

**TENDON EXTRACELLULAR MATRIX: TENOGENIC ACTIVITY ON
MESENCHYMAL STEM CELLS AND UTILITY IN TENDON TISSUE ENGINEERING**

by

Guang Yang

Bachelor of Science, University of Science and Technology of China, 2010

Submitted to the Graduate Faculty of
Swanson School of Engineering in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

University of Pittsburgh

2015

UNIVERSITY OF PITTSBURGH
SWANSON SCHOOL OF ENGINEERING

This dissertation was presented

by

Guang Yang

It was defended on

December 1st, 2015

and approved by

Johnny Huard, Ph.D., Professor, Department of Orthopaedic Surgery

Nam Vo, Ph.D., Assistant Professor, Department of Orthopaedic Surgery

William R. Wagner, Ph.D., Professor, Department of Bioengineering and Chemical
Engineering

Dissertation Director: Rocky S. Tuan, Ph.D., Department of Orthopaedic Surgery and
Bioengineering

Copyright © by Guang Yang

2015

**TENDON EXTRACELLULAR MATRIX: TENOGENIC ACTIVITY ON
MESENCHYMAL STEM CELLS AND UTILITY IN TENDON TISSUE ENGINEERING**

Guang Yang, Ph.D.

University of Pittsburgh, 2015

Because of the limited and unsatisfactory outcomes of clinical tendon repair, tissue engineering approaches using adult mesenchymal stem cells (MSCs) are being considered a promising alternative healing strategy for injured tendon tissues. Successful and functional tendon tissue engineering depends on harnessing the biochemical cues presented by the native tendon extracellular matrix (ECM) and the embedded tissue-specific bio-factors. We have prepared and characterized the biological activities of a soluble extract of decellularized tendon ECM (tECM) on adult adipose derived stem cells (ASCs) on the basis of histological, biochemical, and gene expression analyses. Our results revealed the tenogenic effect of tECM on hASCs cultured in a 3-dimensional (3D) collagen scaffold under uniaxial tension. The presence of tECM also suppressed the osteogenic differentiation of hASCs induced by uniaxial tension and enhanced scaffold mechanical strength, accompanied by reduced expression and activity of matrix metalloproteinases (MMPs). Furthermore, we found that tECM enhanced the proliferation and TGF- β 3 induced tenogenesis of ASCs, and modulated matrix deposition and organization by ASCs seeded in 3D fibrous scaffolds. These findings support the utility of tECM in creating bio-functional scaffolds for tendon tissue engineering. We also report here the development of a novel composite fibrous scaffold as a tendon graft fabricated by co-electrospinning of poly- ϵ -

caprolactone (PCL) and methacrylated gelatin (mGLT), which allowed the encapsulation of ASCs within the scaffold upon visible light induced gelatin photo-crosslinking, as well as the formation of stable, crosslinked multi-layered constructs. This scaffold design may improve cell-based tendon regeneration by serving as an effective reservoir of tECM and tenogenic growth factors to recapitulate the structural and biochemical characteristics of native tendon tissue. Our findings should lead to translational tissue engineering applications that will improve patient outcomes in the context of clinical tendon repair.

TABLE OF CONTENTS

PREFACE.....	XIV
1.0 INTRODUCTION.....	1
1.1 TENDON BIOLOGY.....	1
1.1.1 Development of tendon tissues.....	1
1.1.2 The structure and composition of tendon tissue	7
1.1.3 Tendon injury and natural healing.....	12
1.2 TENDON TISSUE ENGINEERING	15
1.2.1 Cells.....	16
1.2.2 Scaffolds.....	19
1.2.3 Bioactive molecules.....	22
1.2.4 Mechanical stimulation	24
1.3 RESEARCH OVERVIEW	26
2.0 TENDON-DERIVED EXTRACELLULAR MATRIX ENHANCES TENOGENIC DIFFERENTIATION OF HUMAN ADIPOSE-DERIVED STEM CELLS CULTURED IN 3-DIMENSIONAL COLLAGEN SCAFFOLD UNDER TENSION.....	28
2.1 ABSTRACT.....	28
2.2 INTRODUCTION	29
2.3 MATERIALS AND METHODS.....	32

2.3.1	Tissue harvest.....	32
2.3.2	Extraction of tECM.....	32
2.3.3	SDS-PAGE and Western blot assay.....	33
2.3.4	Cell isolation and culture	34
2.3.5	3D culture of cells under static tension.....	34
2.3.6	Scanning electron microscopy (SEM).....	35
2.3.7	Cell viability analysis.....	35
2.3.8	Histology	36
2.3.9	Real-time PCR analysis.....	37
2.3.10	Mechanical test	38
2.3.11	MMP activity assay	39
2.3.12	Statistical analysis.....	39
2.4	RESULTS.....	40
2.4.1	Characteristics of tendon-derived ECM.....	40
2.4.2	Constructs and cell morphology.....	43
2.4.3	Cell viability and proliferation	46
2.4.4	Cell differentiation.....	47
2.4.5	Mechanical properties of the scaffold.....	50
2.4.6	Gene expression and activity of MMPs	51
2.5	DISCUSSION.....	52
2.6	CONCLUSIONS	58
3.0	TENDON-DERIVED EXTRACELLULAR MATRIX ENHANCES TGF-BETA 3 INDUCED TENOGENIC DIFFERENTIATION OF HUMAN ADIPOSE-DERIVED STEM CELLS.....	59

3.1	ABSTRACT.....	59
3.2	INTRODUCTION	60
3.3	MATERIALS AND METHODS.....	63
3.3.1	Cell isolation and culture	63
3.3.2	Colony forming unit-fibroblast assay	63
3.3.3	Flow cytometry	64
3.3.4	Preparation of tendon ECM	64
3.3.5	Preparation of scaffold.....	65
3.3.6	Scanning electron microscopy (SEM).....	65
3.3.7	Differentiation of hASCs.....	65
3.3.8	Cell proliferation tests	66
3.3.9	Real-time PCR analysis of gene expression.....	67
3.3.10	Protein extraction and Western blot assay	67
3.3.11	Mechanical testing.....	68
3.3.12	Matrix deposition and characterization	68
3.3.13	Immunofluorescent staining.....	69
3.3.14	Statistical analysis.....	69
3.4	RESULTS.....	70
3.4.1	Characterization of human ASCs	70
3.4.2	Effect of tendon ECM on ASC behavior in 2D	72
3.4.3	Characterization of the aligned microfiber scaffolds.....	75
3.4.4	Effect of tendon ECM on ASC behavior in 3D scaffolds	77
3.5	DISCUSSION.....	80

3.6	CONCLUSIONS	83
4.0	MULTILAYERED POLYCAPROLACTONE/GELATIN FIBER-HYDROGEL COMPOSITE FOR TENDON TISSUE ENGINEERING	84
4.1	ABSTRACT.....	84
4.2	INTRODUCTION	85
4.3	MATERIALS AND METHODS	87
4.3.1	Synthesis of methacrylated gelatin.....	87
4.3.2	Fabrication of composite scaffolds.....	88
4.3.3	Imaging of composite scaffolds.....	89
4.3.4	Histological examination of composite scaffolds.....	90
4.3.5	Biochemical composition of composite scaffolds	90
4.3.6	Cell isolation and culture	90
4.3.7	Creation of multilayer constructs	91
4.3.8	Mechanical testing	91
4.3.9	Creation of a tendon-mimetic construct by multilayer scaffolds	92
4.3.10	Cell proliferation tests.....	92
4.3.11	Cytoskeleton fluorescent staining	93
4.3.12	Real-time PCR analysis.....	93
4.3.13	Statistical analysis.....	94
4.4	RESULTS	95
4.4.1	Organization of fibers in composite scaffold.....	95
4.4.2	Biochemical and mechanical analysis of composite scaffold	97
4.4.3	Multilayered construct created by photocrosslinking.....	99
4.4.4	Cell incorporation and activity in multilayered construct.....	101

4.4.5	Characterization of tendon phenotype induced by aligned multilayered constructs.....	102
4.5	DISCUSSION.....	103
4.6	CONCLUSION	106
5.0	DISCUSSION, ADDITIONAL FINDINGS, AND FUTURE PERSPECTIVE..	107
5.1	ADIPOSE STEM CELL ISOLATION AND CHARACTERIZATION....	108
5.1.1	hASC isolation.....	108
5.1.2	hASC characterization	109
5.2	TENOGENIC DIFFERENTIATION OF ADIPOSE STEM CELLS	110
5.2.1	Induction of ASC tenogenesis via growth factor treatment	111
5.2.2	Induction of ASC tenogenesis via tensioned 3D culture	113
5.3	THE BIOACTIVITY OF TENDON EXTRACELLULAR MATRIX.....	114
5.3.1	Determination of the functional composition of tendon ECM	115
5.3.2	Understanding the interaction between tendon ECM and cells.....	116
5.4	SCAFFOLD DEVELOPED FOR TENDON TISSUE ENGINEERING ...	117
5.4.1	Possible improvements of hydrogel scaffold	118
5.4.2	Possible improvements of fibrous scaffold	119
5.4.3	Possible improvements for composite scaffold	120
5.5	CONCLUSIONS	121
	LIST OF ABBREVIATIONS	123
	BIBLIOGRAPHY	125

LIST OF TABLES

Table 1. Non-collagenous proteins in tendon tissue	11
Table 2. Bioactive molecules involved in natural tendon healing process	14
Table 3. Primer sequences of genes analyzed by real-time PCR.....	38
Table 4. Primer sequences of genes analyzed by real-time PCR.....	67
Table 5. Primer sequences of genes analyzed by real-time PCR.....	94

LIST OF FIGURES

Figure 1. Factors involved in limb tendon development and their spatial distribution	3
Figure 2. Somite structure depicted in transverse section.....	4
Figure 3. Summary of genes involved in regulating scleraxis (SCX) expression in somites and the subsequent mediators of collagen expression in tendons	6
Figure 4. The hierarchical structure of collagen fibers.....	8
Figure 5. The hierarchical architecture of tendon tissue.....	9
Figure 6. An overview of repair approaches for tendon injuries	16
Figure 7. Decellularization of tendon tissue	40
Figure 8. Characterization of tECM proteins by SDS-PAGE and Western blot	42
Figure 9. Contraction of constructs.....	43
Figure 10. Examination of scaffold structure, cell morphology and distribution	45
Figure 11. Cell viability assays.....	47
Figure 12. Real time PCR analysis of gene expression	49
Figure 13. Mechanical properties of constructs	50
Figure 14. Matrix metalloproteinase (MMP) expression and activity	52
Figure 15. Characterization of human adipose stem cells (hASC).....	71
Figure 16. Assay of hASC proliferation in 2D cultures	72
Figure 17. Tenogenesis of hASCs in 2D cultures.....	74
Figure 18. Real-time PCR analysis of SOX9 expression	74

Figure 19. Characterization of polycaprolactone (PCL) scaffold by SEM.....	75
Figure 20. Characterization of PCL scaffold by mechanical tests.....	76
Figure 21. Morphology of hASCs cultured on scaffolds.....	76
Figure 22. Behavior of hASCs seeded on aligned scaffolds.....	77
Figure 23. Tenogenic differentiation of hASCs seeded on aligned scaffolds.....	78
Figure 24. Matrix deposition by hASCs cultured on aligned scaffolds.....	79
Figure 25. Synthesis and crosslinking of methacrylated gelatin (mGLT).....	88
Figure 26. Composite scaffold preparation.....	89
Figure 27. Characterization of scaffold architecture.....	96
Figure 28. Examination of gelatin retention.....	98
Figure 29. Picrosirius red staining of scaffolds.....	98
Figure 30. Laminar integration of scaffold sheets.....	99
Figure 31. Preparation and characterization of multilayer construct.....	100
Figure 32. Analysis of cell distribution and metabolic activity in multilayer constructs.....	101
Figure 33. Tendon-like features of cell impregnated multilayer constructs.....	102
Figure 34. DAPI-stained cross-sections of ASC seeded scaffolds.....	105
Figure 35. The location of the infrapatellar fat pad in knee joint.....	109
Figure 36. Dose effect of growth factors on the tenogenesis of hASCs.....	112
Figure 37. Western blot of phosphorylated Smad2 (P-SMAD2) and total SMAD2/3.....	117
Figure 38. Real time PCR analysis.....	117

PREFACE

Scientific acknowledgements

I acknowledge my advisor, Dr. Rocky Tuan first and foremost for providing me a scientific home to explore my scientific interests and encouraging me through all the setbacks and frustrations. This work would not have been done without the help, advice, support, and guidance of Dr. Tuan and all members of his lab, Center for Cellular and Molecular Engineering (CCME). I thank my dissertation committee for their guidance and support in finishing my dissertation project. I also thank the Center of Biological Image (CBI) for technical support of microscopy.

Personal acknowledgements

I cannot thank my parents enough, Junhe Yang and Lingyun Hu for their emotional, intellectual, and financial support that afford me the opportunity to get to this point. I thank all my friends for always encouraging me to pursue my goals in times of doubt. To my fiancée, Xiaoxiao Lu, words fail me to express enough thanks to you for your company in my Ph.D. journey. Your optimism, love and patience will continue to impress and support me in my life.

1.0 INTRODUCTION

This chapter includes materials excerpted from the following publication:

Guang Yang, Benjamin B. Rothrauff, and Rocky S. Tuan. Tendon and Ligament Regeneration and Repair: Clinical Relevance and Developmental Paradigm. *Birth Defects Res C Embryo Today*. 2013 Sep; 99(3): 203–222. doi: 10.1002/bdrc.21041

1.1 TENDON BIOLOGY

1.1.1 Development of tendon tissues

Tendons are fibrous tissues composed of densely packed collagen fiber bundles that connect muscle to bone, and function in force transmission from muscle to bone. Tendon development has been intensively studied in multiple animal models, and was historically divided into two domains of the developing embryos—(1) tendons of the limb, and (2) tendons of the axial skeleton and abdomen. Early studies on limb tendons revealed that distal limb tendons appear to first arise from the lateral plate mesoderm and consist of a single dorsal and ventral blastema. With the progress of development, the dorsal blastema differentiates into the extensor tendons of each phalange, while the ventral blastema splits to form the flexor tendons of the digits [1, 2]. Although eventually connected to muscle tissues, distal tendon primordia can

segregate into individual tendons in a normal temporal and spatial pattern in the absence of limb muscles. In contrast, the proximal tendon blastema must develop in close proximity to muscle precursors [3].

A number of tendon-specific genes dynamically coordinate to give rise to an ultimate specification of tendon tissues. For instance, the murine homeobox-containing genes *Six 1* and *Six 2* are involved in the patterning of mouse dorsal extensor and ventral flexor tendons of the phalangeal elements in a complementary manner [4]. Likewise, *Eya1* and *Eya2*, two mouse homologs of *Drosophila* eyes absent (*Eya*) gene, are associated with cell condensations that form limb tendons and may as well function along with *Six* to pattern dorsal and ventral limb tendon morphogenesis [2]. *Follistatin* and *Eph-A4* are expressed in tendon-forming regions of developing chick limbs to coordinate cartilage and tendon development [5].

In the past decade, the identification of several new gene markers that are selectively associated with early tendon primordial and mature tendon has enabled tracking of the origin and formation of tendon progenitor cells. Most notably, *scleraxis (SCX)*, a basic helix-loop-helix transcription factor originally found in all muscle-to-bone attachment sites in chick embryos, is used as a marker to identify tendon cell fate [6, 7]. Expressed from Hamburger-Hamilton (HH) stage 21 onward, *SCX* expression in the limb bud reveals the superficial proximomedial limb mesenchyme as the origin site of limb tendon formation. Signals from the ectoderm are necessary for the induction and proliferation of the *SCX*-expressing tendon cells, although no direct ectodermal interference with *SCX* expression exists. Conversely, bone morphogenetic protein (BMP) secreted from surrounding areas suppress *SCX*-expression to restrict the location and scale of tendon cell phenotype adoption. At later stages, the repressive effects of BMP signaling are partially blocked by the BMP antagonist *Noggin* (Fig. 1) [7].

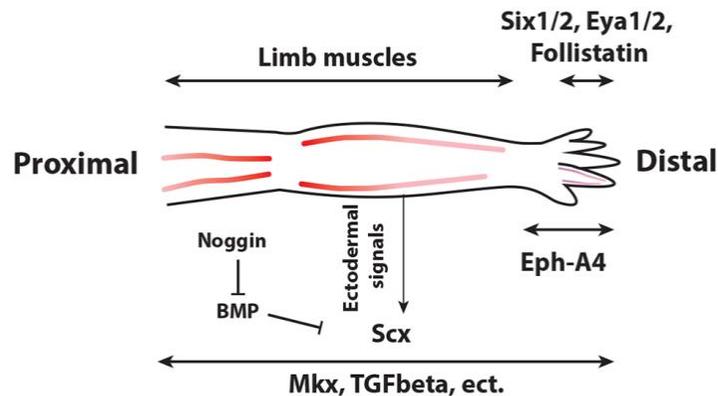


Figure 1. Factors involved in limb tendon development and their spatial distribution with respect to proximal–distal axis. Scleraxis (SCX), Mohawk (MKX), and TGF- β are functional in the formation of most limb tendons. Morphogenesis of proximal tendon is coupled with muscle precursors. Genes including Six1/2, Eya1/2, and Follistatin are expressed in distal regions and are thought to pattern flexor and extensor tendon morphogenesis, whereas Eph-A4 transcripts are localized in a more proximal region and later in the body of the tendon.

Unlike limb tendons, somite is the origin of the tendons that attach the back muscles to the vertebrae, and the intercostal muscles to the ribs [8]. Through tracking SCX expression, axial and trunk tendon progenitor cells were found to constitute a previously undetected fourth somitic compartment, the “syndetome” (Fig. 2), that derives from the early sclerotome [9]. SCX-expressing progenitor cell population lies between adjacent myotomes, both dorsomedial and ventrolateral to sclerotome [9]. SCX null mutant mice (SCX^{-/-}) displayed severe tendon loss and defects, reflected by limited use of paws and tail. The residual tendons that persisted in SCX^{-/-} mutants showed disrupted compartmentalization of differentiated tendon cells, known as tenocytes (TCs), and endotenon cells due to less-organized tendon matrix. Interestingly, unlike disrupted force-transmitting and intermuscular tendons, other categories of tendons were less affected or fully functional in SCX^{-/-} mutants [10]. Such variety of tendon phenotype exemplifies the diversity of tendon tissue origin and the complexity of tendon development.

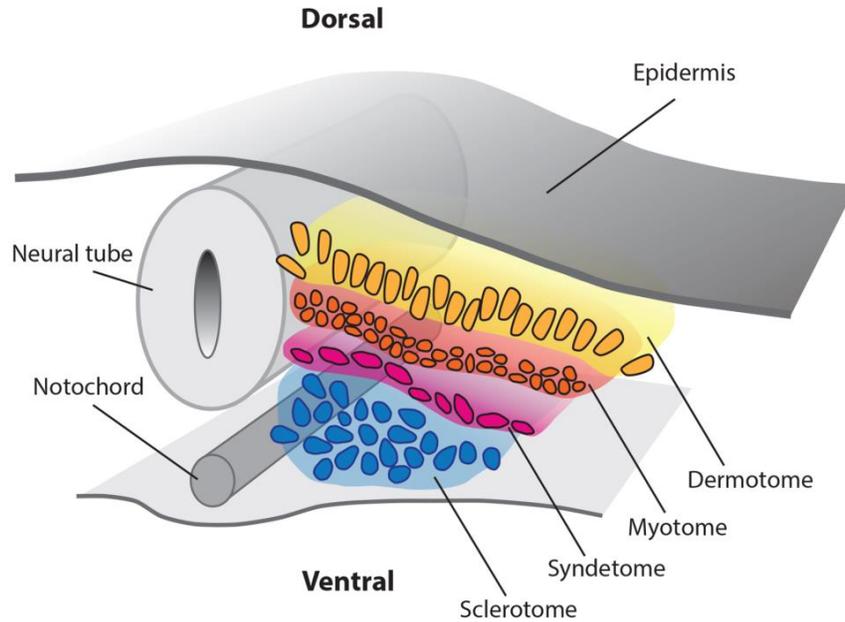


Figure 2. Somite structure depicted in transverse section. Scleraxis (SCX)-expressing cells (purple) are found between the sclerotome and myotome and constitute a fourth somitic compartment, syndetome.

In addition to attempts in understanding the origin and identity of tendon progenitor cells, considerable efforts have been made to elucidate the instructive cues of these cells. Notably, SCX expression is initiated later than markers of other somitic compartments, [10] such temporal pattern implies the involvement of signals from other compartments in tendon morphogenesis. For instance, fibroblast growth factor (FGF) secreted from the myotome was necessary and sufficient for SCX induction through the activation of Ets transcription factors polyomavirus enhancer activator 3 (PEA3) and Ets-related molecule (ERM) [2]. The induction of SCX expression is also dependent on signals from the sclerotome. In fact, although tendon progenitor cells and cartilage progenitor cells initially overlap in dorsolateral sclerotome domains, their distribution becomes distinct with the progress of differentiation. Cells constituting the axial tendon and cartilage are exclusive lineages: cartilage differentiation represses tendon development in the dorsolateral sclerotome [11]. Ventral midline expression of sonic hedgehog

(SHH) might also be involved in SCX expression, for mis-expression of SHH led to loss of SCX [11]. In addition to FGF and SHH, several members of the transforming growth factor- β (TGF- β) superfamily are identified in the tenogenesis cascade. For instance, in the chick embryo, TGF- β 2/3 ligands and their receptors were detected in mid-substance and endotenon of tertiary bundles during the intermediate stages of tendon development [12]. Likewise, murine patellar tendon cells were found to be responsive to TGF- β and BMP signaling at all stages examined starting at E17.5 and ending at P14 [13]. The *in vivo* involvement of TGF- β signaling in tenogenesis was confirmed in a mouse model in which the disruption of TGF- β signaling resulted in the loss of most tendons and ligaments [14]. Equally important, TGF- β signaling coordinates cartilage and tendon differentiation in the developing limb mesenchyme [15]: TGF- β 1 or TGF- β 2 treatment on microassay culture of primary chicken mesenchymal cells transformed prechondrogenic aggregates into fibrous tendon tissue through activation of tenogenic gene SCX and inhibition of chondrogenic genes SOX9.

In addition to SCX, two other putative markers attracting growing research attention are tenomodulin (TNMD), and mohawk homeobox (MKX). TNMD encodes a type II transmembrane glycoprotein that contains a C-terminal anti-angiogenic domain and is dominantly expressed in tendons and ligaments. Overexpression of SCX caused significant increase in TNMD level of cultured TCs but not chondrocytes, indicating that the impact of SCX on TNMD is restricted to the tendon lineage [16]. Unlike TNMD, MKX might act as the upstream regulator of SCX, for overexpression of MKX led to higher SCX expression through activation of the TGF- β signaling [17]. However, on the other hand, no obvious change was found in the level or spatiotemporal pattern of SCX expression in MKX^{-/-} mutants compared to wild-type embryos (Fig. 3) [18].

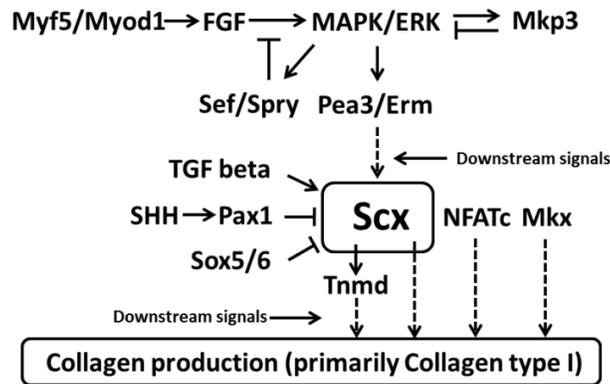


Figure 3. Summary of genes involved in regulating scleraxis (SCX) expression in somites and the subsequent mediators of collagen expression in tendons.

While a complex signaling network of tendon differentiation begins to emerge, a key to successful adult tendon regeneration through recapitulation of developmental events is to understand the functions of the molecular markers in shaping the biochemical/physical feature of tendons. For instance, the transcription factors SCX and NFATc4 coordinate to activate the gene encoding $\alpha 1$ chain of collagen type I (COL I A1) in tendon fibroblasts through binding to two short tendon-specific elements: TSE1 and TSE2. Conversely, inhibition of the nuclear translocation of NFATc proteins inhibited the expression of collagen type I (COL I) [19]. MKX is another possible regulator of collagen production in TCs as a transcriptional repressor [17]. MKX^{-/-} mice exhibited smaller tendons and disrupted tendon collagen fibrils with significantly reduced amounts of COL I, fibromodulin, and TNMD compared with wild-type animals. Lastly, recent research has partially revealed the role of TNMD in tendon collagen organization: The variation in collagen fibrils diameter was significantly higher in TNMD deficient mice. Loss of TNMD expression also led to reduced TC density and proliferation rate [20].

1.1.2 The structure and composition of tendon tissue

Fibrillar collagens, including types I, II, III, V, and XI, constitute the basic structural framework of tendons and ligaments [21]. Synthesis and deposition of these collagenous fibrils was initiated in both longitudinal and lateral directions by tendon progenitors during the embryo stage and continues after birth [22]. Gradual decrease in the cell-to-matrix ratio occurs along with maturation and aging, and collagen ultimately accounts for 65–80% of the dry mass of tendon tissue [23]. Fibrillar collagen molecules consist of three α chains that are wrapped around one other to form a triple stranded helical rod. Each α chain is arranged in the repeating structure Gly-X-Y, in which X and Y are frequently proline and hydroxyproline, respectively [24]. Glycine allows the three helical α chains to pack tightly due to the absence of side chain, meanwhile the conformation of helix is stabilized by the ring structure of proline (Fig. 4).

Synthesis of the mature collagen protein is a multiple-step process. Intracellularly formed alpha chains are assembled into triple helix molecules as procollagen molecules that have additional propeptide domains at the COOH- and NH₂-terminal ends of each chain. The propeptide domains solubilize procollagen molecules and thereby prevent intracellular assembly and precipitation. After partial hydroxylation and glycosylation of the proline and lysine residues, procollagen molecules are transported to cell surface and secreted into the extracellular space, where the propeptide domains are removed by specific telopeptidase cleavage to form less-soluble tropocollagen. Modified tropocollagen molecules self-assemble into fibrils that have periodic cross-striation every 67 nm, reflecting the staggered packing of individual collagen molecules in each fibril. Collagen fibrils are often 100 to 500-nm in diameter, and further bundled into fibers between which tenocytes (TCs) reside and maintain the extracellular matrix (ECM) (Fig. 4) [23]. The number and diameter of collagen fibers in tendon tissues are highly

variable among sites and species. Geometrically speaking, while most fibers are aligned longitudinally along tendon tissue, collagen fibers may also be oriented transversely. The heterogeneity in fiber orientation allows tendon to resist forces from various directions, although principally functioning in uniaxial tension [25].

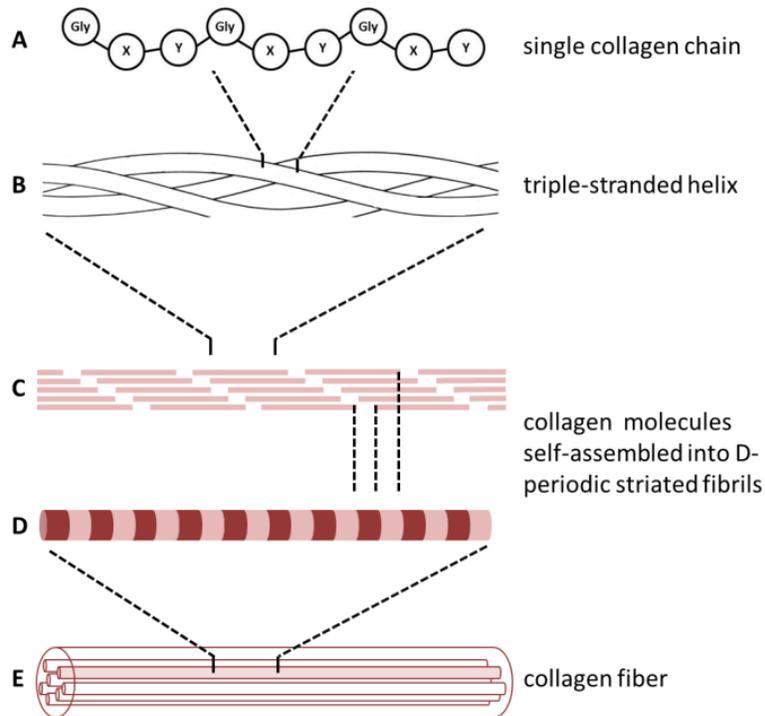


Figure 4. The hierarchical structure of collagen fibers. (A) Single collagen α chain is arranged as a helix with three amino acids per turn. (B) Collagen molecule consists of three α chains coiled to form a triple-stranded helix. (C and D) Collagen molecules self-assemble into quarter-staggered, periodic striated fibrils every 67 nm. (E) A group of collagen fibrils further bundle to form a collagen fiber.

In native tendon tissues, a group of collagen fibers are wrapped by a layer of connective tissue known as endotenon that contains blood vessels, lymphatics, and nerves, to form fascicles. Yet another connective tissue layer, the epitenon, surrounds fascicles to outline the tendon tissue. Some types of tendons are further surrounded by smooth surface connective tissue called

paratenon that functions as an elastic sleeve to allow free movement of the tendon against the surrounding tissues (Fig. 5) [26]. Collagen type I protein (Col I) is the dominant fibril-forming collagen in tendons as a heterotrimer consisting of two $\alpha 1$ chains and one $\alpha 2$ chain. Col I copolymerizes with collagen type V [27], as well as other nonfibrillar collagens to regulate the fibrillogenesis during tendon development. These collagens include the fibril-associated collagens with interrupted triple helices (FACIT) such as collagen types XII and XIV [22, 28].

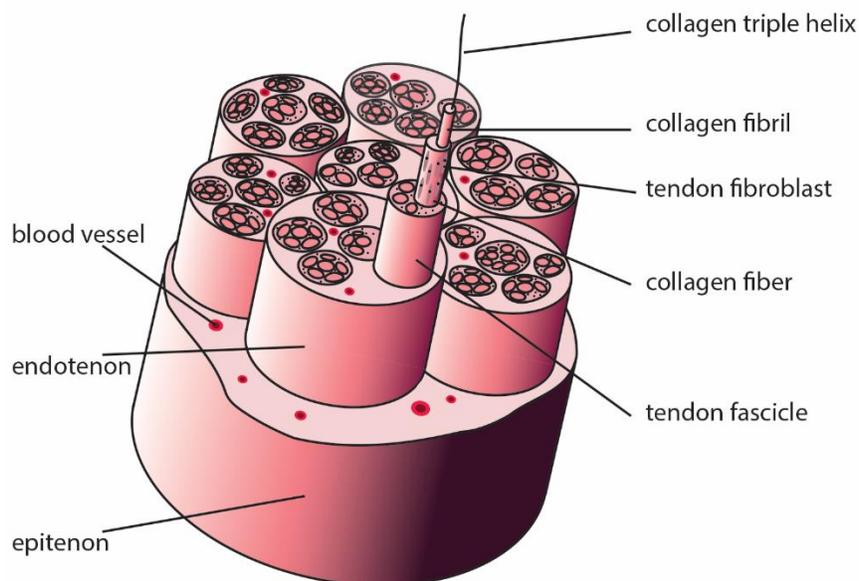


Figure 5. The hierarchical architecture of tendon tissue. Collagen triple-helices self-assemble into fibrils. Bundles of fibrils form fibers constitute tendon fascicles. Tendon fibroblasts (tenocytes, TCs) reside between collagen fibers. Fascicles are wrapped by endotenon, a layer of connective tissue containing blood vessels, nerves, and lymphatics. Multiple fascicles are further surrounded by another connective tissue layer, epitenon, to form the tendon tissue.

Non-collagenous ECM proteins such as glycoproteins, proteoglycans, and elastic fibers are of great importance in maintaining TC phenotype and tissue homeostasis, despite relatively low abundance compared to collagen [25]. Proteoglycans are the most abundant class of glycoproteins within tendon, consisting of a core protein and at least one glycosaminoglycan

(GAG) as polysaccharide chain. Small leucine rich proteoglycan (SLRP) is the major type of tendon proteoglycans, including decorin, biglycan, fibromodulin, and lumican. SLRPs are able to facilitate fibril assembly and ultrastructural organization of the tendon through binding non-covalently to the gap region of collagen fibrils at specific sites [29]. Table 1 summarizes the function of major non-collagenous proteins found in tendon tissue.

As the development and growth proceeds, tendon tissue stiffens due to accumulating collagen deposition and structural arrangement alteration. Proper formation of covalent cross-links occurs at the overlapping ends of adjacent collagen molecules during maturation to enhance collagen interconnectivity and fibril stability, primarily driven by the lysyl oxidase that acts on specific lysine and hydroxylysine residues [30-32]. Equally important, SLRPs have been proposed to stabilize the collagen network by either functioning as aforementioned crosslinkers between adjacent collagen fibrils (Table 1); or via regulating lysyl oxidase-mediated cross-link formation. For example, when binding to collagen, fibromodulin sterically hinders the access of lysyl oxidase to telopeptides [33]. Beyond activity in cross-link formations, hydroxyproline also contributes to the strength of fibrils by forming hydrogen bonds between polypeptide chains between tropocollagen molecules. Nevertheless, in spite of increasing knowledge in biochemical composition and mutual interaction between components of tendon tissue, the structure–function relationship between these biochemical characteristics and mechanical properties remains inconclusive [34].

Table 1. Non-collagenous proteins in tendon tissue

ECM protein	Category	Function	Animal Study
decorin (DCR)	SLRP	Interacts with the side chain of an adjacent DCR molecule to form interfibrillar bridge between adjacent collagen fibrils [35]	DCR-deficient mice developed structurally impaired tendons with abnormal, irregular fibril contours and altered distribution of collagen fibril diameters [36].
biglycan (BGC)	SLRP	Binds to gap regions between sequential collagen fibrils; may compensate for decorin loss [36, 37]; maintains the niche of TSPC [38].	BGC-deficient mice exhibited disorganized collagen fibrils with irregular cross-sectional profiles, increased prevalence of torn cruciate ligaments, and ectopic ossification in their patella tendon [37].
fibromodulin (FMOD)	SLRP	Stabilizes small-diameter fibrils due to the ability to inhibit fibril fusion [39] ; maintains the niche of TSPC [38].	FMOD deficiency led to reduced tendon stiffness, with further stiffness loss when combined with LMC knock out. FMOD and LMC may coordinate in a temporal order: FMOD is involved in early tendon development and LMC later stages [39].
lumican (LMC)	SLRP	Stabilizes small-diameter fibrils due to the ability to inhibit fibril fusion [39].	
fibronectin (FNC)	glycoprotein	Presents binding sites for integrins, collagen, and other ECM proteins, as well as for self-association [21].	FNC affects collagen type III fibril assembly and act as a template for neo-collagen formation during the remodeling phase after injury [31].
collagen oligomeric matrix protein (COMP)	glycoprotein	Interacts with free collagen types I and II molecules, bringing several molecules into close proximity so that these assemblies can further aggregate [40].	The distribution of collagen fibril diameters of tendons and ligaments in COMP ^{-/-} mice shifted toward larger median, with the tendons becoming more lax in cyclic strain tests [41].
tenascin-C (TNC)	glycoprotein	Unclear, directly regulated by mechanical stress; induction of its expression in stretched fibroblasts is rapid [42].	High levels of TNC expression have been found in both developing and diseased tendons. TNC deposition is highest in tissues of high mechanical loading [43].
elastin	fibrous protein	May contribute to the resiliency and elasticity of tendon by promoting the recovery of the wavy collagen configuration after tendon stretch [29].	Elastin is known to situate between fascicles in the canine cruciate ligament. Rat tail tendon fascicles are surrounded by a thin sheath of elastin [44, 45].

1.1.3 Tendon injury and natural healing

Tendon injuries can be broadly categorized into chronic degenerative tendinopathies and acute ruptures. Degenerative tendinopathy is often followed by acute ruptures, with the former characterized by hypervascularity, mucoid degeneration, ectopic bone and cartilage nodules, and disorganized extracellular matrix [46]. The natural healing process of an injured tendon tissue is traditionally divided into three overlapping stages – (1) inflammation, (2) proliferation/repair, and (3) remodeling [47].

In the inflammatory stage, blood clots form immediately following rupture of tendon blood vessels to activate the release of chemo-attractants and serve as a provisional scaffold for invading cells. Inflammatory cells such as neutrophils, monocytes, and lymphocytes migrate from surrounding tissues into the wound site [48], along with the recruitment and activation of TCs until peaks in the subsequent stage known as the proliferative or reparative phase. Roughly two days after the occurrence of injury, fibroblasts from paratenon or surrounding synovial sheath are recruited to the wounded area and proliferate [49]. Meanwhile, intrinsic TCs residing in the endotenon and epitenon migrate to the wound site and begin to proliferate. TCs from both origins are involved in the synthesis of extracellular matrix and establishment of an internal neovascular network [50]. Concurrently, macrophages continue to release growth factors that direct cell recruitment and activity with a declining level of neutrophils. In this stage of healing, the matrix synthesized by TCs is rich in increased amount of collagen type III, water and glycosaminoglycan [51]. Lastly, the remodeling phase begins 1–2 months after injury, in which TCs and collagen fibers become aligned in the direction of tension. A higher proportion of collagen type I is found concomitant with decrease in cellularity, collagen type III and glycosaminoglycan contents [52, 53]. 10 weeks post-injury, the transformation of fibrous tissue

into scar-like tissue commences and continues for years. It should be remembered that repaired tissue never completely restores the mechanical strength it originally possessed before injury and the biochemical and structural characteristics remain abnormal for over one year [54].

Numerous bioactive molecules are involved in orchestrating tissue regeneration during tendon healing. A variety of growth factors are markedly upregulated following tendon injury and are active at multiple stages of the healing process, including insulin-like growth factor-I (IGF-I), transforming growth factor β (TGF- β), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), bone morphogenetic protein (BMP), and connective tissue growth factor (CTGF) (Table 2).

In addition to the spatial and temporal coordination of growth factors in tendon healing, several other families of bioactive molecules have been recently identified as mediators of repair. For instance, matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are known to respond to tendon injury and repair [55, 56]: MMP-9 and MMP-13 participated in collagen degradation in rat flexor tendon laceration while MMP-2, MMP-3, and MMP-14 mediated both collagen degradation and remodeling. Likewise, markers of re-innervation such as growth associated protein 43 (GAP-43) and protein gene product 9.5 (PGP9.5), were found active during tendon healing [57]. Lastly, nitric oxide appears to be involved in tendon healing. Nitric oxide synthase (NOS) levels elevated following Achilles tendon tenotomy in a rat model, while inhibition of NOS reduced the cross-sectional area and the failure load of healing tendons [58]. The increasing understanding of the molecular basis of tendon healing has led to improved quality of clinically repaired tendons through supplementation or inhibition of these bioactive molecules.

Table 2. Bioactive molecules involved in natural tendon healing process [59, 60]

Growth factor	Roles	Animal study
insulin-like growth factor-I (IGF-I)	Promotes the proliferation and migration of cells, stimulates matrix production	IGF-I mRNA levels were significantly higher 3 weeks after injury to rabbit medial collateral ligament [61]. IGF-I protein was increased and peaked in weeks 4-8 following equine flexor tendons lesion [62].
transforming growth factor β (TGF- β)	Regulates cell migration, proteinase expression, fibronectin binding interactions, termination of cell proliferation, and stimulation of collagen production	Rabbit flexor tendons subjected to transection and repair exhibited increased signal for TGF- β 1 mRNA [63]. In rat tendon injury model, TGF- β 1 expression was highest in the early phases of healing and TGF- β 3 was upregulated later [51].
basic fibroblast growth factor (bFGF)	Promotes cell migration, proliferation, angiogenesis, and matrix synthesis	Tendons subjected to transection and repair exhibited an increased signal for bFGF mRNA in both resident tenocytes and in the fibroblasts and inflammatory cells located in the tendon sheath [64].
platelet-derived growth factor (PDGF)	Regulates protein and DNA synthesis at the injury site, regulates the expression of other growth factors	PDGF was detected in canine digital flexor tendons 3, 10, and 17 days after tendon repairs. It is a potent chemoattractant for macrophages and fibroblasts and may stimulate these cells to express other growth factors [65].
vascular endothelial growth factor (VEGF)	Promotes angiogenesis	Significant expression of VEGF occurred at the canine flexor tendon repair site at 7 days post-operatively, with expression localized to cells within the repair site itself [66].
bone morphogenetic protein (BMP)	Maintains tendon homeostasis, promotes matrix synthesis and cell proliferation	BMP-12, -13, and -14, were dramatically elevated at 1 week and gradually decreased thereafter in rat supraspinatus tendon detachment and repair model [67].
connective tissue growth factor (CTGF)	Regulates matrix synthesis and organization	CTGF showed high level of gene expression over a 21-day period of early chicken flexor tendon healing [68]. CTGF was moderately expressed across all time points in both the insertion and mid-substance in a rat supraspinatus tendon repair model [67].

Another noteworthy issue concerns tendon healing is the identification of individual element of the adult tissue that hinders tendon regeneration. Controversial discoveries have been made in attempts to elucidate individual negative elements in adult tendon healing. For instance, the adult microenvironment appears not an impediment to scarless tendon regeneration in some cases, for fetal tenocytes used to fill the adult partial tenotomy defect displayed no scar formation

[69]. This finding suggests that the lack of sufficient healing is an issue intrinsic to aged cells. Conversely, embryonic-like fibrillogenesis by adult human tendon fibroblasts was achieved when cultured in a contracted fibrin gel, indicating an opposing conclusion: the adult biochemical/mechanical milieu inhibits the regenerative potential of tendon cells [70]. Taken together, it remains unclear why adult tendons fail to regenerate.

1.2 TENDON TISSUE ENGINEERING

In cases of severe tendon injury, surgical intervention may be employed to repair or replace the damaged tendon with autografts, allografts, xenografts, or prosthetic devices. In the U.S. alone, around 45% of the 32 million musculoskeletal injuries each year involve tendon, ligament and joint capsule, in which tendon injuries are especially common and require over 120,000 surgical repairs annually [71]. However, the clinical outcomes remain unsatisfactory due to limitations including donor site morbidity, failure rates, risk of injury recurrence and limited long-term function recovery [72-74]. These limitations have spurred the development of tissue engineering strategies, which combine therapeutic cells, biomimetic scaffolds, bioactive molecules, and *ex vivo* mechanical stimulation to create functional replacements or to amplify the innate healing capacity of injured tendon tissue (Fig. 6). Ultimately, tendon tissue engineering aims at improving the quality of healing in order to achieve sufficient restoration of tendon function.

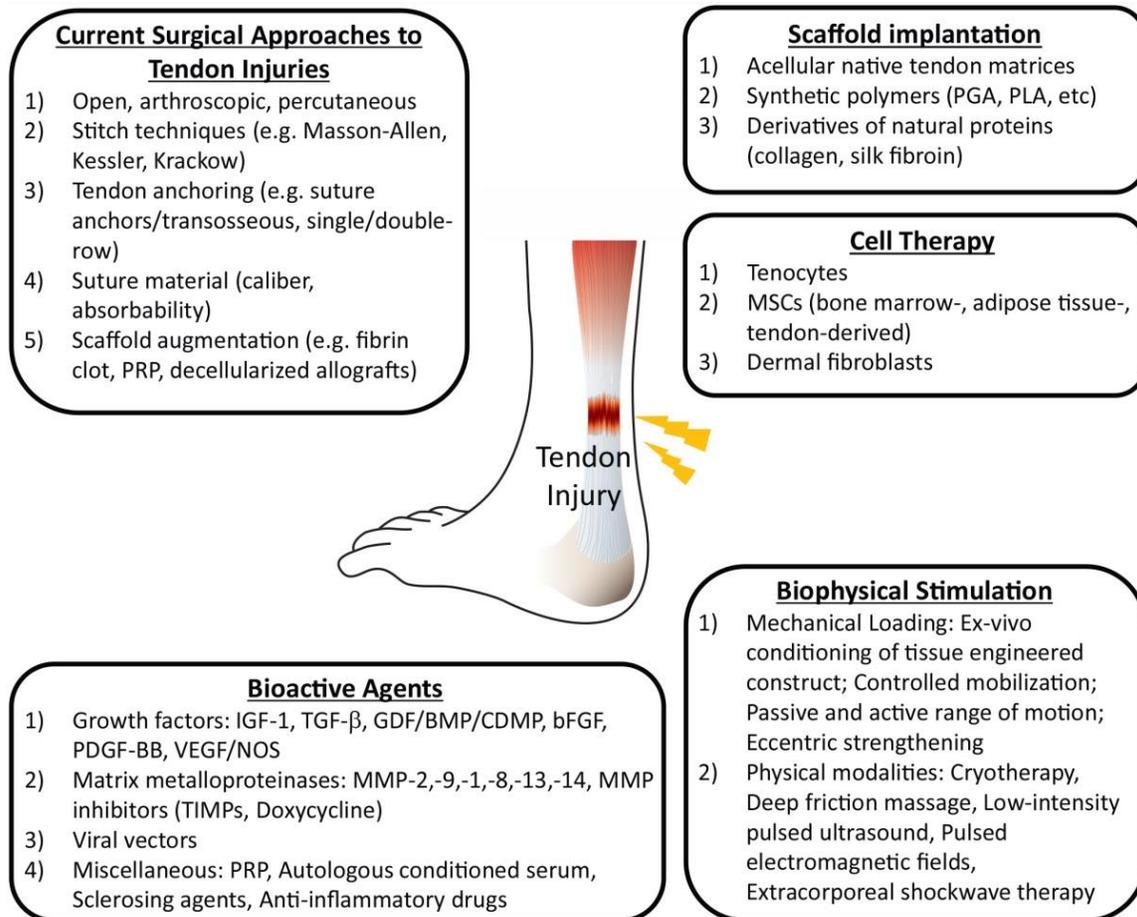


Figure 6. An overview of repair approaches for tendon injuries. Surgical interventions and biophysical stimulation are currently used in clinical care. Meanwhile, tissue engineering strategies are the cutting-edge of tendon healing and regeneration. Engineered replacement of injured tendon using a combination of cells, bioactive molecules, and scaffolds is under intensive investigation.

1.2.1 Cells

Cells that have been used in tendon tissue engineering include TCs, tendon-derived stem/progenitor cells (TSPCs) and mesenchymal stem cells (MSC). TCs, as discussed above, are responsible for building and maintaining the unique tendon ECM consisting of collagen, proteoglycans and glycoproteins. TCs were found to be able to deposit collagen and express

tendon markers *in vitro* including SCX, TNMD, and DCR in response to growth factors [75]. Tendon defects bridged by an autologous TC-engineered graft demonstrated improved mechanical strength and matrix deposition compared to the cell-free groups [76]. Compared to terminally differentiated TCs, dormant tendon-specific adult stem cells, known as TSPCs, appear to have a higher regenerative potential. These cells possess common stem cell characteristics such as clonogenicity, multipotency and self-renewal capacity [38]. In this regard, TSPCs have been intensively studied and applied to tendon tissue engineering. TSPCs seeded in fibrin glue led to superior tendon repair both histologically and biomechanically compared to fibrin glue alone [77]. However, despite the growing knowledge in the functionality of TCs and TSPCs in tendon tissue engineering, harvesting autologous tendon cells could lead to secondary tendon defects at the donor site. Moreover, the relatively hypocellular nature of tendon tissue impedes the use of autologous tendon cells in large scale.

To this end, adult MSCs have been considered as an alternative cell source to address this limitation, for they are easily accessible, expandable to large quantity, and do not cause major donor site morbidity. Due to the shared self-renewal and multi-lineage differentiation potential, MSCs from a variety of tissues have been applied to tendon tissue engineering. Bone marrow MSCs (BMSCs), for instance, has been extensively used to populate various graft materials and demonstrated promising outcomes including significantly increased tendon-related gene expression, higher DNA content, and enhanced mechanical strength [78-81]. While the tendon tissue engineering potential of BMSCs has been carefully explored, less attention has been paid to adipose-derived mesenchymal stem cells (ASCs). Compared to BMSCs, ASCs are (1) harvested by less invasive procedures, and (2) available in greater quantities, for human adipose tissue is ubiquitous and can be isolated in large quantities with little donor site morbidity or

patient discomfort [82]. Moreover, the greater stem cell yield rate of adipose tissue than that of other stem cell reservoirs is another factor that contributes to the suitability of ASCs for regenerative medicine: 1×10^7 adipose stromal/stem cells are reported being isolated from 300 ml of lipoaspirate [83]. In other words, ASCs account for approximately 2% of nucleated cells in processed lipoaspirate, leading to an estimated yield rate of 5,000 fibroblast colony-forming units (CFU-F) per gram of adipose tissue, in contrast to only 100–1,000 CFU-F per milliliter of bone marrow [84].

For the purpose of facilitating tendon healing and regeneration, ASCs need to proliferate and then undergo appropriate tenogenic differentiation to adopt tendon cell phenotype and deposit tendon-specific matrix in order to develop into a functional replacement tissue at sites of injury. Nevertheless, despite the extensive exploration of the differentiation potential of ASCs, the tendon cell lineage commitment of ASCs remains elusive and controversial. For instance, murine and canine ASCs demonstrated up to 6-fold of upregulation in SCX expression when treated with 100 ng/ml of GDF-5 for 1 week, whereas equine ASCs cultured in 3D collagen scaffold displayed no such trend toward tendon cell lineage with GDF-5 treatment [85-87]. Furthermore, as described below, the contradictory findings regarding the tenogenesis potential of human ASCs (hASCs) adds uncertainty to the established differentiation protocol. Goncalves et al. succeeded in acquiring tendon phenotype of abdominal hASCs along with aligned collagen pattern that resembles the native tendon ECM through TGF- β 1 treatment. Meanwhile, this research team also reported the efficacy of a group of other growth factors on partial induction of tendon phenotype of hASCs [88]. However, Eagan et al. questioned the suitability of hASCs for tendon tissue engineering and showed the lack of any significant and consistent up-regulation in the expression of COL I, TNC, or SCX, in hASCs treated for up to 4 weeks with TGF- β 1 or

IGF-I [89]. Lastly, hASCs cultured in standard growth medium demonstrated lower SCX expression compared to TSPCs, suggesting the lack of spontaneous tenogenesis of hASCs in the absence of proper induction [90]. Due to the inconsistent tenogenesis manifested by ASCs, their potential as the cellular component for tendon tissue engineering is not sufficiently exploited. To date, ASCs are used for tendon tissue engineering primarily as a means to improve vascularization, matrix deposition, and implant integration, with little consideration has been paid to their potential ability to recapitulate tendon cell phenotype if provided with proper tendon microenvironment. There is thus a critical need to explore and develop a robust tenogenesis protocol for hASCs.

1.2.2 Scaffolds

Scaffolds are another crucial element for tendon tissue engineering. They provide temporary biomechanical support to the healing tissue until deposition of the newly synthesized native matrix, and thereby preventing tendon re-rupture. In addition, scaffolds possessing desired features should be able to enhance tendon healing through directing cell proliferation, promoting matrix production, and organizing the matrix into aligned pattern. Scaffolds may be modified to improve regenerative events through approaches such as surface modification, growth factor adsorption, and mechanical stimulation-mediated cellular remodeling [91]. Commonly used scaffolds for tendon tissue engineering can be divided into three categories: (1) native tendon matrices, (2) synthetic polymers, and (3) derivatives of naturally occurring proteins.

Scaffolds derived from native tendon tissue theoretically retain natural biomechanical and biochemical properties, and thereby may serve as a biomaterial to support tendon healing. [92]. The effective removal of antigenic epitopes presented by residing cells within xenogeneic and

allogeneic tissues and organs is essential to avoid or minimize the adverse immune response or overt immune-mediated rejection by recipients of ECM scaffold material [92, 93]. In contrast, most molecules that constitute the ECM are conserved across species and therefore well tolerated in recipients. Commonly used methods of decellularization have been carefully reviewed by Badylak et al., including a combination of physical and chemical treatments [92, 94]. For instance, cell lysis agents such as tri(n-butyl)phosphate (TnBP), sodium dodecyl sulfate (SDS) and Triton X-100 in combine with sonication, agitation and freeze-thaw cycles are often used to disrupt cell membranes, release cell contents and facilitate the subsequent rinsing and removal of cell remnants from the ECM. The goal of any decellularization protocol is to remove the cellular materials while preserve the composition, mechanical integrity and biological activity of the remaining ECM. The decellularized allografts or xenografts are then developed into biocompatible and functional delivery vehicles for cells, therapeutic gene vectors, and other biological agents [95-97].

Scaffolds prepared from natural proteins and derivatives are an alternative option for tendon tissue engineering. For instance, as the major structural protein of tendon tissue, collagen and its derivative, gelatin, exhibit bio-functionality in supporting cell adhesion and cell proliferation [98, 99]. Cells encapsulated within collagen/gelatin hydrogels are easily distributed homogeneously by mixing during gel preparation without the need for any further scaffold modification. Collagen-derived scaffolds may be further modified by cross-linking or co-fabricating with other materials to improve the mechanical strength or hydrophobicity [30, 100, 101]. For instance, methacrylate pendant groups can be conjugated to the amine groups on gelatin molecules and covalently crosslinked in the presence of proper initiator. In this regard, our lab has succeeded in creating a methacrylated gelatin (mGLT) hydrogel by using cell

compatible, visible light (VL)-activated initiator to crosslink methacrylate groups [102]. More importantly, the crosslinkable nature of this material allows the integration between mGLT and other forms of scaffold, suggesting the utility of mGLT in developing composite scaffold consisting of multiple components. The details of scaffold development and characterization will be presented in the following chapters.

Beyond modified native materials, a great number of biodegradable/biocompatible synthetic polyester materials have been applied to tendon tissue engineering, including poly- ϵ -caprolactone (PCL), polyglycolic acid (PGA), poly-L-lactic acid (PLLA) and their copolymer poly(lactic-co-glycolic acid) (PLGA). Featured by relatively simple and well-defined chemical composition, scaffolds fabricated from these materials are tunable in physical and chemical properties and thereby highly versatile for clinical commitments. In terms of tendon tissue engineering, polyester scaffolds are often used to bridge the defect and serve as a vehicle to deliver and immobilize therapeutic cells to the site of injury [76, 103, 104]. Nevertheless, synthetic polyester materials suffer from several limitations, including absence of biochemical motifs for cellular attachment and accompanying inability to fully support cell activity.

The presentation of topographical cues must be carefully designed when engineering cell-interacting scaffold, for the micro/nano-structure of material-cell interface is known to modulate cellular behavior. As far as tendon tissue engineering is concerned, alignment is an important topographical characteristic to create in order to reproduce the aligned fibrous feature of native tendon tissue. The impact of scaffold alignment on tendon tissue engineering is manifold: Firstly, placing cells on substrate with alignment pattern leads to increased expression of tendon markers, such as SCX and TNMD [105, 106]. Secondly, cell extension and matrix deposition are also found to align along the orientation of fibers that constitute scaffold mesh [104, 107]. It is

noteworthy that the tenogenic influence of substrate alignment is observed across various mammalian species and cell types, implying a shared underlying molecular basis of cell substrate-sensing. It is thought that integrin, the primary cell surface receptor family for ligands provided by scaffolds, functionally mediate this effect due to their pivotal role in cell-substrate interaction and highly conserved protein sequence and conformation across species.

1.2.3 Bioactive molecules

As reviewed earlier, numerous growth factors have been found to be actively involved in tendon development and healing. Nevertheless, the application of growth factors to clinical tendon repair remains challenging, due to the paucity of knowledge in the spatiotemporal interaction and distribution of growth factors that would produce the best effects. We highlight here some successes that have been achieved in accelerating the healing process and subsequently enhancing the quality of repaired tissue through exogenous application of growth factors.

The application of IGF-I to the inflammation site in rat Achilles tendon mitigated inflammation-induced functional deficits [108]. IGF-I also stimulated the proliferation and migration of fibroblasts at the site of injury, leading to increased production of collagens and other ECM components during the remodeling stages [52, 109]. Like IGF-I, TGF- β is involved in matrix synthesis in tendon healing [110]. Equally important, TGF- β signaling is able to drive tenogenic differentiation through the induction of SCX, indicating its potential in stem cell-based tendon tissue engineering (See **1.1.1** and **1.1.2**). TGF- β is therefore of particular research interest to us in attempts to explore the tenogenic potential of MSCs. Other TGF- β super family members, growth differentiation factor-5 (GDF-5, also known as BMP-14) and GDF-7 (BMP-

12) are reportedly effective in healing injured tendon tissue [111, 112]. Another frequently used growth factor in tendon tissue engineering is bFGF. Administered either through injection or scaffold-based releasing, bFGF was able to significantly increase tenogenesis and collagen type III level [113-115]. As was seen with IGF-I, PDGF stimulated tendon ECM protein production and DNA synthesis in a dose-dependent manner, and therefore improves healing [116, 117].

In addition to growth factors, the extracellular matrix (ECM), a complex network of interacting macromolecules secreted and maintained by the residing cells, is responsible for both biomechanical and microenvironmental signaling functions, and is therefore considered as potential bioactive molecules for tissue engineering. While there are many common ECM components, each tissue and organ possesses a unique ECM composition tailored to tissue-specific physiologic and mechanical requirements in order to maintain a particular cell phenotype and functionality. In this regard, properly prepared ECM that has a complex composition of diverse molecules may support the cellular processes necessary for optimal function of the tissue and organ from which the ECM is harvested [92]. Badylak et al. has extensively studied and reported the bioactivity of ECM prepared from a variety of tissues, including urinary bladder matrix (UBM), small intestinal submucosa (SIS), brain, bone, esophagus, and muscle on the basis of their influence in angiogenesis, cell proliferation, cell migration, cell differentiation, and host tissue remodeling [118-121]. As far as tendons are concerned, these tissues are rich in both collagenous and non-collagenous ECM proteins (Table 1). The influence of tendon ECM on tendon cell behavior is manifold, including regulation of proliferation and differentiation, as depletion of key ECM components impairs the self-renewal and tendon-specific gene expression of tendon progenitor cells [38]. Furthermore, hASCs and rabbit TSPCs seeded on decellularized tendon/ligament ECM showed improved proliferation and

differentiation in 2D cultures [122, 123]. However, challenges remain in understanding the mechanism and utility of ECM as bioactive factors for tissue engineering. On one hand, the combined effects of ECM composition and other environmental cues, such as tissue architecture, mechanical loading and presence of growth factors, are worth studying to gain further insights into the role of ECM biochemistry in cell activity regulation. On the other hand, the method employed to dissociate tissue has substantial impact on the bioactivity of subsequent homogeneous ECM extract. To date, acid-pepsin based enzymatic digestion on decellularized ECM is the most commonly used solubilization approach to obtain ECM solution containing peptides fragments of parent ECM molecules such as collagen, fibronectin along with embedded growth factors. However, the drawbacks of digestive enzyme treatment such as fragmentation of functional motifs and unwanted dissociation between interactive molecules might lead to the loss of activity of a fraction of ECM molecules. In this regard, treatment with chaotropic agent, such as urea, to disrupt lipid-lipid, lipid-protein, and protein-protein interactions in the ECM network without enzymatic digestion is considered as a promising approach to obtain a water soluble extract of ECM rich in non-collagenous proteins that would have otherwise been significantly degraded by enzyme treatment. Using this method, we have prepared a soluble fraction of tendon ECM (tECM) in this study and will explore the composition and bioactivity of tECM in the following chapters.

1.2.4 Mechanical stimulation

As tendons transmit force from muscles to bone, the role of mechanical loading in tendon biology has been extensively studied. In the context of tendon injury and repair, it is recognized that controlled motion of healing tendons is required to improve clinical outcomes [124].

Although the optimal time span and magnitude of loading for tendon rehabilitation protocol is not clearly understood, it is well accepted that healing tendons should be subjected to loading in a controlled manner to drive favorable remodeling and functional outcomes [124]. More recently, the cellular and molecular basis for loading-induced healing of tendons and ligaments has been explored. Fibroblasts and MSCs exposed to static or cyclic uniaxial tension manifested increased cellular proliferation, ECM production, and tenogenesis [125-127]. Integrin-dependent signaling is pivotal in conveying external mechanical stimulation to downstream intrinsic targets. For instance, Xu et al. revealed that RhoA/ROCK and focal adhesion kinase coordinate to drive tenogenesis of MSCs in response to mechanical stretch through the regulation of cytoskeletal organization [128]. Other signaling pathways, such as TGF- β , are believed to be relevant to force induced tenogenesis independently of cytoskeletal tension [129]. In addition to improving the biochemical and material properties of healing tendons, mechanical stimulation is also able to precondition tissue engineered constructs prior to implantation for improved *in vivo* outcomes. For example, cell-repopulated ECM demonstrated higher ultimate stress and elastic moduli after being cyclically stretched in bioreactors [130]. The improved mechanical properties can be explained in part by increased deposition of collagen fibrils in the direction of strain [131].

Although recent research has begun to elucidate the effect of mechanical stimulation in improving natural healing and properties of cellular constructs prepared *ex vivo*, a comprehensive understanding is lacking regarding the influence of mechanical loading on tendon healing. For instance, the effect of mechanical stretch on the integration between tendon tissue engineering constructs and native tissue is largely unknown [132]. Moreover, the efficacy of growth factors injected into a tendon injury site to improve healing seems dependent on the mechanical environment of the tissue [133]. For example, minimal or no bone formation was

observed as a consequence of GDF-6 treatment when the healing rat tendons were loaded. In contrast, GDF-6 led to an increased formation of cartilage or bone in unloaded healing rat tendons [134]. Taken together, a thorough understanding of the impact of mechanical stimulation will be needed to realize the ultimate promise of regenerative medicine for tendon injuries.

1.3 RESEARCH OVERVIEW

The objective of this dissertation research is to understand the bioactivity of a soluble ECM fraction extracted from native tendon tissue (tECM) on the tenogenic potential of human adipose stem cells (hASCs). **We hypothesize that tECM enhances proliferation and tenogenic differentiation of hASCs induced by either static tension or growth factor treatment.** To achieve the proposed goal, this research is divided into three relevant aims.

Specific Aim 1: Prepare and characterize the composition of tECM. Determine the activity of tECM on the tenogenesis of hASCs induced by mechanical loading using a 3D tension-bearing scaffold.

Specific Aim 2: Determine the activity of tECM on the proliferation and TGF- β 3 induced tenogenesis of hASCs cultured in 2D and 3D. Examine the influence of tECM on collagen synthesis and organization by hASCs cultured on 3D aligned fibrous scaffolds.

Specific Aim 3: Develop a novel composite scaffold as a tendon graft consisting of electrospun PCL microfibers and methacrylated gelatin (mGLT). A cell-scaffold construct will be prepared by this scaffold seeded with hASCs to mimic the architecture and cell phenotype of

native tendon tissues; and also serve as a reservoir of tECM as well as other tenogenic cues for tendon tissue engineering.

The following chapters detail the results of each specific aim.

2.0 TENDON-DERIVED EXTRACELLULAR MATRIX ENHANCES TENOGENIC DIFFERENTIATION OF HUMAN ADIPOSE-DERIVED STEM CELLS CULTURED IN 3-DIMENSIONAL COLLAGEN SCAFFOLD UNDER TENSION

This chapter includes material from the following publication:

Yang G, Rothrauff BB, Lin H, Gottardi R, Alexander PG, Tuan RS. Enhancement of tenogenic differentiation of human adipose stem cells by tendon-derived extracellular matrix. *Biomaterials*. 2013 Dec;34(37):9295-306. doi: 10.1016/j.biomaterials.2013.08.054.

2.1 ABSTRACT

Mesenchymal stem cells (MSCs) have gained increasing research interest for their potential in improving healing and regeneration of injured tendon tissues. Developing functional three-dimensional (3D) scaffolds to promote MSC proliferation and differentiation is a critical requirement in tendon tissue engineering. Tendon extracellular matrix has been shown to maintain the tenogenic potential of tendon stem cells and stimulate tenogenesis of human adipose stem cells (hASCs) in 2D culture. This study aims at characterizing the biological composition of urea-extracted fraction of tendon ECM (tECM) and its tenogenic effect on hASCs cultured in a 3D collagen scaffold under uniaxial tension. The tECM obtained was cell-free and rich in ECM proteins. hASCs seeded in tECM supplemented scaffold exhibited

significantly increased proliferation and tenogenic differentiation. The presence of tECM also greatly suppressed the osteogenic differentiation of hASCs triggered by uniaxial tension. In addition, tECM-supplemented constructs displayed enhanced mechanical strength, accompanied by reduced expression and activity of MMPs in the seeded hASCs, indicating a regulatory activity of tECM in cell-mediated scaffold remodeling. These findings support the utility of tECM in creating bio-functional scaffolds for tendon tissue engineering.

2.2 INTRODUCTION

Tendons are fibrous tissues composed of densely packed collagen fiber bundles that connect muscle to bone and function in transmitting force from muscle to bone [50]. These tissues bear high tensile force and are therefore prone to injuries such as rupture and laceration. In the U.S. alone, tendon, ligament, and joint capsular injuries account for 45% of the 32 million musculoskeletal injuries each year [71]. It was estimated that about 200,000 tendon and ligament repair surgeries are performed annually in the U.S. [135]. Unfortunately, the natural healing process of tendons is slow and the resulting tissue is inferior in structure and function due to their hypocellular and hypovascular nature [74]. In cases of severe tendon injury, surgical treatments may be used to repair or replace the damaged tendon with autografts, allografts, xenografts, or prosthetic devices [136]. However, the clinical outcomes remain unsatisfactory due to limitations including donor site morbidity, high failure rates, risk of injury recurrence and limited long-term function recovery [48, 137, 138]. These limitations have spurred the development of tissue engineering strategies, which use a combination of cells, scaffolds and bioactive molecules, to treat tendon injuries [91, 112, 115, 139].

The use of adult mesenchymal stem cells (MSCs) as the cellular component for tendon tissue engineering has been of particular research interest in recent years [136]. While the most common approach to direct MSCs to differentiate into a specific lineage *in vitro* is through growth factors stimulation, differentiation of MSCs into tendon cells (tenogenesis) can be induced through uniaxial tension in three-dimensional (3D) culture [91, 126, 140]. For this purpose, a variety of biomaterials have been used as the scaffold to convey desired mechanical loads to the seeded cells. For example, when cultured under cyclic stretch, MSCs seeded on polyester fibrous scaffolds demonstrated enhanced tenogenesis [141]. Similarly, collagen gel has been used as a scaffold for MSC culture under uniaxial tension to create engineered tissues mimicking native tendons and ligaments [125, 126, 142]. However, none of these load-transmitting scaffolds possesses ideal bio-functionality for successful tendon tissue engineering, which refers to the ability to support cell proliferation, differentiation into tendon-forming cells without ossification, and matrix remodeling to generate a functional replacement at the wound site [50, 143, 144]. Therefore, the need remains in tendon tissue engineering to develop supportive scaffolds with desired bio-functionality.

To date, a number of extracellular matrices have been used in various approaches to address the lack of bio-functionality in tissue engineering scaffolds [92]. In native tissues, the extracellular matrix (ECM), a complex network of interacting macromolecules that occupies the space between cells, is principally responsible for both biomechanical and microenvironmental signaling functions [145]. While there are many common ECM components, each tissue and organ possesses a unique ECM composition tailored to tissue-specific physiologic and mechanical requirements in order to maintain a particular cell phenotype and functionality [92]. Tendon tissues are rich in ECM components and contain fewer cells than most tissues, and many

of the tendon ECM components as well as growth factors embedded in the ECM appear to be retained in decellularized tendons [146]. The influence of ECM on tendon cell behavior is many-fold, including regulation of proliferation and differentiation, as depletion of key ECM components impairs the self-renewal and tendon-specific gene expression of tendon progenitor cells [38]. Thus, the use of native tissue matrices in scaffold synthesis may be very beneficial in tissue engineering approaches to tendon repair. For example, both human adipose derived stem cells (hASCs) and rabbit tendon stem/progenitor cells (rTSPCs) seeded on decellularized tendon/ligament ECM showed improved proliferation and differentiation in a 2D format [122, 123]. These findings, taken together, suggest that tendon ECM may be included in 3D scaffold to enhance tenogenic differentiation of encapsulated MSCs, particularly in constructs cultured under tensile conditions, to produce a more functional neo-tendinous tissue.

In this study, we hypothesize that incorporation of native tendon ECM components will enhance the bio-functionality of 3D tension-bearing scaffold. To test this hypothesis, tendon ECM was extracted with urea and incorporated into a 3D collagen scaffold into which hASCs were seeded, and the construct was placed in culture under uniaxial tension for over 7 days. Cell proliferation, differentiation, and matrix remodeling capacity within these tECM-enhanced constructs were analyzed, and the influence of tECM on the mechanical properties of the constructs was assessed.

2.3 MATERIALS AND METHODS

2.3.1 Tissue harvest

Bovine Achilles tendons were harvested from eight 2-3 week old bovine hind legs (Research 87). The midsubstance of the tendinous portion between the distal end of gastrocnemius muscle and the calcaneal insertion was dissected and immediately immersed in phosphate buffered saline (PBS; Gibco) supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich), 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) and 1 x Penicillin-Streptomycin (P/S; Gibco), and stored at -20°C until processing. EDTA, PMSF, and P/S were added for their metalloproteinase inhibition, serine protease inhibition and anti-bacterial effect, respectively.

2.3.2 Extraction of tECM

To obtain tECM powder, Achilles tendons were frozen in PBS at -80 °C and then thinly sliced at 40 µm thickness using a cryotome (Leica CM 1850). The tendon sections were pulverized in liquid nitrogen with a mortar pestle, and the tissue powder was then decellularized by incubation in 1% Triton X-100 (Sigma-Aldrich) in PBS under continuous agitation for 24 h at 4°C, followed by three washes, 30 min each in PBS. The decellularized material was treated with 200 U/ml DNase and 50 U/ml RNase (Worthington) solution at 37°C for 12 h and then washed in PBS 6 times, 30 min for each wash. After nuclease digestion, the preparation was extracted with 3 M urea (Sigma-Aldrich) in water with gentle agitation for 3 days at 4°C. The suspension was then centrifuged for 20 min at 1,500 g to collect the extract supernatant. Urea was removed

by dialysis of the extract contained in dialysis cassettes (3,500 MWCO, Thermo Scientific) against deionized water for 2 days at 4°C. Water was changed every 4 h. The dialyzed tECM extract was transferred into centrifugal filter tubes (3,000 MWCO, Millipore) and spin-concentrated by 10 folds for 30 min at 1,500 g. The final ECM solution was filter-sterilized through PVDF syringe filter units (0.22 µm, Millipore), protein concentration determined using the BCA assay (Thermo Scientific), and then stored at 4°C until further use. Total proteins were extracted from homogenized original tendon tissues by TM buffer (Total Protein Extraction Kit, Millipore) and served as a control.

2.3.3 SDS-PAGE and Western blot assay

For SDS-PAGE and Western blotting, protein samples of the same concentration was mixed with loading buffer and reducing agent (Life Technologies), and heated at 70°C for 10 min. Protein samples were then loaded into wells of pre-cast NuPAGE gels (NuPAGE Bis-tris Mini Gel, Life Technologies) and separated by electrophoresis in MOPS SDS running buffer for 50 min at constant 200 V. Proteins were transferred onto PVDF membranes using the iBlot dry blotting system (Invitrogen). After incubating in 5% powder milk in TBST for blocking, membranes were incubated at 4°C overnight with the following primary antibodies: mouse anti-fibronectin, sheep anti-decorin, goat anti-biglycan or rabbit anti-fibromodulin (Abcam). The membranes were then washed in TBST 6 times and incubated with corresponding horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Blots were allowed to react with chemiluminescence substrate (West Dura Extended Duration Substrate, Thermo Scientific) for 5 min and imaged with a CCD camera gel imaging system. (FOTODYNE)

2.3.4 Cell isolation and culture

hASCs were isolated from the lipoaspirate-derived fat tissue from three female donors (28, 32, and 36 years old) with Institutional Review Board approval (University of Pittsburgh) using an automated cell isolation system (Tissue Genesis). hASCs were cultured in growth medium (DMEM-high glucose, 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin, Gibco). At 80% confluence, cells were detached with 0.25% trypsin in 1 mM EDTA (Gibco) and passaged. All experiments were performed with passage 3 (P3) hASCs. To reduce donor to donor variation, cells from the three donors were pooled in equal number.

2.3.5 3D culture of cells under static tension

To study the combined effect of mechanical stimulation and tECM on hASCs, the Flexcell Tissue Train Culture System (Flexcell) was employed. Briefly, hASCs at P3 were trypsinized from cell culture flasks and mixed at 1×10^6 cells/ml with 0.5% collagen type I solution at neutral pH (Purecol EZ, Advanced Biomatrix) containing either 10% (v/v) 800 $\mu\text{g/ml}$ collagen type I solution (Purecol EZ, Advanced Biomatrix) or 10% (v/v) 800 $\mu\text{g/ml}$ tECM solution. To set up 3D gel constructs, linear Trough LoadersTM were placed in baseplates beneath the flexible membrane of Tissue TrainTM culture plate so that the anchor stems were aligned with the long axis of the Trough Loader. A vacuum was applied to deform the membrane into the conformation of the trough. 300 μl of cell-gel suspension was then pipetted into each well and allowed to gel at 37°C for 2 h. After the gel set, the vacuum was released and the culture plates were dissociated from the base plates. Gels appeared as linear bands attached at each anchor end in the well (Fig. 9 A), and uniaxial tension was generated as a result of cell traction that caused

the gel to contract. hASCs were also seeded into 6-well tissue culture plates as 2D controls at 1×10^5 cells/well. Growth medium was added to each well and changed every 3 days. Contraction rate was determined from digital images taken on days 1, 3 and 7 of culture analyzed in Image J (NIH). Total construct area between the anchors divided by the length between anchors was defined as the average width. Constructs were also harvested at the same time points for other analyses.

2.3.6 Scanning electron microscopy (SEM)

hASC-seeded 3D constructs were collected at day 1, 3 and 7, washed twice in PBS, fixed with 2.5% glutaraldehyde in PBS for 2.5 h at room temperature, and then rinsed in PBS and dehydrated in a graded ethanol series. After critical point drying, samples were mounted on aluminum stubs, sputter-coated with 3.5 nm gold, and examined by SEM (field emission, JEOL JSM6335F), operated at 3 kV accelerating voltage and 8 mm working distance. The external surface of the central part of the constructs was selected for imaging.

2.3.7 Cell viability analysis

On days 1, 3 and 7, MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) was performed according to the manufacturer's instructions to determine cell viability for cells from each group. Briefly, the MTS reagent was thawed and diluted 1:5 in phenol red free medium (Gibco). 3 ml working solution was added into each flexcell well and incubated for 3 h at 37°C. The reduced reagent was then transferred to a 96 well plate and A_{490} determined spectrophotometrically using a microplate reader (BioTek), with reagent blank as

control. The readings were normalized to the pure collagen group at each time point. For Picogreen (Quant-iT PicoGreen dsDNA Reagent, Invitrogen) assay, samples were first washed twice in PBS for 5 min and then digested in 500 µg/ml papain (Sigma-Aldrich) at 60°C for 2 h. 100 µl 1x Picogreen working solution in TE buffer was added into 100 µl papain solution containing one digested gel construct and incubated for 2 min protected from light. The fluorescence of samples and standards were measured using a fluorescence microplate reader (excitation 480 nm, emission 520 nm, BioTek), with reagent blank as control. The fluorescence values were compared against a lambda phage DNA standard curve to determine the DNA concentration. The calcein acetoxymethyl (calcein-AM)/ethidium homodimer-1 (EthD-1) based Live/Dead assay (Invitrogen) was performed to confirm the cell viability. At days 1 and 7, constructs from each group were incubated in calcein-AM and EthD-1 (diluted 1:1000 in growth medium) for 1 h, and then visualized by fluorescence microscopy. For nuclear staining, tendon tissues were placed on glass slides and incubated with 500 µl 300 nM diamidino-2-phenylindole, dilactate (DAPI, D3571 Invitrogen) in PBS for 1 min. The samples were then washed 3 times in PBS and viewed using fluorescence microscopy.

2.3.8 Histology

Tendon tissues and Flexcell constructs were washed 3 times in PBS, fixed for 12 h in 10% phosphate buffered formalin (Fisher Scientific) at room temperature, dehydrated in serial ethanol solutions followed by xylene, and embedded in paraffin. The embedded samples were sectioned at 10 µm thickness, deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin (H&E), or picosirius red for visualization.

2.3.9 Real-time PCR analysis

At each time point, total RNA was isolated using an RNA extraction Kit (Qiagen) according to the manufacturer's protocol. RNA concentrations were determined based on A_{260} using the Nanodrop spectrophotometer (Thermo Scientific). First-strand cDNA was synthesized with oligo(dT) primers using a cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was performed using SYBR green Supermix in a Step One Plus real-time PCR system (Applied Biosystem, Life Technologies) and then analyzed by comparative Ct quantification ($\Delta\Delta$ -Ct method). Primers were used for tenogenesis marker genes including scleraxis (SCX) and tenomodulin (TNMD), osteogenesis marker genes including runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP) and osteocalcein (OCN), ECM protein genes including collagen I (COL I), collagen III (COL III), decorin (DCR) and tenascin-C (TNC), and matrix metalloproteinase (MMP) genes including MMP-1, -2, -8, -9 and -13. The targets and sequences of primers are shown in Table 3 except tenomodulin, which was purchased from Qiagen (Hs TNMD, QuantiTect Primer Assay, Qiagen). Primer specificity and product sizes were confirmed by melting curve analysis and agarose gel electrophoresis. The expression level of each gene was normalized to GAPDH.

Table 3. Primer sequences used in assessment of gene expression by real time PCR analysis

Gene		Primer sequence (5'-3')	Product size (bp)
GAPDH	Forward	CAAGGCTGAGAACGGGAAGC	194
	Reverse	AGGGGGCAGAGATGATGACC	
SCX	Forward	ACACCCAGCCCAAACAGA	65
	Reverse	GCGGTCCTTGCTCAACTTTC	
COL I	Forward	GGCTCCTGCTCCTCTTAGCG	123
	Reverse	CATGGTACCTGAGGCCGTTC	
COL III	Forward	CAGCGGTTCTCCAGGCAAGG	179
	Reverse	CTCCAGTGATCCCAGCAATCC	
DCR	Forward	CGCCTCATCTGAGGGAGCTT	205
	Reverse	TACTGGACCGGGTTGCTGAA	
TNC	Forward	GGTGGATGGATTGTGTTCTGAGA	328
	Reverse	CTGTGTCTTGTCAAAGGTGGAGA	
RUNX2	Forward	CAACCACAGAACCACAAGTGC	196
	Reverse	TGTTTGATGCCATAGTCCCTCC	
ALP	Forward	TGGAGCTTCAGAAGCTCAACACCA	454
	Reverse	ATCTCGTTGTCTGAGTACCAGTCC	
OCN	Forward	ATGAGAGCCCTCACACTCCTC	294
	Reverse	GCCGTAGAAGCGCCGATAGGC	
MMP-1	Forward	GGGGCTTTGATGTACCCTAGC	142
	Reverse	TGTCACACGCTTTTGGGGTTT	
MMP-2	Forward	GATACCCCTTTGACGGTAAGGA	112
	Reverse	CCTTCTCCAAGGTCCATAGC	
MMP-8	Forward	TTTTGATGCCGAAGAAACATGGA	97
	Reverse	GTGAGCGAGCCCCAAAGAA	
MMP-9	Forward	TGTACCGCTATGGTTACTCTCG	97
	Reverse	GGCAGGGACAGTTGCTTCT	
MMP-13	Forward	ACTGAGAGGCTCCGAGAAATG	103
	Reverse	GAACCCCGCATCTTGGCTT	

2.3.10 Mechanical test

Mechanical testing was performed using a mechanical tester (Bose 3230) consisting of a 2.45 N (250 g) load cell with accuracy of 0.004 N. At days 3 and 7, constructs were washed twice in PBS for 5 min, and then separated from the rubber membrane, while remaining attached

to the anchors. The construct was then fixed between two clamps through the anchors (Fig. 13 A). Samples were pre-loaded to remove slack and then stretched 10 mm at an elongation rate of 0.02 mm/sec. The load/displacement curve was recorded. For each construct, maximum force recorded at the peak of the curve was defined as maximum load (N), and the slope of the linear region in load/displacement curve as stiffness (N/mm).

2.3.11 MMP activity assay

MMP activity in conditioned medium was assayed. Briefly, hASC-seeded constructs were cultured in serum- and phenol red-free medium (DMEM + 1% ITS (Gibco) + penicillin/streptomycin) for up to 7 days. At day 1, 3 and 7, conditioned media were collected and incubated with an equal volume of 1 mM 520 MMP FRET substrate XI working solution (AnaSpec) in a black 96-well plate at 37 °C for 1 h. Fluorescence was measured every 5 min (excitation 485 nm, emission 528 nm). The reaction rate calculated from the slope of the linear portion of the kinetics curve represented the activity level of MMPs secreted from hASCs in scaffolds.

2.3.12 Statistical analysis

Data are presented as mean \pm standard deviation (SD). Each experiment was performed independently at least three times. Two-tailed Student's t-test and one-way ANOVA followed by Bonferroni post hoc test were used for determining statistical significance of two-group comparisons and multiple-group comparisons, respectively. Results with $p < 0.05$ were considered statistically significant.

2.4 RESULTS

2.4.1 Characteristics of tendon-derived ECM

Decellularization was verified by the lack of cell nuclei using H&E and DAPI staining (Fig. 7 A-D) and by the reduction in DNA content after Triton X-100 and subsequent nuclease treatment compared to that of original tendon tissues (Fig. 7 E, n=4). The combination of Triton X-100 and subsequent nuclease treatment significantly reduced double-stranded DNA content compared to untreated tendon tissues ($p < 0.01$).

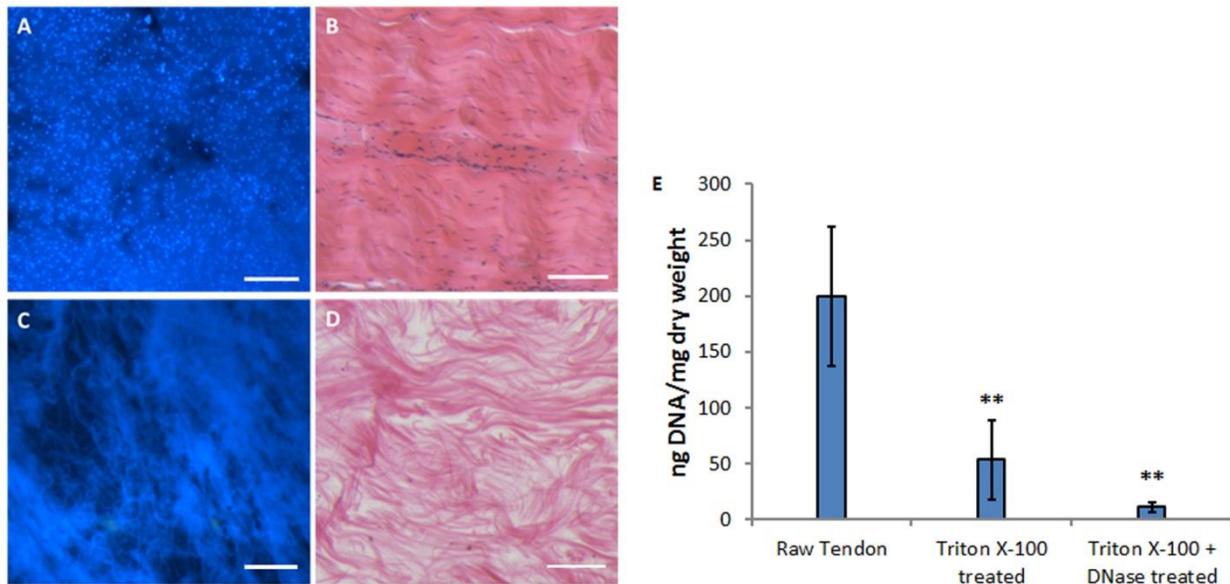


Figure 7. Decellularization of tendon tissue. Tendon tissue was stained with DAPI (A, C) or H&E (B, D). Prior to decellularization (A, B), abundant cell nuclei are visible. After decellularization (C, D), nuclei are rarely seen in the tissue, whereas ECM remains. (E) Triton X-100 treatment alone (Triton X-100 treated) and with subsequent nuclease treatment (Triton X-100 + DNase treated) both significantly reduce the double stranded DNA content compared to untreated tendon tissues (Raw Tendon). Scale bar=100 μ m; **, $p < 0.01$ compared to Raw Tendon group, n=4

SDS-PAGE analysis revealed that tECM contained a number of low molecular weight (<100 KD) protein components that were absent in commercial bovine collagen type I solution (Fig. 8 A), but present in total tendon extract. This observation indicated the preservation of biochemical composition of tendon tissue after decellularization and protein extraction. Western blot assay showed that at least four types of critical tendon ECM protein components were present in this tECM extract: decorin, biglycan, fibromodulin and fibronectin (Fig. 8 C). In contrast, none of these four types of proteins was detected in the collagen type I sample. To confirm the consistency of the extraction method, the compositions of tECM extracts harvested independently from 4 calves were analyzed by SDS-PAGE and Western blot assay (Fig. 8 B, D). The yield, expressed as the final protein content in the extract solution divided by the wet weight of starting tissue, was $0.125\% \pm 0.019\%$.

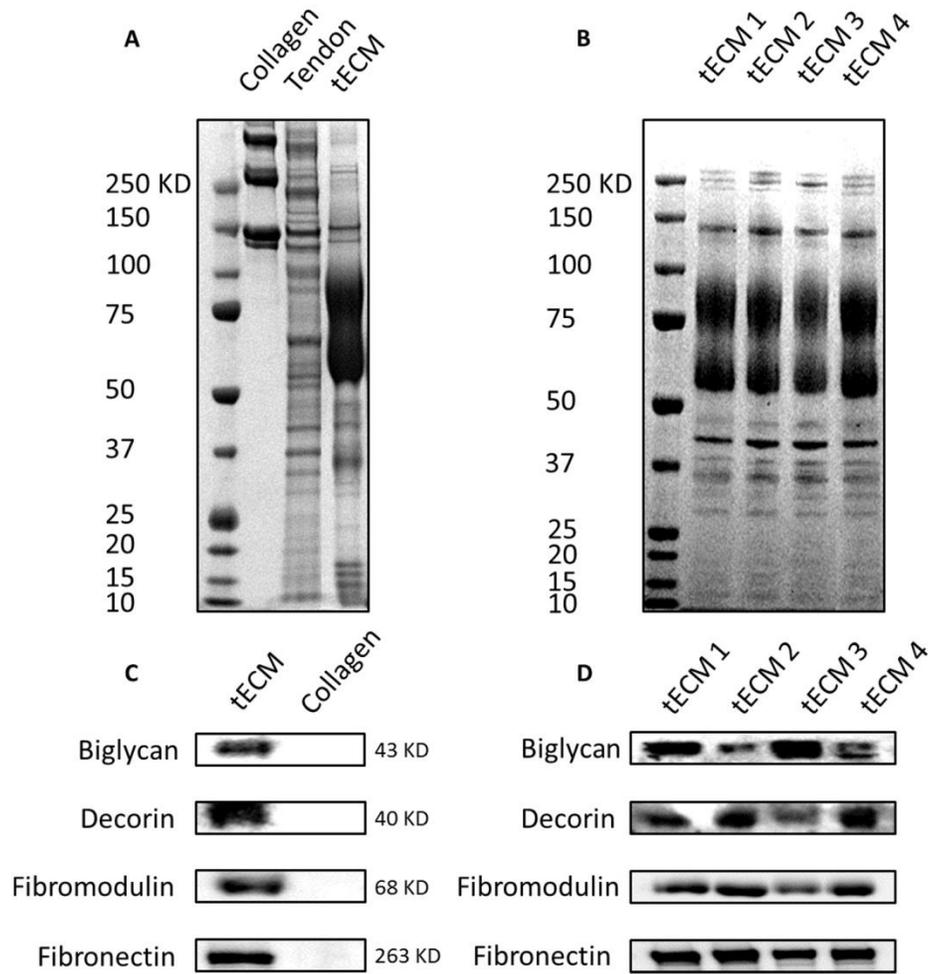


Figure 8. Characterization of tECM by SDS-PAGE (A, B) and Western blot assay (C, D). (A) Both original tendon tissue (Tendon) and tECM contain abundant low molecular weight proteins (<100 KD) that are absent in collagen type I solution (Collagen). (C) At least four types of matrix proteins, biglycan, decorin, fibromodulin and fibronectin, are detected in ECM solution. (B, D) The consistency of the extraction procedure is confirmed by a nearly identical protein profile and immunoreactivity between four independent extracts of tECM (tECM 1-4) from tissues of 4 different calves.

2.4.2 Constructs and cell morphology

Under static tension, all hASC-gel complexes gradually formed cylindrical 3D constructs with each end attached to an anchor stem (Fig. 9 A). With the passage of time, all constructs contracted to form narrower bands, presumably due to the combined effect of cell traction and matrix remodeling (Fig. 9 B). The width of the tECM supplemented constructs was significantly greater than that of pure collagen constructs at days 1, 3 and 7 (Fig. 9 C, $n=6$, $p<0.05$). Three days after gel setting, the pure collagen constructs contracted their diameter by 62.6% and tECM-supplemented constructs by 53.6% on average. Over a 7-day period, the final diameters were 69.9% and 64.4% smaller than the starting values for pure collagen constructs and tECM-supplemented constructs, respectively.

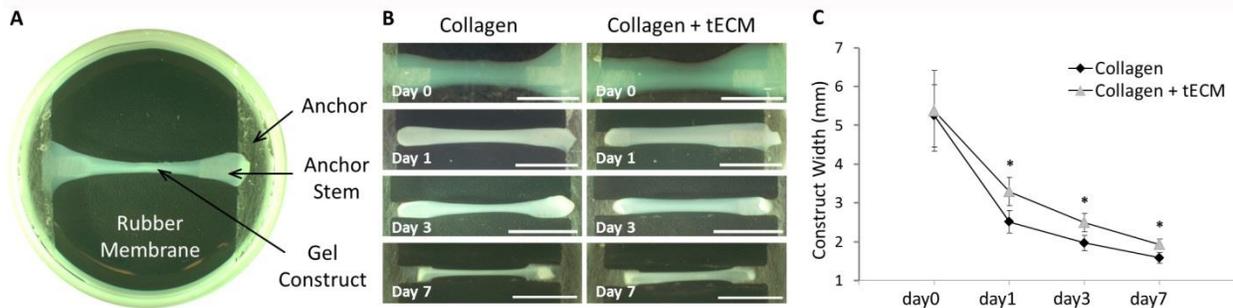


Figure 9. Contraction of constructs. (A) Top view of Tissue Train culture plate together with gel construct shows that the cell-collagen construct was fixed at each end by bonding to the nylon anchor stem. (B) Reduction in contraction is seen in the tECM-supplemented collagen constructs (right panels) compared to the collagen gel constructs (left panels). (C) The average width of the tECM-supplemented constructs is significantly higher than that of pure collagen constructs at days 1, 3 and 7. Scale bar=10 mm; *, $p<0.05$, $n=6$

Cell morphology within the constructs was analyzed by SEM and histological staining. SEM revealed the presence of wavy, randomly oriented collagen fibrils in both collagen only and tECM-supplemented scaffolds. At day 1, cells in the tECM-supplemented scaffold demonstrated well aligned cell bodies and extensions along the longitudinal axis of the scaffold (Fig. 10 D, white arrows), whereas cells in the pure collagen scaffold were more randomly oriented (Fig. 10 A, white arrows). Higher magnification images showed that cells within tECM-supplemented scaffolds projected more fine extensions attached to collagen fibrils (Fig. 10 E) than that of pure collagen scaffolds, where extensions were fewer and only appeared at the extremities of cells (Fig. 10 B). The difference in cell orientation between scaffold types was no longer evident at day 7, when most cells were longitudinally aligned, and cell density was dramatically higher than that of day 1 in each group (Fig. 10 C, F). H&E staining of longitudinally sectioned samples revealed uniform cell distribution within the constructs on day 1 (Fig. 10 G, J). At day 7, the difference in construct width was apparent between the pure collagen construct (Fig. 10 H) and the tECM-supplemented construct (Fig. 10 K), in agreement with the results of gross width measurement. Longitudinally elongated cytoplasm and nuclei were apparent in cells in both construct types. Higher cell number was found in the peripheral region of the tECM-supplemented constructs, resembling an epitendinous sheath surrounding native tendon fibers (Fig. 10 L).

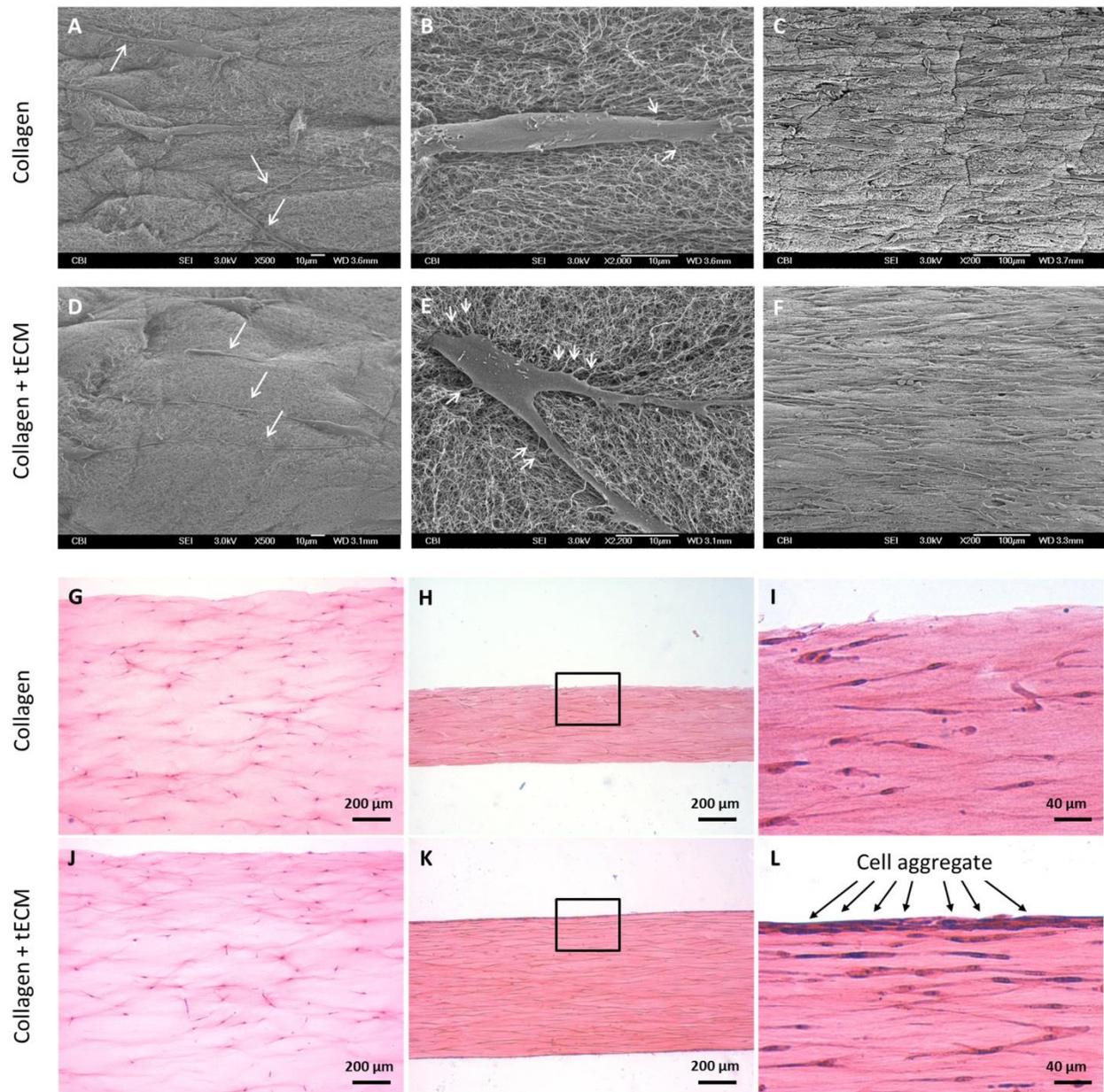


Figure 10. Examination of scaffold structure, cell morphology and distribution by SEM (A-F) and H&E staining (G-L). Cells in the tECM-supplemented scaffolds exhibit aligned cell bodies and extensions along the longitudinal axis of the scaffold (D, white arrows), whereas cells in the pure collagen scaffold are more randomly oriented (A, white arrows). Higher magnification images show more abundant thin extensions from the cell body attached to the collagen fibrils in tECM-supplemented scaffolds (E, white arrows) compared to pure collagen scaffolds (B, white arrows). (C, F) After 7 days of culture, most cells assume an elongated spindle shape along the longitudinal axis of the scaffold. (G, J) H&E staining demonstrates uniform cell distribution along the depth of the

constructs 1 day after gel setting. (H, I, K, L) At day 7, cells in each group are longitudinally aligned. (I, L) Interestingly, intense hematoxylin nuclear staining is evident at the peripheral region of tECM-supplemented scaffolds (L, black arrows). Such superficial cell aggregate is not seen in pure collagen scaffolds.

2.4.3 Cell viability and proliferation

At days 1, 3 and 7, cell metabolic activity and cell number were determined using MTS assay and Picogreen staining, respectively (n=3). Cells seeded on tECM-supplemented scaffolds demonstrated significantly increased metabolic activity ($p<0.05$) and cell number ($p<0.01$) compared to that of pure collagen scaffolds at day 7, suggesting that the presence of tECM greatly promoted cell proliferation and cell activity in a 3D culture under uniaxial tension (Fig. 11 A, B). A high percentage of viable cells were seen by Live/Dead assay in both pure collagen and tECM-supplemented scaffolds 24 hours after gel setting, indicating that the tECM solution was non-toxic to cells. At day 1, most cells were round in shape, and evenly distributed inside the scaffolds. After 7 days of culture, cells retained a high rate of viability and exhibited longitudinal elongation, in agreement with histological analysis (Fig. 11 C).

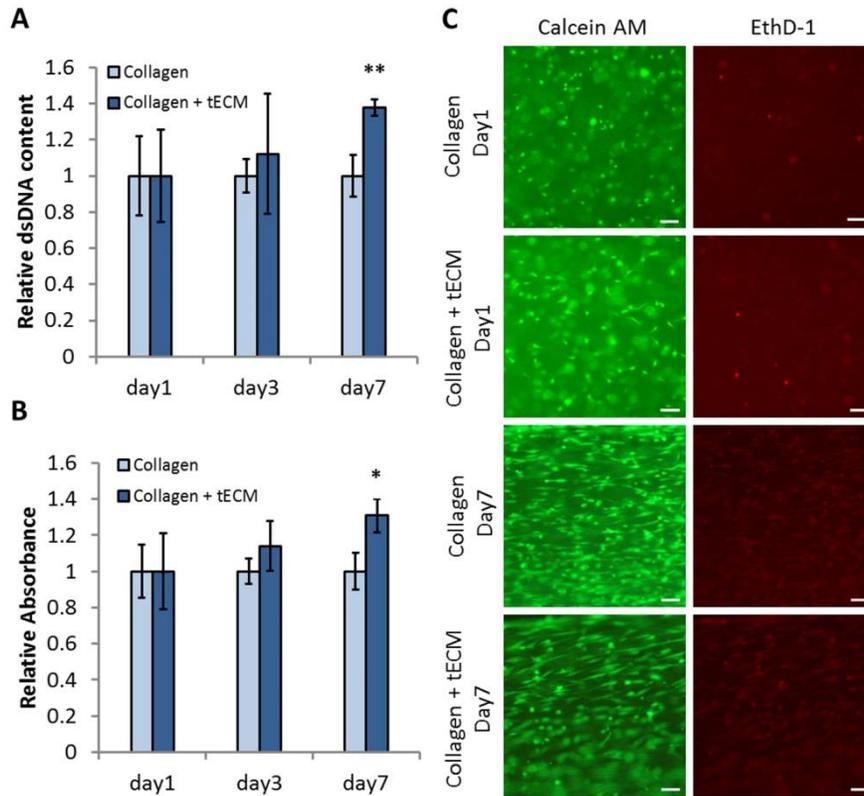


Figure 11. Cell viability assays. (A) After 7 days of culture, cell number, represented by DNA content, is significantly higher in the tECM group. (B) Similarly, cells in the tECM group demonstrate increased metabolic activity, as evidenced by elevated level of reduced MTS reagent. (C) Live/Dead cell viability staining (green, live cells; red, dead cells) showed a high percentage of viable cells, round shaped and evenly distributed, in each group 1 day after seeding. After 7 days of culture, cells retain a high rate of viability and exhibit longitudinally elongated cell bodies. *, $p < 0.05$; **, $p < 0.01$; scale bar=100 μm , $n=3$

2.4.4 Cell differentiation

Gene expression of ASCs in both 2D and 3D culture ($n=5$) were quantified at the mRNA level by real-time PCR. The results showed that the 3D scaffolds under uniaxial tension promoted tenogenic differentiation of hASCs, as compared to 2D controls, and that tenogenesis was further enhanced by tECM supplementation (Fig. 12 A-C). The expression of SCX was

significantly higher in the tECM group than the pure collagen group at days 1 and 3. Both 3D groups demonstrated an up-regulation of SCX at all three time points compared to 2D culture. The expression of TNMD was found significantly higher in the tECM group than the 2D and the 3D collagen group at day 3, but the difference between the two 3D groups was negligible at day 7, when they were both higher than the 2D group. The expression pattern of TNC was similar among all three groups at the first two time points, but was significantly higher in the tECM group at day 7. Taken together, these findings demonstrated that hASCs underwent tenogenic differentiation in 3D culture under tension, and that exposure to tECM improved this lineage-specific differentiation. In contrast, the gene expression of other matrix proteins remained similar (Fig. 12 D-F). There was no significant difference for COL I or COL III between the tECM and the pure collagen group. The expression of COL I in 3D culture was lower than that in 2D, which could be attributed to the collagen rich environment. DCR was up-regulated in 3D cultures at all time points, with expression in the tECM group lower than the collagen group at day 7.

To evaluate the influence of tECM on osteogenic differentiation, the expression of osteogenesis markers, including RUNX 2, ALP and OCN (Fig. 12 G-I), was analyzed. At days 1 and 3, the level of RUNX2 gene expression in hASCs in the tECM group was significantly lower than that of the pure collagen group. hASCs in the tECM group also expressed lower amount of ALP and OCN than in pure collagen at days 3 and 7. Taken together, these results suggested that the exposure to tECM in 3D scaffold promoted tenogenesis of hASCs and partially suppressed osteogenesis.

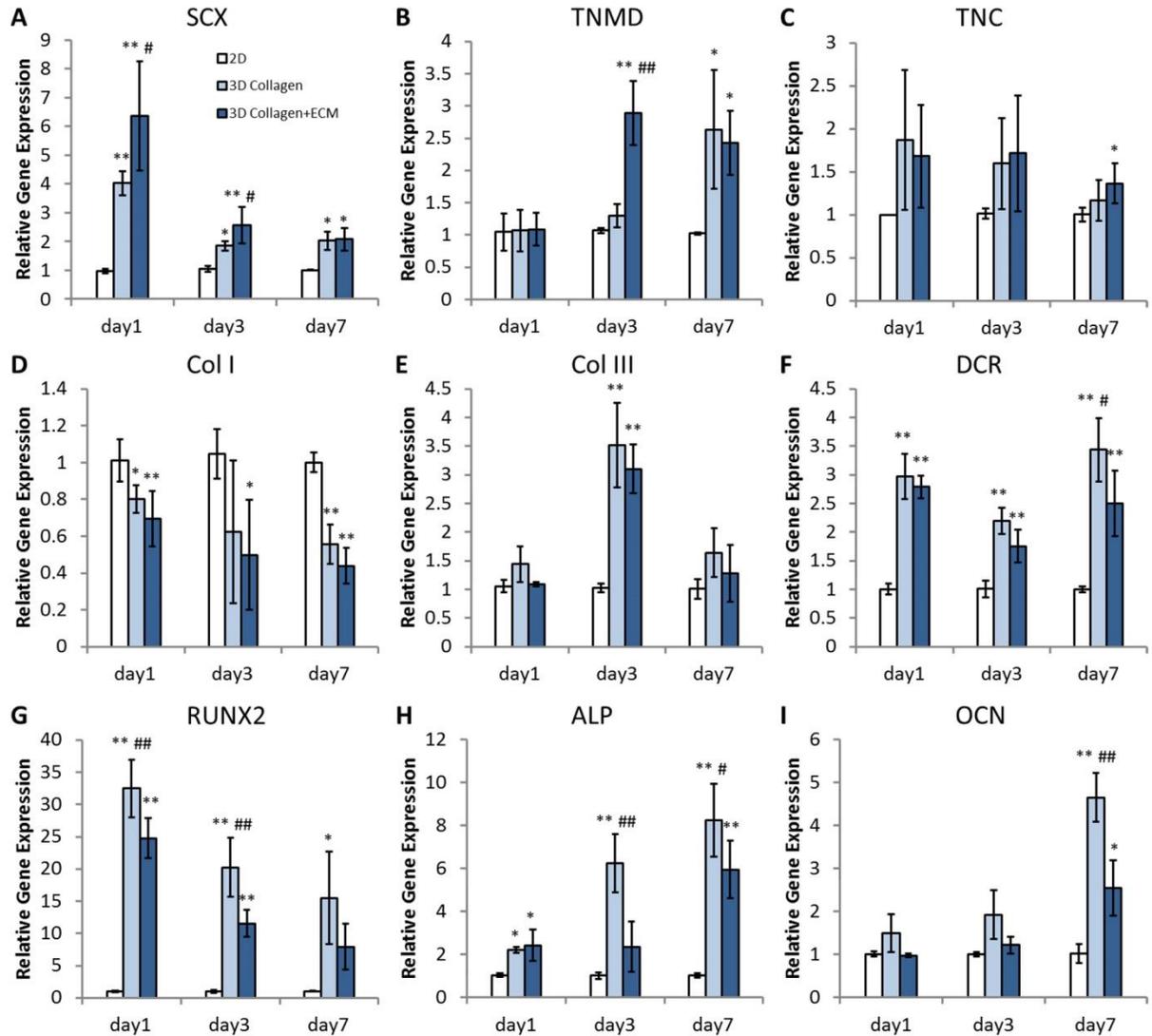


Figure 12. Real time PCR analysis of gene expression. (A-C) hASCs seeded in tECM-supplemented scaffolds (3D Collagen+ECM) show higher expression levels of tendon-specific genes (SCX, TNMD, and TNC) compared to both pure collagen scaffolds (3D Collagen) and 2D culture group (2D). (D) Collagen type I expression level in 3D culture is lower than that in 2D culture. (F) Decorin expression is up-regulated in 3D cultures throughout the experiment, but differs between collagen and tECM groups only at day 7. (G-I) Osteogenesis-related genes (RUNX2, ALP, and OCN) are expressed at lower levels in hASCs seeded in tECM-supplemented scaffolds compared to the pure collagen scaffold group. *, $p < 0.05$ compared to 2D; **, $p < 0.01$ compared to 2D; #, $p < 0.05$ compared to the other 3D group; ##, $p < 0.01$ compared to the other 3D group; $n = 5$

2.4.5 Mechanical properties of the scaffold

We tested the mechanical properties of constructs by clamping the anchor pieces of the plate membrane during elongation (Fig. 13 A, black arrows). A representative load/displacement curve for day 3 samples showed a steeper linear slope of the tECM-supplemented scaffold when compared with the pure collagen scaffold, indicating better resistance to deformation (Fig. 13 B). Maximum load of tECM-supplemented scaffold was significantly higher than that of pure collagen scaffold at both time points (Fig. 13 C, $n=5$, $p<0.05$). The stiffness of tECM-supplemented scaffolds was 2.37 and 4.11 times that of the pure collagen scaffolds at days 3 and 7, respectively (Fig. 13 D, $p<0.01$). In summary, these results showed that the addition of tECM enhanced the mechanical properties of hASC-seeded scaffolds under static tension.

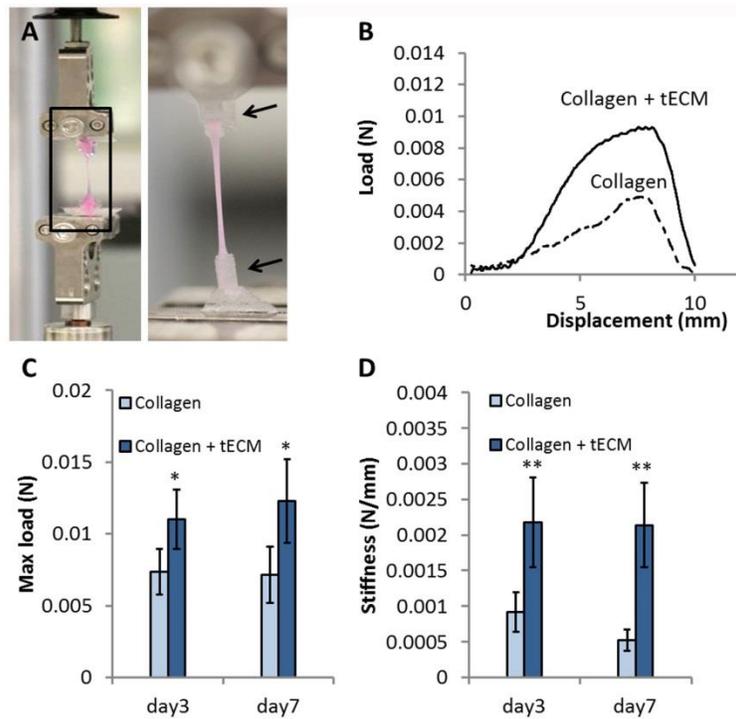


Figure 13. Mechanical properties of constructs. (A) At days 3 and 7, constructs were excised from the rubber membrane together with the anchors and fixed between two clamps (black arrows), so that the integrity of scaffold was not compromised by clamping. (B) A representative load/displacement curve for day 3 samples shows

steeper linear slope and higher failure force of the tECM-supplemented scaffold (Collagen + tECM) compared to the pure collagen scaffold (Collagen). (C, D) Higher maximum load (C) and stiffness (D) of tECM-supplemented scaffolds (Collagen+ECM) were found than that of cell seeded pure collagen scaffolds (Collagen) at each time point.

*, $p < 0.05$; **, $p < 0.01$; $n = 5$

2.4.6 Gene expression and activity of MMPs

Interestingly, none of the cell-seeded scaffolds demonstrated improved mechanical strength compared to cell free controls (data not shown). Moreover, we found little difference in ECM protein-encoding gene expression patterns between 3D groups. Combined, these results implied that the difference in mechanical properties between the cellular scaffold groups might be due to remodeling of the matrix rather than a net increase in matrix deposition. To test this hypothesis, we investigated the expression and activity of matrix metalloproteinases (MMPs), an endopeptidase family responsible for degradation and remodeling the ECM. Real-time PCR analysis revealed that gene expression level of MMPs significantly increased in 3D groups compared to 2D, indicating highly active matrix remodeling occurred in 3D culture under tension (Fig. 14 A-E, $n = 5$). MMP-8, -9 and -13 were down-regulated in the tECM-supplemented group by day 3 or 7 compared to the pure collagen group (Fig. 14 C-E, $p < 0.01$). Finally, the total combined MMP activities in conditioned medium collected from the tECM group was found to be significantly lower than that of the collagen group at both days 3 and 7 (Fig. 14 F, $n = 5$).

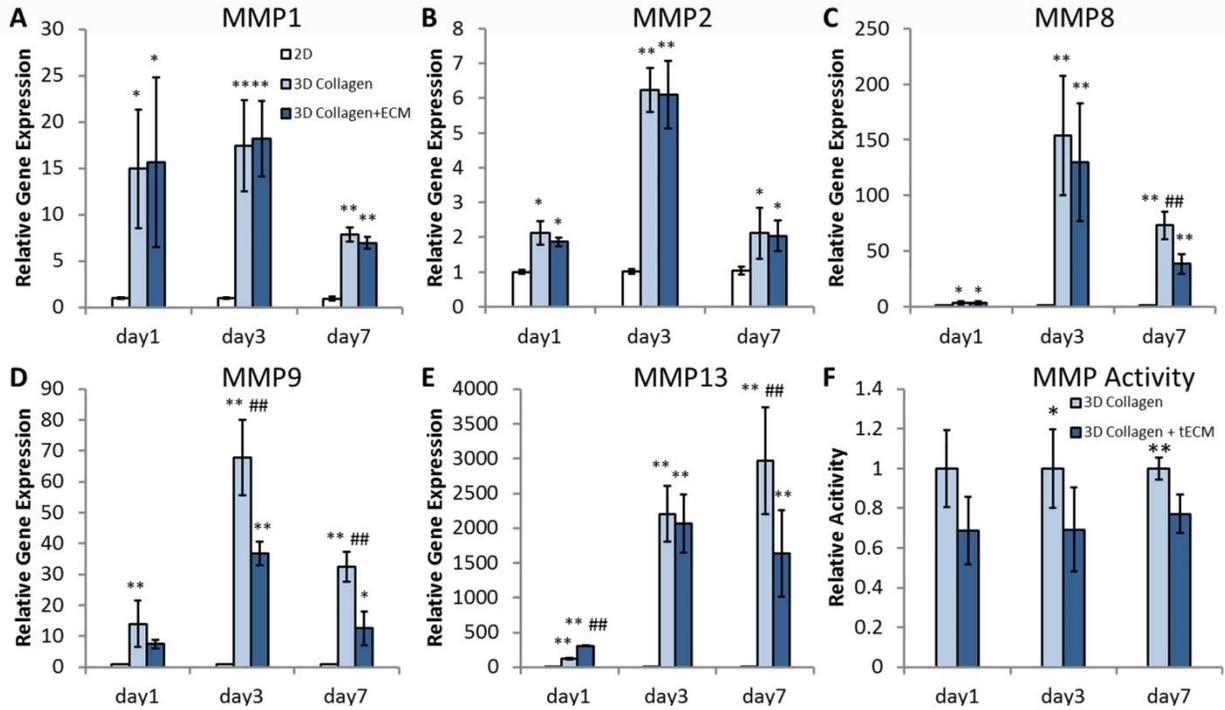


Figure 14. Matrix metalloproteinase (MMP) expression and activity. (A-E) hASCs seeded in 3D scaffolds expressed significantly higher amounts of MMPs compared to 2D culture group. (C-E) Expression levels of MMP-8, -9 and -13 were down-regulated in the tECM group (3D Collagen+tECM) compared to the collagen group (3D Collagen). (F) MMP activity in conditioned medium collected from the tECM group (3D Collagen+tECM) was significantly lower than that of the collagen group (3D Collagen). *, $p < 0.05$ compared to 2D; **, $p < 0.01$ compared to 2D; #, $p < 0.05$ compared to the other 3D group; and ##, $p < 0.01$ compared to the other 3D group; $n = 5$

2.5 DISCUSSION

In this chapter, we have investigated the influence of a soluble ECM extract from bovine tendon tissue, used as a supplement to collagen scaffold, on hASCs cultured in 3D collagen gel under uniaxial tension. We hypothesize that incorporation of tECM will enhance the bio-functionality of 3D tension-bearing scaffolds. Our results show that the tECM contains a variety of tendon matrix proteins. The inclusion of tECM within collagen scaffolds promotes hASC

proliferation and tenogenic differentiation, as compared with pure collagen scaffold. Moreover, hASCs in tECM-containing scaffolds demonstrate reduced osteogenesis compared to hASCs in pure collagen scaffolds. The addition of tECM also results in changes of the mechanical properties of the constructs, including contraction rate, maximum load, and stiffness, partially by altering the expression level and activity of MMPs of the seeded hASCs. Collectively, these findings reveal the bio-functionality of tECM on hASC behaviors in 3D culture under tension, and strongly suggest the feasibility of using tECM to facilitate hASC-based tendon healing and regeneration.

ASCs, a multipotent mesenchymal stem cell population derived from adipose tissues, possess the potential to differentiate into a variety of cell lineages [147]. As far as the regeneration of mesodermal tissues are concerned, ASCs can undergo adipogenic, osteogenic, chondrogenic [148], myogenic [149], and tenogenic differentiation [82, 86, 150]. Given their availability and tenogenic capacity, the use of ASCs thus expands potential approaches to tendon tissue engineering and tendon repair. Tenogenesis of MSCs can be induced through uniaxial tension in 3D culture [126, 142, 151]. In this study, we have used the Flexcell Strain Unit with the Tissue Train culture plates to generate 3D cylindrical constructs under tension [125]. Collagen type I gel was used as the material to cast the scaffold for two reasons. First, collagen type I is the primary component of tendon tissue, and has been widely used as a scaffold in tendon tissue engineering. Second, cells could be easily suspended in collagen solution before setting, ensuring uniform distribution within the constructs. In contrast, previous studies have shown that cells re-seeded on acellular tendon tissue attached only to the surface but did not penetrate the matrix easily. In these studies, few cells were observed to migrate into the center of acellular flexor tendons even after 8 weeks in culture [95, 152].

For the purpose of facilitating tendon healing and regeneration, MSCs need to proliferate and then undergo appropriate differentiation and matrix remodeling on the scaffold in order to develop into a functional replacement tissue at sites of injury [50], [136]. The ECM plays important roles in regulating cell behavior. Tissue-derived ECMs have been found to induce corresponding tissue-specific differentiation of MSCs, and are therefore considered as attractive candidate biomaterials to support stem cell-based regeneration of the tissue from which it is derived. For example, soluble ECM derived from skeletal muscle tissue induced myogenesis of muscle progenitor cells [153]. Similarly, acellular cartilage sheets were found to stimulate BMSCs to undergo chondrogenesis [154]. In tendon, tenocytes are surrounded primarily by ECM in their native microenvironment. BMSC seeded on acellular tendon scaffold expressed TNMD, a tendon phenotype marker [155]. Tendon-derived stem/progenitor cells (TSPCs) were also found to express more TNMD when seeded on tendon ECM film than on tissue culture plastic [123]. In this study, we have used urea, a nonionic chaotropic reagent that disrupts lipid-lipid, lipid-protein, and protein-protein interactions, for tECM extraction, producing a soluble extract that is easily miscible in aqueous solutions. SDS-PAGE and western blot assay revealed that soluble tECM isolated from tendons contains not only collagen but also numerous non-collagenous proteins, including fibronectin, fibromodulin, biglycan and decorin, all of which are known to regulate MSC behavior. For example, fibronectin is a multi-domain protein that contains binding sites for integrins, collagen and other ECM proteins, glycosaminoglycans, as well as self-association sites, thereby influencing cell shape and adhesion to the matrix [156]. Moreover, fibronectin has also been reported to induce chemotaxis and mitogenic activity for human MSCs [157]. These characteristics of fibronectin may contribute to the improved interaction between cells and tECM-containing scaffold, as evidenced by increased extensions

from the cell body connected to the collagen fibrils, and increased retention of the seeded cells (data not shown). Decorin greatly affects the assembly of collagen fibrils during tendon development [36], and also prevents aberrant lateral fusion of collagen fibrils [158]. Biglycan and fibromodulin appear to have substantial impacts on cell fate. BMSCs from biglycan- and decorin-deficient mice were unable to sequester TGF- β in ECM, resulting in a switched fate from growth to apoptosis [159]. Human skin fibroblasts exposed to human fibromodulin formed embryonic stem cell-like colonies expressing pluripotency markers including NANOG, SOX2 and OCT4 [160]. Biglycan and fibromodulin were also identified as critical components of the tendon stem cell niche controlling the fate of tendon stem cells [38]. It is thus reasonable to hypothesize that the known activities of these ECM molecules, and of other yet-to-be-determined components in the tECM, are responsible for the observed bioactivity of the tECM on the hASCs seeded in the composite 3D scaffold.

Our hypothesis is supported by the results of the cell proliferation assay, real-time PCR analysis, and mechanical testing. DNA content and cell metabolic activity are significantly higher in the tECM-supplemented constructs than pure collagen constructs after 7 days of culture, indicating more rapid proliferation in the tECM group. It is noteworthy that DNA content measured immediately after gel setting (defined as day 0) showed no significant difference between the two groups (data not shown), indicating minimal residual, tissue-derived nuclear remnants. Consistent with our results, rabbit TSPCs have been shown to grow faster in tECM than on plastic surfaces, as indicated by the population doubling times [123]. Furthermore, hASC cultures seeded in constructs containing ligament-derived matrix were also found to contain more dsDNA than in collagen constructs [122].

Real-time PCR analysis revealed enhanced tenogenesis of hASCs in the tECM group based on tendon-specific gene expression compared to tECM free groups (Fig. 12). Furthermore, despite the efficacy of 3D culture under tension to promote tenogenesis, simultaneous osteogenesis had been reported for hASCs cultured under mechanical stimulation, an undesirable effect that compromises the application of hASCs for tendon repair. For instance, cyclic mechanical loading was found to increase RUNX2 expression of rat ASCs [161, 162]. In another study, up-regulation of ALP and OCN expression in hASCs was achieved by 1 and 2 hour cyclic strain [163]. It has been suggested that such tension-induced osteogenesis is the cause of pathological ossification seen in mechanically overused tendon, such as in tendinosis [164]. Therefore, we asked whether hASCs cultured under uniaxial tension undergo osteogenesis and if this differentiation can be attenuated by incorporation of tECM. Our findings show that tECM significantly reduces osteogenic differentiation of hASCs when cultured in 3D under tension (Fig. 12 G-I). Based on the results reported here, we speculate that the reduced osteogenesis is the result of exposure to a more tenogenic niche provided by the tECM incorporated into the 3D scaffold, implying a tendon tissue-specificity of the pro-differentiation effect of the tECM on hASCs.

Since tendons are primarily load-bearing tissues, the mechanical properties of engineered tendons need careful consideration. In this study, we investigated the mechanical properties of the constructs from two perspectives: contraction rate and mechanical strength. As the cells reorganized and remodeled the collagen matrix, macroscopic radial contraction of the construct was evident (Fig. 9). Similar to our results, constructs made from collagen type I gel seeded by BMSCs at 1 million cells/mL were found to contract by 61% of its initial diameter after 3 days [142]. In another study, the contraction rate peaked 1 day after gel setting with 82% reduction of

the cross-sectional area of the construct after 8 days [125]. To our knowledge, this study is the first to provide quantitative data for the effect of tECM on the contraction rate of cell-seeded collagen gels. In addition, tECM-supplemented constructs demonstrates improved mechanical strength, evidenced by significantly higher maximum load and stiffness of the constructs. Greater scaffold stiffness may result in greater tactile forces placed upon the encapsulated cells during contraction, causing tenogenic differentiation through the induction of SCX expression, which is known to be responsive to change in mechanical stimulus [165, 166].

Interestingly, we have observed no increase in mechanical properties of each type of cell-seeded scaffold compared to cell-free scaffolds. This observation, combined with the finding that there was little difference in ECM protein gene expression between 3D groups, ruled out the contribution of neo-matrix deposition. Therefore, we hypothesized that the change in mechanical properties was due to cell-mediated remodeling of the scaffold. To test this hypothesis, we investigated the expression and activity of MMPs, endopeptidases capable of degrading and remodeling extracellular matrix [167]. Due to their matrix degrading capability, MMPs are involved in the degradation of a number of collagenous scaffolds. Real-time PCR analysis revealed a dramatic up-regulation of the expression of MMPs in hASCs seeded in the 3D culture under tension compared to 2D culture. Similar to our results, a significant up-regulation of MMP-1 and -13 expression was observed after 14 days of culture, when BMSCs were seeded on collagen type I gels [168]. In the same study, MMP-2 was found only activated in cells cultivated in collagen gels. In addition to studies of *in vitro* models, the alteration of MMP expression has been widely observed in a variety of tendon injuries *in vivo* [55]. For example, increased expression of MMP-1 and -9 was found in supraspinatus tendons in the defect group in a patient study [169]. MMP-2, -9 and -13 were up-regulated in ruptured human Achilles tendons [170,

171]. Similarly, there was a significant increase in MMP-13 mRNA in rotator cuff tendon tissue obtained from patients with rotator cuff tears [172]. MMP-9 and 13 were also found to participate in collagen degradation but not remodeling in a rat flexor tendon laceration model [56]. In our study, MMP-8, -9 and -13 are down-regulated by nearly 50% in the tECM group compared to the pure collagen group at day 7. The total MMP activity in the culture-conditioned medium was also significantly lower at day 3 and day 7 in the tECM group. These findings therefore suggest the possible influence of tECM on suppressing MMP-mediated scaffold degradation, which could contribute to the improvement of tendon healing.

2.6 CONCLUSIONS

A bioactive, soluble fraction of tendon-derived extracellular matrix (tECM) was extracted with urea. Exposure to tECM greatly promoted human adipose stem cell (hASC) proliferation and tenogenic differentiation in 3D collagen gels under static tension. hASCs in tECM-containing scaffolds also demonstrated suppressed osteogenesis compared to hASCs in pure collagen scaffolds. Furthermore, the addition of tECM improved the mechanical properties of the constructs, in part by altering the amount of MMPs expressed by seeded hASCs and their activity in culture medium. These findings suggest a new approach to inducing hASC tenogenesis that may enable and improve tendon tissue engineering to facilitate tendon healing and regeneration.

3.0 TENDON-DERIVED EXTRACELLULAR MATRIX ENHANCES TGF-BETA 3 INDUCED TENOGENIC DIFFERENTIATION OF HUMAN ADIPOSE-DERIVED STEM CELLS

3.1 ABSTRACT

Because of the limited and unsatisfactory outcomes of clinical tendon repair, tissue engineering approaches using adult mesenchymal stem cells (MSCs) are being considered as a promising alternative strategy to heal tendon injuries. Successful and functional tendon tissue engineering depends on harnessing the biochemical cues presented by the native tendon extracellular matrix (ECM) and the embedded tissue-specific bio-factors. In this chapter, we have prepared and characterized the biological activities of a soluble extract of decellularized tendon ECM (tECM) on adult adipose derived stem cells (ASCs), on the basis of histological, biochemical, and gene expression analyses. The results showed that tECM enhances the proliferation and TGF- β 3 induced tenogenesis of ASCs in both 2- and 3-dimensional (2D and 3D) cultures *in vitro*, and modulates matrix deposition and organization of ASCs seeded in 3D scaffolds. These findings suggest the utility of reproducing the native tissue microenvironment as a design principle to elicit desired cellular responses for tissue regeneration, and that combining tissue-specific ECM with growth factor treatment is an effective approach to induce robust tenogenesis for ASCs.

3.2 INTRODUCTION

Tendon injuries occur frequently in sports and daily activities due to excessive load or overuse. In the U.S. alone, around 45% of the 32 million musculoskeletal injuries each year involve tendon, ligament and joint capsule, in which tendon injuries are especially common and require over 120,000 surgical repairs annually [71]. Unfortunately, the natural healing process of tendons is slow and insufficient, resulting in fibrotic scar formation and inferior mechanical strength at the injured sites [21]. Current clinical outcomes of tendon repair remain unsatisfactory due to limitations including autograft donor site morbidity, risk of injury recurrence and limited long-term function recovery [73, 74, 173]. Therefore, tissue engineering approaches, which use a combination of cells, scaffolds and bioactive molecules, are gaining increasing research interest as a promising alternative strategy to treat tendon injuries.

The use of adult mesenchymal stem cells (MSCs) as the cellular component for tendon tissue engineering has been increasingly explored in recent years [38, 85, 86, 174]. Compared with other cell sources, adipose-derived mesenchymal stem cells (ASCs) are abundant and can be isolated by minimally invasive approaches [148, 175]. However, although a variety of growth factors are able to induce expression of tenogenic markers in ASCs, no single growth factor has been found to exclusively promote tenogenic differentiation. Rather, growth factors interact with the evolving microenvironment produced by progenitor cells to induce proliferation and tissue-specific differentiation during development or tissue repair. In terms of tendon tissue, the biochemical and biophysical cues of tendon microenvironment may modulate growth factor bioactivity to promote tendon-specific effects that enhance the efficacy of cell therapies in restoring the native structure and function of tendon following injury [15, 38, 176].

Studies in tendon development have revealed the complexity of tendon differentiation. As shown across several animal models, members of the transforming growth factor- β (TGF- β) superfamily are actively involved in tendon development and healing in a spatiotemporally specific manner. For example, mouse patellar tendon cells were found to respond to TGF- β signaling at developmental stages starting at gestation day 17.5 and ending at postnatal day 14 [13]. Consistent with this finding, micromass culture of chick embryonic limb bud mesodermal cells with TGF- β demonstrated significant up-regulation of tendon markers, scleraxis (SCX) and tenomodulin (TNMD), with concurrent reduction in cartilage markers [15]. Conversely, disruption of TGF- β signaling resulted in the loss of most tendons and ligaments in a SCX-GFP mouse model [14]. When injured, high levels of TGF- β expression and activity were seen throughout the healing period [51, 177, 178]. However, TGF- β signaling is also known to play a crucial role in chondrogenesis [179]. As is increasingly recognized, the tissue-specific bioactivity of TGF- β depends upon additional cues provided by the extracellular microenvironment [15, 159].

Concurrently, recent research has illustrated the pivotal role of the extracellular matrix (ECM) in tendon differentiation [38, 180]. ECM is composed of the structural and signal molecules secreted by the resident cells of each tissue. While the ECM of most tissues share highly conserved structural proteins (e.g., collagen, proteoglycans), it is the unique biophysical arrangement of these proteins, and the highly orchestrated deposition and presentation of soluble cues that serve to promote and maintain a particular cell phenotype [181, 182]. Tendon is rich in ECM components, and many of the tendon ECM proteins and growth factors embedded within the ECM network appear to be retained in decellularized tendons [146, 183]. As proof, tendon-derived stem/progenitor cells (TSPCs) seeded on decellularized tendon/ligament ECM

demonstrated improved proliferation and tendon cell phenotype, indicating the role of tendon ECM in regulating proliferation and differentiation [184]. Taken together, it is reasonable to assume that the presence of native tendon ECM may be beneficial to TGF- β induced tenogenic differentiation of MSCs for tendon repair.

In addition to biochemical cues, scaffolds are also utilized in tendon tissue engineering to provide mechanical support as well as three-dimensional (3D) environment that mimics the architecture of native tendon. Because tendon is primarily composed of aligned collagen fibers, scaffold anisotropy is an important topographical characteristic to consider in tendon tissue engineering. Cell growth and matrix deposition were found to align along the direction of electrospun nanofibers in a scaffold designed for rotator cuff repair [107]. Likewise, TSPCs seeded on aligned nanofibrous scaffolds demonstrated increased tenogenesis and collagen deposition, together with suppressed osteogenesis [104]. In this study, we have therefore prepared and employed aligned poly- ϵ -caprolactone (PCL) scaffolds to reproduce the biophysical cues of native tendon ECM.

The objective of this study is to investigate the effect of native tendon ECM components and TGF- β on the tenogenesis of human ASCs (hASC). A soluble extract of decellularized tendon ECM (tECM) was prepared as described in the previous chapter [183]. The individual and combined effects of tECM and TGF- β 3 on hASC behavior, including proliferation and differentiation, were analyzed by using tECM as a medium supplement for cell culture with or without TGF- β 3 for up to 2 weeks in 2D, and by ASCs seeded on 3D aligned PCL scaffolds. We hypothesize that tECM is able to enhance the proliferation and TGF- β 3 induced tenogenesis of ASCs in both 2D and 3D culture *in vitro*, and that tECM modulates matrix deposition and organization of ASCs in 3D scaffolds.

3.3 MATERIALS AND METHODS

3.3.1 Cell isolation and culture

hASCs were obtained from lipoaspirate-derived fat tissue of two donors (34 years old male and 38 years old female) using an automated cell isolation system (Tissue Genesis Inc.), with University of Pittsburgh Institutional Review Board approval. Isolated hASCs were cultured in growth medium (GM) consisting of DMEM-high glucose (Gibco), 10% fetal bovine serum (FBS), and penicillin/streptomycin (P/S). hASCs between passage 2 and 4 (P2-P4) were used for experiments.

3.3.2 Colony forming unit-fibroblast assay

The colony forming unit-fibroblast (CFU-F) assay was performed using an established method described elsewhere [179]. hASCs from each donor at P2 were plated separately in 100 mm dishes (Falcon) in triplicate at densities of 100 cells per dish and cultured in GM for 2 weeks. The cultures were stained with 0.5% crystal violet solution in methanol and visible colonies scored.

3.3.3 Flow cytometry

hASCs at P2 were detached by trypsin-EDTA and incubated with propidium iodide (PI) and PE- or FITC-conjugated mouse (IgG1, κ) anti-human antibodies for 30 min at 4 °C. Antibodies include mouse anti-human CD31, CD34, CD44, CD45, CD73, CD90, CD105 (BD Biosciences). Dead cells were excluded by positive PI staining. PE- or FITC-conjugated isotype-matched IgGs (BD Biosciences) were used as controls. After washing, the cells were sorted using the FACS Aria II SORP flow cytometer (BD Biosciences), and data analyzed with DiVa v6 software.

3.3.4 Preparation of tendon ECM

A soluble fraction of tendon ECM (tECM) was prepared using our previously reported protocol.[183] Bovine Achilles tendons were harvested from juvenile bovine hind legs (Research 87), pulverized, and decellularized by 1% Triton X-100 (Sigma-Aldrich). After nuclease treatment (200 U/ml DNase, Worthington), the acellular tissue was extracted in 3 M urea (Sigma-Aldrich) for 3 days. Urea was removed by dialysis in 3,500 MWCO cassettes (Thermo Scientific) against water for 2 days, and then the tECM extract was spin-concentrated, sterilized using 0.25 μ m PVDF syringe filter units (Millipore), and stored as 1 mg/ml stock at -20 °C until use.

3.3.5 Preparation of scaffold

Microfibers were fabricated by electrospinning. A solution of PCL (MW = 70k-90k, Sigma-Aldrich) prepared at 18% w/v in 1:1 (v/v) dimethylformamide (DMF) and tetrahydrofuran (THF) (Fisher Scientific) was extruded at 2 mL/h through a 22-gauge blunt-tip needle charged by 10 kV DC potential (Gamma High Voltage) and fibers collected on a custom-designed rotating mandrel. Scaffolds were cut into 20 mm x 5 mm rectangular pieces, rehydrated in ethanol, and then soaked in GM overnight before cell seeding.

3.3.6 Scanning electron microscopy (SEM)

Scaffolds were dried in vacuum, mounted on aluminum stubs, sputter-coated with 3.5 nm gold, and examined by a scanning electron microscope (field emission, JEOL JSM6335F) operated at 3 kV accelerating voltage and 8 mm working distance. The external surface of the central part of the constructs was selected for imaging.

3.3.7 Differentiation of hASCs

Differentiation along mesenchymal lineages, including osteogenesis, adipogenesis and chondrogenesis, was performed to assess the multipotency of the isolated hASCs using established protocol with slight modifications [179]. Osteogenesis was induced by using DMEM supplemented with 10% FBS, 10 mM β -glycerophosphate, 0.1 μ M dexamethasone and 50 μ g/ml ascorbate for 14 days. Adipogenesis was accomplished by treatment with DMEM supplemented with 10% FBS, 1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 1x insulin-

transferrin-selenium (ITS) for 14 days. Control cultures were maintained without osteogenic or adipogenic supplements, respectively. Chondrogenesis of high-density pellet cultures (2.5×10^5 cells/pellet) was induced in serum-free DMEM supplemented with $0.1 \mu\text{M}$ dexamethasone, $40 \mu\text{g/ml}$ L-proline, $50 \mu\text{g/ml}$ ascorbate, and 1x ITS. Recombinant human transforming growth factor- $\beta 1$ (TGF- $\beta 1$; R&D Systems, Minneapolis, MN) and bone morphogenetic protein 6 (BMP-6, R&D Systems) was also added to the chondrogenic medium at a final concentration of 10 ng/ml . Control pellet cultures were maintained without the addition of growth factors. For all groups the medium was changed every 3 days.

To induce tenogenesis, hASCs at P3 were serum-starved overnight at a density of 1×10^4 cells/cm² in 2D culture and at 6×10^4 cells/cm² in 3D culture. Cells were then treated with or without 10 ng/ml TGF- $\beta 3$ (PeproTech) in basal medium (BM) consisting of DMEM, 1x ITS and P/S supplemented with 10% v/v of 1 mg/ml tECM, 1 mg/ml collagen type I solution (Col I, PureCol Advanced Biomatrix) or FBS for up to 14 days.

3.3.8 Cell proliferation tests

hASCs at P3 were plated on 2D culture plastic and 3D scaffolds at a density of 0.5×10^4 cells/cm² and 4×10^4 cells/cm², respectively. Twenty-four hours after initial seeding, cells were fed with BM containing one of the following supplements at 10% v/v: 1 mg/ml tECM, 1 mg/ml Col I solution, or FBS. DMEM supplemented with 10% v/v Hanks' Balanced Salt Solution (HBSS, Gibco) was used as a negative control. On days 0, 3 and 7, MTS assay (CellTiter 96 Assay, Promega) was performed to spectrophotometrically determine metabolic activity of cells from each group.

3.3.9 Real-time PCR analysis of gene expression

Total cellular RNA was isolated (Rneasy, Qiagen) and first-strand cDNA synthesized using SuperScript III First-Strand cDNA synthesis kit (Invitrogen). Real-time PCR was performed using SYBR green Supermix in a Step One Plus real-time PCR system (Applied Biosystem, Life Technologies). The targets and sequences of primers are shown in Table 4. Relative expression level of each gene was normalized to that of 18S rRNA and calculated using the $\Delta\Delta$ -Ct method.

Table 4. Primer sequences of genes analyzed by real-time PCR

Gene		Primer sequence (5'-3')	Product size (bp)
18S rRNA	Forward	GTAACCCGTTGAACCCCAT	151
	Reverse	CCATCCAATCGGTAGTAGCG	
SCX	Forward	ACACCAGCCCAAACAGA	65
	Reverse	GCGGTCCTTGCTCAACTTTC	
TNC	Forward	GGTGGATGGATTGTGTTCTGAGA	328
	Reverse	CTGTGTCCTTGTCAAAGGTGGAGA	
SOX9	Forward	CTGTAGGCGATCTGTTGGGG	85
	Reverse	AGCGAACGCACATCAAGA	
COL II	Forward	GGATGGCTGCACGAAACATACCGG	157
	Reverse	CAAGAAGCAGACCGGCCCTAT	

3.3.10 Protein extraction and Western blot assay

Eight days after differentiation induction, total protein was extracted from each group by TM buffer (Total Protein Extraction Kit, Millipore). Equal loads of reduced protein samples of the same concentration were electrophoretically separated in NuPAGE Bis-Tris Mini Gel (Life Technologies), and transferred onto PVDF membranes (iBlot dry blotting system, Invitrogen) for incubation with rabbit anti-scleraxis (SCX) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody (Abcam) at 4°C overnight. Western blots were developed using

horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary antibodies (GE Healthcare Bio-Sciences) and West Dura Extended Duration Substrate (Thermo Scientific).

3.3.11 Mechanical testing

Tensile properties of scaffolds were analyzed using the Bose 3230 mechanical tester. Scaffolds were securely mounted and loaded with uniaxial force applied at a displacement rate of 0.2 mm/s until 10 mm displacement. The tensile force and the displacement were recorded, and tensile stress was calculated by dividing the measured force by the initial cross-sectional area of the scaffold. Strain was calculated by normalizing the measured displacement against the initial clamp-to-clamp distance (~15 mm). The slope of the linear portion of the stress–strain curve was calculated as Young’s modulus.

3.3.12 Matrix deposition and characterization

hASCs were seeded on PCL scaffolds at a density of 6×10^4 cells/cm² and cultured with BM supplemented with either only 2% FBS or 2% FBS plus 10% tECM (v/v), in the presence of 50 µg/mL ascorbate-2-phosphate (Sigma-Aldrich), for 3 weeks. Negative controls consisted of cell-free scaffolds treated under the same conditions. Collagen content in each group was quantified using the Chloramine T-based hydroxyproline assay.

3.3.13 Immunofluorescent staining

Cell-seeded scaffolds were washed in PBS, fixed in 4% paraformaldehyde, and blocked with 1% bovine serum albumin (BSA) and 22.52 mg/ml glycine in PBS-T. Primary antibodies used included goat anti-tenomodulin (Tnmd, 1:50, sc49325 Santa Cruz), or rabbit anti-Col I (1:500, ab34710 Abcam), with overnight incubation at 4 °C. Alexa Fluor 488 chicken anti-goat or Alexa Fluor 488 goat anti-rabbit were used as secondary antibodies at 1:500 dilution (Life Technologies). For F-actin staining, fixed cells were permeabilized in 0.1% Triton X-100 and then incubated with Alexa Fluor 488 phalloidin for 30 min at room temperature (Life Technologies). After nuclear counterstaining with DAPI (Life Technologies), cells were imaged using a confocal microscope (Olympus FluoView 1000).

3.3.14 Statistical analysis

Data are presented as mean \pm standard deviation (SD). All quantitative assays were performed for no less than three times independently (N equals to the number of independent tests in figure legends). In each replicate, cells from two donors were treated and analyzed separately in duplicate, and data were combined. One-way ANOVA with Bonferroni post hoc test and Student's t-test was performed to determine statistical significance. Significance was considered at $p < 0.05$.

3.4 RESULTS

3.4.1 Characterization of human ASCs

The stromal vascular fraction (SVF) of adipose tissue is composed of a mixture of cell types, including endothelial, smooth muscle, cardiac, immune, stromal cells and ASCs (Fig. 15 A) [185]. After 14 days of culture, $28.83 \pm 3.31\%$ of the adherent cells were found proliferating by CFU assay, indicating a self-renewal capability within the cell population (Fig. 15 B). Upon differentiation induction, the cultured cells at P2 were able to undergo differentiation toward multiple mesenchymal lineages, including adipogenesis, osteogenesis, and chondrogenesis (Fig. 15 C). Moreover, the phenotypic analysis by flow cytometry suggested a relatively homogeneous population that expressed mesenchymal cell markers (CD44, CD73, CD90 and CD105) but was free of hematopoietic and endothelial markers (CD31, CD34, CD45) (Fig. 15 D) [186]. Taken together, these results confirmed that the cell population used for subsequent experiments exhibited characteristics consistent with ASCs derived from subcutaneous lipoaspirate.

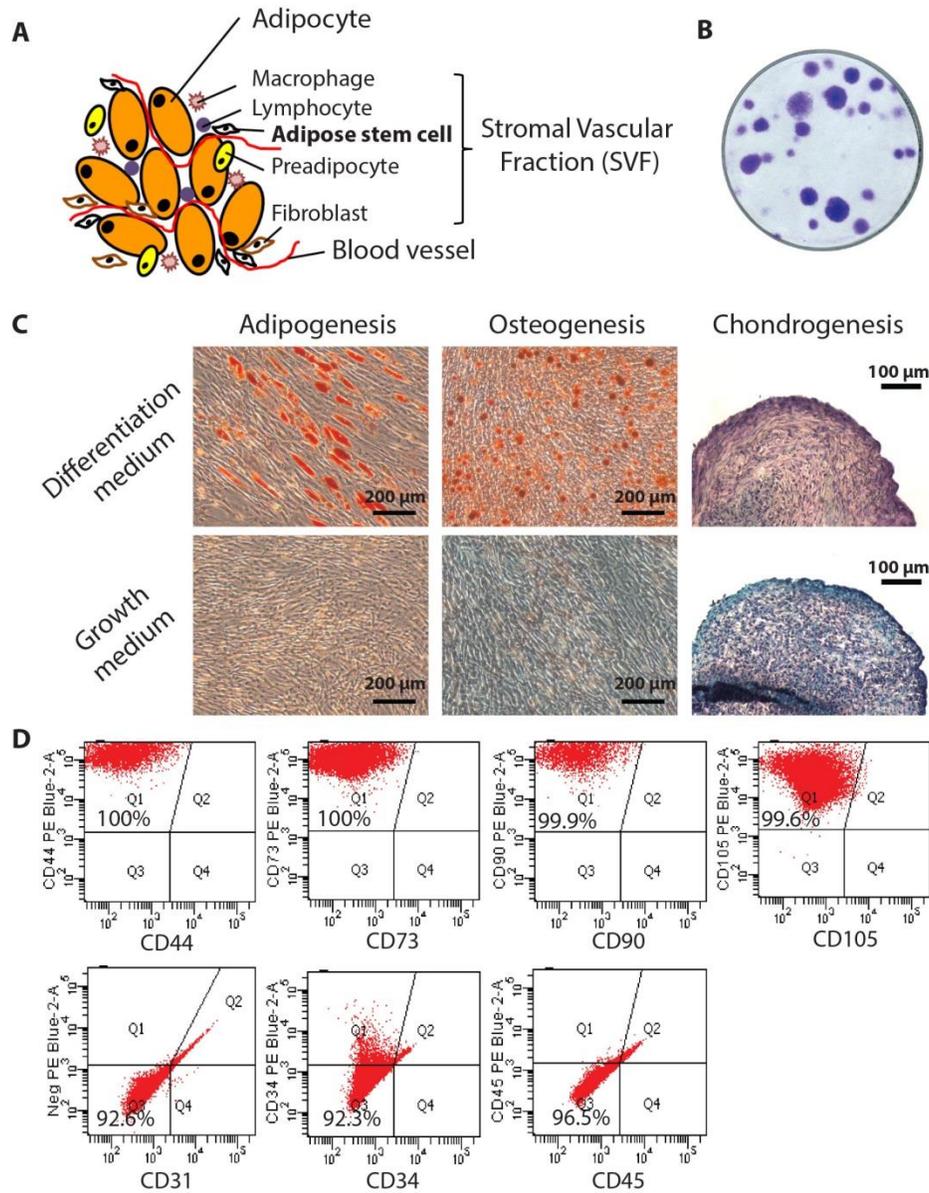


Figure 15. Characterization of hASCs. (A) Composition of adipose tissue, consisting of mature adipocytes, blood vessels and a stromal vascular fraction (SVF). (B) Crystal violet staining visualizes the colonies formed by proliferating cells. (C) Histological detection of ASC multipotency: Adipogenesis by Oil Red staining (red), osteogenesis by Alizarin Red staining (red), and chondrogenesis by Safranin-O staining (red). (D) Flow cytometry analysis of the cell surface markers characteristic for mesenchymal stem cells, hematopoietic and endothelial cells.

3.4.2 Effect of tendon ECM on ASC behavior in 2D

In order to investigate the role of tendon ECM in regulating ASC behavior, we extracted the soluble fraction of decellularized tendon ECM (tECM) from juvenile bovine Achilles tendons. As previously reported, the tECM solution prepared by this method is cell free and rich in non-collagenous ECM proteins, with a constant yield rate and consistent composition (Fig. 8) [183]. tECM or Col I solution were used at 1 mg/ml as medium supplements at 10% v/v to treat ASCs on 2D tissue culture plastic for up to 7 days. ASCs cultured with HBSS-supplemented medium or FBS-supplemented medium (10% v/v) were used as negative and positive control, respectively (Fig. 16 A). At days 0, 3 and 7, cell density and metabolic activity were determined by DAPI staining and MTS assay. After 7 days of culture, higher cell density in tECM and FBS-treated groups was clearly visualized by DAPI staining (Fig. 16 B). As shown in Figure 16 C, ASCs cultured with tECM demonstrated significantly higher metabolic activity than those with Col I, the most abundant structural ECM protein in tendon tissue, but slightly lower than those cultured with FBS.

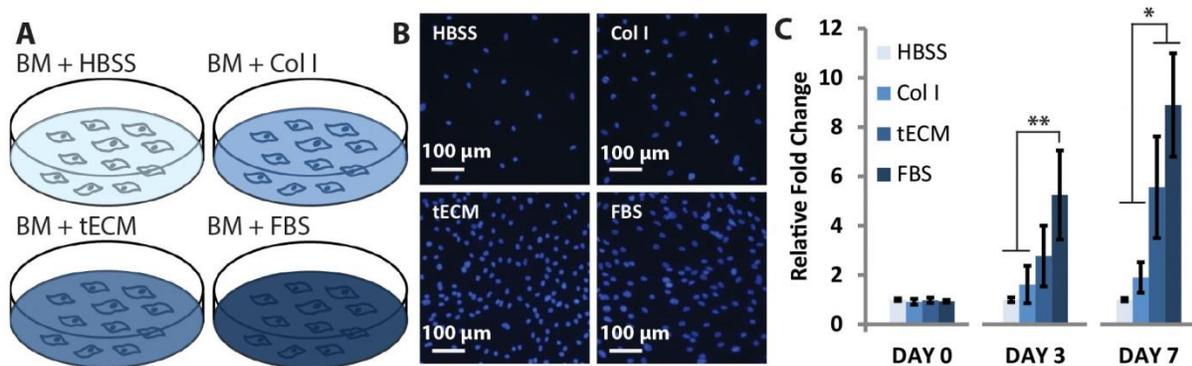


Figure 16. Assay of hASC proliferation in 2D cultures. (A) hASCs were plated on 2D tissue culture plastic, and treated with basal medium (BM) containing one of the following supplements at 10% v/v: HBSS, 1 mg/ml tECM solution (tECM), 1 mg/ml collagen type I solution (Col I) or FBS. (B) After 7 days of culture, DAPI

nuclear staining showed high cell density in tECM- and FBS-treated groups. (C) MTS assay revealed elevated cellular metabolic activity in tECM- and FBS-treated groups. *, $p < 0.05$; **, $p < 0.01$; $N = 3$

The individual and combined effect of tECM and TGF- β 3 on hASC tenogenesis was analyzed at the mRNA and protein level. Three types of medium (BM supplemented with 10% v/v FBS, Col I, or tECM) were prepared, with or without 10 ng/ml TGF- β 3, in which ASCs were cultured for up to 14 days (Fig. 17 A). Real-time PCR analysis showed that treatment with tECM alone did not significantly increase the expression of SCX, the primary marker for early tendon differentiation. However, tECM combined with TGF- β 3 gave rise to significantly higher levels of SCX expression than all other groups tested at days 3 and 7 that were maintained after two weeks of culture. ASCs treated with TGF- β 3 in the presence of Col I showed lower levels of SCX expression on days 3 and 7 compared to the tECM plus TGF- β 3 treatment (Fig. 17 B). The difference in SCX expression among the groups was confirmed at the protein level by Western blot (Fig. 17 D). Interestingly, unlike SCX, TNC expression was up-regulated by tECM treatment in the absence of TGF- β 3 after 7 and 14 days of culture, while the combined treatment of tECM and TGF- β 3 led to the highest level of TNC mRNA among all groups (Fig. 17 C). ASCs treated with TGF- β 3 in the presence of Col I again showed delayed up-regulation of tendon marker compared to the tECM plus TGF- β 3 treatment (Fig. 17 C). Taken together, these data showed that tECM treatment in 2D resulted in partial adoption of the tendon cell phenotype in the absence of other inductive cues, and further enhanced tenogenesis of ASCs induced by TGF- β 3. Moreover, tECM exhibited no such inductive effect on chondrogenesis of ASCs in 2D culture, suggesting a tissue-specific functionality of the tECM (Fig. 18).

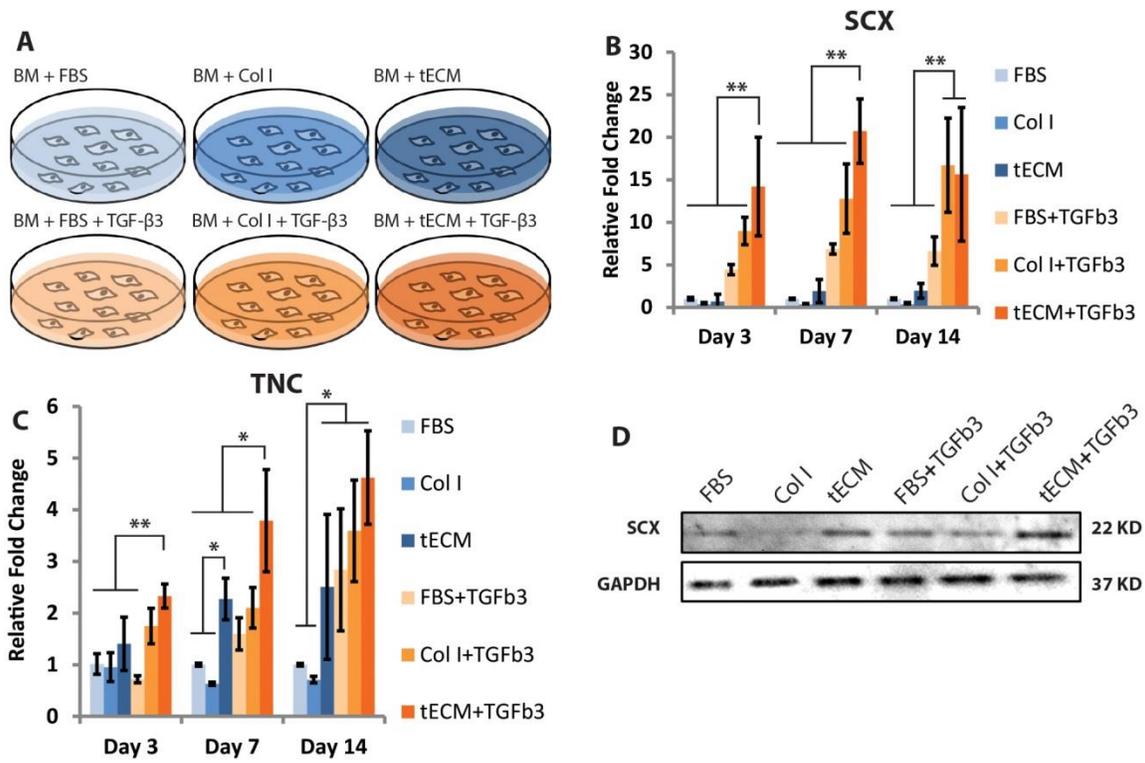


Figure 17. Tenogenesis of hASCs in 2D cultures. (A) Experiment groups. (B, C) Real-time PCR analysis of scleraxis (SCX, B) and tenascin-C (TNC, C) expression levels suggested that tECM treatment up-regulated SCX expression in the presence of TGF- β 3, and increased TNC expression with or without TGF- β 3. (D) Western blot assay showed consistent difference in SCX protein. *, $p < 0.05$; **, $p < 0.01$; N=3

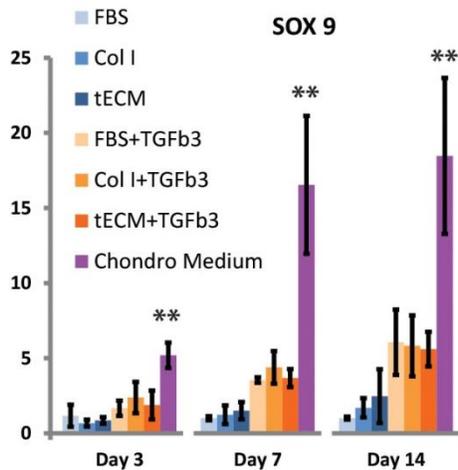


Figure 18. Real-time PCR analysis of SOX9 expression revealed that tECM alone possessed little inductive effect on chondrogenesis of ASCs in 2D culture compared to cell pellets cultured in standard chondrogenic medium (Chondro Medium). **, $p < 0.01$; N=3

3.4.3 Characterization of the aligned microfiber scaffolds

We next attempted to generate a physical scaffold environment that mimics the structural feature of tendon. A 3D microfibrinous PCL scaffold was fabricated by electrospinning. Aligned scaffolds exhibited highly uniaxial fiber orientation: most fibers were oriented at between 80° and 100° with respect to cross axis (Fig. 19 A, B), in contrast to the random orientation seen in the non-aligned scaffolds (Fig. 19 C, D). No significant difference in fiber diameter was found between aligned and random scaffold ($1.26 \pm 0.51 \mu\text{m}$ vs. $1.29 \pm 0.34 \mu\text{m}$). When tension was applied in the direction of fiber alignment, the aligned scaffolds displayed 2.5-fold higher tensile strength as compared to the randomly oriented scaffolds (Fig. 20 A, B). Anisotropy of the aligned scaffold was confirmed by tensile testing along two planes: the elastic modulus along the axis of fibers (longitudinal) was 10-fold higher than that in the perpendicular direction (cross) (Fig. 20 A, B). hASCs seeded on aligned scaffolds adopted elongated morphology and were orientated in the direction of fibers after 3 days of culture. In contrast, hASCs seeded on random scaffolds exhibited polygonal shape without uniformity in orientation (Fig. 21).

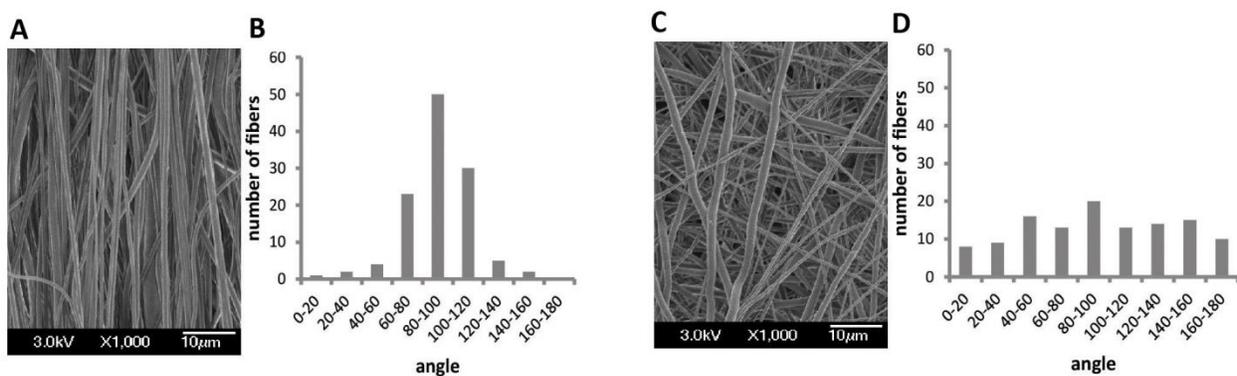


Figure 19. Characterization of PCL scaffold. (A, C) SEM image of aligned and random microfibrinous PCL scaffolds. (B) Most fibers in the aligned scaffold were oriented at between 80° and 100° with respect to cross axis, whereas in non-aligned scaffold (D), fibers were evenly distributed in all directions.

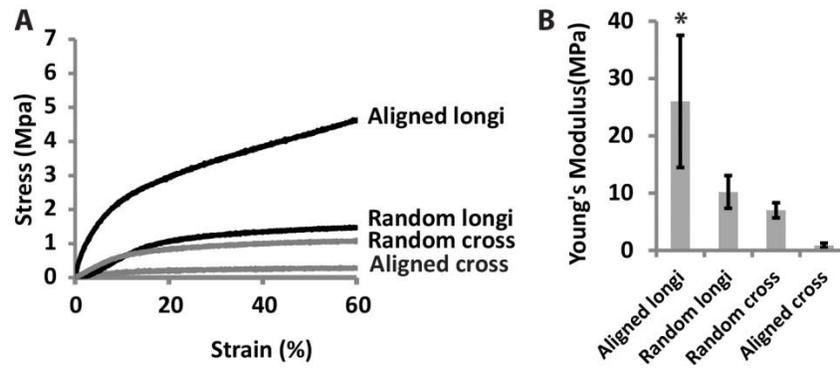


Figure 20. Characterization of PCL scaffold (continued). (A) Strain-stress curves showed substantial difference in tensile strength between aligned and random scaffolds. Anisotropy in tensile strength was noticed in aligned scaffolds (Aligned longi vs. Aligned cross). (B) The elastic modulus was the highest in longitudinal direction (Aligned longi) in aligned scaffolds. *, $p < 0.05$; $N = 3$

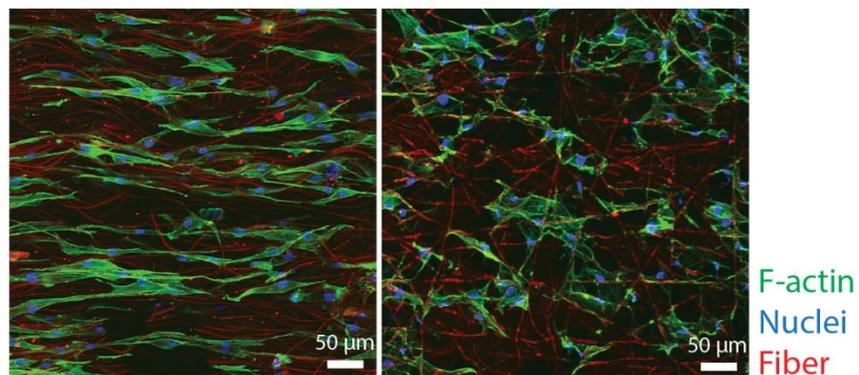


Figure 21. Morphology of ASCs cultured on scaffolds. ASCs seeded on aligned scaffolds adopted an elongated morphology and were orientated in the direction of the fibers (left, confocal microscopy; F-actin, green; nuclei, blue; microfiber, red). In contrast, ASCs seeded on random scaffold exhibited polygonal shape without uniformity in orientation (right).

3.4.4 Effect of tendon ECM on ASC behavior in 3D scaffolds

ASCs seeded on aligned scaffolds were treated for one week in BM supplemented with 10% v/v FBS, Col I, or tECM. No observable difference in cell shape was found among groups: most ASCs cultured on the aligned scaffolds were elongated and aligned in the direction of the surrounding fibers regardless of treatment method, as indicated by immunofluorescent staining of F-actin (Fig. 22 A). Nevertheless, cell metabolic activity differed greatly among groups: the tECM-treated group exhibited enhanced metabolic activity compared to Col I- or HBSS-treated group. At day 7, the metabolic activity of tECM-treated cells remained significantly higher than Col I- or HBSS-treated cells, and was comparable to FBS-treated cells (Fig. 22 B).

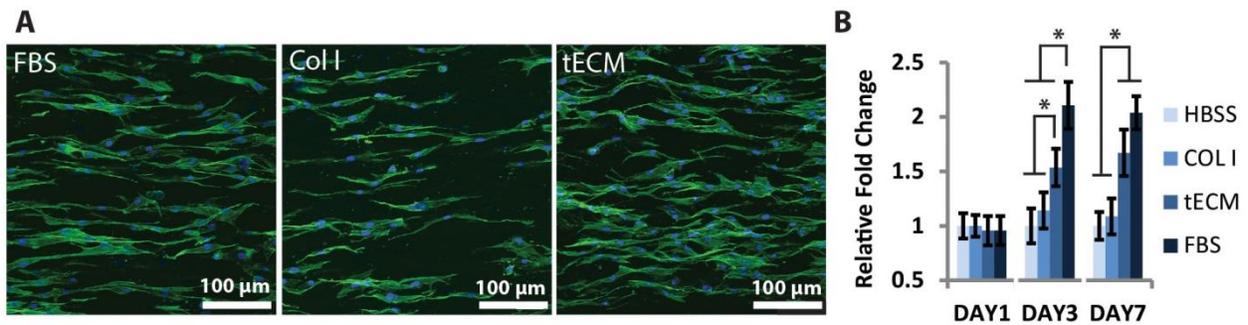


Figure 22. Behavior of hASCs seeded on aligned scaffolds. Cultures were treated with basal medium (BM) containing 10% v/v FBS, Col I, or tECM. (A) After 3 days of culture, most ASCs cultured on aligned scaffolds were elongated and aligned regardless of treatment methods. F-actin, green; nuclei, blue. (B) MTS assay showed enhanced cellular activity in the tECM-treated group compared to Col I- or HBSS-treated groups. *, $p < 0.05$; N=3

Given the established positive influence of fiber alignment on tenogenic differentiation, analysis of gene expression and matrix deposition were only performed on aligned microfibrillar scaffolds. When cultured in BM with TGF- β 3 and 2% FBS, SCX expression in hASCs was increased by the presence of tECM on days 3, 7 and 14, whereas in the Col I group the up-

regulation in SCX was not seen until day 14 (Fig. 23 A, SCX). Similarly, tECM supplementation resulted in significantly higher TNC levels compared to controls at all three time points tested. In contrast, Col I treatment did not significantly up-regulate TNC level although there was a trend of increase (Fig. 23 A, TNC). To further investigate the extent of tECM-mediated tenogenesis, we analyzed the presence of Tnmd, a tendon-specific membrane glycoprotein found in the late phase of differentiation, by immunofluorescent staining. Compared to other treatment groups, an evidently higher density and intensity of staining for Tnmd (green) was seen in the tECM-treated group (Fig. 23 B).

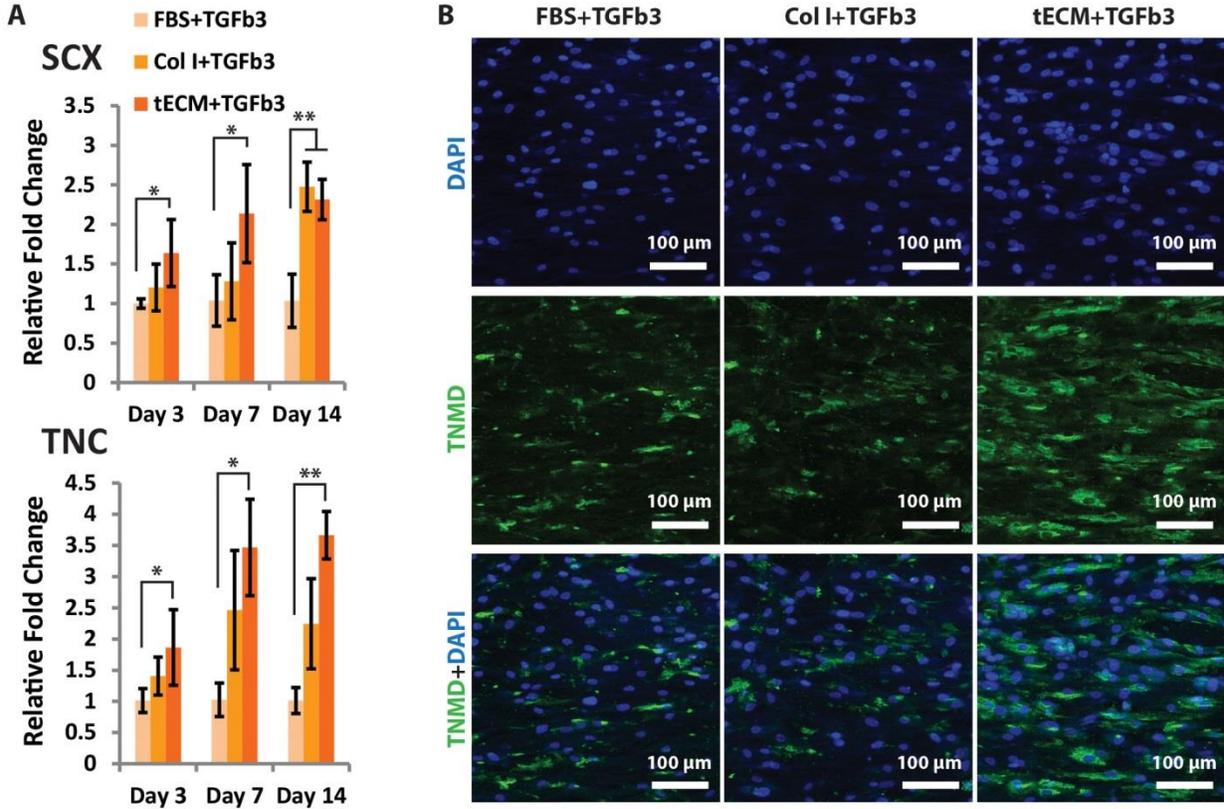


Figure 23. Tenogenic differentiation of hASCs seeded on aligned scaffolds. (A) Real-time PCR assay showed that scleraxis (SCX) and tenascin C (TNC) expression was significantly increased in the presence of tECM. (B) Immunofluorescence showed that staining for tenomodulin (Tnmd) was denser and more intense (green) in tECM-treated group. *, $p < 0.05$; **, $p < 0.01$; $N = 4$

We next examined the influence of tECM on the synthesis and organization of collagen, the primary structural protein of tendon tissue. Cells seeded on PCL scaffolds were treated with L-ascorbate 2-phosphate for 3 weeks to accelerate ECM synthesis. Immunofluorescent staining of collagen type I on scaffolds surface confirmed the presence of newly-synthesized collagen fibrils extended in the direction of the PCL fibers in both groups. Denser collagen fibrils were found on scaffolds treated with tECM, while the amount of collagen presented by tECM alone on the acellular scaffolds was negligible (Fig. 24 A). The observed difference in collagen content was confirmed quantitatively by hydroxyproline assay (Fig. 24 B). In the presence of tECM, hASCs produced a 2.4-fold higher amount of collagen per scaffold than controls (86.54 ± 7.46 vs. 36.79 ± 2.49 $\mu\text{g}/\text{scaffold}$). This pattern persisted when collagen content was normalized against double stranded DNA (dsDNA) content, with a 1.8-fold higher collagen content per unit weight of dsDNA (145.05 ± 17.46 vs. 80.02 ± 17.77 $\mu\text{g}/\mu\text{g DNA}$) in the tECM-treated group.

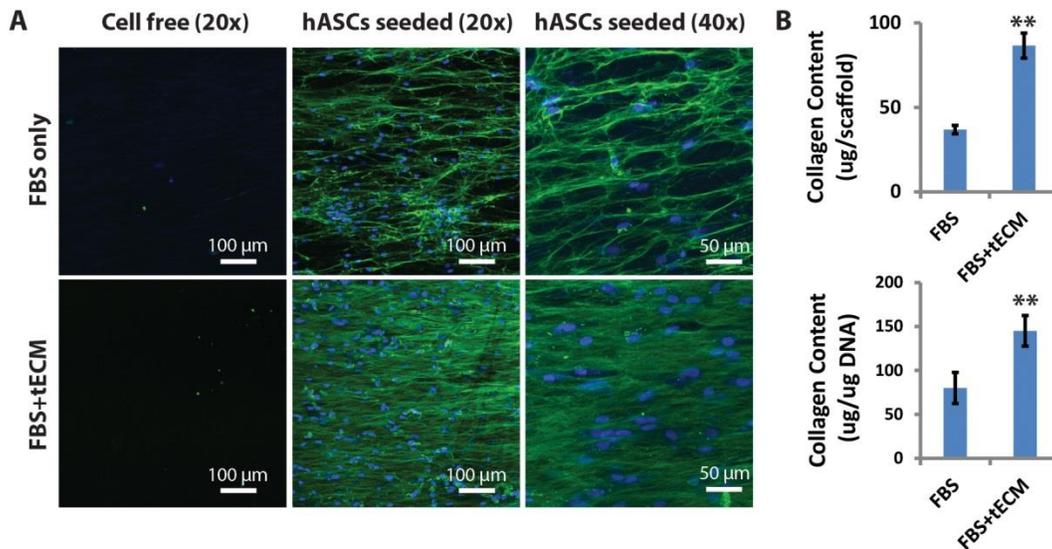


Figure 24. Matrix deposition by ASCs cultured on aligned scaffolds. (A) Immunofluorescent staining for collagen type I (green) found denser collagen fibrils deposited by cells treated with tECM compared to tECM free group. (B) Hydroxyproline assay showed higher collagen content in the tECM-treated group. Collagen content of each group was normalized to that of the corresponding cell-free group. **, $p < 0.01$; $N = 3$

3.5 DISCUSSION

The goal of this study IS to investigate the modulatory effect of soluble tendon ECM (tECM) on known biochemical (i.e. TGF- β 3) and biophysical cues that promote tendon differentiation, in order to advance the development of functional engineered tendon grafts. Human ASCs were prepared and characterized, and individual and combined effects of tECM and TGF- β 3 on cell behavior, including proliferation and differentiation, were examined. We found that: (1) tECM enhanced TGF- β 3-induced tenogenesis of hASCs in both 2D and 3D culture, and (2) tECM favorably modulated matrix deposition and organization by hASC-seeded microfibrinous scaffolds.

In the native tendon microenvironment, dense ECM surrounds the tendon cell. Tendon stem/progenitor cells (TSPCs) exhibited impaired proliferative and tenogenic potential in the absence of critical ECM proteins [38], and showed reduced TNMD expression when seeded on tissue culture plastic than on tendon ECM [123]. The tECM prepared in our study increased hASC proliferation and TNC expression when used as a culture supplement in the absence of other inductive cues. Expression of both TNC and SCX was further enhanced when treated concomitantly with TGF- β 3, as shown in both 2D and 3D cultures. Our findings indicate that the regulatory effects of tECM on tendon cell behavior also apply to ASCs. Consistent with our results, Little et al. [122] found increased proliferation and partial adoption of tendon phenotype by ASCs seeded on acellular tendon/ligament matrix. The tECM contains not only collagen but also a number of non-collagenous proteins, including fibronectin, fibromodulin, biglycan and decorin, all of which are known to regulate MSC activities, such as adhesion, proliferation, stemness and differentiation [156, 157, 159, 160]. How these ECM molecules, along with other yet-to-be-determined components in the tECM, contribute to the bioactivity of the tECM in our

results remains to be investigated. The enhancement of ASC tenogenesis by tECM combined with TGF- β 3 treatment exemplifies the complexity in the fine control of tissue differentiation: the tECM may serve as a reservoir of signals by itself or, not mutually exclusively, exert a regulatory effect on exogenous inductive cues [159]. Moreover, we found little inductive effect of tECM on chondrogenesis. Substantially lower SOX9 expression in 2D culture of ASCs treated with TGF- β 3 in BM, regardless of medium supplement, was found when compared to standard chondrogenic treatment for pellet culture. This finding further highlights the tissue specificity of the derivation of ECM in influencing MSC differentiation.

To date, ASCs are widely used for scaffold repopulation and as a means to improve vascularization, matrix deposition, and implant integration [187-190], whereas optimization of tendon-specific differentiation of seeded ASCs remains elusive. On one hand, ASCs isolated from a variety of animal species have been reported to upregulate tenogenic markers *in vitro* under specific treatment [85-88, 176], suggesting the tenogenic potential of ASCs. On the other hand, Eagan et al. questioned the suitability of hASCs for tendon tissue engineering and reported the lack of any significant and consistent up-regulation in the expression of COL I, TNC, or SCX, in hASCs treated for up to 4 weeks with TGF- β 1 or IGF1 [191]. In addition, hASCs showed lower SCX expression compared to TSPCs when cultured *in vitro* [90]. Incorporating tissue-specific ECM molecules into differentiation protocols represents an alternative approach to develop a robust tenogenesis strategy for ASCs in order to better exploit the regenerative potential of ASCs applied to tendon tissue engineering.

As noted above, the scaffold is another key component in tendon tissue engineering by creating a 3D environment for mechanical support and tissue regeneration. Therefore we prepared electrospun, aligned fibrous PCL scaffolds consisting of microfibers ($\sim 1.3 \mu\text{m}$) to

simulate the size of collagen fibrils in natural tendon tissue (1-300 μm) [192]. The diameter of fibers has been found to have an influence on seeded cells: compared with nanofibers, microfibers promoted the expression of phenotypic markers of tendon fibroblasts, possibly due to the resemblance of the healthy, mature matrix with micron-sized collagen bundles [193, 194]. The aligned scaffold also demonstrated anisotropy similar to that of tendon tissue, but improvements in tensile strength (~ 26 MPa) is clearly needed if intended to be used as a clinical tendon graft (~ 550 MPa) [195].

We examined the influence of tECM on collagen synthesis and organization by hASCs seeded on aligned scaffolds. Consistent with previous studies, collagen fibrils were aligned in the direction of the PCL fibers [196]. More abundant and homogenous distribution of collagen fibrils were found in cells treated with tECM. The tECM-induced enhancement of collagen fibrillogenesis may be due to the bioactivity of small leucine-rich proteoglycans (SLRPs) and glycoproteins in tECM, such as decorin, biglycan, lumican and collagen oligomeric matrix protein. These proteins are able to bind non-covalently to collagen molecules at specific sites in the gap region of fibrils and therefore facilitate collagen fibrillogenesis and stabilization [36, 37, 39, 40]. Acellular scaffolds possessed negligible collagen content when treated with tECM. This confirms that the tECM acts by promoting collagen production in ASCs rather than by merely presenting collagen to the scaffold.

3.6 CONCLUSIONS

In this study, a bioactive, soluble fraction of tendon ECM (tECM) was prepared, characterized and incorporated into growth/differentiation medium to treat ASCs. We demonstrated that tECM treatment enhanced the proliferation and tenogenic capacity of hASCs in both 2D and 3D culture. Moreover, when cultured on scaffolds that mimic the mechanical properties and architecture of native tendon tissue, hASCs treated with tECM exhibited increased Col I matrix synthesis and improved organization. These findings provide new insights into the role of tissue-specific ECM in guiding site-appropriate cell responses in terms of connective tissue differentiation and healing. In addition to serving as an *in vitro* differentiation model, the design attributes of the 3D culture system developed in this study are applicable to functional tendon tissue engineering that aims at simultaneous induction of phenotypic markers and enhanced matrix deposition. Our findings highlight the importance of reproducing the native tissue microenvironment as a design principle for eliciting desired cellular responses for tissue regeneration.

4.0 MULTILAYERED POLYCAPROLACTONE/GELATIN FIBER-HYDROGEL COMPOSITE FOR TENDON TISSUE ENGINEERING

4.1 ABSTRACT

Regeneration of injured tendon and ligament (T&L) remains a clinical challenge due to their poor intrinsic healing capacity. Tissue engineering provides a promising alternative treatment approach to facilitate T&L healing and regeneration. Successful tendon tissue engineering requires the use of three-dimensional (3D) biomimetic scaffolds that possess the physical and biochemical features of native tendon tissue. We report here the development and characterization of a novel composite scaffold fabricated by co-electrospinning of poly- ϵ -caprolactone (PCL) and methacrylated gelatin (mGLT). We found that photocrosslinking retained mGLT, resulted in uniform distribution of mGLT throughout the depth of scaffold and preserved scaffold mechanical strength. Moreover, photocrosslinking integrated stacked scaffold sheets to form multilayered constructs mimicking the structure of native tendon tissues. Cells impregnated into the constructs remained responsive to topographical cues and exogenous tenogenic factors such as TGF- β 3. The excellent biocompatibility and highly integrated structure of the scaffold developed in this study will allow the creation of more advanced tendon graft that recapitulates the architecture and cell phenotype of native tendon tissues.

4.2 INTRODUCTION

Tendons and ligaments are prone to injuries such as rupture and laceration due to their load-bearing nature [71, 135]. In cases of severe tendon injury, surgical intervention is employed to repair or replace the damaged tendon with autografts, allografts, xenografts, or prosthetic devices [197-199], for the natural healing process is slow and insufficient [23, 200]. To date, the clinical outcomes of tendon repair remain limited and unsatisfactory due to donor site morbidity, high failure rates, risk of injury recurrence, and limited long-term function restoration [72, 73, 201]. These limitations have spurred the development of tendon tissue engineering approaches, which apply combination of cells, scaffolds and bioactive molecules, as a promising strategy to create functional tissue replacements or to enhance the innate healing process [139, 202]. Ultimately, tendon tissue engineering aims at improving the quality of healing in order to fully restore tendon structure and function [21].

Tendon tissues are composed of densely packed aligned collagen fibrils that connect muscle to bone [200, 203]. Therefore, aligned nano- and micro-fibrous scaffolds fabricated by electrospinning have been extensively explored in attempts to reproduce the mechanical and topographical characteristics of native tendon tissue [104, 107, 204]. Electrospun poly- ϵ -caprolactone (PCL) scaffolds are frequently used in tendon tissue engineering as well as applications for other soft tissues. PCL is an aliphatic linear polyester approved by the U.S. Food and Drug Administration for clinical use [205]. It is biocompatible, bioresorbable and a low-cost synthetic polymer. Of equal importance, PCL exhibits low degradation rate due to its semi-crystalline and hydrophobic nature [206, 207], making it a suitable graft material to facilitate the relatively slow healing process of injured tendons [47, 54]. However, the hydrophobic nature of PCL often results in poor wettability and low surface protein adsorption [208], leading to

inadequate cell attachment and tissue integration [209]. Moreover, as a synthetic polyester, its lack of bioactivity is a major challenge for PCL to direct cell behavior after seeding due to the absence of cell-binding motifs found in natural ECM proteins [210].

Hydrogels prepared from collagen and its derivative, gelatin, represent another class of scaffolds for regenerating and repairing a wide variety of tissues and organs [211, 212]. Unlike other types of scaffolds, hydrogels retain a large volume of water and thus provide a highly hydrated environment similar to that in native tissues. Cells encapsulated within collagen/gelatin hydrogels can be easily distributed homogeneously by simple mixing during gel preparation compared to electrospun scaffolds, without any further scaffold modification [102, 183]. Importantly, collagen and gelatin, as constituents of natural ECM, better mimic at least in part the native tissue microenvironment, as compared to synthetic polymers [213, 214]. Nevertheless, improvement in the mechanical properties and introduction of topographical cues are needed to apply these hydrogels to tendon grafts that aim at reproducing the mechanical and structural features of native tendon tissues.

The organization of native ECM may be viewed as a cell-containing hydrogel reinforced by structural fibers. An engineered scaffold consisting of hydrogels and electrospun fibers may thus be considered as a biomimetic of the ECM. For example, a microfiber-reinforced silk hydrogel displayed a greatly improved modulus compared to a fiber-free hydrogel [215]. In addition, hydrogels composed of natural proteins could provide the bioactive motifs absent from synthetic polymeric scaffolds to enhance control of cell binding and fate determination [183, 184]. In terms of tendon tissue engineering, an ideal composite scaffold consisting of hydrogel and fibrous scaffold has yet to be developed. Consequently, little is known about the effects such a composite scaffold may have on the activities of encapsulated cells.

In this chapter, we have developed a novel composite scaffold as a tendon graft consisting of electrospun PCL microfibers and methacrylated gelatin (mGLT). We have optimized the retention of mGLT by photocrosslinking and its integration with the fibrous scaffold. Simultaneous cell seeding and photocrosslinking between scaffold layers were performed to create cell-impregnated multilayered constructs, and their mechanical properties and architecture and the activity of encapsulated cells were assessed. Our results showed that this novel cell-scaffold construct combines the advantages of PCL nanofibrous scaffolds and gelatin hydrogels to mimic the mechanical strength, structural architecture and cell phenotype of native tendon tissue.

4.3 MATERIALS AND METHODS

4.3.1 Synthesis of methacrylated gelatin

Methacrylated gelatin (mGLT) was synthesized using an established protocol with slight modification [102, 216]. Gelatin (GLT, Sigma-Aldrich) was dissolved in deionized H₂O at 37 °C (30%, w/v). Methacrylic anhydride (Sigma-Aldrich) was added dropwise into the mixture at 37 °C under mild agitation to react with amine groups on GLT for 24 hours (Fig. 25 A). Reacted mGLT solution was dialyzed against water to completely remove low molecular-weight byproducts using 3,500 NMWCO dialysis cassettes (Thermo Scientific). Dialyzed mGLT was lyophilized and stored desiccated for future use. The methacrylation rate of the product was ~80% [209]. The visible light (VL)-activated photo initiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as described by Fairbanks et al. [217].

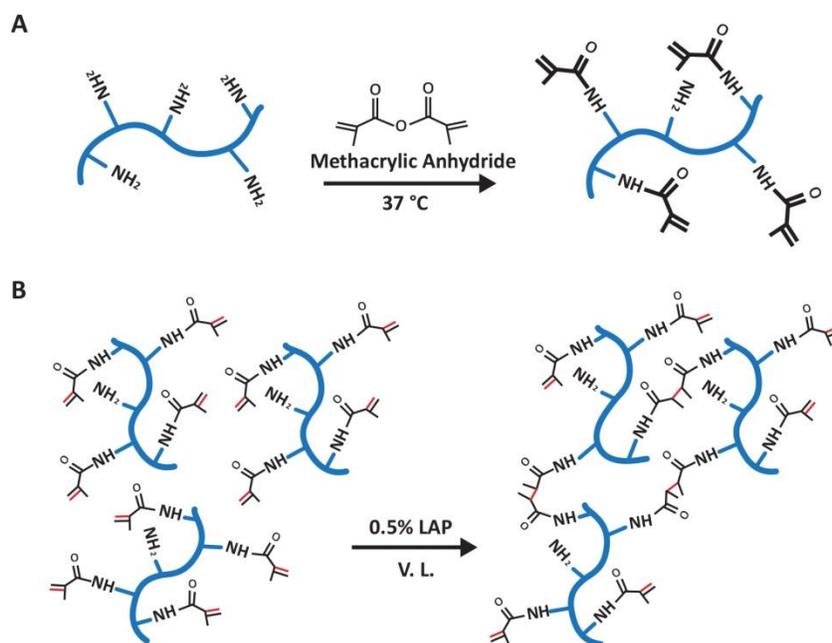


Figure 25. Synthesis and crosslinking of methacrylated gelatin (mGLT). (A) The amine groups on gelatin molecules were reacted with methacrylic anhydride to add methacrylate pendant groups. (B) To create a hydrogel network, mGLT was crosslinked using VL irradiation in the presence of photoinitiator LAP.

4.3.2 Fabrication of composite scaffolds

Composite scaffolds containing interspersed PCL and mGLT fibers were produced using dual electrospinning [218]. PCL particles (70k-90k, Sigma-Aldrich) was dissolved in 2,2,2-trifluoroethanol (TFE, Sigma-Aldrich, 18% w/v). Dehydrated mGLT was dissolved in 95% TFE (20% w/v). Each polymer solution was extruded through a 22-gauge blunt-tip needle at 2 mL/h for 1 hour. The spinnerets were charged with an optimized DC potential (8 kV for PCL and 15 kV for mGLT, respectively) and aligned in opposing positions on each side of the collection mandrel with a distance of 15 cm between the needle tip and mandrel (Fig. 26). The scaffold was stored desiccated under vacuum to remove residual solvent. To retain mGLT in the composite scaffold, 0.5% LAP solution was cast onto dry composite scaffold surface at 15 $\mu\text{l}/\text{cm}^2$ and

allowed to spread until the scaffold was completely wet. LAP was then activated by exposure to VL irradiation (450-490 nm) to photocrosslink the methacrylate groups of the dissolved gelatin in the scaffold (Fig. 25 B, Fig. 26).

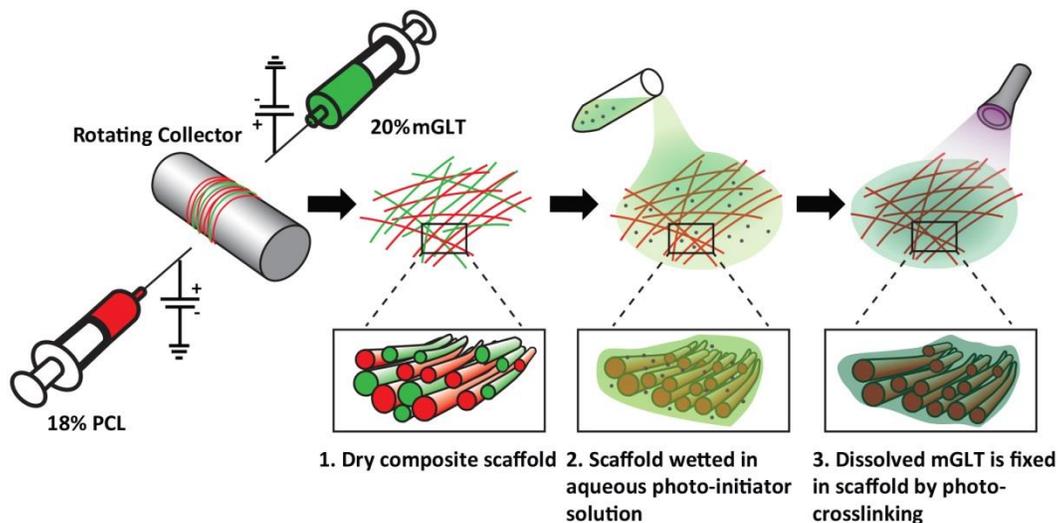


Figure 26. Composite scaffold preparation. Dual electrospinning was employed to fabricate a scaffold containing PCL and mGLT fibers (Insert 1). Dry scaffold was wetted with aqueous photo-initiator solution (Insert 2) and then photocrosslinked by visible light (VL) to retain the gelatin (Insert 3).

4.3.3 Imaging of composite scaffolds

Polymers were fluorescently labeled to track the presence and interspersions of the two distinct fiber populations: PCL solution was mixed with 0.1% (v/v) Vybrant® DiI Cell-Labeling Solution (DiI, Life Technologies), and non-methacrylated GLT was conjugated with fluorescein 5(6)-isothiocyanate (FITC, Sigma-Aldrich) before dissolution, respectively. Fibers were dual-electrospun onto glass slides for 5 min and imaged before and after wetting using an Olympus CKX41 inverted fluorescent microscope equipped with a CCD camera. Additionally, scaffold surface was examined by scanning electron microscopy (SEM, field emission, JEOL JSM6335F) operated at 3 kV accelerating voltage and 8 mm working distance.

4.3.4 Histological examination of composite scaffolds

Picrosirius red staining was employed to assess gelatin retention and distribution within scaffolds. Composite scaffolds before and after photocrosslinking were washed in PBS at 37 °C overnight under mild agitation, frozen-embedded in OCT compound (4583 Scigen Scientific), and cryosectioned at 15 µm thickness using a Leica CM 1850 cryotome. Sections were washed in PBS and stained with 0.1% sirius red in saturated picric acid (Electron Microscopy Sciences) for 1 hour. To visualize impregnated cells, cryosections of fixed, cell-seeded scaffolds were incubated with ethidium homodimer-1 (EthD-1, Life Technologies) to label cells via DNA binding.

4.3.5 Biochemical composition of composite scaffolds

Gelatin content of composite scaffolds was quantified by the Chloramine-T based hydroxyproline assay (Fisher Scientific and Sigma-Aldrich). Reaction product was measured spectrophotometrically at 550 nm using a microplate reader (BioTek). Relative gelatin retention rate was calculated as the reading of washed scaffold divided by that of dry scaffold.

4.3.6 Cell isolation and culture

Human adipose-derived stem cells (hASCs) were obtained from lipoaspirate-derived fat tissue of two donors (34 years old male and 38 years old female) using an automated cell isolation system (Tissue Genesis). The protocol was approved by the Institutional Review Board of the University of Pittsburgh. Isolated hASCs were cultured in growth medium (GM)

consisting of DMEM-high glucose (Gibco), 10% fetal bovine serum (FBS, Gibco), 100 units/ml penicillin and 100 mg/ml streptomycin (P/S, Gibco). Multipotency was confirmed by established differentiation tests [179].

4.3.7 Creation of multilayer constructs

Scaffold pairs were prepared by overlaying one third the area of a rectangular scaffold piece atop another scaffold piece of identical shape. The scaffold pair was then wetted with photo-initiator and crosslinked by VL irradiation (Fig. 29 A), followed by gentle washing in PBS at 37 °C for up to 7 days. To create multilayered structures, 5 sheets of scaffold of identical rectangular shape were wetted with photo-initiator solution, stacked, and exposed to VL for 1 min on each side to crosslink adjacent scaffold layers (Fig. 29 A). For these multilayer constructs, photo-initiator was dissolved in 8% mGLT solution to further reinforce the association between scaffold layers. Sandwich constructs made from alternative layers of fibrous PCL and mGLT solution were also prepared and subjected to the same photocrosslink protocol. After 7 days of washing in PBS at 37 °C, scaffold integrity was examined by picrosirius red staining of orthogonal cryosections.

4.3.8 Mechanical testing

Tensile properties of single layer scaffold and multilayer constructs were both analyzed by using the Bose 3230 mechanical tester. Samples were securely mounted and loaded with uniaxial force applied at a displacement rate of 0.2 mm/s until 10 mm displacement. The tensile

force and the displacement were recorded. The slope of the linear portion of the stress–strain curve was calculated as the elastic modulus.

4.3.9 Creation of a tendon-mimetic construct by multilayer scaffolds

Aligned composite scaffolds were fabricated by dual electrospinning. hASCs were detached and suspended in LAP-containing 8% mGLT solution and cast onto scaffold sheets for hydration before VL irradiation. Multilayer constructs consisting of 5 sheets of cell seeded, aligned scaffolds were then prepared as described above. To induce tenogenic differentiation, constructs were maintained for 7 days in differentiation medium (DM) consisting of DMEM, 2% FBS, P/S, and 10 ng/ml TGF- β 3 (PeproTech).

4.3.10 Cell proliferation tests

Cells encapsulated in multilayer constructs were maintained in standard GM and subject to MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) at 1 day or 7 days after initial seeding. A_{490} was determined spectrophotometrically using a microplate reader (BioTek). Double stranded DNA (dsDNA) content in each group was determined fluorimetrically by Picogreen assay (Quant-iT PicoGreen, Invitrogen, excitation 480 nm, emission 520 nm). All readings were normalized to the cell free control group at each time point.

4.3.11 Cytoskeleton fluorescent staining

Cell seeded, single layer scaffold sheets were washed in PBS, fixed in 4% paraformaldehyde and incubated with 1% bovine serum albumin (BSA). Cells were permeabilized in 0.1% Triton X-100 for 5 min and then incubated with phalloidin for 30 min at room temperature (Alexa Fluor 488 phalloidin, Life Technologies). Lastly, cells were rinsed with PBS, nuclear counterstained with DAPI (Life Technologies), and imaged using a confocal microscope (Olympus FluoView 1000).

4.3.12 Real-time PCR analysis

Total cellular RNA was isolated using an RNA extraction Kit (Qiagen) and first-strand cDNA synthesized with random hexamer primers (SuperScript III First-Strand cDNA synthesis kit, Invitrogen). Quantitative real-time PCR was performed using SYBR green Supermix in a Step One Plus real-time PCR system (Applied Biosystem, Life Technologies) to analyze expression of tenogenic markers, scleraxis (SCX) and tenascin-C (TNC). The targets and sequences of primers are shown in Table 5. The relative level of each gene was normalized to that of 18S rRNA and calculated using the $\Delta\Delta C_t$ method.

Table 5. Primer sequences of genes analyzed by real-time PCR

Gene		Primer sequence (5'-3')	Product size (bp)
18S rRNA	Forward	GTAACCCGTTGAACCCCAT	151
	Reverse	CCATCCAATCGGTAGTAGCG	
SCX	Forward	ACACCCAGCCCAAACAGA	65
	Reverse	GCGGTCCTTGCTCAACTTTC	
TNC	Forward	GGTGGATGGATTGTGTTCTGAGA	328
	Reverse	CTGTGTCCTTGTCAAAGGTGGAGA	

4.3.13 Statistical analysis

Data are presented as mean \pm standard deviation (SD). All quantitative assays were performed for no less than three times independently (n equals to the number of samples tested). One-way ANOVA with Bonferroni post hoc tests and Student's t-test was performed to determine statistical significance. Significance was considered at $p < 0.05$.

4.4 RESULTS

4.4.1 Organization of fibers in composite scaffold

Microscopic observation of the co-electrospun scaffold showed that the fluorescently labeled fibers were interspersed within the dry scaffold (Fig. 27 A, Composite Dry, PCL: red; mGLT: green). Hydration of the scaffold in an aqueous environment resulted in rapid dissolution of mGLT fibers, whereas PCL fibers remained intact (Fig. 27 A, Composite Wet, PCL: red; mGLT: green). SEM revealed a fraction of fibers with distinct ribbon-like morphology in addition to ordinary cylindrically shaped fibers in the dry composite scaffold mesh (Fig. 27 B, Composite Dry, indicated by arrow in insert). After hydration, the ribbon portion was no longer detectable; instead, adjacent fibers were found bridged or bundled by deposited sheaths (Fig. 27 B, Composite Wet, indicated by arrows in insert). The alteration in scaffold organization is likely caused by the dissolution and deposition of incorporated gelatin during hydration, due to its high aqueous solubility. In contrast, scaffold made only of PCL showed no observable difference between the dry and wet state, exhibiting uniform shaped fibers without fused contacts (Fig. 27 B, PCL Dry vs. PCL Wet).

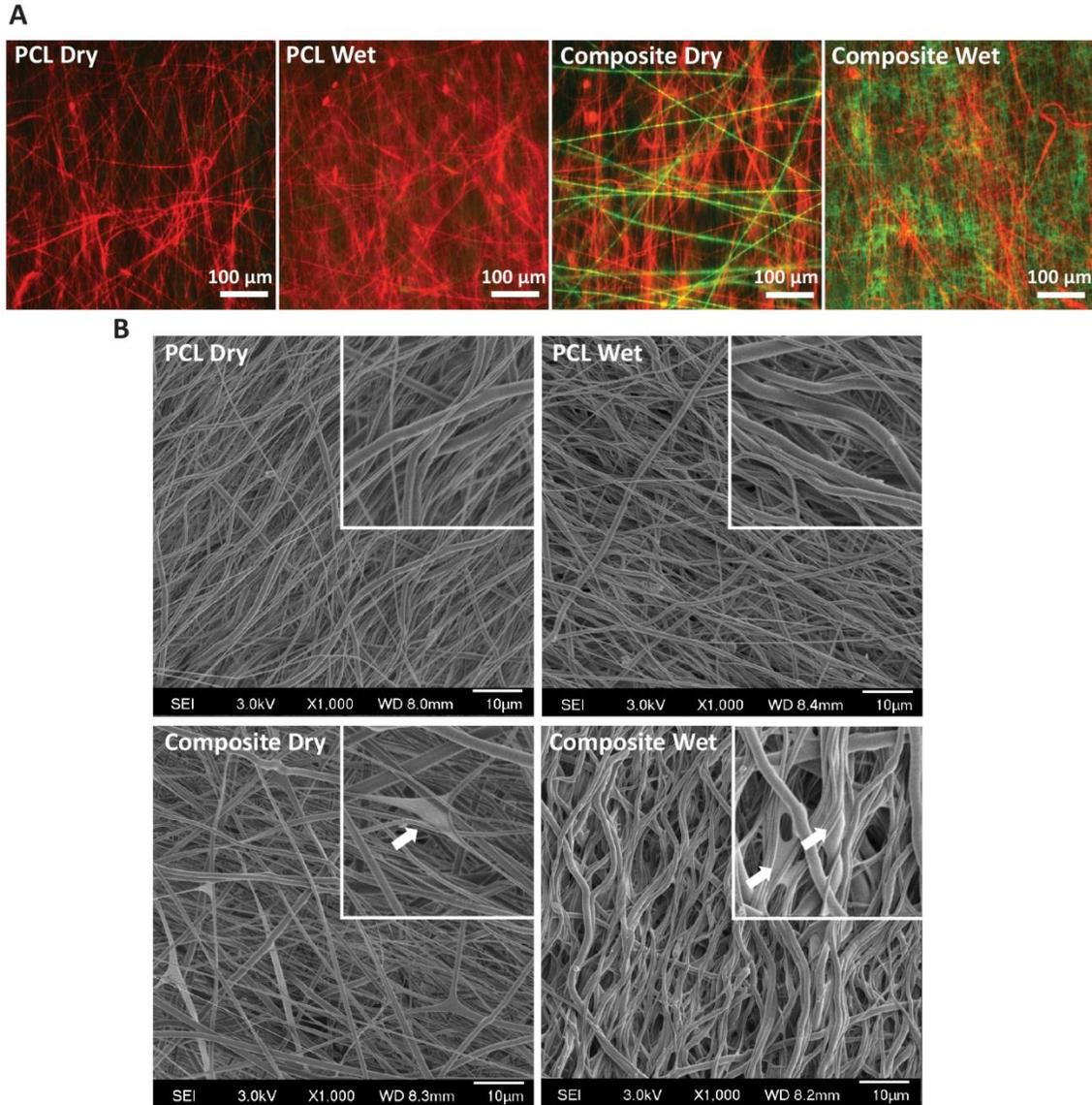


Figure 27. Characterization of scaffold architecture. (A) Fluorescently labeled PCL (red) and gelatin (green) displayed a fibrous morphology and interspersed distribution within the dry composite scaffold prepared by dual electrospinning (Composite Dry). Wetting of the scaffold with an aqueous solution resulted in dissolution of the gelatin fibers (green), whereas the PCL fibers (red) remained intact (Composite Wet). (B) SEM revealed ribbon-like bands in addition to predominant cylindrical fibers in composite scaffold (Composite Dry, indicated by arrow in insert). After wetting, adjacent fibers were bridged or wrapped with a sheath of gelatin (Composite Wet, indicated by arrows in insert). Unlike the composite mesh, scaffolds made of only PCL showed negligible differences in morphology before and after wetting (PCL Dry vs. PCL Wet).

4.4.2 Biochemical and mechanical analysis of composite scaffold

Hydrated composite scaffold was exposed to LV irradiation for crosslinking of mGLT in the presence of photo-initiator (Fig. 26). Picrosirius staining of gelatin showed a pronounced difference in gelatin retention between non-crosslinked and crosslinked scaffolds (Fig. 28 A). Without crosslinking, the scaffold displayed little red staining, indicating loss of the majority of incorporated gelatin after washing (Fig. 28 A, Composite non-crosslinked). In contrast, crosslinked scaffolds showed a large amount of gelatin retained within the scaffold mesh (Fig. 28 A, Composite crosslinked, red). Gelatin deposition was also evenly distributed within the fiber mesh, suggesting excellent integration between gelatin and PCL fibers. As a control, mGLT solution was cast atop PCL scaffold, resulting in a distinct boundary between gelatin hydrogel and PCL that suggested the separation of two scaffold formats (Fig. 29).

Scaffolds were dried in an oven and weighed before and after gentle washing in PBS for 1 day at 37 °C to estimate the gelatin loss (Fig. 28 B, $p < 0.01$). After washing, non-crosslinked scaffolds lost one third of their initial weight ($32.87\% \pm 3.89$, $n=5$), whereas crosslinked scaffolds lost only 16% ($15.89\% \pm 3.50$, $n=5$). This finding is consistent with the gelatin content data estimated by hydroxyproline assay (Fig. 28 C, $p < 0.01$): crosslinked scaffold retained half of its initial gelatin content ($44.53\% \pm 1.58$, $n=4$), while non-crosslink scaffold preserved less than 3%. ($2.93\% \pm 0.46$, $n=4$). We then analyzed the impact of crosslinking on the mechanical strength of scaffolds, and found a 16.0% reduction in maximum load of non-crosslinked material after washing. In contrast, crosslinked scaffold demonstrated consistent maximum load with no significant alteration compared to dry composite scaffold (Fig. 28 D).

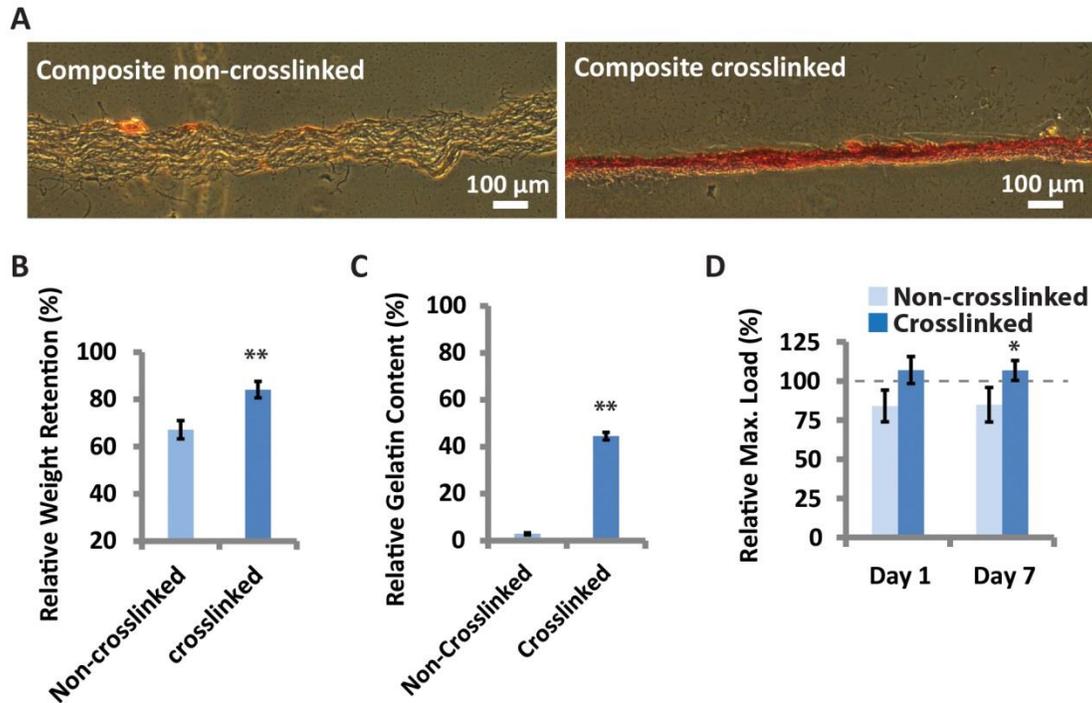


Figure 28. Examination of gelatin retention. (A) Picrosirius red staining showed that non-crosslinked scaffolds lost most gelatin after washing (Composite non-crosslinked), whereas photo-crosslinked scaffolds retained a portion of incorporated gelatin within the scaffold mesh (Composite crosslinked, red). (B) Non-crosslinked scaffolds showed significantly higher weight loss compared with crosslinked scaffolds. (C) Difference in gelatin retention between crosslinked and non-crosslinked scaffolds was quantified by hydroxyproline assay. (D) Mechanical testing of crosslinked scaffolds demonstrated equivalent maximum load to that of dry composite scaffolds, while non-crosslinked scaffolds showed ~16% reduction after washing. *, $p < 0.05$; **, $p < 0.01$; $n = 4$

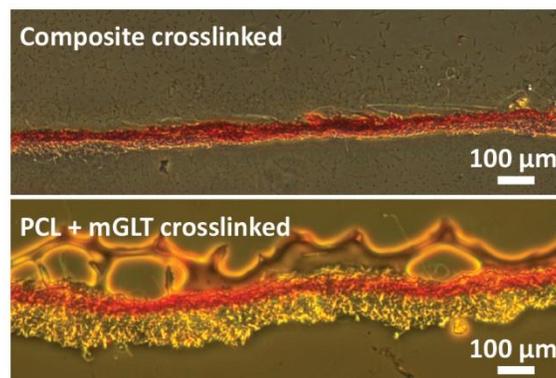


Figure 29. Picrosirius red staining of scaffolds. Unlike the crosslinked composite scaffold (upper), mGLT solution cast atop PCL scaffold aggregated on the surface of PCL fibers after crosslinking (lower).

4.4.3 Multilayered construct created by photocrosslinking

The crosslinkable nature of composite scaffold combined with the excellent integration between gelatin deposition and fibrous PCL suggests the possibility of preparing multilayered constructs by stacking and photocrosslinking individual scaffold layers. To test the feasibility of this approach, two scaffold sheets were partially overlapped with or without subsequent photocrosslinking (Fig. 30 A). Some of the non-crosslink scaffold pairs no longer adhered to each other after washing, whereas all crosslinked scaffold layers remain intact (Fig. 30 B). After washing in PBS at 37 °C, unseparated scaffold layers were subjected to uniaxial stretch. The load-displacement curve revealed markedly higher resistance to tension in the crosslinked scaffold pair, indicating that the stacked layers of sheets can bond with each other (Fig. 30 C).

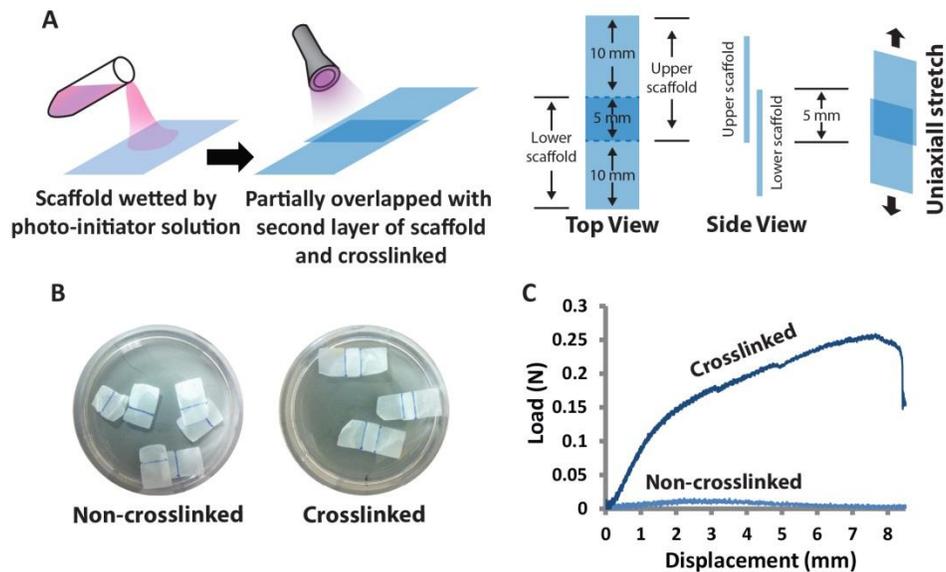


Figure 30. Lamina integration of scaffold sheets. (A) Scaffold pairs were prepared by overlaying 1/3 the area of one scaffold sheet atop another and then photocrosslinked. (B) Without crosslinking, the scaffold pairs fell apart after washing, whereas crosslinked scaffold pairs remained intact. Blue lines suggested the boundary of overlapping regions. (C) Representative load-displacement curve of crosslinked scaffold pairs showed higher resistance to tension. n=6

To create multilayered constructs, 5 sheets of scaffold were wetted with photo-initiator solution, stacked and exposed to VL to crosslink adjacent scaffold layers (Fig. 31 A). After 7 days of washing in PBS, a substantial difference in scaffold integrity was identified by picrosirius red staining (Fig. 31 B). Without crosslinking (left, CN), the scaffold layers did not adhere to each other. Sandwich constructs made from alternative layers of fibrous PCL and mGLT showed partial failure (center, PC), while crosslinked multilayer composite scaffolds remained fully integrated with negligible separation between layers (right, CC). Mechanical testing showed significantly higher tensile strength of crosslinked scaffolds than non-crosslinked scaffolds (Fig. 31 C, CC vs. CN, $p < 0.05$, $n = 6$), while the integration between layers exerted little impact on overall tensile strength (Fig. 31 C, PC vs. CC, $n = 6$).

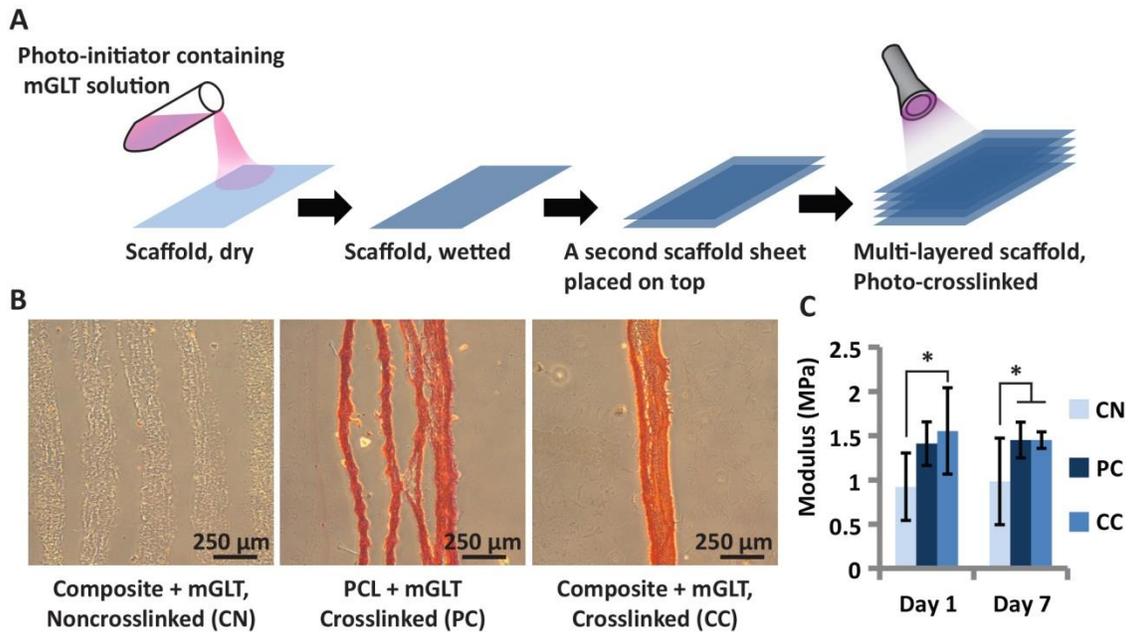


Figure 31. Preparation and characterization of multilayer construct. (A) Five scaffold sheets were wetted, stacked and exposed to VL to crosslink adjacent scaffold layers. (B) Picrosirius red staining showed pronounced difference in scaffold integrity after washing among the three groups: stacked composite scaffold without crosslinking (CN, left), sandwich construct made from alternative layers of fibrous PCL and mGLT (PC, center), and crosslinked composite scaffold (CC, right). (C) Higher tensile strength was found in crosslinked scaffolds than

in non-crosslinked scaffolds (CC vs. CN), while the integration between fibrous scaffolds had little impact on overall tensile strength (CC vs. PC). *, $p < 0.05$; $n = 6$

4.4.4 Cell incorporation and activity in multilayered construct

hASCs were impregnated into the crosslinked PCL/mGLT sandwich constructs (PC) or crosslinked composite constructs (CC) to generate a cell-laden graft. After 7 days of culture, EthD-1 staining showed that most hASCs were localized in the interstitial space between adjacent layers (Fig. 32 A; red). Crosslinked composite constructs again showed better structural integrity than that of sandwich constructs (Fig. 32 A; dark red). Moreover, hASCs impregnated in composite scaffold displayed significantly higher metabolic activity (Fig. 32 B), while total cell number, reflected by the dsDNA content, remained comparable between two groups (Fig. 32 C). These two findings suggest that constructs made from composite scaffolds promote cell metabolism but not proliferation.

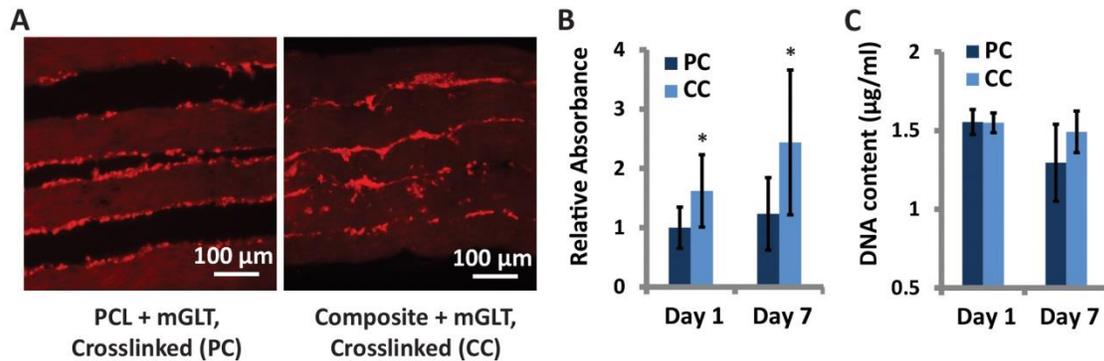


Figure 32. Analysis of cell distribution and metabolic activity in multilayer constructs. (A) Ethidium homodimer-1 (EthD-1, red) staining indicated that most cells were localized between two adjacent layers. Crosslinked composite constructs (CC) demonstrated evidently higher structural integrity than sandwich constructs (PC). (B) MTS assay revealed significantly higher metabolic activity of cells impregnated in composite scaffold (CC), whereas (C) the total cell number remained comparable to that of sandwich constructs (PC). *, $p < 0.05$; $n = 8$

4.4.5 Characterization of tendon phenotype induced by aligned multilayered constructs

To create tendon-mimicking constructs, aligned composite scaffolds were prepared and anisotropy confirmed by SEM (Fig. 33 A) and mechanical testing (Fig. 33 C, 0.12 ± 0.01 vs. 2.51 ± 1.33 MPa). F-actin fluorescent staining showed that impregnated hASCs adopted elongated morphology in the direction of fibers (Fig. 33 B; green, F-actin; blue, nuclei). For tendon cell lineage commitment, hASCs were treated with 10 ng/ml TGF- β 3 for 7 days. Real-time PCR assay showed pronounced upregulation of tendon markers SCX and TNC (Fig. 33 D), indicating that encapsulated cells remained responsive to soluble tenogenic factors, and that the construct possessed sufficient porosity for cells to receive exogenous biochemical cues.

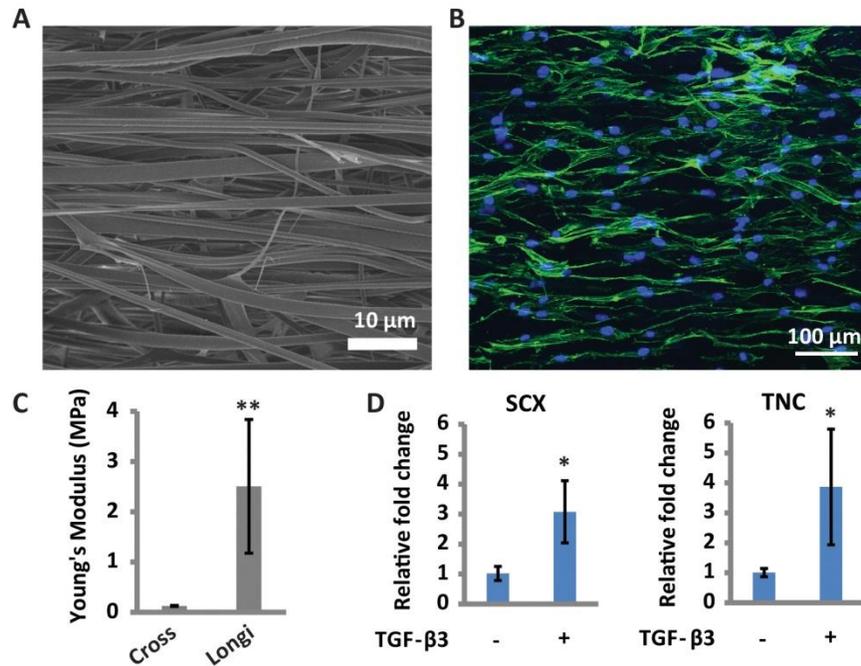


Figure 33. Tendon-like features of cell-impregnated multilayer constructs. (A) Fiber alignment observed by SEM. (B) Anisotropy in tensile strength were measured by mechanical testing along two directions (Longi. vs. Cross). (C) Elongated morphology of hASCs aligned in the direction of fibers (green, F-actin; blue, nuclei). (D) Significant upregulation of tendon markers scleraxis (SCX) and tenascin C (TNC) upon treatment with exogenous tenogenic factor TGF- β 3 were measured by real-time PCR analysis.

4.5 DISCUSSION

Scaffolds are of critical importance in the context of tissue engineering, serving to provide a physical substrate that can recapitulate the *in vivo* milieu of healthy tissues and thus orchestrate the activity of therapeutic cells in a tissue-specific fashion [219]. In terms of tendon tissue engineering, a number of scaffold designs have been developed to reproduce one or multiple structural/compositional characteristics of tendon tissues, among which aligned, electrospun fibrous scaffolds and the 3D hydrogels have been frequently implemented in preclinical models due to their possession of respective topographical cues and native biochemical cues to seeded therapeutic cells [104, 107, 183, 220]. Therefore, a composite scaffold consisting of fibrous scaffold sheets and hydrogel is a potentially promising approach for the mimicry of physical and biological features in combine of tendon tissue.

To achieve this goal, we firstly prepared PCL/mGLT composite scaffolds by dual electrospinning. Upon VL irradiation, the free radicals generated from the photo-initiator resulted in crosslinking via methacrylate on the gelatin backbone [221], thereby generating a hydrogel network within the PCL fiber mesh. We chose gelatin rather than collagen as the bioactive building block of the construct for two reasons: (1) as a product of denatured collagen [222], gelatin possesses a portion of the amino acid sequence and bioactive motifs of the parent collagen and is consequently chemically similar to native collagen chains; and (2) gelatin is far less costly compared to purified collagen (~25 USD/100 g vs. 650 USD/100 mg, pricing from Sigma-Aldrich) while more stable to organic solvent dissolution [223]. These features make gelatin suitable for industry-scale scaffold manufacturing.

For electrospinning, PCL and mGLT were dissolved and extruded separately to create intercalated PCL and mGLT fibers so that the mechanical strength of the PCL scaffold mesh is

not compromised by the change in mGLT structure upon hydration. In contrast, electrospinning of a mixed solution of gelatin and PCL led to a collection of fibers comprised of alternating segments of gelatin and PCL, in which the dissolution of gelatin will presumably lead to fiber dissociation at gelatin regions and sequential loss of overall mechanical strength [224]. Concentrations of PCL and mGLT ranging from 10% to 22% were tested, and 18% PCL and 20% mGLT were chosen based on the stability of the solution stream during electrospinning [225]. However, improvement in tensile strength is clearly needed if the scaffold is intended to be used to construct a clinical tendon graft (~550 MPa) [195]. Notably, although mGLT fibers rapidly dissolved upon wetting and therefore possibly increased the pore size of the scaffold, we did not observe any improved cell infiltration into the interior region of those crosslinked scaffolds (Fig. 34) [226]. The gelatin hydrogel formed within the PCL fiber mesh may occupy the pores and impede cell migration. To address this potential issue, a peptide linker containing a matrix metalloproteinase (MMP)-sensitive motif might be incorporated to generate a more cell-cleavable hydrogel within a fibrous scaffold to improve cell infiltration [227, 228].

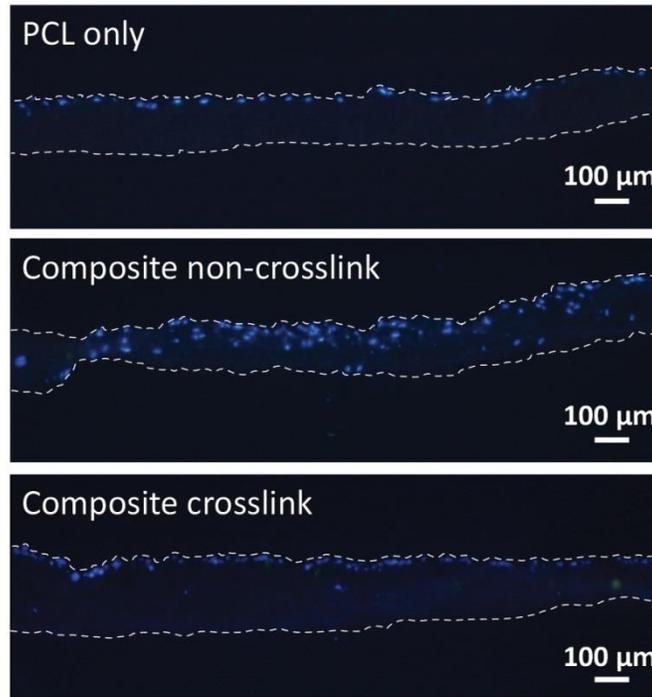


Figure 34. DAPI-stained cross-sections of ASC-seeded scaffolds after one week of *in vitro* culture. Dissolution of gelatin without crosslinking increased scaffold porosity and therefore improved cell infiltration (Composite non-crosslink) compared to cells seeded on PCL scaffold. However, retention of gelatin via crosslinking appeared to impede ASC migration into the interior region of scaffold (Composite crosslink). Blue, nuclei; scaffolds were outlined by white dash lines.

In addition to presenting multiple environmental cues, multi-layering of fibrous scaffold sheets can be employed to reconstitute the 3D architecture of a native tissue [219]. To achieve this goal, the rapid, robust integration between scaffold layers and sufficient incorporation of therapeutic cells must be carefully balanced [229-231]. In the scaffold described here, the gelatin component was found evenly distributed throughout the depth of PCL mesh, and therefore enabled integration of multiple sheets along the z axis upon photocrosslinking. Moreover, the VL-based photocrosslinking technique employed in our study is biocompatible, as our previous study found less than 6% cell death [232]. By combining these two characteristics, we have

achieved a robust and instant integration of scaffold layers that is compatible with simultaneous, sufficient cell encapsulation. For future study, the composite scaffold is open to further modifications that aim at eliciting the tenogenesis of impregnated cells. For instance, heparin may be conjugated to the gelatin backbone to sequester and deliver exogenous tenogenic growth factors into the constructs [233]. Tendon tissue derived-ECM may also be incorporated into the hydrogel portion of the constructs to recapitulate the proteinaceous microenvironment of native tendon tissue [183]. Finally, we believe that the scaffold engineered in this study as a building block for multilayer constructs should have applications beyond tendon tissue engineering in the fabrication of tissue grafts that contain both a fibrous component and a hydrogel portion

4.6 CONCLUSION

In this study, we developed a novel composite scaffold consisting of fibrous PCL and methacrylated gelatin (mGLT) interspersed by dual-electrospinning. The crosslinkable nature of the composite scaffold, together with the excellent integration of the gelatin within the PCL mesh, allowed the creation of a multilayered construct as a tendon graft through photocrosslinking of stacked scaffold sheets. Human ASCs were impregnated into the scaffold to generate a cell-laden construct and were seen to align along the orientation of fibers. Seeded cells adopted tendon cell phenotype upon treatment with TGF- β 3. These findings provide information for the development of more advanced tendon grafts that can mimic both structural and cellular characteristics of native tendon tissues.

5.0 DISCUSSION, ADDITIONAL FINDINGS, AND FUTURE PERSPECTIVE

This dissertation study revealed the tenogenic effect of tendon derived extracellular matrix (tECM) on human adipose stem cells (hASCs) cultured under various conditions. In a 3-dimensional (3D) collagen scaffold under uniaxial tension, the presence of tECM improved tenogenic differentiation while suppressed the osteogenic differentiation of hASCs induced by uniaxial tension and enhanced scaffold mechanical strength, accompanied by reduced expression and activity of matrix metalloproteinases (MMPs). Furthermore, tECM enhanced the proliferation and TGF- β -induced tenogenesis of ASCs in 2D culture, and modulated matrix deposition and organization by ASCs seeded in 3D fibrous scaffolds. We also reported the development of a novel composite fibrous scaffold as a tendon graft fabricated by co-electrospinning of poly- ϵ -caprolactone (PCL) and methacrylated gelatin (mGLT), which allowed the encapsulation of ASCs within the scaffold upon visible light induced gelatin photocrosslinking, as well as the formation of crosslinked multilayer constructs. This scaffold design can serve as an effective reservoir of tECM and tenogenic growth factors to recapitulate the structural and biochemical characteristics of native tendon tissue. In addition to all the findings presented, we propose the following future studies to address the limitations of current study and obtain more insights into the research subject.

5.1 ADIPOSE STEM CELL ISOLATION AND CHARACTERIZATION

5.1.1 hASC isolation

Adipose tissue is composed mainly of fat cells, which constitute more than 90% of the cellular component, and a stromal vascular fraction (SVF) consisting of a mixture of cell phenotypes, including ASCs [83]. A number of earlier studies have suggested correlation between the characteristics of ASCs and their site of harvest. For instance, ASCs harvested from superficial abdominal regions demonstrate greater resistance to apoptosis than those isolated from the upper arm, medial thigh and deep abdominal depots [234]. Likewise, the density of stem cells reserved within adipose tissue is also a function of anatomical location, tissue type, and animal species. For example, for white adipose tissue, ASC yields are higher in subcutaneous depots compared with visceral fat [235]. Of relevance to the subject of this dissertation research, tendon tissue engineering, the ASC population isolated from infrapatellar fat pad (IPFP) is perhaps particularly worthy of studying in the future as a potential cell source, for previous studies have shown considerable MSC activity from IPFP-derived cells [236], which have been speculated to be involved in the healing of patellar tendons [237]. However, current studies of IPFP ASCs are primarily restricted to their chondrogenic capacity, while their tenogenic potential remains largely unknown. For instance, cells derived from the IPFP were found to possess undiminished chondrogenic potential (comparable to control BMSCs) even isolated from osteoarthritic donors, or after a significant number of population doublings *in vitro* [238, 239]. Of equal importance is that the IPFP is an intracapsular, extrasynovial structure within the knee that lies directly posterior to the patellar tendon. The close proximity between resident cells of IPFP and surrounding patellar tendon tissues implies possible interaction

between IPFP ASCs and tendon ECM (Fig. 35). It is therefore reasonable to hypothesize that IPFP ASCs will respond rapidly to tECM treatment as well as other tenogenic factors by undergoing tenogenesis. This assumption is indirectly evidenced by the finding that IPFP ASCs expanded on ECM deposited by synovium-derived stem cells (SDSCs) displayed intensive chondrogenic markers, on the basis of histology [240].

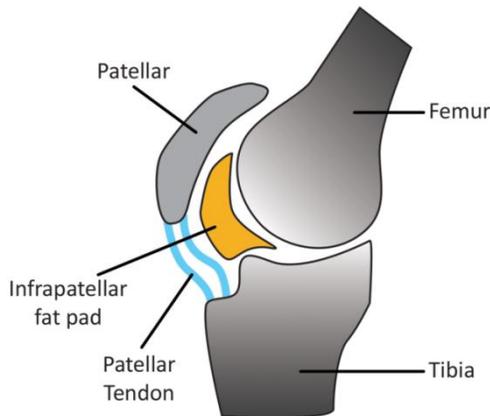


Figure 35. The location of the infrapatellar fat pad in knee joint (orange).

5.1.2 hASC characterization

The phenotypic characterization of ASCs is at an early stage. To date, a precise definition of the ASC phenotype is lacking, compromising a clear distinction between these cells and fibroblasts. Therefore, a combination of multiple cluster of differentiation (CD) markers are often targeted to distinguish ASCs (CD31⁻, CD34^{+/-}, CD45⁻, CD90⁺, CD105⁻ and CD146⁻) from other heterogeneous cell populations found in freshly isolated SVF, including endothelial cells (CD31⁺, CD34^{+/-}, CD45⁻, CD90⁺, CD105⁻, and CD146⁺), vascular smooth muscle cells (CD31⁻, CD34^{+/-}, CD45⁻, CD90⁺, CD105⁻, and CD146⁺), and hematopoietic stem cells (CD45⁺) [185]. ASCs share many cell surface markers with pericytes and bone marrow MSCs. Besides those listed above, pericyte markers are expressed by ASCs, including smooth muscle β -

actin, platelet-derived growth factor (PDGF) receptor- β , and neuro-glial proteoglycan 2, while the markers shared by ASCs and bone marrow MSCs include CD13, CD29, CD44, CD58, and CD166 [241]. In addition to similarity of CD marker profile among these cell types, culture conditions may also markedly affect ASC gene expression, such as culture medium, mechano-physiological environment, and degree of oxygenation [148]. Taken together, CD marker profiling must be coupled with other characterization approaches to accurately identify the ASC population from heterogeneous SVF preparations based on proliferation and multi-lineage differentiation potentials.

ASCs possess the potential to differentiate into a variety of cell lineages [147]. As far as the regeneration of mesodermal tissues is concerned, ASCs can undergo adipogenic, osteogenic, chondrogenic, and myogenic differentiation [149, 186, 242, 243]. Compared to BMSCs and tendon-derived stem/progenitor cells (TSPCs), ASCs displayed reduced chondrogenic potential under standard differentiation conditions driven by TGF- β , which may be improved by co-treatment with BMP-6 to restore the functions of TGF- β receptor I [244].

5.2 TENOGENIC DIFFERENTIATION OF ADIPOSE STEM CELLS

As noted in INTRODUCTION, the tendon cell lineage commitment of ASCs remains inconclusive. To date, an effective and putative protocol is lacking for tenogenesis of ASCs across species, underscoring the need to optimize the type and dose of inductive factor for tenogenesis through a case-dependent fashion.

5.2.1 Induction of ASC tenogenesis via growth factor treatment

We tested the tenogenic effect of several growth factors on hASCs, including bFGF, GDF-5 (BMP-14), GDF-7 (BMP-12), and TGF- β 3, due to their known active involvement in either tendon morphogenesis or physiological tendon healing. For instance, members of the transforming growth factor- β (TGF β) superfamily are involved in regulating tendon development, as has been found in various species. TGF β -2/3 ligand and its receptors were detected throughout the tertiary bundles in the tendon midsubstance and endotenon during the intermediate stages of tendon development in the chick embryo [12]. Growth and differentiation factors (GDF), members of the bone morphogenetic protein (BMP) family, are additional regulators of tendon development and healing. Subcutaneous implantation of GDF-5, 6, and 7 leads to the formation of neotendon-like connective tissue in rats [245]. Mice deficient in GDF-5 demonstrate inferior matrix composition and mechanical strength in their Achilles tendons [246]. From a regeneration perspective, rat tendon injury model exhibited a biphasic pattern in which TGF- β 1 expression was highest in the early phases of healing and gradually decreased thereafter, when TGF- β 3 was upregulated [51]. Similarly, tendons subjected to transection and repair exhibited an increased signal for bFGF mRNA in both resident tenocytes and in the fibroblasts and inflammatory cells located in the tendon sheath [64]. Besides, bFGF significantly accelerated wound closure of a rat patellar tendon defect *in vitro* [247].

To explore the possible dose effect of growth factors on tenogenesis of hASCs, cells were cultured with each type of growth factor at a range of serial concentrations for 7 days and then analyzed. Real-time PCR analysis suggested that GDF-5 or GDF-7 treatment was not able to significantly upregulate the expression of SCX or TNC regardless of the amount used in the test. No evident dose effect on tenogenesis of ASCs was found for these two growth factors (Fig. 36).

In contrast, bFGF and TGF- β 3 were found to be capable of inducing tenogenic differentiation to distinct extents (Fig. 36). When treated with 1 ng/ml of bFGF, the expression level of SCX and TNC was increased by 2.02- and 2.79-fold, respectively. Nevertheless, higher concentrations of bFGF did not result in any pronounced tendon lineage commitment. In comparison, tendon marker expression levels were markedly increased by TGF- β 3 and plateaued when exposed to 10 ng/ml TGF- β 3, in which SCX and TNC levels were 21.65- and 3.89 -fold higher than the untreated control. From these initial findings, TGF- β 3 at 10 ng/ml was selected as the primary tenogenic factor for this dissertation study. To further characterize the regulation of tendon lineage commitment, the combined effects and specific spatiotemporal coordination of the activities of multiple growth factors on ASC tenogenesis must be carefully studied.

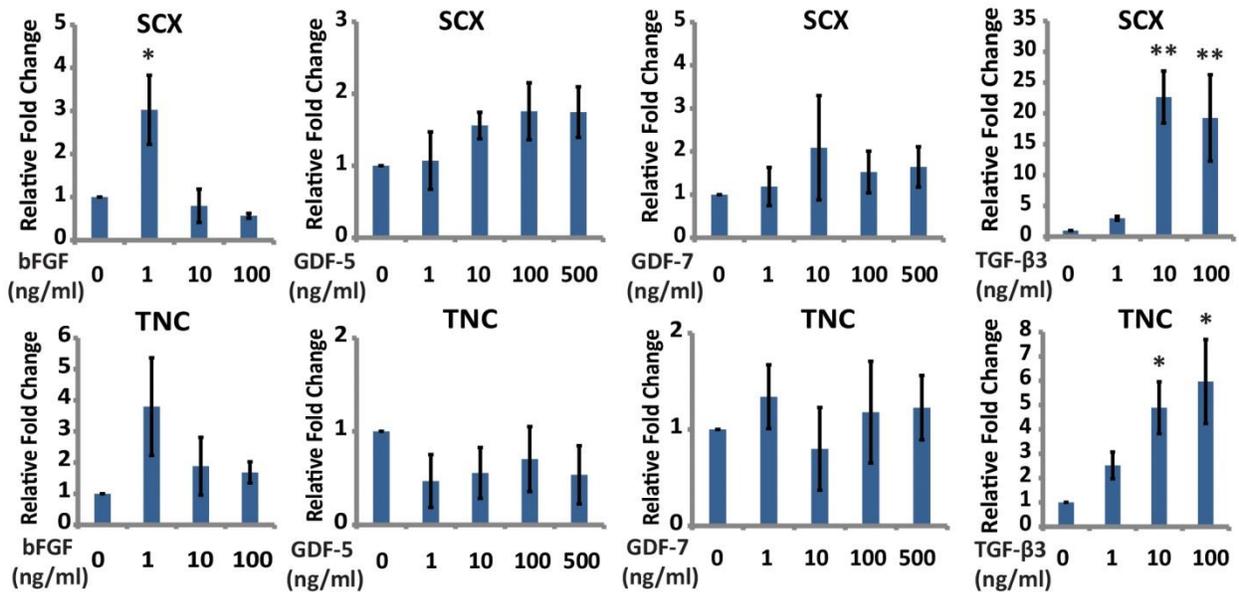


Figure 36. Dose effect of growth factors on the tenogenesis of hASCs analyzed by real time PCR analysis.

bFGF and TGF- β 3 treatment was able to significantly upregulate SCX and TNC level. *, $p < 0.05$; **, $p < 0.01$; $n =$

5.2.2 Induction of ASC tenogenesis via tensioned 3D culture

While the most common approach to direct MSCs to differentiate into a specific lineage *in vitro* is through growth factor stimulation, tenogenesis of MSCs can also be induced through uniaxial tension in 3D culture [87, 126, 142]. In this study, we used the Flexcell Strain Unit with the Tissue Train culture plates to generate 3D cylindrical constructs under tension, and confirmed that hASCs underwent tenogenesis in response to static mechanical stimuli. Collagen type I (Col I) gel was used as the scaffold material for many reasons. First, Col I is the primary structural component of tendon tissue and is thus widely used in tendon tissue engineering. Second, collagen hydrogel is contractible when interacting with cells, and can thereby create tension without the need to apply exogenous force. Furthermore, compared to synthetic polymer mesh, tensioned collagen hydrogel is soft enough to reflect cell-mediated matrix remodeling by changes in macroscopic shape and strength.

A promising future prospect based on this work is the application of dynamic/cyclic stretch to the construct besides static tension to simulate the postnatal loading of tendon during motions. In this regard, cyclic uniaxial tension has been found to promote greater tenogenic effects, and that dynamic stretching is required to maintain expression of SCX over time [126]. However, tenogenic effect of dynamic loading on encapsulated cells in the presence of tECM is yet to be explored. Likewise, dynamic loading may be combined with other known pro-tenogenesis factors, such as growth factor treatment or scaffold alignment, to recapitulate the pattern of tendon morphogenesis precisely *in vitro*.

5.3 THE BIOACTIVITY OF TENDON EXTRACELLULAR MATRIX

Extracellular matrix (ECM), a network of proteins secreted by cells that provides structural and biochemical support to the surrounding cells, also plays important roles in regulating cell behavior. Tissue-derived ECMs have been generally found to induce corresponding differentiation of MSCs toward the phenotype of cells in the tissue from which the ECM is derived, and are therefore considered as attractive candidate biomaterials to support stem cell-based tissue-specific regeneration. [38, 122, 123, 153, 154]. These findings represent the principal rationale of the core hypothesis of this dissertation study: **Tendon ECM has a positive influence on the proliferation and tenogenic differentiation of hASCs.**

To test our hypothesis, we used the chaotropic reagent, urea, to disrupt lipid-lipid, lipid-protein, and protein-protein interactions in the tendon ECM network without enzymatic digestion of ECM components [156]. By using this approach, we succeeded in obtaining a water soluble extract of tendon ECM rich in non-collagenous proteins that would have otherwise been significantly degraded if enzyme treatment was used to dissociate the tissue. We then examined the effect of biochemical components of tECM on ASCs maintained in standard 2D culture. Unlike other studies that aimed at exploring the bioactivity of ECM, the tECM in our study was used as a medium supplement rather than as a coating of the culture substrate [144, 248]; for immediate cell binding to a rigid ECM substrate coating could affect cell shape and initial cell-seeding density compared to interaction with soluble, free ECM. Our results showed that exposure to the soluble tECM significantly enhanced tenogenic activity of the ASCs, particularly in terms of the influence of mechanoactivation. Further exploration of the underlying mechanism of the bioactivity of tECM will require: (1) determination of the effective composition of tECM, and (2) understanding the interaction between tECM and cells.

5.3.1 Determination of the functional composition of tendon ECM

The quest for a comprehensive understanding of the molecular mechanisms of ECM regulation in cell activity has become a research hotspot in recent years via high throughput analytical approaches such as proteomics. Mass spectrometry (MS) based proteomic technologies, in particular, provide the opportunity for rapid and thorough analyses of ECM composition [249].

In terms of the in-depth analysis of tECM, the samples can be enzymatically digested into peptides and then analyzed by tandem MS, the core technology used in ‘bottom-up’ proteomics that results in the fragmentation of peptide ions to provide structural information about the ions and subsequently enables the determination of their amino acid sequence. With the availability of completed genome sequences, protein sequence databases can be queried with MS data using database search tools to infer the identity of peptides, and thus their corresponding proteins present in the samples [250-252]. In fact, many proteomic studies have been conducted on healthy or diseased whole tendon tissues. For instance, tandem MS-based proteomics analysis comparing healing tendons to uninjured tendons revealed significant increases in proteins associated with inflammation, stress response, and matrix degradation [253]. Likewise, significant differences were found by tandem MS between male and female in components of the tendon proteome critical for response to mechanical loading and injury [254]. These studies have suggested the feasibility of using proteomic techniques to analyze the composition of tECM.

5.3.2 Understanding the interaction between tendon ECM and cells

We found that tECM treatment on ASCs increased proliferation and TNC expression in the absence of other inductive cues. Expression of both TNC and SCX was further enhanced when treated concomitantly with TGF- β 3. The enhancement of ASC tenogenesis by tECM combined with TGF- β 3 treatment may be attributed to two possible events: (1) the tECM serves as a reservoir of signals distinct from TGF- β ; or, not mutually exclusively, (2) the tECM exerts a regulatory effect on exogenous inductive cues. We believed that the latter assumption is more dominant, as exposure to tECM substantially increased Smad phosphorylation in response to exogenous TGF- β 3 (Fig. 37). In addition, the content of TGF- β 3 carried in tECM solution is negligible compared to the amount added into the medium (160 pg/ml vs. 10 ng/ml). We propose that particular research attention should be paid to the interaction between cell surface integrins and tECM as the initial step of an outside-in signal transduction (Fig. 38). In our view, integrin mediated cross talk between TGF- β signaling pathway and the tECM is a promising approach for future studies. Signal induction initiated by ECM-integrin interactions at focal adhesion sites may affect TGF- β signaling either through enzymatic action of protein complex recruited to the cytoplasmic domain of integrins or influence interaction with the actin cytoskeleton [255]. We speculate that there is functional cross talk between integrin mediated signaling pathways and TGF- β signaling pathways.

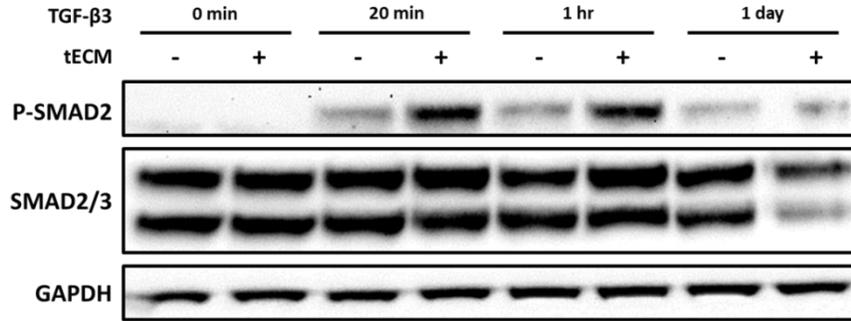


Figure 37. Western blot of phosphorylated Smad2 (P-SMAD2) and total SMAD2/3 revealed the kinetics of cell response to TGF-β3. Exposure to tECM increased SMAD2 phosphorylation. GAPDH is used as an endogenous control. n=3

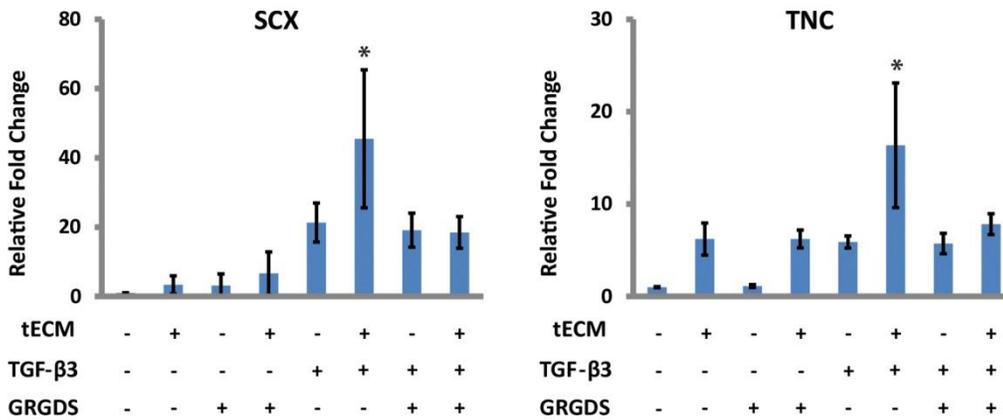


Figure 38. Real time PCR analysis revealed that blockade of tECM-integrin interaction with integrin antagonist (GRGDS) led to a drop in the expression level of tendon markers to the similar level as those in TGF-β3+/tECM- group. *, p<0.05, compared with all other groups; n=6

5.4 SCAFFOLD DEVELOPMENT FOR TENDON TISSUE ENGINEERING

Properly differentiated cell population alone is not sufficient to address clinical challenges in tissue engineering based tendon repair. First of all, therapeutic cells need to be immobilized to the site of injury to function in a site-appropriate manner. Secondly, specific topographical characteristics along with other physical cues must be presented to the

microenvironment in order to induce and maintain the desired phenotype of the seeded cells. Lastly, seeded cells are not able to restore the mechanical strength of injured tendon tissue until adequate amounts of tissue specific ECM have been produced and deposited in a structurally relevant manner, and are therefore unable to prevent secondary rupture or reduction in tissue integrity during early rehabilitation stages. These concerns motivate in part the desire to develop bioactive/biocompatible scaffolds capable of bridging the gap between bench side study and clinical repair.

In this dissertation research, two basic types of scaffold were studied: (1) hydrogel made of auto-assembled collagen macromolecules; and (2) electrospun polyester microfibrinous mesh. The use of these scaffold culture models not only represented a platform for studying the bioactivity of tECM within tendon-mimicking physical microenvironment, but also allowed us to develop a novel, advanced tendon-like construct consisting of hydrogel and scaffold mesh composite. We propose here the following respective adjustments and improvements to address the limitations of the scaffold formats used and developed in this study.

5.4.1 Possible improvements of hydrogel scaffold

Although the study on collagenous hydrogel-based culture is fruitful, the current collagen hydrogel system is insufficient in a number of aspects. Firstly, we are concerned that a collagen-rich microenvironment, possibly via biological feedback regulation, may influence the natural, early sequence of mesenchymal tenogenesis, since the natural process would necessarily arise prior to matrix saturation with collagen. Secondly, identification and detection of the deposition and organization of newly produced collagen fibrils is complicated by the abundant collagenous matrix. In this regard, constructs made of hyaluronic acid (HA) or fibrin are possibly better

alternative platforms if the aim is to track collagen fibril formation. Secondly, as noted in INTRODUCTION, the proper formation of covalent cross-links between adjacent collagen molecules deserves research attention in order to enhance collagen interconnectivity and subsequently reinforce the constructs if intended to use as a clinical graft. Characterization of crosslinking, e.g., activity of lysyl oxidase and quantitation of pyridinoline, would be helpful for this assessment.

5.4.2 Possible improvements of fibrous scaffold

Unlike hydrogels, the fibrous PCL scaffold is engineered to provide the necessary topographical characteristics and mechanical strength. However, we chose to use tECM as a medium supplement for cells seeded on PCL scaffold rather than as a coating material to pre-treat the scaffold surface, for a rapid loss of protein coating was observed during incubation despite significant initial adsorption. Moreover, unlike collagen, PCL lacks the motifs and residues that mediate cell interaction, leading to unsatisfactory cell attachment rate and viability. Although medium supplementation of tECM is sufficient to rescue the absence of bioactivity of scaffolds in an *ex vivo* culture system, functional *in vivo* implant requires a scaffold that retains bioactive agents without large-scale diffusion. For this purpose, scaffold surface modification seems a promising approach to address the challenge. For instance, amino groups can be covalently introduced onto PCL scaffold surface by reacting 1,6-hexanediamine with the ester groups of PCL, known as aminolysis [256]. The added amino groups may be targeted to immobilize bioactive macromolecules contained in tECM on the aminolyzed PCL scaffold using bifunctional cross-linking agents [257]. In addition, the introduction of reactive amino groups enables covalent crosslinking between adjacent fibers, and therefore reinforces the mechanical

strength of scaffolds. Another prospective approach is to blend PCL polymer with biologically active materials prior to electrospinning [258-260]. However, denaturation of bioactive molecules in organic solvent is a serious concern [261]. Moreover, at present, the functional bioactive molecules that drive tendon regeneration remain unclear.

5.4.3 Possible improvements for composite scaffold

The fact that the cell-containing hydrogel reinforced by structural fibers exhibits an organization similar to that of native ECM motivated us to develop a composite construct consisting of both fibrous scaffold mesh and ECM hydrogel. When wetted and exposed to visible light (VL) irradiation, the free radicals generated from the photo-initiator crosslink methacrylate groups presented by gelatin molecules, thereby forming hydrogel within the network of PCL fibers to retain water and ECM proteins.

Notably, although mGLT fibers rapidly dissolved upon wetting and therefore possibly increased the pore size of the scaffold, we did not observe any improved cell infiltration into the interior region of those crosslinked scaffolds [226]. The gelatin hydrogel formed within the PCL fiber mesh may occupy the pores and impede cell migration. In the future, to address this potential issue, a molecular linker containing an MMP-degradable peptide sequence might be used to generate a more cell-degradable hydrogel within a fibrous scaffold to improve cell infiltration [227, 228]. In fact, depending on the specific requirements, appropriate peptide sequences that correspond to specific MMPs may be adopted to generate specific bio-responsiveness to the microproteolytic environment. It should be point out that the construct we have prepared so far was made of no more than five layers of scaffold sheet to ensure the orthogonal penetration of light into the construct for sufficient crosslinking. Higher degree of

stacking may result in inadequate crosslinking in the center zone of the construct due to the accumulated opacity. To address such restriction on construct thickness, thermo-responsive or chemically initiated crosslinkers may be considered in order to overcome the limitation of light penetration. For future studies that aim at eliciting the tenogenesis of impregnated cells, the composite scaffold is open to further modifications. For instance, heparin can be conjugated to the gelatin backbone in order to sequester and subsequently deliver exogenous tenogenic growth factors into the constructs [233]. In a similar manner, the tECM can be incorporated into the hydrogel portion of the constructs during crosslinking to “reconstitute” the protein profile of native tendon tissue.

5.5 CONCLUSIONS

In conclusion, this dissertation research has demonstrated the bioactivity of a soluble fraction of tendon-derived extracellular matrix (tECM) on the proliferation and tenogenic differentiation of human adipose stem cell (hASC) cultured in 2D with TGF- β 3 or within 3D scaffolds. Moreover, hASCs in tECM-containing 3D cultures demonstrated suppressed osteogenesis under tension and improved matrix deposition and organization on aligned fibrous scaffold. In addition, we developed a novel multilayered construct as a tendon graft through photo-crosslinking of stacked composite scaffold sheets, and demonstrated tendon cell phenotype of impregnated hASCs.

We believe that these findings will have considerable utility in the field of tendon tissue engineering. As mesenchymal stem cells (MSCs) continue to be a research focus in tissue engineering, incorporating tendon specific ECM molecules into differentiation protocols

represents an alternative approach to achieve robust tenogenesis of ASCs to better exploit their regenerative potential. Furthermore, the design attributes of the 3D scaffolds developed in this study are applicable not only to functional tendon tissue engineering, but also to reproducing structural and cellular characteristics of other native tissues. The scaffold design is open to further modifications to elicit the tissue-specific differentiation of impregnated cells. We believe that the successful, translational application of our findings is likely to improve patient outcomes in the context of clinical tendon repair.

APPENDIX A

LIST OF ABBREVIATIONS

Abbreviation	Full term
ASC	adipose derived mesenchymal stem cell/adipose stem cell
bFGF	basic fibroblast growth factor
BGC	biglycan
BM	basal medium
BMP	bone morphogenetic protein
BMSC	bone marrow derived mesenchymal stem cell
BSA	bovine serum albumin
CFU-F	colony forming unit-fibroblast
COL I	collagen type I gene
Col I	collagen type I protein
COL I A1	collagen type I $\alpha 1$ chain-encoding gene
COMP	collagen oligomeric matrix protein
CTGF	connective tissue growth factor
DCR	decorin
DM	differentiation medium
DMF	dimethylformamide
ECM	extracellular matrix
FBS	fetal bovine serum
FGF	fibroblast growth factor
FMOD	fibromodulin
FNC	fibronectin
GAG	glycosaminoglycan
GDF	growth differentiation factor
GM	growth medium
hASC	human adipose stem cell
HBSS	Hanks' balanced salt solution
HRP	horseradish peroxidase

Abbreviation	Full term
IGF	insulin-like growth factor
IPFP	infrapatellar fat pad
ITS	insulin-transferrin-selenium
LAP	phenyl-2,4,6-trimethylbenzoylphosphinate
LMC	lumican
mGLT	methacrylated gelatin
MKX	mohawk homeobox
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell
P/S	penicillin/streptomycin
PBS	phosphate buffered saline
PCL	poly- ϵ -caprolactone
PDGF	platelet-derived growth factor
PGA	polyglycolic acid
PLLA	poly-L-lactic acid
SCX	scleraxis gene
Scx	scleraxis protein
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SHH	sonic hedgehog
SIS	small intestinal submucosa
SLRP	small leucine-rich proteoglycan
SVF	stromal vascular fraction
TC	tenocyte
tECM	soluble fraction of tendon ECM
TFE	2,2,2-trifluoroethanol
TGF- β	transforming growth factor- β
THF	tetrahydrofuran
TNC	tenascin-C
TNMD	tenomodulin gene
Tnmd	tenomodulin protein
TSPC	tendon stem/progenitor cell
UBM	urinary bladder matrix
VEGF	vascular endothelial growth factor
VL	visible light

BIBLIOGRAPHY

- [1] Benjamin M, Ralphs JR. The cell and developmental biology of tendons and ligaments. *International review of cytology* 2000;196:85-130.
- [2] Xu PX, Cheng J, Epstein JA, Maas RL. Mouse Eya genes are expressed during limb tendon development and encode a transcriptional activation function. *Proceedings of the National Academy of Sciences of the United States of America* 1997;94:11974-9.
- [3] Kardon G. Muscle and tendon morphogenesis in the avian hind limb. *Development* 1998;125:4019-32.
- [4] Oliver G, Wehr R, Jenkins NA, Copeland NG, Cheyette BN, Hartenstein V, et al. Homeobox genes and connective tissue patterning. *Development* 1995;121:693-705.
- [5] D'Souza D, Patel K. Involvement of long- and short-range signalling during early tendon development. *Anatomy and embryology* 1999;200:367-75.
- [6] Cserjesi P, Brown D, Ligon KL, Lyons GE, Copeland NG, Gilbert DJ, et al. Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. *Development* 1995;121:1099-110.
- [7] Schweitzer R, Chyung JH, Murtaugh LC, Brent AE, Rosen V, Olson EN, et al. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* 2001;128:3855-66.
- [8] Huang R, Zhi Q, Brand-Saberi B, Christ B. New experimental evidence for somite resegmentation. *Anatomy and embryology* 2000;202:195-200.
- [9] Brent AE, Schweitzer R, Tabin CJ. A somitic compartment of tendon progenitors. *Cell* 2003;113:235-48.
- [10] Murchison ND, Price BA, Conner DA, Keene DR, Olson EN, Tabin CJ, et al. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development* 2007;134:2697-708.
- [11] Brent AE, Braun T, Tabin CJ. Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development. *Development* 2005;132:515-28.

- [12] Kuo CK, Petersen BC, Tuan RS. Spatiotemporal protein distribution of TGF-betas, their receptors, and extracellular matrix molecules during embryonic tendon development. *Developmental dynamics : an official publication of the American Association of Anatomists* 2008;237:1477-89.
- [13] Liu CF, Aschbacher-Smith L, Barthelery NJ, Dymant N, Butler D, Wylie C. Spatial and temporal expression of molecular markers and cell signals during normal development of the mouse patellar tendon. *Tissue Eng Part A* 2012;18:598-608.
- [14] Pryce BA, Watson SS, Murchison ND, Staverosky JA, Dunker N, Schweitzer R. Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. *Development* 2009;136:1351-61.
- [15] Lorda-Diez CI, Montero JA, Martinez-Cue C, Garcia-Porrero JA, Hurlé JM. Transforming growth factors beta coordinate cartilage and tendon differentiation in the developing limb mesenchyme. *The Journal of biological chemistry* 2009;284:29988-96.
- [16] Shukunami C, Takimoto A, Oro M, Hiraki Y. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. *Developmental biology* 2006;298:234-47.
- [17] Liu H, Zhang C, Zhu S, Lu P, Zhu T, Gong X, et al. Mohawk promotes the tenogenesis of mesenchymal stem cells through activation of the TGFbeta signaling pathway. *Stem Cells* 2015;33:443-55.
- [18] Liu W, Watson SS, Lan Y, Keene DR, Ovitt CE, Liu H, et al. The atypical homeodomain transcription factor Mohawk controls tendon morphogenesis. *Molecular and cellular biology* 2010;30:4797-807.
- [19] Lejard V, Brideau G, Blais F, Salingcarnboriboon R, Wagner G, Roehrl MH, et al. Scleraxis and NFATc regulate the expression of the pro-alpha1(I) collagen gene in tendon fibroblasts. *The Journal of biological chemistry* 2007;282:17665-75.
- [20] Docheva D, Hunziker EB, Fassler R, Brandau O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. *Molecular and cellular biology* 2005;25:699-705.
- [21] Yang G, Rothrauff BB, Tuan RS. Tendon and ligament regeneration and repair: clinical relevance and developmental paradigm. *Birth defects research Part C, Embryo today : reviews* 2013;99:203-22.
- [22] Zhang G, Young BB, Ezura Y, Favata M, Soslowsky LJ, Chakravarti S, et al. Development of tendon structure and function: regulation of collagen fibrillogenesis. *Journal of musculoskeletal & neuronal interactions* 2005;5:5-21.
- [23] O'Brien M. Structure and metabolism of tendons. *Scandinavian journal of medicine & science in sports* 1997;7:55-61.

- [24] Kadler KE, Holmes DF, Trotter JA, Chapman JA. Collagen fibril formation. *The Biochemical journal* 1996;316 (Pt 1):1-11.
- [25] Chansky HA, Iannotti JP. The vascularity of the rotator cuff. *Clinics in sports medicine* 1991;10:807-22.
- [26] Kastelic J, Galeski A, Baer E. The multicomposite structure of tendon. *Connective tissue research* 1978;6:11-23.
- [27] Wenstrup RJ, Florer JB, Brunskill EW, Bell SM, Chervoneva I, Birk DE. Type V collagen controls the initiation of collagen fibril assembly. *The Journal of biological chemistry* 2004;279:53331-7.
- [28] Ansorge HL, Meng X, Zhang G, Veit G, Sun M, Klement JF, et al. Type XIV Collagen Regulates Fibrillogenesis: PREMATURE COLLAGEN FIBRIL GROWTH AND TISSUE DYSFUNCTION IN NULL MICE. *The Journal of biological chemistry* 2009;284:8427-38.
- [29] Thorpe CT, Birch HL, Clegg PD, Screen HR. The role of the non-collagenous matrix in tendon function. *International journal of experimental pathology* 2013;94:248-59.
- [30] Fessel G, Gerber C, Snedeker JG. Potential of collagen cross-linking therapies to mediate tendon mechanical properties. *Journal of shoulder and elbow surgery / American Shoulder and Elbow Surgeons [et al]* 2012;21:209-17.
- [31] Bailey AJ. Molecular mechanisms of ageing in connective tissues. *Mechanisms of ageing and development* 2001;122:735-55.
- [32] Eyre DR, Weis MA, Wu JJ. Advances in collagen cross-link analysis. *Methods* 2008;45:65-74.
- [33] Kalamajski S, Liu C, Tillgren V, Rubin K, Oldberg A, Rai J, et al. Increased C-telopeptide cross-linking of tendon type I collagen in fibromodulin-deficient mice. *The Journal of biological chemistry* 2014;289:18873-9.
- [34] Connizzo BK, Yannascoli SM, Soslowsky LJ. Structure-function relationships of postnatal tendon development: a parallel to healing. *Matrix biology : journal of the International Society for Matrix Biology* 2013;32:106-16.
- [35] Vesentini S, Redaelli A, Montevicchi FM. Estimation of the binding force of the collagen molecule-decorin core protein complex in collagen fibril. *Journal of biomechanics* 2005;38:433-43.
- [36] Zhang G, Ezura Y, Chervoneva I, Robinson PS, Beason DP, Carine ET, et al. Decorin regulates assembly of collagen fibrils and acquisition of biomechanical properties during tendon development. *J Cell Biochem* 2006;98:1436-49.

- [37] Kilts T, Ameye L, Syed-Picard F, Ono M, Berendsen AD, Oldberg A, et al. Potential roles for the small leucine-rich proteoglycans biglycan and fibromodulin in ectopic ossification of tendon induced by exercise and in modulating rotarod performance. *Scandinavian journal of medicine & science in sports* 2009;19:536-46.
- [38] Bi Y, Ehrchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* 2007;13:1219-27.
- [39] Chakravarti S. Functions of lumican and fibromodulin: lessons from knockout mice. *Glycoconjugate journal* 2002;19:287-93.
- [40] Halasz K, Kassner A, Morgelin M, Heinegard D. COMP acts as a catalyst in collagen fibrillogenesis. *J Biol Chem* 2007;282:31166-73.
- [41] Pirog KA, Jaka O, Katakura Y, Meadows RS, Kadler KE, Boot-Handford RP, et al. A mouse model offers novel insights into the myopathy and tendinopathy often associated with pseudoachondroplasia and multiple epiphyseal dysplasia. *Human molecular genetics* 2010;19:52-64.
- [42] Chiquet M, Renedo AS, Huber F, Fluck M. How do fibroblasts translate mechanical signals into changes in extracellular matrix production? *Matrix biology : journal of the International Society for Matrix Biology* 2003;22:73-80.
- [43] Chiquet-Ehrismann R, Tucker RP. Connective tissues: signalling by tenascins. *The international journal of biochemistry & cell biology* 2004;36:1085-9.
- [44] Smith KD, Vaughan-Thomas A, Spiller DG, Innes JF, Clegg PD, Comerford EJ. The organisation of elastin and fibrillins 1 and 2 in the cruciate ligament complex. *Journal of anatomy* 2011;218:600-7.
- [45] Korol RM, Finlay HM, Josseau MJ, Lucas AR, Canham PB. Fluorescence spectroscopy and birefringence of molecular changes in maturing rat tail tendon. *Journal of biomedical optics* 2007;12:024011.
- [46] Riley G. Tendinopathy--from basic science to treatment. *Nature clinical practice Rheumatology* 2008;4:82-9.
- [47] Hope M, Saxby TS. Tendon healing. *Foot and ankle clinics* 2007;12:553-67, v.
- [48] Voleti PB, Buckley MR, Soslowsky LJ. Tendon healing: repair and regeneration. *Annual review of biomedical engineering* 2012;14:47-71.
- [49] Garner WL, McDonald JA, Koo M, Kuhn C, 3rd, Weeks PM. Identification of the collagen-producing cells in healing flexor tendons. *Plastic and reconstructive surgery* 1989;83:875-9.

- [50] James R, Kesturu G, Balian G, Chhabra AB. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. *The Journal of hand surgery* 2008;33:102-12.
- [51] Juneja SC, Schwarz EM, O'Keefe RJ, Awad HA. Cellular and molecular factors in flexor tendon repair and adhesions: a histological and gene expression analysis. *Connective tissue research* 2013;54:218-26.
- [52] Abrahamsson SO. Similar effects of recombinant human insulin-like growth factor-I and II on cellular activities in flexor tendons of young rabbits: experimental studies in vitro. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 1997;15:256-62.
- [53] Sharma P, Maffulli N. Biology of tendon injury: healing, modeling and remodeling. *Journal of musculoskeletal & neuronal interactions* 2006;6:181-90.
- [54] Miyashita H, Ochi M, Ikuta Y. Histological and biomechanical observations of the rabbit patellar tendon after removal of its central one-third. *Archives of orthopaedic and trauma surgery* 1997;116:454-62.
- [55] Garofalo R, Cesari E, Vinci E, Castagna A. Role of metalloproteinases in rotator cuff tear. *Sports medicine and arthroscopy review* 2011;19:207-12.
- [56] Oshiro W, Lou J, Xing X, Tu Y, Manske PR. Flexor tendon healing in the rat: a histologic and gene expression study. *The Journal of hand surgery* 2003;28:814-23.
- [57] Ackermann PW, Ahmed M, Kreicbergs A. Early nerve regeneration after achilles tendon rupture--a prerequisite for healing? A study in the rat. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2002;20:849-56.
- [58] Murrell GA, Szabo C, Hannafin JA, Jang D, Dolan MM, Deng XH, et al. Modulation of tendon healing by nitric oxide. *Inflammation research : official journal of the European Histamine Research Society [et al]* 1997;46:19-27.
- [59] Molloy T, Wang Y, Murrell G. The roles of growth factors in tendon and ligament healing. *Sports Med* 2003;33:381-94.
- [60] Branford OA, Klass BR, Grobbelaar AO, Rolfe KJ. The growth factors involved in flexor tendon repair and adhesion formation. *J Hand Surg-Eur Vol* 2014;39:60-70.
- [61] Sciore P, Boykiw R, Hart DA. Semiquantitative reverse transcription-polymerase chain reaction analysis of mRNA for growth factors and growth factor receptors from normal and healing rabbit medial collateral ligament tissue. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 1998;16:429-37.
- [62] Dahlgren LA, Mohammed HO, Nixon AJ. Temporal expression of growth factors and matrix molecules in healing tendon lesions. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2005;23:84-92.

- [63] Chang J, Most D, Stelnicki E, Siebert JW, Longaker MT, Hui K, et al. Gene expression of transforming growth factor beta-1 in rabbit zone II flexor tendon wound healing: evidence for dual mechanisms of repair. *Plastic and reconstructive surgery* 1997;100:937-44.
- [64] Chang J, Most D, Thunder R, Mehrara B, Longaker MT, Lineaweaver WC. Molecular studies in flexor tendon wound healing: the role of basic fibroblast growth factor gene expression. *The Journal of hand surgery* 1998;23:1052-8.
- [65] Duffy FJ, Jr., Seiler JG, Gelberman RH, Hergrueter CA. Growth factors and canine flexor tendon healing: initial studies in uninjured and repair models. *The Journal of hand surgery* 1995;20:645-9.
- [66] Bidder M, Towler DA, Gelberman RH, Boyer MI. Expression of mRNA for vascular endothelial growth factor at the repair site of healing canine flexor tendon. *J Orthopaed Res* 2000;18:247-52.
- [67] Wurgler-Hauri CC, Dourte LM, Baradet TC, Williams GR, Soslowsky LJ. Temporal expression of 8 growth factors in tendon-to-bone healing in a rat supraspinatus model. *J Shoulder Elb Surg* 2007;16:198s-203s.
- [68] Chen CH, Cao Y, Wu YF, Bais AJ, Gao JS, Tang JB. Tendon Healing In Vivo: Gene Expression and Production of Multiple Growth Factors in Early Tendon Healing Period. *J Hand Surg-Am* 2008;33A:1834-42.
- [69] Favata M, Beredjikian PK, Zgonis MH, Beason DP, Crombleholme TM, Jawad AF, et al. Regenerative properties of fetal sheep tendon are not adversely affected by transplantation into an adult environment. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2006;24:2124-32.
- [70] Bayer ML, Yeung CY, Kadler KE, Qvortrup K, Baar K, Svensson RB, et al. The initiation of embryonic-like collagen fibrillogenesis by adult human tendon fibroblasts when cultured under tension. *Biomaterials* 2010;31:4889-97.
- [71] Butler DL, Juncosa N, Dressler MR. Functional efficacy of tendon repair processes. *Annual review of biomedical engineering* 2004;6:303-29.
- [72] Bach BR, Jr., Tradonsky S, Bojchuk J, Levy ME, Bush-Joseph CA, Khan NH. Arthroscopically assisted anterior cruciate ligament reconstruction using patellar tendon autograft. Five- to nine-year follow-up evaluation. *The American journal of sports medicine* 1998;26:20-9.
- [73] Duquin TR, Buyea C, Bisson LJ. Which method of rotator cuff repair leads to the highest rate of structural healing? A systematic review. *Am J Sports Med* 2010;38:835-41.
- [74] Liu CF, Aschbacher-Smith L, Barthelery NJ, Dymont N, Butler D, Wylie C. What we should know before using tissue engineering techniques to repair injured tendons: a developmental biology perspective. *Tissue engineering Part B, Reviews* 2011;17:165-76.

- [75] Qiu Y, Wang X, Zhang Y, Rout R, Carr AJ, Zhu L, et al. Development of a refined tenocyte differentiation culture technique for tendon tissue engineering. *Cells, tissues, organs* 2013;197:27-36.
- [76] Cao Y, Liu Y, Liu W, Shan Q, Buonocore SD, Cui L. Bridging tendon defects using autologous tenocyte engineered tendon in a hen model. *Plastic and reconstructive surgery* 2002;110:1280-9.
- [77] Lui PP, Chan KM. Tendon-derived stem cells (TDSCs): from basic science to potential roles in tendon pathology and tissue engineering applications. *Stem cell reviews* 2011;7:883-97.
- [78] Tucker BA, Karamsadkar SS, Khan WS, Pastides P. The role of bone marrow derived mesenchymal stem cells in sports injuries. *Journal of stem cells* 2010;5:155-66.
- [79] Van Eijk F, Saris DB, Riesle J, Willems WJ, Van Blitterswijk CA, Verbout AJ, et al. Tissue engineering of ligaments: a comparison of bone marrow stromal cells, anterior cruciate ligament, and skin fibroblasts as cell source. *Tissue engineering* 2004;10:893-903.
- [80] Liu H, Fan H, Toh SL, Goh JC. A comparison of rabbit mesenchymal stem cells and anterior cruciate ligament fibroblasts responses on combined silk scaffolds. *Biomaterials* 2008;29:1443-53.
- [81] Yokoya S, Mochizuki Y, Natsu K, Omae H, Nagata Y, Ochi M. Rotator cuff regeneration using a bioabsorbable material with bone marrow-derived mesenchymal stem cells in a rabbit model. *The American journal of sports medicine* 2012;40:1259-68.
- [82] Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circulation research* 2007;100:1249-60.
- [83] Boquest AC, Shahdadfar A, Brinchmann JE, Collas P. Isolation of stromal stem cells from human adipose tissue. *Methods Mol Biol* 2006;325:35-46.
- [84] Strem BM, Hicok KC, Zhu M, Wulur I, Alfonso Z, Schreiber RE, et al. Multipotential differentiation of adipose tissue-derived stem cells. *The Keio journal of medicine* 2005;54:132-41.
- [85] Shen H, Gelberman RH, Silva MJ, Sakiyama-Elbert SE, Thomopoulos S. BMP12 induces tenogenic differentiation of adipose-derived stromal cells. *PloS one* 2013;8:e77613.
- [86] Park A, Hogan MV, Kesturu GS, James R, Balian G, Chhabra AB. Adipose-derived mesenchymal stem cells treated with growth differentiation factor-5 express tendon-specific markers. *Tissue Eng Part A* 2010;16:2941-51.
- [87] Raabe O, Shell K, Fietz D, Freitag C, Ohrndorf A, Christ HJ, et al. Tenogenic differentiation of equine adipose-tissue-derived stem cells under the influence of tensile strain, growth differentiation factors and various oxygen tensions. *Cell and tissue research* 2013;352:509-21.

- [88] Goncalves AI, Rodrigues MT, Lee SJ, Atala A, Yoo JJ, Reis RL, et al. Understanding the role of growth factors in modulating stem cell tenogenesis. *PloS one* 2013;8:e83734.
- [89] Eagan MJ, Zuk PA, Zhao KW, Bluth BE, Brinkmann EJ, Wu BM, et al. The suitability of human adipose-derived stem cells for the engineering of ligament tissue. *Journal of tissue engineering and regenerative medicine* 2012;6:702-9.
- [90] Stanco D, Vigano M, Perucca Orfei C, Di Giancamillo A, Peretti GM, Lanfranchi L, et al. Multidifferentiation potential of human mesenchymal stem cells from adipose tissue and hamstring tendons for musculoskeletal cell-based therapy. *Regenerative medicine* 2015:1-15.
- [91] Liu Y, Ramanath HS, Wang DA. Tendon tissue engineering using scaffold enhancing strategies. *Trends in biotechnology* 2008;26:201-9.
- [92] Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: Structure and function. *Acta biomaterialia* 2009;5:1-13.
- [93] Erdag G, Morgan JR. Allogeneic versus xenogeneic immune reaction to bioengineered skin grafts. *Cell transplantation* 2004;13:701-12.
- [94] Brown BN, Badylak SF. Extracellular matrix as an inductive scaffold for functional tissue reconstruction. *Transl Res* 2014;163:268-85.
- [95] Pridgen BC, Woon CY, Kim M, Thorfinn J, Lindsey D, Pham H, et al. Flexor tendon tissue engineering: acellularization of human flexor tendons with preservation of biomechanical properties and biocompatibility. *Tissue engineering Part C, Methods* 2011;17:819-28.
- [96] Cartmell JS, Dunn MG. Development of cell-seeded patellar tendon allografts for anterior cruciate ligament reconstruction. *Tissue engineering* 2004;10:1065-75.
- [97] Tischer T, Vogt S, Aryee S, Steinhauser E, Adamczyk C, Milz S, et al. Tissue engineering of the anterior cruciate ligament: a new method using acellularized tendon allografts and autologous fibroblasts. *Archives of orthopaedic and trauma surgery* 2007;127:735-41.
- [98] Awad HA, Boivin GP, Dressler MR, Smith FN, Young RG, Butler DL. Repair of patellar tendon injuries using a cell-collagen composite. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2003;21:420-31.
- [99] Juncosa-Melvin N, Boivin GP, Gooch C, Galloway MT, West JR, Dunn MG, et al. The effect of autologous mesenchymal stem cells on the biomechanics and histology of gel-collagen sponge constructs used for rabbit patellar tendon repair. *Tissue engineering* 2006;12:369-79.
- [100] An K, Liu H, Guo S, Kumar DN, Wang Q. Preparation of fish gelatin and fish gelatin/poly(L-lactide) nanofibers by electrospinning. *International journal of biological macromolecules* 2010;47:380-8.

- [101] Panzavolta S, Gioffre M, Focarete ML, Gualandi C, Foroni L, Bigi A. Electrospun gelatin nanofibers: optimization of genipin cross-linking to preserve fiber morphology after exposure to water. *Acta biomaterialia* 2011;7:1702-9.
- [102] Lin H, Cheng AWM, Alexander PG, Beck AM, Tuan RS. Cartilage Tissue Engineering Application of Injectable Gelatin Hydrogel with In Situ Visible-Light-Activated Gelation Capability in Both Air and Aqueous Solution. *Tissue Eng Pt A* 2014;20:2402-11.
- [103] Ouyang HW, Goh JC, Mo XM, Teoh SH, Lee EH. The efficacy of bone marrow stromal cell-seeded knitted PLGA fiber scaffold for Achilles tendon repair. *Annals of the New York Academy of Sciences* 2002;961:126-9.
- [104] Yin Z, Chen X, Chen JL, Shen WL, Hieu Nguyen TM, Gao L, et al. The regulation of tendon stem cell differentiation by the alignment of nanofibers. *Biomaterials* 2010;31:2163-75.
- [105] Zhu J, Li J, Wang B, Zhang WJ, Zhou G, Cao Y, et al. The regulation of phenotype of cultured tenocytes by microgrooved surface structure. *Biomaterials* 2010;31:6952-8.
- [106] Kishore V, Bullock W, Sun X, Van Dyke WS, Akkus O. Tenogenic differentiation of human MSCs induced by the topography of electrochemically aligned collagen threads. *Biomaterials* 2012;33:2137-44.
- [107] Moffat KL, Kwei AS, Spalazzi JP, Doty SB, Levine WN, Lu HH. Novel nanofiber-based scaffold for rotator cuff repair and augmentation. *Tissue engineering Part A* 2009;15:115-26.
- [108] Kurtz CA, Loebig TG, Anderson DD, DeMeo PJ, Campbell PG. Insulin-like growth factor I accelerates functional recovery from Achilles tendon injury in a rat model. *The American journal of sports medicine* 1999;27:363-9.
- [109] Hansen M, Boesen A, Holm L, Flyvbjerg A, Langberg H, Kjaer M. Local administration of insulin-like growth factor-I (IGF-I) stimulates tendon collagen synthesis in humans. *Scandinavian journal of medicine & science in sports* 2013;23:614-9.
- [110] Klein MB, Yalamanchi N, Pham H, Longaker MT, Chang J. Flexor tendon healing in vitro: effects of TGF-beta on tendon cell collagen production. *The Journal of hand surgery* 2002;27:615-20.
- [111] Forslund C, Aspenberg P. Tendon healing stimulated by injected CDMP-2. *Medicine and science in sports and exercise* 2001;33:685-7.
- [112] James R, Kumbar SG, Laurencin CT, Balian G, Chhabra AB. Tendon tissue engineering: adipose-derived stem cell and GDF-5 mediated regeneration using electrospun matrix systems. *Biomed Mater* 2011;6:025011.

- [113] Chan BP, Fu S, Qin L, Lee K, Rolf CG, Chan K. Effects of basic fibroblast growth factor (bFGF) on early stages of tendon healing: a rat patellar tendon model. *Acta orthopaedica Scandinavica* 2000;71:513-8.
- [114] Durgam SS, Stewart AA, Pondenis HC, Yates AC, Evans RB, Stewart MC. Responses of equine tendon- and bone marrow-derived cells to monolayer expansion with fibroblast growth factor-2 and sequential culture with pulverized tendon and insulin-like growth factor-I. *American journal of veterinary research* 2012;73:162-70.
- [115] Sahoo S, Ang LT, Cho-Hong Goh J, Toh SL. Bioactive nanofibers for fibroblastic differentiation of mesenchymal precursor cells for ligament/tendon tissue engineering applications. *Differentiation; research in biological diversity* 2010;79:102-10.
- [116] Yoshikawa Y, Abrahamsson SO. Dose-related cellular effects of platelet-derived growth factor-BB differ in various types of rabbit tendons in vitro. *Acta orthopaedica Scandinavica* 2001;72:287-92.
- [117] Hildebrand KA, Woo SL, Smith DW, Allen CR, Deie M, Taylor BJ, et al. The effects of platelet-derived growth factor-BB on healing of the rabbit medial collateral ligament. An in vivo study. *The American journal of sports medicine* 1998;26:549-54.
- [118] Wolf MT, Daly KA, Reing JE, Badylak SF. Biologic scaffold composed of skeletal muscle extracellular matrix. *Biomaterials* 2012;33:2916-25.
- [119] Sicari BM, Dziki JL, Siu BF, Medberry CJ, Dearth CL, Badylak SF. The promotion of a constructive macrophage phenotype by solubilized extracellular matrix. *Biomaterials* 2014;35:8605-12.
- [120] Medberry CJ, Crapo PM, Siu BF, Carruthers CA, Wolf MT, Nagarkar SP, et al. Hydrogels derived from central nervous system extracellular matrix. *Biomaterials* 2013;34:1033-40.
- [121] Keane TJ, DeWard A, Londono R, Saldin LT, Castleton AA, Carey L, et al. Tissue-Specific Effects of Esophageal Extracellular Matrix. *Tissue Eng Pt A* 2015;21:2293-300.
- [122] Little D, Guilak F, Ruch DS. Ligament-derived matrix stimulates a ligamentous phenotype in human adipose-derived stem cells. *Tissue engineering Part A* 2010;16:2307-19.
- [123] Zhang J, Li B, Wang JH. The role of engineered tendon matrix in the stemness of tendon stem cells in vitro and the promotion of tendon-like tissue formation in vivo. *Biomaterials* 2011;32:6972-81.
- [124] Killian ML, Cavinatto L, Galatz LM, Thomopoulos S. The role of mechanobiology in tendon healing. *Journal of shoulder and elbow surgery / American Shoulder and Elbow Surgeons [et al]* 2012;21:228-37.
- [125] Garvin J, Qi J, Maloney M, Banes AJ. Novel system for engineering bioartificial tendons and application of mechanical load. *Tissue engineering* 2003;9:967-79.

- [126] Kuo CK, Tuan RS. Mechanoactive tenogenic differentiation of human mesenchymal stem cells. *Tissue engineering Part A* 2008;14:1615-27.
- [127] Yang G, Crawford RC, Wang JH. Proliferation and collagen production of human patellar tendon fibroblasts in response to cyclic uniaxial stretching in serum-free conditions. *Journal of biomechanics* 2004;37:1543-50.
- [128] Xu B, Song G, Ju Y, Li X, Song Y, Watanabe S. RhoA/ROCK, cytoskeletal dynamics, and focal adhesion kinase are required for mechanical stretch-induced tenogenic differentiation of human mesenchymal stem cells. *Journal of cellular physiology* 2012;227:2722-9.
- [129] Maeda T, Sakabe T, Sunaga A, Sakai K, Rivera AL, Keene DR, et al. Conversion of mechanical force into TGF-beta-mediated biochemical signals. *Current biology : CB* 2011;21:933-41.
- [130] Androjna C, Spragg RK, Derwin KA. Mechanical conditioning of cell-seeded small intestine submucosa: a potential tissue-engineering strategy for tendon repair. *Tissue engineering* 2007;13:233-43.
- [131] Nguyen TD, Liang R, Woo SL, Burton SD, Wu C, Almarza A, et al. Effects of cell seeding and cyclic stretch on the fiber remodeling in an extracellular matrix-derived bioscaffold. *Tissue engineering Part A* 2009;15:957-63.
- [132] Ambrosio F, Wolf SL, Delitto A, Fitzgerald GK, Badylak SF, Boninger ML, et al. The emerging relationship between regenerative medicine and physical therapeutics. *Physical therapy* 2010;90:1807-14.
- [133] Aspenberg P. Stimulation of tendon repair: mechanical loading, GDFs and platelets. A mini-review. *International orthopaedics* 2007;31:783-9.
- [134] Forslund C, Aspenberg P. CDMP-2 induces bone or tendon-like tissue depending on mechanical stimulation. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2002;20:1170-4.
- [135] Pennisi E. Tending tender tendons. *Science* 2002;295:1011.
- [136] Lui PP, Rui YF, Ni M, Chan KM. Tenogenic differentiation of stem cells for tendon repair-what is the current evidence? *Journal of tissue engineering and regenerative medicine* 2011;5:e144-63.
- [137] Krueger-Franke M, Siebert CH, Scherzer S. Surgical treatment of ruptures of the Achilles tendon: a review of long-term results. *British journal of sports medicine* 1995;29:121-5.
- [138] Klepps S, Bishop J, Lin J, Cahlon O, Strauss A, Hayes P, et al. Prospective evaluation of the effect of rotator cuff integrity on the outcome of open rotator cuff repairs. *The American journal of sports medicine* 2004;32:1716-22.

- [139] Kuo CK, Marturano JE, Tuan RS. Novel strategies in tendon and ligament tissue engineering: Advanced biomaterials and regeneration motifs. *Sports medicine, arthroscopy, rehabilitation, therapy & technology* : SMARTT 2010;2:20.
- [140] Riehl BD, Park JH, Kwon IK, Lim JY. Mechanical stretching for tissue engineering: two-dimensional and three-dimensional constructs. *Tissue engineering Part B, Reviews* 2012;18:288-300.
- [141] Subramony SD, Dargis BR, Castillo M, Azeloglu EU, Tracey MS, Su A, et al. The guidance of stem cell differentiation by substrate alignment and mechanical stimulation. *Biomaterials* 2013;34:1942-53.
- [142] Awad HA, Butler DL, Harris MT, Ibrahim RE, Wu Y, Young RG, et al. In vitro characterization of mesenchymal stem cell-seeded collagen scaffolds for tendon repair: effects of initial seeding density on contraction kinetics. *Journal of biomedical materials research* 2000;51:233-40.
- [143] Harris MT, Butler DL, Boivin GP, Florer JB, Schantz EJ, Wenstrup RJ. Mesenchymal stem cells used for rabbit tendon repair can form ectopic bone and express alkaline phosphatase activity in constructs. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2004;22:998-1003.
- [144] Lin H, Yang G, Tan J, Tuan RS. Influence of decellularized matrix derived from human mesenchymal stem cells on their proliferation, migration and multi-lineage differentiation potential. *Biomaterials* 2012;33:4480-9.
- [145] Discher DE, Mooney DJ, Zandstra PW. Growth factors, matrices, and forces combine and control stem cells. *Science* 2009;324:1673-7.
- [146] Ning LJ, Zhang Y, Chen XH, Luo JC, Li XQ, Yang ZM, et al. Preparation and characterization of decellularized tendon slices for tendon tissue engineering. *Journal of biomedical materials research Part A* 2012;100:1448-56.
- [147] Mizuno H. Adipose-derived stem and stromal cells for cell-based therapy: current status of preclinical studies and clinical trials. *Current opinion in molecular therapeutics* 2010;12:442-9.
- [148] Mizuno H, Tobita M, Uysal AC. Concise review: Adipose-derived stem cells as a novel tool for future regenerative medicine. *Stem Cells* 2012;30:804-10.
- [149] Mizuno H, Zuk PA, Zhu M, Lorenz HP, Benhaim P, Hedrick MH. Myogenic differentiation by human processed lipoaspirate cells. *Plastic and reconstructive surgery* 2002;109:199-209; discussion 10-1.
- [150] Uysal AC, Mizuno H. Tendon regeneration and repair with adipose derived stem cells. *Current stem cell research & therapy* 2010;5:161-7.

- [151] Raabe O, Shell K, Fietz D, Freitag C, Ohrndorf A, Christ HJ, et al. Tenogenic differentiation of equine adipose-tissue-derived stem cells under the influence of tensile strain, growth differentiation factors and various oxygen tensions. *Cell and tissue research* 2013.
- [152] Kryger GS, Chong AK, Costa M, Pham H, Bates SJ, Chang J. A comparison of tenocytes and mesenchymal stem cells for use in flexor tendon tissue engineering. *The Journal of hand surgery* 2007;32:597-605.
- [153] Stern MM, Myers RL, Hammam N, Stern KA, Eberli D, Kritchevsky SB, et al. The influence of extracellular matrix derived from skeletal muscle tissue on the proliferation and differentiation of myogenic progenitor cells ex vivo. *Biomaterials* 2009;30:2393-9.
- [154] Xue JX, Gong YY, Zhou GD, Liu W, Cao Y, Zhang WJ. Chondrogenic differentiation of bone marrow-derived mesenchymal stem cells induced by acellular cartilage sheets. *Biomaterials* 2012;33:5832-40.
- [155] Omae H, Zhao C, Sun YL, An KN, Amadio PC. Multilayer tendon slices seeded with bone marrow stromal cells: a novel composite for tendon engineering. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2009;27:937-42.
- [156] Singh P, Schwarzbauer JE. Fibronectin and stem cell differentiation - lessons from chondrogenesis. *J Cell Sci* 2012;125:3703-12.
- [157] Thibault MM, Hoemann CD, Buschmann MD. Fibronectin, vitronectin, and collagen I induce chemotaxis and haptotaxis of human and rabbit mesenchymal stem cells in a standardized transmembrane assay. *Stem cells and development* 2007;16:489-502.
- [158] Chen B, Wang B, Zhang WJ, Zhou G, Cao Y, Liu W. In vivo tendon engineering with skeletal muscle derived cells in a mouse model. *Biomaterials* 2012;33:6086-97.
- [159] Bi Y, Stuelten CH, Kilts T, Wadhwa S, Iozzo RV, Robey PG, et al. Extracellular matrix proteoglycans control the fate of bone marrow stromal cells. *J Biol Chem* 2005;280:30481-9.
- [160] Zheng Z, Jian J, Zhang X, Zara JN, Yin W, Chiang M, et al. Reprogramming of human fibroblasts into multipotent cells with a single ECM proteoglycan, fibromodulin. *Biomaterials* 2012;33:5821-31.
- [161] Yang X, Cai X, Wang J, Tang H, Yuan Q, Gong P, et al. Mechanical stretch inhibits adipogenesis and stimulates osteogenesis of adipose stem cells. *Cell proliferation* 2012;45:158-66.
- [162] Yang X, Gong P, Lin Y, Zhang L, Li X, Yuan Q, et al. Cyclic tensile stretch modulates osteogenic differentiation of adipose-derived stem cells via the BMP-2 pathway. *Archives of medical science : AMS* 2010;6:152-9.

- [163] Diederichs S, Bohm S, Peterbauer A, Kasper C, Scheper T, van Griensven M. Application of different strain regimes in two-dimensional and three-dimensional adipose tissue-derived stem cell cultures induces osteogenesis: implications for bone tissue engineering. *Journal of biomedical materials research Part A* 2010;94:927-36.
- [164] Shi Y, Fu Y, Tong W, Geng Y, Lui PP, Tang T, et al. Uniaxial mechanical tension promoted osteogenic differentiation of rat tendon-derived stem cells (rTDSCs) via the Wnt5a-RhoA pathway. *Journal of cellular biochemistry* 2012;113:3133-42.
- [165] Scott A, Danielson P, Abraham T, Fong G, Sampaio AV, Underhill TM. Mechanical force modulates scleraxis expression in bioartificial tendons. *Journal of musculoskeletal & neuronal interactions* 2011;11:124-32.
- [166] Mendias CL, Gumucio JP, Bakhurin KI, Lynch EB, Brooks SV. Physiological loading of tendons induces scleraxis expression in epitenon fibroblasts. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2012;30:606-12.
- [167] Somerville RP, Oblander SA, Apte SS. Matrix metalloproteinases: old dogs with new tricks. *Genome biology* 2003;4:216.
- [168] Heckmann L, Fiedler J, Mattes T, Dauner M, Brenner RE. Interactive effects of growth factors and three-dimensional scaffolds on multipotent mesenchymal stromal cells. *Biotechnology and applied biochemistry* 2008;49:185-94.
- [169] Robertson CM, Chen CT, Shindle MK, Cordasco FA, Rodeo SA, Warren RF. Failed healing of rotator cuff repair correlates with altered collagenase and gelatinase in supraspinatus and subscapularis tendons. *The American journal of sports medicine* 2012;40:1993-2001.
- [170] Karousou E, Ronga M, Vigetti D, Passi A, Maffulli N. Collagens, proteoglycans, MMP-2, MMP-9 and TIMPs in human achilles tendon rupture. *Clinical orthopaedics and related research* 2008;466:1577-82.
- [171] de Mos M, van El B, DeGroot J, Jahr H, van Schie HT, van Arkel ER, et al. Achilles tendinosis: changes in biochemical composition and collagen turnover rate. *The American journal of sports medicine* 2007;35:1549-56.
- [172] Lo IK, Marchuk LL, Hollinshead R, Hart DA, Frank CB. Matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase mRNA levels are specifically altered in torn rotator cuff tendons. *The American journal of sports medicine* 2004;32:1223-9.
- [173] Bach BRJ, Tradonsky S, Bojchuk J, Levy ME, Bush-Joseph CA, Khan NH. Arthroscopically assisted anterior cruciate ligament reconstruction using patellar tendon autograft. Five- to nine-year follow-up evaluation. *Am J Sports Med* 1998;26:20-9.
- [174] Violini S, Ramelli P, Pisani LF, Gorni C, Mariani P. Horse bone marrow mesenchymal stem cells express embryo stem cell markers and show the ability for tenogenic differentiation by in vitro exposure to BMP-12. *BMC Cell Biol* 2009;10:29.

- [175] Konno M, Hamabe A, Hasegawa S, Ogawa H, Fukusumi T, Nishikawa S, et al. Adipose-derived mesenchymal stem cells and regenerative medicine. *Dev Growth Differ* 2013;55:309-18.
- [176] Munger JS, Sheppard D. Cross Talk among TGF-beta Signaling Pathways, Integrins, and the Extracellular Matrix. *Cold Spring Harbor Perspectives in Biology* 2011;3.
- [177] Natsu-ume T, Nakamura N, Shino K, Toritsuka Y, Horibe S, Ochi T. Temporal and spatial expression of transforming growth factor-beta in the healing patellar ligament of the rat. *J Orthop Res* 1997;15:837-43.
- [178] Chen CH, Cao Y, Wu YF, Bais AJ, Gao JS, Tang JB. Tendon healing in vivo: gene expression and production of multiple growth factors in early tendon healing period. *J Hand Surg Am* 2008;33:1834-42.
- [179] Tuli R, Tuli S, Nandi S, Wang ML, Alexander PG, Haleem-Smith H, et al. Characterization of multipotential mesenchymal progenitor cells derived from human trabecular bone. *Stem Cells* 2003;21:681-93.
- [180] Marturano JE, Arena JD, Schiller ZA, Georgakoudi I, Kuo CK. Characterization of mechanical and biochemical properties of developing embryonic tendon. *Proc Natl Acad Sci U S A* 2013;110:6370-5.
- [181] Watt FM, Huck WT. Role of the extracellular matrix in regulating stem cell fate. *Nature reviews Molecular cell biology* 2013;14:467-73.
- [182] Faulk DM, Johnson SA, Zhang L, Badylak SF. Role of the extracellular matrix in whole organ engineering. *J Cell Physiol* 2014;229:984-9.
- [183] Yang G, Rothrauff BB, Lin H, Gottardi R, Alexander PG, Tuan RS. Enhancement of tenogenic differentiation of human adipose stem cells by tendon-derived extracellular matrix. *Biomaterials* 2013;34:9295-306.
- [184] Yin Z, Chen X, Zhu T, Hu JJ, Song HX, Shen WL, et al. The effect of decellularized matrices on human tendon stem/progenitor cell differentiation and tendon repair. *Acta biomaterialia* 2013;9:9317-29.
- [185] Zimmerlin L, Donnenberg VS, Pfeifer ME, Meyer EM, Peault B, Rubin JP, et al. Stromal vascular progenitors in adult human adipose tissue. *Cytometry A* 2010;77:22-30.
- [186] Lin G, Garcia M, Ning H, Banie L, Guo YL, Lue TF, et al. Defining stem and progenitor cells within adipose tissue. *Stem cells and development* 2008;17:1053-63.
- [187] Raghavan SS, Woon CY, Kraus A, Megerle K, Pham H, Chang J. Optimization of human tendon tissue engineering: synergistic effects of growth factors for use in tendon scaffold repopulation. *Plast Reconstr Surg* 2012;129:479-89.

- [188] Schmitt T, Fox PM, Woon CY, Farnebo SJ, Bronstein JA, Behn A, et al. Human flexor tendon tissue engineering: in vivo effects of stem cell reseeded. *Plast Reconstr Surg* 2013;132:567e-76e.
- [189] Vindigni V, Tonello C, Lancerotto L, Abatangelo G, Cortivo R, Zavan B, et al. Preliminary report of in vitro reconstruction of a vascularized tendonlike structure: a novel application for adipose-derived stem cells. *Ann Plast Surg* 2013;71:664-70.
- [190] Martinello T, Bronzini I, Volpin A, Vindigni V, Maccatrozzo L, Caporale G, et al. Successful recellularization of human tendon scaffolds using adipose-derived mesenchymal stem cells and collagen gel. *Journal of tissue engineering and regenerative medicine* 2014;8:612-9.
- [191] Eagan MJ, Zuk PA, Zhao KW, Bluth BE, Brinkmann EJ, Wu BM, et al. The suitability of human adipose-derived stem cells for the engineering of ligament tissue. *J Tissue Eng Regen Med* 2011.
- [192] Silver FH, Kato YP, Ohno M, Wasserman AJ. Analysis of mammalian connective tissue: relationship between hierarchical structures and mechanical properties. *J Long Term Eff Med Implants* 1992;2:165-98.
- [193] Erisken C, Zhang X, Moffat KL, Levine WN, Lu HH. Scaffold fiber diameter regulates human tendon fibroblast growth and differentiation. *Tissue engineering Part A* 2013;19:519-28.
- [194] Cardwell RD, Dahlgren LA, Goldstein AS. Electrospun fibre diameter, not alignment, affects mesenchymal stem cell differentiation into the tendon/ligament lineage. *J Tissue Eng Regen Med* 2014;8:937-45.
- [195] Louis-Ugbo J, Leeson B, Hutton WC. Tensile properties of fresh human calcaneal (Achilles) tendons. *Clin Anat* 2004;17:30-5.
- [196] Baker BM, Shah RP, Silverstein AM, Esterhai JL, Burdick JA, Mauck RL. Sacrificial nanofibrous composites provide instruction without impediment and enable functional tissue formation. *Proc Natl Acad Sci U S A* 2012;109:14176-81.
- [197] Griffin M, Hindocha S, Jordan D, Saleh M, Khan W. An overview of the management of flexor tendon injuries. *The open orthopaedics journal* 2012;6:28-35.
- [198] Calleja M, Connell DA. The Achilles tendon. *Seminars in musculoskeletal radiology* 2010;14:307-22.
- [199] Derwin KA, Baker AR, Spragg RK, Leigh DR, Iannotti JP. Commercial extracellular matrix scaffolds for rotator cuff tendon repair. Biomechanical, biochemical, and cellular properties. *The Journal of bone and joint surgery American volume* 2006;88:2665-72.
- [200] Kannus P. Structure of the tendon connective tissue. *Scandinavian journal of medicine & science in sports* 2000;10:312-20.

- [201] Maffulli N, Sharma P, Luscombe KL. Achilles tendinopathy: aetiology and management. *Journal of the Royal Society of Medicine* 2004;97:472-6.
- [202] Rodrigues MT, Reis RL, Gomes ME. Engineering tendon and ligament tissues: present developments towards successful clinical products. *Journal of tissue engineering and regenerative medicine* 2013;7:673-86.
- [203] Lavagnino M, Wall ME, Little D, Banes AJ, Guilak F, Arnoczky SP. Tendon mechanobiology: Current knowledge and future research opportunities. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2015;33:813-22.
- [204] Yin Z, Chen X, Song HX, Hu JJ, Tang QM, Zhu T, et al. Electrospun scaffolds for multiple tissues regeneration in vivo through topography dependent induction of lineage specific differentiation. *Biomaterials* 2015;44:173-85.
- [205] Cipitria A, Skelton A, Dargaville TR, Dalton PD, Hutmacher DW. Design, fabrication and characterization of PCL electrospun scaffolds-a review. *J Mater Chem* 2011;21:9419-53.
- [206] Middleton JC, Tipton AJ. Synthetic biodegradable polymers as orthopedic devices. *Biomaterials* 2000;21:2335-46.
- [207] Nair LS, Laurencin CT. Biodegradable polymers as biomaterials. *Prog Polym Sci* 2007;32:762-98.
- [208] Ma ZW, Mao ZW, Gao CY. Surface modification and property analysis of biomedical polymers used for tissue engineering. *Colloid Surface B* 2007;60:137-57.
- [209] Vanwachem PB, Beugeling T, Feijen J, Bantjes A, Detmers JP, Vanaken WG. Interaction of Cultured Human-Endothelial Cells with Polymeric Surfaces of Different Wettabilities. *Biomaterials* 1985;6:403-8.
- [210] Ruoslahti E, Pierschbacher MD. New Perspectives in Cell-Adhesion - Rgd and Integrins. *Science* 1987;238:491-7.
- [211] 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;33:6020-41.
- [212] El-Sherbiny IM, Yacoub MH. Hydrogel scaffolds for tissue engineering: Progress and challenges. *Global cardiology science & practice* 2013;2013:316-42.
- [213] Antoine EE, Vlachos PP, Rylander MN. Review of collagen I hydrogels for bioengineered tissue microenvironments: characterization of mechanics, structure, and transport. *Tissue engineering Part B, Reviews* 2014;20:683-96.
- [214] Gasperini L, Mano JF, Reis RL. Natural polymers for the microencapsulation of cells. *J R Soc Interface* 2014;11:20140817.

- [215] Yodmuang S, McNamara SL, Nover AB, Mandal BB, Agarwal M, Kelly TA, et al. Silk microfiber-reinforced silk hydrogel composites for functional cartilage tissue repair. *Acta biomaterialia* 2015;11:27-36.
- [216] Nichol JW, Koshy ST, Bae H, Hwang CM, Yamanlar S, Khademhosseini A. Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials* 2010;31:5536-44.
- [217] Fairbanks BD, Schwartz MP, Bowman CN, Anseth KS. Photoinitiated polymerization of PEG-diacrylate with lithium phenyl-2,4,6-trimethylbenzoylphosphinate: polymerization rate and cytocompatibility. *Biomaterials* 2009;30:6702-7.
- [218] Baker BM, Gee AO, Metter RB, Nathan AS, Marklein RA, Burdick JA, et al. The potential to improve cell infiltration in composite fiber-aligned electrospun scaffolds by the selective removal of sacrificial fibers. *Biomaterials* 2008;29:2348-58.
- [219] Atesok K, Doral MN, Karlsson J, Egol KA, Jazrawi LM, Coelho PG, et al. Multilayer scaffolds in orthopaedic tissue engineering. *Knee surgery, sports traumatology, arthroscopy : official journal of the ESSKA* 2014.
- [220] Awad HA, Boivin GP, Dressler MR, Smith FNL, Young RG, Butler DL. Repair of patellar tendon injuries using a cell-collagen composite. *J Orthopaed Res* 2003;21:420-31.
- [221] Yue K, Trujillo-de Santiago G, Alvarez MM, Tamayol A, Annabi N, Khademhosseini A. Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. *Biomaterials* 2015;73:254-71.
- [222] Veis A, Anesey J, Cohen J. The long range reorganization of gelatin to the collagen structure. *Archives of biochemistry and biophysics* 1961;94:20-31.
- [223] Zeugolis DI, Khew ST, Yew ES, Ekaputra AK, Tong YW, Yung LY, et al. Electrospinning of pure collagen nano-fibres - just an expensive way to make gelatin? *Biomaterials* 2008;29:2293-305.
- [224] Zhang YZ, Ouyang HW, Lim CT, Ramakrishna S, Huang ZM. Electrospinning of gelatin fibers and gelatin/PCL composite fibrous scaffolds. *J Biomed Mater Res B* 2005;72B:156-65.
- [225] Leach MK, Feng ZQ, Tuck SJ, Corey JM. Electrospinning fundamentals: optimizing solution and apparatus parameters. *Journal of visualized experiments : JoVE* 2011.
- [226] Baker BM, Shah RP, Silverstein AM, Esterhai JL, Burdick JA, Mauck RL. Sacrificial nanofibrous composites provide instruction without impediment and enable functional tissue formation. *Proceedings of the National Academy of Sciences of the United States of America* 2012;109:14176-81.
- [227] Jha AK, Tharp KM, Ye JQ, Santiago-Ortiz JL, Jackson WM, Stahl A, et al. Enhanced survival and engraftment of transplanted stem cells using growth factor sequestering hydrogels. *Biomaterials* 2015;47:1-12.

- [228] Khetan S, Guvendiren M, Legant WR, Cohen DM, Chen CS, Burdick JA. Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels. *Nat Mater* 2013;12:458-65.
- [229] Fisher MB, Henning EA, Soegaard N, Bostrom M, Esterhai JL, Mauck RL. Engineering meniscus structure and function via multi-layered mesenchymal stem cell-seeded nanofibrous scaffolds. *Journal of biomechanics* 2015;48:1412-9.
- [230] Omi R, Gingery A, Steinmann SP, Amadio PC, An KN, Zhao C. Rotator cuff repair augmentation in a rat model that combines a multilayer xenograft tendon scaffold with bone marrow stromal cells. *Journal of shoulder and elbow surgery / American Shoulder and Elbow Surgeons [et al]* 2015.
- [231] Hakimi O, Mouthuy PA, Zargar N, Lostis E, Morrey M, Carr A. A layered electrospun and woven surgical scaffold to enhance endogenous tendon repair. *Acta biomaterialia* 2015;26:124-35.
- [232] Lin H, Cheng AW, Alexander PG, Beck AM, Tuan RS. Cartilage tissue engineering application of injectable gelatin hydrogel with in situ visible-light-activated gelation capability in both air and aqueous solution. *Tissue engineering Part A* 2014;20:2402-11.
- [233] Manning CN, Schwartz AG, Liu W, Xie J, Havlioglu N, Sakiyama-Elbert SE, et al. Controlled delivery of mesenchymal stem cells and growth factors using a nanofiber scaffold for tendon repair. *Acta biomaterialia* 2013;9:6905-14.
- [234] Schipper BM, Marra KG, Zhang W, Donnenberg AD, Rubin JP. Regional anatomic and age effects on cell function of human adipose-derived stem cells. *Annals of plastic surgery* 2008;60:538-44.
- [235] Prunet-Marcassus B, Cousin B, Caton D, Andre M, Penicaud L, Casteilla L. From heterogeneity to plasticity in adipose tissues: site-specific differences. *Experimental cell research* 2006;312:727-36.
- [236] Wickham MQ, Erickson GR, Gimble JM, Vail TP, Guilak F. Multipotent stromal cells derived from the infrapatellar fat pad of the knee. *Clinical orthopaedics and related research* 2003:196-212.
- [237] Liu YP, Li SZ, Yuan F, Xia J, Yu X, Liu XQ, et al. Infrapatellar fat pad may be with tendon repairing ability and closely related with the developing process of patella Baja. *Med Hypotheses* 2011;77:620-3.
- [238] English A, Jones EA, Corscadden D, Henshaw K, Chapman T, Emery P, et al. A comparative assessment of cartilage and joint fat pad as a potential source of cells for autologous therapy development in knee osteoarthritis. *Rheumatology* 2007;46:1676-83.
- [239] Liu YR, Buckley CT, Almeida HV, Mulhall KJ, Kelly DJ. Infrapatellar Fat Pad-Derived Stem Cells Maintain Their Chondrogenic Capacity in Disease and Can be Used to

- Engineer Cartilaginous Grafts of Clinically Relevant Dimensions. *Tissue Eng Pt A* 2014;20:3050-62.
- [240] He F, Pei M. Extracellular matrix enhances differentiation of adipose stem cells from infrapatellar fat pad toward chondrogenesis. *Journal of tissue engineering and regenerative medicine* 2013;7:73-84.
- [241] Bailey AM, Kapur S, Katz AJ. Characterization of adipose-derived stem cells: an update. *Current stem cell research & therapy* 2010;5:95-102.
- [242] Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. *Molecular biology of the cell* 2002;13:4279-95.
- [243] Guilak F, Awad HA, Fermor B, Leddy HA, Gimple JM. Adipose-derived adult stem cells for cartilage tissue engineering. *Biorheology* 2004;41:389-99.
- [244] Hennig T, Lorenz H, Thiel A, Goetzke K, Dickhut A, Geiger F, et al. Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6. *Journal of cellular physiology* 2007;211:682-91.
- [245] Wolfman NM, Hattersley G, Cox K, Celeste AJ, Nelson R, Yamaji N, et al. Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-beta gene family. *The Journal of clinical investigation* 1997;100:321-30.
- [246] Mikic B, Schalet BJ, Clark RT, Gaschen V, Hunziker EB. GDF-5 deficiency in mice alters the ultrastructure, mechanical properties and composition of the Achilles tendon. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2001;19:365-71.
- [247] Chan BP, Chan KM, Maffulli N, Webb S, Lee KK. Effect of basic fibroblast growth factor. An in vitro study of tendon healing. *Clinical orthopaedics and related research* 1997;239-47.
- [248] Decaris ML, Binder BY, Soicher MA, Bhat A, Leach JK. Cell-derived matrix coatings for polymeric scaffolds. *Tissue engineering Part A* 2012;18:2148-57.
- [249] Byron A, Humphries JD, Humphries MJ. Defining the extracellular matrix using proteomics. *Int J Exp Pathol* 2013;94:75-92.
- [250] Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature* 2003;422:198-207.
- [251] Han XM, Aslanian A, Yates JR. Mass spectrometry for proteomics. *Curr Opin Chem Biol* 2008;12:483-90.

- [252] Nesvizhskii AI, Vitek O, Aebersold R. Analysis and validation of proteomic data generated by tandem mass spectrometry. *Nat Methods* 2007;4:787-97.
- [253] Gelberman RH, Shen H, Kormpakis I, Rothrauff B, Yang G, Tuan RS, et al. Effect of adipose-derived stromal cells and BMP12 on intrasynovial tendon repair: A biomechanical, biochemical, and proteomics study. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2015.
- [254] Little D, Thompson JW, Dubois LG, Ruch DS, Moseley MA, Guilak F. Proteomic Differences between Male and Female Anterior Cruciate Ligament and Patellar Tendon. *PloS one* 2014;9.
- [255] Munger JS, Sheppard D. Cross talk among TGF-beta signaling pathways, integrins, and the extracellular matrix. *Cold Spring Harbor perspectives in biology* 2011;3:a005017.
- [256] Zhu Y, Mao ZW, Shi HY, Gao CY. In-depth study on aminolysis of poly(epsilon-caprolactone): Back to the fundamentals. *Sci China Chem* 2012;55:2419-27.
- [257] Zhu Y, Gao C, Liu X, Shen J. Surface modification of polycaprolactone membrane via aminolysis and biomacromolecule immobilization for promoting cytocompatibility of human endothelial cells. *Biomacromolecules* 2002;3:1312-9.
- [258] Schenke-Layland K, Rofail F, Heydarkhan S, Gluck JM, Ingle NP, Angelis E, et al. The use of three-dimensional nanostructures to instruct cells to produce extracellular matrix for regenerative medicine strategies. *Biomaterials* 2009;30:4665-75.
- [259] Heydarkhan-Hagvall S, Schenke-Layland K, Dhanasopon AP, Rofail F, Smith H, Wu BM, et al. Three-dimensional electrospun ECM-based hybrid scaffolds for cardiovascular tissue engineering. *Biomaterials* 2008;29:2907-14.
- [260] Zhang Y, Ouyang H, Lim CT, Ramakrishna S, Huang ZM. Electrospinning of gelatin fibers and gelatin/PCL composite fibrous scaffolds. *Journal of biomedical materials research Part B, Applied biomaterials* 2005;72:156-65.
- [261] Zeugolis DI, Khew ST, Yew ESY, Ekaputra AK, Tong YW, Yung LYL, et al. Electrospinning of pure collagen nano-fibres - Just an expensive way to make gelatin? *Biomaterials* 2008;29:2293-305.