INFLUENCE OF MICROENVIRONMENT ON TISSUE ENGINEERING APPLICATIONS

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES OF MIDDLE EAST TECHNICAL UNIVERSITY

BY

ESEN SAYIN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

SEPTEMBER 2017

Approval of the thesis:

INFLUENCE OF MICROENVIRONMENT ON TISSUE ENGINEERING APPLICATIONS

submitted by ESEN SAYIN in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Department of Biotechnology, Middle East Technical University by,

Prof. Dr. Gülbin Dural Ünver Dean, Graduate School of Natural and Applied Sciences	
Assoc. Prof. Dr. Çağdaş Son Head of Department, Department of Biotechnology, METU	
Prof. Dr. Vasıf Hasırcı Supervisor, Department of Biological Sciences, METU	
Assoc. Prof. Dr. Erkan Türker Baran Co-Supervisor, Program of Biomedical Device Technologies , University of Health Sciences	
Examining Committee Members:	
Prof. Dr. İhsan Gürsel Dept. of Molecular Biology and Genetics, Bilkent University	
Prof. Dr. Vasıf Hasırcı Dept. of Biological Sciences, METU	
Prof. Dr. Alp Can Faculty of Medicine, Ankara University	
Assoc. Prof. Dr. Çağdaş Son Dept. of Biological Sciences, METU	
Assoc. Prof. Dr. İrem Erel Göktepe Dept. of Chemistry, METU	
	Date. 08.09.2017

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: Esen Sayın

Signature:

ABSTRACT

INFLUENCE OF MICROENVIRONMENT ON TISSUE ENGINEERING APPLICATIONS

Sayın, Esen Ph.D., Department of Biotechnology Supervisor: Prof. Dr. Vasıf Hasırcı Co-Supervisor: Assoc. Prof. Dr. Erkan Türker Baran

September 2017, 156 pages

Cues of microenvironment that guide both mature and stem cells determine the success of tissue engineered constructs. To prove and emphasize this expectation, various parameters such as surface topography, scaffold (cell carrier, scaffold) chemistry, 2D vs 3D microenvironments and mechanical stimulation were included into the microenvironment. Surfaces with two distinct physical cues pillar and groove-ridge type micropatterns were transferred to the surfaces of the films by casting the collagen type I and silk fibroin biopolymers on poly(dimethylsiloxane) (PDMS) templates which were replicated from photolithographically produced micropatterns on silicon wafers. Bombyx mori silk fibroin a structural biopolymer, was blended with collagen type I protein, to obtain high mechanical properties and biodegradability. Adipose derived stem cells (ADSCs) were cultured on collagen-silk fibroin films with microchannel and micropillar patterns to investigate the effects of cell morphology changes on osteogenic differentiation. While higher ADSC proliferation profiles were obtained on micropillar patterned film, microchannel patterned films, however, caused twice higher aspect ratio and effective orientation of cells. Alkaline phosphatase activity of ADSCs was several times higher on microchannel surface when the measured activities were normalized to cell number. Effective deposition of collagen type I and mineral after cell culture was observed for patterned and unpatterned films and these extracellular matrix (ECM) components were oriented along the axis of the microchannels. The use of collagenfibroin blend film with microchannel topography increased the aspect ratio and alignment of cells significantly, and it was also effective in the differentiation of ADSCs into osteogenic lineage.

As an additional biochemical cue of microenvironment defining element, elastinlike recombinamer (ELR) with a hydroxyapatite depositing amino acid sequence was incorporated into films of collagen-silk fibroin blend carrying microchannel patterns to stimulate anisotropic cell growth and osteogenesis. The Young's modulus and the ultimate tensile strength (UTS) of unseeded films were 0.58 ± 0.13 MPa and 0.18 ± 0.05 MPa, respectively. After 28 days of cell culture, ADSC seeded films had a Young's modulus of 1.21 ± 0.42 MPa and UTS of 0.32 ± 0.15 MPa which were about 3 fold higher than human osteoblast (HOB) seeded films. The difference in Young's modulus was statistically significant (p = 0.02). ADSCs attached, proliferated and produced calcium phosphate mineral on films better than the HOBs. In the light of these results, ADSCs served as a better cell source than HOBs for bone tissue engineering of collagen-fibroin-ELR based constructs used in this study.

In vitro systems generally rely on 2D test media whereas *in vivo* can best be mimicked if a 3D test medium is used. This is important because the maturation of a tissue engineered product should be different than on a 2D surface due to the kind of interactions and the accumulation of molecular signals in 2D and 3D systems are expected to be different. 3D scaffolds were created by folding long strips of engineered films on a rod to investigate the contributions of 3D microenvironment over mesenchymal stem / multipotent stromal cells (MSCs) by comparing with 2D films. Additionally, the contribution of hypoxia and arterial oxygen pressure was studied further to look into the concept of oxygen limited microenvironments of the native MSCs and osteoblast niche. Hypoxia maintained the stemness of the MSCs on TCPS and 2D scaffold. Interestingly, MSCs had elevated VEGFA level and osteogenic differentiation on 3D construct while preserving their stemness at normoxic conditions. Strikingly, osteogenic and angiogenic marker expressions were 13200 and 266 fold

higher, respectively on 3D construct than 2D scaffold at 21% oxygen. At all of the oxygen tensions that were tested, UTS was found to be similar with the unseeded scaffolds at less preferable 2D scaffolds by MSCs. Distinctively, during the transition from hypoxia to normoxia, Young's modulus and UTS of the MSC seeded 3D scaffolds were enhanced from 1.13 ± 0.33 MPa to 2.16 ± 0.81 MPa and 0.51 ± 0.12 MPa to 1.82 ± 0.27 MPa, respectively, owing to the contribution of cells by secreting their ECM onto the surface of 3D scaffold surface.

MSC proliferation was dependent both on oxygen tension and cyclic strain exposure (10%) on collagen hydrogel embedded scaffolds. On the other hand, strain was able to alter MSC shape by elongating them on the force direction and inhibit angiogenic activity both at hypoxia and normoxia.

In summary, this study showed that mimicking the bone ECM was possible through the use of microchannel patterns especially in 3D microenvironments that overcome the limitations of 2D substrates. The transition from 2D to 3D microenvironment enhanced the osteogenic and angiogenic activity of MSCs and along with the tensile properties both at normoxia and oxygen limited physiological environments.

Keywords: Microenvironment, Tissue Engineering, 2D vs 3D, Oxygen, Stem Cells.

viii

MİKROÇEVRENİN DOKU MÜHENDİSLİĞİ UYGULAMALARINA ETKİSİ

Sayın, Esen Doktora, Biyoteknoloji Bölümü Tez Yöneticisi: Prof. Dr. Vasıf Hasırcı Ortak Tez Yöneticisi: Doç. Dr. Erkan Türker Baran

Eylül 2017, 156 sayfa

Hem olgun hem de kök hücreleri yönlendiren mikroçevre kaynaklı işaretler, doku mühendisliği yapılarının başarısını belirlemektedir. Bu beklentiyi ispatlamak ve vurgulamak için, yüzey topoğrafyası, iskele (hücre taşıyıcı, scaffold) kimyası, 2 boyuta karşı 3 boyutlu mikroçevreler ve mekanik uyarım gibi çeşitli parametreler mikroçevreye dahil edilmiştir. Fotolitografik olarak üretilen mikro desenlere sahip silikon çiplerden kopyalanan poli(dimetilsiloksan) (PDMS) kalıpları kullanılarak sütun ve kanallı iki adet farklı fiziksel isaret içeren yüzeyler cözücü uçurma yöntemi ile kollajen tip I ve ipek fibroini biyopolimerlerinden üretilmiştir. Bombyx mori ipek fibroini ve kollajen tip I proteini ile optimum mekanik özellikler ve biyobozunma elde etmek için karıştırılmıştır. Adipöz kaynaklı kök hücreleri (ADSCs) mikrokanal ve mikrosütun desenli kollajen-ipek fibroini filmlerinin üstünde hücre morfolojisindeki değişimlerin osteojenik farklılaşmaya olan etkisini araştırmak için kültür edilmiştir. Yüksek mikrosütun desenli film üstünde yüksek ADSC çoğalma profili elde edilirken, ancak mikrokanal desenli filmler iki kat daha yüksek en-boy oranına ve hücrelerin etkili yönlendirilmesine neden olmuştur. ADSC'lerin alkalin fosfataz aktivitesi ölcülen aktiviteler hücre sayısına göre normalize edildiğinde, mikrokanal yüzeyinde sütunlara oranla birkaç kat daha yüksek olduğunu göstermiştir. Desenli ve desensiz filmlerle kültür sonrasında verimli bir etkili kollajen tip I ve mineral birikimi gözlemlenmiş ve bu ekstraselüler matriks (ECM) bileşenlerinin mikrokanalların ekseni boyunca yönlendirilmesini sağlanmıştır. Mikrokanal

topografyayla birlikte kollajen-fibroin karışım filmi kullanımı, en-boy oranını ve hücre hizalamasını önemli ölçüde arttırmış ve ADSC'lerin osteojenik kökene farklılaştırılmasında da etkili olmuştur.

Mikroçevreyi tanımlayan unsurlara ek bir biyokimyasal işaret olarak, anizotropik hücre büyümesi ve kemik oluşumunu uyarmak için mikrokanal desenleri taşıyan kollajen-ipek fibroin karışımı filmlerine hidroksiapatit biriktiren amino asit dizisine sahip elastin benzeri rekombinant proteini (ELR) dahil edilmiştir. Hücresiz filmlerin Elastik Modülü ve maksimum gerilme mukavemeti (UTS) sırasıyla 0.58 ± 0.13 MPa ve 0.18 ± 0.05 MPa ölçülmüştür. 28 günlük hücre kültüründen sonra ADSC ekilmiş filmlerin Elastik Modülü 1.21 ± 0.42 MPa ve UTS'i 0.32 ± 0.15 MPa olup ve bu değerler insan osteoblast (HOB) ekilmiş filmlerden yaklaşık 3 kat fazladır. Elastik Modülündeki fark istatistiksel olarak anlamlı bulunmuştur (p = 0.02). ADSC'ler, filmler üzerine HOB'lardan daha iyi tutunmuş ve çoğalmış ve daha çok kalsiyum fosfat minerali üretmiştir. Bu sonuçların ışığında ADSC'ler bu çalışmada kullanılan kollajen-fibroin-ELR tabanlı yapıların kemik dokusu mühendisliği için HOB'lardan daha iyi bir hücre kaynağı olarak görev yapmıştır.

In vitro sistemler genel olarak 2 boyutlu test ortamına dayanırken *in vivo* ortam en iyi 3 boyutlu test ortamı ile taklit edilebilmektedir. Bu durum önemlidir çünkü doku mühendisliği ile üretilmiş olan ürünün olgunlaşması 2 boyutlu yüzeyin üzerinde etkileşimlerin tipi nedeniyle farklı olmalıdır ve 2 boyutlu ve 3 boyutlu sistemlerde moleküler sinyallerin birikiminin farklı olması beklenmektedir. 3 boyutlu iskeleler uzun film şeritlerini bir çubuk üzerinde spiral oluşturmak üzere yuvarlayarak yapılmış ve 2 boyutlu, spiral olmayan, filmlerle karşılaştırılarak mezenkimal kök / multipotent stroma hücrelere (MSC'ler) 3 boyutlu mikroçevrenin katkılarını araştırmak için tasarlanmıştır. Ek olarak, hipoksiya ve artere ait oksijen basıncının katkısı, MSC'lerin ve osteoblast nişinin oksijen açısından sınırlı mikroçevre kavramı çerçevesinde çalışılmıştır. Hipoksiya, MSC'lerin TCPS ve 2D iskele üzerinde iken kök hücre özelliğini korumasını sağlamıştır. İlginç olan bir nokta ise MSC'ler 3 boyutlu yapıda normoksik koşullarda kök hücre özelliğini korurken VEGFA ve osteojenik farklılaşma düzeyini yükseltmiştir. Dikkat çekici bir şekilde, normoksiya koşulunda osteojenik ve anjiyojenik gen ifadeleri, 3 boyutlu yapıda 2 boyutlu iskeleye kıyasla sırasıyla 13200 ve 266 kat daha fazla ölçülmüştür. Test edilen bütün oksijen seviyelerinde MSC'ler tarafından daha az tercih edilen 2 boyutlu iskelelerde UTS hücresiz örneklere yakın bulunmuştur. Bundan farklı olarak, hipoksiyadan normoksiyaya geçiş esnasında, MSC'lerin ECM'lerini 3 boyutlu iskele yüzeyine salgılamaları ile yaptıkları katkı sayesinde 3 boyutlu iskelelerinin Elastik Modülü ve UTS'si sırasıyla 1.13 ± 0.33 MPa'dan 2.16 ± 0.81 MPa'ya ve 0.51 ± 0.12 MPa'dan 1.82 ± 0.27 MPa'ya yükselmiştir.

MSC çoğalması kollajen hidrojel içinde olan iskeleler üzerinde hem oksijen seviyesi hem de dinamik olarak, tekrarlı gerilime (% 10) bağımlı olduğu gösterilmiştir. Diğer bir taraftan, gerilim MSC'lerin şeklini uygulanan kuvvet yönünde uzatmış ve aynı zamanda hipoksiya ve normoksiyada anjiyojenik aktiviteyi engellemiştir.

Özet olarak, bu çalışma, kemik ECM'sini taklit etmenin, mikrokanallı desenlerin özellikle 2 boyutlu substratların sınırlamalarını aşan 3 boyutlu mikroçevreler içinde kullanılması yoluyla ile mümkün olduğunu göstermiştir. Normoksik ve oksijeni sınırlı fizyolojik ortamların ikisinde de, 2 boyuttan 3 boyutlu mikroçevreye geçiş MSC'lerin osteojenik ve anjiyojenik aktivitelerini ve gerilim mekanik özelliklerini arttırmıştır.

Anahtar Kelimeler: Mikroçevre, Doku Mühendisliği, 2 Boyuta Karşı 3 Boyut, Oksijen, Kök Hücre.

Dedicated to my lovely family...

ACKNOWLEDGEMENTS

I would like to express my most sincere thanks and gratitude to my supervisor Prof. Dr. Vasif Hasirci for his advices, support, encouragement, and patience throughout my thesis. I fell very lucky to work under the direction of Prof. Hasirci and I deeply appreciate his great comments and suggestions, and also the time and effort he spent during my Ph.D. study.

I am also grateful to my co-advisor, Assoc. Prof. Dr. Erkan Türker Baran for his guidance and valuable suggestions that improved this thesis. I would like to sincerely thank his continuous help not only at the writing stage of the thesis but also at the experimental part that he put his support from the very beginning.

I deeply indebted to Dr. Umber Cheema for giving me the opportunity to study in the Institute of Orthopaedics & Musculoskeletal Science (University College London, UK) for six months. Her amazing leadership, support, help and encouragement throughout this time helped me to accomplish much more.

I am grateful to Prof. Dr. Vivek Mudera (University College London, UK) for his support, guidance and valuable comments that took my study a step further.

I wish to express my gratitude to Prof. Dr. Ahmed Elsheikh (University of Liverpool, UK) for providing me the opportunity to carry out mechanical tests and teaching me the calculations.

I am also grateful to Prof. Dr. Carlos Rodríguez-Cabello (Universidad de Valladolid, Spain) for kindly sending the elastin-like recombinamer that contributed to my studies.

I would like to thank Prof. Dr. Muharrem Demirogullari (Cag Hospital, Turkey) and Prof. Dr. Cemil Yıldız (GATA, Turkey) for providing me lipoaspirate fat sample and bone sections, respectively.

I would like to express my thanks to Prof. Dr. Ihsan Gürsel and Assoc. Prof. Dr. Çağdaş Son for serving on my thesis monitoring committee and for their useful suggestions and discussions.

xiii

I would like to thank my special friend and lab partner Dr. Selcen Alagöz for her continuous support and encouragement throughout this long path and her valuable friendship.

I wish to thank my all friends in BIOMATEN especially Dr. Menekşe Ermiş Şen, Cemile Bektaş, Aylin Kömez, Gözde Eke, Tuğba Dursun, Dr. Arda Büyüksungur, Senem Büyüksungur, Gökhan Bahçecioğlu, Deniz Sezlev Bilecen, Ezgi Antmen, and our technician Zeynel Akın for their support in this study.

I also would like to express my thanks to my all friends in IOMS especially Judith Pape, Wollis Vas, Julia Jones, Rawiya Al Hosni and Mittal Shah for their help in this study.

I gratefully acknowledge the Scientific and Technological Research Council of Turkey (TUBITAK) for the Ph.D. fellowships (2214A and 2211C).

I also would like to acknowledge the Taylor & Francis Group for the permission to use the published material.

This study was supported by METU-BAP-07.02.2013.101 and DPT2011K120350.

Last but definitely not least, I would like to express my profound gratitude to my family for their love and patience and, continuous support and trust in me, without which it would not be possible to succeed.

TABLE OF CONTENTS

ABSTRACT	v
ÖZ	ix
ACKNOWLEDGEMENTS	xiii
TABLE OF CONTENTS	.XV
LIST OF TABLES	xxi
LIST OF FIGURES	xxii
LIST OF ABBREVIATIONSx	xxi
CHAPTERS	
1. INTRODUCTION	1
1.1 Cell Microenvironment	1
1.1.1 Components of Cell Microenvironment	1
1.1.1.1 Cell - Cell Interactions	3
1.1.1.2 Cell - Matrix Interactions	3
1.1.1.2.1 Importance of the ECM Dimension	4
1.1.1.3 Soluble Factors	6
1.1.1.4 Physiochemical Factors	6
1.1.1.4.1 Oxygen as a Microenvironmental Factor	7
1.2 Tissue Engineering	9
1.2.1 Cell Sources	11
1.2.2 Materials Used in Tissue Engineering	13
1.2.2.1 Synthetic Polymers	13
1.2.2.2 Natural Polymers	14
1.2.2.2.1 Collagen	14
1.2.2.2.2 Silk Fibroin	15
1.2.2.2.3 Elastin	16

1.2.2.3 Recombinant Polymers	17
1.2.2.3.1 Elastin Like Recombinamer (ELR)	18
1.2.3 Processing, Modification and Characterization of Scaffolds	19
1.2.3.1 The Importance of the Cell Guidance	22
1.3 Structure and Function of Bone Tissue	24
1.4 Approaches and Materials Used in Bone Tissue Engineering	27
1.5 Approach and Novelty of the Study	31
2. MATERIALS and METHODS	35
2.1 Materials	35
2.2 Methods	37
2.2.1 Scaffold Preparation	
2.2.1.1 Isolation of Collagen	37
2.2.1.1.1 SDS-PAGE Analysis of Isolated Collagen	
2.2.1.2 Isolation of Silk Fibroin	
2.2.1.3 Preparation of Templates for Patterned Substrate Surfaces	
2.2.1.4 Preparation of Scaffolds	39
2.2.1.4.1 Micropatterned Films	39
2.2.1.4.2 ELR Bearing Films	41
2.2.1.4.3 2D and 3D Scaffolds	41
2.2.1.5 Characterization of Scaffolds	43
2.2.1.5.1 Films with Different Compositions of Collagen and Fibroin	43
2.2.1.5.1.1 In Vitro Degradation Test	43
2.2.1.5.1.2 Atomic Force Microscopy (AFM)	43
2.2.1.5.1.3 Scanning Electron Microscopy (SEM)	43
2.2.1.5.1.4 FTIR-ATR Spectra	44
2.2.1.5.1.5 Swelling Test	44
2.2.1.5.1.6 Differential Scanning Calorimetry	44
2.2.1.5.2 ELR Bearing Film	44
2.2.1.5.3 2D and 3D Scaffolds	45

2.2.1.5.3.1 Stereo Microscopy	45
2.2.1.5.3.2 Fluorescence Microscopy, CLSM and SEM	45
2.2.2 In Vitro Studies	46
2.2.2.1 Osteogenic Differentiation of Human Adipose Derived	Stem Cells
(ADSCs) on Collagen - Fibroin Blend Films	46
2.2.2.1.1 Isolation of ADSCs	46
2.2.2.1.2 Culture of Cells	47
2.2.2.1.3 Characterization of ADSCs	47
2.2.2.1.3.1 Fluorescence Microscopy	47
2.2.2.1.3.2 Flow Cytometry	48
2.2.2.1.4 Studies with Undifferentiated ADSCs on Films	
2.2.2.1.4.1 Cell Proliferation	49
2.2.2.1.4.2 ADSC Viability	49
2.2.2.1.4.3 Microscopy (Fluorescence, CLSM, SEM)	49
2.2.2.1.4.4 Quantification of Cell Morphology	
2.2.2.1.5 Osteogenic Differentiation of ADSCs	
2.2.2.1.5.1 Cell Proliferation of Differentiated ADSCs	51
2.2.2.1.5.2 Alkaline Phosphatase (ALP) Activity	51
2.2.2.1.5.3 Microscopy (Phase Contrast, CLSM, SEM)	51
2.2.2.2 Human ADSCs and Human Osteoblasts (HOBs) on Collage	en - Fibroin
- ELR Blend Films	53
2.2.2.2.1 Isolation of HOB	53
2.2.2.2 Culture of Cells	53
2.2.2.3 Characterization of HOB	54
2.2.2.2.4 Studies with ADSCs and HOBs	55
2.2.2.4.1 Cell Proliferation	55
2.2.2.4.2 Microscopy (CLSM, SEM)	55
2.2.2.4.3 Quantification of Mineralization	56
2.2.2.4.4 Mechanical Test	56

	3.2.3	2D and 3D Scaffolds	74
3.3	Ost	eogenic Differentiation of Human ADSCs on Micropatterned	Collagen -
Fib	oroin Blei	nd Films	76
	3.3.1	Characterization of ADSCs	76
	3.3.2	Live-Dead Assay for Viability and Alamar Blue Test for Proli	iferation of
ADSCs on Micropatterned Films		on Micropatterned Films	79
	3.3.3	Morphology Changes of ADSCs on Micropatterned Films	81
	3.3.4	Osteogenic Differentiation of ADSCs on Micropatterned Films .	85
3.4	Co	mparison of Human ADSCs and HOBs on Collagen – Fibroin –	ELR Blend
Fil	ms		92
	3.4.1	Characterization of HOBs	92
	3.4.2	Proliferation of ADSCs and HOBs	94
	3.4.3	Surface Characterization and Cell Morphology	95
	3.4.4	Cell Alignment	96
	3.4.5	Quantification of Mineralization	97
	3.4.6	Mechanical Testing	97
3.5	Hu	man MSCs on 2D and 3D Collagen - Fibroin Blend Scaffe	olds Under
Dif	fferent O	xygen Tensions	101
	3.5.1	Cell Proliferation	101
	3.5.2	Gene Expression	
	3.5.3	Microscopy (Fluorescence, SEM)	107
	3.5.4	Oxygen Monitoring	110
	3.5.5	Mechanical Testing	111
3.6	Bel	navior of Human MSCs in 3D Microenvironment Exposed to Cyc	clic Tensile
Foi	rce and 5	% Oxygen	113
	3.6.1	Cell Proliferation	113
	3.6.2	Viability of MSCs	114
	3.6.3	Gene Expression	114
	3.6.4	Microscopy (Fluorescence, SEM)	115

4. CONCLUSION	119
REFERENCES	121
APPENDICES	141
A. ETHICAL COMMITTEE APPROVAL	141
B. EQUATION FOR ALAMAR BLUE CALCULATIONS	145
C. CALIBRATION CURVES FOR ALAMAR BLUE ASSAY	147
D. CALIBRATION CURVE FOR ALP ASSAY	149
E. CALIBRATION CURVE FOR CALCIUM QUANTIFICATION	151
F. CALIBRATION CURVE FOR DNA QUANTIFICATION	
CURRICULUM VITAE	155

LIST OF TABLES

TABLES

Table 1.1: Constituents of the bone (Murugan and Ramakrishna, 2005)26
Table 1.2: Processing methods and mechanical properties of different protein scaffolds
designed for bone tissue engineering
Table 1.3: Cyclic tensile strain contributed to osteogenic differentiation of MSCs and the
experimental parameters used in the literature were summarized
Table 2.1: Predetermined fibroin and collagen quantities for composite film preparation.
Table 3.1: Weight loss of crosslinked micropatterned films with different collagen-
fibroin ratios, incubated in collagenase type II for 3h at 37 $^{\circ}\mathrm{C}$ (n=3)67
Table 3.2: Weight loss in PBS was determined during 4 weeks for crosslinked collagen,
fibroin and blend films with microchannel and micropillar patterns (37 $^{\circ}$ C, n=3)68
Table 3.3: Expression of surface markers of two different passages of ADSC phenotype
(flow cytometry)
Table 3.4: Mean and standard deviation values of the Fig. 3.20 (n=3)

LIST OF FIGURES

FIGURES

Figure 1.3: 3D microenvironment mimics better the *in vivo* niches. A) Cells on a smooth surface organize focal adhesions and actin stress fibers at the basal side of the cells and transmit the contractile forces to their surface and the rest of the cell. Cells can attach to ECM via the basal side and interact with the secreted factors in the medium via the apical side. B) The curvature and softness of the 3D microenvironment decreases the formation of actin stress fibers. Mechanical stress is exerted to the cells both in directions planar and perpendicular to the cell basal surface (Gattazzo et al., 2014).......5 Figure 1.4: Various types of stem cell are located at environments measured to have low oxygen tension (dark grey colored areas). Scheme shows the currently available data obtained by direct measurements or mathematical modeling carried out for niches of mesenchymal, hematopoietic, and neural stem cells (in red color) that are found in adipose tissue, bone marrow and the subventricular zone, respectively (Mohyeldin et al., 2010).

Figure 1.5: The scheme summarizes the influence of oxygen on the functions of the bone cell. The physiological normoxia is accepted to be 12% oxygen for the osteoblasts to maintain the osteoblast activity. Hypoxia inhibits the proliferation, differentiation and collagen production while it stimulates the formation of osteoclasts and so bone resorption. Acidosis along with the hypoxia lead to more severe bone resorption by

preventing matrix mineralization and inducing osteoclasts for resorption (Marenzana and
Arnett, 2013)
Figure 1.6: Tissue engineering principles and a summary for the advances in the field
(Khademhosseini and Langer, 2016)
Figure 1.7: Mesenchymal stem / multipotent stromal cells in the sinusoidal cavity of
bone marrow can self-renew or differentiate into mesodermal lineage in vivo. They are
also reported to differentiate into endoderm and ectoderm lineages in vitro (Uccelli et
al., 2008)
Figure 1.8: Multilineage differentiation possibilities for ADSCs to be utilized in tissue
engineering and regenerative medicine (Bunnell et al., 2008)
Figure 1.9: The structure of the collagen type I (http://www.iupui.edu/~bbml/boneintro.
html)16
Figure 1.10: The antiparallel β -sheet structure at regions comprised mostly of glycine
and alanine (red: oxygen; blue: nitrogen; dark gray: carbon; light gray: hydrogen). The
spacing between the β -sheets depends on the side chains (Sayin et al., 2014)17
Figure 1.11: Different forms of tissue engineered scaffolds produced by various
processing methods. (A) SEM image of wet spun poly(3-hydroxybutyrate-co-3-
hydroxyvalerate), magnification x100, side view. (B) SEM image of electrospun
collagen fibers crosslinked with dehydrothermal treatment, magnification x3000. (C)
SEM image of electrospun silk fibroin fibers, magnification x500. (D) SEM image of
collagen sponge produced via lyophilization, magnification x250. (E) SEM image of 3D
PCL construct made with rapid prototyping, magnification x50. (F) SEM images of
micropatterned silk film stabilized with methanol showing groove and ridge patterns,
magnification x700 (Sayin et al., 2014)
Figure 1.12: Anatomical structure of the bone (Nguyen et al., 2012)
Figure 1.13: Sketches for the hierarchical levels of cortical bone (Dorozhkin, 2010)27
Figure 2.1: Monomeric structure of the ELR with an amino acid sequence that has
affinity to calcium and phosphate ions (Barbosa et al., 2009)

Figure 2.2: Scheme for the explanation of film preparation. Same steps were applied for
smooth, microchannel and micropillar patterned films40
Figure 2.3: The production scheme for 2D/3D collagen-fibroin blend scaffolds was presented.
Figure 2.4: Collagenase type I digested fat tissue
Figure 2.5: HOB isolation from human bone. A) Bone samples in T175 flasks. B) HOBs
migrated away from the explants. Phase contrast microscopy on Day 33 (scale bar: 200 µm)
Figure 2.6: The experimental setup. Collagen hydrogel was cast inside a teflon mold.
Film was immersed within the hydrogel to enable the force transmission to MSCs.
Floatation bars were placed at both ends to hook the bars to culture force monitor
(CFM). A) Top view. B) Scheme for the explanation of the setup
Figure 3.1: SDS-PAGE of type I collagen isolated from rat tail. Lane 1: the protein
markers (ladder); lane 2: commercial type I collagen (1 mg/mL); lanes 3 and 4: isolated
collagen type I at 1 mg/mL and 2 mg/mL concentrations, respectively
Figure 3.2: SEM micrographs of 2:1 collagen/fibroin (w/w) patterned films in A)
microchannel, and B) micropillar form after 4 weeks of in situ degradation in PBS (37
°C)69
Figure 3.3: SEM images of film (2:1 collagen/fibroin (w/w) patterned films) surfaces
were shown for A) microchannel, B) micropillar patterned blend films before
crosslinking. Atomic force microscopy images of C) uncrosslinked microchannel and D)
micropillar patterned blend films
Figure 3.4: FTIR-ATR spectra of collagen, fibroin, methanol treated fibroin and
methanol treated collagen-fibroin films. β -sheet band is shown with the arrow71
Figure 3.5: Characterization of pure and blend (2:1 collagen/fibroin (w/w)) films. A)
Degree of swelling of collagen, fibroin and collagen-fibroin films (n=3). Statistically
significant differences were determined between collagen film and other groups (p
values: *) and also between fibroin and collagen-fibroin blend films (p values: #). B)
DSC spectra of films. Tg values for each sample are marked with arrows on the DSC

spectra. C) Swelling ratios (%) and T_g of unseeded films. Pattern dimensions of D) collagen-fibroin, and E) collagen-fibroin-ELR blend films with microchannel patterns Figure 3.6: Characterization of the 2D/3D collagen-fibroin blend scaffolds. A) Stereo microscopy images of the 2D and 3D scaffolds in wet state (scale bar: 5 mm), B) Fluorescence microscopy (scale bar: 2 mm) and CLSM (scale bar: 200 µm) of the crosssection of the 3D scaffold. The 3D layered construct was suitable to examine the MSC behavior due to the swelling of the layers which created a close packing of tissue engineered matrix C) SEM of the 2D and 3D scaffolds showing the uniformity of the microchannel patterns (scale bar: 20 µm). The gaps between the PDMS pieces were transferred to long film strip as smooth surfaces (red dashed line). Cross-section image of the 3D scaffold (scale bar: 200 µm) and the marks showing the dimension of the Figure 3.7: Flow cytometry readings of passages 2 and 4 for ADSC phenotype Figure 3.8: Fluorescence microscopy images of ADSCs after immunostaining for CD105 (green) (A-C) and CD90 (green) (D) a positive marker for ADSCs and DAPI (blue) for cell nuclei. Passages A) 3, B) 4 and C) 5. D) Cells immunostained for CD90 Figure 3.9: Isotype and DAPI staining of ADSCs at passage 5 (scale bar: 100 µm).79 Figure 3.10: Calcein AM (green) and ethidium homodimer-1 (red) double stained ADSCs (initial seeding density of 3 x 10^4) on Day 3, on: A) coverslip, B) unpatterned Figure 3.11: ADSC proliferation on films and TCPS control (Alamar Blue assay) (n=3, Figure 3.12: Fluorescence and SEM micrographs of ADSCs on unpatterned, microchannel and micropillar patterned films on Day 3. A) Fluorescence microscopy images of ADSCs stained for actin filaments and nuclei with FITC-phalloidin (green) and DAPI (blue for nucleus), respectively. B) CLSM images of anti a-Tubulin immunostaining showed cell microtubule arrangement on unpatterned, channel and pillar micropatterned films (white dashed lines indicate the layers that signals were acquired from and reflected to their parallel 3D side views, scale bar: 250 µm). C) SEM micrographs of the same surfaces.

 micrographs of HOBs isolated on coverslips. C) Anti-collagen immunostaining (green). D) Anti-osteopontin immunostaining (green) (scale bar: 100 µm) (Passage 5). Nuclei Figure 3.18: Immunostaining of HOBs for negative and positive MSC markers was carried out (Passage 7). Cells were seeded on coverslip and stained for nucleus (green) with DRAQ5 and propidium iodide (green). Isotype, anti-CD34, anti-CD45 and anti-CD90 immunostaining (green) were presented at the second row (Scale bar: 100 µm). 93 Figure 3.19: HOB and ADSC proliferation on collagen-fibroin-ELR blend films and TCPS control (Alamar Blue assay) (n=3). On Day 7, an osteogenic medium was added for ADSCs. Statistical differences were indicated with color codes: black (HOB seeded to TCPS vs film), blue (ADSC seeded to TCPS vs film) and green (HOB seeded vs Figure 3.20: SEM micrographs of unseeded collagen-fibroin-ELR blend films on Days A) 1, B) 28 and C) HOB and D) ADSC seeded collagen-fibroin-ELR blend films on Day Figure 3.21: Fluorescence micrographs of actin filament staining with Phalloidin (green) on Day 28 indicated the cell alignment along the microchannel direction (white arrow). A) HOB and B) ADSC on collagen-fibroin-ELR blend films (scale bar: 250 μ m). C) Calcium amounts of unseeded collagen-fibroin-ELR blend films, HOB and ADSC seeded collagen-fibroin-ELR blend films on were quantified by o-cresol phthalein complexone method on Day 28. Statistical differences were determined and differences between each groups were analyzed (*: unseeded film on Day 1 vs other groups, [#]: unseeded film on Day 28 vs HOB and ADSC seeded films on Day 28, ⁺: HOB vs ADSC seeded films on Day 28). D) Calcium amounts of HOB and ADSC seeded films were normalized to cell number. E) Young's modulus of the unseeded and seeded films were presented with the statistical analysis. F) The average ultimate tensile strength (UTS), Young's modulus (E) and elongation at break (EB) values of unseeded and seeded

Figure 3.22: The proliferation of the MSCs at 5%, 12% and 21% oxygen tensions was studied on different time points. For the proliferation dsDNA content was quantified by Figure 3.23: Gene expression levels of the MSCs (passage number 2) that were seeded on TCPS and 2D scaffold and in 3D construct were determined with real-time RT-PCR to detect the transcripts of osteogenic differentiation with early markers, RUNX2 and osterix (OSX) (n=3). Cells were cultured for 3 days at the oxygen tensions of 5%, 12% Figure 3.24: Gene expression levels of the MSCs (passage number 2) that were seeded on TCPS and 2D scaffold and in 3D construct were determined with real-time RT-PCR to detect the transcripts of stemness with BST1 and CD90 (n=3). Cells were cultured for Figure 3.25: Gene expression levels of the MSCs (passage number 2) that were seeded on TCPS and 2D scaffold and in 3D construct were determined with real-time RT-PCR to detect the transcripts of angiogenic factor with VEGFA (n=3). Cells were cultured for Figure 3.26: Epifluorescence microscope images of MSCs on a coverslip and 2D scaffolds were taken to observe the intensity profile of RUNX2 (red). Nuclei (blue) were stained with Hoechst (scale bar: 100 µm). Coverslip was used as a substitute of TCPS since TCPS has autofluorescence that can lead to misinterpretation for the fluorescence intensity and also lower the quality of images. MSCs were elongated on the microchannels. Depending on the surface chemistry and topography, the RUNX2 intensity was the highest at 12% oxygen on coverslip and 5% oxygen on 2D scaffold.

Figure 3.28: Partial oxygen measurements of the MSC seeded 3D constructs. Oxygen Figure 3.29: Ultimate tensile strength (UTS) and Young's modulus (E) of unseeded and MSC seeded 2D/3D scaffolds were determined on the 35th day at 5% and 21% oxygen tensions. Unlike the 2D scaffolds exposed to any of the oxygen tension, 3D microenvironment and the 21% oxygen simultaneously helped MSCs to contribute to Figure 3.30: PicoGreen® assay was used to determine the dsDNA amount on 2D scaffolds after ensile strain application on the second day of 5% or 21% oxygen......113 Figure 3.31: MSCs exposed to 5% oxygen during a) No and b) 10% tensile strain Figure 3.32: RUNX2 and VEGFA expression levels after exposure to 24 h of uniaxial cyclic strain at 5% and 21% oxygen. 115 Figure 3.33: RUNX2 immunostaining (red) and nuclei staining (blue) of MSCs on 2D scaffolds after 0% or 10% tensile strain application at 5% or 21% oxygen (scale bar: 100 Figure 3.34: SEM micrographs on the 2nd day of osteogenic medium treatment showing

LIST OF ABBREVIATIONS

2D	Two Dimensional
3D	Three Dimensional
ADSC	Adipose Derived Stem Cell
AFM	Atomic Force Microscope
ALP	Alkaline Phosphatase
bFGF	Basic Fibroblast Growth Factor
BMSC	Bone Marrow Mesenchymal Stem Cell
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
BST1	Bone Marrow Stromal Cell Antigen 1
CAD	Computer Aided Design
CD	Cluster of Differentiation
CLSM	Confocal Laser Scanning Microscopy
DAPI	4',6-diamine-2-phenylindole drochloride
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
EDC	1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
EGF	Epidermal Growth Factor
ELR	Elastin Like Recombinamer
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FDM	Fused Deposition Modeling
FTIR-ATR	Fourier Transform Infrared-Attenuated Total Reflectance
TGF-β	Transforming Growth Factor Beta

HAP	Hydroxyapatite
HOB	Human Osteoblast
IGF	Insulin-like Growth Factors
FGF	Fibroblast Growth Factors
ITT	Inverse Temperature Transition
LCST	Lower Critical Solution Temperature
MSC	Mesenchymal Stem / Multipotent Stromal Cell
N-cadherin	Neural Cadherin
NHS	N-hydroxysulfosuccinimide
OSX	Osterix
PAA	Poly(acrylic acid)
PCL	Poly(ɛ-caprolactone)
PDMS	Poly(dimethylsiloxane)
PEG	Poly(ethylene glycol)
PFA	Paraformaldehyde
PGA	Polyglycolic Acid
PHEMA	Poly(2-hydroxyethyl methacrylate)
PLA	Polylactic Acid
PGA	Poly(glycolic acid)
PLGA	Poly(lactic acid-co-glycolic acid)
PPF	Poly(propylene fumarate)
PVA	Polyvinyl Alcohol
RGD	Arginine, Glycine, Aspartic Acid
RUNX2	Runt-Related Transcription Factor 2
SEM	Scanning Electron Microscope
TCPS	Tissue Culture Polystyrene
UTS	Ultimate Tensile Strength
v/v	volume/volume
VE-cadherins	Vascular Endothelial Cadherin

VEGF	Vascular Endothelial Growth Factor
W/V	weight/volume

CHAPTER 1

INTRODUCTION

1.1 Cell Microenvironment

Spatial and temporal cues in the cell microenvironment operate to drive tissue morphogenesis and differentiation. It is the environment in which cell resides and there is a need in regulating the factors such as cell-cell contacts, cell-matrix interactions, levels of soluble factors and specific physiochemical cues. All of these conditions are tightly controlled in the surroundings of the cell and these control the cell behavior. The dynamic environment is not limited to these microenvironmental cues because the cells are changing and modifying their environment, therefore, the knowledge about the *in vivo* cell microenvironment need to mimic *in vitro* are crucial parameters needed to advance tissue engineering applications. Well controlled microenvironments are also required in cancer research, drug screening, and immunological studies.

1.1.1 Components of Cell Microenvironment

Cell microenvironment can be maintained or modified with factors that act directly or indirectly on the cell behavior by using biophysical, biochemical, or other routes (Barthes et al., 2014). Multifactorial effects that compose the *in vivo* microenvironment of a single cell are:

- composition of the extracellular matrix (ECM),
- neighbor cells that can be either homotypic or heterotypic,
- cytokines, hormones and the rest of the bioactive agents found at juxtaposition of cells owing to autocrine, endocrine, and paracrine secretions,

- physical cues of the ECM and adjuvant cells at nano/microscale,
- and mechanical forces exerted due to the movement of the body or the physiological fluids such as blood (Fig. 1.1).

These factors can be detailed further and presented in four subsections: cell-cell contacts, cell-matrix interactions, soluble agents and specific physiochemical factors.



Figure 1.1: The schematic illustration of the complex factors that define the cell microenvironment include: surface topography and chemistry, matrix stiffness, mechanical stress, the molecular composition of the physiological fluids, physiochemical parameters that can be in the form of gradient or on/off like manner (https://patricwallin.wordpress.com/science/cell-microenvironment/).
1.1.1.1 Cell - Cell Interactions

Cells are found indirect contact with other cells *in vivo* through cell junctions. This type of interaction is called juxtacrine signaling. A widely known example of these junctions are cadherins that are dependent on calcium to adhere. They are in the form of transmembrane proteins and the type of the cadherin that is expressed specifically dependent on the cell type and location of the cell. For example, endothelial cells connect dominantly via VE-cadherins but use N-cadherins to attach themselves to the underlying layer of pericytes. Tight junctions can also be mentioned as a type of junction that brings the cells very close to each other, separated by an almost impermeable barrier. Specifically, for stem cell fate and differentiation, the notch signaling pathway has a particular importance (Artavanis-Tsakonas et al., 1999). It is partially responsible for the position of the heart which is on the left hand side of the body during the embryogenesis. A different phenomenon for the bone marrow hematopoietic stem cells is that if they lose contact with the osteoblasts that they are found in close proximity, they start to differentiate (Calvi et al., 2003).

1.1.1.2 Cell - Matrix Interactions

Cells interact with and attach to the ECM in the native tissue. Apart from attachment, they also grow on the ECM which is essential for the formation of tissue. The ECM serves as a specific topography and provides a chemical composition to regulate cellular fate. The ECM consists of many proteins such as collagen, elastin, laminin along with various glycosaminoglycans like hyaluronic acid and heparin sulfate. Every component has different physical properties such as stiffness, elasticity, and tensile and compressive strength because the composition and ratios of these macromolecules are specific for each tissue (Fig. 1.2). Studies showed that these physical properties are enough to alter stem cell commitment (Chaudhuri et al., 2015; Das et al., 2015a).



Figure 1.2: Elasticity of solid tissues have a range of elastic moduli (E) (Engler et al., 2006).

Cell adhesion onto a substrate is achieved via integrin family. Integrins are transmembrane proteins that connect the outside of the cell with the cytoplasmic components, mainly the actin filaments. Several molecules are in charge of cell binding and regulating cell spreading, migration, and stress fiber formation (Ermis et al., 2014).

1.1.1.2.1 Importance of the ECM Dimension

Mimicking the complexity of the cell microenvironment in a tissue is not possible through conventional 2D culture systems simply because the contribution of the third dimension includes signal accumulation originating from microenvironmental processes (Yamada and Cukierman, 2007) (Fig. 1.3). Introduction of an additional dimension alters cell function (Green and Yamada, 2007; Lund et al., 2009; Lee et al., 2013b; Sung et al., 2013), mechanotransduction (Kraning-Rush et al., 2011), diffusion trend of the oxygen and glucose, and additionally, it enables the formation of tissue like gradients of growth factors and enzymes by binding to them (Griffith and Swartz, 2006). A study showed the reversion of the malignant phenotype of human breast cancer cells in 3D culture by using integrin blocking antibodies (Weaver et al., 1997). However, the unclarity about the underlying mechanisms that are associated with the contribution of the third dimension on cell adhesion, migration, proliferation, and differentiation still persists. This question also addresses the 3D constructs that are widely used in tissue engineering applications, especially those implemented on the bone, which has an intricate developmental process.



Figure 1.3: 3D microenvironment mimics better the *in vivo* niches. A) Cells on a smooth surface organize focal adhesions and actin stress fibers at the basal side of the cells and transmit the contractile forces to their surface and the rest of the cell. Cells can attach to ECM via the basal side and interact with the secreted factors in the medium via the apical side. B) The curvature and softness of the 3D microenvironment decreases the formation of actin stress fibers. Mechanical stress is exerted to the cells both in directions planar and perpendicular to the cell basal surface (Gattazzo et al., 2014).

1.1.1.3 Soluble Factors

Cells can communicate with each other over long distances through molecules. There are three types of communication: autocrine, paracrine and endocrine. Autocrine signaling is achieved by the cell secretions. The cell is both the source and the target, and in turn, the molecule binds to a receptor found on the outside of the same cell's membrane. Paracrine signaling is limited to the neighborhood of the cell. The source and the target cell can be of the same type or different. The distance that the signal can be transmitted depends on the half-life of the molecule, composition of the ECM surrounding the cells and, size and composition of the molecule. On the other hand, endocrine signaling helps cells to communicate over longer distances by using the bloodstream. Source cell inside the gland secretes the molecule which can travel the whole body to be received by the target cell. The duration of the travel through the circulation slows the response.

1.1.1.4 Physiochemical Factors

Temperature and pH are kept in a rigid regimen in the body (37 °C, 7.35 - 7.45). Several buffering systems help for the preservation of pH. One specific example is the bicarbonate in the blood. The amount of the bicarbonate depends on the CO₂ concentration. CO₂ and H₂O react to form HCO₃⁻ and H⁺, therefore, CO₂ that is exhaled with respiration keeps the chemical equilibrium. In contrast to stable pH and temperature, oxygen level differs between the arterial and venous blood vessels. Partial oxygen level is higher at the arteries due to the oxygenation of the blood. Depending on the distance between the blood vessel and the cell, the partial oxygen level that the cell is exposed to varies and affect the cell behavior. *In vitro* systems does not take this difference in the partial oxygen level seen *in vivo* into account.

1.1.1.4.1 Oxygen as a Microenvironmental Factor

Recent studies showed the importance of the oxygen in defining and regulating the cell microenvironment in the maintenance of the tissue function. For example, as a progenitor of the skeleton, bone marrow mesenchymal stem cells (BMSCs) can transform into osteoblasts depending on the signals gathered during the fetal development. The resulting bone tissue has vasculature for arterial blood that keeps the oxygen level around 12% (Marenzana and Arnett, 2013). On the other hand, BMSCs still reside in the sinusoidal cavity of bone marrow, where the oxygen level is in the range of 1-6% which can be considered as hypoxia (Eliasson and Jönsson, 2010). The fact that "low oxygen tension is a characteristic of stem cell microenvironment" is now widely accepted for several other stem cell types such as embryonic, hematopoietic and neural stem cells (Mohyeldin et al., 2010) (Fig. 1.4). As a microenvironmental cue, hypoxia helps to preserve the stem cell phenotype by keeping the cells in an undifferentiated state and maintaining the self-renewal capacity. In contrast to the stem cells, it is important for osteoblasts to receive enough oxygen from arteries since their functions such as proliferation, differentiation, and collagen production are inhibited under hypoxic condition (Marenzana and Arnett, 2013). Impairment in the oxygen transport heavily delays bone healing and slows down bone turnover (Wang et al., 2007). Overall, approximately 12% oxygen tension should be maintained in the bone for the continuum of the bone tissue formation (Fig. 1.5).



Figure 1.4: Various types of stem cell are located at environments measured to have low oxygen tension (dark grey colored areas). Scheme shows the currently available data obtained by direct measurements or mathematical modeling carried out for niches of mesenchymal, hematopoietic, and neural stem cells (in red color) that are found in adipose tissue, bone marrow and the subventricular zone, respectively (Mohyeldin et al., 2010).



Figure 1.5: The scheme summarizes the influence of oxygen on the functions of the bone cell. The physiological normoxia is accepted to be 12% oxygen for the osteoblasts to maintain the osteoblast activity. Hypoxia inhibits the proliferation, differentiation and collagen production while it stimulates the formation of osteoclasts and so bone resorption. Acidosis along with the hypoxia lead to more severe bone resorption by preventing matrix mineralization and inducing osteoclasts for resorption (Marenzana and Arnett, 2013).

1.2 Tissue Engineering

The need for organs and tissues is ever increasing because of the aging of the population, diseases, accidents and the limited availability of donors for transplantation. Tissue engineering and regenerative medicine seek to construct substitutes to revitalize compromised tissues and improve organ functionality. Tissue engineering idea is based on harnessing *in vitro* cell culture methods and bioactive agents with the goal of

replacing or supporting the function of defective or injured body parts (Atala, 2012) (Fig. 1.6). In the biological tissues, cells reside on and interact with the ECM which is composed of glycosaminoglycans and a fibrillar network of collagen and elastin. ECM not only provides a structural integrity to tissues but it also interacts with the progenitor cells at the cell membrane level to induce proliferation and differentiation in the process of tissue regeneration (Beachley and Wen, 2010). Polymeric scaffolds carry great importance in tissue engineering as they protect the cells against dynamic forces exerted by the body and provide appropriate surfaces for cell adhesion, proliferation and they guide tissue formation with their inherent biochemical and topographical cues.



Figure 1.6: Tissue engineering principles and a summary for the advances in the field (Khademhosseini and Langer, 2016).

1.2.1 Cell Sources

The sources used in tissue engineering are autologous, allogeneic and xenogeneic, and they are from the same individual, another person or different species, respectively. Except for autologous cells, however, they are prone to risks such as infections and immunological responses that are associated with allogeneic and xenogeneic isolates. For this reason, autologous adult cells and stem cells (mesenchymal stem / multipotent stromal cells (MSC), embryonic stem cells (ESC)) have become an increasingly preferred source to use with tissue engineered substitutes. MSCs can be isolated from bone marrow and dental pulp and differentiate into several osteogenic, chondrogenic and neurogenic lineage (Fig. 1.7).



Figure 1.7: Mesenchymal stem / multipotent stromal cells in the sinusoidal cavity of bone marrow can self-renew or differentiate into mesodermal lineage *in vivo*. They are

also reported to differentiate into endoderm and ectoderm lineages *in vitro* (Uccelli et al., 2008).

Adipose derived stem cells (ADSCs) have surface markers and differentiation capacity which are similar to bone marrow stem cells (BMSCs) (Zuk et al., 2002; Kern et al., 2006). ADSC is an advantageous cell source due to lower invasiveness of tissue harvesting procedure, and reduced pain (Chai and Leong, 2007). Due to increasing obesity liposuction procedure become widespread and the material is generally being discarded as bio-waste which can be useful cell source. The capacity of ADSC to differentiate into osteogenic, chondrogenic and adipogenic cell lines is well known. Besides, ADSCs can differentiate into cell types other than those of mesenchymal lineages such as endothelial cells (Planat-Benard et al., 2004), hepatocytes (Banas et al., 2007) and neurons (Ashjian and Elbarbary, 2003) (Fig. 1.8). In addition, newly discovered cell types such as induced pluripotent stem cells (iPSCs) are also started to be used. There are, however, ethical issues and concern over cancerogenesis with the use of embryonic stem cells and the induced stem cells achieved by gene transfer rise respectively, thereby adult stem cells become increasingly preferred cell source for cell therapy.



Figure 1.8: Multilineage differentiation possibilities for ADSCs to be utilized in tissue engineering and regenerative medicine (Bunnell et al., 2008).

1.2.2 Materials Used in Tissue Engineering

1.2.2.1 Synthetic Polymers

In contrast to natural materials, mechanical and chemical properties of synthetic polymers can be customized according to the properties of the targeted implant site in the body. Synthetic polymers are more abundant and cost effective when compared to natural tissue substitutes and polymers. These materials should degrade through the time and provide physical support as the new tissue is regenerated in order to apply tissue engineering principle. Biocompatible but not biodegradable poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(acrylic acid) (PAA), poly(2-hydroxyethyl

methacrylate) (PHEMA) and biodegradable poly (lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ε -caprolactone) (PCL) and poly(propylene fumarate) (PPF) are the most commonly utilized synthetic polymers which have been used for tissue engineering (Place et al., 2009). PLA, PGA, PCL and their homo and copolymers have been used in the production of degradable medical devices such as sutures, stents, wound dressings that were approved by FDA. The most synthetic polymers have no cell adhesion property and usually require bulk or surface modification with biological moieties, such as immobilization of cell attachment peptide (arginine-glycine-aspartic acid, RGD) onto surfaces.

1.2.2.2 Natural Polymers

Since biopolymers constitute the native ECM of tissues, they are preferred materials for tissue engineering as they bear significant bioactive properties. They have intrinsic biological roles in maintenance of cell adhesion and differentiation, along with controlled degradation by enzymes. However, in a biotechnological sense, their production efficiency varies between batches, their mechanical properties can not be tuned like the synthetic polymers and they are prone to degradation at high temperatures and pressures during processing (Malafaya et al., 2007). Although, natural polymers have these disadvantages, they are widely used for tissue engineering applications due to their high performances. Collagen, silk fibroin, gelatin, fibrin, elastin and other structural proteins and polysaccharides like chitosan, alginate, starch, hyaluronan and chondroitin sulfate are widely employed as scaffold materials.

1.2.2.2.1 Collagen

Collagen is a structural protein that is found abundantly in bone, tendon, ligament, and skin. It has high tensile strength, adhesiveness and cohesiveness properties. Collagen is composed of three left handed helices twisted to form a right handed triple helix (Fig. 1.9). Gly-X-Y repeats are found in the fibrous structure where one of X and Y is generally proline or hydroxyproline (Nair and Laurencin, 2007). It carries cell adhesion sequences such as the arginine-glycine-aspartic acid (RGD). Collagen isolated from xenogenic tissues can trigger very low levels of antigenic responses (Pati et al., 2012). Recently, commercial recombinant human collagen has come into use in tissue engineering applications (Merrett et al., 2009).

1.2.2.2.2 Silk Fibroin

Silk has been employed in the production of sutures, membranes, scaffolds, controlled release systems and substrates for cell growth due to its low inflammatory and antithrombotic nature. Crystalline β -sheets formed by self-assembly are the reason for its superior mechanical properties (Fig. 1.10). Silk is a fibrous protein and has high strength and toughness with limited extensibility. It is obtained from insects and spiders. Silk has a high molecular weight but this value significantly changes depending on the source. The major source of silk is the silkworm *Bombyx mori* and is made up of two components, namely sericin, and fibroin. Sericin is a glycoprotein and keeps the two fibroin fibers attached to each other in the natural silk structure. Removal of sericin is required since it can elicit adverse immune reactions (Teuschl et al., 2014), therefore, elimination of the sericin with hot detergent treatment increases its biocompatibility when used as films, porous matrices and hydrogels (Mieszawska et al., 2010).



Figure 1.9: The structure of the collagen type I (http://www.iupui.edu/~bbml/boneintro. html).

1.2.2.2.3 Elastin

Elastin can be isolated from animal skin and also can be produced by recombinant DNA technology. It has a great importance in terms of bringing in elasticity to vascular tissues, skin, and cartilage. From the mechanical point of view, this adds an extension and elastic recoil features to tissues and durability against cyclic loading. In its formation, soluble tropoelastin molecules covalently bind to each other and crosslink elastin.



Figure 1.10: The antiparallel β -sheet structure at regions comprised mostly of glycine and alanine (red: oxygen; blue: nitrogen; dark gray: carbon; light gray: hydrogen). The spacing between the β -sheets depends on the side chains (Sayin et al., 2014).

1.2.2.3 Recombinant Polymers

The use of recombinant DNA technology in tailoring physical and biological attributes of proteins is a significant development in biomaterials science. In this approach gene sequences of different proteins from various organisms can be combined in a single synthetic DNA and subsequently, it is expressed in high yield when it is transferred into a suitable vector. In designing high strength protein scaffolds, the desired properties of certain proteins, such as the strength, elasticity and active cell adhesion, can be mimicked on a chimera protein. Researchers have produced elastin-like recombinamers (ELRs) (Costa et al., 2011), silk-like proteins (SLPs) (Teulé et al.,

2009), collagen-like proteins (CLPs) (Yoshizumi et al., 2009), gelatin-like proteins (GLKs) (Werten et al., 2009) and resilin-like proteins (RLPs) (Li et al., 2011) and they all have sequences repeated in their primary structure which introduce them various capabilities. For example, chimeric proteins combine different functions when used in the design of synthetic ECMs and these can help us control cell migration, differentiation and adhesion (Gomes et al., 2012). Additionally, many alternative fusion proteins have been designed such as resilin-elastin-collagen like polypeptide that has sequences from each of these proteins (Bracalello et al., 2011) or vitronectin-insulin like growth factor fusion protein that promotes cell growth and migration (Van Lonkhuyzen et al., 2007). Similarly, other ECM protein fused variants that enhance cell survival, proliferation, spreading and adhesion are available by using the sequences of MAP integrated fibronectin, laminin, type IV collagen derived peptides (Choi et al., 2010) as well as cell adhesion sequence RGD (Bini et al., 2006).

1.2.2.3.1 Elastin Like Recombinamer (ELR)

Elastin-like recombinamers (ELRs) are recombinant proteins coded in a synthetic DNA and expressed via the use of high yield vectors. VPGXG repeating sequences, where X is a natural or modified amino acid except L-proline originates from elastin which is an ECM protein found in many tissues including bone and is responsible for their elasticity. The amino acid sequences are combined in repeating fashion to form the backbone of ELRs (Trabbic-Carlson et al., 2003). Most ELRs are thermoresponsive materials and aggregate at temperatures higher than their inverse transition temperature (T_t). This feature aids purification of ELRs via insolubilization-precipitation (Rodríguez-Cabello et al., 2011). ELRs have earlier been used in bone tissue engineering and shown to be biocompatible (Amruthwar and Janorkar, 2013). Use of ELRs with special sequences in bone tissue engineering were reported to increase cell adhesion (Ozturk et al., 2009), to form an antifouling coat on titanium implants (Salvagni et al., 2014) or to enhance nucleation of HAP on the implant surfaces.

1.2.3 Processing, Modification and Characterization of Scaffolds

Scaffolds are biodegradable and biocompatible biomaterial structures in which cells proliferate and tissue forms. They are produced in fiber mesh, porous foam, membrane and rapid prototyped/additive forms depending on the type of engineered tissue.

Various techniques are being employed for production of scaffolds for tissue engineering. Wet spinning (Fig. 1.11A) and electrospinning (Fig. 1.11B, C) techniques were used in the production of micro/nanofibers and designed natural microarchitectures to enable cell ingrowth and effective cellularization of the scaffold. Spinning has been the most effectively used method to produce fiber form of biomaterials. In this method, a polymer melt or a viscous solution is ejected through an orifice to form the fiber structures. Electrospinning generates micro or nano scaled fibers and meshes that have high surface area to volume ratio. Additionally, these meshes have high void space interconnectivity which facilitates mass transfer in culture media. With these properties, nutrient and waste transport throughout the scaffold, an interior organization at cell level and drug delivery can be achieved. During electrospinning, polymer solution is ejected from a syringe via high voltage application to a metal capillary tube. As the electric field ejects the solution at a constant rate, the liquid component evaporates leaving dry fibers in air before they reach to a collector or surface. The fiber morphology is an important parameter that depends on several factors such as applied potential, injector-collector distance, ejection rate and solution properties such as polymer concentration, solvent volatility and solution conductivity (McManus et al., 2007; Sill and von Recum, 2008). Also, the orientation of the fibers has a significant influence on cellular responses, and especially on their alignment. Fibers can be used to obtain a woven or knitted scaffold manufactured from biocompatible fibers and present unique mechanical properties and improved mechanical strength by providing effective interconnecting voids for tissue ingrowth. Nonwoven fiber scaffolds, especially when prepared using random nanofibers, mimic the ECM proteins (50-500 nm diameter fibers) and can create in vivo like condition by enabling cells a three dimensional orientation (Barnes et al., 2007).

Freeze drying is a widely used technique due to the resultant high porosity and mechanical strength features that are desirable for load bearing scaffolds (Fig. 1.11D). Moreover, freeze drying can provide a controllable average pore diameter within the scaffold structure. Solutions of water soluble polymers can be directly poured into a mold and freeze dried in contrast to the preparation of hydrophobic polymers which require an organic solvent. For the latter type of polymer solutions, aqueous solution should be mixed with them in order to obtain a homogeneous emulsion that can provide even pore distribution. Finally, application of vacuum and sublimation of water results in high porosity structures.

Solvent casting and particulate leaching is a convenient method to obtain controlled sized pores within the scaffold. In order to introduce this porosity to the scaffold, porogen particles are added into the polymer solution and the polymer solution is cast into a mold. After drying by evaporation of the solvent, the porogen is dissolved out by a suitable solvent to expose the pores. Residual organic solvents can cause cytotoxicity and loss of bioactivity of agents such as growth factors incorporated into the scaffold (Mooney et al., 1996). Dimensions and the quantity of the porogen particles determine the size and the porosity respectively.

Scaffold design is a crucial part of tissue engineering process. The microarchitecture of the constructs is important in the tissue formation as the physical and biochemical cues guide cell commitment and correct tissue morphology. With the advances in the additive techniques or rapid prototyping technology, it is possible to make scaffolds with precise geometries and finely controlled physical properties (Fig. 1.11E) which can be customized according to the needs of a patient (Melchels et al., 2012). By using rapid prototyping, horizontal layers are added on top of each other to build up a scaffold by a computer-controlled mechanical process. Usually, 3D models that are derived from imaging data is the source for the design of 2D layers (Billiet et al., 2012). Projection stereolithography (PSL) based on computer aided design (CAD) is another type of additive technology used to engineer 3D scaffolds that mimic the microarchitecture of tissues. The PSL system has been used successfully in the fabrication of 3D scaffolds from photocrosslinkable gelatin methacrylate (GelMA) (Gauvin et al., 2012). Multilayered tissue engineering principle was applicable to the construction of human skin by mimicking skin layers. For this purpose, three dimensional free form fabrication with direct cell dispensing was applied for collagen hydrogel incorporating fibroblasts and keratinocytes (Lee et al., 2009).

Micropatterning can help cellular organizations needed to achieve complex tissue formation in the micron to nanoscale level. Especially, the soft lithography, at which biomaterials are patterned by a poly(dimethylsiloxane) (PDMS) mold, has become a convenient microfabrication tool for biomaterials (Fig. 1.11F). In this technique, a wafer is processed by photolithography on which PDMS is formed to obtain the replicate of pattern. Subsequently, PDMS mold is used for the production of patterned collagen films (Vrana et al., 2007).



Figure 1.11: Different forms of tissue engineered scaffolds produced by various processing methods. (A) SEM image of wet spun poly(3-hydroxybutyrate-co-3-

hydroxyvalerate), magnification x100, side view. (B) SEM image of electrospun collagen fibers crosslinked with dehydrothermal treatment, magnification x3000. (C) SEM image of electrospun silk fibroin fibers, magnification x500. (D) SEM image of collagen sponge produced via lyophilization, magnification x250. (E) SEM image of 3D PCL construct made with rapid prototyping, magnification x50. (F) SEM images of micropatterned silk film stabilized with methanol showing groove and ridge patterns, magnification x700 (Sayin et al., 2014).

1.2.3.1 The Importance of the Cell Guidance

Even though the natural microenvironment of most cells in the tissues involve a 3D organization including fibers, hydrogels and meshes (Flemming et al., 1999), a large number of studies employ 2D surfaces to create a more controlled environment to study the influence of certain parameters such as surface decorations (Wojciak-Stothard et al., 1995; den Braber et al., 1998). In recent studies, it has been shown that topography of cell microenvironment plays an important stimulatory role in human MSC differentiation, especially when the area and aspect ratio of the cells are controlled (Kolind et al., 2014; Lee et al., 2013a).

Cells interact with substrates via focal contact points constituted of cytoskeletal and membrane components, which make the contact with the ECM components such as fibronectin and all these interactions are at the nano scale. Physical cues play a role in mimicking ECM even when no biomolecules are present. Since in *in vivo*, cells have no smooth surface around themselves due to pits, pores, protrusions, striations, particulates, and fibers (Kim et al., 2013), they are being sent extracellular signals. Cells adapt their orientation by the participation of actin filaments and microtubules (Chen et al., 2014) and align and migrate on these topographies. Also localization of focal adhesion proteins may have an influence on alignment (Natale et al., 2014). It was shown that the guidance of the cell alignment with the use of nanopatterns much lower in size than that of the cells (332.5 and 650 nm) was possible and these topographies helped to improve the

mechanical properties of cell seeded films (Zorlutuna et al., 2009b) and human microvascular endothelial cell adhesion under shear stress (Zorlutuna et al., 2009a). Patterned surfaces at micro and nano level affect cell adhesion, morphology, proliferation and differentiation (Lim and Donahue, 2007; Thian et al., 2008). Furthermore, patterns can affect protein adsorption (Huang et al., 2010) and it is known that presence of specific protein sites can alter cell functionality (Tran et al., 2004). Macromolecules such as collagen that form the extracellular matrix (ECM) show particular orientation to create a tissue microarchitecture and to carry out tissue functions. Corneal stroma and bone have highly aligned collagen fibers which are responsible from tensile and compressive strength (Skedros et al., 2006; Ruberti and Zieske, 2008), and circumferential alignment of collagen fibers at media layer of artery gives resistance to high loads in the same direction (Gasser et al., 2006).

Various methods are being employed for cellular organization and alignment in 2D and 3D such as photolithography (Ross and Lahann, 2013) and electrospinning (Yang et al., 2005). Along with topographical control of tissue regeneration, chemical patterning with ECM components can also restrict the localization of desired cell types to a specific location (Thickett et al., 2012). Peptides and ECM components such as fibronectin and collagen that contain arginine-glycine-aspartate (RGD) cell adhesion sequences have been examined for their suitability in controlled confinement of cells (Kenar et al., 2008; Chen et al., 2014). Microcontact printing (μ CP) (McBeath et al., 2004) and dip-pen lithography (Wilson et al., 2001) are micro and nano scale biomolecule deposition techniques to achieve chemical patterning. However, chemical patterns on biodegradable surfaces may be not stable as material erosion may wash away the chemical cues. Instead, topographical patterning over cytocompatible surfaces by soft lithography can be used to form durable patterns that may withstand cell culture and *in vivo* conditions (Nikkhah et al., 2012).

1.3 Structure and Function of Bone Tissue

Cortical (compact) and trabecular (cancellous or spongy) bone make up the bone by forming the outer and the inner structure of bone, respectively (Fig. 1.12). Cortical bone consists the 80% of the total bone mass (Murugan and Ramakrishna, 2005). It is highly dense due to low porosity (20%) and it provides high mechanical strength (130-190 MPa). Trabecular bone (20%) that forms the rest of the bone, has a higher porosity (50-90%) and enables space for vasculature. Its mechanical strength is the one tenth of the cortical bone. Osteon is the functional unit of compact bone that is formed as concentric layers of mineralized matrix. At the center of the osteon, haversian canals enable the access of nerves and blood vessels (Nguyen et al., 2012).

Bone is a dynamic tissue and undergoes continuous remodeling of organic collagen fibers and inorganic hydroxyapatite. Collagen provides flexibility and resistance to cracking and hydroxyapatite $(Ca_{10}(PO_4)_6(OH)_2)$ introduces stiffness. Additionally, elastin is found in bone in minute quantities (Table 1.1). The mechanical properties and densities of different type bone vary according to collagen and nano hydroxyapatite composition.



Figure 1.12: Anatomical structure of the bone (Nguyen et al., 2012).

Bone is a mineralized connective tissue that houses nerves, blood vessels, and specialized cells: osteoblasts (bone forming cells), osteocytes (fully differentiated bone cells) and osteoclasts (bone resorbing cells). Osteoblasts synthesize collagen type I and other matrix proteins (osteopontin, osteocalcin, bone sialoprotein) during the ossification process. Osteoblasts deposit the calcium phosphate, which is amorphous at the beginning, transforms into more crystalline forms in time. As the osteoblasts secrete denser matrix around themselves, they are entrapped within the matrix and turn into osteocytes. Osteoprogenitor cells that can differentiate to bone cells reside in the periosteum and the marrow. Bone not only functions as a mechanical support for body and protect organs from outer damages but also house growth factors, cytokines and hematopoietic stem cells for formation of blood cellular components, mineral homeostasis, and acid-base balance (Clarke, 2008).

Inorganic phase	w/w %	Organic phase	w/w %
Hydroxyapatite	~60	Collagen	~20
Carbonate	~4	Water	~9
Citrate	~0.9	Non-collagenous proteins	~3
		(osteocalcin, osteonectin,	
		osteopontin, thrombospondin,	
		morphogenetic proteins,	
		sialoprotein, serum proteins)	
Sodium	~0.7		
Magnesium	~0.5		
Other trace elements: Cl^- , F^- , K^+ ,		Other trace elements:	
Sr ²⁺ , Pb ²⁺ , Zn ²⁺ , Cu ²⁺ , Fe ²⁺		Polysaccharides, lipids,	
		cytokines	
		Primary bone cells:	
		Osteoblasts, osteocytes,	
		osteoclasts	

Table 1.1: Constituents of the bone (Murugan and Ramakrishna, 2005).

Bone assembles into distinct levels of hierarchical units from macro to micro and micro to nano scales in order to fulfill its functions (Fig. 1.13). In bone, collagen fibrils are reinforced by plate-shaped HAP like nanocrystals 10–20 nm in length and 2–3 nm width (Kane and Ma, 2013) and also grow along the long axis of the collagen fibrils (Dorozhkin, 2010).



Figure 1.13: Sketches for the hierarchical levels of cortical bone (Dorozhkin, 2010).

1.4 Approaches and Materials Used in Bone Tissue Engineering

Tissue engineering (TE) can still be an appropriate alternative for small and medium sized bone defects because TE can introduce properties like osteoconduction, osteoinduction, and osteointegration to the scaffolds. The main constituents of bone tissue engineering are porous scaffolds with biocompatible and biodegradable properties and autologous cells to be seeded into the scaffolds to obtain biologically and mechanically adequate tissue substitutes (Rajzer et al., 2014).

For bone tissue constructs various stem cell sources are used alternatively such as BMSCs, adipose tissue-derived stem cells (ADSCs), umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs), muscle-derived stem cells (MDSCs), dental pulp stem cells (DPSCs) and embryonic stem cells (ESCs) (Seong et al., 2010). Besides, several growth factors such as transforming growth factor beta 1 (TGF β 1), bone morphogenetic protein 2 (BMP-2) and BMP-7 are used *in vitro* to induce bone forming cell lineage.

Synthetic polymers utilized for the bone tissue engineering release their degradation products in time. Unfortunately, degradation products of polyesters such as poly(lactic acid) (PLA) (Badami et al., 2006), poly(glycolic acid) (PGA) (Boland et al., 2001) and their copolymers were shown to create an acidic environment. In addition, the synthetic polymers do not possess cell signaling sequences that are naturally present in the structure of biological polymers such as fibronectin, collagen, vitronectin, and fibrinogen. Recently, protein based scaffolds gained importance in load bearing sites in the body such as bone, cartilage, tendon, meniscus, vessels, skin, bladder and cornea (Sayin et al., 2014), but since they lacked the required mechanical strength, additional materials were needed to be added to improve their mechanical strength (Table 1.2). Generally, for bone tissue engineering ceramics such as hydroxyapatite (HAP) (Arafat et al., 2011) are used together with the protein to give the scaffolds the fundamental features of the native bone. Composites in a foam form were used, for example, by adding sintered and non-sintered HAP into the gelatin and chitosan foams to increase their mechanical strength and make the composition similar to the bone (Isikli et al., 2012).

Collagen is widely used as scaffold material due to its higher biocompatibility, however, its mechanical properties are not high enough and it is degraded rapidly to use in hard tissue engineering. Reconstituted collagen has a much lower mechanical strength than the bone to be substituted (Burg et al., 2000). The reason for this is partly the lack of fibrillar arrangement due to hydrolysis during reconstitution. Additionally, collagen denatures during sterilization and this decreases its resistance to enzymes and

mechanical strength (Parenteau-Bareil et al., 2010). Several strategies have been tried to remedy this situation, such as incorporation of interdigitated collagen and polycaprolactone struts (Ahn et al., 2012) into collagen matrix (Rodrigues et al., 2003; Pallela et al., 2011; Hoyer et al., 2012). Alternatively, silk fibroin has been proposed as protein biomaterial for load bearing tissue engineering applications due to its unique mechanical properties. Self assembled *Bombyx mori* fibroin molecules have a crystalline β -sheet structure which gives its high tensile strength and toughness and, the remaining amorphous region provides the needed elasticity (Agrawal et al., 2013). However, handling of pure silk fibroin scaffolds is an issue and therefore, it is advantageous to blend it with more flexible materials such as collagen, to achieve optimum mechanical strength and ease of use.

Material	Scaffold	Processing method	References
Spider silk/bone sialoprotein fusion protein	morphology Film	Solvent casting	(Gomes et al., 2011)
Collagen with chitosan and HAP	Sponge	Lyophilization	(Pallela et al., 2011)
Silk with nano HAP or/and BMP- 2	Fiber	Electrospinning	(Li et al., 2006)
Gelatin with chitosan and sintered HAP	Sponge	Lyophilization	(Isikli et al., 2012)
Silk fibroin– gelatin	Hydrogel	3D bioprinting	(Das et al., 2015b)

Table 1.2: Processing methods and mechanical properties of different protein scaffolds

 designed for bone tissue engineering.

Besides the choice of polymers to produce scaffolds, mechanobiology can be utilized as an approach to facilitate the *in vitro* development of a bone tissue engineered construct by exposing MSCs to mechanical stimuli (Sumanasinghe et al., 2006). Low to moderate tensile strains and low hydrostatic stresses were shown to promote the intramembranous bone formation (Carter et al., 1998; Claes and Heigele, 1999; Lacroix and Prendergast, 2012). 10-12% tensile strain stimulated intramembraneous bone formation in a rat model (Loboa et al., 2004, 2005). Additionally, the utility of the tensile strain was observed for the bone tissue engineering as summarized in Table 1.3.

Material	Scaffold	Strain	Strain –	Osteogenic	References
	morphology	level	culture	medium	
			duration		
Collagen	Hydrogel	10% -	4 h/day - 7 days	+	(Sumanasinghe
type I		1 Hz			et al., 2006)
Collagen	Strip	2.5% -	Continuous - 6	-	(Kearney et al.,
type I		0.17	days		2010)
coated		Hz			
silicone					
Plastic	Strip	2000	40 min - 0, 0.5,	-	(Qi et al.,
		μΕ -	1, 2, 6, 12 and		2008)
		0.5 Hz	24 h after		
			mechanical		
			loading		
Plastic	Smooth	2400	A 15-sec rest	+	(Ye et al.,
	surface	με - 1	after every 5-		2012)
		Hz	sec working		
			duration for a		
			total of 2 h/day		
			– 4 days		

Table 1.3: Cyclic tensile strain contributed to osteogenic differentiation of MSCs and

 the experimental parameters used in the literature were summarized.

1.5 Approach and Novelty of the Study

Influence of the microenvironmental factors on bone tissue engineering was studied in many ways by employing specific physical and chemical cues, comparing 2D

and 3D matrices along with the physiologically relevant oxygen tensions and exerting tensional force at hypoxic and normoxic conditions.

In order to study microenvironmental factors firstly, ADSCs were tested on 2D composite films for their potential to obtain engineered bone tissue. Various collagen type I and fibroin compositions were tested for high stability that is required during in *vitro* studies by performing degradation test. Additionally, films were examined for their capacity to be rolled for further 3D experiments. Because of its low degradation level and ease of rolling (elasticity), 33% (w/w) fibroin was used for in vitro experiments. Channel and pillar type micropatterns, which can affect cell morphology and orientation differently, were evaluated to determine the effect of topographical cues on ADSC proliferation and differentiation. ADSC viability, short term cell proliferation profile (3 days), cell alignment, morphology were investigated as the preliminary study. ADSCs were cultured in osteogenic medium to investigate the effect of the micropatterns on differentiation to bone tissue. For detection of osteogenesis ALP activity (as an osteogenic marker), immunostaining for collagen type I and calcium deposition by alizarin red were performed. The microchannel patterned blend films were more successful in driving the ADSCs to osteogenic differentiation and so at the rest of the study, this micropattern type was employed.

Involving the chemical cues in as an additional microenvironment defining an element, ELR was added to the blend composition to alter the surface chemistry and the suitability of the stem cells and primary osteoblasts were compared for bone tissue engineering. In this study, an ELR with [(VPGIG)₂ (VPGKG) (VPGIG)₂]₂ DDDEEKFLRRIGRFG [(VPGIG)₂ (VPGKG) (VPGIG)₂]₂ repeats was used. The DDDEEKFLRRIGRFG region is responsible for nucleation of hydroxyapatite. The aim was to maintain an anisotropic cell growth with a motive to mimic the osteon organization. Microchannel pattern decorated films were seeded with human osteoblasts (HOBs) and human adipose-derived stem cells (ADSCs) to align the cells and expected them to secrete ECM in a parallel fashion as in cortical bone. Mature and stem cells

were compared with respect to their proliferation potential and influence on the mechanical strength of the scaffold.

After testing the ADSCs and HOBs on 2D scaffolds, the advantage of the 3D matrix was examined by using the same material composition and topographical cue. Microchannel patterned 2D and 3D blend matrices made of collagen and fibroin were tested with a motive to support the osteogenic differentiation by guiding the MSCs and so forming aligned extra cellular matrix. In order to examine the functionality of a 2D substrate and a novel 3D bone microenvironment, the MSC responses such as osteogenic differentiation, stemness, and angiogenic factor expression were compared with a widely used in vitro test surface, TCPS. In summary, a 3D substrate that can overcome the limitations of flat surfaces and 2D materials was examined. Furthermore, given the variability of oxygen tensions that are physiologically associated with the phenotypes of MSCs (5%) and osteoblasts (12%), MSC behavior on 2D/3D microenvironments was examined at these oxygen tensions with a comparison to *in vitro* test ambient (21%). Oxygen level inside the 3D construct was also monitored at the 5% and 21% oxygen levels. The preference of MSCs to 3D microenvironment and 21% oxygen was identified with regard to enhanced stemness, osteogenic differentiation, angiogenic marker expression and tensile properties. Additionally, if the 3D construct is used as an implant, it can be utilized as a support by rolling the long film strip on a screwed broken bone.

In the final stage, as an approach to promote osteogenic differentiation of MSCs, cyclic tensile strain application in an osteogenic medium was carried out. 5% and 21% oxygen were compared for their capacity to stimulate osteogenic differentiation and angiogenic factor expression under 10% cyclic tensile strain by using a 3D microenvironment capable of guiding the cells.

The novelty of the study: This is the first study in the literature that compares mature and stem cells with respect to their proliferation potential and influence on the mechanical strength of the scaffold for their potential use in bone tissue engineering. In addition, the advantage of 3D matrices over 2D scaffolds was explored at the oxygen tensions of MSC and bone niches and, *in vitro* test environment. These two aspects were used as microenvironmental cues that were not explored thoroughly in the tissue engineering applications.

CHAPTER 2

MATERIALS and METHODS

2.1 Materials

Spraque-Dawley rat tails were kindly received from Tayfun İde, (DVM), GATA Animal Experiments Laboratory (Turkey). Bombyx mori silk threads were gift from Prof. Esra Karaca, Uludag University Textile Engineering Department (Turkey). ELR ([(VPGIG)₂ (VPGKG) (VPGIG)₂]₂ DDDEEKFLRRIGRFG [(VPGIG)₂ (VPGKG) (VPGIG)₂]₂) carrying an HAP nucleating sequence was sent kindly and characterized by Prof. José Carlos Rodríguez-Cabello (Universidad de Valladolid, Spain) (Fig. 1.2). The theoretical mass of the ELR was calculated according to recombinamer design and found as 31,877 Da (Barbosa et al., 2009). Briefly, E. coli system was used for the oligopeptide synthesis. Cells were lysed by ultrasonication and protein was purified by applying a series of cold and warm centrifugation steps and dialysis. Purification was carried out by using aggregation of the recombinamer with a lower critical solution temperature (LCST) above its transition temperature. ITT was found to be 32 °C at pH = 7.36 as determined by size measurement between 20 °C and 40 °C by using Nano-ZS (Malvern, UK). Molecular weight and purity of protein were confirmed by matrixassisted laser desorption/ionization time-of-flight (MALDI-TOF) with a sharp peak at 31,857.21 Da and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a distinctive band around 30 kDa that was close to the theoretical mass.

Poly(dimethylsiloxane) (PDMS) prepolymers and curing agent were purchased from Dow Corning (Sylgard 184 Elastomer Kit, USA). 1-ethyl-3-[3dimethylaminopropyl]carbodiimide hydrochloride (EDC) was supplied by Pierce (USA). N-hydroxysulfosuccinimide (NHS), DMEM high glucose, 10X MEM, amphotericin B, bovine serum albumin (BSA), sodium cacodylate trihydrate, glutaraldehyde (25%), 4',6-diamine2-phenylindole dihydrochloride (DAPI), FITC-

35

conjugated phalloidin, acrylamide/bisacrylamide, paraformaldehyde (37%), trypsin-EDTA (0.25%), sodium azide, β -glycerophosphate, L-ascorbic acid, dexamethasone, ocresol phthalein complexone, 8-hydroxyquinone, 2-amino-2-methyl-1,3 propanediol and 5(6)-carboxyfluorescein were obtained from Sigma-Aldrich (USA). Bicoll separating solution and Dulbecco's Modified Eagle Medium (DMEM) low glucose were purchased from Biochrom (Germany). Recombinant human epidermal growth factor (EGF) 100 µm and 40 µm cell strainers were bought from BD Biosciences (USA). Basic fibroblast growth factor (bFGF), fetal bovine serum (FBS), fetal calf serum (FCS) and penicillinstreptomycin were purchased from HyClone (USA). Coomassie brilliant blue was obtained from Fisher Scientific (USA). HEPES, and collagenase type I and II were supplied from Gibco Invitrogen Co. (USA). NucleoCassette was purchased from ChemoMetec (Denmark). Alamar blue solution, Quant-iT PicoGreen Assay and Hoechst 33342 were purchased from Invitrogen Inc. (USA). Calcein AM and ethidium homodimer-1 were supplied from Molecular Probes (USA). Triton X-100 and dimethyl sulfoxide (DMSO) were obtained from AppliChem (USA). Tween 20 and acetic acid were bought from Merck KGaA (Germany). SnakeSkin dialysis tubing (CO 10000) and Fermentas Spectra multicolor broad range protein ladder were obtained from Thermo Scientific (USA). All primary and monoclonal isotype antibodies were purchased from Abcam (UK). Anti-CD44, anti-CD-90, anti-collagen type I, anti- α -Tubulin and anti-RUNX2 were monoclonal. Anti-CD45, anti-CD105 and anti-osteopontin were polyclonal. Phalloidin-Alexa Fluor 532 and secondary antibodies were bought from Invitrogen (USA). Alexa Fluor 488 conjugated anti-CD34, Alexa Fluor 488 conjugated anti-CD45, Alexa Fluor 647 conjugated anti-CD90 and Alexa Fluor 488 conjugated Mouse IgG1 K Isotype Control (FC) were purchased from BioLegend (USA). SensoLyte pNPP Alkaline Phosphatase Assay Kit was obtained from AnaSpec Inc. (USA). McCoy's 5A medium was purchased from (Switzerland). Colorless high glucose DMEM was bought from HyClone (USA). Trizol was purchased from Life Technologies (USA). Direct-zol RNA Miniprep was bought from Zymo Research (USA). High-Capacity cDNA Reverse Transcription Kit (without RNase inhibitor) was

purchased from Applied Biosystems (USA). iTaqTM Universal SYBR® Green Supermix, and real-time RT-PCR primers for HPRT1 (qHsaCID0016375), RUNX2 (qHsaCID0006726), BST1 (qHsaCID0013947) and VEGFA (qHsaCED0043454) were bought from Biorad (USA). Primers for 18S rRNA (QT00199367), Osterix (QT00213514) and CD90 (QT00023569) were purchased from Qiagen (Germany). Rest of the chemicals were analytical grade and used as received. Sterile rat tail collagen type I was bought from First Link (UK).



Figure 2.1: Monomeric structure of the ELR with an amino acid sequence that has affinity to calcium and phosphate ions (Barbosa et al., 2009).

2.2 Methods

2.2.1 Scaffold Preparation

2.2.1.1 Isolation of Collagen

Collagen type I was isolated from tendons of the Sprague–Dawley rat tails. Tail skins were removed and tendons pulled away and dissolved in cold acetic acid (0.5 M). After 3 days, solution was filtered with glass wool to remove any insoluble material and then solution was dialyzed against phosphate buffer (12.5 mM sodium phosphate dibasic, 11.5 mM sodium phosphate monobasic, pH 7.2, 4 °C) for 6 days. The solution was centrifuged at 12200 rpm for 10 min at 4 °C and the pellet was dissolved in acetic acid (0.15 M, 4 °C). Protein was precipitated by adding NaCl (5%) and the next day it

was centrifuged. Pellets were dissolved in acetic acid (0.15 M, 4 °C) and dialyzed for 7 days. After centrifugation, pellets were sterilized in 70% ethanol (4 °C) for 2 days. Solution was centrifuged and pellets were lyophilized at -80 °C (Labconco Freezone 6, USA). Collagen purity was studied with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.1.1.1 SDS-PAGE Analysis of Isolated Collagen

Collagen purity was checked with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Collagen was dissolved in acetic acid (0.15 M) and denatured in mercaptoethanol at 95 °C for 5 min. Separating and stacking gel concentrations were 10% and 4% acrylamide/bisacrylamide, respectively. Solution was loaded and gel was run at 30 mA for 2.5 h and stained with Coomassie Brilliant Blue (0.1% w/v) and destained with water:methanol:acetic acid (4:5:1).

2.2.1.2 Isolation of Silk Fibroin

Fibroin is isolated from silk by washing out the sericin part. *Bombxy mori* silk threads were cut into small pieces and 12.5 g silk was weighted. It was washed in boiled plenty of Na₂CO₃ (0.02 M) for 30 min. Threads were dried overnight at 37 °C and dry threads were weighted (approximately 9 g). Silk was dissolved at 60 °C in preheated LiBr solution (9.3 M, 112 mL). Solution was filtered with filter paper and dialyzed against water and lyophilized at -80 °C.

2.2.1.3 Preparation of Templates for Patterned Substrate Surfaces

Microchannel and micropillar patterned silicon templates with 1 cm² designed area were developed by using reactive-ion etching (RIE) method. Smooth silicon templates were used for the production of the unpatterned film. PDMS negative replicas were prepared by slow mixing of prepolymer and curing agent (Sylgard 184 Elastomer
Kit, Dow Corning, USA) in 10:1 ratio. After degassing of the mixture in vacuum to remove air bubbles it was poured on the patterned silicon template carefully. The prepolymer mixture was cured at 70 °C for 3 h and PDMS mold was peeled carefully from silicon template. Five PDMS pieces were joined for 3D scaffold production by attaching the parts onto glass slide surface. To connect the pieces, glass slide was stuck by a double sided tape onto glass petri plate and melted paraffin wax was poured into petri dish to stabilize molds. When the wax was solidified PDMS:curing agent mix was poured into the petri dish and PDMS was left to polymerize at room temperature.

2.2.1.4 Preparation of Scaffolds

2.2.1.4.1 Micropatterned Films

Films of pure collagen, fibroin and their blends were produced via solvent casting (Fig. 2.2). In order to prepare blend films different ratios of collagen and fibroin were mixed in the collagen:fibroin ratios of 4:1, 2:1, 1:1 and 1:9 (w/w) (Table 2.1). Films were prepared by solvent casting the protein solution ($250 \mu L/cm^2$, in 0.5 M acetic acid) on PDMS replicas. After drying, films were carefully peeled off. For stabilizing fibroin films they were incubated in 90% methanol for 1 h. Afterwards, the films containing collagen were crosslinked in EDC (12.5 mM) and NHS (5.2 mM) solution (90% methanol) at room temperature overnight. Usage of EDC and NHS provided the crosslinking between the amino and carboxyl groups of collagen and fibroin by forming amide bonds between collagen-collagen, fibroin-fibroin and collagen-fibroin. They were then washed with distilled water and air dried. Crosslinked films were stored in a desiccator until use.



Figure 2.2: Scheme for the explanation of film preparation. Same steps were applied for smooth, microchannel and micropillar patterned films.

Collagen:fibroin	Collagen	Collagen	Fibroin	Fibroin
(w/w)	(mg/mL)	(w/w %)	(mg/mL)	(w/w %)
1:0	16	100	0	0
4:1	20	80	5	20
2:1	16	66.7	8	33.3
1:1	12	50	12	50
1:9	2.4	10	21.6	90
0:1	0	0	80	100

Table 2.1: Predetermined fibroin and collagen quantities for composite film preparation.

2.2.1.4.2 ELR Bearing Films

In order to use for *in vitro* studies, 2.6% (w/v) collagen-fibroin-ELR blend films were produced in a ratio of collagen:fibroin:ELR 6:3:1. Materials were dissolved in 0.5 M acetic acid and microchannel patterned films were produced and crosslinked as explained in the section 2.2.1.4.1.

2.2.1.4.3 2D and 3D Scaffolds

Microchannel patterned 2D and 3D scaffolds were prepared (Fig 2.3) on the PDMS replicates by using solvent casting technique. A solution (250 μ L/cm²) of collagen type I (1.6% (w/v)) and fibroin (0.8% (w/v)) in HFIP was used on PDMS templates. The solution was dried at room temperature to let the formation of film layer on the PDMS. Dried films were then stabilized for 1 h in methanol (90% (v/v)) by inducing the formation of β -sheet structure in the fibroin part. After stabilization, films were crosslinked with EDC (12.5 mM) and NHS (5.2 mM) in methanol solution (90% (v/v)) for overnight. Films were washed with distilled water and dried at room

temperature. To create 3D microenvironment, a long strip of microchannel patterned film was rolled around a teflon rod that has 2 mm diameter. In order to stick the end of the roll, cyanoacrylate glue was used.



Figure 2.3: The production scheme for 2D/3D collagen-fibroin blend scaffolds was presented.

2.2.1.5 Characterization of Scaffolds

2.2.1.5.1 Films with Different Compositions of Collagen and Fibroin

2.2.1.5.1.1 In Vitro Degradation Test

Stability of microchannel and micropillar patterned films were tested against collagenase type II as was previously described (Vrana et al., 2007). Briefly, films were incubated in 0.1 mg/m collagenase type II in PBS at 37 °C for 3 h, washed with distilled water and freeze dried for gravimetric measurement.

Degradation test was carried out by leaving the micropatterned films in the phosphate buffered saline (10 mM, pH 7.4, 0.02% (w/v) sodium azide) at 37 °C. Films were weighed after 1, 2 and 4 weeks and weight loss was calculated by dividing the weight loss with the initial weight. The blend 2:1 (collagen:fibroin) was selected upon degradation test due to its mid level degradation and relative softness of the film which was important for the rolling the long pieces of strips with the same composition.

2.2.1.5.1.2 Atomic Force Microscopy (AFM)

In order to determine pattern fidelity of microchannel and micropillar patterned films, surface of the uncrosslinked blend films were characterized with Atomic Force Microscopy (AFM, Ambios Technology Inc., U.S.A.) by using noncontact mode.

2.2.1.5.1.3 Scanning Electron Microscopy (SEM)

Microchannel and micropillar patterned films were also observed with SEM to confirm the pattern uniformity before crosslinking. Additionally, the effect of 28 days of degradation in PBS was observed for the crosslinked films. Samples were sputter coated with Au and examined with SEM (Bruker, USA).

2.2.1.5.1.4 FTIR-ATR Spectra

FTIR-ATR spectra (Perkin Elmer, USA) of collagen, fibroin, methanol treated fibroin and methanol treated collagen:fibroin 2:1 films were studied for β -sheet formation. Films were scanned in the range of 1000-4000 cm⁻¹ with the interval of 4 cm⁻¹.

2.2.1.5.1.5 Swelling Test

The swelling degrees of pure collagen, pure fibroin and collagen-fibroin blend films were determined via gravimetric method. Pre-weighed, crosslinked films were incubated in 10 mM PBS for 24 h at 37 °C, rinsed with distilled water and blotted lightly. Swelling was calculated as follows:

Swelling (%) =
$$\frac{w_{\rm S} - w_{\rm D}}{w_{\rm D}} \ge 100$$

where w_S is the swollen weight and w_D is the dry weight.

2.2.1.5.1.6 Differential Scanning Calorimetry

Glass transition temperatures of crosslinked collagen, fibroin and collagenfibroin blend films were determined by differential scanning calorimetry (DSC) (Perkin Elmer Diamond, USA). Samples were heated under nitrogen gas at a rate of 10 °C/min in the range 0-250 °C.

2.2.1.5.2 ELR Bearing Film

After the crosslinking procedure, pattern dimensions (without and with ELR films) were measured with a profilometer (NewView[™] 73003D Optical Surface Profiler, Zygo, USA) and compared.

2.2.1.5.3 2D and 3D Scaffolds

2.2.1.5.3.1 Stereo Microscopy

The overall morphology of the crosslinked 2D/3D scaffolds was imaged with stereomicroscope. Scaffolds were wetted with the distilled water before imaging in order to observe the swollen structure during the *in vitro* testing.

2.2.1.5.3.2 Fluorescence Microscopy, CLSM and SEM

Cross section of the 3D scaffold was observed after conjugation of the 5(6)carboxyfluorescein with collagen – fibroin blend with the help of EDC/NHS that crosslinks between the carboxyl and amine groups. The concentration of the fluorophore was 6.4 mM in the collagen – fibroin solution. After addition of the 5(6)carboxyfluorescein, production, stabilization and crosslinking were carried out as explained in section 2.2.1.4.3. Before the imaging, the scaffold was wetted with distilled water. The roll was cut in half with scissors and examined with fluorescence microscopy (Zeiss, Jena, Germany) and CLSM (Leica DM2500, Germany). 2D/3D scaffolds were sputter coated with Au-Pd (3 nm) and visualized with QUANTA 400F field emission SEM (Netherland). The dimension of the resulting micropatterns was calculated with ImageJ software (NIH).

2.2.2.1 Osteogenic Differentiation of Human Adipose Derived Stem Cells (ADSCs) on Collagen - Fibroin Blend Films

2.2.2.1.1 Isolation of ADSCs

Lipoaspirate fat sample was a kind gift of Prof. Muharrem Demirogullari (Cag Hospital, Ankara Turkey). Human ADSCs were isolated by using the procedure by Francis et al. (Francis et al., 2010) with the consent of the patient and approval of Middle East Technical University Human Ethics Committee (Appendix A). Briefly, after washing, the fat was digested with 150 μ g/mL collagenase type I for 1 h at 37 °C (Fig. 2.4) and Bicoll separating solution was used to separate the cell layer. After centrifugation, cell suspension was filtered through 100 μ m and 40 μ m cell strainers and cultured in DMEM supplemented with 40% FBS, 1% penicillin-streptomycin, 250 ng/mL amphotericin B and 10 ng/mL epidermal growth factor (EGF) for 1 day to enhance cell adhesion. The freezing medium for the cells was used as 10 % DMSO in standard medium.



Figure 2.4: Collagenase type I digested fat tissue.

2.2.2.1.2 Culture of Cells

ADSCs were cultured in DMEM supplemented with 10% FBS, 1% penicillinstreptomycin, 250 ng/mL amphotericin B and 10 ng/mL EGF. Flasks were placed in in a carbon dioxide incubator (5 % CO₂, MCO-17AIC, Sanyo Electric Co. Ltd., Japan) operated at 37 °C. The cells were passaged using 0.25% trypsin-EDTA solution at subconfluency. All *in vitro* tests were carried out with ADSCs at passages between 2 and 5.

2.2.2.1.3 Characterization of ADSCs

2.2.2.1.3.1 Fluorescence Microscopy

Cells were detached and suspended with 0.25% trypsin. Standard medium was added for enzyme inactivation and cells were centrifuged at 3000 rpm for 5 min. Cells were counted by using NucleoCounter (ChemoMetec A/S, Denmark). 30000 cells were seeded on coverslips. ADSCs were immunostained against CD105 marker and their nuclei were stained with DAPI. Coverslips were washed with PBS and fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature. Then, cells were washed twice and permeabilized with 1% (v/v) Triton X-100 for 12 min at room temperature. After washing twice, samples were incubated at 37 °C for 30 min in blocking solution (1% BSA, 0.1% Tween 20, 0.1% FCS, 0.1% sodium azide in PBS) and consequently incubated with human anti CD105 solution (final antibody concentration 10 μ g/mL in PBS with 0.1% BSA) for 2 h at 37 °C. Then cells were washed with PBS twice and they were incubated with secondary anti rabbit antibody (final antibody concentration 20 μ g/mL in PBS with 0.1% BSA) for 1 h at 37 °C. Cells were washed with PBS twice and cell nuclei were stained with DAPI. Finally, samples were washed twice and visualized under fluorescence microscope (Zeiss, Germany).

2.2.2.1.3.2 Flow Cytometry

For characterization of ADSCs, cells were trypsinized at passage 2 and 4 and flow cytometry measurements were taken through determination of surface antigens (CD44, CD45 and CD105) of cell suspensions. Initially, suspended cells were washed with PBS (with 1% BSA, 0.1% (w/v) sodium azide) and fixed with 4% (w/v) paraformaldehyde for 15 min at room temperature. Then, cells were washed with PBS and resuspended in PBS (with 1% BSA, 0.1% sodium azide). Primary antibodies were added to cell suspensions and incubated for 30 min at room temperature. After washing steps with PBS, cells were resuspended in PBS with 1% BSA and 0.1% sodium azide. Secondary antibodies and isotype controls were mixed with cell suspensions in eppendorf tubes and incubated for 30 min at room temperature. Finally, cells were washed with PBS at least three times and signals were measured with flow cytometry (BD Accuri C6, USA).

2.2.2.1.4 Studies with Undifferentiated ADSCs on Films

Crosslinked films were sterilized in 70% (v/v) ethanol for 2 h and washed with PBS. For preparation of cell suspension cells near confluence in T-flasks were detached with trypsin. Consequently, standard medium was added for enzyme inactivation and cells were centrifuged at 3000 rpm for 5 min. Cell number was determined with NucleoCounter and 30000 cells were seeded to each surface. Suspended ADSCs were seeded onto each type of film in a 24-well plate and tissue cultured plate (TCPS) was used as positive control. 2 mL of growth medium was added to samples and changed three times in a week. After 1 day of incubation, films with adhered cells were transferred to new culture plate wells.

2.2.2.1.4.1 Cell Proliferation

Alamar Blue assay measures cell number with respective to cell redox activity of metabolically active cells (Appendix B). Before the measurement, films were washed with colorless DMEM twice. At day 1 and 3, triplicate samples were incubated with Alamar Blue[®] solution (10% in colorless DMEM) for 1 h at 37 °C. Absorbance values were measured at 570 and 595 nm by using Multi-Well Plate Reader (Molecular Devices, USA). From percent reduction values and calibration curve, cell number conversion was performed (Appendix C).

2.2.2.1.4.2 ADSC Viability

Cell viability was examined by live-dead cell staining. Films which were cultured for 3 days were washed with PBS twice. For visualizing live and dead cells on films, cells were double stained with calcein AM (4 μ M in PBS) and ethidium homodimer-1 (10 μ M in PBS) and washed with PBS. The stained ADSCs were then visualized under fluorescence microscope (Olympus IX70, Japan).

2.2.2.1.4.3 Microscopy (Fluorescence, CLSM, SEM)

In order to observe cell alignment on patterned films, cell nucleus and cytoskeleton were stained with DAPI and FITC-phalloidin, respectively. Films were treated the same pre-staining steps as described in immunostaining procedure. After PBS washing, cells were incubated in FITC labeled phalloidin solution (1 μ g/mL, prepared in PBS with 0.1% BSA) for 1 h at 37 °C. Finally, after washing with PBS, cells were counterstained with DAPI for and washed with PBS. Stained cells were then visualized under fluorescence microscope (Zeiss, Germany).

For 3D scanning of cell orientations, cells were immunostained with α -Tubulin by carrying out the same experimental procedure with CD105 immunostaining. Final

primary and secondary antibody concentrations were 50 µg/mL and 20 µg/mL in blocking solution. Images were taken with Confocal Laser Scanning Microscopy (CLSM, Leica DM2500, Germany).

Films were washed with PBS and cacodylate buffer (0.1 M, pH: 7.4) and cells were fixed with glutaraldehyde solution (2.5%, in cacodylate buffer) for 2 h at room temperature. Films were washed twice with cacodylate buffer and freeze dried (Sanyo MDF-U53865, Japan) for 2.5 h at -80 °C. Films on metal stubs were coated with gold thin layer for 3 min (MCM-100 SEC). Unseeded and seeded films were examined at day 3 with SEM (Bruker, USA).

2.2.2.1.4.4 Quantification of Cell Morphology

Micrographs that were taken for the calcein staining of ADSCs were used for cell alignment, cell area, Feret's diameter and aspect ratio measurements. Quantitative image analysis was performed with the ImageJ Java software (National Institute of Health, USA) after acquiring random area with stained cells.

2.2.2.1.5 Osteogenic Differentiation of ADSCs

Films were immersed into 70% ethanol (v/v) for 2 h and then washed with sterile PBS twice. Films were then dried inside of a laminar flow cabinet. 10000 cells were seeded to each type of film and positive TCPS controls. Standard medium (high glucose DMEM, 10% FBS, 1% penicillin-streptomycin and 250 ng/mL amphotericin B) was used for 7 days of cell culture. After 1 week of incubation with growth medium, it was switched to osteogenic medium (standard medium containing 100 nM dexamethasone, 10 mM β -glycerophosphate, 50 μ M L-ascorbic acid) and cells were cultured for an additional 21 days in osteogenic medium. 2 mL of osteogenic medium was added to samples (in 12-well plate) and changed three times in a week.

2.2.2.1.5.1 Cell Proliferation of Differentiated ADSCs

Cell proliferation was determined at days 1, 7, 14, 21 and 28. Alamar blue assay was performed as explained at section 2.2.2.1.4.1.

2.2.2.1.5.2 Alkaline Phosphatase (ALP) Activity

SensoLyte pNPP Alkaline Phosphatase Assay Kit was used for the test done at days 7, 14, 21 and 28. In principle, p-Nitrophenyl phosphate (pNPP) is a chromogenic substrate and after dephosphorylation absorption maximum is 405 nm. Kit manual was used for ALP assay. Briefly, films were washed with colorless DMEM and component B. Films were cut with lancet and 400 μ L lysis buffer. Samples were frozen and thawed three times at -80 °C and 37 °C, respectively. Contents were centrifuged at 2000 rpm for 10 min. Supernatant was transferred to new eppendorf and 50 μ L supernatant was added to 96-well plates. 50 μ L ALP dilution buffer and Component A were added to each well. Samples were incubated for 1 h at 37 °C and 50 μ L stop solution was transferred to each well and absorbance was measured at 405 nm by using Shimadzu 2100-S UV-Vis Spectrophotometer (Japan). ALP concentration was calculated by employing the calibration curve formula (Appendix D). ALP levels per cell were found by dividing cell number that were measured with Alamar blue assay.

2.2.2.1.5.3 Microscopy (Phase Contrast, CLSM, SEM)

After osteogenic induction, films were stained for calcium deposition. At day 28 cells were washed with colorless medium and PBS. Cells were fixed with neutral formaldehyde (10%, pH 6.8) for 30 min at room temperature. Samples were washed with distilled water. Alizarin red S (2%, pH 4.2) was added and films were kept at dark for 45 min at room temperature. Films were washed with distilled water at least four

times and PBS was added to wells. Red colored calcium deposits were imaged at phase contrast microscopy (Zeiss, Germany).

At day 28 cells were immunostained for detection of collagen type I secretion which an indication of osteogenic differentiation of ADSCs. Additionally, cells were counterstained with phalloidin-Alexa Fluor 532 for cytoskeleton imaging. Cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Samples were washed with PBS and cells were permeabilized with 1% Triton X-100 for 12 min at room temperature. After PBS washing step, cells were incubated with blocking solution (10% BSA, 0.1% Tween 20, 0.1% FCS, 0.1% sodium azide in PBS) for 30 min at 37 °C. Cells were incubated with anti collagen type I antibody (50 µg/mL in blocking solution) for 2 h at 37 °C. Cells were washed with PBS and incubated with blocking solution (5% normal goat serum, 1% Tween 20, 0.1% sodium azide in PBS) for 30 min at 37 °C. Secondary antibody (20 µg/mL in blocking solution) was added and cells were incubated for 1 h at 37 °C. Cells were washed with PBS and cells were incubated with blocking solution (1 μ g/mL, prepared in PBS with 0.1% BSA) for 30 min at 37 °C. Cells were incubated with Alexa Fluor labeled phalloidin (59 µg/mL in blocking solution) for 1 h at 37 °C. Samples were washed with PBS and cells were examined with fluorescence microscope (Zeiss, Germany).

Micropatterned film surface and cell morphologies were analyzed with SEM. Cultured films were washed with PBS and cacodylate buffer (0.1 M, pH: 7.4) and cells were fixed with 2.5% gluteraldehyde solution (in cacodylate buffer) for 2 h at room temperature. Films were washed twice with cacodylate buffer and cells were fixed further 1% osmium tetroxide (in cacodylate buffer) for 1 h. Films were washed and dehydration was carried out in a series of 10 min 50%, 70%, 80%, 95% (twice) and 15 min 100% ethanol treatment. Samples were dried with lyophilizator and examined with SEM and its EDX component (FEI, Quanta 400F, Eindhoven, Netherlands). Samples were mounted on aluminum stubs and sputter-coated with Au-Pd prior to analysis.

2.2.2.2 Human ADSCs and Human Osteoblasts (HOBs) on Collagen - Fibroin -ELR Blend Films

2.2.2.1 Isolation of HOB

Bone samples were obtained from the Gulhane Medical Military Academy (GATA) (Ankara, Turkey) with their and the Middle East Technical University Ethical Committee's approval and with patient consent (Appendix A). Bone pieces received from elective joint replacement surgery were transferred to sterile transport medium (DMEM high glucose supplemented with 2% penicillin-streptomycin, 500 ng/mL amphotericin B). Fragments were washed with PBS and broken into 2-3 mm pieces. Pieces were placed in T175 flasks and medium (DMEM high glucose supplemented with 10% FBS, 1% penicillin-streptomycin, 250 ng/mL amphotericin B) was added (Fig. 2.5A). Flasks were not moved for 1 week and after that the medium was renewed twice a week. Cells migrated out of the explants and at the end of 34 days cells reached 90% confluency and were trypsinized with 0.25% trypsin–EDTA for 2 min at 37 °C. Some of the flasks were used for cryopreservation for future use and others were used for immunostaining for phenotype characterization.

2.2.2.2.2 Culture of Cells

Proliferation medium was used as McCoy's 5A containing 10% FBS, 1% penicillin-streptomycin, 0.5% L-glutamine, 250 ng/mL amphotericin B and 24 μ g/mL L-ascorbic acid because it was reported to have a positive effect on osteoblast proliferation (Pradel et al., 2008). HOBs with passage numbers up to 5 were used.

2.2.2.3 Characterization of HOB

For HOB characterization, the isolated cells were immunostained with anticollagen type I and anti-osteopontin antibodies and their nuclei were stained with DAPI. HOBs were fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature. Cells were washed twice and permeabilized with 1% (v/v) Triton X-100 for 12 min at room temperature. After washing, samples were incubated at 37 °C for 30 min in the blocking solution (1% BSA, 0.1% Tween 20, 0.1% FCS, 0.1% sodium azide in PBS) and samples were incubated with anti-collagen type I and anti-osteopontin antibody solutions (final antibody concentration 10 µg/mL in 0.1% BSA in PBS) for 2 h at 37 °C. Then, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG as secondary antibody (final antibody concentration 20 µg/mL in 0.1% BSA in PBS) for 1 h at 37 °C to complete the immunostaining of collagen type I and osteopontin markers, respectively. Cell nuclei were stained with DAPI. Samples were visualized with fluorescence microscopy (Zeiss, Germany). HOBs with passage numbers up to 5 were used in *in vitro* studies.



Figure 2.5: HOB isolation from human bone. A) Bone samples in T175 flasks. B) HOBs migrated away from the explants. Phase contrast microscopy on Day 33 (scale bar: 200 µm).

For the confirmation of the absence of contaminating MSCs, cells (Passage 7) were immunostained for CD34 (#343518), CD45 (#304017) and CD90 (#328116) along with isotype control (#400129). After blocking step, cells were incubated with conjugated primary antibodies for 1 h at 37 °C. Nucleus was stained with DRAQ5 (50 μ M) and propidium iodide (50 μ g/mL) for 1 h and 10 min, respectively.

2.2.2.4 Studies with ADSCs and HOBs

Films were sterilized in 70% (v/v) ethanol for 2 h and then air dried inside the laminar flow hood. 10000 cells were seeded on the films and after one day in growth medium, films were placed into new wells. ADSCs were cultured in the growth medium for 1 week and then the medium was changed with osteogenic medium in which ADSCs were cultured for the next 3 weeks. Since HOBs are originally bone cells no osteogenic medium was used.

2.2.2.4.1 Cell Proliferation

Proliferation of ADSCs and HOBs were measured with Alamar Blue assay on Days 1, 7, 14, 21 and 28 as explained at section 2.2.2.1.4.1. Separate calibration curves for ADSCs and HOBs were prepared and cell numbers were determined from the individual calibration curves (Appendix C).

2.2.2.4.2 Microscopy (CLSM, SEM)

On Day 28, cell seeded samples were stained with Phalloidin Alexa Fluor 532. Cells were fixed and permeabilized as explained earlier, incubated in blocking solution (1% BSA in PBS) at 37 °C for 30 min and in Phalloidin solution (118 μ g/mL final concentration prepared in PBS with 0.1% BSA) at 37 °C for 1 h. Images were obtained with Confocal Laser Scanning Microscopy (Leica DM2500, Germany).

Morphologies of ADSCs and HOBs on film surfaces were examined with SEM on Day 28. Additionally, surfaces of unseeded films were examined on Days 1 and 28. SEM and EDX analysis were carried out as detailed in section 2.2.2.1.5.3.

2.2.2.4.3 Quantification of Mineralization

Quantity of calcium in samples was measured with a colorimetric assay based on o-cresol phthalein complexone that forms a violet colored complex with calcium. Films of Day 28 were washed with PBS and calcium was extracted from them by immersing in 0.6 N HCl overnight at 4 °C. Supernatant (10 μ L) was added to a solution (190 μ L) containing equal volumes of calcium binding reagent (0.024% o-cresol phthalein complexone and 0.25% 8-hydroxyquinone in water) and calcium buffer (500 mmol/L 2-amino-2-methyl-1,3 propanediol in water). Absorbance was measured at 570 nm with a multiwell plate reader. The amount of calcium in each sample was determined with a standard curve (Appendix E). Normalized calcium calculations were performed by subtracting the background calcium reading, unseeded film of Day 28, from each value and then dividing with the cell numbers measured with Alamar Blue assay.

2.2.2.4.4 Mechanical Test

ADSC and HOB seeded collagen-fibroin-ELR blend films were tested after 28 days of culture to investigate the effect of ECM secretion of cells on the tensile behavior of blend films. None of the samples were fixed. Films (n=3-5) were cut into 4 mm x 10 mm strips and tested at room temperature by Instron 3366 Uniaxial Testing Machine (Instron, Norwood, MA) with a 10 N load cell. Films were placed in a longitudinal direction parallel to the microchannel axis with custom made clamps in a Perspex chamber, which was filled with PBS. Preload (0.01 N) was applied in uniaxial tension mode to prevent loose layout of films between clamps. Preload rate was 0.4 mm/min and

no data was recorded during preload. After preload, uniaxial tension was applied up to a maximum load of 10 N at an elongation rate of 0.04 mm/min which corresponded to 1.0%/min strain rate. Testing was continued until failure.

2.2.2.3 Human Bone Marrow Mesenchymal Stem Cells (MSCs) on 2D and 3D Collagen – Fibroin Blend Scaffolds

2.2.2.3.1 Culturing MSCs

Human MSCs were isolated by Michelle Korda and colleagues from the iliac crest of the patients that undergone total hip replacement surgery (Royal National Orthopaedic Hospital, Stanmore, UK) (Reissis et al., 2013). Written and signed patient consent and ethical committee approval from NHS Health Research Authority (National Research Ethical Committee, London) were obtained. Characterization of the cells were also carried out by showing the capacity of the MSCs to differentiate into osteogenic and adipogenic cell lines by Reissis et al. Cells between the passages of 2 and 5 were used for the experiments. MSCs were cultured in high glucose DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 5 ng/mL bFGF (basic fibroblast growth factor).

2.2.2.3.2 Studies with MSCs

Films were sterilized in ethanol (70% (v/v)) for 2 h and washed with PBS twice. Cells were detached with 0.1% trypsin and counted with a hemocytometer. 50000 cells were seeded to tissue culture polystyrene (TCPS) and normal sized films (2D). For the longer films, which were used in the preparation of 3D scaffolds, 250000 cells were seeded to maintain the same cell density as the 2D films. During cell seeding to the 3D scaffold, 50000 cells were seeded to each 5 parts of the long film strip. TCPS was used as a control surface to include the conditions that are employed at most of the *in vitro* test environment. 3D microenvironment was set by rolling the long film after cell seeding. 3 mL of osteogenic medium (high glucose DMEM medium containing 10% FBS, 1% penicillin-streptomycin, 100 nM dexamethasone, 10 mM β -glycerophosphate, 50 μ g/mL ascorbate 2-phosphate) was added to samples (in 12-well plate) and changed every other day. MSCs were cultured for 3 days in a CO₂ incubator (Sanyo, Japan) that was adjustable to 5%, 12% and 21% oxygen tensions for each set of experiments. This incubator could be set for any oxygen tension by displacing the oxygen through taking nitrogen in.

2.2.2.3.2.1 Cell Proliferation

The total DNA content on TCPS and 2D/3D scaffolds were determined with Quant-iT PicoGreen® Assay on Days 1 and 3. Samples were washed with colorless DMEM to remove phenol red. The cellular DNA was released with 1 mL lysis buffer (10 mM Tris pH 8, 1 mM EDTA and 0.2 % (v/v) Triton X-100) and samples were stored in -80 °C. After thawing, scaffolds were cut into smaller pieces with scalpel blade (#20). Lysis buffer was mixed thoroughly and 10 μ L of sample solution was added to 190 μ L of assay reagent (1:200 diluted PicoGreen in 1 x TE buffer) in 96-well plate. Fluorescence was measured with multiplate reader (Tecan, Switzerland) by applying the settings of 480 nm as excitation wavelength and 533 nm as emission wavelength with an emission cut off at 515 nm. Fluorescence intensity was converted to total DNA content according to manufacturer's protocol by using the known concentrations of lambda DNA for the standard curve (Appendix F).

2.2.2.3.2.2 SEM

After 3 days of incubation, samples were fixed with 2.5% glutaraldehyde for 2 h at room temperature. Cells were stained with 1% osmium tetroxide (in cacodylate buffer) for 1 h and dehydrated with a series of ethanol. Micrographs were taken with high resolution Field Emission SEM (Jeol, USA).

2.2.2.3.2.3 Real-Time RT-PCR

In order to obtain required RNA levels for real-time RT-PCR, the number of seeded cells was increased. Cell number was 2×10^5 for TCPS and 2D scaffolds, and 1×10^5 for TCPS and 2D scaffolds. 10⁶ for 3D scaffolds. On Day 3, 120 µL, 200 µL and 300 µL Trizol was added to TCPS, 2D and 3D scaffolds, respectively. Control and scaffolds were kept in -80 °C. After thawing the samples, an equal volume of ethanol was added to the suspension. For RNA extraction Direct-zol RNA Miniprep® was used and manufacturer's protocol was carried out. Total RNA of each sample was reverse transcribed to complementary DNA (cDNA) with High-Capacity cDNA Reverse Transcription Kit. cDNA levels (n=3) were quantified by using the iTaq[™] Universal SYBR[®] Green Supermix. HPRT1 and 18S rRNA were employed as housekeeping genes. Osterix, RUNX2, BST1, CD90, and VEGFA were the primers studied with real-time RT-PCR. Real-time RT-PCR conditions were 2 min at 50 °C, 10 min at 95 °C, and then 50 cycles at 95 °C for 15 s, and 1 min at 60 °C and it was performed with CFX96 Real Time System (Biorad, USA). Results were normalized to housekeeping genes with regard to the same primer brand by employing the $\Delta\Delta c_{T}$ method. The level of housekeeping gene expression of the TCPS sample that was incubated at 21% oxygen for 3 days, was assigned as 1 and the rest of the calculations for gene expression levels were performed accordingly.

2.2.2.3.2.4 Fluorescence Microscopy

Samples were fixed with 4% paraformaldehyde for 30 min at room temperature. MSCs were immunostained for an osteogenic marker, RUNX2. Cells were washed twice and permeabilized with 1% (v/v) Triton X-100 for 1 h at room temperature. After washing, samples were incubated at 37 °C for 30 min in the blocking solution (1% BSA, 0.1% Tween 20, 0.1% FCS, 0.1% sodium azide in PBS) and then incubated with anti RUNX2 antibody solution (final antibody concentration was 10 μ g/mL in 0.1% BSA/PBS) for 2 h at 37 °C. Scaffolds were washed with PBS and incubated with

secondary antibody (final antibody concentration was 20 µg/mL in 0.1% BSA/PBS) for 1 h at 37 °C. Cell nuclei were stained with Hoechst 33342 and they were imaged with epifluorescence microscope (Zeiss, Germany).

2.2.2.3.2.5 Oxygen Diffusivity of 2D and 3D Scaffolds

Fiber-optic oxygen probes (Oxford Optronix, Oxford, UK) were placed between the layers (termed as interlaminar) of MSC seeded 3D scaffolds at 5% and 21% oxygen tensions on Day 0. Partial pressure of oxygen was measured for at least 9 h at a rate of 1000 sampling/min. No difference in the oxygen level was determined at the core of the seeded 3D scaffolds in ambient oxygen tension on Day 0 (data not shown.). The same procedure was carried out for the 5% oxygen tension. Measurements were recorded according to the same procedure with Cheema et al. (Cheema et al., 2012). Since oxygen diffusivity and solubility are sensitive to temperature fluctuations, all of the equipment and 3D constructs in the medium were kept at 37 °C before oxygen monitoring in order to equilibrate the environment.

2.2.2.3.2.6 Mechanical Properties

The tensile properties of the unseeded and MSC seeded 2D/3D collagen-fibroin blend scaffolds were examined after 35 days of cell culture. This time point was chosen to observe the effect of 5% oxygen and 2D/3D microenvironment on the tensile behavior of blend scaffolds and also to give time to osteogenically-induced MSCs for the extra cellular matrix secretion that can strengthen the scaffold structure. 2D/3D scaffolds that were exposed to 5% and 21% oxygen conditions were preferred for the test since MSCs expressed osteogenic markers higher at these oxygen tensions than 12% oxygen in general. Same number of cells with the real-time RT-PCR was seeded and none of the samples were fixed chemically before the test. 3D scaffolds were unrolled for the measurement and sections from the outer regions were removed and then sections (n=3-

5) were cut into 4 mm x 10 mm strips. Tensile testing was performed by using Instron 3366 Uniaxial Testing Machine (Instron, Norwood, MA) with a 10 N load cell at room temperature. The film ends were placed between the two clamps and then put in a Perspex chamber, which was filled with PBS. Samples were placed in a longitudinal direction parallel to the microchannel axis. In order to maintain flat layout of films between the clamps, preload (0.01 N, 1 mm/min) was carried out in uniaxial tension mode and no data was recorded during the process. Lastly, uniaxial tension was exerted with a maximum load of 10 N and elongation rate of 0.04 mm/min (which was equal to 1.0%/min strain rate). Testing was stopped at the point of failure. The measurements of applied force (F), cross-sectional area (A), initial length (l) and length of displacement (Δ l) were used for the calculation of the stress ($\sigma = F/A$), strain ($\varepsilon = \Delta l/l$), ultimate tensile strength (maximum σ) and, Young's modulus (σ/ε).

2.2.2.4 Human MSCs Subjected to Cyclic Tensile Force in 3D Collagen - Fibroin Based Microenvironments

Human MSCs were seeded as explained in section 2.2.2.3.2. 200000 cells were seeded on 2D scaffolds. After 2 h of incubation, films (2D) were entrapped in collagen hydrogels. Collagen hydrogels were produced by adding 10X MEM to collagen type I (rat tail) according to RAFTTM Kit Protocol. In order to neutralize collagen solution 1 M HEPES and 10 M NaOH were mixed in 5.048:1 ratio and added to the mixture. The hydrogel was casted in a teflon mold after placing 2 floatation bars to both ends (Fig. 2.6). After hydrogel solidification, MSCs were cultured in osteogenic medium for 2 days in a CO₂ incubator (Sanyo, Japan) that was adjusted to 5% and 21% oxygen tensions for each set of experiments.

2.2.2.4.1 Cyclic Tensile Force Test System

After 1 day of incubation, for tension application, half of the samples were connected to culture force monitor (CFM). In order to connect floatation bars were found in the teflon molds, the wires within the floatation bars were hooked to custom made platform. The tensile strain was applied by a motor (Parker, UK) that run at 1 Hz to generate 10% strain. Coding was executed via EASI-V software.



Figure 2.6: The experimental setup. Collagen hydrogel was cast inside a teflon mold. Film was immersed within the hydrogel to enable the force transmission to MSCs. Floatation bars were placed at both ends to hook the bars to culture force monitor (CFM). A) Top view. B) Scheme for the explanation of the setup.

2.2.2.4.2 Cell Proliferation

On Day 2 cell numbers on films were determined with Quant-iT PicoGreen® Assay as explained in section 2.2.2.3.2.1.

2.2.2.4.3 Cell Viability

After 2 days of incubation at 5% oxygen tension Live-Dead staining was carried out for unstrained and strained samples. Calcein AM and ethidium homodimer-1 staining was performed as detailed in section 2.2.2.1.4.2. and cells were imaged with epifluorescence microscope (Zeiss, Germany).

2.2.2.4.4 Microscopy (Fluorescence, SEM)

On Day 2 cells were fixed with 2.5% glutaraldehyde for 2 h at room temperature. Samples were processed as in section 2.2.2.1.5.3. and examined with SEM (FEI, Quanta 400F, Eindhoven, Netherlands). Some of the samples were fixed with 4% paraformaldehyde for 30 min at room temperature. hMSCs were immunostained for an osteogenic marker, RUNX2 and cell nuclei was stained with Hoechst 33342. They were imaged with epifluorescence microscope (Zeiss, Germany).

2.2.2.4.5 Real-Time RT-PCR

On Day 2, the expression of RUNX2 and VEGFA were determined as in section 2.2.2.3.2.3. by using a real-time RT-PCR. HPRT1 was employed as housekeeping gene. Results were analyzed with regard to 0% tension sample that was incubated in 21% oxygen tension.

2.2.3 Statistical Analysis

All data were obtained at least in triplicate and expressed as mean value \pm standard deviation. The significance of differences between smooth, micropillar and microchannel patterned surfaces were analyzed using one-way ANOVA with Tukey Test. In this analysis, p values which were equal or smaller than 0.05 were considered as statistically significant. The analysis was performed for cell area, Feret's diameter, aspect ratio and ALP assay. Origin software from OriginLab (USA) was used for the statistical analysis and significant differences were denoted with *p \leq 0.05, **p \leq 0.01 and ***p \leq 0.001.

Rest of the data was analyzed with Graphpad Prism 6 software from Graphpad Software Inc. (USA) for the statistical analysis. $p \le 0.05$ was taken as statistically significant. Statistical differences were presented as $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, and $****p \le 0.0001$. Student's *t*-test was employed for the statistical analysis of the normalized mineralization assay results. Two-way ANOVA was performed for the cell proliferation results of the ELR part, comparison of tensile properties between 2D and 3D scaffolds and comparison of unstrained and strained 3D microenvironments at hypoxic and normoxic conditions. After the two-way ANOVA, Tukey's post hoc test was performed. Significant differences between the groups belong to the rest of the data were examined with one-way ANOVA followed by Tukey's post hoc test.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 SDS-PAGE of Isolated Collagen Type I

The purity of the isolated collagen type I was assessed with SDS-PAGE. Lanel had protein markers that corresponded to bands at 260 kDa, 140 kDa, 100 kDa and 70 kDa (Fig. 3.1). The commercial (lane 2) and isolated collagen (lane 3 and 4) had doublets at molecular weights of 115 and 130 kDa, and at 215 and 235 kDa, respectively. These bands were the characteristic pattern of type I collagen. There were no additional bands other than these which pointed out to the purity of isolated type I collagen.



Figure 3.1: SDS-PAGE of type I collagen isolated from rat tail. Lane 1: the protein markers (ladder); lane 2: commercial type I collagen (1 mg/mL); lanes 3 and 4: isolated collagen type I at 1 mg/mL and 2 mg/mL concentrations, respectively.

3.2 Characterization of Scaffolds

3.2.1 Characterization of Films with Different Compositions of Collagen and Fibroin

3.2.1.1 Degradation

Different compositions of collagen and fibroin were assessed for their suitability to use in *in vitro* tests. Low degrees of swelling and high degradation resistance are essential properties of a substrate to preserve topographical cues created on it in micrometer scale. Rifas et al. showed that human osteoblasts secrete collagenase that breaks down the ECM (Rifas et al., 1989). In this study degradation of protein based films in collagenase type II solution in 3 h was decreased by increasing the fibroin content of the collagen-fibroin films (Table 3.1). With the absence of the fibroin, films degraded fully. The increased fibroin content of the films mostly prevented weight loss if it were 50% or higher. For films with lower fibroin contents more than 30% of the weight was lost after 3 h of enzyme activity. Table 3.1 shows that as the fibroin fraction in the film increases the resistance to collagenase type II activity is improved. This is important especially for when the material is implanted and is exposed to the hydrolytic activity of local enzymes. Degradation of the composite films was also studied in PBS. The uncrosslinked films disintegrated in 1 h. The data presented in Table 3.2 shows that crosslinked films retain their integrity for at least 4 weeks. With the exception of the pure fibroin film, all the other films showed a high level of degradation during the first week. The blend film with 33% (w/w) fibroin composition was selected for further use in cell culture because of its observed stability against degradation and ease of rolling to be used for 3D microenvironment testing. Pure fibroin film was stabilized by self assembly in methanol and was not preferred as a material in this study because of its stiffness. Pattern variation did not cause any difference in degradation level that could be seen from SEM images (Fig. 3.2.A-B). Surface erosion of films was confirmed by

measuring the features of degraded films with NIH-ImageJ software. Ridge and groove widths were 4 μ m and 13 μ m, respectively. Pillar area was 6 x 6 μ m², and interpillar distance was 4.5 μ m.

Table 3.1: Weight loss of crosslinked micropatterned films with different collagenfibroin ratios, incubated in collagenase type II for 3h at $37 \degree C$ (n=3).

Film	Cumulative Weight Loss (%)			
Composition Col:Fib Ratio	Microchannel	Micropillar		
1:0	100	100		
4:1	30.00 ± 4.24	19.00 ± 6.93		
2:1	23.40 ± 4.39	18.75 ± 5.19		
1:1	12.33 ± 2.52	12.75 ± 2.63		
1:9	8.17 ± 2.04	7.00 ± 2.19		

Film	Cumulative Weight Loss (%)						
Composition	Microchannel			Micropillar			
Col:Fib Ratio	Week 1	Week 2	Week 4	Week 1	Week 2	Week 4	
1:0	15.33 ±	14.33 ±	16.33 ±	17.67 ±	20.00 ±	24.50 ±	
	2.31	2.89	3.78	4.04	2.00	3.54	
4:1	13.67 ±	$14.67 \pm$	19.00 ±	13.00 ±	17.00 ±	17.33 ±	
	0.58	1.15	3.00	3.00	1.41	0.58	
2:1	17.67 ±	19.33 ±	20.33 ±	19.33 ±	17.33 ±	21.33 ±	
	0.58	0.58	1.53	1.53	2.89	0.58	
1:1	11.67 ±	$11.50 \pm$	17.33 ±	13.50 ±	16.00 ±	$18.00 \pm$	
	4.04	0.71	4.04	3.53	2.83	2.83	
1:9	8.33 ±	$10.33 \pm$	$10.67 \pm$	9.33 ±	14.67 ±	12.00 ±	
	0.58	1.53	2.08	0.58	2.89	1.41	
0:1	1.50 ±	6.67 ±	15.00 ±	$1.00 \pm$	3.00 ±	8.50 ±	
	0.71	1.53	0	1.00	1.73	2.12	

Table 3.2: Weight loss in PBS was determined during 4 weeks for crosslinked collagen, fibroin and blend films with microchannel and micropillar patterns (37 °C, n=3).



Figure 3.2: SEM micrographs of 2:1 collagen/fibroin (w/w) patterned films in A) microchannel, and B) micropillar form after 4 weeks of *in situ* degradation in PBS (37 °C).

3.2.1.2 Microscopic Characterization

Parallel channels and isotropically distributed pillars were studied for their effect on cell morphology and cell orientation. For this purpose, initially, collagen-fibroin blend film was formulated to obtain biocompatible and mechanically stable scaffolds that can present micropatterns over prolonged culture conditions for continuous cell guidance. SEM imaging confirmed that patterns had sharp contours before crosslinking of films (Fig. 3.3A-B). The surface of uncrosslinked blend films with 33% (w/w) fibroin composition were studied with AFM to obtain more precise knowledge about pattern dimensions as well as depths. Ridge and groove widths, and depth were 4.8 μ m, 10.8 μ m and 4 μ m, respectively (Fig. 3.3C) and pillar area was 7.8 x 7.8 μ m² with 4 μ m height and 2 μ m spacing (Fig. 3.3D).



Figure 3.3: SEM images of film (2:1 collagen/fibroin (w/w) patterned films) surfaces were shown for A) microchannel, B) micropillar patterned blend films before crosslinking. Atomic force microscopy images of C) uncrosslinked microchannel and D) micropillar patterned blend films.

3.2.1.3 FTIR-ATR Analysis

FTIR-ATR spectroscopy indicated that collagen had the characteristic band patterns of amide A (N-H stretching, 3280.6 cm⁻¹), amide B (C=O stretching, 2928.3 cm⁻¹), amide I (N-H stretching, 1632.5 cm⁻¹), amide II (C-N stretching, 1534 cm⁻¹) and amide III (N-H stretching, 1203.9 cm⁻¹) (Fig. 3.4). Absorbance at 1621.8 cm⁻¹, is due to the absorption by the beta sheet conformation (Zhang et al., 2012), and it was 1.6 fold higher with the collagen-fibroin films crosslinked with EDC-NHS than the untreated film.

Collagen and fibroin based films were crosslinked with carbodiimide (EDC) and NHS in a mixture of methanol and water to limit swelling, prevent loss of pattern sharpness and decrease degradation rate. EDC/NHS was also shown as a method for

crosslinking the scaffold without any impairment in the biocompatibility (Vrana et al., 2008; Zorlutuna et al., 2010). Stabilization in methanol solution induced beta-sheet formation for the fibroin part of blend film as it was seen at FTIR-ATR spectra.



Figure 3.4: FTIR-ATR spectra of collagen, fibroin, methanol treated fibroin and methanol treated collagen-fibroin films. β -sheet band is shown with the arrow.

3.2.1.4 Swelling Test

ECM is known for its ability to carry water needed for the metabolism (Cooper, 2000) and therefore, scaffolds also need to contain a comparable amount of water to create an appropriate microenvironment for the cells. Swelling ratio (water uptake capacity of the films) was determined for crosslinked collagen, fibroin and collagen-fibroin blend films. Collagen film gained significantly more water than fibroin film (163% vs 47%) (Fig. 3.5A, C). It is known that the amorphous regions of the scaffold

swell more than the crystalline regions due to fewer contact points between the polymeric chains allowing liquid influx (Sakurada, 1985). The blend (2:1 collagen/fibroin (w/w)) swelled less than the pure collagen film because of the fibroin in the composition of the blend. The presence of collagen twice as much as fibroin led to a significantly higher swelling capacity (139%) than pure fibroin (47%).

3.2.1.5 Thermal Analysis

Glass transition temperature (T_g) provides information about the organization of a material. The thermal properties of the collagen and fibroin reported by several studies. Lu et al. found higher T_g value with the increase in the β -sheet structure of the fibroin after stabilization by the use of water annealing process (Lu et al., 2011). Additionally, formation of crystalline structure was stimulated by the application of EDC crosslinking to collagen-fibroin blend hydrogels which in turn elevated the T_g of the blend material (Lv et al., 2008). DSC revealed that T_g of pure collagen (55.5 °C) was significantly lower than that of pure fibroin (81.5 °C) (Fig. 3.5B, C) due to the β -sheet rich content of fibroin (Das et al., 2015b). The Tg of the blend (58.8 °C) on the other hand is closer to the collagen due to the high collagen content in the blend and much more smaller than fibroin because the crystallinity of fibroin is decreased due to the more amorphous collagen (Shoulders and Raines, 2009). In the rest of the study, collagen-fibroin-elastin like recombinamer (ELR) blend was employed since collagen-fibroin blend has superior properties such as optimum water uptake due to collagen and enhanced mechanical strength owing to presence of fibroin. Additionally, ELR has an HAP nucleation sequence that can help the formation of mineralized tissue at *in vitro* conditions.





spectra. C) Swelling ratios (%) and T_g of unseeded films. Pattern dimensions of D) collagen-fibroin, and E) collagen-fibroin-ELR blend films with microchannel patterns were studied with a profilometer (x20).

3.2.2 ELR Bearing Film

The depth and width of the grooves and ridges of the microchannels were determined as $4.3\pm0.1 \mu m$, $10.0\pm0.3 \mu m$ and $7.4\pm0.3 \mu m$, respectively for the collagenfibroin blend film by using a profilometer (Fig. 3.5D). For the ELR-added films, dimensions were $4.6\pm0.2 \mu m$, $10.3\pm0.4 \mu m$ and $6.8\pm0.3 \mu m$ (Fig. 3.5E) showing that the presence of ELR does not lead to a distinct change in the dimensions and the topography of micropatterns.

3.2.3 2D and 3D Scaffolds

The 2D and rolled 3D scaffolds were easy to handle in the wet state due to swelling of the collagen – fibroin blend. As a gross observation during the optimization of the collagen and fibroin blend, fibroin only films were decided to be stiff in rolling the film. On the contrary, blending collagen with fibroin enabled less stiff structure that was desirable to obtain closer layers. Crosslinking with EDC/NHS was useful for the production of 3D constructs from long film strips since they were easy to be rolled into 3D construct when wetted. The size of the 2D rectangular scaffold in the wet state was 1.5 cm in width and 1.7 cm in length (Fig. 3.6A). The unrolled long strip used to prepare the 3D scaffold was in the same width as the 2D scaffold and 5.7 cm in length. When the rolling was applied along the strip to form the 3D scaffold, the diameter and the width was 0.5 cm and 1.5 cm, respectively.

Constructs were conjugated with fluorescein (green) to observe the microchannels within the 3D scaffold structure showed the number of the layers. Rolled 3D scaffold had fully completed 5 layers and the swelling of the 3D scaffold in liquid led to decrease in the distance between the layers (Fig. 3.6B). The close proximity of the
layers was suitable to form a 3D microenvironment to test the MSC behavior. The microchannel patterns were also evenly distributed into the 3D scaffold and that could help to maintain the MSC alignment throughout the construct.

Also, microchannels on the 2D/3D scaffolds were examined with SEM. Despite of some irregularities on both 2D and 3D, patterns were satisfactory to continue with the *in vitro* cell culture tests (Fig. 3.6C). The gaps between the fixated PDMS molds were cast as smooth surfaces as SEM micrographs of the flat long films showed. When the long films were rolled to form a 3D matrix, the gaps were still visible yet the vast majority of the surface belonged to patterned regions. The sizes of the topographical cues were measured with ImageJ software as 2 μ m, 2 μ m, and 10 μ m respectively for the width and height of the ridges and the width of grooves.



Figure 3.6: Characterization of the 2D/3D collagen-fibroin blend scaffolds. A) Stereo microscopy images of the 2D and 3D scaffolds in wet state (scale bar: 5 mm), B) Fluorescence microscopy (scale bar: 2 mm) and CLSM (scale bar: 200 µm) of the cross-

section of the 3D scaffold. The 3D layered construct was suitable to examine the MSC behavior due to the swelling of the layers which created a close packing of tissue engineered matrix C) SEM of the 2D and 3D scaffolds showing the uniformity of the microchannel patterns (scale bar: 20 μ m). The gaps between the PDMS pieces were transferred to long film strip as smooth surfaces (red dashed line). Cross-section image of the 3D scaffold (scale bar: 200 μ m) and the marks showing the dimension of the micropatterns (scale bar: 20 μ m).

3.3 Osteogenic Differentiation of Human ADSCs on Micropatterned Collagen -Fibroin Blend Films

3.3.1 Characterization of ADSCs

A lipoaspirate was used as the MSC source since its extraction is less invasive and has similar differentiation capacities with the cells from the bone marrow. After isolation, cells were studied for known surface markers of ADSCs CD44, CD45 and CD105 with flow cytometry. Endoglin (CD105) is one of the best known mesenchymal stem cell (MSC) markers and it is commonly used in the identification of ADSCs. Expression of CD105 marker was increased as the passage number increased from 2 to 4 (Fig. 3.7, Table 3.3) which is in agreement with Mitchell et al. (Mitchell et al., 2006). The amounts of negative markers CD44 and CD45 were low. Low CD44 expression was reported by another study that employed ADSCs and silk based scaffolds for bone tissue engineering (Correia et al., 2012). In order to confirm characterization and preservation of CD105 in the Passages 3 to 5, ADSCs were immunostained for this antigen (Fig. 3.8A-C). There were no passages with only DAPI stain, indicating that there was no dedifferentiation of ADSC. Additionally, staining for positive marker CD90 (Fig. 3.8D) and absence of nonspecific primary antibody binding via negative isotype staining (Fig. 3.9) confirmed ADSC phenotype. Fig 3.8 proved that ADSCs did not have specific sites for goat IgG. These results, therefore, proved the successful isolation and expansion of ADSCs up to Passage 5.



Figure 3.7: Flow cytometry readings of passages 2 and 4 for ADSC phenotype characterization.

Table 3.3: Expression of surface markers of two different passages of ADSC phenotype (flow cytometry).

Passage	Expression of Surface Markers (%)			
	CD44	CD45	CD105	
2	0.8	5.6	41.7	
4	3.7	7.8	70.2	



Figure 3.8: Fluorescence microscopy images of ADSCs after immunostaining for CD105 (green) (A-C) and CD90 (green) (D) a positive marker for ADSCs and DAPI (blue) for cell nuclei. Passages A) 3, B) 4 and C) 5. D) Cells immunostained for CD90 (green) another positive ADSC marker, and DAPI for nuclei at Passage 5.



Figure 3.9: Isotype and DAPI staining of ADSCs at passage 5 (scale bar: 100 µm).

3.3.2 Live-Dead Assay for Viability and Alamar Blue Test for Proliferation of

ADSCs on Micropatterned Films

The viability of ADSCs on collagen-fibroin film was studied on patterned surfaces and the unpatterned control. Calcein AM stained cells, which indicates viability, were high in number while there was no ethidium homodimer-1 stained dead cells on the films and the control surface (coverslip) on Day 3 (Figures 3.10A-D). These results indicated that EDC/NHS-crosslinked collagen-silk blend films with or without micropatterns did not have a negative effect on the viability of ADSC. Literature also indicates that collagen and silk fibroin are biocompatible materials as shown by high cell viability of human microvascular endothelial cells on EDC/NHS crosslinked collagen films (Zorlutuna et al., 2009a) and feline fibroblasts (90% viability on Day 3) on biospun *Antheraea mylitta* silk fibers (Mandal and Kundu, 2010).

Alamar Blue assay was carried out for upto 14 days to study the cell proliferation on the films in EGF supplemented medium. EGF was used in medium to shorten the doubling time of the ADSCs without affecting the differentiation capacity (Francis et al., 2010). As Day 1 results indicate initial cell attachment was slightly higher on micropatterned films (Figure 3.11). ADSC numbers were similar to that of TCPS indicating successful attachment of the cells. The micropillar surfaces showed significantly high cell proliferation like the cells on TCPS and proliferation rate increased at later time points. Unpatterned and microchannel patterned surfaces, however, showed relatively lower proliferation rates and their numbers did not increase during the whole duration. In the literature, it was reported that ADSC proliferation on microchannel patterned poly(butylene succinate) film was constant for three days (Coutinho et al., 2012). Higher cell number on micropillar blend films was a result of increased adherence of cells on narrow pillars. This preference was also reported by a recent study which showed adherence of Saos-2 cells onto micropillars mostly by avoiding pillar-free smooth surfaces (Özçelik et al., 2014). Results in the Figure 3.10 shows that collagen and fibroin blend at 2:1 ratio is a cytocompatible scaffold material by sustaining cell viability.



Figure 3.10: Calcein AM (green) and ethidium homodimer-1 (red) double stained ADSCs (initial seeding density of 3 x 10^4) on Day 3, on: A) coverslip, B) unpatterned film, C) microchannel patterned film, D) micropillar patterned film.



Figure 3.11: ADSC proliferation on films and TCPS control (Alamar Blue assay) (n=3, initial seeding density of 3 x 10⁴).

3.3.3 Morphology Changes of ADSCs on Micropatterned Films

Orientation of bone forming cells is especially important as the natural bone tissue is composed of hierarchically organized, anisotropic extracellular matrix and attached nano-sized hydroxyapatite crystals. Figure 3.12 shows that cell alignment and morphology were different with each micropattern as shown by fluorescence and confocal laser scanning microscopy (CLSM). While on the unpatterned film the cells had no orientation (Fig. 3.12A). Majority of the cells on the microchannels were aligned with the channel axis. While cell nuclei and cytoplasm were localized mainly in the grooves of microchannels, with the micropillars, the cells were localized within interspace of the micropillars; their cytoskeleton was stretched. Staining of the microtubule also indicated that ADSCs were in the grooves of the microchannels (Fig. 3.12B) while cells were extended within the gaps of the micropillars. Orientation on

micropillar surfaces is probably the result of the organization of micropillars in x and y directions. That, in turn, may have diverted cell extension from a single direction. Previously, guidance of ADSCs was demonstrated on electrospun thermoresponsive polymer fibers (Rifas et al., 1989). Likewise, other recent studies have also indicated that ADSC could align on physical cues (Tandon et al., 2009; Choi et al., 2012). SEM micrographs supported the observations made by fluorescence microscopy. It is clearly visible that cells were well aligned on (and in) the grooves of microchannels and micropillars (Fig. 3.12C).



Figure 3.12: Fluorescence and SEM micrographs of ADSCs on unpatterned, microchannel and micropillar patterned films on Day 3. A) Fluorescence microscopy

images of ADSCs stained for actin filaments and nuclei with FITC-phalloidin (green) and DAPI (blue for nucleus), respectively. B) CLSM images of anti α -Tubulin immunostaining showed cell microtubule arrangement on unpatterned, channel and pillar micropatterned films (white dashed lines indicate the layers that signals were acquired from and reflected to their parallel 3D side views, scale bar: 250 µm). C) SEM micrographs of the same surfaces.

Quantitative image analysis was used to clarify and compare the morphology changes of ADSCs on microchannel and micropillar patterned blend films. While microchannels oriented 90% of the ADSCs (orientation within $\pm 10^{\circ}$ respect to micropattern axis), unpatterned and micropillar surfaces oriented only 13% and 20% of cells, respectively (Fig. 3.13A). Micropatterns led to significantly longer and smaller cells when compared to those on the unpatterned film (Fig. 3.13B-C). While cell area on unpatterned surfaces was about 300 μ m² this was about 200 μ m² for microchannels and micropillars. This indicates that due to the unobstructed surface, the cells were able to spread better on the unpatterned surfaces. Foldberg et al. also showed decreased ADSC area on nanopatterned polylactic acid films (Foldberg et al., 2012). Aspect ratio of ADSCs was calculated to assess adaptation of cells on these micropatterns by morphology change. Higher cell length, in turn, meant higher aspect ratio on microchannels (Fig. 3.13D). Aspect ratio on microchannels was two times higher than that on micropillar and on unpatterned films. Similarly, rat (Wilson et al., 2001) and canine MSCs (Wood et al., 2012) showed increased alignment and higher aspect ratio on channel patterned surfaces. Human BMSCs have been also tested on channels (pitch length 1.4-4 µm) (Watari et al., 2012). Similar to the present results, cell length and alignment increased significantly on channels but the cell areas did not change significantly.



Figure 3.13: Quantification of cell morphologies on unpatterned, microchannel and micropillar patterned films (minimum number of cells counted = 35). A) alignment, B) length, C) area, and D) aspect ratio. Statistical differences were determined between unpatterned and other groups and between channeled and pillar results.

3.3.4 Osteogenic Differentiation of ADSCs on Micropatterned Films

Osteogenic activity of ADSCs on micropatterned films was tested in osteogenic medium. Cell proliferation results showed that unpatterned and micropillar films supported cell adhesion and proliferation at a higher rate than the microchannel patterned film (Fig. 3.14A). ADSCs were mainly localized in the grooves of the microchannel surface which might have led to restricted spreading which lowered the proliferation rates. High ALP activity on microchannel patterned films indicated that the microchannel topography promoted osteogenic differentiation of ADSCs. The low cell density but high ALP activity might be due to the crowding effect. The low cell density on microchannels might have increased the extension and elongation of ADSC. Similar to our results, McBeath et al. reported human MSCs plated at low densities adopted well spread morphologies which led to differentiation into bone cells, while at high densities, cells adopted round morphology and adipogenic phenotype (Yang et al., 2005). Also, cell spreading has been shown to increase the differentiation of preosteoblastic progenitors into osteoblasts as shown by increased osteopontin and osteocalcin expression (Thomas et al., 2002). After Day 14, cell proliferation rates decreased for all samples due to osteogenic differentiation as mineralized ECM was produced. This was also shown with BMSCs by Coelho et al (Coelho and Fernandes, 2000).

ALP, which is an important marker of early osteogenic activity, was increased for all type of films after 3 weeks of osteogenic medium exposure (Fig. 3.14B). ALP activity of TCPS control, on the other hand, started in the second week and showed an increase until the 4th week. ALP level of ADSCs was found to be higher on microchannel patterned blend film than other type of films at the end of 4 weeks. Notably, when the ALP level was normalized to cell number microchannel patterned film appeared to be the template inducing the highest osteogenic differentiation on the third and fourth weeks. As seen in Fig. 3.14C, specific ALP activity on microchannel patterned film was several times higher than those on other films and the positive control (p<0.001). We can conclude that the high aspect ratio of the cells might have induced by microchannels increased the osteogenic differentiation of ADSCs. In the literature, there is also one study that reported the induction of osteogenic differentiation of stem cells by high aspect ratio (Banas et al., 2007). Similar to our results, this study showed that the high aspect ratio of MSCs, which were achieved chemically by creating long, cell adhesive microislands over a cell repellent surface (polyethylene), led to higher osteogenic markers in contrast to low aspect ratio surfaces which induced adipogenesis. As a consequence, physical micropatterning of a cytocompatible material is a durable surface modification method to control stem cell aspect ratio. Conveniently, the rate of differentiation of cells can be increased without immobilization of cell adhesion factors by using methods such as micro contact printing, which might be not durable on biodegradable surfaces.

The pattern stability during osteogenic activity is an important parameter since these physical cues should be maintained during experiment timeline for effective cell guidance. In this study the micropillars and microchannels preserved their morphology and dimensions even after 28 days in the cell culture medium (Fig. 3.15A). On the unpatterned film ADSCs were spread without any orientation (Fig. 3.15B). Microchannel patterned films on the other hand could not provide adhesion points for pseudopodia as they have a smoother surface than micropillar patterned film. SEM micrographs revealed that cells were confined into microchannel grooves by avoiding the narrower ridges, which may explain the lower proliferation rate on microchannel surfaces. Cells were seen to be localized in certain areas instead of spreading on the whole micropillar surface as detected after 28 days. Presence of a comparatively better cell adhesion environment created by the micropillars was also shown by the SEM micrographs where extensive cell stretching over a number of micropillars simultaneously by the pseudopodia can be seen.



Figure 3.14: The initial seeding amount of ADSCs was 10^4 . Proliferation and ALP activity were monitored on Days 1, 7, 14, 21 and 28. Seeded TCPS control, unpatterned, microchannel and micropillar patterned films were first cultured in the growth medium and then, on Day 7, introduced to osteogenic medium (n=3). Red arrow indicates the beginning of osteogenic induction with osteogenic medium after obtaining Day 7 cell proliferation data. A) ADSC numbers were determined with Alamar Blue assay. B) Cumulative ALP levels were measured for all type of surfaces (n=3), and C) it is normalized to cell number (n=3). Statistical differences between TCPS and other groups were analyzed by one-way ANOVA (*: TCPS vs unpatterned, microchannel and

micropillar; #: unpatterned vs microchannel and micropillar; +: microchannel vs micropillar).

Osteogenic differentiation of ADSCs on micropatterned collagen-fibroin blend films was further investigated by immunostaining of deposited collagen type I and minerals, which are important markers of osteogenic activity of osteoblastic cells. Collagen type I secretion is an early indicator of osteogenic differentiation since osteoblasts stack their collagen in the extracellular matrix (ECM) space where they are entrapped and become mature bone cells. Our results indicate that cells deposited collagen type I effectively on unpatterned and micropatterned collagen-fibroin films (Fig. 3.15C). For micropillar patterned film collagen type I deposition and actin filaments were determined mainly between pillars. Clearly, microchannel surfaces induced effective collagen type I synthesis and orientation which is an indication of anisotropic ECM formation. In the case of unpatterned film, however, random orientation was evident for both deposited collagen I and actin fluorescence emission it is much clear that deposited collagen type I was in microchannels and localized between micropillar space, indicating the pattern guidance on ECM deposition (Fig. 3.15D).

Hydroxyapatite (HAP) mineral deposition was analyzed to determine late osteogenic activity of cells following collagen deposition and ALP activity. For this purpose unpatterned and micropatterned blend films were stained with Alizarin red S which is specific to calcium compounds. It is apparent that all surfaces showed staining independent of topography (Fig. 3.15E). In contrast to unpatterned and micropillar surfaces, which showed even staining, mineral staining was localized into the grooves of the microchannels which indicated anisotropy of ECM deposition by the aligned cells. This is an important feature of functional bone tissue because this tissue is composed of collagen fibers and hydroxyapatite nano crystals which are organized anisotropically for superb mechanical performance (Skedros et al., 2006).





CLSM images of collagen type I immunostaining (green) of cells. D) overlay CLSM image of the collagen type I staining and cytoskeleton staining (red) E) Phase contrast microscopy images of Alizarin red S stained films (scale bar 100 µm).

Furthermore, SEM at high magnification revealed deposited crystals on each type of film surface (Fig. 3.16A). Characteristically, micropillar surface presented rather smaller but numerous crystal agglomerates which were homogenously dispersed. However, on unpatterned and microchannel patterned surfaces relatively larger minerals were deposited. As it happens in the natural bone, hydroxyapatite crystals must be formed on pre-deposited collagen matrix which was oriented by cells aligned on topographies. Parallel to SEM analysis these crystals were also analyzed with Energy Dispersive X-Ray Spectroscopy (EDAX) for elemental analysis of deposited minerals (Fig. 3.16B). According to this analysis Ca/P ratios were determined as 1.34, 1.42 and 1.36 for the unpatterned, microchannel and micropillar film surfaces, respectively. Thus, for all surfaces the Ca/P ratio was close to 1.33 of the in vivo hydroxyapatite precursor octacalcium phosphate (OCP) (Suzuki et al., 1991) and 1.5 of the natural hydroxyapatite component tricalcium phosphate. As a summary, these results show that bone formation on collagen-fibroin blend films was guided efficiently by the micropatterns on the engineered surface through orientation of deposited extracellular matrix and bone minerals.



Figure 3.16: Mineral analysis of unpatterned, microchannel and micropillar patterned blend films' surfaces on Day 28 A) SEM micrographs and b) EDAX area analysis of the SEM field marked with green box for P and Ca elements.

3.4 Comparison of Human ADSCs and HOBs on Collagen – Fibroin – ELR Blend Films

3.4.1 Characterization of HOBs

Human osteoblasts (HOBs) were isolated from the tissues of patients who underwent joint replacement surgery with consent of the patients. HOBs were smaller in size in comparison to ADSCs as the phase contrast microscopy images show (Fig. 3.17A, B). According to literature, HOBs can be half the size of ADSCs but a definite comparison is not possible due to the heterogeneity of the size of ADSCs (Ryu et al., 2013; Wheeless et al., 2016). Additionally, HOB phenotype was confirmed by immunostaining for common human osteoblast markers secreted by HOB: anti-collagen type I for collagen type I and anti-osteopontin for osteopontin (Aubin et al., 1995; El-Amin et al., 2006). These proteins constitute different components of the extracellular matrix in bone. The isolation of HOBs in this study is considered successful because no cells were observed with DAPI-only staining; this indicates that contaminating cells like fibroblasts originating from the isolation step are not present (Fig. 3.17C, D). Immunostaining also indicated that HOBs preserved their phenotype during passaging up to 5 which was the highest number of passage used in this study.

During the HOB isolation periosteum skinned from the bone fragments before cutting them into small pieces. In order to confirm the absence of the MSCs that might be left from the periosteum, cells were immunostained for positive (CD90) and negative (CD34 and CD45) MSC markers. Nucleus was stained with DRAQ5 and propidium iodide depending on the choice of the fluorophore conjugated antibody and the available lasers. There were no stained cells with any of the antibodies including the isotype control (Fig. 3.18). This result showed the absence of MSCs among the isolated HOBs.



Figure 3.17: Cell micrographs. Phase contrast images of A) HOBs and B) ADSCs in tissue culture flask. Individual cells were framed with green boxes. Fluorescence micrographs of HOBs isolated on coverslips. C) Anti-collagen immunostaining (green). D) Anti-osteopontin immunostaining (green) (scale bar: 100 μ m) (Passage 5). Nuclei were stained with DAPI (blue).



Figure 3.18: Immunostaining of HOBs for negative and positive MSC markers was carried out (Passage 7). Cells were seeded on coverslip and stained for nucleus (green)

with DRAQ5 and propidium iodide (green). Isotype, anti-CD34, anti-CD45 and anti-CD90 immunostaining (green) were presented at the second row (Scale bar: 100 µm).

3.4.2 Proliferation of ADSCs and HOBs

Alamar Blue assay was performed to study cell proliferation for 28 days. The osteogenic differentiation of the ADSCs was induced with the introduction in a differentiation medium. The attachment and proliferation rate of the ADSCs and HOBs were compared for their potential to form an engineered bone tissue on biodegradable films. Collagen-fibroin-ELR blend films supported ADSC adhesion and growth (Fig. 3.19). Application of osteogenic medium on Day 7 slowed down the increase in the ADSC proliferation rate and ended in a plateau (on Days 14 and 21) on both TCPS and collagen-fibroin-ELR blend surfaces. No osteogenic medium was used for HOBs since they already possessed osteoblast phenotype (Fig. 3.17C, D) and they showed a proliferation trend as the ADSCs on film. HOBs tested on the same surfaces showed 2fold lower adhesion on both surfaces than ADSCs (Day 1). Cell numbers of both HOBs and ADSCs were higher on TCPS (3.9 cm²) than on film (2.55 cm²) surfaces. Interestingly, number of ADSC on films was approximately 2-fold higher than that of the HOB at each time point. For this reason, it can be stated that ADSCs were more potent to proliferate on collagen-fibroin-ELR blend films, despite the low rate due to differentiation. Previously, HOBs were shown to proliferate more on silk fibroin coated PCL - biphasic calcium phosphate (BCP) scaffolds than uncoated ones (Roohani-Esfahani et al., 2012). Additionally, Gronthos et al. showed that HOBs adhered on the collagen type I surface as well as the surfaces coated with other ECM components (collagen types IV, V, fibronectin, laminin, and vitronectin) (Gronthos et al., 1997). According to these studies HOBs have a preference for both collagen and fibroin materials which is an advantage of the blend film that was discussed in this paper. Similarly, another study pointed out that while ADSCs attached on collagen scaffolds slightly at a higher rate than silk fibroin scaffolds, bone marrow stem cells (BMSCs) attached on both scaffolds without any particular preference for either surface (Mauney et al., 2007).



Figure 3.19: HOB and ADSC proliferation on collagen-fibroin-ELR blend films and TCPS control (Alamar Blue assay) (n=3). On Day 7, an osteogenic medium was added for ADSCs. Statistical differences were indicated with color codes: black (HOB seeded to TCPS vs film), blue (ADSC seeded to TCPS vs film) and green (HOB seeded vs ADSC seeded TCPS).

3.4.3 Surface Characterization and Cell Morphology

Collagen-fibroin-ELR blend films patterned with microchannels were produced with predetermined groove and ridge topographies as SEM micrographs showed (Fig. 3.120A). Additionally, features were mainly preserved as a result of crosslinking with EDC/NHS in methanol and blend films could withstand 27 days of cell culture conditions (Fig. 3.20B). However, some holes in the films could be seen due to film degradation during cell culture in the medium.

HOBs were lower in number than ADSCs and both cells (Fig. 3.20C, D) were found to be guided on the film surfaces. Energy Dispersive X-Ray Spectrometry (EDX) showed no Ca and P atoms on the unseeded (Days 1 and 28) and HOB seeded (Day 28) films. ADSCs, on the other hand, deposited calcium and phosphorus containing compounds on the collagen-fibroin-ELR blend films with a Ca/P ratio of 0.47. This value is lower than that of HAP (1.67) and; its *in vivo* precursor octacalcium phosphate (OCP) (1.33) (Causa et al., 2006) and also tricalcium phosphate (1.5). The reason for the 0.47 needs to be further investigated.



Figure 3.20: SEM micrographs of unseeded collagen-fibroin-ELR blend films on Days A) 1, B) 28 and C) HOB and D) ADSC seeded collagen-fibroin-ELR blend films on Day 28. Magnification: x5,000; x25,000 (inset).

3.4.4 Cell Alignment

Guidance of bone cells is important because it helps an anisotropic ECM deposition by aligned cells. On Day 28, actin filament staining of seeded ADSCs and HOBs showed that cytoskeletons of these cells were aligned along the microchannel direction on patterned collagen-fibroin films (Fig. 3.21A, B). It can be reported that ADSCs and HOBs were aligned on ridges and grooves smaller than the cells as seen in

different types of cells and surfaces (Kenar et al., 2006; Vrana et al., 2007; Zorlutuna et al., 2007). BMSCs were also a supporting example for cell alignment on a similar sized microchannels composed of thermoresponsive poly(N-isopropylacrylamide) films with ELR adsorbed on the surface (Ozturk et al., 2009). Additionally, the guidance of HOBs was reported by Biggs et al. with the best alignment on microchannels of poly(methylmethacrylate) (PMMA) that were in 10 μ m size amongst the ones that had 25 and 100 μ m pattern width (Biggs et al., 2008). Previously, ADSCs were also reported to get aligned on graphene oxide (ridge: 30 μ m, groove: 15 μ m) and collagen-fibroin (ridge: 7.4 μ m, groove: 10 μ m) microchannels with a tendency to differentiate into osteogenic cell line (Kim et al., 2015; Sayin et al., 2015).

3.4.5 Quantification of Mineralization

Minerals deposited by the HOB and ADSC on collagen-fibroin-ELR blend films were quantified with o-cresol phthalein assay on Day 28 in order to get a chemical and more sensitive measurement than EDX. Unseeded collagen-fibroin-ELR blend film, incubated for 28 days, was the control. Calcium was extracted from the film structure by HCl treatment. There was no significant difference between unseeded on Day 28 (14 μ g) and HOB seeded films (24 μ g) (Fig. 3.21C). The low levels of calcium were also supported by EDX analysis. The calcium content on ADSC seeded ELR blend films on Day 28 (274 μ g) was significantly higher than on similar films seeded with HOB. Calcium amount was normalized to cell number and ADSCs deposited significantly higher mineral than HOBs (Fig. 3.21D). This result indicated that ADSCs during the osteogenic differentiation produced more minerals than HOBs.

3.4.6 Mechanical Testing

Films that were used in this study were designed to mimic bone lamellae and were tested along the microchannel direction to investigate the contribution of ECM synthesized by the seeded cells to the tensile properties. Tensile properties of scaffolds that were applied in bone tissue engineering were reported in the literature. Amruthwar et al. studied the effect of ELR addition to collagen hydrogels for bone tissue engineering. Improvement in the UTS and E were observed in ELR-collagen (25:8 mg/mL) hydrogels from 0.34 MPa to 0.99 MPa and 4.06 MPa to 11.41 MPa, respectively (Amruthwar and Janorkar, 2013). These values are higher than the UTS and E values measured in this study. The reason could be due to the use of concentrated protein solutions by Amruthwar et al. Causa et al. reported that addition of 13% (v/v) HAP to PCL scaffolds enhanced the UTS from 0.93 MPa to 2.19 MPa (Causa et al., 2006). For that reason, as far as we know the mechanical properties of bone tissue engineered scaffolds has been enhanced by concentrated solutions of synthetic polymer or with the addition of ceramics. These results in our study showed the importance of seeded cell type on the mechanical strength of bone tissue engineered scaffolds for the first time.

Tensile properties of unseeded collagen-fibroin-ELR blend films on Days 1 and 28 and ADSC and HOB seeded collagen-fibroin-ELR blend films on Day 28 were determined. UTS, E (Fig. 3.21E) and EB were also calculated and the average values were presented in Fig. 3.21F. There was no significant difference for UTS and EB for all groups. On Day 28, unseeded collagen-fibroin-ELR blend film had a higher UTS and E than unseeded film on Day 1. This result could be the stiffening of the film structure due to glycation of collagen in medium as suggested also by Girton et al. (Girton et al., 1999). In this relevant study, 30 mM glucose was present in the medium that resulted in increase in Young's modulus at the end of 10th week. Hu et al. employed films formulated with recombinant human like collagen and fibroin for hepatic tissue engineering purpose. This study showed the elevated EB from 28.7% to 30.9% with the increase of fibroin content by 10% (w/w) in films (Hu, 2006). In our work, on Day 1, E was 0.58 ± 0.13 MPa for unseeded collagen-fibroin-ELR blend film. This value is higher than the literature value of 502±575 kPa which was measured from crosslinked and microchannel patterned HAP nucleated ELR membrane after 7 days of incubation in simulated body fluid (Tejeda-Montes et al., 2014). By taking this result into account, it can be suggested that collagen and fibroin promoted the mechanical strength of the film, even though, no CaP nucleation in simulated body fluid was performed.

ADSC seeding showed no negative effect on the UTS and E however, HOB seeding decreased these properties substantially and led to highest EB. Significantly enhanced E in ADSC seeded film (1.21 MPa) when compared to HOB seeded film (0.41 MPa) could be explained by matrix metalloproteinase-2 (MMP-2) secretion by HOBs *in vitro* as Rifas et al reported (Rifas et al., 1989). A relevant work also showed that MMP-2 is downregulated during osteogenic differentiation of ADSCs (Egusa et al., 2007). Consequently, degradation by MMP-2 might have lowered E significantly for HOB seeded film and helped protein chains to stretch by loosening them. In return, this effect could lead to increase in the EB up to 2-fold (71.32%) when compared to other films. Additionally, Ascenzi et al. applied tensile test in longitudinal direction for wet human fully calcified osteon and measured EB as 6.84% (Ascenzi and Bonucci, 1967) which is fivefold lower than of Day 28 film that ADSCs seeded on $(35.53 \pm 19.18\%)$.



Figure 3.21: Fluorescence micrographs of actin filament staining with Phalloidin (green) on Day 28 indicated the cell alignment along the microchannel direction (white arrow). A) HOB and B) ADSC on collagen-fibroin-ELR blend films (scale bar: 250 μ m). C) Calcium amounts of unseeded collagen-fibroin-ELR blend films, HOB and ADSC seeded collagen-fibroin-ELR blend films on were quantified by o-cresol

phthalein complexone method on Day 28. Statistical differences were determined and differences between each groups were analyzed (*: unseeded film on Day 1 vs other groups, [#]: unseeded film on Day 28 vs HOB and ADSC seeded films on Day 28, ⁺: HOB vs ADSC seeded films on Day 28). D) Calcium amounts of HOB and ADSC seeded films were normalized to cell number. E) Young's modulus of the unseeded and seeded films were presented with the statistical analysis. F) The average ultimate tensile strength (UTS), Young's modulus (E) and elongation at break (EB) values of unseeded and seeded and seeded collagen-fibroin-ELR blend films (n=3-5).

3.5 Human MSCs on 2D and 3D Collagen – Fibroin Blend Scaffolds Under Different Oxygen Tensions

3.5.1 Cell Proliferation

Human MSC content of TCPS, 2D and 3D scaffolds was determined on Days 1 and 3 by using PicoGreen® staining and the results are presented as DNA amount. For samples that were incubated under 5% and 12% oxygen tensions a special incubator was employed. The DNA amount on TCPS was higher than it is on 2D scaffold surface because of the smaller area and dissimilar chemistry of the scaffold (Fig. 3.22, Table 3.4). Cell proliferation on 2D and 3D scaffolds under 12% oxygen concentration was similar to that on TCPS. When the overall results were evaluated, application of the osteogenic medium from the beginning of the cell culture slowed down the cell proliferation in 3D scaffold at a higher extent at 5% and 21% oxygen tensions between Day 1 and 3.



Figure 3.22: The proliferation of the MSCs at 5%, 12% and 21% oxygen tensions was studied on different time points. For the proliferation dsDNA content was quantified by PicoGreen® staining.

Time (Days)	5% O ₂	12% O ₂	21% O ₂	Samples
1	283.28 ± 36.63	326.66 ± 36.43	275.47 ± 39.47	TCPS
3	341.56 ± 29.35	356.58 ± 61.87	269.26 ± 53.32	1015
1	60.24 ± 9.79	176.61 ± 24.02	87.33 ± 38.45	2D
3	88.80 ± 21.98	187.67 ± 53.51	191.03 ± 71.98	
1	442.68 ± 5.42	266.69 ± 31.63	488.59 ± 86.87	3D
3	248.72 ± 63.53	358.78 ± 20.99	355.71 ± 26.19	

Table 3.4: Mean and standard deviation values of the Fig. 3.20 (n=3).

3.5.2 Gene Expression

Relative expression of the RUNX2, osterix, BST1, CD90, and VEGFA were quantified with real-time RT-PCR. The results were reported for each marker according to up or down regulation of gene expression relative to its TCPS control under 21% oxygen tension on Day 3. We employed RUNX2 and osterix as the osteogenic markers in this study. Osteoblast differentiation requires the activation of a molecular switch: RUNX2 (Karsenty et al., 2009) and osterix is regulated at the downstream of the RUNX2 to enhance collagen type I production (Ortuño et al., 2013). MSCs on TCPS and 2D scaffold showed a similar level of expression of the osteogenic markers at all oxygen tensions except for 12% oxygen on TCPS sample which showed a significant increase in RUNX2 (Fig. 3.23). On the other hand, osterix expression on 3D constructs under 21% oxygen was 8 - 9 fold higher than the other oxygen tensions. Additionally, at the 21% oxygen, MSCs expressed osterix 13200 fold higher in 3D construct than on 2D scaffold. The increment in the latter marker, osterix, at 3D showed that MSCs in the 3D construct was ahead in osteogenic differentiation than those on the 2D scaffold for all of the oxygen tensions.



Osteogenic Transcript Levels

Figure 3.23: Gene expression levels of the MSCs (passage number 2) that were seeded on TCPS and 2D scaffold and in 3D construct were determined with real-time RT-PCR to detect the transcripts of osteogenic differentiation with early markers, RUNX2 and osterix (OSX) (n=3). Cells were cultured for 3 days at the oxygen tensions of 5%, 12% and 21%.

The natural bone marrow niche is a hypoxic environment that was reported to induce self-renewal of mesenchymal stem cells by upregulating telomerase and Oct4

activity (Mohyeldin et al., 2010). In our study CD90, a well known MSC marker, and BST1, bone marrow stromal cell antigen 1, were used as indicators of MSC stemness. In the literature, BST1 was found to preserve the self-renewal potential and stem cell marker expression of the MSCs, and it is downregulated as the MSCs proceed in osteogenic differentiation (Aomatsu et al., 2014) as same as the CD90 (Granchi et al., 2010). However, BST1 is preferred more for the interpretation of the MSC multipotency (Kolf et al., 2007; Lv et al., 2014). Owing to being the native MSC marker in vivo unlike the CD90 which is an *in vitro* MSC marker (Deschaseaux et al., 2010), BST1 gives a better insight for the stemness of MSCs at in vivo condition. Additionally, the knowledge of the complex formation between the receptor of the BST1 and β 1 integrin suggested the existence of a correlation between BST1 activity and signal transduction in ECM recognition (Docheva et al., 2007). In our study, as the BST1 and CD90 markers were looked into, hypoxia driven stemness was prominent for TCPS and 2D scaffold (Fig. 3.24). MSCs differentiated more in 3D construct than on 2D scaffold due to the significantly lower expression of stemness markers in 3D. To be more specific, the expression of CD90 at 5% and 12% oxygen tensions and the expression of BST1 at 5% oxygen tension decreased significantly in 3D constructs at all oxygen tensions. Keeping in mind the effect of BST1 on integrin mediated signaling pathways (Aomatsu et al., 2014), here we can see the effect of 3D construct on signal transduction in MSCs. At this point, the influence and importance of cell-matrix and cell-cell interactions on MSC stemness were pointed out by using a 3D construct made of concentric layers. Furthermore, MSCs in 3D construct strongly expressed CD90 under 21% not the 5% oxygen tension. This behavior was quite the opposite that was seen on TCPS and 2D scaffold therefore, MSCs were indeed exposed to lower oxygen tensions due to the limitation of oxygen diffusion throughout the 3D construct by leading higher expression of stemness markers at 21% oxygen.

Stemness Transcript Levels



Figure 3.24: Gene expression levels of the MSCs (passage number 2) that were seeded on TCPS and 2D scaffold and in 3D construct were determined with real-time RT-PCR to detect the transcripts of stemness with BST1 and CD90 (n=3). Cells were cultured for 3 days at the oxygen tensions of 5%, 12% and 21%.

Low oxygen tension activates hypoxia-inducible factor (HIF) (Semenza, 2000) as a key transcriptional regulator of neovessel formation (Pugh and Ratcliffe, 2003). Potier et al. showed that temporary exposure to 1% oxygen induced the VEGF expression at BMSCs (Potier et al., 2007). In our study, mRNA level of VEGFA increased with the 5% oxygen on TCPS control and 2D scaffold in a similar fashion with the literature (Fig. 3.25). We also showed the positive effect of 3D over the 2D regarding significantly higher VEGFA expression on 3D constructs than 2D scaffolds up to 6 – 266 fold at every oxygen tension. Interestingly, 21% oxygen significantly promoted the VEGFA level in 3D, and yet the 5% pO₂ resulted with a statistically higher VEGFA expression than 12% pO₂.

Angiogenic Transcript Level



Figure 3.25: Gene expression levels of the MSCs (passage number 2) that were seeded on TCPS and 2D scaffold and in 3D construct were determined with real-time RT-PCR to detect the transcripts of angiogenic factor with VEGFA (n=3). Cells were cultured for 3 days at the oxygen tensions of 5%, 12% and 21%.

In summary, the contribution of 3D construct on MSC signaling was shown with the enhancement in the osteogenic differentiation and VEGFA expression when compared to TCPS and 2D scaffold at all oxygen tensions. The ECM dimension also altered the MSC behavior between the 2D scaffold and 3D construct at a given oxygen tension. At 21% oxygen, markers of osteogenic differentiation, stemness, and angiogenic differentiation were expressed strongly, whereas, on TCPS and 2D scaffold, 5% oxygen was more effective than the 21% oxygen. Although, the expression of stemness markers are downregulated at 3D construct with the upregulation of osteogenic markers when compared to the 2D scaffold, at 21% oxygen the case was not the same for 3D construct. The elevated expression of stemness and osteogenic differentiation markers at the same time suggested the existence of heterogeneous MSC populations within the 3D construct at 21% oxygen. The most possible answer here the oxygen gradients within the 3D construct can create a phenotype variety among the MSCs by leaving some of them more differentiated while the others not. It can be concluded that the dimension of the scaffold and oxygen tension can simultaneously regulate the stemness, osteogenic differentiation and VEGFA expression capacity of MSCs.

3.5.3 Microscopy (Fluorescence, SEM)

MSCs were seeded on 2D scaffolds and also coverslips instead of TCPS control since TCPS has autofluorescence which makes it unsuitable for fluorescence microscopy imaging. On Day 3, cells were immunostained for RUNX2 which is an early osteogenic marker. The brighter signal intensity of coverslip control that was subjected to 12% oxygen indicated a higher RUNX2 level (Fig. 3.26). On the other hand, on 2D films, the RUNX2 intensity was observed to be higher at 5% and 12% oxygen tensions which indicated the importance of the surface chemistry and topography on osteogenic differentiation of MSC. These results were also supported by the real-time RT-PCR data.

After 3 days of cell culture, SEM was carried out to show the MSCs on the 2D and the core of the 3D scaffolds. On 2D scaffolds, MSCs formed a layer of cells both on ridges and in the grooves of microchannels at all oxygen tensions (Fig. 3.27). When 3D scaffolds were unrolled after 3 days of incubation, it was observed that 21% O_2 led to cell proliferation at the most inner part of the 3D scaffold which was not the case for the 5% and 12% oxygen tensions. The absence of MSCs in the core of 3D scaffolds at hypoxia and arterial blood oxygen pressure indicated the oxygen diffusion was not sufficient to fulfill oxygen demand of the cells at the inner region.



Figure 3.26: Epifluorescence microscope images of MSCs on a coverslip and 2D scaffolds were taken to observe the intensity profile of RUNX2 (red). Nuclei (blue) were stained with Hoechst (scale bar: 100 μ m). Coverslip was used as a substitute of TCPS since TCPS has autofluorescence that can lead to misinterpretation for the fluorescence intensity and also lower the quality of images. MSCs were elongated on the microchannels. Depending on the surface chemistry and topography, the RUNX2 intensity was the highest at 12% oxygen on coverslip and 5% oxygen on 2D scaffold.



Figure 3.27: SEM micrographs on Day 3 showed the presence of MSCs on 2D scaffold at all oxygen tensions. However, except the 21% oxygen, cells were missing at the inner part of the 3D scaffolds at 5 and 12% oxygen tensions despite the homogenous cell seeding to the long film strip. This result showed the importance of the oxygen diffusion within a 3D scaffold at oxygen limited niches.

3.5.4 Oxygen Monitoring

Availability of oxygen inside a 3D construct should be monitored since the oxygen in the medium is not at the same level with the air due to its limited solubility. Oxygen probes were inserted into the core and between the layers of the rolled 3D constructs to measure the oxygen levels. The oxygen level in the core of the 3D construct under 21% oxygen remained the same (data not shown). The region between the layers was also monitored (Fig. 3.28) and readings were obtained between the 124-127 mm Hg which corresponds to 16.3-16.7%. As suggested, these levels were smaller than the ambient oxygen level. On the other hand, at hypoxia, the pO₂ concentration at the core and between the layers decreased continuously and reached an equilibrium around 4%. This behavior was typical thanks to restriction of the oxygen solubility that made the oxygen level even lower than 5%.



Figure 3.28: Partial oxygen measurements of the MSC seeded 3D constructs. Oxygen monitoring was started on Day 0 until there was no change.
3.5.5 Mechanical Testing

Given the complexity of the microenvironmental cues that have an effect on the osteogenic differentiation of stem cells, the quality of the secreted extracellular matrix was evaluated for 2D/3D scaffolds. 5% and 21% oxygen tensions were selected to test the mechanical strength since MSCs exhibited enhanced osteogenic differentiation at these oxygen tensions on 3D construct. MSCs were kept in osteogenic medium for 35 days to permit extracellular matrix production by cells that can alter the mechanical strength of the scaffolds. Testing the scaffolds on Day 35 was also important if the 2D and 3D constructs were implanted as bone grafts and so this time frame was giving the time to MSCs that was needed for osteogenic differentiation. Samples were tested along the microchannel direction to investigate the contribution of the aligned extra cellular matrix to the tensile properties. In order to represent the 3D scaffolds, the outer section was tested for each oxygen conditions. The reason for this preference was the presence of the MSCs at this region when exposed to both oxygen tensions, unlike the inner sections at 5% oxygen. Cells were missing at the inner section of the 3D scaffold at 5% oxygen condition as observed with the SEM micrographs (Fig. 3.29). After 35 days of hypoxic treatment, both ultimate tensile strength (UTS) $(0.46 \pm 0.23 \text{ MPa})$ and Young's modulus (E) $(0.99 \pm 0.19 \text{ MPa})$ of unseeded 2D scaffold was lower than 21% oxygen treated unseeded 2D scaffold (1.89 \pm 0.17 MPa and 1.00 \pm 0.24 MPa) (Fig. 3.26). The decrease in the UTS and E at 5% oxygen tension pointed out the elevated hydrolysis of the blend material with the decreased oxygen availability. On the other hand, there was no negative effect of 5% oxygen on the UTS and E of the unseeded 3D scaffold which pointed out the preservation of the mechanical properties in the case of rolled 3D scaffold due to hindrance against medium hydrolysis. When the seeded scaffolds were compared with the unseeded ones, 21% oxygen led to an improvement in the E for 2D and 3D scaffolds, unlike the 5% oxygen where the mechanical properties remained as same with the unseeded scaffolds. In the case of the comparison between the MSC seeded 3D scaffolds at 21% oxygen, there was a significant increase in UTS and E with regard to unseeded scaffolds from 0.59 ± 0.15 MPa to 1.82 ± 0.27 MPa and 1.06 ± 0.22 MPa to 2.16 ± 0.81 MPa. This enhancement in the mechanical properties of the 3D scaffold is also another indication of the contribution of 3D microenvironments over the maturation of the bone tissue engineered scaffolds. Our study clearly demonstrates the utility of 3D scaffolds to serve a dynamic environment for MSCs to deposit their extra cellular matrix.



Figure 3.29: Ultimate tensile strength (UTS) and Young's modulus (E) of unseeded and MSC seeded 2D/3D scaffolds were determined on the 35th day at 5% and 21% oxygen tensions. Unlike the 2D scaffolds exposed to any of the oxygen tension, 3D microenvironment and the 21% oxygen simultaneously helped MSCs to contribute to UTS and E significantly when compared to the unseeded counterpart.

3.6 Behavior of Human MSCs in 3D Microenvironment Exposed to Cyclic Tensile Force and 5% Oxygen

3.6.1 Cell Proliferation

Mechanical stimulus was applied to MSCs at 1 Hz by stretching the samples 10% in order to drive the cell to osteogenic lineage. The cyclic strain exposure was started for the strained samples on the last day of MSC culture. Cell number on the scaffolds was determined on Day 2 (Fig. 3.30). According to Picogreen staining, the DNA amount was approximately 2-fold higher at 21% oxygen when no tension was applied. Similar result was also observed on Day 3 for 2D scaffolds were not entrapped in collagen hydrogels and 3D collagen-fibroin blend construct (Fig. 3.22). As expected, 10% strain led to significantly lower MSCs under 21% oxygen. This result was also supported by Kearney et al observed the decrease in MSC number on Day 2 after applying 2.5% tensile cyclic strain on collagen coated silicone substrate (Kearney et al., 2010). On the other hand, the trend was the reverse at 5% oxygen. Hypoxia led to a 2-fold higher cell amount than 21% oxygen after exposure to 10% cyclic strain.



Figure 3.30: PicoGreen® assay was used to determine the dsDNA amount on 2D scaffolds after ensile strain application on the second day of 5% or 21% oxygen.

3.6.2 Viability of MSCs

Calcein AM and ethidium homodimer-1 staining showed that 5% oxygen (Fig. 3.31A) and 24 h of 10% tensile strain application (Fig. 3.31B) had no negative effect on cell viability inside the collagen gel as the low cell number stained with ethidium homodimer-1 was observed.



Figure 3.31: MSCs exposed to 5% oxygen during a) No and b) 10% tensile strain application (scale bar: $100 \mu m$).

3.6.3 Gene Expression

The expression of the osteogenic marker, RUNX2 and angiogenic marker, VEGFA were studied with real-time RT-PCR on Day 2. RUNX2 expression was similar for all conditions which could be a result of the short (48 h) cell culture duration (Fig. 3.32). According to literature, the culture time can be prolonged by decreasing the duration of cyclic tension exposure (Sumanasinghe et al., 2006; Kearney et al., 2010). Cyclic tensile strain however, decreased the angiogenic factor expression significantly both at 5% and 21% oxygen. Wilson et al. showed that the formation of endothelial network by microvascular endothelial cells was inhibited under a series of tensile strain exposure (24 h) including 10% (Wilson et al., 2009).



Figure 3.32: RUNX2 and VEGFA expression levels after exposure to 24 h of uniaxial cyclic strain at 5% and 21% oxygen.

3.6.4 Microscopy (Fluorescence, SEM)

The extent of the osteogenic differentiation of MSCs was demonstrated by RUNX2 immunostaining on Day 2. RUNX2 immunostaining showed no difference between the osteogenic activity of the scaffolds dynamically compressed under hypoxic and 21% oxygen conditions (Fig. 3.33) which was also supported by real-time RT-PCR results.

Application of tensile strain which was exerted uniaxially with the microchannel direction changed the morphology of the MSCs (Fig. 3.34). Cells were stretched in the direction of mechanical stimulation due to the force make them to grip microchannels. On the other hand, unstrained MSCs were more circular in shape due to absence of uniaxial tension.



Figure 3.33: RUNX2 immunostaining (red) and nuclei staining (blue) of MSCs on 2D scaffolds after 0% or 10% tensile strain application at 5% or 21% oxygen (scale bar: 100 μ m).





CHAPTER 4

CONCLUSION

Engineering of microenvironmental cues within scaffolds is an advanced approach in tissue engineering field and a key strategy for a guided tissue formation to meet the requirements of natural bone tissue formation. The contributions of 3D microenvironment on bone tissue regeneration were investigated by testing various biochemical and physical factors and comparing the 3D scaffolds with 2D scaffolds. Initially, 2D scaffolds were tested for their efficiency in the formation of bone tissue. Microchannel patterned collagen-fibroin surfaces were more potent surfaces to induce or augment the differentiation of ADSCs into osteogenic lineage by increasing the aspect ratio of the cells. Elastin-like recombinamer (ELR) protein carrying a hydroxyapatite nucleation sequence was used as an additional biochemical cue and ADSCs served as a better cell source on the films than HOBs. Next, the physiological oxygen tensions of bone microenvironment were modeled and assessed on MSCs by testing the 3D scaffold as well as 2D scaffold. The stimulation of osteogenesis, angiogenesis and mechanical properties by 3D microenvironment in comparison to 2D was significant for all of the oxygen tensions that were tested. Although, cyclic tensile strain (10%) did not affect the osteogenic activity on collagen hydrogel embedded films, exposure to strain elongated cell morphology and lowered angiogenic factor expression independent of oxygen level.

As a conclusion, microenvironmental factors that were covered in this study affected the functionality of the bone tissue engineered constructs. Transition from 2D to 3D microenvironment was an instructive cue for bone progenitor cells towards bone tissue. Although the *in vitro* test results proved the utility of the use of micropatterned 3D constructs that could better mimic the *in vivo* for the bone tissue engineering, the functionality of the 3D microenvironments can further be studied in *in vivo* settings.

REFERENCES

Agrawal, C. M.; Ong, J. L.; Appleford, M. R.; Mani, G. *Introduction to Biomaterials: Basic Theory with Engineering Applications*; Cambridge University Press: New York, NY, USA, 2013.

Ahn, S.; Kim, Y.; Lee, H.; Kim, G. A New Hybrid Scaffold Constructed of Solid Freeform-Fabricated PCL Struts and Collagen Struts for Bone Tissue Regeneration: Fabrication, Mechanical Properties, and Cellular Activity. *J. Mater. Chem.* **2012**, *22* (31), 15901.

Amruthwar, S. S.; Janorkar, A. V. In Vitro Evaluation of Elastin-like Polypeptide-Collagen Composite Scaffold for Bone Tissue Engineering. *Dent. Mater.* **2013**, *29* (2), 211–220.

Aomatsu, E.; Takahashi, N.; Sawada, S.; Okubo, N.; Hasegawa, T.; Taira, M.; Miura, H.; Ishisaki, A.; Chosa, N. Novel SCRG1/BST1 Axis Regulates Self-Renewal, Migration, and Osteogenic Differentiation Potential in Mesenchymal Stem Cells. *Sci. Rep.* **2014**, *4*, 1–9.

Arafat, M. T.; Lam, C. X. F.; Ekaputra, A. K.; Wong, S. Y.; Li, X.; Gibson, I. Biomimetic Composite Coating on Rapid Prototyped Scaffolds for Bone Tissue Engineering. *Acta Biomater.* **2011**, *7* (2), 809–820.

Artavanis-Tsakonas, S.; Rand, M. D.; Lake, R. J. Notch Signaling: Cell Fate Control and Signal Integration in Development. *Science*. **1999**, *284* (5415), 770-776.

Ascenzi, A; Bonucci, E. The Tensile Properties of Single Osteons. *Anat. Rec.* **1967**, *158* (4), 375–386.

Ashjian, P.; Elbarbary, A. In Vitro Differentiation of Human Processed Lipoaspirate Cells into Early Neural Progenitors. *Plast Reconstr Surg.* **2003**, *6*, 1922–1931.

Atala, A. Regenerative Medicine Strategies. J. Pediatr. Surg. 2012, 47 (1), 17–28.

Aubin, J. E.; Lui, F.; Malaval, L.; Gupta, A. K. Osteoblast and Chondroblast Differentiation. *Bone* **1995**, *17*, 77S-83S.

Badami, A. S.; Kreke, M. R.; Thompson, M. S.; Riffle, J. S.; Goldstein, A. S. Effect of Fiber Diameter on Spreading, Proliferation, and Differentiation of Osteoblastic Cells on Electrospun Poly(lactic Acid) Substrates. *Biomaterials* **2006**, *27*, 596–606.

Banas, A.; Teratani, T.; Yamamoto, Y.; Tokuhara, M.; Takeshita, F.; Quinn, G.; Okochi,
H.; Ochiya, T. Adipose Tissue-Derived Mesenchymal Stem Cells as a Source of Human
Hepatocytes. *Hepatology* 2007, *46* (1), 219–228.

Barbosa, J. S.; Costa, R. R.; Testera, A. M.; Alonso, M.; Rodríguez-Cabello, J. C.; Mano, J. F. Multi-Layered Films Containing a Biomimetic Stimuli-Responsive Recombinant Protein. *Nanoscale Res. Lett.* **2009**, *4* (10), 1247–1253.

Barnes, C. P.; Sell, S. A.; Boland, E. D.; Simpson, D. G.; Bowlin, G. L. Nanofiber Technology: Designing the next Generation of Tissue Engineering Scaffolds. *Adv. Drug Deliv. Rev.* 2007, *59* (14), 1413–1433.

Barthes, J.; Ozcelik, H.; Hindie, M.; Hasan, A.; Engin, N.; Cell, V.; Özçelik, H.; Hindié, M.; Ndreu-halili, A.; Vrana, N. E. Cell Microenvironment Engineering and Monitoring for Tissue Engineering and Regenerative Medicine: The Recent Advances. *Biomed Res. Int.* **2014**, *2014*.

Beachley, V.; Wen, X. Polymer Nanofibrous Structures: Fabrication, Biofunctionalization, and Cell Interactions. *Prog. Polym. Sci.* **2010**, *35* (7), 868–892.

Biggs, M. J. P.; Richards, R. G.; McFarlane, S.; Wilkinson, C. D. W.; Oreffo, R. O. C.; Dalby, M. J. Adhesion Formation of Primary Human Osteoblasts and the Functional Response of Mesenchymal Stem Cells to 330 nm Deep Microgrooves. *J. R. Soc. Interface* **2008**, *5* (27), 1231–1242.

Billiet, T.; Vandenhaute, M.; Schelfhout, J.; Van Vlierberghe, S.; Dubruel, P. A Review of Trends and Limitations in Hydrogel-Rapid Prototyping for Tissue Engineering. *Biomaterials* **2012**, *33* (26), 6020–6041.

Bini, E.; Foo, C. W. P.; Huang, J.; Karageorgiou, V.; Kitchel, B.; Kaplan, D. L. RGD-Functionalized Bioengineered Spider Dragline Silk Biomaterial. *Biomacromolecules* **2006**, *7* (11), 3139–3145.

Boland, E. D.; Wnek, G. E.; Simpson, D. G.; Pawlowski, K. J.; Bowlin, G. L. Tailoring Tissue Engineering Scaffolds Using Electrostatic Processing Techniques: A Study of Poly(Glycolic Acid). *J. Macromol. Sci. Part A* **2001**, *38* (12), 1231–1243.

den Braber, E. T.; de Ruijter, J. E.; Ginsel, L. A.; von Recum, A. F.; Jansen, J. A. Orientation of ECM Protein Deposition, Fibroblast Cytoskeleton, and Attachment Complex Components on Silicone Microgrooved Surfaces. *J. Biomed. Mater. Res.* **1998**, *40* (2), 291–300.

Bracalello, A.; Santopietro, V.; Vassalli, M.; Marletta, G.; Del Gaudio, R.; Bochicchio,
B.; Pepe, A. Design and Production of a Chimeric Resilin-, Elastin-, and Collagen-like
Engineered Polypeptide. *Biomacromolecules* 2011, *12* (8), 2957–2965.

Bunnell, B. A.; Flaat, M.; Gagliardi, C.; Patel, B.; Ripoll, C. Adipose-Derived Stem Cells: Isolation, Expansion and Differentiation. *Methods* **2008**, *45* (2), 115–120.

Burg, K. J.; Porter, S.; Kellam, J. F. Biomaterial Developments for Bone Tissue Engineering. *Biomaterials* **2000**, *21* (23), 2347–2359.

Calvi, L.; Adams, G.; Weibrecht, K.; Weber, J.; Olson, D.; Knight, M.; Martin, R.; Schipani, E.; Divieti, P.; Bringhurst, F.; et al. Osteoblastic Cells Regulate the Haematopoietic Stem Cell Niche. *Nature* **2003**, *425* (6960), 841–846.

Carter, D. R.; Beaupré, G. S.; Giori, N. J.; Helms, J. A. Mechanobiology of Skeletal Regeneration. *Clin. Orthop. Relat. Res.* **1998**, *355*.

Causa, F.; Netti, P. A.; Ambrosio, L.; Ciapetti, G.; Baldini, N.; Pagani, S.; Martini, D.; Giunti, A. Poly-Epsilon-Caprolactone/hydroxyapatite Composites for Bone Regeneration: In Vitro Characterization and Human Osteoblast Response. *J. Biomed. Mater. Res. - Part A* **2006**, *76* (1), 151–162.

Chai, C.; Leong, K. W. Biomaterials Approach to Expand and Direct Differentiation of

Stem Cells. Mol. Ther. 2007, 15 (3), 467-480.

Chaudhuri, O.; Gu, L.; Klumpers, D.; Darnell, M.; Bencherif, S. A.; Weaver, J. C.; Huebsch, N.; Lee, H.; Lippens, E.; Duda, G. N.; et al. Hydrogels with Tunable Stress Relaxation Regulate Stem Cell Fate and Activity. *Nat. Mater.* **2015**, *15* (3), 326–334.

Cheema, U.; Rong, Z.; Kirresh, O.; Macrobert, A. J.; Vadgama, P.; Brown, R. A. Oxygen Diffusion through Collagen Scaffolds at Defined Densities : Implications for Cell Survival in Tissue Models. **2012**, No. February 2011, 77–84.

Chen, W.; Shao, Y.; Li, X.; Zhao, G.; Fu, J. Nanotopographical Surfaces for Stem Cell Fate Control: Engineering Mechanobiology from the Bottom. *Nano Today* **2014**, *9* (6), 759–784.

Choi, B. H.; Choi, Y. S.; Kang, D. G.; Kim, B. J.; Song, Y. H.; Cha, H. J. Cell Behavior on Extracellular Matrix Mimic Materials Based on Mussel Adhesive Protein Fused with Functional Peptides. *Biomaterials* **2010**, *31* (34), 8980–8988.

Choi, Y. S.; Vincent, L. G.; Lee, A. R.; Kretchmer, K. C.; Chirasatitsin, S.; Dobke, M.K.; Engler, A. J. The Alignment and Fusion Assembly of Adipose-Derived Stem Cells on Mechanically Patterned Matrices. *Biomaterials* 2012, *33* (29), 6943–6951.

Claes, L. E.; Heigele, C. A. Magnitudes of Local Stress and Strain along Bony Surfaces Predict the Course and Type of Fracture Healing. *J. Biomech.* **1999**, *32* (3), 255–266.

Clarke, B. Normal Bone Anatomy and Physiology. *Clin. J. Am. Soc. Nephrol.* 2008, *3 Suppl 3*, S131-9.

Coelho, M. J.; Fernandes, M. H. Human Bone Cell Cultures in Biocompatibility Testing. Part II: Effect of Ascorbic Acid, β -Glycerophosphate and Dexamethasone on Osteoblastic Differentiation. *Biomaterials* **2000**, *21* (11), 1095–1102.

Cooper, G. M. *The Cell - A Molecular Approach*, 2nd Edition.; Sunderland (MA): Sinauer Associates, 2000.

Correia, C.; Bhumiratana, S.; Yan, L. P.; Oliveira, A. L.; Gimble, J. M.; Rockwood, D.; Kaplan, D. L.; Sousa, R. A.; Reis, R. L.; Vunjak-Novakovic, G. Development of Silk-

Based Scaffolds for Tissue Engineering of Bone from Human Adipose-Derived Stem Cells. *Acta Biomater.* **2012**, *8* (7), 2483–2492.

Costa, R. R.; Custódio, C. A.; Arias, F. J.; Rodríguez-Cabello, J. C.; Mano, J. F. Layerby-Layer Assembly of Chitosan and Recombinant Biopolymers into Biomimetic Coatings with Multiple Stimuli-Responsive Properties. *Small* **2011**, *7* (18), 2640–2649.

Coutinho, D. F.; Gomes, M. E.; Neves, N. M.; Reis, R. L. Development of Micropatterned Surfaces of Poly(butylene Succinate) by Micromolding for Guided Tissue Engineering. *Acta Biomater.* **2012**, *8* (4), 1490–1497.

Das, R. K.; Gocheva, V.; Hammink, R.; Zouani, O. F.; Rowan, A. E. Stress-Stiffening-Mediated Stem-Cell Commitment Switch in Soft Responsive Hydrogels. *Nat. Mater.*2015a, 15 (3), 318–325.

Das, S.; Pati, F.; Choi, Y. J.; Rijal, G.; Shim, J. H.; Kim, S. W.; Ray, A. R.; Cho, D. W.; Ghosh, S. Bioprintable, Cell-Laden Silk Fibroin-Gelatin Hydrogel Supporting Multilineage Differentiation of Stem Cells for Fabrication of Three-Dimensional Tissue Constructs. *Acta Biomater.* **2015b**, *11* (1), 233–246.

Deschaseaux, F.; Pontikoglou, C.; Sensébé, L. Bone Regeneration: The Stem/progenitor Cells Point of View. J. Cell. Mol. Med. 2010, 14 (1–2), 103–115.

Docheva, D.; Popov, C.; Mutschler, W.; Schieker, M. Human Mesenchymal Stem Cells in Contact with Their Environment: Surface Characteristics and the Integrin System. *J. Cell. Mol. Med.* **2007**, *11* (1), 21–38.

Dorozhkin, S. V. Nanosized and Nanocrystalline Calcium Orthophosphates. *Acta Biomater.* **2010**, *6* (3), 715–734.

Egusa, H.; Iida, K.; Kobayashi, M.; Lin, T. Y.; Zhu, M.; Zuk, P. A; Wang, C. J.; Thakor, D. K.; Hedrick, M. H.; Nishimura, I. Downregulation of Extracellular Matrix-Related Gene Clusters during Osteogenic Differentiation of Human Bone Marrow- and Adipose Tissue-Derived Stromal Cells. *Tissue Eng.* **2007**, *13* (10), 2589–2600.

El-Amin, S. F.; Botchwey, E.; Tuli, R.; Kofron, M. D.; Mesfin, A.; Sethuraman, S.;

Tuan, R. S.; Laurencin, C. T. Human Osteoblast Cells: Isolation, Characterization, and Growth on Polymers for Musculoskeletal Tissue Engineering. *J. Biomed. Mater. Res. - Part A* **2006**, *76* (3), 439–449.

Eliasson, P.; Jönsson, J. I. The Hematopoietic Stem Cell Niche: Low in Oxygen but a Nice Place to Be. *J. Cell. Physiol.* **2010**, *222* (1), 17–22.

Engler, A. J.; Sen, S.; Sweeney, H. L.; Discher, D. E. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* **2006**, *126* (4), 677–689.

Ermis, M.; Baran, E. T.; Dursun, T.; Antmen, E.; Hasirci, V. Contribution of Physical Forces on the Design of Biomimetic Tissue Substitutes. *Bio-inspired Mater. Biomed. Eng.* **2014**, *9781118369*, 59–76.

Flemming, R. G.; Murphy, C. J.; Abrams, G. A.; Goodman, S. L.; Nealey, P. F. Effects of Synthetic Micro- and Nano-Structured Surfaces on Cell Behavior. *Biomaterials* **1999**, *20* (6), 573–588.

Foldberg, S.; Petersen, M.; Fojan, P.; Gurevich, L.; Fink, T.; Pennisi, C. P.; Zachar, V. Patterned Poly(lactic Acid) Films Support Growth and Spontaneous Multilineage Gene Expression of Adipose-Derived Stem Cells. *Colloids Surf. B. Biointerfaces* **2012**, *93*, 92–99.

Francis, M. P.; Sachs, P. C.; Elmore, L. W.; Holt, S. E. Isolating Adipose-Derived Mesenchymal Stem Cells from Lipoaspirate Blood and Saline Fraction. *Organogenesis* **2010**, 6(1), 11–14.

Gasser, T. C.; Ogden, R. W.; Holzapfel, G. A. Hyperelastic Modelling of Arterial Layers with Distributed Collagen Flbre Orientations. *J. R. Soc. Interface* **2006**, *3* (6), 15–35.

Gattazzo, F.; Urciuolo, A.; Bonaldo, P. Extracellular Matrix: A Dynamic Microenvironment for Stem Cell Niche. *Biochimica et Biophysica Acta* **2014**, *1840* (8), 2506–2519.

Gauvin, R.; Chen, Y. C.; Lee, J. W.; Soman, P.; Zorlutuna, P.; Nichol, J. W.; Bae, H.; Chen, S.; Khademhosseini, A. Microfabrication of Complex Porous Tissue Engineering Scaffolds Using 3D Projection Stereolithography. *Biomaterials* 2012, *33* (15), 3824–3834.

Girton, T. S.; Oegema, T. R.; Tranquillo, R. T. Exploiting Glycation to Stiffen and Strengthen Tissue Equivalents for Tissue Engineering. *J. Biomed. Mater. Res.* **1999**, *46* (1), 87–92.

Gomes, S.; Leonor, I. B.; Mano, J. F.; Reis, R. L.; Kaplan, D. L. Spider Silk-Bone Sialoprotein Fusion Proteins for Bone Tissue Engineering. *Soft Matter* **2011**, *7* (10), 4964.

Gomes, S.; Leonor, I. B.; Mano, J. F.; Reis, R. L.; Kaplan, D. L. Natural and Genetically Engineered Proteins for Tissue Engineering. *Prog. Polym. Sci.* **2012**, *37* (1), 1–17.

Granchi, D.; Ochoa, G.; Leonardi, E.; Devescovi, V.; Baglio, S. R.; Osaba, L.; Baldini, N.; Ciapetti, G. Gene Expression Patterns Related to Osteogenic Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells during Ex Vivo Expansion. *Tissue Eng. Part C. Methods* **2010**, *16* (3), 511–524.

Green, J. A.; Yamada, K. M. Three-Dimensional Microenvironments Modulate Fibroblast Signaling Responses. *Adv. Drug Deliv. Rev.* **2007**, *59* (13), 1293–1298.

Griffith, L. G.; Swartz, M. A. Capturing Complex 3D Tissue Physiology in Vitro. *Nat. Rev. Mol. cell Biol.* **2006**, *7* (3), 211–224.

Gronthos, S.; Stewart, K.; Graves, S. E.; Hay, S.; Simmons, P. J. Integrin Expression and Function on Human. *J. Bone Miner. Res.* **1997**, *12* (8), 1189–1197.

Hoyer, B.; Bernhardt, A.; Heinemann, S.; Stachel, I.; Meyer, M.; Gelinsky, M. Biomimetically Mineralized Salmon Collagen Scaffolds for Application in Bone Tissue Engineering. *Biomacromolecules* **2012**, *13* (4), 1059–1066.

Hu, K. Biocompatible Fibroin Blended Films with Recombinant Human-like Collagen for Hepatic Tissue Engineering. *J. Bioact. Compat. Polym.* **2006**, *21* (1), 23–37.

Huang, Y.; Lü, X.; Qian, W.; Tang, Z.; Zhong, Y. Competitive Protein Adsorption on Biomaterial Surface Studied with Reflectometric Interference Spectroscopy. *Acta* Biomater. 2010, 6 (6), 2083–2090.

Isikli, C.; Hasirci, V.; Hasirci, N. Development of Porous Chitosan – Gelatin / Hydroxyapatite Composite Scaffolds for Hard Tissue-Engineering Applications. *J. Tissue Eng. Regen. Med.* **2012**, *6*, 135–143.

Kane, R.; Ma, P. X. Mimicking the Nanostructure of Bone Matrix to Regenerate Bone. *Mater. Today* **2013**, *16* (11), 418–423.

Karsenty, G.; Kronenberg, H. M.; Settembre, C. Genetic Control of Bone Formation. *Annu. Rev. Cell Dev. Biol.* **2009**, *25*, 629–648.

Kearney, E. M.; Farrell, E.; Prendergast, P. J.; Campbell, V. A. Tensile Strain as a Regulator of Mesenchymal Stem Cell Osteogenesis. *Ann. Biomed. Eng.* **2010**, *38* (5), 1767–1779.

Kenar, H.; Köse, G. T.; Hasirci, V. Tissue Engineering of Bone on Micropatterned Biodegradable Polyester Films. *Biomaterials* **2006**, *27* (6), 885–895.

Kenar, H.; Kocabas, A.; Aydinli, A.; Hasirci, V. Chemical and Topographical Modification of PHBV Surface to Promote Osteoblast Alignment and Confinement. *J. Biomed. Mater. Res. A* **2008**, *85* (4), 1001–1010.

Kern, S.; Eichler, H.; Stoeve, J.; Klüter, H.; Bieback, K. Comparative Analysis of Mesenchymal Stem Cells from Bone Marrow, Umbilical Cord Blood, or Adipose Tissue. *Stem Cells* **2006**, *24* (5), 1294–1301.

Khademhosseini, A.; Langer, R. A Decade of Progress in Tissue Engineering. *Nat. Protoc.* **2016**, *11* (10), 1775–1781.

Kim, H. N.; Jiao, A.; Hwang, N. S.; Kim, M. S.; Kang, D. H.; Kim, D. H.; Suh, K. Y. Nanotopography-Guided Tissue Engineering and Regenerative Medicine. *Adv. Drug Deliv. Rev.* **2013**, *65* (4), 536–558.

Kim, T. H.; Shah, S.; Yang, L.; Yin, P. T.; Hossain, M. K.; Conley, B.; Choi, J. W.; Lee,
K. B. Controlling Differentiation of Adipose-Derived Stem Cells Using Combinatorial
Graphene Hybrid-Pattern Arrays. ACS Nano 2015, 9 (4), 3780–3790.

Kolf, C. M.; Cho, E.; Tuan, R. S. Mesenchymal Stromal Cells: Biology of Adult Mesenchymal Stem Cells: Regulation of Niche, Self-Renewal and Differentiation. *Arthritis Res. & Therapy* **2007**, *9* (1), 204.

Kolind, K.; Kraft, D.; Bøggild, T.; Duch, M.; Lovmand, J.; Pedersen, F. S.; Bindslev, D. A.; Bünger, C. E.; Foss, M.; Besenbacher, F. Control of Proliferation and Osteogenic Differentiation of Human Dental-Pulp-Derived Stem Cells by Distinct Surface Structures. *Acta Biomater.* **2014**, *10* (2), 641–650.

Kraning-Rush, C. M.; Carey, S. P.; Califano, J. P.; Smith, B. N.; Reinhart-King, C. A. The Role of the Cytoskeleton in Cellular Force Generation in 2D and 3D Environments. *Phys. Biol.* **2011**, *8* (1), 15009.

Lacroix, D.; Prendergast, P. J. A Mechano-Regulation Model for Tissue Differentiation during Fracture Healing: Analysis of Gap Size and Loading. *J. Biomech.* **2012**, *35* (9), 1163–1171.

Lee, J.; Abdeen, A. A.; Zhang, D.; Kilian, K. A. Directing Stem Cell Fate on Hydrogel Substrates by Controlling Cell Geometry, Matrix Mechanics and Adhesion Ligand Composition. *Biomaterials* **2013a**, *34* (33), 8140–8148.

Lee, J. M.; Mhawech-Fauceglia, P.; Lee, N.; Parsanian, L. C.; Lin, Y. G.; Gayther, S. A.; Lawrenson, K. A Three-Dimensional Microenvironment Alters Protein Expression and Chemosensitivity of Epithelial Ovarian Cancer Cells in Vitro. *Lab. Invest.* **2013b**, *93* (5), 528–542.

Lee, W.; Debasitis, J. C.; Lee, V. K.; Lee, J. H.; Fischer, K.; Edminster, K.; Park, J. K.; Yoo, S. S. Multi-Layered Culture of Human Skin Fibroblasts and Keratinocytes through Three-Dimensional Freeform Fabrication. *Biomaterials* **2009**, *30* (8), 1587–1595.

Li, C.; Vepari, C.; Jin, H.; Joo, H.; Kaplan, D. L. Electrospun Silk-BMP-2 Scaffolds for Bone Tissue Engineering. **2006**, *27*, 3115–3124.

Li, L.; Teller, S.; Clifton, R. J.; Jia, X.; Kiick, K. L. Tunable Mechanical Stability and Deformation Response of a Resilin-Based Elastomer. *Biomacromolecules* **2011**, *12* (6),

2302-2310.

Lim, J. Y.; Donahue, H. J. Cell Sensing and Response to Micro- and Nanostructured Surfaces Produced by Chemical and Topographic Patterning. *Tissue Eng.* **2007**, *13* (8), 1879–1891.

Loboa, E. G.; Fang, T. D.; Warren, S. M.; Lindsey, D. P.; Fong, K. D.; Longaker, M. T.; Carter, D. R. Mechanobiology of Mandibular Distraction Osteogenesis: Experimental Analyses with a Rat Model. *Bone* **2004**, *34* (2), 336–343.

Loboa, E. G.; Fang, T. D.; Parker, D. W.; Warren, S. M.; Fong, K. D.; Longaker, M. T.; Carter, D. R. Mechanobiology of Mandibular Distraction Osteogenesis: Finite Element Analyses with a Rat Model. *J. Orthop. Res.* **2005**, *23* (3), 663–670.

Van Lonkhuyzen, D. R.; Hollier, B. G.; Shooter, G. K.; Leavesley, D. I.; Upton, Z. Chimeric Vitronectin:insulin-like Growth Factor Proteins Enhance Cell Growth and Migration through Co-Activation of Receptors. *Growth Factors* **2007**, *25* (5), 295–308.

Lu, Q.; Zhang, B.; Li, M.; Zuo, B.; Kaplan, D. L.; Huang, Y.; Zhu, H. Degradation Mechanism and Control of Silk Fibroin. *Biomacromolecules* **2011**, *12*, 1080–1086.

Lund, A. W.; Yener, B.; Stegemann, J. P.; Plopper, G. E. The Natural and Engineered 3D Microenvironment as a Regulatory Cue during Stem Cell Fate Determination. *Tissue Eng. Part B. Rev.* **2009**, *15* (3), 371–380.

Lv, F. J.; Tuan, R. S.; Cheung, K. M. C.; Leung, V. Y. L. Concise Review: The Surface Markers and Identity of Human Mesenchymal Stem Cells. *Stem Cells* **2014**, *32* (6), 1408–1419.

Lv, Q.; Hu, K.; Feng, Q.; Cui, F. Fibroin/collagen Hybrid Hydrogels with Crosslinking Method: Preparation, Properties, and Cytocompatibility. *J. Biomed. Mater. Res. Part A* **2008**, *84A* (1), 198–207.

Malafaya, P. B.; Silva, G. A.; Reis, R. L. Natural-Origin Polymers as Carriers and Scaffolds for Biomolecules and Cell Delivery in Tissue Engineering Applications. *Adv. Drug Deliv. Rev.* **2007**, *59*, 207–233.

Mandal, B. B.; Kundu, S. C. Biospinning by Silkworms: Silk Fiber Matrices for Tissue Engineering Applications. *Acta Biomater.* **2010**, *6* (2), 360–371.

Marenzana, M.; Arnett, T. R. The Key Role of the Blood Supply to Bone. *Bone Res.* **2013**, *1* (3), 203–215.

Mauney, J. R.; Nguyen, T.; Gillen, K.; Kirker-Head, C.; Gimble, J. M.; Kaplan, D. L. Engineering Adipose-like Tissue in Vitro and in Vivo Utilizing Human Bone Marrow and Adipose-Derived Mesenchymal Stem Cells with Silk Fibroin 3D Scaffolds. *Biomaterials* **2007**, *28* (35), 5280–5290.

McBeath, R.; Pirone, D. M.; Nelson, C. M.; Bhadriraju, K.; Chen, C. S. Cell Shape, Cytoskeletal Tension, and RhoA Regulate Stem Cell Lineage Commitment. *Dev. Cell* **2004**, *6* (4), 483–495.

McManus, M.; Boland, E.; Sell, S.; Bowen, W.; Koo, H.; Simpson, D.; Bowlin, G. Electrospun Nanofibre Fibrinogen for Urinary Tract Tissue Reconstruction. *Biomed. Mater.* **2007**, *2* (4), 257–262.

Melchels, F. P. W.; Domingos, M. A. N.; Klein, T. J.; Malda, J.; Bartolo, P. J.; Hutmacher, D. W. Additive Manufacturing of Tissues and Organs. *Prog. Polym. Sci.* **2012**, *37* (8), 1079–1104.

Merrett, K.; Liu, W.; Mitra, D.; Camm, K. D.; McLaughlin, C. R.; Liu, Y.; Watsky, M. A.; Li, F.; Griffith, M.; Fogg, D. E. Synthetic Neoglycopolymer-Recombinant Human Collagen Hybrids as Biomimetic Crosslinking Agents in Corneal Tissue Engineering. *Biomaterials* **2009**, *30* (29), 5403–5408.

Mieszawska, A. J.; Nadkarni, L. D.; Perry, C. C.; Kaplan, D. L. Nanoscale Control of Silica Particle Formation via Silk-Silica Fusion Proteins for Bone Regeneration. *Chem. Mater.* **2010**, *22* (20), 5780–5785.

Mitchell, J. B.; McIntosh, K.; Zvonic, S.; Garrett, S.; Floyd, Z. E.; Kloster, A.; Di Halvorsen, Y.; Storms, R. W.; Goh, B.; Kilroy, G.; et al. Immunophenotype of Human Adipose-Derived Cells: Temporal Changes in Stromal-Associated and Stem Cell-

Associated Markers. Stem Cells 2006, 24 (2), 376–385.

Mohyeldin, A.; Garzón-Muvdi, T.; Quiñones-Hinojosa, A. Oxygen in Stem Cell Biology: A Critical Component of the Stem Cell Niche. *Cell Stem Cell* **2010**, *7*, 150– 161.

Mooney, D. J.; Baldwin, D. F.; Suh, N. P.; Vacanti, J. P.; Langer, R. Novel Approach to Fabricate Porous Sponges of Poly(d,l-Lactic-Co-Glycolic Acid) without the Use of Organic Solvents. *Biomaterials* **1996**, *17* (14), 1417–1422.

Murugan, R.; Ramakrishna, S. Development of Nanocomposites for Bone Grafting. *Compos. Sci. Technol.* **2005**, *65* (15–16), 2385–2406.

Nair, L. S.; Laurencin, C. T. Biodegradable Polymers as Biomaterials. *Prog. Polym. Sci.* **2007**, *32* (8–9), 762–798.

Natale, C. F.; Ventre, M.; Netti, P. A. Tuning the Material-Cytoskeleton Crosstalk via Nanoconfinement of Focal Adhesions. *Biomaterials* **2014**, *35* (9), 2743–2751.

Nguyen, L. H.; Annabi, N.; Nikkhah, M.; Bae, H.; Binan, L.; Park, S.; Kang, Y.; Yang, Y.; Khademhosseini, A. Vascularized Bone Tissue Engineering: Approaches for Potential Improvement. *Tissue Eng. Part B Rev.* **2012**, *18* (5), 363–382.

Nikkhah, M.; Eshak, N.; Zorlutuna, P.; Annabi, N. Directed Endothelial Cell Morphogenesis in Micropatterned Gelatin Methacrylate Hydrogels. *Biomaterials* **2012**, *33*, 9009–9018.

Ortuño, M. J.; Susperregui, A. R. G.; Artigas, N.; Rosa, J. L.; Ventura, F. Osterix Induces Col1a1 Gene Expression through Binding to Sp1 Sites in the Bone Enhancer and Proximal Promoter Regions. *Bone* **2013**, *52* (2), 548–556.

Ozturk, N.; Girotti, A.; Kose, G. T.; Rodríguez-Cabello, J. C.; Hasirci, V. Dynamic Cell Culturing and Its Application to Micropatterned, Elastin-like Protein-Modified poly(N-Isopropylacrylamide) Scaffolds. *Biomaterials* **2009**, *30* (29), 5417–5426.

Özçelik, H.; Padeste, C.; Hasirci, V. Systematically Organized Nanopillar Arrays Reveal Differences in Adhesion and Alignment Properties of BMSC and Saos-2 Cells. *Colloids*

Surf. B. Biointerfaces 2014, 119, 71–81.

Pallela, R.; Venkatesan, J.; Janapala, V. R.; Kim, S. K. Biophysicochemical Evaluation of Chitosan-Hydroxyapatite-Marine Sponge Collagen Composite for Bone Tissue Engineering. *J. Biomed. Mater. Res. A* **2011**, 486–495.

Parenteau-Bareil, R.; Gauvin, R.; Berthod, F. Collagen-Based Biomaterials for Tissue Engineering Applications. *Materials*. **2010**, *3* (3), 1863–1887.

Pati, F.; Datta, P.; Adhikari, B.; Dhara, S.; Ghosh, K.; Das Mohapatra, P. K. Collagen Scaffolds Derived from Fresh Water Fish Origin and Their Biocompatibility. *J. Biomed. Mater. Res. A* **2012**, *100* (4), 1068–1079.

Place, E. S.; George, J. H.; Williams, C. K.; Stevens, M. M. Synthetic Polymer Scaffolds for Tissue Engineering. *Chem. Soc. Rev.* **2009**, *38* (4), 1139–1151.

Planat-Benard, V.; Silvestre, J. S.; Cousin, B.; André, M.; Nibbelink, M.; Tamarat, R.; Clergue, M.; Manneville, C.; Saillan-Barreau, C.; Duriez, M.; et al. Plasticity of Human Adipose Lineage Cells toward Endothelial Cells: Physiological and Therapeutic Perspectives. *Circulation* **2004**, *109* (5), 656–663.

Potier, E.; Ferreira, E.; Andriamanalijaona, R.; Pujol, J. P.; Oudina, K.; Logeart-Avramoglou, D.; Petite, H. Hypoxia Affects Mesenchymal Stromal Cell Osteogenic Differentiation and Angiogenic Factor Expression. *Bone* **2007**, *40* (4), 1078–1087.

Pradel, W.; Mai, R.; Gedrange, T.; Lauer, G. Cell Passage and Composition of Culture Medium Effects Proliferation and Differentiation of Human Osteoblast-like Cells from Facial Bone. *J. Physiol. Pharmacol.* **2008**, *59*, 47–58.

Pugh, C. W.; Ratcliffe, P. J. Regulation of Angiogenesis by Hypoxia: Role of the HIF System. *Nat. Med.* **2003**, *9* (6), 677–684.

Qi, M.; Hu, J.; Zou, S.; Chen, H.; Zhou, H.; Mechanical, L. H. Mechanical Strain Induces Osteogenic Differentiation: Cbfa1 and Ets-1 Expression in Stretched Rat Mesenchymal Stem Cells. **2008**, 453–458.

Rajzer, I.; Menaszek, E.; Kwiatkowski, R.; Planell, J. A.; Castano, O. Electrospun

Gelatin/poly(ε-Caprolactone) Fibrous Scaffold Modified with Calcium Phosphate for Bone Tissue Engineering. *Mater. Sci. Eng. C* **2014**, *44*, 183–190.

Reissis, Y.; García-Gareta, E.; Korda, M.; Blunn, G. W.; Hua, J. The Effect of Temperature on the Viability of Human Mesenchymal Stem Cells. *Stem Cell Res. Ther.* **2013**, *4* (6), 139.

Rifas, L.; Halstead, L. R.; Peck, W. A.; Avioli, L. V; Welgus, H. G. Human Osteoblasts In Vitro Secrete Tissue Inhibitor of Metalloproteinases and Gelatinase but Not Interstitial Collagenase as Major Cellular Products. *J Clin Invest.* **1989**, *84*, 686–694.

Rodrigues, C. V. M.; Serricella, P.; Linhares, A. B. R.; Guerdes, R. M.; Borojevic, R.; Rossi, M. A.; Duarte, M. E. L.; Farina, M. Characterization of a Bovine Collagen–hydroxyapatite Composite Scaffold for Bone Tissue Engineering. *Biomaterials* **2003**, *24* (27), 4987–4997.

Rodríguez-Cabello, J. C.; Martín, L.; Girotti, A.; García-Arévalo, C.; Arias, F. J.; Alonso, M. Emerging Applications of Multifunctional Elastin-like Recombinamers. *Nanomedicine (Lond).* **2011**, *6* (1), 111–122.

Roohani-Esfahani, S. I.; Lu, Z. F.; Li, J. J.; Ellis-Behnke, R.; Kaplan, D. L.; Zreiqat, H. Effect of Self-Assembled Nanofibrous Silk/polycaprolactone Layer on the Osteoconductivity and Mechanical Properties of Biphasic Calcium Phosphate Scaffolds. *Acta Biomater.* **2012**, *8* (1), 302–312.

Ross, A. M.; Lahann, J. Surface Engineering the Cellular Microenvironment via Patterning and Gradients. *J. Polym. Sci. Part B Polym. Phys.* **2013**, *51* (10), 775–794.

Ruberti, J. W.; Zieske, J. D. Prelude to Corneal Tissue Engineering - Gaining Control of Collagen Organization. *Prog. Retin. Eye Res.* **2008**, *27* (5), 549–577.

Ryu, Y. J.; Cho, T. J.; Lee, D. S.; Choi, J. Y.; Cho, J. Phenotypic Characterization and In Vivo Localization of Human Adipose-Derived Mesenchymal Stem Cells. *Mol. Cells* **2013**, *35* (6), 557–564.

Sakurada, I. Polyvinyl Alcohol Fibers; CRC Press, 1985; Vol. 6.

Salvagni, E.; Berguig, G.; Engel, E.; Rodriguez-Cabello, J. C.; Coullerez, G.; Textor, M.; Planell, J. A.; Gil, F. J.; Aparicio, C. A Bioactive Elastin-like Recombinamer Reduces Unspecific Protein Adsorption and Enhances Cell Response on Titanium Surfaces. *Colloids Surfaces B Biointerfaces* **2014**, *114*, 225–233.

Sayin, E.; Baran, E. T.; Hasirci, V. Protein-Based Materials in Load-Bearing Tissue-Engineering Applications. *Regen. Med.* **2014**, *9* (5), 687–701.

Sayin, E.; Türker Baran, E.; Hasirci, V. Osteogenic Differentiation of Adipose Derived Stem Cells on High and Low Aspect Ratio Micropatterns. *J. Biomater. Sci. Polym. Ed.* **2015**, *26* (18), 1402–1424.

Semenza, G. L. Expression of Hypoxia-Inducible Factor 1: Mechanisms and Consequences. *Biochem. Pharmacol.* **2000**, *59* (99), 47–53.

Seong, J. M.; Kim, B. C.; Park, J. H.; Kwon, I. K.; Mantalaris, A.; Hwang, Y. S. Stem Cells in Bone Tissue Engineering. *Biomed. Mater.* **2010**, *5* (6), 62001.

Shoulders, M. D.; Raines, R. T. Collagen Structure and Stability. *Annu. Rev. Biochem.* **2009**, *78*, 929–958.

Sill, T. J.; von Recum, H. a. Electrospinning: Applications in Drug Delivery and Tissue Engineering. *Biomaterials* **2008**, *29* (13), 1989–2006.

Skedros, J. G.; Dayton, M. R.; Sybrowsky, C. L.; Bloebaum, R. D.; Bachus, K. N. The Influence of Collagen Fiber Orientation and Other Histocompositional Characteristics on the Mechanical Properties of Equine Cortical Bone. *J. Exp. Biol.* **2006**, *209* (Pt 15), 3025–3042.

Sumanasinghe, R. D.; Bernacki, S. H.; Loboa, E. G. Osteogenic Differentiation of Human Mesenchymal Stem Cells in Collagen Matrices: Effect of Uniaxial Cyclic Tensile Strain on Bone Morphogenetic Protein (BMP-2) mRNA Expression. *Tissue Eng.* **2006**, *12* (12), 3459–3465.

Sung, K. E.; Su, X.; Berthier, E.; Pehlke, C.; Friedl, A.; Beebe, D. J. Understanding the Impact of 2D and 3D Fibroblast Cultures on In Vitro Breast Cancer Models. *PLoS One*

2013, 8 (10), 1–13.

Suzuki, O.; Nakamura, M.; Miyasaka, Y.; Kagayama, M.; Sakurai, M. Bone Formation on Synthetic Precursors of Hydroxyapatite. *Tohoku J. Exp. Med.* **1991**, *164*, 37–50.

Tandon, N.; Goh, B.; Marsano, A.; Chao, P. H. G.; Montouri-Sorrentino, C.; Gimble, J.; Vunjak-Novakovic, G. Alignment and Elongation of Human Adipose-Derived Stem Cells in Response to Direct-Current Electrical Stimulation. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* **2009**, *2009*, 6517–6521.

Tejeda-Montes, E.; Klymov, A.; Nejadnik, M. R.; Alonso, M.; Rodriguez-Cabello, J. C.; Walboomers, X. F.; Mata, A. Mineralization and Bone Regeneration Using a Bioactive Elastin-like Recombinamer Membrane. *Biomaterials* **2014**, *35* (29), 8339–8347.

Teulé, F.; Cooper, A. R.; Furin, W. A.; Bittencourt, D.; Rech, E. L.; Brooks, A.; Lewis, R. V. Nat Protoc. **2009**, *4* (3), 341–355.

Teuschl, A. H.; van Griensven, M.; Redl, H. Sericin Removal from Raw Bombyx Mori Silk Scaffolds of High Hierarchical Order. *Tissue Eng. Part C. Methods* **2014**, *20* (5), 431–439.

Thian, E. S.; Ahmad, Z.; Huang, J.; Edirisinghe, M. J.; Jayasinghe, S. N.; Ireland, D. C.; Brooks, R. A.; Rushton, N.; Bonfield, W.; Best, S. M. The Role of Electrosprayed Apatite Nanocrystals in Guiding Osteoblast Behaviour. **2008**, *29*, 1833–1843.

Thickett, S. C.; Moses, J.; Gamble, J. R.; Neto, C. Micropatterned Substrates Made by Polymer Bilayer Dewetting and Collagen Nanoscale Assembly Support Endothelial Cell Adhesion. *Soft Matter* **2012**, *8* (39), 9996.

Thomas, C. H.; Collier, J. H.; Sfeir, C. S.; Healy, K. E. Engineering Gene Expression and Protein Synthesis by Modulation of Nuclear Shape. *Proc. Natl. Acad. Sci.* **2002**, *99* (4), 1972–1977.

Trabbic-Carlson, K.; Setton, L. A.; Chilkoti, A. Swelling and Mechanical Behaviors of Chemically Cross-Linked Hydrogels of Elastin-like Polypeptides. *Biomacromolecules* **2003**, *4*, 572–580.

Tran, K. T.; Griffith, L.; Wells, A. Extracellular Matrix Signaling through Growth Factor Receptors during Wound Healing. *Wound Repair Regen.* **2004**, *12* (3), 262–268.

Uccelli, A.; Moretta, L.; Pistoia, V. Mesenchymal Stem Cells in Health and Disease. *Nat Rev Immunol* **2008**, *8* (9), 726–736.

Vrana, N. E.; Elsheikh, A.; Builles, N.; Damour, O.; Hasirci, V. Effect of Human Corneal Keratocytes and Retinal Pigment Epithelial Cells on the Mechanical Properties of Micropatterned Collagen Films. *Biomaterials* **2007**, *28* (29), 4303–4310.

Vrana, N. E.; Builles, N.; Justin, V.; Bednarz, J.; Pellegrini, G.; Ferrari, B.; Damour, O.;
Hulmes, D. J. S.; Hasirci, V. Development of a Reconstructed Cornea from Collagen-Chondroitin Sulfate Foams and Human Cell Cultures. *Invest. Ophthalmol. Vis. Sci.*2008, 49 (12), 5325–5331.

Wang, Y.; Wan, C.; Deng, L.; Liu, X.; Cao, X.; Gilbert, S. R.; Bouxsein, M. L.; Faugere,
M. C.; Guldberg, R. E.; Gerstenfeld, L. C.; et al. The Hypoxia-Inducible Factor α
Pathway Couples Angiogenesis to Osteogenesis during Skeletal Development. J. Clin.
Invest. 2007, 117 (6), 1616–1626.

Watari, S.; Hayashi, K.; Wood, J. A.; Russell, P.; Nealey, P. F.; Murphy, C. J.; Genetos,D. C. Modulation of Osteogenic Differentiation in hMSCs Cells by SubmicronTopographically-Patterned Ridges and Grooves. *Biomaterials* 2012, *33* (1), 128–136.

Weaver, V. M.; Petersen, O. W.; Wang, F.; Larabell, C. A.; Briand, P.; Damsky, C.; Bissell, M. J. Reversion of the Malignant Phenotype of Human Breast Cells in Three-Dimensional Culture and In Vivo by Integrin Blocking Antibodies. *J. Cell Biol.* **1997**, *137* (1), 231–245.

Werten, M. W. T.; Teles, H.; Moers, A. P. H. A; Wolbert, E. J. H.; Sprakel, J.; Eggink,G.; de Wolf, F. A. Precision Gels from Collagen-Inspired Triblock Copolymers.*Biomacromolecules* 2009, *10* (5), 1106–1113.

Wheeless, C. R.; Nunley, J. A.; Urbaniak, J. R.; Surgery, Wheeless' Textbook of Orthopaedics; 2016.

Wilson, C. J.; Kasper, G.; Schütz, M. A.; Duda, G. N. Cyclic Strain Disrupts Endothelial Network Formation on Matrigel. *Microvasc. Res.* **2009**, *78* (3), 358–363.

Wilson, D. L.; Martin, R.; Hong, S.; Cronin-Golomb, M.; Mirkin, C. A.; Kaplan, D. L. Surface Organization and Nanopatterning of Collagen by Dip-Pen Nanolithography. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98* (24), 13660–13664.

Wojciak-Stothard, B.; Curtis, A. S.; Monaghan, W.; McGrath, M.; Sommer, I.; Wilkinson, C. D. Role of the Cytoskeleton in the Reaction of Fibroblasts to Multiple Grooved Substrata. *Cell Motil. Cytoskeleton* **1995**, *31* (2), 147–158.

Wood, J. A.; Ly, I.; Borjesson, D. L.; Nealey, P. F.; Russell, P.; Murphy, C. J. The Modulation of Canine Mesenchymal Stem Cells by Nano-Topographic Cues. *Exp. Cell Res.* **2012**, *318* (19), 2438–2445.

Yamada, K. M.; Cukierman, E. Modeling Tissue Morphogenesis and Cancer in 3D. *Cell* **2007**, *130* (4), 601–610.

Yang, F.; Murugan, R.; Wang, S.; Ramakrishna, S. Electrospinning of Nano/micro Scale poly(L-Lactic Acid) Aligned Fibers and Their Potential in Neural Tissue Engineering. *Biomaterials* **2005**, *26* (15), 2603–2610.

Ye, Y.; Du, Y. U.; Guo, F.; Gong, C.; Yang, K.; Qin, L. Comparative Study of the Osteogenic Differentiation Capacity of Human Bone Marrow- and Human Adipose-Derived Stem Cells under Cyclic Tensile Stretch Using Quantitative Analysis. **2012**, 1327–1334.

Yoshizumi, A.; Yu, Z.; Silva, T.; Thiagarajan, G.; Ramshaw, J. A. M.; Inouye, M.; Brodsky, B. Self-Association of Streptococcus Pyogenes Collagen-like Constructs into Higher Order Structures. *Protein Sci.* **2009**, *18* (6), 1241–1251.

Zhang, H.; Li, L. L.; Dai, F.-Y.; Zhang, H. H.; Ni, B.; Zhou, W.; Yang, X.; Wu, Y. Z. Preparation and Characterization of Silk Fibroin as a Biomaterial with Potential for Drug Delivery. *J. Transl. Med.* **2012**, *10* (1), 117.

Zorlutuna, P.; Builles, N.; Damour, O.; Elsheikh, A.; Hasirci, V. Influence of

Keratocytes and Retinal Pigment Epithelial Cells on the Mechanical Properties of Polyester-Based Tissue Engineering Micropatterned Films. *Biomaterials* **2007**, *28*, 3489–3496.

Zorlutuna, P.; Rong, Z.; Vadgama, P.; Hasirci, V. Influence of Nanopatterns on Endothelial Cell Adhesion: Enhanced Cell Retention under Shear Stress. *Acta Biomater*. **2009a**, *5* (7), 2451–2459.

Zorlutuna, P.; Elsheikh, A.; Hasirci, V. Nanopatterning of Collagen Scaffolds Improve the Mechanical Properties of Tissue Engineered Vascular Grafts. *Biomacromolecules* **2009b**, *10*, 814–821.

Zorlutuna, P.; Vadgama, P.; Hasirci, V. Both Sides Nanopatterned Tubular Collagen Scaffolds as Tissue-Engineered Vascular Grafts. **2010**, *4* (8), 628–637.

Zuk, P. A.; Zhu, M.; Ashjian, P.; Ugarte, D. A. De; Huang, J. I.; Mizuno, H.; Alfonso, Z.
C.; Fraser, J. K.; Benhaim, P.; Hedrick, M. H. Human Adipose Tissue Is a Source of Multipotent Stem Cells. *Mol. Biol. Cell* 2002, *13* (12), 4279–4295.

APPENDIX A

ETHICAL COMMITTEE APPROVAL

FEN BILIMLERI ENSTITÜSÜ GRADUATE SCHOOL OF NATURAL AND A		JĜU TEKNIK ÜNIVERSITESI
	MIDDLE	EAST TECHNICAE UNIVERSI
DUMLUPINAR BULVARI 06800 ÇANKAYA/ANKARA T: +90 312 210 22 92 F: +90 312 210 79 59 wwwbb@metu.edu.tr www.fbe.metu.edu.tr		
Say	1: B.30.2.ODT.0.AH.00.00/126/95	-1585
		8 Ağustos 2012
Gönderilen: F	Prof. Dr. Vasıf Hasırcı	
E	Biyolojik Bilimler Bölümü	A
Gönderen : F	Prof. Dr. Canan Özgen	Morgen
L	AK Başkan Yardımcısı	0
İlgi : E	tik Onayı	
"Mikro Çevre	nin Doku Mühendisliği Uygulamala	rına Etkisi" isimli
araştırmanız	"İnsan Araştırmaları Komitesi" t	tarafından uygun
görülerek gere	kli onay verilmiştir.	
Bilgilerinize sa	ygılarımla sunarım.	
	Etik Komite Onayı	
	Uygundur	
	08/08/2012	Hormiz. Bily
	Canan Drees	(2.08.2012
	Prof.Dr. Canan ÖZGEN Uygulamalı Etik Araştırma Merke (UEAM) Başkanı ODTÜ 06531 ANKARA	zi
	SETO WOOT ANNANA	

Figure A.1: Ethical committee approval from METU Human Ethics Committee for human ADSC isolation

ORTA DOĞU TEKNİK ÜNİVERSİTESİ UYGULAMALI ETİK ARAŞTIRMA MERKEZİ APPLIED ETHICS RESEARCH CENTER MIDDLE EAST TECHNICAL UNIVERSITY DUMLUPINAR BULVARI 06800 ÇANKAYA ANKARA/TURKEY T: +90 312 210 22 91 F: +90 312 210 79 59 ueam.metu.edu.tr Sayı: 28620816/203 - 598 18.04.2014 Gönderilen : Prof. Dr. Vasıf HASIRCI Biyoteknoloji Gönderen : Prof. Dr. Canan Özgen IAK Başkanı : Etik Onayı İlgi Danışmanlığını yapmış olduğunuz Biyoteknoloji Bölümü öğrencisi Esen Sayın'ın "Mikro Çevrenin Doku Mühendisliği Uygulamalarına Etkisi" isimli araştırması "İnsan Araştırmaları Komitesi" tarafından uygun görülerek gerekli onay verilmiştir. Bilgilerinize saygılarımla sunarım. Etik Komite Onayı Uygundur 18/04/2014 anan Prof.Dr. Canan Özgen Uygulamalı Etik Araştırma Merkezi (UEAM) Başkanı ODTÜ 06531 ANKARA 09 22.04.2014

Figure A.2: Ethical committee approval from METU Human Ethics Committee for HOB isolation

HIZMETE ÖZEL		T.C.	
	GENELKUF GÜLHANE ASKERI TI	MAY BAŞKANLIĞI	NI IĞI
	ETİK KURUL	TOPLANTI RAPORU	
	. 34		
OTURUM TARIHI	: 06 Mart 2014		
OTURUM BAŞKANI OTURUM SEKRETERI	: Prof.Diş Tbp.Alb.Ya : Doç.Tbp.Alb.Muhar	aşar Meriç TUNCA rrem UÇAR	
GATA Etik Kurulu'nu	ın 06 Mart 2014 günü	yapılan 34'üncü oturumu	ında, ODTÜ Biyomalzeme
Doku Muhendisliği M araştırmacılığını, GATA	ükemmeliyet Merkezi'i Ortopedi ve Travmato	nde görevli Prof. Dr. bloji AD'da görevli Prof. T	Vasıf HASIRCI'nın sorur Top. Kd. Alb. Cemil YILDIZ
yardımcı araştırmacılığ çok merkezli, yeni teda	ını yaptığı "Mikro Çevre vi geliştirme çalışması o	nin Doku Mühendisliği U olan araştırma doşvaşı de	ygulamalarına Etkisi" başlı eğerlendirildi
Araştırma dosyasınır	amac, vöntem ve vakl	asım bakımından etik ilkı	elere UYGUN olduğuna ka
verildi.	entendinimese sociality		
BAŞKAN	ÜYE	ÜYE	
and AD Card YEE	11	11	1 m
Y MOTO TUNCA		-Mar	///
Prof.Diş Tbp.Alb.	Prof.Tbp.Alb.	Prof.Tbp.Alb.	Mahir GULEÇ Prof.Tbp.Alb
Rica ederim.	•		
ÜYE	ÜYE	ÜYE	ÜYE
Made	TOPLANTIYA	AL	
Cengiz BASOĞLU	KATILMADI	Error TO KODADAD	
Prof.Tbp.Alb.	Cemil YILDIZ Prof. Tbp. Alb.	Prof.Tbp.Alb.	Doç.Tbp.Alb.
ÜYE	ÜYE	ÜYE	
A		2:0	
Emine IYIGÜN -	Harun TUĞCU		
	Doç.Tbp.Alb	Doç.Tbp.Alb.	
Doç.Dr.Hv.Sağ.Alb.			
Doç.Dr.Hv.Sağ.Alb.			
Doç.Dr.Hv.Sağ.Alb.			

Figure A.3: Ethical committee approval from Gulhane Medical Military Academy (GATA) Ethics Committee for HOB isolation

APPENDIX B

EQUATION FOR ALAMAR BLUE CALCULATIONS

Percent reduction of the dye =
$$\frac{((\varepsilon_{ox})_{\lambda_2} \ge A_{\lambda_1}) - ((\varepsilon_{ox})_{\lambda_1} \ge A_{\lambda_2})}{((\varepsilon_{red})_{\lambda_1} \ge A'_{\lambda_2}) - ((\varepsilon_{red})_{\lambda_2} \ge A'_{\lambda_1})} x 100$$

$\lambda_1 = 570 \text{ nm}$	$\lambda_2 = 595 \text{ nm}$
$(\varepsilon_{\rm ox})_{\lambda 2} = 117.216$	$(\epsilon_{red})_{\lambda 1} = 155.677$
$(\varepsilon_{\rm ox})_{\lambda 1} = 80.586$	$(\epsilon_{\rm red})_{\lambda 2} = 14.652$

 $A_{\lambda 1}$ = Observed absorbance reading for test well

 $A_{\lambda 2}$ = Observed absorbance reading for test well $A'_{\lambda 1}$ = Observed absorbance reading for negative control well (blank)

 $A'_{\lambda 2}$ = Observed absorbance reading for negative control well
APPENDIX C

CALIBRATION CURVES FOR ALAMAR BLUE ASSAY



Figure C.1: Calibration curve of ADSCs for Alamar Blue Assay.



Figure C.2: Calibration curve of HOBs for Alamar Blue Assay.

APPENDIX D

CALIBRATION CURVE FOR ALP ASSAY



Figure D.1. Calibration curve for ALP assay

APPENDIX E

CALIBRATION CURVE FOR CALCIUM QUANTIFICATION



Figure E.1: Calibration curve for mineralization assay

APPENDIX F

CALIBRATION CURVE FOR DNA QUANTIFICATION



Figure F.1: Calibration curve for PicoGreen® Assay.

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Sayın, Esen Nationality: Turkish Date and Place of Birth: 10 October 1984, Ankara, Turkey Marital Status: Single Phone: +90 312 210 5193 Fax: +90 312 210 1542 Email: esensyn@gmail.com

EDUCATION

DegreeInstitution		Year of Graduation
M.Sc.	METU, Dept. of Biotechnology	2011
B.Sc.	ITU, Molecular Biology and Genetics	2007
High School	Eskişehir Kılıçoğlu Anadolu High School	2002

ACADEMIC EXPERIENCE

Year	Place	Enrollment
May 2012 Present	BIOMATEN METU CoE in Biomaterials and Tissue Engineering	Full time Researcher
December 2009 Present	METU Department of Biological Sciences	Teaching Assistant
November 2015 May 2016	University College London Institute of Orthopaedics & Musculoskeletal Science	Visiting Research Scholar
March 2009 September 2011	METU Central Laboratory Molecular Biology, Biotechnology R&D Center	Full time Researcher

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

1. Sayin E., Hama Rashid R., Elsheikh A., Rodríguez-Cabello J. C., Baran E. T., Hasirci V. "Human adipose derived stem cells are superior to human osteoblasts (HOB) in bone tissue engineering on a collagen-fibroin-ELR blend." Bioactive Materials, 2(2), 71-81.

2. Sayin E., Baran E.T., Hasirci V. (2015). "Osteogenic differentiation of adipose derived stem cells on high and low aspect ratio micropatterns." Journal of Biomaterials Science, Polymer Edition, 26(18), 1402-1424.

3. Sayin E., Baran E.T., Hasirci V. (2014). "Protein-based materials in load-bearing tissue engineering applications." Regenerative Medicine, 9(5), 687–701.