolved. Here we used “cell viability” obtained from AlamarBlue™ Assay to estimate the overall cell growth profile in order to evaluate the effects of different factors, and a simple RsM to validate our findings. As discussed in Section 3.1, AlamarBlue™ Assay was the main approach applied in this study for the assessment of cell growth rate and metabolism indices for singly as well as co-cultured cells owing to its applicability for long term monitoring studies. For the study of different culturing parameters in co-culture systems (such as cell ratio and physical interaction), other studies applied alternative approaches to analyze cell proliferation and metabolism, including Picogreen assay [55], counting electronically after trypsinization [147], thymidine assay [148], Hoechst 33258 dye [120], all of which requires sacrificing the cells in one way or another and compromise the purpose of long term monitoring for cells. Also, this is the first time effects of medium composition, cell ratio, and culturing time are evaluated in any co-culture system.

3.2.2 Analysis of growth dynamics of MSC and HUVEC co-culture using a differential model

We have found that the Lotka-Volterra model, commonly used in population studies to describe the dynamics of two species (prey-and-predator) sharing a closed ecological niche [149, 150], can be modified to fit complex mammalian co-culture systems [29].

Lotka-Volterra model, one of the most classic mathematical model for competition was first developed during World War I to model the population dynamics of two competing fish populations in Adriatic Sea, and still dominates the theory of ecologic competition. This model demonstrates the effect of interaction within the same species and with the other species on the population dynamics of two species competing in the prey-and-predator pattern within an enclosed economic niche. Lotka-Volterra model has been well studied and used in ecological applications [151]. Sherratt applied Lotka-Volterra model as a generic model for competition kinetics of two cell populations in his study for wavefront propagation [152]. Same theory was also applied in a study on bacterial competition for attachment space to verify the application of a simple biofilm model [153]. To the best of our knowledge never before has this model been used in the context of co-culture, the same basic ‘ecological niche’ rules apply to the tissue culture plate and to the reseeded tissue-engineered constructs, We therefore speculated that it could be of added value to explore this model fitting and predictions in the context of the co-culture of two most valuable tissue engineering cell sources such as the MSCs and HUVECS as presented below.

As explained in Section 2.3.2, by fitting the viability data from AlamarBlueTM assay to the Lotka-Volterra model, we have managed to evaluate the k (the self-effect) and l (the other-effect) parameters for each cell type (MSC and HUVEC) with R^2 values of 0.81, 0.80 and 0.76 under complete α MEM, 1:1 α MEM and EGM-2 mix and complete EGM-2, respectively. As can be seen from Figure 7a, MSC did not exhibit a significant self-effect in either medium ($k_{MSC}=0$, $p>0.05$), whilst HUVEC exhibited a self-inhibitory effect

($k_{\text{HUV}} < 0$, $p < 0.01$) in all medium compositions. Although, this self-inhibitory effect was much weaker in complete HUVEC designated medium EGM-2 ($k_{\text{HUV}, \text{EGM-2}} = -0.02$ [1000·cell·day] $^{-1}$) than under other medium compositions ($k_{\text{HUV}} = -0.16 \pm 0.01$ [1000·cell·day] $^{-1}$). As for the ‘other-effect’ and as can be seen from Figure 7b, MSCs were only affected (and in particular inhibited) by HUVECs in complete MSC designated medium (α MEM) with $l_{\text{MSC}, \alpha\text{MEM}} = -0.15$ [1000·cell·day] $^{-1}$ ($p < 0.05$). The effect of HUVEC on MSC under the two other medium compositions was also insignificant ($l_{\text{MSC}} = 0$, $p > 0.05$). However, the growth of HUVECs was significantly encouraged ($p < 0.05$) by MSCs in both complete EGM-2 and 1:1 mix of complete α MEM and EGM-2 ($0 \approx l_{\text{HUV}, \alpha\text{MEM}} < l_{\text{HUV}, \text{MIX}} < l_{\text{HUV}, \text{EGM-2}} = 0.075$ [1000·cell·day] $^{-1}$).

In this model, “self-effect” and “other-effect” were defined respectively to describe the mutual effects each cell type exerted on itself as well as the other. The inhibitory self-effect of HUVEC and “silent” self-effect of MSC in all medium types tested revealed by fitting the Lotka-Volterra model to our empirical data can be easily explained with the high proliferative nature of MSC and the self-limiting tendency of HUVEC to not overgrow and ensure monolayer formation on vascular intima [154, 155]. For other-effect, again owing to MSC’s “stemness” and self-regenerative attributes [156], its growth was not susceptible to the presence of HUVEC except in pure α -MEM medium, which was unexpected. Nevertheless, with much higher basal growth rate, MSCs were still found to dominate the co-culture over time. A strong enhancing other-effect exerted on HUVEC by MSC was observed, which agrees with the regulatory effect of MSC on

HUVEC and its function in promoting the vascular network formation and stabilization [66, 135].

After elucidating the model coefficients (i.e., k and l), for each cell type under each medium composition, it was then possible to evaluate the size of each population. This is demonstrated for initial seeding density of 3000 cells per well (in a 96-well plate) using a 1:1 MSC to HUVEC ratio grown for seven days under complete α MEM (Figure 7c), 1:1 α MEM and EGM-2 mix (Figure 7d) and complete EGM-2 (Figure 7e). With negative self-effect and positive other-effect, our model predicts that in EGM-2, MSCs will undergo gradual depletion over the course of 7 days, while the overall co-culture will be eventually dominated by HUVEC population (Figure 7e). Cell numbers in Figure 7c, d, and e are presented in exponential scale. A FACS analysis for the phenotypic properties of cultured cells was later performed to verify this prediction by the model (Section 3.2.2).

Figure 7. Population dynamics of MSCs and HUVECs co-cultured under different medium compositions. Self- (a) and other-effect (b) of co-cultured MSCs and HUVECs according to the Lotka-Volterra model under various medium compositions. Significant effects are indicated by asterisks ($p < 0.05$). Predicted cell numbers using the model under α -MEM (c), 1:1 (v/v) mix (d), and EGM-2 (e, $n=3$).

A differential model corresponding with the Lotka-Volterra model (or “prey-and-predator” model), was fitted to explain the variance in the fluorescence intensities of the AlamarBlueTM assay as a function of time and seeding ratios under each medium composition. The correlation coefficients (R^2), significance levels (P values) and the 95% confidence intervals for the real fluorescence intensities (Real FI) against the fluorescence intensities estimated using the model (Model FI) were plotted for each medium compositions: α MED (Figure 8a), 1:1 mix (V/V) of complete α MED and EGM-2 (Figure

8b) and complete EGM-2 (Figure 8c). Highly correlative and significant fits were found under all medium compositions.

Figure 8. Model correlations for MSC and HUVEC co-cultures growth dynamics. (a) α MEM, (b) 1:1 mix (V/V) of complete α MEM and EGM-2, (c) and complete EGM-2.

3.2.3 Phenotypic properties of MSC and HUVEC grown in co-culture

Since MSCs lack unique distinguishing markers [47, 157, 158], and application of CD31 or vWF to distinguish endothelial cells from MSCs was demonstrated in other studies [159, 62]. The expression of common MSC (CD105 and CD44) and unique HUVEC (CD31 and vWF) markers [38] was investigated for phenotyping purposes. The two cell types were cultured singly and in a 1:1 co-culture for 7 days in complete HUVEC medium (EGM-2) that was previously shown to best support both cell types. As expected, MSCs expressed two common markers for both cell types: CD105 and CD44, but exhibited negative expression for the unique endothelial markers CD31 and vWF (Figure 9); whilst all four markers were positive for HUVECs (Figure 10). However, the expression profiles of the co-cultures were almost identical to those of HUVECs alone (Figure 11) showing no decrease in the expression of unique HUVEC markers. This result revealed a final population for 1:1 co-culture in EGM-2 medium, which was almost entirely dominated by HUVECs with little trace of MSC. This phenomenon agrees strikingly with the model prediction (Figure 7e) and is based on the assumption that all cells maintained their original phenotypic properties with no trans-differentiation occurred for MSC.

If there were no dynamic interactions between the two co-cultured cells and due to the higher basal growth rate of MSC in complete EGM-2 (0.68 1/day compared to 0.44 1/day for HUVEC), we would have expected the proportion of MSC in the final co-culture to be higher than 50% or at least 50% (owing to the initial 1:1 seeding ratio). The fact that the FACS analysis of the co-culture reveals no traces of the CD31 and vWF negative MSC

was in accordance with our model prediction. The predicted final proportion of MSCs was around 15% (based on population size of MSC (7,000 cells) and HUVEC (41,000 cells) on day 7, Figure 7e), revealing a final population entirely dominated by endothelial markers expressing cells. Unlike the separately cultured cells, which maintained their original markers, MSCs seemed to disappear when co-cultured with HUVECs under these conditions. Though it is very unlikely that MSC underwent trans-differentiation within a short period of 7 days of culture [62, 71]. In vitro differentiation of MSC into endothelial cells was achieved only after 7 days using differentiation medium [71, 74, 160]. Co-culturing with EC either failed to induce stem cells to express endothelial markers, or differentiated stem cells to a smooth muscle or pericyte fate [62, 161, 162]. To further verify the MSC depletion instead of trans-differentiation in co-culture, cell tracking using membrane labeling dye was carried out and explained in the next section.

Figure 9. Phenotypic analysis for MSC. Flow cytometry analysis of CD105 (a), CD44 (b), CD31(c) and Von-Willebrand factor (vWF) (d) expression by MSC. Representative results (n = 3) depicted as forward scatter versus fluorescence intensity. Percentages refer to positive populations (blue) under identical gates (per marker) determined according to the isotype control (red) 7 days post culture in complete EGM-2 medium. This set of data is representative out of 3 independent sample sets.

Figure 10. Phenotypic analysis for HUVEC. Flow cytometry analysis of CD105 (a), CD44 (b), CD31(c) and vWF (d) expression by HUVECs. Representative results (n = 3) depicted as forwards scatter versus fluorescence intensity. Percentages refer to positive populations (blue) under identical gates (per marker) determined according to the isotype control (red), 7 days post culturing in EGM-2 medium. This set of data is representative out of 3 independent sample sets.

Figure 11. Phenotypic analysis for MSC and HUVEC co-culture. Flow cytometry analysis of CD105 (a), CD44 (b), CD31(c) and vWF (d) expression by HUVECs and MSCs 1:1 co-culture. Representative results (n = 3) depicted as forwards scatter versus fluorescence intensity. Percentages refer to positive populations (blue) under identical gates (per marker) determined according to the isotype control (red), 7 days post co culture in EGM-2 medium. This set of data is representative out of 3 independent sample sets.

3.2.4 DiI staining of MSC and HUVEC co-culture

MSC and HUVEC co-cultures, under the same conditions as stated above, were also followed up by cell tracking with non-specific membrane labeling dye DiI to further answer the question whether the absence of MSCs in the co-cultures was due to MSCs under-growth (and/or HUVEC hyper-growth) or MSCs differentiation into HUVECs. Only MSCs were pre-stained with fluorescent membrane cell tracker DiI (red), while HUVECs were un-stained. The co-cultured and separately cultured DiI stained MSCs and un-stained HUVECs were imaged under fluorescent and phase contrast light microscopy

after 3 and 7 days. In Figure 12, images were merged from phase contrast images and fluorescent images. Images are representative out of 3 regions of interest (ROI) taken from each culture on each day from 3 independent experiments.

As shown in fluorescent labeling and the distinct morphology of MSCs, their presence in the co-culture, after 3 days, was quite scarce (compared to co-cultured HUVECs or singly cultured MSCs) and they were almost completely lost after 7 days. As revealed in Section 3.2.2, the “silent” self-effect of MSC in EGM-2 medium prevented its fast proliferation. Even though the “stemness” nature of MSC spared them from getting significantly influenced by the presence of other cell types in the same niche with its slightly negative other-effect, this balance was broken by the robust other-effect exerted on HUVEC by MSC, i.e. the presence of MSC in the niche significantly promoted the proliferation of HUVECs, and eventually lead to over dominance of HUVEC and depletion of MSC in the closed niche over the period of 7 days. This phenomenon verified the self- and other-effect of the two cell types from the model and coincides strikingly with the model prediction as shown in Figure 7 (e, with cell number presented in y axis in exponential scale).

The fact that the co-cultured HUVECs did not exhibit DiI labeling implies that they did not originate from differentiated MSCs that would have otherwise kept their membrane labeling regardless of their differentiated state. As with the above FACS analysis, the microscope images showing much less MSCs than HUVECs in the co-cultures further corroborate our suggested model. .

Figure 12. Cell tracking using membrane labeling dye. MSC (stained with DiI, red) and un-stained HUVEC in single and 1:1 co-cultures, imaged after 3 and 7 days under fluorescent and phase contrast light microscopy. All images are presented as an overlay of phase contrast light images with the fluorescent red components. Images are representative out of three regions of interest taken from each culture on each day from three independent experiments ($n = 3$).

3.2.5 Effect of low seeding density on population dynamics

It is expected that in our model, the final proportion of each population component (out of the entire population) will depend not only on the growth time and the initial ratio but also on the absolute population size at time zero. In particular, it is imperative to the validation of the model that under the same conditions, growth time and initial MSC to HUVEC ratio, the final proportion of MSC to HUVEC will differ with different seeding densities. To prove that this theoretical hypothesis, which is directly derived from the

model, agrees with the de-facto behavior of the co-cultures, we studied the population dynamics of MSC and HUVEC co-cultures following low seeding densities.

MSCs were pre-stained with fluorescent membrane cell tracker DiI. Following staining, MSCs, HUVECs and their co-culture with 1:1 ratio were seeded at densities 95% lower than previous experiments, cultured for 7 days in complete EGM-2 medium and analyzed by fluorescence-activated cell sorting (FACS). As shown in Figure 13, two distinct populations (in terms of DiI labeling) can be identified when overlaying the results for unlabeled HUVECs and DiI-labeled MSCs. However, the analysis of the co-culture (Figure 13) demonstrates only one population with 56% fraction of DiI positive cells. Dividing the DiI-positive fraction of the co-culture (56%) by the DiI-positive fraction of the control (85%), a fraction of 66% MSCs was yielded in the co-culture after 7 days. Not only that this proportion of MSCs is much higher than the one previously observed for high seeding densities, it also strikingly agrees with MSC proportion anticipated by the model (71%) in Figure 14 (based on population sizes of 15,000 MSCs and 6000 HUVECs on day 7. Supporting fluorescent microscope images of the same co-cultures, after 7 days, also reveal high proportion of DiI-labeled MSCs (Figure 15). Although unlabeled cells are still apparent, HUVECs can no longer be clearly identified and distinguished from the MSCs based on the cells typical morphologies.

Figure 13. MSCs and HUVECs co-cultured under low seeding densities. FACS analysis of separately grown unstained HUVECs and DiI-labeled MSCs (overlay) (a) and a 1:1 co-culture (b). This set of data is representative out of 3 sample sets.

Figure 14. Simulation of population dynamics under low initial seeding densities. Prediction was made for 7 days of co-culture using the Lotka-Volterra model.

To identify and distinguish sub-populations within co-cultures, MSCs were stained with DiI and co-cultured with un-stained HUVECs under different conditions. The proportion of the stained or labeled cells in the population was then evaluated using flow cytometry and fluorescent microscopy. However, the applicability of this method should be validated in order to ensure that the cells in the co-culture maintain their fluorescent labeling and

that the extent of dye leakage is not significant enough to affect the results. MSCs alone were separately stained with fluorescent membrane cell trackers DiI (red) and DiO (green). Both dyes have similar affinity and chemical properties while having different emission and excitation wavelengths, which allow us to distinguish them using appropriate fluorescent analytical equipment. Following staining, MSCs stained with DiI, DiO and co-cultures of them with ratio of 1:1 were cultured for 7 days and analyzed by FACS for DiI and DiO labeling, with unstained MSCs as control. Additional control samples were also analyzed, composed of a 1:1 mixture of DiI and DiO stained MSC (grown for 7 days) that were mixed immediately prior to the analysis. As can be seen from Figure 15a, 3 distinct populations can be identified when overlaying the results for DiI-stained (with 97% cell population), DiO-stained (97%) and unstained MSC (96%). These three populations are used as indicators for the gates (quadrants) that were later used to analyze the proportion of DiI and DiO stained MSCs in the co-cultures and mixtures. As can be seen from Figure 15b, using the same gates (quadrants) identified from Figure 15a for DiI and DiO stained cell populations, the gate statistics for the co-cultured samples (43% for DiI and 50% for DiO stained cells) were quite similar to those of the mixed samples (48% for DiI and 49% for DiO stained cells) indicating a minimal amount (<5%) of dye loss or leakage. Hence, the percentage of “artifact events”, appearing as double negative or double positive, is low enough and shouldn't hinder the validity of this method for our proposed applications.

Figure 15. Validation of MSC staining by fluorescent membrane cell trackers using flow cytometry analysis. FACS results for DiI-stained, DiO-stained and unstained MSC (a) and 1:1 co-culture of DiI and DiO stained MSC as well as DiI and DiO stained MSC that were mixed at a 1:1 ratio immediately prior to the analysis (b) are presented as overlaid dot plots. FACS results are representative of three independent experiments (n=3) showing similar results and are depicted as dot plots of DiO staining vs. DiI staining (both as fluorescence intensity on a bi-exponential scale). The FACS analysis gates are identical for all cultures.

3.2.6 Effect of medium volume and seeding density on population dynamics

To evaluate the population dynamics with different seeding densities, by changing the

seeding area, in turn the seeding density of 1:1 MSC and HUVEC co-culture, the viability profile varies with time. Two different medium volumes (0.2mL and 1mL) were used for the co-culture for 7 days. The growth of co-culture over time was found to be inversely proportional to the seeding densities in both medium volumes, excluding the high density in low medium volume showing no growth over time, probably due to depletion of nutrient for large cell number (Figure 16).

Figure 16. Seeding density effect on co-culture growth. The overall average viability of cells seeded 1:1 (HUVEC-to-MSC) on plates with different culturing areas to achieve three seeding densities: LOW: 1000, MED: 1500, and HIGH: 3000 cells/cm² and grown for 7 days in 1 (a) or 0.2ml (b, n=3).

The different population dynamics due to varying seeding density demonstrate the importance of counting initial seeding density when evaluating cell-cell interaction in co-culture models, and verify the “biological suitability” of our model for such applications. The difference in subpopulation growth in the co-culture can be attributed to the paracrine effect between cells in close proximity, and highly affected by both the distance between two neighboring cells and the short half-life of chemokine secreted by the cells [68]. Similar results were shown by Hirschi’s study, in which they suggested that the EC-derived transforming growth factor beta (TGF- β) has direct impact on the recruitment and differentiation of MSCs when they were close to each other [34].

Other interesting mathematical models describing dynamic growth of MSCs and other cell types exist in the literature taking into consideration only cellular factors without much explanation on external factors such as culturing time, medium and seeding densities, let alone mutual effects among sub-populations, and are mostly restricted to 2D environment [163, 164]. To our best knowledge, this is the first time such a delicate interplay between two cell types has been described in such a comprehensive manner. This model was developed in but not limited to simple 2D environment. It remains clear that more applicable tissue engineering attempts require MSC and HUVEC co-culture to be performed on more complex 3D substrates [62, 66, 68, 69]. As such, preliminary co-culture studies on the surface of complex 3D ECM scaffold was performed with results presented in Section 3.4.2. As important as geometry of complex 3D substrate may be, other factors such as the ones addressed here also substantially influence the population dynamics of co-cultured cells. This model can serve as an insightful guideline for more

complicated 3D studies with the extension to other cell-cell or cell-scaffold interaction studies, which will definitely be beneficial for numerous engineering applications.

3.3 HUVEC attachment and growth on decellularized ECM

3.3.1 HUVEC attachment and growth on ECM slices

Previous studies have demonstrated the suitability and supportability of decellularized ECM as scaffold for cell attachment and proliferation for various cell types [14, 110]. Following decellularization, histological images from H&E staining proved the absence of cells in decellularized ECM (Figure 17a) in contrast to native tissue (Figure 17b), which is similar to results presented before by our group and others [106, 110].

Figure 17. H&E staining for acellular and native ECM vasculature. Acellular (A) and native tissue (B) (pink-red: eosin staining of acellular ECM fibers and cell cytoplasm (when applicable), dark blue: cell nuclei). Cross-sectional images were taken randomly from more than 3 ROIs for each sample slice.

For any tissue engineering application, rapid and effective integration of cells with tissue scaffold is important and one of its requisites is the process of initial adhesion and proliferation [165]. In order to optimize the seeding and culture conditions for HUVECs on ECM scaffold for the purpose of achieving vascularization with EC monolayer on ECM vasculature, and to simplify the process for large scale screening, HUVECs were

seeded and cultured on the surface of small (0.5cm^2) acellular ECM samples, cut from myocardial side of decellularized porcine left ventricle, to assess their attachment and growth over time under static culture condition. HUVECs were seeded onto the small ECM with pipette, and allowed to attach for 0, 45, 90, 135, and 180 minutes followed by a brief washing in PBS to remove any non-bounded cells from the scaffold before assessment. The attachment of HUVECs on ECM slices was assessed by both AlamarBlueTM assay and H&E staining. The percentage of attachment from AlamarBlueTM result was calculated based on known cell quantities' calibration curve and divided to the total initial seeding number. Highest degree of attachment (about 30%) was observed 45 to 90 minutes after seeding. Only 20% attachment was achieved immediately after seeding or with 180 minutes incubation. AlamarBlueTM reading after 135 minutes incubation time fell in the middle showing 26% attachment rate (Figure 18). This set of results demonstrated the crucial effect of initial seeding time for cell attachment on ECM scaffold suggesting that enough attachment time should be allowed for cells to interact and attach to scaffold, probably via integrin mediated collagen binding. Too long an attachment time however may compromise cell viability and metabolism due to complete consumption of nutrients in a limited seeding volume (used to ensure high seeding density) over time.

Figure 18. HUVEC percentage attachment on ECM slices. Best attachment efficiency of about 30% was achieved after static incubation of 45 to 90 minutes (Significant effects are indicated by asterisks.* $p < 0.05$, ** $p < 0.005$, $n=4$).

H&E staining for recellularized ECM samples after different attachment time appeared to be consistent with the AlamarBlueTM assay. Very few cells with low density were spotted in samples immediately after seeding (Figure 19a, b) and with 180 minutes incubation time (Figure 19i, j). Majority of the sample was found with no cell attached. Much more densely attached cells were observed on samples with 45 (Figure 19c, d) and 90 minutes (Figure 19e, f) incubation time. In some parts of the sample, almost confluent monolayers were formed. Samples with 135 minutes incubation time (Figure 19e, f) exhibited moderate cell attachment, which also agreed with the AlamarBlueTM results (Figure 18).

Figure 19. H&E images for HUVEC attachment to ECM utilizing various attachment times. After static incubation for 0 minutes (A: 4x, B: 10x), 45 minutes (C: 4x, D: 10x), 90 minutes (E: 4x, F: 10x), 135 minutes (G: 4x, H: 10x), and 180 minutes (I: 4x, J: 10x) (pink: acellular ECM fiber, dark blue: cell nuclei). Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

After initial attachment test, the next step was to find the optimal seeding number to achieve long-term growth of HUVECs on ECM slices. From the result of previous experiment, different seeding numbers (100K, 500K, 1M, and 2M cells) were tried with the optimized 90 minute attachment time (Figure 18). One day after attachment, as expected, higher FI was found by AlamarBlueTM assay with higher initial seeding number, as shown in Figure 20. However, during subsequent 7 days of culture, the number of viable cells kept dropping and reached steady state on day 5 with no growth observed for all seeding numbers. These HUVEC growth curves on ECM with different seeding numbers appeared to be similar and merging toward the low end after 7 days of culture. This decreasing trend could result from the use of a relatively high seeding density at the initial time point, which is higher than the actual density that the scaffold's lumen can actually support. Multiple possible factors may affect this observation and other studies on HUVECs. On all occasions, the initial seeding density was far above the steady-state density. Studies of MSCs were conducted by some of my colleagues to further characterize this phenomena. Applying the calibration curve for ECM (shown in appendix), estimation of cell density after 7 days with initial seeding number of 2M on ECM surface can be made $((8.4 \pm 1.7) \times 10^4 \text{ cells/cm}^2)$, which is in the same order of magnitude as that measured for HUVEC-GFP on gelatin coated ECM vasculature after long term dynamic culture $((2.5 \pm 0.6) \times 10^4 \text{ cells/cm}^2)$, Figure 44a), as well as cell density of native porcine vasculature $((5 \pm 0.7) \times 10^4 \text{ cells/cm}^2)$, Figure 44b), suggesting the steady-state density of HUVEC on ECM surface. Another assumption of this decline in cell number is the inadequate surface protein available for cell attachment and growth. To verify this assumption, effect of ECM treatment with different protein solutions on both

HUVECs and MSCs was investigated, and discussed in Section 3.4.

Figure 20. HUVEC growth on ECM scaffold. AlamarBlue™ reading of HUVECs with different initial seeding numbers over 7 days (n=3).

3.3.2 Cell morphology on ECM

Confocal microscopy imaging showed that HUVECs attached and formed self-alignment on ECM surface 7 days after seeding (Figure 21a) while MSCs were relatively less arranged but more densely packed against each other (Figure 21b). When co-cultured together (Figure 21c), DiI-labeled HUVECs were seen even more uniformly arranged, better spread and aligned than singly cultured ones. HUVECs cultured on tissue culture dish do not exhibit such cell alignment and morphology. They are randomly distributed with their typical cobblestone shape (Figure 22d), while MSCs appear to be highly elongated and aligned on TCP when reaching confluence under EGM-2 medium (Figure 22e). The high density might have “forced” MSCs to form the alignment on TCP, which appeared to be similar for them on ECM, HUVECs on the other hand, did not only change their morphology on ECM, but also aligned to the ECM fibers by themselves.

Figure 21. Morphological properties of HUVEC, MSC, and their co-culture. DiI (red) and DAPI (blue) staining for HUVEC (a) and MSC (b), and 1:1 co-culture with labeled HUVEC and unstained MSC (c) on ECM using confocal microscope; HUVECs on TCP with typical cobblestone morphology (d) and MSC on TCP under EGM-2 medium culture (e) with phase contrast light microscope. Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

With the understanding of population dynamics of the two species in co-culture model derived from 2-D culture environment, cell co-cultures on 3-D acellular ECM scaffold were also attempted as cells behaves differently on more complex 3-D environment

compared to 2-D culture plate [92]. For practical reasons and to allow efficient and broad screening of a variety of factors, the cells in this case were seeded onto the surface of small ECM tissue (0.5cm^2) derived from acellular porcine left ventricle, and results will serve as valuable reference when we moved on to studies of cells on much more complex ECM vasculature scaffold.

Scanning electron microscopy further showed the morphologies of co-cultured HUVECs and MSCs on the surface of ECM. Compared with acellular ECM (Figure 22a), relatively dense cells were founds firmly attached to the re-seeded ECM with elongated morphology and a certain degree of cell alignment (Figure 22b).

In the context of this study, the cell morphological properties can be influenced by a few factors including cell density, HUVEC-MSC interaction, as well as structural properties of the scaffold. Higher cell density indicates higher degree of cell-cell interaction, which affects the way individual cell spread and align. Cell mobility is discouraged with inhibited actin polymerization and flow at the cell-cell contact [166]. As cells tried to migrate away from the group, they were forced to move along the structural pathway provided by fibrous layout of ECM, which explains why HUVECs form alignment on ECM surface but not on TCP. As same initial seeding densities were used for singly and co-cultured cells, cell proliferation study on ECM surface unveiled similar cell densities for the 3 groups after 7 days of culture (Figure 25a). Hence higher degree of HUVEC alignment in co-culture compared with single culture on ECM can be attributed to the interaction with MSCs, which were demonstrated with potential to facilitate formation of

tubular structures of endothelial cells [62, 63]. Another influencing factor for cell morphology is cell-scaffold interaction. Numerous studies have demonstrated the effects of structural properties of scaffold, such as porosity, spatial architecture, stiffness, and signaling factors, on cell attachment, morphology and proliferation [167-169]. On 2D TCP, though pre-coating with gelatin was performed in attempt to minimize the difference of chemical clue compared to 3D scaffold, cell attachment and spreading were restricted to flat surface and its stiffness is primarily contributed by the rigid culture plate underneath the gelatin layer. In contrast, on 3D ECM scaffold, cell morphology was greatly affected by the porosity, stiffness, and unique collagen fiber alignment serving as “guiding rails” for cells to anchor, spread and migrate. Further studies are required to confirm and understand how these properties of ECM scaffold affect the morphological properties of MSCs, HUVECs and their co-cultures.

Figure 22. SEM images for acellular and re-seeded ECM. Acellular (a) and ECM seeded with HUVEC and MSC co-culture (b). Representative cells morphologies on ECM surface were marked with dashed circles. Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

3.4 Protein Treatment for Acellular ECM

3.4.1 Effect of treatment on attachment and growth of HUVEC and MSC

The availability of attachment proteins present in native ECM network is another important factor facilitating the initial cell attachment and growth on the scaffold. These could probably affect the limited space restrictions by allowing higher cell densities at the reseeded site. The successful retaining of some ECM adhesion proteins (at least in part) within the decellularized matrix was previously reported for collagen type I and IV [14, 110]. However, the sufficiency of those for cell attachment and proliferation and the effect of additional treatments on the cellular behavior when reseeded on ECM are still not clear. Nonetheless, previous literature has shown the positive effects of protein treatment for scaffold material in tissue engineering applications [84, 85, 89, 97, 170]. Alternative approaches to better “prepare” the ECM surface for endothelialization includes co-culture model [62, 63]. Hence, to improve cell survival and proliferation on acellular ECM scaffold, two parallel approaches can be explored. As already shown in static 2D model, MSC promotes HUVEC growth under controlled co-culture conditions (Section 3.2). The scaling up to 3D under static culture conditions (Section 3.6) demonstrated the need for dynamic cultivation to increase cell densities, lumen coverage and supportability. Thus these two approaches (protein treatment and co-culture as discussed in Sections 3.4 and 3.6 respectively) were carried out under both static and dynamic culture conditions.

Even though our previous studies demonstrated the preservation of collagen type I, III, and fibronectin on decellularized ECM scaffold with comparable quantities to native

tissue [110], the relatively low attachment efficiency and proliferation of HUVECs suggested that additional protein molecules might be required to enhance those qualities on the thick decellularized ECM scaffold [170]. It was previously demonstrated that ECM proteins, with various types of collagen, laminin, and fibronectin being the most common, have substantial effect on EC attachment, morphology, as well as neovascularization process [171-175]. It is also a common practice to coat tissue culture plate with gelatin prior to HUVECs plating to enhance their attachment and growth.

AlamarBlueTM assay was used to assess the effect of protein treatment on ECM using three representative treatments: laminin, fibronectin and gelatin. HUVEC and MSC attachment and proliferation were compared over time on treated and untreated ECM scaffolds. Statistical significance was shown for improvement of HUVEC proliferation on all treated ECM compared to untreated ones, with fibronectin and gelatin treated samples demonstrating the best results ($p < 0.001$, Figure 23a). Over the 21 days culture, MSCs showed improved growth ($p < 0.005$) only on fibronectin treated ECM (Figure 23b). This set of results indicates that one or two types of protein coatings may assist in increasing the initial attachment and subsequently the proliferation of MSCs and HUVECs over time, probably by allowing better anchoring sites on the ECM. Since a certain degree of ECM protein loss during any decellularization process is expected [98, 176], it is reasonable that some reversal of this damage can be achieved through these biochemical modifications.

Figure 23. Proliferation over 21 days on biochemically modified ECM scaffold. Growth curve for HUVEC (a) and MSC (b). Cell viability is presented as Fluorescent Intensity (FI) from AlamarBlue™ assay (n=3).

Other than the beneficial effect on cell attachment and growth on ECM surface, collagen is also a substantial factor for mechanical property of the ECM structure. This is critical for blood vessels as they are required to withstand stress under physiological conditions [177, 178]. The mechanical properties such as strength and elasticity of acellular and re-cellularized ECM were investigated in another study by our group [136].

3.4.2 Gelatin staining with CBP-TAMRA

In order to verify that gelatin coating remains on ECM for longer time frames relevant for future static and dynamic experiments, gelatin staining with collagen binding protein (CBP) conjugated with TAMRA dye was used to stain the gelatin coating. Prior to staining, an optimized dilution factor was determined by plotting a titration curve of fluorescent signal according to a series of dilution factors applied. This step was done to ensure efficient staining and marking of the gelatin solution (prior to coating) while avoiding leakage of unbound dye to neighboring pcECM matrix, which may result in false positive signals. A dilution factor of 1:32 of CBP-TAMRA in 0.2% gelatin solution was shown to be optimal (i.e. an equivalent point) for both bulk stained gelatin solutions (measured through fluorescence intensity signals of filled 96 well plates, Figure 24a) and surface coating staining (measured on the well bottom after solution removal and PBS wash, Figure 24b).

Figure 24. CBP-TAMRA staining for gelatin coating on ECM. Bulk solution titration curve for CBP-TAMRA dilution factor in gelatin solution (a), and surface coating titration curve following overnight incubation with gelatin solution, removal of bulk solution and PBS washes (b). Confocal image for control CBP (non-conjugated) blocked ECM stained with CBP-TAMRA (c), and with double the gain (d). Confocal image for CBP (non-conjugated) blocked ECM which was coated with gelatin containing CBP-TAMRA solution at 1 day (e) and 17 days (f) following gelatin treatment. Gain and acquisition parameters for c, e and f were kept identical for comparison. Images are representative out of three regions of interest taken from each culture from three independent experiments ($n = 3$).

Since CBP-TAMRA is non-specific to gelatin, non-conjugated CBP was used to block the ECM prior to gelatin treatment and CBP-TAMRA staining. The fact that CBP blocked pcECM samples stained with CBP-TAMRA showed no signal (Figure 24c) and only background noise with double the gain (Figure 24d) suggested effective blocking of non-conjugated CBP. Thus all signals obtained for CBP pre-blocked and gelatin treated pcECMs would be expected to arise from gelatin presence only, particularly as equivalent point dilutions were used. Indeed, one day after gelatin treatment and staining, bright TAMRA signal was observed from treated pcECMs under confocal microscope (Figure

24e). Slightly weaker signal was observed even 17 days post treatment (Figure 24f), suggesting the gelatin treatment has a stable attachment to our acellular pcECM, and can be used for long term cell studies.

3.4.3 Effect of seeding sequence on co-culture

Another approach to improve HUVEC attachment and proliferation on the pcECM employed the use of co-cultures. In this approach MSCs were used as a supporting cell type with the aim of mediating HUVEC interaction with the ECM, through possible remodeling of the ECM matrix and production of natural components that may be required by the HUVECs to better attach and proliferate. Other reasons for MSC usage were the possible usage of these cells as pericytes [62] to support blood vessel maturation in any future applications, in addition to their ‘standalone’ regenerative capacity and immune-tolerance properties.

Two co-culture approaches were applied, simultaneous and sequential co-culture to evaluate the importance of seeding order on population growth over time. No variations were found in the population sizes between separately and simultaneously co-cultured cells ($p > 0.05$) grown on complex 3D pcECM scaffolds. In both cases, population sizes decreased until day 4, from which point they remained constant (Figure 25a). However, in the sequential co-culture, where HUVECs were seeded on top of MSCs after 7 days, significant growth ($p < 0.01$) of more than two fold was observed on the following 7 days (Figure 25b). This improvement demonstrates that on such highly geometrically and

chemically complex material, the order of seeding is also of high significance. Whereas some cell survival was observed with separately cultured or simultaneously co-cultured cells, only a sequential co-culture of MSCs topped up with HUVECs exhibited significant growth. These results may suggest that on such scaffolds, MSCs not only support HUVEC growth but are also required to prepare the physiological niche before HUVEC establishment, probably by rearranging the ECM and providing the HUVECs with better “docking sites”. Previous studies have reported the application of simultaneous co-culture approach with endothelial and mural precursor cell in mice model, and demonstrated the importance of EC-mural cell interaction [34]. Since no surrounding mural cells are available in the neighboring tissue to be recruited in our *in vitro* model, pre-formed MSC layer or MSC-derived supportive cells were critical for long-term survival and stability of EC [179]. The supporting function of MSC on HUVEC in co-culture also strongly agrees with the result set obtained from Lotka-Volterra model on 2D environment. Indeed, MSC simultaneously co-cultured with HUVEC in EGM-2 medium, exhibited strong enhancing “other effect” on HUVECs and prohibited the “self-effect” on themselves through probably a paracrine mechanism (Figure 7). However, the fact that only co-culture in a sequential mode exhibited significant effect on 3D ECM implies that on scaffold with such complex fiber morphologies, ECM remodeling induced by MSC in addition to paracrine effect was necessary to prepare the environment prior to EC engraftment.

Figure 25. Proliferation of single and co-cultured cells. Simultaneous co-culture of HUVEC and MSC on ECM scaffold, compared to separately cultured cells (a) ($p > 0.05$); Simultaneous co-culture compared with sequential one grown separately for 7 days (b) ($p < 0.01$, $n=3$).

3.4.4 Effect of treatment on co-culture

With the knowledge that protein treatment with coating proteins significantly enhanced

the growth of HUVEC and MSC on ECM surface, we attempted this method on co-culture models. For HUVEC and MSC co-cultures, the proliferation curve was similar to HUVEC alone, demonstrating results on fibronectin and gelatin coated ECM (Figure 26a). Even though sequential co-culture also led to improved cell attachment and growth compared to simultaneous co-culture and single culture (Figure 25a, b) from day 7 onward (by ANOVA test), no additional improvement was observed with ECM treatment (Figure 26b), indicating that preparing ECM by pre-seeding with MSCs and by coating with protein solutions might serve the same function in terms of HUVECs' support, and hence combining these two approaches does not give any further improvement. Based on this conclusion, we decided to use either sequential co-culture or treatment with gelatin/fibronectin approach to enhance the attachment and growth of HUVECs when conducting dynamic studies on thick vasculature containing ECM scaffold (section 3.6 below).

Figure 26. Cell proliferation on ECM surface with protein treatment. Growth curve over 21 days on treated ECM for HUVEC and MSC simultaneous (a) and sequential (b) co-culture (n=3).

3.5 HUVEC-GFP and MSC-mTomato expression and culture

To effectively label the cells with long term stability in co-culture system for tracking and monitoring purposes, transfection studies with Lenti-viraTM system were carried out. After transfection, growth curves were generated for stabilized transfected HUVEC-GFP cells (Figure 27b) using AlamarBlueTM assay to compare their proliferation rate with untransfected HUVECs (Figure 27a). The estimated cell number in this case was derived from FI of AlamarBlueTM reading using standard calibration curve. Growth fold serves as the estimated population size, or viability of cells normalized by the value on day 1. The

growth rate of HUVEC-GFP (0.5839) gave a doubling time of 1.2 days ($\ln(2)/0.5839$), which is 41% shorter than un-transfected HUVECs ($\ln(2)/0.3446 = 2$ days). Though cell proliferation varies with passage, culturing medium and condition, the observed doubling time was comparable with ones in the literature [180-182]. It is possible that during the transfection process, the viral plasmids were incorporated into cell genomic DNA sequence randomly, which might have induced a more rapid division. Since the transfected cells were to be used strictly *in vitro* only, no detailed examination on the viral plasmid incorporation was carried out. However, morphological study on HUVEC-GFP did show their typical cobblestone shape (Figure 28a) on tissue culture dish, and elongated spindle shape on ECM (Figure 28b, c), which appeared to be the same as un-transfected ones (Figure 21a).

Figure 27. Proliferation curve for HUVEC. Un-transfected HUVECs (a) and GFP transfected HUVECs (b, n=3).

After Lenti-viral transfection with GFP carrying virus for HUVECs and mTomato carrying virus for MSCs, cells were seeded on TCP as well as ECM scaffold and cultured in standard static condition over time. The expression efficiency and stability of cells were assessed using fluorescent microscope and confocal microscope after 15 days. Both transfected HUVECs (Figure 28a) and MSCs (Figure 29a, b) expressed GFP and mTomato respectively with more than 90% efficiency on TCP. The expression efficiency and fluorescent intensity did not decrease compared to the ones on day one, indicating

that both cell types were efficiently transfected with stable expression over time. HUVEC-GFP cells grown on ECM after 15 days, like un-transfected ones, formed self-alignment and exhibited elongated morphology (Figure 28b, c). MSC-mTomato was further counterstained with DAPI and observed using confocal microscopy after 60 days of culture (Figure 29c). Though only HUVEC-GFP was actively used in studies described in this thesis as an effective way for cell observation (especially in thick tissue) and subgroup distinguishment in co-culture, successful transfection of both HUVEC and MSC to express signals in two distinct wavelengths allows much higher flexibility and more direct characterization over long term culture in future studies.

Figure 28. Expression of HUVEC-GFP (green). On TCP (a), and on ECM surface (b, c) after 15 days of culture. Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

Figure 29. Expression of MSC-mTomato. MSC-mTomato (red) on TCP (a, b), and on ECM surface counterstained with DAPI (blue) staining after 60 days of culture (c). Images are representative out of three regions of interest taken from each culture from three independent experiments ($n = 3$).

3.6 Endothelialization of acellular ECM

3.6.1 Static culture of HUVEC on ECM vasculature

Our ultimate goal of growing HUVECs and other cell types on ECM is to achieve vascularization and provide medium supply to most of the cardiac patch tissue construct. Previous experiments demonstrated that decellularized porcine ECM, with their protein compositions and mechanical structures, can support the attachment and initial survival of HUVECs. To test the suitability of the preserved vasculature [14, 111] within the thick ECM, in supporting the attachment and growth of HUVECs, cells were seeded onto the inner vasculature walls by perfusion. AlamarBlueTM assay was used to monitor the proliferation and viability of cells over 5 days. The overall size of the population increased over the first 2 days but decreased afterward reaching steady state levels (Figure 30).

The cross-section of ECM vasculature re-seeded with HUVECs was also studied using H&E (Figure 31) and Masson's Trichrome (Figure 32) histological stains, following 7 day culture under static culture conditions with EGM-2 medium. Acellular non-reseeded ECM scaffolds served as control. The expression of GFP was further confirmed with confocal microscopy and DAPI counterstaining (Figure 33). As observed from all three staining methods, after seeding, HUVECs were confined and attached forming partial coverage of the ECM vascular lumens. This partial coverage demonstrated the maximal support available by the ECM vasculature under these culture conditions (as depicted by above mentioned representative figures). Though this set of static experiments showed promising results, the degree of coverage of vasculature and cell density was far from

desired. Effective nutrient and waste exchange and shear stress through medium perfusion with dynamic culturing system might be advantageous in maintaining cell viability and enhancing endothelialization [70, 126, 183, 184].

Figure 30. HUVEC growth on ECM vasculature under static culture conditions. AlamarBlueTM reading was taken for 5 days of culture (n=3).

Figure 31. H&E staining for static study on ECM vasculature. Acellular ECM (a) and ECM vasculature statically seeded with HUVECs (b) (pink: acellular ECM fiber, dark blue: cell nuclei). Arrows point to EC location within the artery lumen. Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

Figure 32. Masson's Trichrome staining for static study on ECM vasculature. Acellular ECM (a) and ECM vasculature statically seeded with HUVECs (b) (blue: acellular ECM fiber, dark brown: cytoplasm). Arrows point to EC location within the artery lumen. Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

Figure 33. Confocal imaging for cross-section of ECM vasculature seeded with HUVEC-GFP. GFP (green) and DAPI (blue). Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

3.6.2 HUVEC attachment and growth using perfusion bioreactor

Under physiological conditions, ECs require and constantly experience shear stress, which is essential for their proliferation and functionality [184-186]. All these factors might have contributed to the fact that under static culture conditions lumen coverage achieved was only partial concomitantly with decreased total HUVEC viability reaching a stabilized steady state without growing beyond their initial attachment densities (Section 3.3.1). Hence, we speculated that in order to achieve confluence of HUVECs on ECM, dynamic culture condition may be required.

For dynamic study using bioreactor system, HUVECs injected into the vasculature embedded in bulk ECM slab were allowed to attach statically for the initial 2 hours before medium perfusion was started. From the proliferation curve over 21 days of culture assessed using AlamarBlue™ assay, similar trend to the static cultures was observed

whereby the overall cell viability gradually decreased until a stabilized steady state condition was reached (Figure 34).

Figure 34. HUVEC growth on ECM vasculature under dynamic culture condition. AlamarBlue™ reading was taken for 21 days of culture (n=3).

As is also suggested by our mathematical model, the initial seeding density and the culture media volume both play a critical role in determining the proliferative profile of reseeded cells (either alone or in co-cultures). These two key parameters, identified in a simple tissue culture plate experiment, may also influence the growth profile of reseeded cells under dynamic culture conditions (Figure 16). Hence, the continuous decrease in the early days of the dynamic HUVECs-ECM cultures might be related to a biological limit of matrix support ability in terms of maximal cell density / capacity for the endothelial cells due to the limited surface area of the vascular network. This phenomenon, which was also mathematically modeled for other cell types in our lab (data not shown), may be even more striking here, as HUVECs do not tend to penetrate into the tissue mass and are rather forming only monolayer coatings of the vascular conduits. This ‘behavior’ is an

expected one due to their biological role and is also apparent from our histological observations (Figure 35). Alternatively, the dilution of nutrients and signaling molecules, which is inherent to this dynamic cultivation system, may also play a role in determining the HUVECs growth profile. Nevertheless, several seeding densities and culture media dilutions were evaluated in our lab and they have all led to a similar growth profile under dynamic culture conditions of HUVEC monocultures.

Interestingly, this representative dynamic growth profile (Figure 35) was considerably different from that measured under static culture conditions (Figure 30) where fluctuations (rather than a sharp decrease) of cell quantities around a steady state value were more readily observed (Figure 30). It should be however noted that *in vivo* only 0.01% of the endothelial cells divide while the rest are usually quiescent [187, 188]. This is at least partially modulated through the Notch ligand pathway, which activation can stimulate or inhibit proliferation by modulating cell cycle regulation in a cell type-specific and context-dependent manner [189]. It may be that the decrease in cell quantities to a stabilized steady state under dynamic culture conditions observed through AlamarBlue™ measurements may reflect a delicate homeostasis which is not directly related to cell numbers. Furthermore, such homeostasis may be governed by the combination of ECM-affected complex signaling pathways and dynamic culturing dependent stimulations that in turn regulate endothelial cell proliferation. Currently, the contribution of ECM signals under physiological mimicking conditions to endothelial cell proliferation is still poorly studied and understood. We therefore suggest our dynamic culture system can be further utilized as a model system to study this interesting interplay *in vitro*.

In addition, it is nowadays recognized that, consistent with the ECM many roles in affecting cell biology [190], multiple regulatory mechanisms exist to ensure its proper function and dynamics within the correct physiological range. It has thus been suggested that disruption to such control mechanisms deregulates and disorganizes the ECM leading to abnormal behaviors of cells and ultimately failure of organ homeostasis and function [191]. In that respect, though the decellularization process is expected to cause some extent of damage to the ECM proteins [192], the pcECM matrix isolated and characterized in our lab seemed to maintain not only its ultra-structural biomechanical properties [14] but also its homeostatic effect on reseeded endothelialized cells, at least when cultivated under proper dynamic culture conditions.

Besides our previous attempts [31], other groups have demonstrated the benefits of applying various types of bioreactor systems to enhance the development of functional regenerated tissue including engineered vascular construct [116, 193, 194]. One study done by Niklason and group managed to generate cultured tissue with histologic appearance closer to native arteries with a pulsatile perfusion bioreactor system [195]. Seliktar et. Al. also revealed the advanced blood vessel functionality in term of mechanical strength and histological distribution when dynamic mechanical conditioning was in force [128]. Another bioreactor model was developed to provide a sterile dynamic environment simulating the physiological condition for both cell seeding and follow-up conditioning of tissue engineered blood vessel. In our study, developed from a commercially available model, a customized bioreactor system was engaged for

homogenous cell seeding and long term cultivation of the regenerated tissue. The customized porous fitting chamber holds the thick ECM patch firmly in place while allowing medium to perfuse through the vasculature embedded within. The entire bioreactor chamber was made of transparent and autoclavable polycarbonate material for easy observation and repeatable sterilization. The tunable rotational and perfusion functions allows adjustable mode of initial cell attachment and proliferation. As a result, a larger number of cells with more homogenous coverage on the vascular lumen were achieved with the assistant of this bioreactor system compared to static culturing condition.

Though gradual decrease in cell number was observed with AlamarBlueTM assay (Figure 30) till a stable condition was reached due to HUVEC's own self-modulating mechanism through Notch ligand pathway and other regulation mechanisms to reach homeostasis, such homeostasis was affected by external stimulations, such as culturing environment. Under both static and dynamic culturing with bioreactor system, two equally stable yet different homeostatic states were reached, at which the number of HUVECs remains relatively constant on ECM scaffold. The dynamic stimulation shifted the original growth curve and regulated cells toward a new balance state. Cross-sectional view of HUVEC seeded vasculature using H&E staining (Figure 35a) and longitudinal view with pre-staining HUVECs (Figure 35b) both showed much denser cell attachment at the end of dynamic study compared to static culture without medium perfusion (Figure 31, Figure 32). However, full coverage of the lumen with confluent mono layer of cells was yet to be achieved. Previous studies showed that both co-culture of HUVEC and MSC with

sequential approach and protein treatment for ECM scaffold lead to better proliferation of cells. Hence, both of the methods were tried on ECM vasculature under dynamic culturing condition to further improve the endothelialization of ECM slab.

Figure 35. H&E staining of dynamic study for HUVEC seeded ECM vasculature. Cross-sectional view (a) (pink: acellular ECM fiber, dark blue: cell nuclei), and live longitudinal optical-sectioning view with DiI pre-staining using confocal microscopy (b). Images are representative out of three regions of interest taken from each culture from three independent experiments ($n = 3$).

Before co-culture experiment was carried out, MSCs alone were seeded on the ECM vasculature under the same condition as HUVEC single culture for a better understating of their behavior. MSCs were seeded and cultured with perfusion in the bioreactor system under the same condition as HUVECs for 11 days. Their proliferation curve (Figure 36) was different from HUVECs, but more similar to their fluctuating behavior in the static studies, suggesting that here too the combined effect of matrix signaling and dynamic culture conditions on cell proliferation is cell type- dependent. One major parameter that may be responsible to this different behavior is shear stress that under physiological conditions, affects normally only ECs while MSCs and their derivatives are embedded inside the tissue away from blood vessels. Confocal laser scanning microscopy also showed that a dense layer of DiI pre-stained MSCs remained after dynamic culture. Even though no obvious morphological arrangement was shown, the coexistence of cells and neighboring scaffold expressing strong green autofluorescent signal verified their distribution along the ECM vasculature (Figure 37).

Figure 36. MSC growth on ECM vasculature under dynamic culture condition. AlamarBlue™ reading was taken for 11 days of culture (n=3).

Figure 37. Longitudinal view with DiI pre-stained MSCs using confocal microscopy. Image was taken after 14 days of dynamic culture. Green channel indicates auto-fluorescence from surrounding ECM scaffold. Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

3.6.3 Dynamic co-culture of HUVEC and MSC on vasculature

With the understanding of the behavior for both HUVEC and MSC over time on ECM vasculature under perfusion, we applied the knowledge gained from co-culture study under static culture condition to perform sequential seeding of HUVEC 6 days after MSC. As clearly shown in the AlamarBlueTM proliferation curve over 21 days of dynamic co-culture (Figure 38), the overall viability of cells (MSCs only) dropped at the beginning, followed by a sharp increase with additional HUVEC seeding, concomitant with stabilization of the overall population size with time around a steady state value.

Figure 38. HUVEC-MSC co-culture growth on ECM vasculature under dynamic culture condition. MSCs were seeded 6 days before HUVECs. AlamarBlueTM reading was taken for 21 days of culture (n=3).

After dynamically culturing the re-seeded ECM vasculature with HUVECs after MSCs seeding, cross-sectional images of the vasculature were acquired using Masson's Trichrome staining at the end point of 21 days culture. A nice monolayer of cells was observed lining the major lumen of ECM with complete coverage of cells (Figure 39a), which shows substantial improvement from static experiment (Figure 31 and Figure 32), and better and more uniform coverage than single dynamic culture (Figure 35). Similar coverage of cells was also found on medium sized (Figure 39b) and small sized (Figure 39c) lumens. This complete confluent mono-layer of endothelial cells resembles very much to the healthy and functional blood vessels [76].

Our group has previously demonstrated the advantage of applying dynamic culturing environment over static culturing condition using a similar approach by cultivating HUVECs on porcine small diameter arterial ECM. Even though no significant difference

in cell number was observed between static and dynamic culturing condition, HUVECs exhibited “healthier” morphological properties, i.e. more homogeneous morphology and uniform coverage on the artery surface [31]. This phenomenon verified the effect of shear stress on EC morphology and the process of endothelialization, which were already proven in literatures [183-185]. In our model, more importantly, since the vasculature was embedded within the relatively thick ECM slab, sufficient medium and waste exchange for the cells seeded on the vasculature wall are critical for cell survival and vascular formation. In addition to shear stress, perfusion bioreactor also provides sufficient medium supply to the cells during cultivation in our case. Hence significant higher cell number as well as better coverage and morphological properties were achieved using bioreactor system.

The absence of MSCs at the end point in co-culture was verified by specific staining on entire population lining the vasculature with specific EC marker, CD31. Confocal imaging further revealed the coexistence of CD31 (Figure 400a), GFP (Figure 40b), which was inheritably bound and expressed by HUVECs, and DAPI (Figure 40c), which stained the entire cellular population. The overlay image of all 3 channels indicated the expression of CD31 of all cells lining the vasculature after dynamically cultured for 21 days (Figure 40d).

In this study with co-culture approach, MSCs were seeded prior to HUVECs to “prepare” the scaffold for better HUVEC attachment and growth. However, given the physiological functions of endothelial cells and anatomical properties of tunica intima, the innermost

layer of arteries and veins, no MSCs should be present on the wall of the final functional blood vessels in regenerated cardiac patch. As suggested in the model derived from population dynamic studies on 2D tissue culture dish, after 6 days of culture, the population of MSCs in the co-culture was dominated by HUVECs, and the number of MSCs decreased exponentially over time when co-cultured with HUVECs. Other literatures also suggested that MSCs, being co-cultured with endothelial cells, or in vascular endothelial growth factor (VEGF) containing medium, could be differentiated into endothelial like cells [71, 196] or supporting mural cells [34, 179] eventually. It is therefore hypothesized here that this sequential seeding strategy may be beneficial in MSC selectively preparing the ground for re-endothelialization of the thick engineered cardiac construct while subsequently getting either trans-differentiated toward endothelial phenotype or being selectively removed out of the system. Similar results were demonstrated by another group showing the presence of MSCs in co-culture model with EC enhanced the formation and stability of the blood vessels, and were critical for long-term blood vessel creation [179].

Figure 39. Masson's Trichrome staining for cross-sections of ECM vasculature sequentially reseeded with MSCs followed by HUVECs with a 7 day interval under dynamic culture condition. Image was taken after 21 days of culture. Major lumen (a), medium lumen (b), small lumen (c), and negative control (d, blue: acellular ECM fiber, dark brown: cytoplasm). Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

Figure 40. Confocal imaging for cross section of ECM vasculature sequentially re-seeded with HUVECs and MSCs under dynamic culture condition. With CD31 staining (red, a), HUVEC-GFP (green, b), DAPI staining (blue, c), and merged picture of 3 channels (d). Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

3.6.4 Effect of protein treatment on vascularization

Our evaluation of the different effect of various protein treatment to the ECM samples indicated that gelatin treatment significantly enhanced the attachment and proliferation of HUVEC compared to untreated scaffold under static conditions. For dynamic study, ECM was treated by injecting gelatin solution into the vasculature and perfusing through the entire tissue slab. Proliferation curve over 12 days of culture exhibited initial decrease (as expected from previous dynamic studies of HUVECs) followed by an unexpected growth

(Figure 41, after day 8). This suggests that the addition of gelatin which is a hydrogel of disrupted ECM collagen may change the homeostatic effect induced by the isolated ECM through alternation of endothelial cell proliferation, even under dynamic culture conditions. Same as co-culture experiment, a full layer of cells was observed lining the major blood vessels from Masson's Trichrome staining (Figure 42a, b). However, for some medium sized lumen, the cellular layer appeared to be thicker and denser with gelatin coating (Figure 43b), which resembled the native porcine blood vessel (Figure 43a). The longitudinal view of a very dense layer of GFP expressing HUVECs covering the protein rich ECM vasculature wall was shown in tiled confocal images (Figure 44a) with branching toward the end, displaying dense 'cobblestone' morphology of ECs on the vasculature lumens.

Figure 41. HUVEC growth on gelatin treated ECM vasculature under dynamic culture condition. AlamarBlue™ reading was taken for 12 days of culture (n=3).

Figure 42. Masson's Trichrome staining for cross-section of gelatin treated ECM vasculature seeded with HUVECs under dynamic culture condition. With small (a), and large magnification (b) (blue: acellular ECM fiber, dark brown: cytoplasm). Arrows point to EC layer location within the artery lumen. Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

Figure 43. Masson's Trichrome staining for cross-sections of native porcine and gelatin treated ECM vasculature. Native (a) and treated ECM vasculature seeded with HUVECs and MSCs under dynamic culture condition (b) (blue: acellular ECM fiber, dark brown: cytoplasm). Arrows point to EC layer location within the artery lumen. Images are representative out of three regions of interest taken from each culture from three independent experiments (n = 3).

Figure 44. Confocal image for longitudinal view of vasculature. Gelatin treated ECM vasculature seeded with HUVEC under dynamic culture condition. GFP (green) and blood vessel ECM auto-fluorescent signal (red) (a); Native porcine vasculature with Hoechst staining (blue) (b). Images are representative out of three independent experiments (n = 3).

Four areas were randomly chosen in each picture (representative pictures are presented herein) and cell number in each ROI were counted to calculate the average cell density within the vasculature lumens. Same analysis was done on longitudinal images of native porcine vasculature (freshly harvested from local slaughter house) with Hoechst staining

(Figure 44b). The obtained HUVEC-GFP cell density on gelatin coated pcECM vasculature wall was approximately $(2.5 \pm 0.6) \times 10^4$ cells/cm², which was smaller but in the same order of magnitude as that measured for native vasculature $(5 \pm 0.7) \times 10^4$ cells/cm², which indicates likely resemblance of our regenerated vasculature to native blood vessels, yet additional studies need to be carried out to further confirm this statement. Porcine native blood vessels was used here as a valid reference for comparison as porcine cardiovascular system shares similar anatomical and physiological properties as humans and were the ideal positive control for the porcine isolated ECM scaffold. The cell density achieved here, which is comparable to the native condition and is also supported by previous characterization of HUVEC surface densities [197], verifies the applicability of the pcECM as a model for future achievement of functional regenerated vascular infrastructure in this or similar ECM based thick constructs.

As suggested by our preliminary studies on static tissue culture and supported by previous literatures, ECM attachment proteins are required for EC integration and growth on ECM, as well as vascular development [198, 199]. The monolayer of EC covering the vasculature lumen is critical to functional blood vessel by serving as a mechanical barrier, blood cell traffic mediator, and vascular remodeler [200]. Furthermore, pathological conditions such as atherosclerosis can arise from the loss of integrity of endothelium monolayer [201]. We have demonstrated the improvement of cell coverage on the ECM lumen compared with static results with the assistance of dynamic perfusion using bioreactor system. However, fully and evenly covered ECM vasculatures, which resemble native blood vessels with monolayer of cells, were achieved only when MSCs were

applied in a co-culture model or additional protein treatment was implemented. This phenomenon is in accordance to the set of results obtained from simplified static studies on small (0.5cm^2) ECM scaffold indicating the suitability of these tissues as appropriate scaffold for screening purposes in tissue cardiovascular engineering applications. The fact that similar results were obtained from both dynamic approaches: sequential co-culture of MSC and HUVEC, and protein treatment of ECM, indicated the supporting role of MSC in EC attachment and growth. Even though MSC-EC co-cultures have been reported for tissue regeneration applications [34], sometimes with other cell types like cardiomyocyte, SMC, and fibroblast [55, 59, 202, 203], the environment and mutual effect on population dynamics have never been comprehensively analyzed, let alone a model for quantitative prediction. ECM scaffolds have been used [109] with limited regeneration capacity and suffering from a lack of vascular system for long-term implant survival and prevention of coagulation. This is, to the best of our knowledge, the first time the importance of co-culture sequence was analyzed in detail, and the co-culture model was applied in a complex 3-D thick ECM scaffold.

Chapter 4 Conclusions and future recommendations

We applied the Lotka-Volterra model to analyze the effects of culturing conditions on the co-culture of MSC and HUVEC, and their population dynamics, which can now be predicted and elucidated. HUVEC and MSC exhibited the prey-and-predator behavior when being co-cultured in a closed niche, where under certain preset culturing environments, the growth of HUVEC can be greatly promoted by the presence of MSC through physical contact or paracrine signaling in very close proximity. The model prediction for cell mutual interaction can be extended to more complex 3-D environment, where similar results were found with additional impact of seeding sequence. We discovered that HUVECs attached but did not proliferate on 3-D myocardial ECM over time, however their growth was significantly enhanced by MSCs when co-cultured sequentially (MSC seeded 7 days before HUVEC). This phenomenon indicates the supporting function of MSC in “preparing” the ECM scaffold for HUVEC integration and expansion. Interestingly, after additional protein treatment was applied to coat ECM with common adherent proteins, the advantage of co-cultured HUVEC with MSC became diminished. We hence hypothesized that one of the two approaches, sequential co-culture or protein treatment for the pcECM scaffold, is necessary for improved endothelialization when we move on to the vascularization of the thick pcECM construct.

Sufficient oxygen and nutrient supply was provided with a perfusion bioreactor after cells were seeded onto the vasculature embedded inside the thick pcECM. Dynamic culture

environment effectively improved the endothelialization process compared to static study, but ECM lumens that were fully and evenly covered with cell monolayer were only achieved by applying one of the two approaches developed from 2-D and simplified 3-D studies. The nice and stable vasculature, which quantitatively resembled the native blood vessels, can serve as a promising indicator for the feasibility of combining thick decellularized pcECM, right cell combinations, and dynamic culture environment in numerous tissue regeneration applications.

This current study demonstrated the potential feasibility of regenerating viable and functional vascular supply within thick cardiac patch, which is essential for the post transplant survival and functioning of cardiac construct. This indication for feasibility is still at *ex-vivo* level, and more critical questions need to be answered in the future studies. Firstly, we've demonstrated viable vascular structured with monolayer of endothelial coverage, the functionality of blood vessels are yet to be proven. Functional vascular supply should demonstrate unobstructed perfusion of blood with no leakage and minimal thrombosis to provide sufficient oxygen, nutrient and waste exchange to the surrounding tissue. Sufficient and continuous perfusion should be demonstrated *ex vivo* within thick cardiac construct to support long term survival of surrounding cells, such as cardiomyocytes and fibroblasts. To achieve successful integration of functional blood vessels and viable myocardial tissue, once populated with competent thickness, a more advanced bioreactor system with pulsatile perfusion, electrical and mechanical stimulation should be used to better mimic the natural physiological condition. Subsequently, *in vivo* studies should be carried out in large animal models to demonstrate

similar or more superior functionality than *ex vivo* condition, with angiogenesis and integration to host environment before potential clinical studies on transplantation in human can be carried out to treat patients with myocardia infarction.

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Appendix

Supplementary data- Calibration curve for AlamarBlue™ assay

Figure S1. Example of cell calibration curve. HUVECs were cultured on 24-well plate with 2ml of EGM-2 medium in each well with different densities. The linearly ascending trendline indicates unsaturation of most densely seeded well, and suitability of the selection of cell density range. The slope (0.0004) of the fitted trendline can be thereby used for conversion of fluorescent density reading to cell number under the same culturing condition (n=4).