IMPACT OF MECHANICAL STIMULATION ON TENDON TISSUE IN A BIOREACTOR SYSTEM

TAO WANG

This thesis is submitted to The University of Western Australia in fulfillment of the requirement for degree of

DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL ENGINEERING

School of Surgery

The University of Western Australia

2014

The work presented in this thesis was performed in The University of Western Australia, School of Surgery, Centre for Orthopaedic Translational Research
Declaration

This is to certify that all work contained herein was performed by myself, except where indicated otherwise.

Tao Wang (PhD candidate)

W/Prof. Minghao Zheng (Supervisor)

A/Prof. Bruce Gardiner (Co-supervisor)

Prof. Brett Kirk (External supervisor)
# TABLE OF CONTENTS

## ABSTRACT 9

## PUBLICATIONS 11

## LIST OF AWARDS & PRESENTATIONS 12

## ABBREVIATIONS 14

### CHAPTER 1: INTRODUCTION 18

#### 1.1 Tendon Biology 18
- 1.1.1 Anatomy of human Achilles tendon 19
- 1.1.2 Histology of healthy tendon 20
- 1.1.3 Aetiology of tendon injuries 24
  - 1.1.3.1 Acute traumatic injury on Achilles tendon 24
  - 1.1.3.2 Chronic Achilles tendinopathy 25
- 1.1.4 Pathology of tendon injuries 30
  - 1.1.4.1 Pathology of tendinopathy 30
  - 1.1.4.2 Healing process 31
  - 1.1.4.3 Molecules involved in tendon remodeling 34
- 1.1.5 Treatments 43
  - 1.1.5.1 Surgical treatment 43
  - 1.1.5.2 Conservative treatment 44
- 1.1.6 Summary 56

#### 1.2 Introduction to Bioreactor Design for Tendon/Ligament Engineering 56
- 1.2.1 Common key elements for tendon/ligament tissue engineering 57
- 1.2.2 Bioreactor design specific to tendon/ligament engineering 63
  - 1.2.2.1 Actuator and culture chamber design 66
  - 1.2.2.2 Environmental control and medium circulation systems 71
  - 1.2.2.3 Monitoring and feedback systems 75
- 1.2.3 Commercial bioreactor systems for tendon/ligament engineering 79
- 1.2.4 Ideal bioreactors for tendon/ligament engineering 81
- 1.2.5 Previous work in this field 83
- 1.2.6 Conclusion 89

#### 1.3 Hypothesis and aims 90
- 1.3.1 Hypothesis 90
  - 1.3.1.1 Overall hypothesis 90
  - 1.3.1.2 Specific hypothesis 91
- 1.3.2 Aims 91

### CHAPTER 2 MATERIALS AND METHODS 94

#### 2.1 Material 94
- 2.1 94
  - 2.1.1 Tissue Culture reagents 94
  - 2.1.2 Chemical reagents 95
  - 2.1.3 Molecular products 96
  - 2.1.4 Oligonucleotide Primers 96
  - 2.1.5 Antibodies 98
4.3.3 TUNEL assay 138
4.3.4 Immunostaining for type III collagen 138
4.3.5 Quantitative Real-time polymerase chain reaction (Q-PCR) 138
4.3.6 Statistical analyses 139

4.4 Results 139
4.4.1 Histological examination of tendon undergoing different cyclic tensile strains 139
4.4.2 Impact of cyclic tensile strain on apoptosis of tenocytes 144
4.4.3 Type III collagen turnover in Achilles tendon under dynamic culture 146
4.4.4 Impact of cyclic tensile strain on ECM remodeling gene expression 147

4.5 Discussion 148

4.6 Conclusion 154

CHAPTER 5 (RESULTS): CYCLIC MECHANICAL STIMULATION RESCUES RABBIT ACHILLES TENDON FROM DEGENERATION IN A BIOREACTOR SYSTEM 156

5.1 Abstract 156

5.2 Introduction 157

5.3 Material and method 158
5.3.1 Tissue preparation and programmable mechanical stimulation 158
5.3.2 Histological preparation and assessment 161
5.3.3 TUNEL assay 161
5.3.4 Biomechanics testing 161
5.3.5 Real-time polymerase chain reaction 162
5.3.6 Immunohistochemistry 162
5.3.7 Statistical analyses 162

5.4 Results 162
5.4.1 Cyclic mechanical stimulation improved the histological structure and cell viability of cultured tendon 162
5.4.2 Cyclic mechanical stimulation improved mechanical properties of cultured tendon 167
5.4.3 Rescue effect of cyclic mechanical stimulation on ECM remodeling gene expression 169
5.4.4 Cyclic mechanical stimulation decreased Type III collagen expression of cultured tendon 171

5.5 Discussion 172

5.6 Conclusion 178

CHAPTER 6 GENERAL DISCUSSION AND FUTURE DIRECTIONS 180

6.1 General discussion 180
6.1.1 The custom-made bioreactor (Chapter 3) 181
6.1.2 Mechanical stimulation in bioreactor system (Chapter 4 and 5) 183
6.1.3 Effect of various mechanical stimulation on tendon homeostasis (Chapter 4) 184
6.1.4 Rescue of degenerated tendon with mechanical stimulation. 187

6.2 Limitation of this work 188
6.2.1 Chapter 3 188
6.2.2 Chapter 4 189
6.2.3 Chapter 5 190
6.3 Future directions

6.3.1 Gene profile of tenocyte subjected to 2D or 3D mechanical stimulation 190
6.3.2 The effect of mechanical stimulation on tendon nutrient infiltration 192
6.3.3 The effect of mechanical stimulation on stem cell tenogenic differentiation 193
6.3.4 Therapeutic guideline 194
6.3.5 Robot-driven orthosis 195
6.3.6 Tendon engineering 196

6.4 Conclusion 197

REFERENCE 198

APPENDIX 225
Dedicated to

My Awesome parents, Jihong Wang and Junhua Xu

and beautiful lovely wife, Xiang Jiang

for their love and support
ACKNOWLEDGEMENTS

The work described in this thesis was performed at the Center for Orthopaedic Translational Research, School of Surgery, The University of Western Australia. I would like to express my appreciation to my supervisor, Winthrop Professor Minghao Zheng, co-supervisor Associate Professor Bruce Gardiner and External supervisor Professor Brett Kirk. Without their support and guidance, this work would not have been finished.

Sincere thanks to Dr. Zhen Lin and Ms. Euphemie Landao-Bassonga for their valuable contributions, technical training and support.

I would love to thanks one of my best friend, Weiming Zeng, for the technical support of bioreactor construction.

At last, I would also like to extend my appreciation to my colleagues and the administrative officer for their assistance, friendship and encouragement.
ABSTRACT

Tendons are force-transmitting tissues connecting muscle to bone. Because of this physiological function, biomechanics plays an essential role in maintaining tendon homeostasis. Indeed tenocytes have the ability to sense and respond to different mechanical loads by remodeling the tendon tissue. Studies reported that load deprivation can cause tendinopathy-like morphology such as disorientated collagen fibers and rounded cell nuclei, while mechanical overloading can result in tears and rupture of the tendon. In tendon engineering studies, mechanical stimulation has been shown to improve the cell viability, proliferation, and neo-tendon structure and mechanical properties. Obviously, the biomechanical environment has various effects on tendon tissue; and yet the specific effects of different loading conditions on tendon have not been well elucidated. Mechanical loading is both a source of degradation (e.g. through damage and indirectly through protease activity) and a stimulus for tissue synthesis/repair. The hypothesis of this PhD project is that there is some optimal mechanical load to maintain or restore functional tendon tissue in a bioreactor.

Firstly, in order to study the mechanical stimulation on tendon tissue, a functional bioreactor is necessary. A uniaxial bioreactor was designed and fabricated. This bioreactor system is able to provide pre-programmable mechanical simulation and sterilized environment for a maximum 6 individual tendons simultaneously.

Secondly, rabbit Achilles tendon were cultured under different mechanical environments including no loading, 3% strain, 6% strain and 9% strain at 0.25Hz for 8h/day for 6 days
in the bioreactor system developed in this study. Histological and gene assessment showed that, of the strains applied, only at 6% strain was the rabbit model able to maintain the tendon homeostasis, other loading regimes resulted in pathological changes to the tendon as observed through histology testing.

Thirdly, due to the fact that loading deprivation of tendon can lead to progressive tendon degeneration; static cultured rabbit Achilles tendons was used as a degradation model. Application of 6% mechanical stimulation for 6 days followed by 6 days loading deprivation culture significantly improved the morphology of degenerative tendon and successfully restored the mechanical properties.

In summary, this study identified the effect of various loading intensities on tendon homeostasis in bioreactor system, and proposed a hypothetical model that there is only a narrow range of mechanical stimulation can maintain tendon homeostasis. We then further estimated the therapeutic effect of mechanical stimulation on a degenerative model, and found out accurate mechanical stimulation was able to reverse the early-stage degradation of Achilles tendon.

This leads to the conclusion that proper mechanical stimulation may have therapeutic benefits and clinical application.
Publications

Publications arising from this thesis


List of Awards & Presentations

Awards


2. 2013 China Distinguished International student scholarship

Oral Presentations


**Poster Presentations**


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS</td>
<td>A Disintegrin and Metalloproteinase with Thrombospondin Motifs</td>
</tr>
<tr>
<td>ADSCs</td>
<td>Adipose-derived stem cells</td>
</tr>
<tr>
<td>AT</td>
<td>Achilles tendon</td>
</tr>
<tr>
<td>ATT</td>
<td>Autologous tenocyte therapy</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMSCs</td>
<td>Bone marrow-derived stem cells</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic proteins</td>
</tr>
<tr>
<td>COX-2</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>ESCs</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>GDF-5</td>
<td>Growth differentiation factor 5</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin-eosin</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>LE</td>
<td>Lateral epicondylitis</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor-I</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>LVDT</td>
<td>Linear variable differential transformers</td>
</tr>
<tr>
<td>MCP1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage inhibitory factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroid anti-inflammation drug</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethylemethacrylate</td>
</tr>
<tr>
<td>PMS</td>
<td>Programmable mechanical stimulation</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>Scx</td>
<td>Scleraxis</td>
</tr>
<tr>
<td>SDSCs</td>
<td>Synovium-derived stem cells</td>
</tr>
<tr>
<td>SMBS</td>
<td>Step motor-ball screw transmission system</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TDSCs</td>
<td>Tendon-derived stem cells</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
CHAPTER 1: INTRODUCTION

1.1 Tendon Biology

The Achilles tendon (AT), also known as the calcaneal tendon, is the strongest and thickest tendon in human body. It connects the gastrocnemius and soleus muscles to calcaneus bone. As a force transferring tissue, AT serves the function of transferring the power generated by the muscle to the calcaneus, allowing plantar flexion about the ankle joint. It also helps store elastic energy while walking/running, increasing locomotion efficiency (Bressel and McNair 2001).

AT injury is a serious and common injury. AT rupture was first reported in 1575 by Ambrose Paré. The AT injuries rate is estimated at between 2 to 12 in 100,000 people (So and Pollard 1997; Schepsis, Jones et al. 2002; Suchak, Bostick et al. 2005). Typically, this injury affects males between 30 to 50 years old (Gebauer, Beil et al. 2007). It is thought that due to the specific geometry of the AT, the mechanical weak point is 2 to 6 cm from the calcaneal insertion, that is the thinnest part of the AT and the common rupture site(Wren, Lindsey et al. 2003). In addition, 11% of regular runners suffer from Achilles tendinopathy(Rees, Wilson et al. 2006), which can cause significant pain and restricts the activities in daily living, and potentially tendon rupture (Leppilahti and Orava 1998; Smith 2000). Traditionally, overuse and/or overloading has been considered to be the cause of tendinopathy, 30% to 50% of sports injuries were reported to be an overuse injury, and tendon injuries comprise a large part of that(Schechtman and Bader 1997; Khan and Cook 2003).
In the United States, injuries to tendons and ligaments represent about half of the 33 million musculoskeletal injuries (Huang, Qureshi et al. 2000). Each year more than 33,000 tendon reconstructions occur in U.S., costing $30 billion USD (Butler, Gooch et al. 2010; Chen, Yin et al. 2010). In Australia, $250 million is spent annually just on rotator cuff repair (Chen, Xu et al. 2009). However, despite the high prevalence of tendon injury and associated tendinopathy worldwide, treatment options remain poorly defined.

1.1.1 Anatomy of human Achilles tendon

The Achilles tendon is the extension of two muscles, the gastrocnemius and soleus muscles (Suchak, Bostick et al. 2005). Depending on knee positioning, these separate muscles have their own movement (Schepsis, Jones et al. 2002), but mainly control the plantar flexion of the ankle, allowing for locomotion at the ankle (Suchak, Bostick et al. 2005). These muscles form together at the mid-calf region of the lower leg, where the AT begins. The AT then merges into a single tendon at around 5 to 6 cm from the calcaneal insertion (So and Pollard 1997). The AT is covered by a peritenon, a single-cell layer tissue, which act as a lubricant during dynamic activity and provides vascular supply to the tendon tissue (Schepsis, Jones et al. 2002). Beside the peritenon, the main nutrient supply is from the muscle-tendon junction and bone tendon junction, therefore, the nutrient delivery to the AT is very limited (Schepsis, Jones et al. 2002).
1.1.2 Histology of healthy tendon

Tendons are relatively hypovascular and hypocellular tissues. Tendon cells, primarily tenocytes and fibroblasts, comprise less than 5% of the total volume. The morphology of tenocytes and fibroblasts are “sharp” and usually elongated along collagen fibers in normal tissues, but some rounded tenocytes are found occasionally (Manske 1988; Bray, Rangayyan et al. 1996; Lo, Ou et al. 2002; Hildebrand, Frank et al. 2004). Tendons consist of collagens, cells, proteoglycans, elastin, glycolipids and water. Although roughly 65-70% of the total weight is water (Lin, Cardenas et al. 2004), tendon is a highly organized hierarchical structure. Collagen type I is the main structural/functional component, and comprises around 70-80% of the dry weight, and is generally aligned along the long axis of tendon (Buckwalter and Hunziker 1996; Jarvinen, Jarvinen et al. 2004). Type III collagen comprises less than 5% of total collagen, and is mainly present...
in the endotenon and epitenon, but is also present in the early phase of tendon repair (Buckwalter and Hunziker 1996; Jarvinen, Jarvinen et al. 2004). The hierarchical structure of tendon is shown in Figure 1.2, which is modified from the study of Kastelic et al. (Kastelic, Galeski et al. 1978).

Type 1 collagen molecules synthesized by tenocytes bind together into a triple helix tropocollagen (Kuhn 1969), which then self-assemble into microfibril and bind to adjacent helixes by molecules such as decorin and biglycan, so forming collagen fibrils (Tkocz and Kuhn 1969). This helixes structure is able to provide high resistance to tensile strain while maintaining its flexibility (Screen, Lee et al. 2004). Due to this structural characteristic, the tendon is able to efficiently transfer force from muscle to bone (Magnusson, Hansen et al. 2003). The collagen fibrils align parallel to each other assemble into collagen fiber with a waveform structure, enabling the tendon to absorb the sudden initial force generate by muscle (Kastelic, Palley et al. 1980). As shown as Figure 1.3, when unloaded the collagen fiber appears ‘wavy’. The wavy structure disappears at around 4% strain, micro and partial rupture starts at ~8% strain, beyond that, complete rupture is likely to occur (Maffulli 1998).

Tenocytes adhere to the collagen fiber; and sense the mechanical loading on the tendon (Benjamin and Ralphs 1998; Murata, Nishizono et al. 2000). In the haematoxylin-eosin (H&E) stained normal and healthy tendon section, tenocytes are evenly distributed between the straight and parallel collagen fibrils with sharp and elongate nuclei (Chen,
Although proteoglycans comprised only ~1% dry mass of tendon, they still play an essential role in tendon biomechanics (Thompson 2013). Small proteoglycans such as decorin control collagen fibril assembly and alignment (Parkinson, Samiric et al. 2011), and the hydrophilic nature of the large proteoglycans has a strong effect on tissue mechanical properties (Screen, Chhaya et al. 2006). Moreover, proteoglycan concentration varies within and between tendon due to different local loading characteristics (Birch 2007).

Elastin and microfibrils form elastic fibers, which comprise around 0.1% to 2% dry mass of tendon (Kannus 2000). They play a role in the tissue low strain and resilience property by bridging between collagen fiber bundles with a network structure (Smith, Vaughan-Thomas et al. 2011).
Figure 1.2 The structure of a healthy tendon. figure modified from Kastrlic et al. (Kastelic, Galeski et al. 1978)

Figure 1.3 Force versus strain curve of tendon (figure modified from the study of Wang et al.) (Wang 2006)
1.1.3 Aetiology of tendon injuries

Tendon injuries can be classified into acute and chronic. In acute traumatic injury extrinsic factors predominate, whereas in chronic tendinopathy both intrinsic and extrinsic factors interact (Williams 1993; Khan and Maffulli 1998).

1.1.3.1 Acute traumatic injury on Achilles tendon

Traumatic AT injuries are often associated with sport activities, although AT is the strongest tendon in human body, sudden, excessive strain of AT from athletic or recreational activities can cause tears or even rupture the AT (Kannus and Natri 1997). Statistics show that 60-75% of AT ruptures happen during sport activities, especially in soccer, badminton and basketball (Kannus and Natri 1997). Most of the AT ruptures are associated with sudden acceleration and jumping, that is those activities which require massive sudden force generation from muscle, and the rupture site tend to locate at 2 to 6 cm above the calcaneus-Achilles tendon junction. Interestingly the left tendon is more commonly affected than the right tendon (Hattrup and Johnson 1985; Leppilahti and Orava 1998).

In acute traumatic injuries, AT failure is mainly due to excessive strain caused by sudden explosive power produced by the triceps surae (Langberg, Skovgaard et al. 1999). Hoffmeyer et al. found some pathological changes including increased lipid droplet and ultrastructural muscle changes indicating partial ischemia in the triceps
surae muscle after AT rupture, and this might contribute to the abnormal stiffness in the triceps muscle causing excessive muscle contraction. (Hoffmeyer, Freuler et al. 1990)

1.1.3.2 Chronic Achilles tendinopathy

Tendinopathy of the AT is a chronic non-inflammatory, degenerative condition, which mainly affect male runners between 35 and 45 years old(Alfredson and Lorentzon 2000). Tendinopathy is normally characterized by matrix disorganization, hypercellularity and vascular hyperplasia (Teitz, Garrett et al. 1997). In some cases heterotopic mineralization was considered as a feature of tendinopathy as well (Jarvinen, Jozsa et al. 1997). The clinical symptom of tendinopathy in the AT is pain, which generally happens at the beginning and the end of training (Maffulli, Sharma et al. 2004). As the pathological process progresses, it can affect daily activities. Approximately 3-10% of chronic Achilles tendinopathy develop to AT ruptures (Khan, Cook et al. 1999).

Traditionally, overuse is considered as an extrinsic factor of Achilles tendinopathy. Therefore, tendinopathy mainly affects runner, dancers and gymnasts, who constantly and repetitively overload their tendon (Teitz, Garrett et al. 1997). During slow walking, human AT is subjected to ~3kN force, and it can reach up to 9kN at a speed of 6m/s, which corresponds to 12.5 times body weight (Komi, Fukashiro et al. 1992). Moreover, the peak stress of AT is more than twice that of other tendons in human body (Wren, Yerby et al. 2001). Although tendinopathy affects such a large population, the exact aetiology remains uncertain beyond the catch-all ‘overuse’. There are several theories
proposed, including vascular supply, hypoxia, hyperthermia, apoptosis and abnormal biomechanical environment.

1.1.3.2.1 **Vascular supply**

When tendon is subjected to mechanical loading, microruptures or damage of collagen fibrils start to accumulate, and need to be repaired by tenocytes. However, the vascular supply of the “high risk, high stress zone” (2-6 cm above the calcaneus insertion) is relatively poor compared to the other parts of the tendon, presumably due to the hostile mechanical environment (Hattrup and Johnson 1985; Carr and Norris 1989; Leppilahti and Orava 1998). Under the situation of repetitive damage without enough blood supply, the repair mechanism may be unable to maintain the tendon’s structural integrity, and then the AT undergoes degeneration (Kannus and Jozsa 1991).

1.1.3.2.2 **Hypoxia**

The oxygen supply of tendon and ligament is only 13% that of skeletal muscles (Kannus and Jozsa 1991). The degree and duration of the hypoxia plays a key role in cellular viability (Nathan 2002). Several studies suggest that a hypoxic environment can induce inflammatory cytokines, including interleukin-6 (IL-6), interleukin-8 (IL8), monocyte chemotactic protein 1 (MCP1) and Platelet-derived growth factor (PDGF), and so might significantly disrupt the balance between reparative and degenerative processes in the tendon (Berse, Hunt *et al.* 1999; Zamara, Galastri *et al.* 2007; Millar, Reilly *et al.* 2012).
Moreover, hypoxia has been shown to increase the total collagen production but with a ‘shift’ in production to type III collagen instead of type I, thus disturbing the original collagen composition ratio (Millar, Reilly et al. 2012). Lastly, when tendon repair mechanisms are activated, tendon requires oxidative energy metabolism to maintain cellular ATP levels. That is local tissue hypoxia may result in reduced repair capacity and/or tenocyte death (Birch, Rutter et al. 1997).

1.1.3.2.3 Hyperthermia

During locomotion, 5% to 10% of energy stored in tendons is converted into heat (Ker 1981; Riemersma and Schamhardt 1985). Due to the hypovascular structure of tendon, the blood supply to the structure is not sufficient to dissipate the heat generated. The temperature of equine superficial digital flexor tendon has been recorded to reach 45°C during galloping (Wilson and Goodship 1994). The in vitro study of Birch et al. indicates that tenocytes appear to have high thermal tolerance; even under 45°C culture condition the viability of tenocyte is still unaffected. However, compromised cellular function might be induced by repeated exposure to short periods of hyperthermia, as it has been shown to be associated with reduced collagen synthesis and disturbed cell metabolism (Arancia, Crateri Trovalusci et al. 1989; Birch, Wilson et al. 1997).
1.1.3.2.4 Apoptosis

Significant tenocyte apoptosis has been found in tendinopathy in various tendons, including rotator cuff and AT (Yuan, Wang et al. 2003; Chen, Wang et al. 2010; Wang, Lin et al. 2013). Deformation of the cytoskeleton of tenocytes can produce stress-activated protein kinase that triggers apoptosis (Arnoczky, Tian et al. 2002; Skutek, van Griensven et al. 2003). In ruptured tendon, apoptotic cell number is elevated compared to normal tendon (Chen, Willers et al. 2007). For example, quadriceps femoris tendons with tendinopathy exhibited a 1.6 times higher apoptosis rate than normal tendon (Machner, Baier et al. 2003).

1.1.3.2.5 Biomechanical environment

As a force transferring tissue, it is unsurprising that the biomechanical environment is essential to tendon homeostasis; overloading and underloading can both lead to tendon abnormality. Repetitive and excessive loading may result in release of cytokines by tenocytes leading to abnormal cellular activities (Leadbetter 1992). The release of cytokines may induce the expression of MMPs, which can degrade the extracellular matrix and eventually causes tendinopathy (Chen, Yu et al. 2011). However, loading deprivation of tenocyte can cause direct upregulation of MMP-1 (Choi, Kondo et al. 2002). It has been reported that mechanical stimulation is able to reduce MMP-1 expression (Lavagnino, Arnoczky et al. 2003). Due to the different protocols that have been adopted in tendon studies on the effect of mechanical stimulation on tendon, simple comparison is often difficult.
Compared to the tendon-muscle junction and the mid-tendon, the biomechanical environment of the bone-tendon junction is more complicated. The attachment of tendon is through fibrocartilage to mineralized fibrocartilage to bone in relatively a short distance (<2mm), which is called as ‘the enthesis organ’ (Benjamin, Moriggl et al. 2004). Between the tendon and the bone is a bursa, and fibrocartilage is expressed on the opposing bone and tendon surface to absorb the compression of the tendon against the bone, as shown in Figure 1.4 (Cook and Purdam 2012). In most of the clinical cases, the abnormal imaging findings and the reports of tendinopathy on tendon-bone insertion are more likely at the site of compression proximal to the tendon insertion, which indicate that abnormal compression might be able to trigger tendinopathy (Ohberg and Alfredson 2003; Kong, Van der Vliet et al. 2007). The study of Grigg et al. suggests that excessive loading on the tendon will cause the loss of bound water normally presented in the transitional zones (Grigg, Wearing et al. 2009), which further increases the loads carried by tenocytes located in these regions. To respond to the excessive compression, tenocyte start to synthesis large water binding proteoglycans to reduce the permeability, thereby protecting against further insult. Further loading on this swollen region may aggravate this situation and potentially result in tendinopathy (Hamilton and Purdam 2004; Parkinson, Samiric et al. 2010).
1.1.4 Pathology of tendon injuries

1.1.4.1 Pathology of tendinopathy

“Tendinitis” has been used for definition of tendon degeneration in tendinopathy for over 20 years (Puddu, Ippolito et al. 1976). Although the fundamental problem of tendinopathy is collagen degeneration instead of inflammatory, many clinicians still use the term of “tendinitis”. Most of the scientists nowadays have advocated the term of “tendinopathy” for description of the clinical degeneration condition in and around tendons caused by overuse, while the term “tendinitis” is only used after histopathological examination (Maffulli, Khan et al. 1998).
Repetitive overloading on tendon was commonly considered as a cause of tendinopathy. Due to repetitive loading during daily activities, Achilles tendon is one of the most common tendons affected by tendinopathy. Histologically, tendinopathy is mainly characterized by collagen fiber disorientation and thinning, scattered vascular ingrowth, cell rounding, changes of cell density and increased expression of type III collagen, in some cases, glycosaminoglycan (GAG) accumulation, lipid droplets accumulation, ossification and increased cell apoptosis (Leadbetter 1992; Khan and Maffulli 1998).

Various types of degeneration can be found in different tendons, however, in AT, mucoid and lipoid are the most common types. In mucoid degeneration, vacuoles and mucoid patches characterized by accumulation of proteoglycan/GAG are found between the collagen fibers (Maffulli, Sharma et al. 2004). However, in lipoid degeneration, the collagen structure of tendon is disrupted by the abnormal accumulation of lipid (Maffulli, Sharma et al. 2004). An abnormally high expression of type III collagen is found in degenerated tendon (Riley, Harrall et al. 1994).

1.1.4.2 Healing process

Tendon injuries can be divided into two categories, traumatic injury and chronic tendinopathy. The tissue response to these two types of injuries are not quite the same (Leadbetter 1992). The healing process of tendon has been studied in both human and animals; however, most are concerned with traumatic injuries healing, whilst the
(inadequate) healing response in degenerative tendon is still poorly understood.

1.1.4.2.1 Acute traumatic injury healing

Tendon healing after acute traumatic injury requires reestablishment of the connection between collagen fibers and the gliding mechanism between tendon and its neighboring structure (Schneewind, Kline et al. 1964; Abrahamsson and Gelberman 1994). Formation of scar tissue provides initial repair at the injury site (Dunphy 1967). However, a lack of mechanical stimulation is thought to lead to an excess of scar tissue, adhesions, and so compromises the normal tendon function. Therefore, the rehabilitation procedure after initial immobilization of the injury site is critical, as the mechanical loading is able to guide organized collagen fiber formation, decrease the formation of postoperative adhesions and increase tendon strength (James, Kesturu et al. 2008).

After acute tendon injury, the body initiates the healing process, which includes three overlapping stages including: (1) acute inflammation; (2) proliferation and (3) remodeling (Goodship, Birch et al. 1994).

The acute inflammation phase begins right after the injury and lasts 1 to 2 week depending on the severity of the injury. Clinically, the inflammation phase is characterized by well-known signs of inflammation such as heat, pain and swelling.
(Goodship, Birch et al. 1994). Histologically, injuries on the tendon cause the formation of hematomas in the tendon sheath, which release various chemotactic factors and pro-inflammatory molecules. These molecules attract inflammatory cells such as neutrophils, monocytes and macrophages to migrate from surrounding tissue to the wound site where the cellular debris and foreign body matter are engulfed and resorbed by phagocytosis(Maffulli, Sharma et al. 2004). Meanwhile, tenocytes are recruited to the site and start to synthesize various extracellular matrix components and reestablish vascular networks (Lindsay and Birch 1964; Myers and Wolf 1974).

During the proliferation stage, tenocytes continue to be recruited and proliferate to accelerate the repair process at the wound site. However, the newly synthesized ECM is mainly a disorganized type III collagen(Garner, McDonald et al. 1989). An extensive vascular network is formed and the wound exhibits scar-like tissue.

During the remodeling stage, 6-8 weeks after injury, cellular proliferation, matrix synthesis, type III collagen expression start to decrease, and collagen type I synthesis increases to replace the type III collagen (Liu, Yang et al. 1995). Type I collagen fibers provide the long-term mechanical support of the regenerated tissue. However, it is uncertain as to whether the repaired tissue achieves the strength of the original tissue(James, Kesturu et al. 2008).
1.1.4.2.2 Chronic healing

Chronic healing processes in chronic tendinopathy are different from the acute healing case described above. In normal tendon, type I collagen, the predominant collagen, is well organized along the axis of the tendon. However, in tendinopathy, the collagen structure is disorganized and type III collagen is highly expressed. In the normal healing process, type III collagen is synthesized by tenocytes as a temporary “band aid” (Maffulli, Khan et al. 1998). During the remodeling stage, type III collagen is replaced by collagen type I which is more resistant to mechanical loading. However, the repetitive and chronic damage in chronic tendinopathy keeps the high expression of collagen type III in tendon without shifting to collagen type I (Riley, Harrall et al. 1994). Due to the failure to complete this final remodeling stage, the tendon is gradually weakened; and eventually leads to rupture even at ‘low’ daily activities (Hamada, Okawara et al. 1994).

1.1.4.3 Molecules involved in tendon remodeling

1.1.4.3.1 Inflammatory mediators in tendon degeneration

Although tendinopathy is considered to be a degenerative process, rather than inflammation, inflammatory mediators still play an important role. Interleukin (IL)-6 and IL-1β have been identified as two of the most important inflammatory mediators and their functions are similar (Chen, Yu et al. 2011). IL-6 and IL-1 are both able to induce Prostaglandin-endoperoxide synthase 2 (COX2), which stimulates the expression of PGE2 and the acute phase of an inflammatory response in tendon (Chen, Yu et al.
2011). The inflammatory response alters the cell homeostasis causing apoptosis of tenocytes which initiates the pathogenesis of tendon. IL-6 and IL-1β have also been shown to stimulate MMP1 and 3 expression (Archambault, Tsuzaki et al. 2002; Chen, Yu et al. 2011), which can degrade collagen type I and collagen–associated small proteoglycans respectively. Therefore, the upregulation of these inflammatory mediators can cause matrix degradation.

The induction of IL-6 and IL-1β can be divided into chemical and mechanical stimulation. Macrophage inhibitory factor (MIF) and Substance P (SP) have been reported to upregulate IL-6 and IL-1β expression in tenocytes (Hart, Archambault et al. 1998; Nguyen, Lue et al. 2003; Morand, Leech et al. 2006). Tenocytes subjected to cyclic strain increase the production of IL-6 and IL-1β (Skutek, van Griensven et al. 2001; Archambault, Tsuzaki et al. 2002; Tsuzaki, Bynum et al. 2003). The regulatory network between inflammatory cytokines and tenocyte apoptosis remains unclear. It is possible that in a routine process, normal loading induces cytokines in tissues that trigger apoptotic cell death to remove the damaged cells. However, over-loading may cause excessive production of cytokines and lead to excessive apoptosis and ultimate tissue degeneration (Millar, Wei et al. 2009).

1.1.4.3.2 Enzyme in tendon degeneration

There are two kinds of metalloproteinase involved in tendon degeneration, which are Matrix metalloproteinase (MMPs) and A Disintegrin and Metalloproteinase with
Thrombospondin Motifs (ADAMTS).

MMPs are zinc-dependent endopeptidases that are able to degrade all the components of the extracellular matrix. MMPs can be classified into four main groups: collagenase, gelatinases, stromelysins and membranes type MMPs (Bramono, Richmond et al. 2004). Tendon degradation is initiated by MMPs (Riley, Curry et al. 2002). ADAMTS family, which are also known as “aggrecanases”, are able to degrade proteoglycans, however, the precise ADAMTSs that are involved in the degradation of tendon proteoglycans remains unclear.

The regulation network of metalloproteinases is very complicated, but includes transcription, activation and inhibition by tissue inhibitors of metalloproteinase (TIMPs) (Nagase, Visse et al. 2006). There are 23 MMPs and 19 ADAMTS in humans, almost all of which can be detected in Achilles tendon, although the expression levels vary widely. These enzymes are essential regulators in cellular activities, matrix remodeling, and pathologic processes (McCawley and Matrisian 2001). The main members of the MMP family involved in tendon remodeling are summarized in Table 1.1 (Magra and Maffulli 2005). In injured tendon, including acute tears and chronic tendinopathy, increased expression of MMP1, 2, downregulation of MMP3 and TIMP2,3,4 are found (Ireland, Harrall et al. 2001; Choi, Kondo et al. 2002; Riley, Curry et al. 2002; Lo, Marchuk et al. 2004). However, TIMP1, which is upregulated in acute tendon tears, is inhibited in tendinopathy (Ireland, Harrall et al. 2001; Choi, Kondo et al. 2002; Lo, Marchuk et al.
2004). The balance of MMPs and TIMPs is important to tendon homeostasis. Over expression of MMPs will cause the pathogenesis of tendinopathy (Riley, Curry et al. 2002; Lo, Marchuk et al. 2004; Jones, Corps et al. 2006).
<table>
<thead>
<tr>
<th>Name</th>
<th>Synonym</th>
<th>Degrades</th>
<th>Other functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP1</td>
<td>Collagenase-1, Interstitial collagenase, Fibroblast collagenase</td>
<td>Collagen type I, II, III, VII, VIII, and X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP2</td>
<td>72 kDa gelatinase A type IV gelatinase</td>
<td>Collagens type IV, V, VII, X, and XI, Fibronectin, elastin, proteoglycans, gelatin</td>
<td>Synergistic with MMP1</td>
<td>(Goupille, Jayson et al. 1998; Bramono, Richmond et al. 2004; Magra and Maffulli 2005)</td>
</tr>
<tr>
<td>MMP3</td>
<td>Stromelysin-1, Transin</td>
<td>Collagens III, IV, V, and IX, proteoglycans, laminin, fibronectin, gelatin</td>
<td>Broad substrate specificity, Activates pro-MMPs</td>
<td>(Goupille, Jayson et al. 1998; Bramono, Richmond et al. 2004; Magra and Maffulli 2005)</td>
</tr>
<tr>
<td>MMP7</td>
<td>Pump-1</td>
<td>Gelatin, proteoglycans, fibronectin, elastin, casein</td>
<td>Activates pro-MMP1</td>
<td></td>
</tr>
<tr>
<td>MMP8</td>
<td>Neutrophil collagenase</td>
<td>Collagens type I, II, and III, aggrecan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP9</td>
<td>92 kDa gelatinase-B</td>
<td>Collagens type IV, V, X, XI, gelatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP10</td>
<td>Stromelysin-2, Transin-2</td>
<td>Collagens type III, IV, and V, gelatin, fibronectin,</td>
<td>Activates pro-MMPs</td>
<td></td>
</tr>
<tr>
<td>MMP11</td>
<td>Stromelysin-3</td>
<td>Aggrecan, fibronectin, laminin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP12</td>
<td>Macrophage metalloelastase</td>
<td>Collagen types I and IV, aggrecan, fibronectin, laminin, entactin, gelatin type I, vitronectin, fibrillin, elastin, c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP13</td>
<td>Collagenase-3</td>
<td>Collagens types I, II and III, gelatin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.1.4.3.3 Growth factor in tendon repair

PDGF-β is another important cytokine in tendon and ligament repair. It is able to promote chemotaxis, cell proliferation, ECM production, surface integrin expression, and revascularization in tendon and ligament (Nakamura, Shino et al. 1998; Nakamura, Timmermann et al. 1998; Harwood, Goomer et al. 1999). Treatment of PDGF-β in a small animal injury model of tendon significantly improved the enthesis structure and biomechanical properties (Hildebrand, Woo et al. 1998; Chan, Fu et al. 2006). In a sheep rotator cuff injury model, scaffolds infused with PDGF-β achieved a better histologic score and mechanical properties than an empty scaffold group (Hee, Dines et al. 2011).

The Transforming growth factor-β (TGF-β) family is essential for tendon development. It has been shown to stimulate collagen synthesis, cell proliferation and migration. Furthermore, it is a key growth factor that modulates the scar tissue following a wound (Chang, Most et al. 1997; Kashiwagi, Mochizuki et al. 2004; Galatz, Rothermich et al. 2007; Kim, Galatz et al. 2011; Kovacevic, Fox et al. 2011). In tendon healing, TGF-β1 expression elevates during the inflammatory phase and promotes collagen production and cell proliferation (Sporn, Roberts et al. 1986; Kannus and Jozsa 1991), while TGF-β3 is able to reduce scar tissue formation during tendon repair (Galatz, Rothermich et al. 2007; Kim, Galatz et al. 2011; Kovacevic, Fox et al. 2011). In a rat rotator cuff injury model, the tendon-bone junction displayed better mechanical properties in the group supplemented with TGF-β3, compared to
the TGF-β1 group (Kim, Kang et al. 2007; Kim, Galatz et al. 2011; Manning, Kim et al. 2011).

The Bone morphogenetic proteins (BMP) family is part of the TGF-β superfamily. Some BMPs are able to stimulate bone and cartilage formation and induce mesenchymal stem cell to differentiate into the cartilage or bone lineage (Ducy and Karsenty 2000). However, instead of osteogenic and chondrogenic induction, BMP-12 is able to induce tendon formation, and is important for tendon healing (Lou, Tu et al. 2001). Treatment of BMP-12 in a tendon injury model showed a better organized tendon tissue, higher volumes of collagen type I and improved mechanical properties (Forslund, Rueger et al. 2003; Majewski, Betz et al. 2008).

The insulin-like growth factor IGF-1 expression increases during the initial inflammatory phase of tendon healing and is able to stimulate the migration and proliferation of fibroblasts and inflammatory cells to the wound site. Several small animal studies have suggested that IGF-1 has the ability to accelerate the tendon healing by promoting cell proliferation and ECM production (Abrahamsson 1991; Abrahamsson, Lundborg et al. 1991; Dahlgren, Nixon et al. 2001; Dahlgren, van der Meulen et al. 2002).

VEGF is not a common cytokine in human tendon, but is expressed in the areas with
high microvascular density during tendon healing (Petersen, Pufe et al. 2003). VEGF treatment has been shown to increase the vessel formation from 1 to 8 weeks after tendon rupture (Hou, Mao et al. 2009); however, the mechanical properties improvement was not significant except for in the first week (Zhang, Liu et al. 2003).

bFGF is secreted by tenocytes and inflammatory cells at the injury site of tendon. It can stimulate cell proliferation, cell migration, collagen synthesis and angiogenesis (Chan, Chan et al. 1997; Chan, Fu et al. 2000; Thomopoulos, Harwood et al. 2005; Tang, Cao et al. 2008). Increased cellularity associated with reduced collagen type I and III expression in injury tendon was observed with additional bFGF in the early healing process (Chan, Fu et al. 2008; Thomopoulos, Das et al. 2010), while collagen synthesis levels increased after two weeks treatment, suggesting a prolonged phase of proliferation can potentially lead to better tendon repair (Sahoo, Toh et al. 2010).
Table 1. Summary of cytokines involved in tendon healing and the main function

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-β</td>
<td>Promote chemotaxis, cell proliferation, extracellular matrix production, surface integrin expression, and revascularization</td>
<td>(Nakamura, Shino et al. 1998; Nakamura, Timmermann et al. 1998; Harwood, Goomer et al. 1999)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Stimulate collagen synthesis, cell proliferation and migration</td>
<td>(Chang, Most et al. 1997; Kashiwagi, Mochizuki et al. 2004; Galatz, Rothermich et al. 2007; Kim, Galatz et al. 2011; Kovacevic, Fox et al. 2011)</td>
</tr>
<tr>
<td>BMP-12</td>
<td>Induces tendon and ligament tissue formation</td>
<td>(Fu, Wong et al. 2003)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Stimulate the migration and proliferation of fibroblasts and inflammatory cells to the wound site</td>
<td>(Abrahamsson 1991; Abrahamsson, Lundborg et al. 1991; Dahlgren, Nixon et al. 2001; Dahlgren, van der Meulen et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Increasing cellular proliferation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enhance matrix synthesis, improving tendon mechanical properties</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Essential for initial vascular plexus formation and granulation tissue development</td>
<td>(Molloy, Wang et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Stimulate vascular bud formation and endotheliocyte migration during neo-vascularization</td>
<td></td>
</tr>
<tr>
<td>bFGF</td>
<td>Promote cell proliferation, cell migration, collagen production, and angiogenesis</td>
<td>(Chan, Chan et al. 1997; Chan, Fu et al. 2000; Takahasih, Nakajima et al. 2002; Thomopoulos, Harwood et al. 2005; Tang, Cao et al. 2008)</td>
</tr>
</tbody>
</table>
1.1.5 Treatments

Although our understanding of tendon biology and the healing mechanism has improved tremendously recently, and various treatments have been adopted for tendon injury, the outcomes remain unsatisfactory. The goal of treatment for Achilles tendon diseases is to reduce pain, promote healing and restore its functional mechanical properties. Surgical and so-called conservative treatments (e.g. physiotherapy) are the most common procedures. However in recent years achievements in tissue engineering in animal studies has begun to offer hope for an alternative solution to tendon injury.

1.1.5.1 Surgical treatment

1.1.5.1.1 Chronic tendinopathy

Surgery is often considered as a last choice for treating tendinopathy. Patients who fail to improve after a period of conservative treatments tend to be subjected to surgical treatment, but the results are not uniformly satisfactory. Various surgical procedures have been described in different studies. They can be divided into four categories: open tenotomy with abnormal tissue removal, paratenon stripped (Paavola, Orava et al. 2000); open tenotomy with abnormal tissue removal, paratenon not stripped (Leach, Schepsis et al. 1992); open tenotomy with longitudinal tenotomy (Rolf and Movin 1997); and percutaneous longitudinal tenotomy (Maffulli, Testa et al. 1997). In the study of Tallon et al., of the 1648 cases of surgically treated Achilles tendinopathies the success rate was 77.4% (Tallon, Coleman et al. 2001)
1.1.5.1.1 Acute rupture

In acute traumatic injuries such as Achilles tendon rupture, early surgery is preferred. Reconstructive surgery is the common option for athletes, young people, and patients with chronic ruptures (Christensen 1953; Krueger-Franke, Siebert et al. 1995). However there is still no agreed protocol for the management of ruptured Achilles tendons (Maffulli 1995; Nyystonen and Luthje 2000) or which operative technique gives the best outcomes (Bugg and Boyd 1968; Hogsaa, Nohr et al. 1990). Ruptured Achilles tendons can be classified as open operative, percutaneous operative and non-operative based on the modalities of management. In open surgery, some studies reported high complication and skin healing problems (Gillespie and George 1969; Nistor 1981; Bomler and Sturup 1989), while others show little or a low rerupture rate (Goldman, Linscheid et al. 1969; Inglis and Sculco 1981; Cetti and Christensen 1983; Beskin, Sanders et al. 1987; Zell and Santoro 2000). Percutaneous repair is another technique that is able to minimizes skin-healing problems under local anesthetic (Ma and Griffith 1977). However, compared to open repair, percutaneously repaired Achilles tendons had higher rerupture rates and thinner diameter (Bradley and Tibone 1990; Cretnik, Kosanovic et al. 2005).

1.1.5.2 Conservative treatment

Conservative treatments are commonly adopted when the clinical symptoms, including pain and restricted motion, first appear. Current conservative treatments include rest, non-steroid anti-inflammation drug (NSAIDs), glucocorticosteroid
injection, platelet-rich plasma therapy, physiotherapies and physical modalities.

1.1.5.2.1  Rest

In the early stage of injury, rest with proper protection including cast is an effective treatment to prevent repetitive injury caused by mechanical overload and allow the self-repair of tendon (Darlington and Coomes 1977).

1.1.5.2.2  NSAIDs

NSAIDs are commonly used to reduce the pain and inflammation in soft-tissue injuries. NSAIDs are able to block the acute inflammatory response through non-specific cyclo-oxygenase inhibition. Although pain can be reduced by this treatment, the use of NSAIDs in tendon injury remains controversial. Various studies showed that they are potentially deleterious to tissue healing and might decrease the mechanical properties of tendons (Kulick, Smith et al. 1986; Weiler, Unterhauser et al. 2002; Forslund, Bylander et al. 2003).

1.1.5.2.3  Glucocorticosteroid Injection

Glucocorticosteroid injection has been used since the 1950s as a pain management method for tendinopathy. The glucocorticosteroid is injected in and around the chronic tendon injury. They are reported to reduce pain and recover the range of
motion to prevent stiffness (Darlington and Coomes 1977; Blair, Rokito et al. 1996). Although the improvement following glucocorticosteroid injection is significant, the effects are often temporary (Coombes, Bisset et al. 2010). There are also several cases reported that tendon rupture followed corticosteroid injection, including Achilles tendon rupture (Ford and DeBender 1979; Kleinman and Gross 1983). Moreover, complications include weakened mechanical properties (Wiggins, Fadale et al. 1995; Tillander, Franzen et al. 1999) and aggravated pain (Goldfarb, Gelberman et al. 2007). In chronic tendinopathy, the absence of inflammation provides no basic target to adopt steroid injection. It is worth mentioning that the study of Kabata et al. showed steroid injection induced osteonecrosis-like lesions and cell apoptosis around the lesions (Kabata, Kubo et al. 2000).

1.1.5.2.4 Platelet-rich Plasma (PRP) Therapy

Platelet-rich plasma (PRP) therapy is a technology that aims to deliver bioactive agents to the injury site, to enable the activation of proliferative and anabolic cellular response to then enhance the repair mechanism of the tissue (Anitua, Sanchez et al. 2006). PRP has been proposed as a treatment for various orthopaedic disorders and conditions. Clinical use of PRP on tendon-related injuries and disorders is over a decade, but the outcomes remained conflicting. There are 2 different Food and Drug Administration (FDA) approved PRP extraction methods, SMARTPEP (SmartPREP, Harvest Technologies Corp., Norwell, MA) and Platelet Concentrating Collection Systems (3i/Implant Innovations, Palm Beach Gardens, FL)(Arora, Ramanayake et al. 2009). To obtain the PRP clinical trial data on human Achilles tendon, key words
‘PRP’ and ‘Achilles tendon’ were used in Pubmed, initially, 32 articles were identified. Of these, 9 were clinical studies, which were summarized in Table 1.3. The clinical outcome of PRP on Achilles tendon varied widely. In clinical studies, Sanchez et al. reported that PRP injection followed by surgical repair of ruptured Achilles tendon showed better and faster recovery at 6 months(Sanchez, Anitua et al. 2007), whilst Schepull et al. reported no significant improvement in biomechanical tests and a worse clinical outcome compared to the non-injection group over 12 months(Schepull, Kvist et al. 2011). Several case studies suggested that patients receiving PRP treatment for Achilles partial rupture and tendinopathy showed significant reduction in pain and clinical improvement(Filardo, Presti et al. 2010; Gaweda, Tarczynska et al. 2010; Finnoff, Fowler et al. 2011; Owens, Ginnetti et al. 2011; Monto 2012), whilst in randomized trials, de Vos et al. reported clinical improvement in both groups but no significant different between the PRP injection group and saline injection group(de Vos, Weir et al. 2010). Despite the fact that the clinical effect of PRP treatment still remains controversial, no complication has been reported from its clinical application(Mishra, Randelli et al. 2012). The conflicted published data implied that the PRP treatment is still immature, but this technique still has great potential for musculoskeletal medicine and orthopaedic surgery.
Table 1. Clinical PRP treatment in Achilles tendon

<table>
<thead>
<tr>
<th>Authors &amp; year</th>
<th>Level of evidence</th>
<th>Disease</th>
<th>Patients number</th>
<th>Follow up</th>
<th>outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanchez et al. 2007</td>
<td>Case series, level 3</td>
<td>Achilles Tendon rupture</td>
<td>6</td>
<td>6 months</td>
<td>Faster recovery and better clinical outcome for PRP</td>
<td>(Sanchez, Anitua et al. 2007)</td>
</tr>
<tr>
<td>Schepull et al. 2011</td>
<td>Randomized trial, level 2</td>
<td>Achilles Tendon rupture</td>
<td>16 PRP vs. 14 control</td>
<td>12 months</td>
<td>Better clinical outcome in control group, no significant difference in mechanical properties</td>
<td>(Schepull, Kvist et al. 2011)</td>
</tr>
<tr>
<td>de Vos et al. 2010</td>
<td>Randomized trial, level 1</td>
<td>Achilles tendinopathy</td>
<td>27 PRP vs 27 saline solution</td>
<td>12 months</td>
<td>Clinical outcome improved but no intergroup significant difference</td>
<td>(de Vos, Weir et al. 2010)</td>
</tr>
<tr>
<td>Filardo et al. 2010</td>
<td>Case series</td>
<td>Achilles Tendon partial rupture</td>
<td>1</td>
<td>18 months</td>
<td>Quick return to full pre-injury sport</td>
<td>(Filardo, Presti et al. 2010)</td>
</tr>
<tr>
<td>Gaweda et al. 2010</td>
<td>Case series</td>
<td>Achilles tendinopathy</td>
<td>14</td>
<td>18 months</td>
<td>Reduced pain and recovered function</td>
<td>(Gaweda, Tarczynska et al. 2010)</td>
</tr>
<tr>
<td>Finnoff et al. 2011</td>
<td>Case series</td>
<td>Achilles tendinopathy</td>
<td>14</td>
<td>14 months</td>
<td>Clinical improvement</td>
<td>(Finnoff, Fowler et al. 2011)</td>
</tr>
<tr>
<td>Owens et al. 2011</td>
<td>Case series level 4</td>
<td>Achilles tendinopathy</td>
<td>10</td>
<td>24 months</td>
<td>Clinical improvement, no MRI assessment improvement</td>
<td>(Owens, Ginnetti et al. 2011)</td>
</tr>
</tbody>
</table>
Table 1.4 Clinical PRP treatment in Achilles tendon

<table>
<thead>
<tr>
<th>Monto et al. 2012</th>
<th>Case series, level 4</th>
<th>Achilles tendinopathy</th>
<th>30</th>
<th>24 months</th>
<th>Significant clinical improvement in 28 patients</th>
<th>(Monto 2012)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrero et al 2012</td>
<td>Case series</td>
<td>Achilles and patellar tendinopathy</td>
<td>30 Achilles tendon, 28 patellar tendons</td>
<td>6 months</td>
<td>Significant clinical improvement</td>
<td>(Ferrero, Fabbro et al. 2012)</td>
</tr>
</tbody>
</table>
1.1.5.2.5 Physical modalities

Physical modalities such as low-energy shock-wave (Rompe, Furia et al. 2008), laser (Bjordal, Lopes-Martins et al. 2006), therapeutic ultrasound (Baysal, Bilsel et al. 2006) and heat (Giommini, Di Cesare et al. 2006) have also been used as a treatment for tendon and ligament injuries. These modalities were suggested to be able to relieve pain by altering the local vascular system and improve the mechanical properties by simulating collagen production. Studies showed the success rate of physical modalities varies widely; the range is from less than 50% to about 80% (Samilson and Binder 1975; Chard, Sattelle et al. 1988; Hoying and Williams 1996; Morrison, Frogameni et al. 1997; Goldberg, Nowinski et al. 2001). Due to recurrence on longer follow-ups, the success rate was reported to drop to around 50% in recent study (Baysal, Bilsel et al. 2006; Bjordal, Lopes-Martins et al. 2006; Rompe, Furia et al. 2008). It was suggested that better results are achieved in patients with minor symptoms.

1.1.5.2.6 Cell Therapy

Cell therapy is the procedure to deliver new cells into a tissue to stimulate the tissue regeneration. Cell therapy for tendon repair has achieved great success in a rabbit model, however, the argument about the best cell type in tendon therapy remains inconclusive. Stem cells, dermal fibroblasts and tenocytes are the most commonly used cell types for tendon healing (Obaid and Connell 2010). Mesenchymal stem cells are a potential candidate due to their rapid proliferation, hypoimmunogenicity and multilineage
differentiation ability(Uccelli, Moretta et al. 2006). In the past decade promising results have been achieved using bone marrow-derived stem cells (BMSCs) in tendon repair. BMSCs therapy in a rabbit model have been reported to stimulate tendon regeneration, achieve a better neo-tendon morphology and improve tendon biomechanical properties (Awad, Butler et al. 1999; Ouyang, Goh et al. 2004; Chong, Ang et al. 2007; Hankemeier, van Griensven et al. 2007). However, the potential risk of ectopic bone formation needed to be considered(Ross, Duxson et al. 1987). Tendon-derived stem cells (TDSCs) were first discovered by Bi et al. in 2007(Bi, Ehirchiou et al. 2007), and they have multi-differentiation potential into musculoskeletal tissue. Ni et al. report that TDSCs-engineered tendon is able to differentiate into tendon-like tissue and repair the tendon defect (Ni, Lui et al. 2012; Ni, Rui et al. 2013). Adipose-derived stem cells (ADSCs) have the advantage of wide availability and are easy to obtain (Obaid and Connell 2010). Uysal et al. reported that the application of ADSCs in a rabbit tendon repair model exhibited better tendon healing and biomechanical properties (Uysal and Mizuno 2010; Uysal and Mizuno 2011; Uysal, Tobita et al. 2012). Synovium-derived stem cells (SDSCs) were first identified by De Bari et al. in 2001 (De Bari, Dell'Accio et al. 2001), and they have proven effective in a wide range of musculoskeletal disorders (Fan, Varshney et al. 2009). The therapeutic effect of SMSCs in the tendon-bone junction has been reported by Tomita et al. and Ju et al.(Tomita, Yasuda et al. 2001; Ju, Muneta et al. 2008). Dermal fibroblasts have also been shown to form tendon tissue (Liu, Chen et al. 2006; Deng, Liu et al. 2009; Woon, Kraus et al. 2011), although further research has suggested that the healing process using skin-derived fibroblasts is suppressed with a lack of tenocyte markers and histopathologic correlations (Chen, Wang et al. 2010; Obaid and Connell 2010). Clinical trials of dermal
fibroblasts on lateral epicondylitis (LE) suggested that it a safe and effective treatment and no significant complication in the majority of patients (Connell, Datir et al. 2009).

Being the native cell source, tenocytes and in situ fibroblasts are arguably the most ideal cell sources for tendon and ligament therapy respectively. Preclinical and early clinical studies using these native cell sources are promising, however, the potential morbidity to the donor site needs to be considered(Cooper, Lu et al. 2005; Lee, Shin et al. 2005; Webb, Hitchcock et al. 2006; Androjna, Spragg et al. 2007; Chen, Willers et al. 2007; Freeman, Woods et al. 2007; Moffat, Kwei et al. 2009; Saber, Zhang et al. 2010). In the most recent clinical trial of autologous tenocyte injection (ATI), patients with chronic LE showed significant improved function and structural repair after ATI, and no adverse event was reported at the biopsy sites(Wang, Breidahl et al. 2013).
Table 1. A summary of cell therapy cell type for tendon healing.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Source</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenchymal Stem Cells (MSC)</td>
<td>Bone marrow-derived</td>
<td>1. Multi-differentiation ability including tenocyte&lt;br&gt;2. High proliferation rate&lt;br&gt;3. Hypoimmunogenicity</td>
<td>1. Accelerate tendon healing&lt;br&gt;2. Improve mechanical properties of tendon&lt;br&gt;Difficult to manipulate the differentiation of stem cell into desirable cell type.</td>
</tr>
<tr>
<td></td>
<td>Tendon-derived</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adipose tissue-derived</td>
<td>1. Wide availability&lt;br&gt;2. Easy to extract&lt;br&gt;3. No damage to donor site</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synovium-derived</td>
<td>Induce bone-tendon healing</td>
<td></td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Skin</td>
<td>1. Wide availability&lt;br&gt;2. No damage to donor site&lt;br&gt;3. Non-invasive procedure for cell harvest</td>
<td>1. Different cell type&lt;br&gt;2. Uncertain behavior in tendon</td>
</tr>
<tr>
<td>Tenocyte</td>
<td>Tendon</td>
<td>Native cell sources</td>
<td>Potential morbidity to donor site</td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
<td>---------------------</td>
<td>----------------------------------</td>
</tr>
</tbody>
</table>

Table 1. A summary of cell therapy cell type for tendon healing.
1.1.5.2.7 Physiotherapies

Physiotherapy has been accepted as one of the mainstays of conservative treatment for chronic tendon injuries. As a force transferring tissue, the biomechanical environment is essential for tendon homeostasis. Eccentric overloading is a common treatment for chronic Achilles tendinopathy. There are two types of eccentric exercises used, maximize the loading on the calf muscle with a straight knee or eccentrically load the soleus muscle with the knee bent (Alfredson, Pietila et al. 1998). Several studies reported positive effect on symptom relief (Stanish, Rubinovich et al. 1986; Alfredson, Pietila et al. 1998; Silbernagel, Thomee et al. 2001; Alfredson and Lorentzon 2003; Fahlstrom, Jonsson et al. 2003; Roos, Engstrom et al. 2004; Shalabi, Kristoffersen-Wilberg et al. 2004), however, the study of Woodley et al. showed no effect (Woodley, Newsham-West et al. 2007) and Rompe et al. report an inferior results (Rompe, Furia et al. 2008). Although the eccentric overloading may be effective for pain relief and tendon repair, the magnitude of loading and the underlying mechanism remain unclear.

1.1.5.2.8 Tissue engineering

Tissue engineering’s aim is to grow tissue in the laboratory and usually involves a procedure that cultures the engineered tissue using different cell sources combined with various bioscaffolds in vitro. The development of tissue engineering is based on the idea that instead of repairing the damaged tissue, it may be better to replace it. The detail is discussed in 1.2.1
1.1.6 Summary

Achilles tendinopathy is a degenerative disease that results in the loss of the functional mechanical properties of tendon and adversely affects the daily activities of those afflicted. Despite the prevalence of those suffering from this condition, our knowledge about tendinopathy remains relatively poor. Although the basic characteristics of tendinopathy that we do understand enable diagnosis, the aetiology of this degenerative process remains unclear. Moreover, none of the current treatments, either conservative or surgical, are able to completely or reliably repair the tendon integrity and restore the tendon mechanical properties. Therefore, further studies are required to both better understand the disease and to develop effective treatment strategies.

1.2 Introduction to Bioreactor Design for Tendon/Ligament Engineering

(This section has been published in ‘Tissue Engineering Part B’ titled “Bioreactor design for tendon/ligament engineering” PDF refer to appendix 1)

Autograph and allograft transplantations are a common surgical treatments for tendon and ligaments injured or degenerated. However, the risks of damage to the donor site from which the autograft are taken, and the potential immune reaction for allografts are major concerns (Coupens, Yates et al. 1992; Cerullo, Puddu et al. 1995; Harner, Olson et al. 1996). A promising translational approach to the treatment of tendon/ligament
injury or degeneration is through the use of engineered autologous grafts made available through the development of bioreactors that generate tendon/ligament tissue in vitro. One common view is that the key to a successful bioreactor is being able to recreate, in vitro, the cell microenvironments that are experienced by cells in vivo. The cell microenvironments can be defined using cell morphological information with data from molecular biology, biochemistry and biomechanics. This review aims to clarify the requirements for a ‘successful bioreactor’ that may be used for tendon/ligament engineering, and to provide an overview of the range of components found in tendon/ligament bioreactors, including custom-made and commercial products. We will also discuss the studies that have involved the application of tendon/ligament bioreactors.

1.2.1 Common key elements for tendon/ligament tissue engineering

Based on the composition and function of tendon/ligament tissue one must consider the four basic elements for their successful regeneration: the cell source, the characteristics of the scaffold matrix and establishing an appropriate chemical and physical cellular microenvironment.

An ideal cell source should meet the following three requirements: availability, rapid proliferation and the ability to differentiate into in situ cells(Arnsdorf, Jones et al. 2009). The details refer to 1.1.5.2.6.
Apart from the selection of the cell source, cell seeding also plays an essential role in the development of engineered tendon and ligament. Several reports indicate that sufficient cell number and a uniform distribution throughout the scaffold is desirable for achieving a homogeneous ECM deposition \textit{in vitro} (Freed, Marquis \textit{et al.} 1993; Freed, Vunjak-Novakovic \textit{et al.} 1993). Compared to lower initial cell seeding density, high seeding density has been shown to result in increasing ECM deposition rate, a higher final cell number and better cellular morphology (Awad, Butler \textit{et al.} 2000; Wang, Seshareddy \textit{et al.} 2009). However, over-seeding also has potential for negative effects on nutrient delivery, and consequently cellular metabolism and cell viability. Nutrient depletion at high cell seeding densities could lead to spatially in homogenous ECM production (Zhang, Gardiner \textit{et al.} 2008). A study by Issa \textit{et al.} showed mechano-stimulated human umbilical veins seeded with 3 million cells/ml had better cellular proliferation rates than other groups(Issa, Engebretson \textit{et al.} 2011). However, optimal seeding density will vary depending on the cell source and the bioscaffold physical and chemical properties, as these properties will affect nutrient transport and rates of consumption, as well as the mechanical and chemical stimuli provided to the attached cells(Zhang, Gardiner \textit{et al.} 2008).

The construct scaffold plays an important role in engineering the new tendon/ligament tissue. Ideally the scaffold should be able to provide substantial initial mechanical strength for its immediate post-implantation functional role, while providing a suitable biological environment for cell migration and proliferation. Furthermore, the degradation rate of the biomaterial needs to be comparable with the rate of tissue
synthesis, to allow the eventual replacement of the starting scaffold with neo-tissue.

Both synthetic and natural biomaterials are commonly used in tendon/ligament engineering. Synthetic polymer scaffolds have the advantage of reproducible mechanical and chemical properties, and they are relatively easy to fabricate into different sizes (Lee, Shin et al. 2005; Pham, Sharma et al. 2006; Sahoo, Ouyang et al. 2006; Sahoo, Cho-Hong et al. 2007; Moffat, Kwei et al. 2009). However, their rapid degradation rate and potential risk of releasing acidic by-products or toxic polyesters during degradation have limited their application in clinical trials. Given these disadvantages, more researchers have turned their focus on exploring natural biomaterials (Derwin, Baker et al. 2006; Juncosa-Melvin, Shearn et al. 2006; Chen, Willers et al. 2007; Gilbert, Stewart-Akers et al. 2007; Nirmalanandhan, Rao et al. 2008; Nirmalanandhan, Shearn et al. 2008; Fleming, Spindler et al. 2009; Kinneberg, Nirmalanandhan et al.). Being the main component of native tendon, collagen type I is the most obvious choice of material. Although the biocompatibility is excellent, the poor mechanical properties of reconstituted type I collagen scaffolds has limited their further development as a load bearing material. Silk fibroin, on the other hand, has similar biocompatibility as collagen scaffolds and comparable mechanical properties as native tendon/ligament. In several in vivo studies, silk fibroin-based engineered ligaments have been proven their ability to restore the function of injured ligament (Chen, Qi et al. 2008; Fan, Liu et al. 2008; Fan, Liu et al. 2009). Another option is the decellularised tendon/ligament construct. Although the mechanical and biological properties are a better match to native tissue than any other currently available scaffolds,
donor cells may remain in the allograft, even with strict sterilization and cleaning, and thus they can potentially cause inflammatory responses (Malcarney, Bonar et al. 2005; Zheng, Chen et al. 2005).

After choosing an appropriate cell source and scaffold type, the tissue needs to be encouraged to develop the properties of native tissue by providing an appropriate biochemical and biomechanical environment to stimulate ECM synthesis. Regarding the biochemical environment, several growth factors have been found to play an important role in tendon/ligament formation and healing. These include Insulin-like growth factor-I (IGF-I), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGFβ) and growth differentiation factor 5 (GDF-5). The roles of TGFβ and GDF-5 seem to be particularly prominent. TGFβ remains active throughout tendon/ligament healing (Chang, Thunder et al. 2000), and is able to regulate cell migration, proteinase expression, fibronectin binding interactions, cell proliferation and collagen production (Bennett and Schultz 1993; Wojciak and Crossan 1994; Marui, Niyibizi et al. 1997; Chang, Thunder et al. 2000). Recent studies demonstrated that tendon and ligament formation was impaired in TGFβ2 and TGFβ3 knockout mouse embryos, reinforce the importance of TGFβs in tendon development and homeostasis (Pryce, Watson et al. 2009). GDF-5 regulates cell growth and differentiation, with a lack of GDF-5 causing delayed tendon healing, irregular collagen type I fibrils and weakened fibril mechanical properties (Clark, Johnson et al. 2001; Mikic, Schalet et al. 2001; Chhabra, Tsou et al. 2003).
How these various biochemical factors should be introduced into the tissue bioreactor system to shape the chemical environment is an extremely challenging and open question. Specifically, at what concentrations, in what combinations and at what sequence or timing should they be made available? Studies have shown that the expression of these factors in tendon repair change over periods of days (Dahlgren, Mohammed et al. 2005; Kobayashi, Itoi et al. 2006; Chamberlain, Crowley et al. 2009).

Presumably as the engineered tissue progresses, cells begin to control their own biochemical environment, and the role of the bioreactor is to now provide the ‘building block’ nutrients and expected systemic signals, along with mechanical stimulus. The early stage in the tissue-engineered tendon is likely to be the most critical in establishing tendon development along a pathway to resemble native tendon. Finding the correct combination of factors is daunting due to the complexity arising from the multitude of possible combinations and interactions. Systematically varying experimental conditions, coupled with computational modeling of transport processes and signaling molecule pathways leading to cell responses, provide the only conceivable means to both understanding and efficiently optimizing the tissue bioreactor system.

Tendon’s primary function is mechanical. It operates in a varying load environment, both on short timescales (e.g. walking, running) and on longer timescales (e.g. changes in body size with age). Tendon responds to its mechanical environment through changes in ECM biosynthesis and degradation. Unsurprisingly, given its functional role, a suitable mechanical stimulus is vital for tendon/ligament homeostasis. In fact, it has
been shown that after 4 weeks in a load-free culture environment tenocytes lose their native elongated morphology, become increasingly rounded, and the collagen fiber becomes more crimped (Hannafin, Arnoczky et al. 1995). In cell-free reconstructed collagen fibril network systems, a tensile load has been shown to be protective to degradation by MMP-8 (Flynn, Bhole et al. 2010). Furthermore, cyclic stretching has been shown to produce an up to 9 fold increase in the cell number of an engineered tendon compared with a static culture over a 2 week period (Abousleiman, Reyes et al. 2009). Finally, an appropriate mechanical environment could help guide collagen fiber formation, i.e. along the direction of loading, which is able to enhance or optimize the mechanical properties including stiffness, elastic modulus, maximum tensile stress and maximum force (Juncosa-Melvin, Shearn et al. 2006; Saber, Zhang et al. 2010; Woon, Kraus et al. 2011).

Rather than being two separate signals, there is crosstalk between mechanical and chemical signals. Recent studies have shown that gradual and temporary loss of tensile loading leads to reversible loss of Scleraxis (Scx) expression, which is a transcription factor specific for tenocytes and their progenitors. In addition, it has been shown that TGFβ directly induced the expression of Scx in cultured tenocytes isolated from mice (Maeda, Sakabe et al. 2011). In Scx-/- mice, a disordered limb tendon phenotype was observed (Lejard, Brideau et al. 2007), and similar phenomenon happened in TGFβ type II receptor gene knock out (Tgfbr2-/-) mice with dramatic loss of Scx expression (Pryce, Watson et al. 2009).
Providing a suitable biomechanical signal is clearly an important component for the success of tendon/ligament engineering. Generating a suitable mechanical signal within the bioreactor system is critically important for tendon/ligament tissue engineering.

1.2.2 Bioreactor design specific to tendon/ligament engineering

Despite the increasing appreciation of tendon/ligament biology and function, conventional culture methods do not seem to meet the biochemical and biomechanical requirements to generate bioengineered tendon/ligament in vitro. A bioreactor system that subjects the ‘cell culture’ to programmable mechanical stimulation (PMS), mimicking the physiological conditions of tendon/ligament in vivo, while allowing cellular proliferation, differentiation and matrix production in a mechanical environment, may provide a solution.

Bioreactors for tendon/ligament engineering are different to the systems that have been used in various other tissue engineering fields in the past decades, i.e. systems for muscle(Dennis, Smith et al. 2009), liver(Catapano, Patzer et al. 2010) and bone(Rauh, Milan et al. 2011; Salter, Goh et al. 2012). Compared to other bioreactors, the main task of the bioreactor for tendon/ligament engineering is to provide the proper biomechanical and biochemical environment specific to tendon/ligament formation. In order to achieve this, certain basic components are required, i.e. the actuating system and the culture chamber, which can provide the construct’s PMS and controlled culture environment respectively. Furthermore, the bioreactor may also include a medium circulation system,
monitoring system, feedback system, and a medium analysis system, depending on the operational requirements (Figure 1.1). With these facts in mind, several custom-made bioreactors have been developed for tendon/ligament engineering (see Table 1.1), and the aforementioned components of these are now are discussed in detail.

Figure 1.1 Schematic demonstration of connection between different components of tendon/ligament bioreactor system
Table 1.1 Components of custom-made bioreactor systems

<table>
<thead>
<tr>
<th>Reference</th>
<th>Actuating system</th>
<th>Culture chamber</th>
<th>Monitor system</th>
<th>Feedback system</th>
<th>Medium circulation system</th>
<th>Medium analysis system</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Altman, Lu et al. 2002)</td>
<td>Biaxial</td>
<td>Multiple</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>(Webb, Hitchcock et al. 2006)</td>
<td>Uniaxial</td>
<td>Single chamber, multiple samples</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>(Juncosa-Melvin, Shearn et al. 2006), (Nirmalanandhan, Rao et al. 2008), (Nirmalanandhan, Shearn et al. 2008), (Butler, Gooch et al. 2010)</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>Displacement</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>(Androjna, Spragg et al. 2007)</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>Force</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>(Nguyen, Liang et al. 2009)</td>
<td>Uniaxial</td>
<td>Single</td>
<td>Force</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>(Abousleiman, Reyes et al. 2009)</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>(Butler, Hunter et al. 2009)</td>
<td>Uniaxial</td>
<td>Single chamber, multiple samples</td>
<td>Displacement Force</td>
<td>×</td>
<td>×</td>
<td>✓</td>
</tr>
<tr>
<td>(Chen, Yin et al. 2010)</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>(Doroski, Levenston et al. 2010)</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>Displacement</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>(Parent, Huppe et al. 2011)</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>Displacement Force</td>
<td>✓</td>
<td>×</td>
<td>×</td>
</tr>
</tbody>
</table>
1.2.2.1 Actuator and culture chamber design

The actuating system is the main component for providing different PMS to engineered tissue. Pneumatic actuators (Juncosa-Melvin, Shearn et al. 2006; Nirmalanandhan, Rao et al. 2008; Nirmalanandhan, Shearn et al. 2008), linear motors (Nguyen, Liang et al. 2009; Doroski, Levenston et al. 2010) and step motor-ball screws (Altman, Lu et al. 2002; Webb, Hitchcock et al. 2006; Chen, Yin et al. 2010) are the most common actuators used in tendon/ligament bioreactors. Pneumatic actuators have several advantages, including the ease of maintenance, cleanliness, low cost and high power-to-weight ratio (Varseveld and Bone 1997). Unfortunately, by using air as a media, pneumatic actuators are subject to high friction. The sensitivity and response to an input signal is relatively slow because of the “dead band” and “dead time” caused by stiction and air compressibility. Due to these nonlinearities, it is difficult to achieve accurate position control with pneumatic actuators (Varseveld and Bone 1997). Typically, the accuracy of pneumatic actuators is approximately ±0.1mm, which is not insignificant when typical tendon bioreactors require <5% strain on 1-5cm tissues. Compared with pneumatic actuators, electrical actuators are more expensive, but the level of accuracy in their positional control is much higher. A direct-drive linear motor is able to provide high-speed/high-accuracy linear motion by eliminating mechanical transmission (Alter and Tsao 1994; Alter and Tsao 1996; Braembussche, Swevers et al. 1996), and the accuracy is the highest of all three actuators; i.e. ~±1µm. Step motor-ball screw transmission systems (SMBS) are based on a ball-screw linkage and a crank-slider mechanism. In general, SMBS transfer the rotation of the crank to a reciprocating motion of the screw (Liu, Hsu et al. 2001). By choosing different ball screws, the optimal speed range and output force can be selected using Equation (1), i.e.
\[ T = \frac{Fl}{2\pi \nu} \]  

where \( T \) is torque applied to screw, \( F \) is linear force, \( l \) is ball screw lead, and \( \nu \) is ball screw efficiency. Although SMBS is not as accurate as a linear motor, a positional accuracy of around \( \pm 5\mu m \) can still be achieved. In a multi-chamber shared loading system, SMBS is widely used given its high accuracy and relative high loading capacity (Altman, Lu et al. 2002; Webb, Hitchcock et al. 2006; Androjna, Spragg et al. 2007). In independent loading multi-chamber systems, linear motors are more popular due to their small size and relatively simple mechanical arrangement (Nguyen, Liang et al. 2009; Parent, Huppe et al. 2011).

In the addition to the actuator, the connection between the mechanical input and the tissue is vital and often presents a major challenge. During PMS of the tissue in culture an even distribution of force throughout the entire sample is critical, otherwise tissue integrity is compromised by overloading the mechanical connection regions and/or by inhomogeneous mechanical stimulation. Different strategies of applying PMS have been adopted based on different construct dimensions. In a study by Chen et al., cell-seeded knitted silk-collagen sponge scaffolds were fixed on stainless rings and connected to sample hooks (Chen, Yin et al. 2010). The bioreactor system by Juncosa-Melvin et al. applied two posts to fix the construct, punching through the scaffold as shown in Figure 1.2 (Juncosa-Melvin, Shearn et al. 2006). However, non-uniform construct deformation is clearly apparent. Tensile force is focused on the side of constructs, which causes uneven distribution of mechanical stimulation. Although tissue clamps are the most
popular and relatively effective method for holding tendon constructs (Webb, Hitchcock \textit{et al.} 2006; Nguyen, Liang \textit{et al.} 2009; Parent, Huppe \textit{et al.} 2011), the potential for damage at the clamping region needs to be considered. The method of reproducibly applying uniform loads to soft tissue without tissue damage or slippage is a critical problem in need of a satisfactory solution. It is our opinion that a robust clamping region should be designed along with the artificial bioscaffold to ensure the proper connection between the sample and the PMS.
The culture chamber is an essential part of whole bioreactor system. The high humidity of culturing conditions (99% humidity, 37°C and 5% CO₂) and chemistry of the culture medium are corrosive to many materials. Corrosion products may in turn be toxic to the tissue. Therefore, noncorrosive and autoclavable material such as stainless steel, polymethylmethacrylate (PMMA), polyoxymethylene, polycarbonate, glass and silicon are preferred and widely used in culture chamber design (Altman, Lu et al. 2002;
The chamber structure is an important consideration in the bioreactor design, with most currently available culture chambers divided into two groups: integrated (Webb, Hitchcock et al. 2006; Butler, Hunter et al. 2009) and separated chambers (Juncosa-Melvin, Shearn et al. 2006; Chen, Yin et al. 2010; Doroski, Levenston et al. 2010) (Altman, Lu et al. 2002; Androjna, Spragg et al. 2007; Parent, Huppe et al. 2011) (Butler, Gooch et al. 2010). In integrated chambers, multiple samples are cultured while sharing the same culture medium. Conversely, separated chambers can provide separate culture environments for each sample. Although the complexity of design and manufacturing costs may be higher in a separated chamber system, reduced cross-contamination and the option of independent environmental control are distinct advantages.

During tissue culture, sufficient air exchange within the culture chamber is critical. Air exchange in conventional cell culture incubators is through the integrated hydrophobic filter of the culture flask and the gap between the leak and the culture dish/well plate. Therefore, the ideal design for the culture chamber should be similar. Like conventional cell culture, an unsealed chamber bioreactor connects to the outside environment through various ways (Juncosa-Melvin, Shearn et al. 2006; Androjna, Spragg et al. 2007; Nguyen, Liang et al. 2009; Doroski, Levenston et al. 2010).
2010), such as the hydrophobic filter leak (Webb, Hitchcock et al. 2006) and the labyrinth channel (Figure 1.3) (Parent, Huppe et al. 2011). In a study by Webb et al. (Webb, Hitchcock et al. 2006), a modified tissue culture flask was used as an integrated culture chamber, and could culture up to four samples simultaneously. Although there are potential risks of cross-contamination from different samples and toxicity from autoclaving the culture flask, the integrated hydrophobic filter leak can ensure adequate gas exchange without inducing contamination. In the bioreactor system used in the research of Parent et al. (Parent, Huppe et al. 2011), a labyrinth channel was added to improve the air exchange and eliminate contamination as shown as Figure 1.3. However, in closed chambers air exchange mostly depends on medium circulation, which is now discussed in the following section.

Figure 1.3 Schematic drawings of air exchange through the labyrinth channel in the culture chamber. Modified from reference (Parent, Huppe et al. 2011)

1.2.2.2 Environmental control and medium circulation systems

Although to the best of our knowledge, no contamination has been reported in any bioreactor study, transportation of the bioreactor and opening of chamber for medium exchange every three days, especially for multi-chambers system, is still a potential
contamination risk. Therefore, a medium circulation system can be introduced to improve the efficiency and minimize these risks. In addition to the advantages of reduced contamination, a circulating medium may be better able to infiltrate into cultured tissue. For example, perfusion bioreactors used in bone engineering circulate culture medium for better nutrient delivery and subsequent improved cell numbers (Cartmell, Porter et al. 2003; Uemura, Dong et al. 2003; Meinel, Karageorgiou et al. 2004; Holtorf, Jansen et al. 2005; Janssen, Oostra et al. 2006; Olivier, Hivart et al. 2007). Similarly, human umbilical vein cultured under a circulating medium had approximately three times the cellular number as those cultured using a quiescent medium (Abousleiman, Reyes et al. 2009).

A traditional incubator based bioreactor and/or independent bioreactor can be used for tendon/ligament engineering. In an incubator bioreactor system, air exchange is through the hydrophobic filter leak of the medium reservoir, and proper CO2 and temperature level is controlled by the incubator. Then by circulating the culture medium, suitable conditions can be applied to engineered tendon/ligament, as shown in Figure 1.4 (Abousleiman, Reyes et al. 2009). However, an independent bioreactor system, as shown Figure 1.5, does not rely on an incubator to control the culture environment. The percentage of different gases (PO2, CO2 and N2) are control by air valves (Altman, Lu et al. 2002), and the mixed gas is humidified first, and then passed into a medium heater. Waste gas emission is transported through a filter in the case of contamination. The prepared, warm culture medium is circulated through the bioreactor culture chamber. For certain tissues such as cartilage, some specific culture conditions are required. For
example, under a low oxygen environment, engineered cartilage displays faster matrix glycosaminoglycan deposition rate, and better cellular morphology but with less dedifferentiation (Hansen, Schunke et al. 2001; Domm, Schunke et al. 2002; Saini and Wick 2004). The environmental chamber allows researchers to manipulate different culture conditions thereby enabling a systematic study of cell growth and differentiation into functional tissue.
Figure 1.4 Schematic illustration of an incubator bioreactor system. Suitable temperature, humidity and CO₂ level of culture medium are maintained by the incubator in the medium reservoir. The medium is circulated by a pump. The waste valve is closed normally and it will open during medium exchange.
Figure 1.5 Schematic diagram of an independent bioreactor system. Suitable temperature, humidity and CO₂ level of the culture medium are control by the environmental chamber. Cold culture medium is pumped to the environmental chamber for heating and then circulated through the culture chamber. The waste valve is closed normally and it will open during medium exchange.

1.2.2.3 Monitoring and feedback systems

The biomechanical properties of the final engineered tendon/ligament should be a central concern of bioreactor design, as the tendon/ligament’s functional role in the body is primarily mechanical. The maximum load and elastic modulus are essential mechanical properties to evaluate the suitability of engineered tendon/ligament. However, in most studies, mechanical tests are performed only at the end of tissue culture. For example, the stiffness of the constructs at different time points during
culture is rarely recorded or assessed. The correlation between stiffness and tissue maturation may provide a better understanding about how the cell differentiates into functional tissue, and for this reason ‘on-line’ stiffness monitoring is likely to be invaluable.

In tendon/ligament bioreactors force measurement is enabled using sensors called load cells (Butler, Juncosa-Melvin et al. 2008; Nguyen, Liang et al. 2009; Parent, Huppe et al. 2011), which are located differently in various bioreactor designs. Load cells located between the actuator and sample clamps (Butler, Hunter et al. 2009), shown in Figure 1.6A, requires high manufacturing accuracy, as the friction between the shaft and culture chamber can induce error. For fragile material like biological tissues, this friction might be higher than the applied load. Conversely, a load cell placed at the end of the culture chamber, shown in Figure 1.6B, can minimize the fiction between the actuator and culture chamber and also the fluid resistance during stimulation. In order to acquire accurate data, load cell selection should be based on the initial mechanical properties of the constructs. Working with the sensitivity of the load cell, extra attention is needed when manipulating the construct so as to avoid causing damage to the load cell through overloading.
In addition to force monitoring, tissue displacement is another important variable that requires monitoring. Linear variable differential transformers (LVDT) and optical decoders are commonly used position sensors (Juncosa-Melvin, Shearn et al. 2006; Parent, Huppe et al. 2011). Although the motion of actuating systems is preprogrammed, overload of the actuator and manual mis-operation can cause desynchronization between the program and actual stimulation. The real time displacement monitor can produce a full record of stimulation position, which then allows researchers to track if there are any unusual features in the results. Another function of the position sensor is to provide feedback of the displacement information to the actuator control system to correct for any desynchronization (Parent, Huppe et al. 2011). Critically, by monitoring
load and displacement, real time stiffness during engineered tendon/ligament culture can be estimated.

Lastly, an imaging component might be useful in the bioreactor system to monitor the formation of the tissue construct at high resolution and possibly provide information to the feedback system. Two imaging modalities, including the confocal microscope and optical coherence tomography (OCT), are potential candidates (Drexler, Morgner et al. 1999; Goetz, Thomas et al. 2008). Both of these techniques are able to acquire high-resolution image and might be adopted in the dynamic culture environment for observation of cell and tissue morphology (Drexler, Morgner et al. 1999; Goetz, Thomas et al. 2008). The magnification of the confocal microscopy may range from 500 to 2400 fold at a dynamic environment. A miniaturized confocal laser scanning probe has been tested in rodents to provide the visualization of the vascular circulation and cell perfusion. Compared to the confocal microscope, OCT has a higher penetration depth up to 1~2 mm and longer working distance (the distance from the objective lens to the sample surface) up to 10 mm, which is sufficient for distant imaging of the tissue constructs in the bioreactor system (Smithpeter, Dunn et al. 1998; Drexler, Morgner et al. 2001; Burkhardt, Walther et al. 2012). However, there are two major issues to be addressed before these systems could be integrated into the bioreactor design. Firstly, for distant imaging, the optical light needs to penetrate 3 layers of media (chamber cover, air and culture medium) with different refraction index to obtain imaging. Secondly, sample deformation induced by dynamic mechanical loading can cause image shift resulting in poor focus or loss of the image. Imaging dynamic sample is extremely difficult in
conventional bioreactors, as features of interest (e.g. cells) may leave the field of view. Techniques need to be developed to move the microscope alone with the sample to achieve optimal focus on the view of interest. Other than distant imaging, minimally invasive imaging technique might provide another potential strategy for this obstacle. Recent studies show that confocal fluorescence microendoscopy and OCT could be integrated into a hypodermic needle (Pillai, Lorenser et al.; Quirk, McLaughlin et al.), which might be inserted into the tissue constructs and thus creating a relatively static imaging environment. However, the technique is invasive and could damage the tissue integrity. In all, the current imaging technology is not mature enough to acquire the dynamic sub-micrometer image, but an imaging system allows dynamic imaging at high resolution is certainly worth to explore. Cell morphological and tissue structural change coping to the PMS might provide a different respective for studying the effect of PMS on tendon/ligament biology and tendon/ligament engineering.

1.2.3 Commercial bioreactor systems for tendon/ligament engineering

Recently, commercial tendon/ligament engineering bioreactor systems have become available. As discussed above, these basic principles are applied to customized bioreactors; however, with greater design input and advanced manufacturing techniques, commercial products are able to provide more accurate and complex environments for tendon/ligament culture. To our knowledge, two relatively complete commercial bioreactor systems have been developed recently: The Bose® ElectroForce® BioDynamic® system and the LigaGen system.
The Bose® ElectroForce® BioDynamic® test instrument provides an accurate programmable uniaxial stretching stimulation and a controllable medium circulation environment to engineered tendon/ligament, which, theoretically, can be adjusted to mimic the *in vivo* biomechanical environment. With a load cell and optional laser micrometer, this bioreactor system is able to monitor the force/strain curve of engineered tendon/ligament during the culture period. Two different force and displacement ranges are available. Single chamber and multiple chambers systems with shared or independent loading are optional. As there are culture chambers compatible to the Bose® testing devices such as ElectroForce 3200, biomechanical tests can be done at different time points without disruption of the tissue culture (www.bose-electroforce.com).

The LigaGen system is a lightweight (<3kg) incubator compatible bioreactor. It is capable of applying a maximum force of 40N to the tissue sample, and simulate complex, and presumably more physiologically realistic, loading patterns. Two systems are available from LigaGen. L30-1X is a single culture model, which has a 23ml internal volume chamber for single tissue culture, and the L30-4C is a multiple culture model with an 80ml chamber for shared dynamic culture on two or four samples. The standard medium circulation system can reduce contamination risk during medium exchange. Rather than being a comprehensive system, the bioreactor is extensible to suite individual needs, by adding various components to the (universal) basic model as required. With an accessory tissue monitoring sensor, this system is able to achieve real-time measurements of the sample stiffness during culture. If flow control is necessary, extra control systems can be installed (http://www.tissuegrowth.com/).
However, there are some disadvantages in the commercial bioreactors. Firstly, the fixation mechanism to stabilize the tendon tissue in the bioreactor cannot be adjusted. Tissue clamps provided in the commercial bioreactor systems can become less effective when it comes to the use of a cylindrical scaffold. Secondly, capacities of the chamber and mechanical input are limited and they can only host small animal tissues. This may restrict clinical development. Moreover, some ligaments, such as the anterior cruciate ligament, are not only subjected to tensile force, but also to rotational loading, and none of the commercial bioreactor systems provide the addition of torsional loading. Lastly, full-scale commercial bioreactor systems are expensive, and for most of the time, not all of the functions they provide are commonly used in every study.

1.2.4 Ideal bioreactors for tendon/ligament engineering

The ideal bioreactor should be able to culture tendon- and ligament-like constructs, which are well-organized, cell-seeded, assemblies of collagen bundles with mechanical properties functionally similar to the native tissue. Autologous cell-seeded constructs are biological compatible, and able to provide mechanical support similar to native tissue, and consequently they are widely studied in tendon/ligament engineering. However, induction of cell directed collagen fiber reorganization and assembly of collagen bundles are two important impediments to this approach. PMS can help provide the necessary signals to cells to increase collagen synthesis, spatially organize of the collagen along the primary stress direction, and stabilize collagen from collagenase degradation (Lavagnino, Arnoczky et al. 2003; Yang, Im et al. 2005; Nguyen, Liang et al. 2009). Moreover, proper PMS can upregulate different
proteoglycans, such as decorin, biglycan, fibromodulin and fibronectin, which help the cells organize the parallel collagen fibrils forming bundles (Scott 1984; Cribb and Scott 1995; Vogel 2004; Webb, Hitchcock et al. 2006; Franchi, Trire et al. 2007).

The host body is in many ways the ultimate bioreactor for all engineered tissues. The study of Juncosa-Melvin et al. indicated that the maximum force of engineered patellar tendons increased more than 3000 times after 2 weeks implantation in rabbits (Juncosa-Melvin, Shearn et al. 2006), and to date none of the bioreactors have been able to accomplish this outcome. Therefore, an ideal bioreactor should aim to mimic the dynamic biochemical and biophysical environments in vivo. In musculoskeletal tissue engineering, various bioreactors have been developed. For instance, muscle tissues are not only subjected to mechanical stretching but also able to receive the electrical impulses to simulate inputs from the central nervous system (Ross, Duxson et al. 1987), and mechanical and electrical stimulation bioreactors have been developed based on mimicking the in vivo environment (Donnelly, Khodabukus et al. 2010; Sharifpoor, Simmons et al. 2011). Compared to muscle, the in vivo environment is comparatively less complex in tendon/ligament and appears to require only passive mechanical input.

Summarizing, the ideal tendon/ligament engineering bioreactor that enables systemic research should integrate all aforementioned components (Figure 1.1). The bioreactor should have culture chambers and an actuating system, but also be fitted with a medium circulation system, an environmental system, a monitoring system, a feedback system,
and a waste medium analysis system. This bioreactor should first be able to provide not only a multiple, suitably sized and sterilized chambers for tissue culture, but also accurate and programmable mechanical stimulation. Tensile strain and rotation are needed to mimic different *in vivo* tendon/ligament loads. Second, the circulated medium should infiltrate into tissue better than a static medium configuration, and the circulation system needs to reduce the risks of contamination during medium exchange and drug delivery. Moreover, environmental control, such as PO2, CO2 and pH level, allows researchers to explore the impact of different culture conditions on tissue maturation. Third, a monitoring system should provide the real-time status of cultured tendon/ligament, such as force and displacement, and based on these data, adjustment of PMS by use of a feedback system. Fourth, through analysis of the waste medium, nutrient consumption needs to be evaluated, which may enable the changing of the medium base at different stages as required, rather than fixed, regular medium exchange every 3 days. Finally and importantly, the best patterns of PMS for culturing engineered tendon/ligaments need to be defined.

### 1.2.5 Previous work in this field

Since the first 3D engineered tendon/ligament bioreactor system published by Altman *et al.* in 2002, the effect of PMS on the engineered tendon/ligament has drawn a lot of research attention. In the past decade, dynamic loading of culture in bioreactor systems has been proven to have been a significant development in producing an engineered tendon/ligament. Indeed, various studies have been performed using bioreactor systems and have met with considerable success, and these are summarized in Table 1.2.
Compared with static culture, the tissue produced using PMS has better cell morphology, including elongated cellular morphology and increased cell density. The mechanical properties of engineered tendon/ligament, such as tensile strength and elastic modulus, are also greatly improved by cyclic loading of the tissue culture, as is the microstructure of the extracellular matrix, such as collagen fiber alignment. Gene expression is also positively influenced by PMS. For instance, collagen type I expression under dynamic loading is three times higher than static culture in 2 week (Butler, Hunter et al. 2009).

Although cyclic stretching has been proven to be an effective way to stimulate the engineered tendon/ligament culture, the optimal stimulation pattern is still unknown. Nirmalanandhan et al. revealed that a 2.4% strain cycle consisting of 3000 cycles per day, produced the best linear stiffness in rabbit MSC seeded in type I collagen sponge (Nirmalanandhan, Shearn et al. 2008). However from the perspective of Butler et al., the stimulation pattern should be adjusted based on maturation of the engineered tendon/ligament, with higher dose loading applied at the later stage of tissue culture (Butler, Hunter et al. 2009).
<table>
<thead>
<tr>
<th>First author</th>
<th>Bioreactor type (Company)</th>
<th>Parameters of mechanical stimulation</th>
<th>Scaffold material (dimensions)</th>
<th>Cell source</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Altman (2002)</td>
<td>Custom-made step motor bioreactor with environmental chamber</td>
<td>Cyclic stretching 2mm, 90° rotation, 0.0167Hz, 21 days</td>
<td>Collagen type I gels, Bombyxmori silkworm silk fiber matrices (length:30 mm)</td>
<td>Human bone marrow stroma cells (hBMSC)</td>
<td>Elongation of hBMSC, cross-section cell density ↑</td>
<td>(Altman, Lu et al. 2002)</td>
</tr>
<tr>
<td>Juncosa-Melvin (2006)</td>
<td>Custom-made pneumatic cylinder bioreactor with LVDT for displacement monitoring</td>
<td>Cyclic stretching 2.4% strain, 0.0033Hz, 8h/day for 2 weeks</td>
<td>Type I collagen sponge (23 ± 0.8 mm × 9 ± 0.8 mm × 3 ± 0.1 mm)</td>
<td>Rabbit MSC</td>
<td>Maximum force ↑, linear stiffness ↑, maximum stress ↑, linear modulus ↑</td>
<td>(Juncosa-Melvin, Shearn et al. 2006)</td>
</tr>
<tr>
<td>Webb (2006)</td>
<td>Custom-made step motor bioreactor</td>
<td>Cyclic stretching 10% strain, 0.25Hz, 8h/day for 7days</td>
<td>Polyurethane construct (20 × 10 × 2 mm)</td>
<td>Human tracheal fibroblast</td>
<td>Type I collagen ↑, TGF β-1 ↑, CTGF, Elastin ↑, alpha I ↑, Procollagen ↑, Fibronectin ↑, MMP-1 ↑, elastic modulus ↑</td>
<td>(Webb, Hitchcock et al. 2006)</td>
</tr>
<tr>
<td>Study (Year)</td>
<td>Bioreactor System Details</td>
<td>Loading Parameters</td>
<td>Tissue/Cells Used</td>
<td>Results</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>---------------------------</td>
<td>--------------------</td>
<td>-----------------</td>
<td>---------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>Androjna (2007)</td>
<td>Custom-made bioreactor with load-displacement measure system</td>
<td>Cyclic stretching 9% strain, twice daily for 30min each period separated by 8h rest for 2 weeks</td>
<td>Small-intestine submucosa (3cm×5cm×95 μm)</td>
<td>Dog tenocyte</td>
<td>Cell density ↑, stiffness ↑</td>
<td></td>
</tr>
<tr>
<td>(Androjna, Spragg et al. 2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nirmalanandhan (2008)</td>
<td>Custom-made pneumatic cylinder bioreactor</td>
<td>Cyclic stretching 2.4% and 1.2% strain, 1Hz, stimulation period: 8h/day, 100 and 3000 cycles/day for 12 days</td>
<td>Type I collagen sponge</td>
<td>Rabbit iliac crest MSC</td>
<td>The stimulation pattern of 2.4% strain, 3000 cycles/day, 1Hz has best effect on increasing stiffness.</td>
<td></td>
</tr>
<tr>
<td>(Nirmalanandhan, Shearn et al. 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nirmalanandhan (2008)</td>
<td>Custom-made pneumatic cylinder bioreactor</td>
<td>Cyclic stretching 2.4% strain, 0.0033Hz, 8h/day for 12 days</td>
<td>Type I purified bovine collagen gel, type I collagen sponges (length: 11mm and 51 mm)</td>
<td>Rabbit MSC</td>
<td>stiffness ↑</td>
<td></td>
</tr>
<tr>
<td>(Nirmalanandhan, Rao et al. 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nguyen (2009)</td>
<td>Custom-made linear actuator bioreactor with load cell for force measurement</td>
<td>Preloaded with 0.05N, cyclic stretching 10%, 0.5Hz, 2h stimulation-2h rest-2h stimulation-18h rest for 5 days</td>
<td>Porcine small intestine submucosa (length: 2cm and width: 1cm)</td>
<td>Rabbit MCL fibroblast</td>
<td>Improved fiber orientation, fiber angular dispersion ↓, better organized collagen fiber, elongated cell morphology.</td>
<td></td>
</tr>
<tr>
<td>(Nguyen, Liang et al. 2009)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Description</td>
<td>Bioreactor</td>
<td>Cyclic Stretching Parameters</td>
<td>Tissue/Derived Cells</td>
<td>Changes</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>------------</td>
<td>-----------------------------</td>
<td>----------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Abousleiman (2009)</td>
<td>Custom-made linear actuator bioreactor with medium circulation system</td>
<td>Cyclic stretching 2% strain, 1h/day, 0.0167Hz for 1 and 2 weeks.</td>
<td>Human umbilical veins (wall thickness: 0.75mm, outer diameter: 6.75 ± 0.25mm, length: 8.5cm)</td>
<td>Wistar Rat bone marrow MSC</td>
<td>better cell distribution, more elongated cell morphology, Cell proliferation ↑, ultimate stress ↑, elastic modulus↑</td>
<td></td>
</tr>
<tr>
<td>Butler 2009)</td>
<td>Custom-made pneumatic cylinder bioreactor with LVDT for displacement monitoring</td>
<td>Cyclic stretching 2.4% strain, 0.0033Hz, 8h/day for 2 weeks</td>
<td>Type I collagen sponge (94% pore volume; 62 mm mean pore diameter)</td>
<td>Mouse Mesenchymal Stem Cell</td>
<td>Type I collagen ↑, linear stiffness↑</td>
<td></td>
</tr>
<tr>
<td>Chen(2010)</td>
<td>Custom-made step motor bioreactor</td>
<td>Cyclic stretching 10% strain, 2h/day, 1Hz for 14 days</td>
<td>Knitted silk-collagen sponge scaffold (5cm×0.5cm×0.2cm)</td>
<td>Human embryonic stem cell</td>
<td>Collagen I↑, Collagen III↑, EphA4↑, Scx↑, Sox9 ↓, Myosin↑, Integrinα1↑, Integrinα2↑, Integrinβ1↑, Collagen content↑, Collagen diameter↑, better cell alignment</td>
<td></td>
</tr>
<tr>
<td>Doroski (2010)</td>
<td>Custom-made Linear motor bioreactor</td>
<td>Cyclic stretching 10% strain (5% offset, 5% amplitude), 1Hz, 3h/day, 1 7 14 and 21 days</td>
<td>Poly(ethylene glycol)-based hydrogel material oligo (poly(ethylene glycol) fumarate) (12.5 × 9.5 × 1.6mm)</td>
<td>MSCs (PT-2510; Lonza)</td>
<td>Collagen I↑, Collagen III↑, TNC↑, Tenascin-C↑</td>
<td></td>
</tr>
<tr>
<td>Study (Year)</td>
<td>Bioreactor System Details</td>
<td>Cyclic Stretching Parameters</td>
<td>Cell Source</td>
<td>Biomaterial Source</td>
<td>Results</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>---------------------------</td>
<td>-----------------------------</td>
<td>-------------</td>
<td>-------------------</td>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>Saber (2010)</td>
<td>Ligagen L30-4C (Tissue Growth Technologies)</td>
<td>Cyclic stretching 1.25N, 1 cycle/min, 1h/day for 5 days</td>
<td>Acellular rabbit hindpaw tendon (length: 5cm)</td>
<td>Rabbit tenocyte</td>
<td>Ultimate tensile stress ↑ (close to fleshly harvested tendon), elastic modulus ↑</td>
<td>(Saber, Zhang et al. 2010)</td>
</tr>
<tr>
<td>Issa (2011)</td>
<td>Custom-made bioreactor</td>
<td>Cyclic stretching 2% strain, 1h/day, 0.0167Hz for 1 and 2 weeks</td>
<td>Human umbilical veins (wall thickness: 0.41mm) with Wharton’s jelly matrix as central portion (total thickness: 0.75mm)</td>
<td>Wistar rat bone marrow MSC</td>
<td>Cell proliferation ↑, tensile strength ↑</td>
<td>(Issa, Engebretson et al. 2011)</td>
</tr>
<tr>
<td>Woon (2011)</td>
<td>Ligagen L30-1C &amp; Ligagen L30-4C (Tissue Growth Technologies)</td>
<td>L30-1C: dynamic loading, 10N, 1h/day, 0.0167 Hz for 5 days L30-4C: dynamic loading, 0.625N 1.25N 2.5N, 1h/day, 0.0167 Hz for 3-5 and 8 days</td>
<td>Acellular human flexor tendon scaffolds</td>
<td>Human Dermal fibroblast</td>
<td>Ultimate tensile stress ↑, elastic modulus ↑</td>
<td>(Woon, Kraus et al. 2011)</td>
</tr>
</tbody>
</table>
1.2.6 Conclusion

The goal of tendon/ligament bioreactor design should be to create a construct with similar microstructure and mechanical properties, as native tissue, using bioscaffolds and autologous tenocytes. However, a tendon-like uni-orientated collagen structure cannot be achieved without a mechanical stimulus within the culture environment. Traditional culture techniques do not provide this PMS. The use of a bioreactor system is able to bridge the gap between in vitro and in vivo systems by creating suitable biochemical and biomechanical environments. Although it is clearly very difficult to reproduce the in vivo microenvironmental conditions exactly within the bioreactor, the goal is to mimic the in vivo biomechanical condition as closely as possible. The essential components of a tendon/ligament engineering bioreactor are the actuating system and culture chamber, which are responsible for the PMS and providing sterilized environment for tissue culture. For better manipulation of the culture environment, accurate stimulation and more precise reporting of mechanical maturation of engineered tendon/ligament, an environmental control system, medium circulation system, monitor and feedback system need to be included. As bioreactors nowadays are becoming more focused on pre-clinical research, using these clinically in the future still presents a substantial challenge. Tissue engineering requires substantial system optimization to achieve a reproducible functional engineered tendon/ligament consistently. This process is difficult and expensive in animal models, let alone in humans. However, there are additional problems in moving from small animal models to humans related to the physical size of human tissue samples. Issues of nutrient transport, cell source and spatial heterogeneity of scaffold properties and cell stimulation become more prominent in these larger tissues.
However, all the evidence suggests that by using bioreactors as described above it will be possible to successfully produce engineered tendons and ligaments. When this occurs we will be able to manufacture basic multi-chambers bioreactors with accurate and appropriate patterns of PMS, which are affordable, and have low maintenance.

As one of the key elements of tissue engineering, biomechanical signal has been introduced to generate engineered tendon for over a decade, and cyclic tensile strain is commonly used. Positive effects, as summarized as figure 1.1, have been reported based on various loading regimes with tensile strain ranged from 2% to 10% associated with different frequencies and loading duration. These studies have demonstrated that mechanical loading can improve cell proliferation, cell morphology, collagen I synthesis and mechanical properties of engineered tendon. However, most of the studies only examine one strain; the comparison of the effect caused by different mechanical strains is poorly studied. The precise physiological levels of strain, frequency and duration to affect such a response are not well understood.

1.3 Hypothesis and aims

1.3.1 Hypothesis

1.3.1.1 Overall hypothesis

I hypothesize that the biomechanical environment is essential for tendon homeostasis. Further, the application of different mechanical stimulation on tendon
in a bioreactor system can induce different pathological changes and that the biomechanical environment can help manipulate the repair mechanism of tendon tissue.

1.3.1.2 Specific hypothesis

i. Different mechanical environments in a bioreactor have different impact on tissue.

ii. There exists a mechanical strain regime that will maintain tendon homeostasis.

iii. There exists a mechanical strain regime that will lead to adverse changes in a tendon.

iv. A strain regime can be found which restores the functional and histological properties of a tendon.

1.3.2 Aims

i. To design and construct a bioreactor system that is able to provide a programmable biomechanical environment for in vitro tendon culture. (Study I, Chapter 3)

ii. To evaluate the effect of different mechanical stimulation on tendon homeostasis in a bioreactor system. (Study II, Chapter 4)
To evaluate if the mechanical stimulation optimized in the previous study is able to activate the self-repair mechanism to repair the tendon degeneration caused by loading deprivation culture. (Study III, Chapter 5)
Chapter 2

Materials & Methods
CHAPTER 2 MATERIALS AND METHODS

There are a number of histological and biochemical analysis used throughout this thesis. This chapter presents these techniques and methodology.

2.1 Material

All chemical reagents used during the course of this experiment were obtained through the following manufacturers.

2.1.1 Tissue Culture reagents

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>MANUFACTURER/SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F-12</td>
<td>Gibco Life Technologies, USA</td>
</tr>
<tr>
<td>L-Glutamine 200mM 100X</td>
<td>Gibco Life Technologies, USA</td>
</tr>
<tr>
<td>FBS (Fetal Bovine Serum)</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td>Pen-Strep (Penicillin-streptomycin)</td>
<td>Gibco Life Technologies, USA</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Invitrogen, Auckland, New Zealand</td>
</tr>
</tbody>
</table>
### 2.1.2 Chemical reagents

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>MANUFACTURER/SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose LE, analytical grade</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>Baxter water</td>
<td>Baxter Healthcare Pty.Ltd., Australia</td>
</tr>
<tr>
<td>Chloroform</td>
<td>MERCK, Germany</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Hurst Scientific Pty. Ltd., Australia</td>
</tr>
<tr>
<td>Ethanol (molecular grade)</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>MERCK, Germany</td>
</tr>
<tr>
<td>TRIzol® reagent</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td>Xylene</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Tissue embedding medium (Paraplast® Regular)</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Haematoxylin</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Eosin</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Diethyl pyrocarbonate</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>proteinase K</td>
<td>Qiagen, Germany</td>
</tr>
</tbody>
</table>
DPX Mountant | Sigma-Aldrich, USA

2.1.3 Molecular products

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>MANUFACTURER/SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1kb DNA ladder</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>6x blue/orange loading dye</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>100bp DNA ladder</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>dNTPs (5nM)</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>Go-Taq® Green Master Mix 2x</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>M-MLV RT RNase (H-)</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>Oligo dT (100mM)</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>RT-PCR buffer (5x M-MLV RT)</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>SYBR® Safe DNA gel stain</td>
<td>Invitrogen, USA</td>
</tr>
<tr>
<td>iQ™ SYBR® Green Supermix</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
</tbody>
</table>

2.1.4 Oligonucleotide Primers

All oligonucleotide primers used in real-time polymerase chain reaction (PCR) were
purchased from GeneWorks Pty. Ltd. (Australia). The primers were reconstituted in DEPC water to a concentration of 100µM and stored at -20°C until required.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE (5’ to 3’)</th>
<th>PRODUCT SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1A2 Forward</td>
<td>TTGCCCTTCTTGATATTGC</td>
<td>241</td>
</tr>
<tr>
<td>COL1A2 Reverse</td>
<td>CCTCTTTTCGCCCACAATTTA</td>
<td></td>
</tr>
<tr>
<td>COL3A1 Forward</td>
<td>AAGCCCCAGCAGAAAAATTG</td>
<td>160</td>
</tr>
<tr>
<td>COL3A1 Reverse</td>
<td>TGGTGGAACAGCAAAAATCA</td>
<td></td>
</tr>
<tr>
<td>MMP1 Forward</td>
<td>GGCTCAGTTCTCCTCCTACTCT</td>
<td>246</td>
</tr>
<tr>
<td>MMP1 Reverse</td>
<td>CAGGTCCATCAAAGGGAGAA</td>
<td></td>
</tr>
<tr>
<td>MMP3 Forward</td>
<td>GCTTTGCTCAGCCTATCCAC</td>
<td>189</td>
</tr>
<tr>
<td>MMP3 Reverse</td>
<td>ACCTCCAAGCCAAGGACTT</td>
<td></td>
</tr>
<tr>
<td>MMP12 Forward</td>
<td>ATGCCCAGGGAGACCAGTATG</td>
<td>248</td>
</tr>
<tr>
<td>MMP12 Reverse</td>
<td>AAAGCATGGGCTATGACACC</td>
<td></td>
</tr>
<tr>
<td>TIMP1 Forward</td>
<td>CTTGTCATCAGGGCCAAGTT</td>
<td>213</td>
</tr>
<tr>
<td>TIMP1 Reverse</td>
<td>TCCAGCGATGAGAAACTCCT</td>
<td></td>
</tr>
<tr>
<td>TIMP2 Forward</td>
<td>GTGGACTCTGGGAACGACAT</td>
<td>223</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence</td>
<td>Length</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>TIMP2 Reverse</td>
<td>AGTCACAGACGTGATGTGC</td>
<td></td>
</tr>
<tr>
<td>TGFB Forward</td>
<td>TGCTTCAGCTCCACAGAGAA</td>
<td>181</td>
</tr>
<tr>
<td>TGFB Reverse</td>
<td>CCTTGCTGTACTGGGTGTCC</td>
<td></td>
</tr>
<tr>
<td>36B4 Forward</td>
<td>ACCCCAAATGCTTCATCGT</td>
<td>150</td>
</tr>
<tr>
<td>36B4 Reverse</td>
<td>CAGGGTTGTTTCCAGATGC</td>
<td></td>
</tr>
</tbody>
</table>

2.1.5 Antibodies

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>MANUFACTURER/SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal anti-collagen type III</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Biotinylated goat anti-mouse IgG</td>
<td>Sigma-Aldrich, USA</td>
</tr>
</tbody>
</table>

2.1.6 Commercial kits

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>MANUFACTURER/SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNeasy® minikit(250)</td>
<td>QIAGEN, Germany</td>
</tr>
<tr>
<td>Liquid DAB+ Substrate Chromogen System</td>
<td>DAKO, Denmark</td>
</tr>
</tbody>
</table>
### 2.1.7 Other materials and equipment

<table>
<thead>
<tr>
<th>MATERIAL/EQUIPMENT</th>
<th>MANUFACTURER/SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>AND EK-300i scientific weighing scale</td>
<td>A&amp;D Mercury Pty. Ltd., China</td>
</tr>
<tr>
<td>Centrifuge 5810R</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>Corning high speed microcentrifuge</td>
<td>Corning Incorporated, USA</td>
</tr>
<tr>
<td>Corning mini microcentrifuge</td>
<td>Corning Incorporated, USA</td>
</tr>
<tr>
<td>CentriStar™ Cap centrifuge tubes: 15ml and 50ml</td>
<td>Corning Incorporated, Mexico</td>
</tr>
<tr>
<td>Easypet® pipette dispenser</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>Forma Series II water jacker CO₂ incubator</td>
<td>Thermo Scientific, Australia</td>
</tr>
<tr>
<td>Grant W-14 water bath</td>
<td>Grant Instruments, UK</td>
</tr>
<tr>
<td>Homo-polymer, boil-proof microtubes: 0.6ml, 1.5ml and 2.0ml</td>
<td>Axygen® Scientific, USA</td>
</tr>
<tr>
<td>Hot plate model 209-1</td>
<td>IEC Australia Industrial Equipment &amp; Control Pty. Ltd., Australia</td>
</tr>
<tr>
<td>Item</td>
<td>Supplier/Location</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>KaterMaster catering foil</td>
<td>Bunzl Limited, Australia</td>
</tr>
<tr>
<td>Laborlux S microscope</td>
<td>Leitz, Germany</td>
</tr>
<tr>
<td>LabServ™ incubator</td>
<td>Biolab (Aust) Pty. Ltd, Australia</td>
</tr>
<tr>
<td>Olympus CKX41 inverted microscope</td>
<td>Olympus Corporation, Japan</td>
</tr>
<tr>
<td>Nikon digital sight DS-5Mc</td>
<td>Nikon Corporation, Japan</td>
</tr>
<tr>
<td>Nikon Eclipse E200 reflective microscope</td>
<td>Nikon Corporation, Japan</td>
</tr>
<tr>
<td>Parafilm “M” ® laboratory film</td>
<td>Parafilm, USA</td>
</tr>
<tr>
<td>PCR-tube 0.5ml, thin wall</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>pH211 microprocessor pH meter</td>
<td>Hanna Instruments, Australia</td>
</tr>
<tr>
<td>Ratek vortex mixer</td>
<td>Rowe Scientific Pty. Ltd, Australia</td>
</tr>
<tr>
<td>Revco Elite Plus -80°C freezer</td>
<td>Thermo-Fisher Scientific, USA</td>
</tr>
<tr>
<td>Serological pipette: 2, 5, 10ml</td>
<td>Sarstedt, France</td>
</tr>
<tr>
<td>SmartSpec™ 3000</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>SmartSpec™ 3000 cuvette quartz</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>Tissue culture dish 100x20 mm</td>
<td>Sarstedt, USA</td>
</tr>
<tr>
<td>Tissue culture flask: 25cm² and</td>
<td>Corning Incorporated, USA</td>
</tr>
<tr>
<td>75cm²</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Leica TP 1020 tissue processor</td>
<td>Leica, Germany</td>
</tr>
<tr>
<td>Sratfrost® microscope slides</td>
<td>Knittel-Glaser, Germany</td>
</tr>
<tr>
<td>Superfrost ultra plus® slide</td>
<td>Thremo Scientific, Australia</td>
</tr>
<tr>
<td>Microscope cover glass 22x50mm</td>
<td>Hurst scientific Pty. Ltd., Australia</td>
</tr>
<tr>
<td>Leica 819 low profile microtome Blades</td>
<td>Leica, Germany</td>
</tr>
<tr>
<td>Jung Biocut 2035</td>
<td>Leica, Germany</td>
</tr>
<tr>
<td>Transferpipette 3.5ml</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Veriti 96 well thermal cycler</td>
<td>Applied Biosystems™, Singapore</td>
</tr>
<tr>
<td>CFX Connect™ Real-time PCR detection system</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>Hard-Shell® PCR plates 96 well WHT/CLR</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>Labnet Excel™ single electronic pipette</td>
<td>Labnet International, Inc.</td>
</tr>
</tbody>
</table>

### 2.1.8 Software

<table>
<thead>
<tr>
<th>SOFTWARE</th>
<th>MANUFACTURER/SUPPLIER</th>
</tr>
</thead>
</table>
### General solution

All general solution and buffers were prepared using MilliQ double distilled water, unless otherwise indicated.

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>10x PBS stock solution:</td>
</tr>
<tr>
<td></td>
<td>30mM NaH₂PO₄, 70mM Na₂HPO₄, 1.3M NaCl dissolved in MilliQ H₂O</td>
</tr>
<tr>
<td></td>
<td>1x PBS pH 7.4</td>
</tr>
<tr>
<td></td>
<td>Dilution of 10x PBS with MilliQ H₂O and adjusted to pH 7.4</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>4% paraformaldehyde:</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>40g of Paraformaldehyde dissolved in prewarmed 0.75L MilliQ H₂O ~ 60°C with stirring. Cover with foil and stir with low heat, add 5-10 drops of 10M NaOH till the solution become clear. Remove from heat and add 100mL 10x PBS and adjust pH to 7.4. Filter solution through a 0.45μm membrane filter before aliquoting and storing at -20°C.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hematoxylin working solution (Harris)</th>
<th>Hematoxylin solution (Harris): 100g Postassium or ammonium heat dissolves in 1L MilliQ H₂O.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Add 50 ml of 10% alcoholic hematoxylin sol. and heat to boil for 1min. Remove from heat and slowly add 2.5 g of mercuric oxide (red). Heat to the solution and until it becomes dark purple color. Cool the solution in cold water bath and add 20 ml of glacial acetic acid (concentrated). Filter before use.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Eosin working solution</th>
<th>Eosin stock solution: 1g Eosin Y dissolved in 100ml MilliQ H₂O.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phloxine stock solution: 1g Phloxine B dissolved in</td>
</tr>
</tbody>
</table>
2.2 Tissue culture procedures

2.2.1 Isolation of rabbit Achilles tendon

Female New Zealand White rabbits (Oryctolagus cuniculus) aged between 14 and 15 weeks with body weight 3-5kg were sacrificed. The lower legs were wetted and sterilized by 70%[v/v] ethanol. A transection was made between the Achilles tendon and tibia to calcaneus using a sterilized scalpel. The Achilles tendon was then completely exposed. A full length Achilles tendon was removed with part of the calcaneus and approximately 1cm of the soleus muscle.

2.2.2 Culture of rabbit Achilles tendon

The Achilles tendons were briefly rinsed in 70%[v/v] ethanol and temporally stored in serum-free DMEM-F12 supplemented with 50µg/ml Gentamicin after isolation from rabbit. Before the tissue culture procedure, the Achilles tendons were washed in 40ml 1x PBS three times. In the loading deprivation culture group, tendons with muscle and calcaneus were cultured in a T25 culture flask. In the dynamic culture group, the
Achilles tendons were cultured with muscle and calcaneus fixed in the bioreactor using tissue clamps and hook. The whole culture procedure was processed in a standard incubator at 37°C and 5% CO₂, and the culture media was DMEM/F-12 supplemented with 10% fetal bovine serum and 50 µg/mL Gentamicin and changed every three days.

2.3 RNA extraction

2.3.1 Extraction of total RNA

Approximately 200mg of tendon tissue was dissected from the mid-substance of the Achilles tendon, then washed with ice-cold 1x PBS for three times. The tissue was placed into 5ml tube and snap frozen using liquid nitrogen and pulverized using mortar and pestle. The tissue debris was then lysed using 1ml Trizol and homogenized by pipetting in several times followed by 5 minutes incubation at room temperature. The lysate was collected in a microcentrifuge tube, shaken by hand with 200µm chloroform for 15 seconds and incubated at room temperature for 2-3 minutes. The mixture was spin at 12,000 × g for 15mins at 4°C, the RNA was separated in the aqueous phase. The supernatant was transfer to a microcentrifuge tube, vortex mixed thoroughly with 1 volume of 70%[v/v] ethanol and transferred up to 700µL to the spin cartridge (RNeasy® minikit), and centrifuge at 12,000 × g for 15 seconds at room temperature. This procedure was repeated several times till the entire samples has been processed. The spin cartridge was then centrifuged at 12,000 × g for 15 seconds at room temperature with 700µL Wash Buffer I (RNeasy® minikit), and the flow-through and collection tube
were discarded. 500 μL Wash Buffer II (RNeasy® minikit) with ethanol was added into the cartridge and centrifuged at 12,000 × g for 15 seconds at room temperature, and the flow-through was discarded, this procedure was repeated once. The cartridge was centrifuged at 12,000 × g for 1-2 minutes at room temperature to dry the membrane with bound RNA; the collection tube was then discarded. Add 30–100μL RNase-free water to the center of the spin cartridge, incubated at room temperature for 1 minute followed by 2 minutes centrifuge at ≥12,000 × g at room temperature to elute the RNA from the membrane into the recovery tube. The purified RNA was stored at -80°C until required.

2.3.2 RNA concentration reading

200 μL of 1/50 dilution of purified RNA with Baxter water was prepared. The SmartSpec™ 3000 (Bio-Rad) was blanked using 200 μL RNase-free water first, and the samples was then injected into the Quartz Microvolume Cuvette, and read by the Spectrophotometer. The absorbance ratio (A260/A280) provides an estimation of the purity of the RNA. Generally, 1.8-2.0 is the acceptable range, where 2.0 represent “pure”. All the purified RNA used in this study has A260/A280 ratio above 1.8.
2.4 Quantitative polymerase chain reaction (QPCR)

2.4.1 Reverse transcription of mRNA

A total 300ng of RNA was used to convert to equal amounts of cDNA using the following protocol:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>300ng</td>
</tr>
<tr>
<td>Oligo dT</td>
<td>0.25μL</td>
</tr>
<tr>
<td>dNTP (5mM)</td>
<td>2 μL</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>final volume of 15μL</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>15μL</td>
</tr>
</tbody>
</table>

The samples were incubated at 75°C for 3 minutes, and then the following reagents mixture was added:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5x MMLV RT-PCR Buffer</td>
<td>4 μL</td>
</tr>
<tr>
<td>RNase</td>
<td>0.5μL</td>
</tr>
<tr>
<td>MMLV RT RNase</td>
<td>0.5μL</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>5μL</td>
</tr>
</tbody>
</table>
The samples were then incubated at 42°C for 60 minutes, followed by incubation at 92°C for 10 minutes. The cDNA was stored at -20°C until required.

### 2.4.2 Quantitative polymerase chain reaction (QPCR)

Each of the QPCR reaction was performed using CFX Connect™ Real-time PCR detection system (Bio-Rad Laboratories, USA) and prepared according to the following protocol:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>iQ™ SYBR® Green Supermix</td>
<td>5µL</td>
</tr>
<tr>
<td>cDNA</td>
<td>1µL</td>
</tr>
<tr>
<td>Primer Forward (20µM)</td>
<td>0.5µL</td>
</tr>
<tr>
<td>Primer Reverse (20µM)</td>
<td>0.5µL</td>
</tr>
<tr>
<td>RNA free water</td>
<td>3 µL</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>10µL</td>
</tr>
</tbody>
</table>

The QPCR reaction primers were specified in 2.1.4 Oligonucleotide primer. The QPCR conditions were carried out as per manufacturer’s instructions, each with 40 cycles. Housing keeping gene 36B4 was used as internal control. A cycle threshold (Ct) value
was obtained from each reaction. The comparative $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of each target gene. The RT-PCR were repeated in 3 individual samples from each group and performed in triplicate in every RT-PCR setting. The average expression levels were calculated and used for comparison.

2.5 General histology

2.5.1 Tissue preparation

The Achilles tendon was first washed by 1x PBS for three time after culture, and then fixed using 35mL 4% PFA for overnight at 4°C followed by 45 minutes wash with 1x PBS. The samples were then placed in tissue cassettes, and put into the Leica TP 1020 processor, processed using the following protocol:

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>CONDITION</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>70%[v/v] Ethanol</td>
<td>Vacuum</td>
<td>2 h</td>
</tr>
<tr>
<td>80%[v/v] Ethanol</td>
<td>Vacuum</td>
<td>2 h</td>
</tr>
<tr>
<td>95%[v/v] Ethanol</td>
<td>Vacuum</td>
<td>2 h</td>
</tr>
<tr>
<td>100%[v/v] Ethanol</td>
<td>Vacuum</td>
<td>2 h</td>
</tr>
<tr>
<td>100%[v/v] Ethanol</td>
<td>Vacuum</td>
<td>2 h</td>
</tr>
<tr>
<td>Xylene</td>
<td>Vacuum</td>
<td>3 h</td>
</tr>
<tr>
<td>Chemical</td>
<td>Time</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>Xylene</td>
<td>3 h</td>
<td></td>
</tr>
<tr>
<td>Xylene</td>
<td>3 h</td>
<td></td>
</tr>
<tr>
<td>Paraffin Wax</td>
<td>2 h</td>
<td></td>
</tr>
<tr>
<td>Paraffin Wax</td>
<td>2 h</td>
<td></td>
</tr>
<tr>
<td>Paraffin Wax</td>
<td>2 h</td>
<td></td>
</tr>
</tbody>
</table>

TOTAL: 25h

After processing, the sample cassettes were removed from the tissue processor, and the samples were taken out from the cassettes. A little wax was placed in the embedded tray, allowing it to slightly cool. The sample then was placed down, and the tray was filled with wax. The sample and tray was first cool down on the cooling station for 20 minutes and then the embedded block was removed from the tray.

2.5.2 Haematoxylin and Eosin Staining

The paraffin-embedded tissue sample were cut into 5μm thick section, placed onto the microscope slider and processed by following protocol:

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution</td>
<td>Duration</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Xylene</td>
<td>5min</td>
</tr>
<tr>
<td>Xylene</td>
<td>5min</td>
</tr>
<tr>
<td>100%[v/v] Ethanol</td>
<td>2min</td>
</tr>
<tr>
<td>90%[v/v] Ethanol</td>
<td>2min</td>
</tr>
<tr>
<td>70%[v/v] Ethanol</td>
<td>2min</td>
</tr>
<tr>
<td>Distill water</td>
<td>2min</td>
</tr>
<tr>
<td>Haematoxylin</td>
<td>2min</td>
</tr>
<tr>
<td></td>
<td><strong>Washed in running water till clean</strong></td>
</tr>
<tr>
<td>Lithium Carbonate</td>
<td>1min</td>
</tr>
<tr>
<td>Distill Water</td>
<td>1min</td>
</tr>
<tr>
<td>Eosin</td>
<td>30s</td>
</tr>
<tr>
<td></td>
<td><strong>Washed in running water till clean</strong></td>
</tr>
<tr>
<td>70%[v/v] Ethanol</td>
<td>2min</td>
</tr>
<tr>
<td>90%[v/v] Ethanol</td>
<td>2min</td>
</tr>
<tr>
<td>100%[v/v] Ethanol</td>
<td>2min</td>
</tr>
<tr>
<td>100%[v/v] Ethanol</td>
<td>3min</td>
</tr>
<tr>
<td>Xylene</td>
<td>5min</td>
</tr>
</tbody>
</table>
2.5.3 Histological scoring

Three tissue sections from different depth of the paraffin block of each sample were selected. The H&E stained sections were viewed under the microscope, with at least 5 fields (20x magnifications) of each slice randomly selected for histological assessment by 3 individuals blinded to group allocation. Four parameters, including fiber arrangement, fiber structure cell roundness and cell density, were evaluated using a 4 point scoring scale from 0 to 3, where 0 being normal and 3 being severely abnormal. The represented Figures of each score are shown as Figure 2.1. The average score of each parameter was calculated and used for comparison.
2.6 Type III collagen immunohistochemistry

Paraffin-embedded sections were dewaxed and rehydrated using graded ethanol as described in 2.5.2 Haematoxylin and Eosin Staining, and processed by the following procedure.

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>CONDITION</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>3% hydrogen peroxide (H₂O₂)</td>
<td>Room temperature</td>
<td>10min</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>4min</td>
</tr>
<tr>
<td>5% fetal bovine serum in 1xPBS</td>
<td></td>
<td>30min</td>
</tr>
<tr>
<td>PBS</td>
<td>4min</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>monoclonal anti-collagen type III 1:100</td>
<td>damp chamber 4°C</td>
<td>overnight</td>
</tr>
<tr>
<td>PBS</td>
<td>3 x 10min</td>
<td></td>
</tr>
<tr>
<td>Biotinylated goat antimouseIgG 1:200</td>
<td>1h</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>3 x 10 min</td>
<td></td>
</tr>
<tr>
<td>DAKO Dab stain</td>
<td>3 min</td>
<td></td>
</tr>
<tr>
<td>Running water</td>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td>Haematoxylin</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>running water</td>
<td>1min</td>
<td></td>
</tr>
<tr>
<td>70%[v/v] Ethanol</td>
<td>Room temperature</td>
<td>2min</td>
</tr>
<tr>
<td>90%[v/v] Ethanol</td>
<td>2min</td>
<td></td>
</tr>
<tr>
<td>100%[v/v] Ethanol</td>
<td>2min</td>
<td></td>
</tr>
<tr>
<td>100%[v/v] Ethanol</td>
<td>3min</td>
<td></td>
</tr>
<tr>
<td>Xylene</td>
<td>5min</td>
<td></td>
</tr>
<tr>
<td>Xylene</td>
<td>5min</td>
<td></td>
</tr>
<tr>
<td>Mount in DPX</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.7 TUNEL assay

Paraffin-embedded sections were dewaxed and rehydrated using graded ethanol as described in 2.5.2 Haematoxylin and Eosin Staining, and processed by following procedure.

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>20µg/mL proteinase K</td>
<td>15min</td>
</tr>
<tr>
<td>PBS</td>
<td>4min</td>
</tr>
<tr>
<td>TUNEL reaction mixture</td>
<td>1h</td>
</tr>
<tr>
<td>PBS</td>
<td>3 x 4min</td>
</tr>
<tr>
<td>convert-AP</td>
<td>30min</td>
</tr>
<tr>
<td>PBS</td>
<td>4min</td>
</tr>
<tr>
<td>DAKO Dab stain</td>
<td>5min</td>
</tr>
<tr>
<td>Running water</td>
<td>30 min</td>
</tr>
<tr>
<td>Haematoxylin</td>
<td>1 min</td>
</tr>
<tr>
<td>Running water</td>
<td>1min</td>
</tr>
<tr>
<td>70%[v/v] Ethanol</td>
<td>2min</td>
</tr>
<tr>
<td>90%[v/v] Ethanol</td>
<td>2min</td>
</tr>
</tbody>
</table>
Three tissue sections from different depth of the paraffin block of each sample were selected. The TUNEL stained sections were viewed under the microscope, with at least 5 fields (20x magnifications) of each slice randomly selected for histological assessment by 3 individuals blinded to group allocation. The positive staining cell and normal cell were counted. The overall cell apoptotic rate was calculated and used for comparison.

### 2.8 Statistics analyses

All statistics have been analyzed using the prism 5.03 software package (GraphPad Software 5.0, USA). Data are presented as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA). P values less than 0.05 were considered significant.
Chapter 3

Bioreactor Design and Fabrication

This chapter is partly based on the paper

Programmable mechanical stimulation influences tendon homeostasis in a bioreactor system

Tao Wang, Zhen Lin, Robert E Day, Bruce Gardiner, Euphemie Landao-Bassonga, Jonas Rubenson, Thomas B. Kirk, David W. Smith, David G. Lloyd, Gerard Hardisty, Allan Wang, Qiujuan Zheng, Ming H Zheng

Biotechnology and Bioengineering

Volume 110, Issue 5, pages 1495-1507, May 2013
CHAPTER 3 (RESULTS): DESIGN AND CONSTRUCTION OF BIOREACTOR

In this study we focus on the design of a tendon-engineering bioreactor, which is able to provide programmable mechanical stimulation and a sterile culture environment. The specific objective of the current study is to apply the design principles of mechanical and electrical engineering to construct a functional bioreactor for tendon culture. To fulfill this objective, a new bioreactor was designed using Solidworks 3D CAD software (Solidworks Corp., Concord, MA), and constructed by an external mechanical vendor under the supervision of the PhD candidate. This bioreactor system will assist in evaluating the effect of various programmable mechanical stimulation regimes on tendon homeostasis and repair *in vitro*, and ultimately enable optimization of the mechanical stimulus for engineered tendon culture.

3.1 Design criteria

To develop a functional bioreactor several design criteria were formulated:

1. The bioreactor must be able to process more than three independent culture specimens simultaneously.

2. The system should have transparent covers, allowing visual monitoring.

3. The mechanical stimulation must be programmable.

4. The extension resolution should be better than ±100μm with the load varying as
required to produce the set strain

5. The minimum expected load was 20N per sample, which is minimum 120N for the bioreactor system.

6. The system must incorporate a means of holding the specimen ends to apply stimulation without either damaging the specimen or allowing the construct to slip relative to the grips.

7. The bioreactor must be able to operate inside a standard incubator, so all of the electrical and mechanical components have to be able to function in a warm and humid environment.

8. The parts in contact with the tissue culture should be autoclavable.

3.2 Material selection

In our design, the bioreactor is divided into two main components, the frame and the culture chambers. The bioreactor is operated inside an incubator, and the culture medium must be changed regularly. As this would require the operator to move the bioreactor in and out of the incubator, the overall weight was a problem that needed to be considered. For local sterilization, 70%[v/v] ethanol and UV treatment are commonly used; therefore, both parts had to be chemical resistant and UV stable. Moreover, the material used for culture chamber had to withstand a higher level of sterilization such as autoclaving and also be resistant to humidity and chemical attack from the culture medium.
In summary, the material used in the bioreactor had to meet the following requirements:

- Good machinability.
- Corrosion resistant (culture medium and warm humid air)
- UV stable
- Compatible with alcohol (up to 70% [v/v])
- For the frame: as light as possible
- For the culture chamber: compatible with autoclave sterilization

From the criteria listed above, a corrosion resistant metal would be preferred to other materials due to its good machinability, high strength, UV stable and availability in the market. We chose 6063 aluminum for the frame. Although its corrosion resistance is not as good as stainless steel, it is relatively light compared to other metals. For the culture chamber, AISI 316 stainless steel was chosen due to its good resistance to chemical attack and humidity, moreover, it is suited to high pressure steam autoclaving, which is the main sterilization method in our study.

### 3.3 Overall bioreactor design

After considering all the criteria and reviewing the published literature on both custom-made and commercialized bioreactors, the design was finalized. The overall
dimensions of this bioreactor are 260mm × 334mm × 55mm, which is able to fit into a standard incubator easily, and the overall weight is 6.9kg. Programmable mechanical stimulation was generated with a single stepper motor and the motion transferred to the cultured tissue through a ball-screw. This design is able to process dynamic cultures for 6 specimens simultaneously; and frequency, displacement and culture period can be preprogrammed through a computer. Figure 3.1 shows the schematic illustration of the bioreactor system, and Figure 3.2 shows the overview of the bioreactor.

Figure 3.2 Schematic illustration of the bioreactor system
Figure 3.4 Top view of bioreactor.
3.3.1 The Actuating System

The actuating system is the key of the whole bioreactor system, providing mechanical stimulation to the cultured tissue. After reviewing the various designs of published bioreactors, it was found that pneumatic actuators (Juncosa-Melvin, Shearn et al. 2006; Nirmalanandhan, Rao et al. 2008; Nirmalanandhan, Shearn et al. 2008), linear motors (Nguyen, Liang et al. 2009; Doroski, Levenston et al. 2010) and stepper motor-ball screw combinations (Altman, Lu et al. 2002; Webb, Hitchcock et al. 2006; Chen, Yin et al. 2010) are the most common actuating systems. Considering the position accuracy and force output required in our study, a stepper motor-ball screw system was chosen. There are two common types of stepper motors available in the market, 2-phase and 5-phase stepper motors. A 2-phase motor only has 8 magnetic poles, whereas a 5-phase motor has 10. The extra poles provide higher resolution, lower vibration, higher acceleration and less synchronization loss than a 2-phase motor for little extra expense. Therefore, a 5 phase stepper motor (PMC33A3, Oriental Motor, Japan) with 0.72° resolution was selected for this bioreactor. To protect the stepper motor, an aluminum cover was designed, which can eliminate the heat caused by the motor due to its good heat conduction (Figure 3.3).

In order to transfer the rotation of the stepper motor to linear movement, a ball-screw is necessary. Two common forms of ball-screw are commercially available, individual ball-screws and guide actuator assemblies. Further machining and base design would be required if a single ball-screw was chosen. The guide actuator consists of a pre-assembled ball-screw, guide track and moving platform, which reduces the error
caused by further machining. Therefore the KR15 (THK, Japan) guide actuator was chosen in our design. The lead of the KR15 is 1mm, and given the 500 steps per revolution resolution of the stepper motor, this result in 1/500 or 2 micrometer per step linear resolution.

3.3.2 The Culture Chamber

AISI 316 stainless steel was used for culture chamber construction due to its good machinability and good resistance to chemical and high temperature. Multiple chambers are necessary, considering that triplication is the minimum requirement for each experiment. To balance the force distribution, the 6 chambers were uniformly located on the base as shown as Figure 3.3. In order to simplify the operation, the culture chamber was a separable individual design with 30mL capacity, shown as Figure 3.4. Each culture chamber can be separated from the base and autoclaved. A removable glass cover was designed to prevent contamination and enable better observation during the culture period. A 1mm gap was reserved between the shaft of tissue clamps and the glass cover, which allow the sufficient air exchange to the culture medium.
3.3.3 Tissue Fixation

The method of tissue fixation is an important aspect of bioreactor designing needing further consideration, as during mechanical stimulation in culture medium, sample slippage can occur. Most bioreactor designs only have flat jawed tissue clamps that, while suitable for flat membranes, cannot provide effective fixation with thick cylindrical tissues such as tendon, leading to increased slippage. Therefore here two fixation methods were designed for different sample types. The thin tissue or scaffold can be clamped by the clamp cover and tightened using screws shown as figure (Figure 3.5A). Big and slippery tissues can be fixed by the tissue hook or tied to the tissue hook using suture (Figure 3.5B).
3.3.4 The Control System

The two main choices for control systems in other bioreactors are direct computer control or a dedicated controller. Although programming a dedicated controller is more complicated than using a computer, the dedicated controller is much more reliable. Therefore, the selected control system was a programmable logic controller (MELSEC FX1S, Mitsubishi, Japan) and a stepper motor driver (PMD03CA, Oriental Motor,
Japan). This driver is compatible with the PMC33A3 stepper motor. The motion control program was written by the candidate himself using GX Developer (Mitsubishi, Japan), and then downloaded to the PLC via RS232 cable. The PLC is able to provide independent control on the stepper motor without any external requirements except power.

3.4 Bioreactor setup

Before each experiment, the frame of the bioreactor was first sprayed using 70% [v/v] ethanol and air dried in a laminar flow hood followed by sterilization under UV light for 30 minutes. The culture chambers and tissue clamps were double wrapped in paper bags separately and autoclaved followed by drying in a 60°C oven for 2 hours.

The bioreactor was placed in a standard incubator at 37°C and 5% CO₂ shown as Figure 3.6A. A 5-pin plug and cable connected the bioreactor to the control box outside of the incubator through the standard hole at the back of the incubator (Figure 3.6B). Figure 3.7 illustrates the bioreactor setup in the incubator.
Figure 3.7 (A) Bioreactor in the incubator. (B) A five pin plug and cable connects the bioreactor to the control box through the hole at the back of the incubator.
3.5 Discussion

In recent years, many studies of tendon engineering have been conducted using custom-made bioreactor system, and the results have been promising (Juncosa-Melvin, Shearn et al. 2006; Webb, Hitchcock et al. 2006; Androjna, Spragg et al. 2007; Nirmalanandhan, Rao et al. 2008; Nirmalanandhan, Shearn et al. 2008; Abousleiman, Reyes et al. 2009; Nguyen, Liang et al. 2009; Chen, Yin et al. 2010; Doroski, Levenston...
et al. 2010; Saber, Zhang et al. 2010; Issa, Engebretson et al. 2011; Woon, Kraus et al. 2011). Several commercial tendon engineering bioreactors are available now, such as Bose® ElectroForce® BioDynamic® and LigaGen system. To develop a functional bioreactor system, several factors needed to be considered, including material, mechanical design, tissue fixation method and operating environment. Compared with commercial bioreactors in Bose and LigaGen, the current design has several advantages. Firstly, our device can condition multiple samples simultaneously but separately. It can be easily cleaned and sterilized in standard autoclaves. Being constructed from AISI 316 stainless steel it can go through many sterilization cycles without corrosion or degradation. The device can produce a maximum force of 198N, which is sufficient for most tendon scaffold and tendon tissue from small scale animals such as are commonly used in tissue engineering research. Most commercial bioreactor systems have problems in holding thick and slippery tissue during PMS, whereas our novel tissue hook design demonstrated excellent tissue fixation. Finally, the actuation system is designed to operate inside a standard incubator. It is able to function in a humid environment and the horizontal design is easy to move and will only occupy a small space in the incubator. As the tendon’s main function is mechanical, this bioreactor system is able to provide high-accuracy programmable mechanical stimulation (PMS), enabling different loading regimes including displacement, frequency and stimulation period. Once programmed it is able to operate reliably for weeks without computer support.
Chapter 4

Programmable mechanical stimulation influences tendon homeostasis in a bioreactor system

This chapter is partly based on the paper

Programmable mechanical stimulation influences tendon homeostasis in a bioreactor system


Published in

Biotechnology and Bioengineering

Volume 110, Issue 5, pages 1495-1507, May 2013
CHAPTER 4 (RESULTS): PROGRAMMABLE MECHANICAL STIMULATION INFLUENCES TENDON HOMEOSTASIS IN A BIOREACTOR SYSTEM

4.1 Abstract

Identification of functional programmable mechanical stimulation (PMS) on tendon not only provides the insight of the tendon homeostasis under physical/pathological conditions, but also guides a better engineering strategy for tendon regeneration. The aims of the study are to design a bioreactor system with PMS to mimic the in vivo loading conditions, and to define the impact of different cyclic tensile strains on tendon. Rabbit Achilles tendons were loaded in the bioreactor with/without cyclic tensile loading (0.25Hz for 8 hours/day, 0–9% for 6 days). Tendons without loading lost their structural integrity, as evidenced by disorientated collagen fiber, increased type III collagen expression, and increased cell apoptosis. Tendons with 3% of cyclic tensile loading had moderate matrix deterioration and elevated expression levels of MMP-1, 3 and 12, whilst loading regime of 9% caused massive rupture of collagen bundles. However, 6% of cyclic tensile strain was observed to maintain the structural integrity and cellular function. Our data indicated that an optimal PMS exists to maintain tendon homeostasis, although this is only a narrow range of tensile strain that can induce this action. The suggested clinical impact of this study is that optimized eccentric training program, based around targeting specific tendon strain, could be developed to achieve beneficial effects on chronic tendinopathy management.
4.2 Introduction

Tissue engineering methods have great potential in the repair of damaged tendons. However, these methods are still very much in their infancy, due to the lack of an integrated understanding of the key conditions required for successful tendinopathy intervention and repair. Recent tendon research has focused on the development of tendon tissue engineering, with the aim of constructing a bio-substitute material for tendon/ligament repair(Juncosa-Melvin, Shearn et al. 2006; Sahoo, Ouyang et al. 2006; Webb, Hitchcock et al. 2006; Chen, Willers et al. 2007; Chen, Qi et al. 2008; Nirmalanandhan, Rao et al. 2008; Saber, Zhang et al. 2010; Stoll, John et al. 2010; Peach, James et al. 2012; Wang, Gardner et al. 2012; Woon, Farnebo et al. 2012); This strategy involves an integration of technologies from cell biology, material science and mechanical engineering.

Engineered bioreactor systems have been demonstrated to be effective tools for constructing various biological tissues and organs such as muscle(Dennis, Smith et al. 2009), liver (Catapano, Patzer et al. 2010) and bone(Rauh, Milan et al. 2011; Rauh, Milan et al. 2012). Several bioreactor systems have been developed specifically for engineered tendon/ligament formation (Altman, Lu et al. 2002; Juncosa-Melvin, Shearn et al. 2006; Webb, Hitchcock et al. 2006; Nirmalanandhan, Rao et al. 2008; Nirmalanandhan, Shearn et al. 2008; Issa, Engebretson et al. 2011). Owing to force transmission being the primary function role of tendon/ligament, mechanical stimulation plays an essential role in the maintenance of tendon structure and
function(Hannafin, Arnoczky et al. 1995). Achilles tendons endure tensile loading \textit{in vivo} during daily physical activities. More complex structures, like the anterior cruciate ligament, undergo both tensile and torsion loading during weight-bearing knee flexion(Li, Defrate et al. 2005). To restore tendon function after injury, engineered tendon constructs should be compatible with the \textit{in vivo} environment both biologically and mechanically(Calve, Lytle et al. 2010). Cell-scaffold constructs may be ‘exercised’ in an artificial mechanical environment before implantation. Indeed, programmable mechanical stimulation (PMS) is considered to be one of the key components for tendon/ligament forming bioreactor system (Altman, Lu et al. 2002; Juncosa-Melvin, Shearn et al. 2006; Webb, Hitchcock et al. 2006; Nirmalanandhan, Rao et al. 2008; Nirmalanandhan, Shearn et al. 2008; Issa, Engebretson et al. 2011). In this study, a bioreactor system was designed to apply different cyclic tensile strain on isolated rabbit Achilles tendon to establish an \textit{ex vivo} model (see previous Chapter). The present aim is to evaluate the effect of different mechanical stimulation on tendon tissue using this developed bioreactor system.

The mechanical stimulation of engineered tendon constructs can result in different structures. Non-loading of engineered neo-tendons results in loose, disorganized matrices with reduced collagen quantity(Jiang, Liu et al. 2011). Static mechanical stress has been shown to improve the fiber arrangement of engineered tendon constructs, but did not produce a compacted tendon-like collagen structure \textit{in vitro}(Chen, Willers et al. 2007). More promisingly, engineered tendon under cyclic tensile strain exhibited a well-organized collagen fiber arrangement and structure and enhanced tensile strength,
suggesting that PMS is preferable for engineered tendon formation (Jiang, Liu et al. 2011). Even though the importance of providing PMS in tendon bioreactor has been recognized previously (Brown and Carson; Wang, Gardner et al. 2012), the optimal cyclic tensile stimulation parameters have not been defined. It is generally assumed that mimicking the dynamic mechanical loading created by daily physical activity will give the optimal loading regime in tendon bioreactor system, but this is not yet proven.

The goals of this study were to investigate the effect of different mechanical stimulation regimes on tendon integrity. I hypothesized that there is an optimum range of mechanical stimulation required for tendon homeostasis and that under or over-stimulation would reduce tendon integrity.

4.3 Material and Method

4.3.1 Tissue harvest and distribution

Achilles tendons were dissected from the hindlimbs of female New Zealand White rabbits (Oryctolagus cuniculus) aged between 14 and 15 weeks with body weight 3-5kg. The tissue was briefly rinsed in 70%[v/v] ethanol, washed in 1x phosphate buffered saline (PBS) 3 times, and submerged in serum free Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (GIBCO, Invitrogen, Auckland, New Zealand) supplemented with 50 µg/mL Gentamicin before further processing.
In total 24 Achilles tendons from 12 rabbits were allocated to 3 groups (Figure 4.1A): a loading deprivation group (8), a dynamic culture (cyclic tensile strain) group (12) and a control group (4). In the loading deprivation group, the Achilles tendons were cultured in petri dish in growth medium (DMEM/F-12, 10% fetal bovine serum and 50 µg/mL Gentamicin) without mechanical loading for either 6 days (4 tendons) or 2 weeks (4 tendons). In the dynamic strain group, the tendons were fixed on the tissue hooks using suture and cultured under 3%, 6% or 9% cyclic tensile strain for 6 days (8 hours per day, 0.25Hz, Figure 4.1A). The stimulating cycle was as demonstrated in Figure 4.1B. This regimen was based on previously published studies and data on rabbit activity levels (West, Juncosa et al. 2004). The samples were sliced into two half, ones was fixed in 4% paraformaldehyde overnight for histological analysis and the other half directly subjected to RNA extraction. The control group was made up of 4 tendons directly dissected from the animal and immediately fixed.

![Figure 4.1](image)

Figure 4.1 (A) Number and distribution of samples for each of the 12 rabbits. (B) Daily mechanical stimulation pattern used in this study. (C) Mechanical input signal from the control system.
4.3.2 Histological preparation and assessment

Refer to 2.5

4.3.3 TUNEL assay

Refer to 2.7

4.3.4 Immunostaining for type III collagen

Refer to 2.6

4.3.5 Quantitative Real-time polymerase chain reaction (Q-PCR)

Refer to 2.3 and 2.4
Table 4.5 Rabbit specific primer sequences for the genes used.

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (3’ to 5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1A2</td>
<td>TTGCCCTTCCTTGATATTGC</td>
<td>CCTCTTTCGCCCAATAAATTA</td>
</tr>
<tr>
<td>COL3A1</td>
<td>AAGCCCCAGCAGAAAAATTG</td>
<td>TGGTGGAACAGAAAAATCA</td>
</tr>
<tr>
<td>MMP1</td>
<td>GGCTCAGTTCGTCCTCACTC</td>
<td>CAGGTCCATCAAAGGGAGAA</td>
</tr>
<tr>
<td>MMP3</td>
<td>GCTTTGCTCAGCCTATCCAC</td>
<td>ACCTCAAGCCAAGGAACTT</td>
</tr>
<tr>
<td>MMP12</td>
<td>ATGCCAGGGAGACCAGTATG</td>
<td>AAAGCATGGGCTATGACACC</td>
</tr>
<tr>
<td>36B4</td>
<td>ACCAAAAATGCTTCATCGT</td>
<td>CAGGGTTGTGTTCAGATGC</td>
</tr>
</tbody>
</table>

4.3.6 Statistical analyses

Refer to 2.8

4.4 Results

4.4.1 Histological examination of tendon undergoing different cyclic tensile strains

Compared to the native tendon (Figure 4.2 A), the collagen fiber arrangement changed from parallel to moderately wavy after 6 days in the absence of applied loading. Cell density was increased with slightly rounded nuclei. After 2 weeks without loading, progressive extracellular matrix (ECM) disruption was observed. Collagen fiber
arrangement had no discernible pattern. Fragmentation of collagen fibers and rounded tenocyte nuclei were observed microscopically (Figure 4.2C). Histological assessment showed a time dependent increasing grading in fiber arrangement, fiber structure and cell roundness, indicating progressive divergence from the native (control) tissue. Cell density was also increased after 6 days without loading (Figure 4.2D-G).
Figure 4.2 Loading deprivation culture group. (A) Native Achilles tendon structure (day 0). (B) Achilles tendon histology after 6 days loading deprivation culture with minor wavy collagen structure. (C) Achilles tendon histology after 2 weeks culture displays disorganized collagen structure with round tenocytes. (D) Fiber arrangement, scale of 0-3, 0 represents compacted and parallel, and 3 represents no identifiable pattern. (E) Fiber structure, scale of 0-3, 0 represents continuous, long fiber, and 3 represents severely fragmented. (F) Cell roundness, scale of 0-3, 0 represents long spindle shape, and 3 represents severe rounding. (G) Cell density, scale of 0-3, 0 represents normal pattern, and 3 represents severely increase Result are expressed as the mean±S.D. (*p<0.05, **p<0.01, ***p<0.001). Scale bar=50 micrometers.
In the dynamic culture (cyclic tensile strain) group, histology revealed that the 3% strain stimulated group had slightly disrupted extracellular matrix structure and significant cellular morphology changes (Figure 4.3B). These tendon samples graded 1.5 in fiber arrangement, 1.1 in fiber structure and 1.3 in cell roundness (Figure 4.3E-G). However, in the tendons that underwent 6% strain, the extracellular matrix orientation and cellular morphology appeared normal (Figure 4.3A, C). That is, there was no statistical difference of the histology scoring between the 6% group and native tissue (Figure 4.3E-H). In the 9% strain stimulated group, the tendon samples were found to be partially torn and graded 1.45 in fiber structure and 0.8 in fiber arrangement (Figure 4.3E, F). Increased cell number and slightly rounded tenocyte nuclei were observed around the rupture site (Figure 4.3D, G, H).
Figure 4.3 6 days dynamic culture group. (A) Native Achilles tendon structure. (B) 3% stimulated group with wavy collagen structure and minor rounded tenocytes. (C) 6% stimulated group with similar morphology compared with the native group. (D) 9% stimulated group with severe tear collagen structure, and rounded nuclei around the tear region (arrow). (E) Fiber arrangement, scale of 0-3, 0 represents compacted and parallel, and 3 represents no identifiable pattern. (F) Fiber structure, scale of 0-3, 0 represents continuous, long fiber, and 3 represents severely fragmented. (G) Cell roundness, scale of 0-3, 0 represents long spindle shape, and 3 represents severe rounding. (H) Cell density, scale of 0-3, 0 represents normal pattern, and 3 represents severely increase. Result are expressed as the mean±S.D. (*p<0.05, **p<0.01, ***p<0.001). Scale bar=50 micrometers.
4.4.2 Impact of cyclic tensile strain on apoptosis of tenocytes

To investigate the impact of mechanical force on the apoptosis of tenocytes, I used the TUNEL assay to examine the cellular apoptosis rate in specimens. In the native tendons, the average cell apoptosis fraction (as a proportion of total cell number) was less than 10% (Figure 4.4A, G). The apoptosis fraction progressively increased over time when the tissues were cultured without loading (35% in 6 days and 95% in 2 weeks) (Figure 4.4G). Cell viability in 3% and 6% stimulated Achilles tendon was similar to that in native tendon (Figure 4.4D, E) without statistical significance (Figure 4.4G), whilst cell apoptosis rate increased to 45% when the stimulation strain reach 9% (Figure 4.4F, G).
Figure 4.4 TUNEL Assay (A) Native Achilles tendon with less than 10% cell apoptosis. (B) 6 days loading deprivation group has increased apoptotic tenocytes pointed out by arrows. (C) In 2 weeks loading deprivation group, almost all tenocytes are under apoptosis (D) 3% stimulated group with minor increased apoptotic cells (arrow). (E) 6% stimulated group with similar cell apoptosis rate as native sample (F) 9% stimulated group with massive cell apoptosis around the damaged site (pointed by arrows). (G) Quantified cell apoptosis fraction of each group. Result are expressed as the mean±S.D. (*p<0.05, **p<0.01, ***p<0.001). Scale bar=50 micrometers
4.4.3 Type III collagen turnover in Achilles tendon under dynamic culture

To investigate the extracellular matrix turnover in tendon undergoing different cyclic tensile strain, type III collagen immunohistochemistry was done. No positive type III collagen was observed in the core of healthy tendon using immunochemistry (Figure 4.5A). After 6 days of either no loading or 3% strain, the Achilles tendons expressed only minor and moderate collagen type III respectively (Figure 4.5B,C), whilst relatively higher positive staining were found in the 9% strain group (Figure 4.5E). No type III collagen expression was observed in the 6% cyclic strain stimulated group (Figure 4.5D), which was similar to the native tendon.

![Collagen type III immunohistochemistry](image)

Figure 4.5 Collagen type III immunohistochemistry (A) Native Achilles tendon with minor collagen type III expression. (B) Loading deprivation group has minor increased collagen type III expression and some positive staining tenocytes pointed out by arrow. (C) 3% stimulated group with increased collagen type III expression. (D) 6% stimulated group with similar collagen type III expression as native sample (E) 9% stimulated group with massive increased collagen type III expression and positive staining tenocytes (pointed by arrows). Scale bar=50 micrometers
4.4.4 Impact of cyclic tensile strain on ECM remodeling gene expression

Q-PCR was carried out to examine the gene expression of type I (COL1A2), type III collagen (COL3A1) and MMP1, 3, 12 (matrix metalloproteinases). As shown in Figure 8, the tissues subjected to 6% cyclic tensile strain had the highest expression of COL1A2 (Figure 8A), whereas 3% stimulated group had the lowest expression. Type III collagen (COL3A1) was suppressed by minor and moderate loading (3% and 6%), yet upregulated in the 9% strain stimulated group (Figure 8B). Gene expression of MMP1, 3 and 12 were highly upregulated by 3% strain stimulation compared to the other groups.

![Figure 4.6](image)

Figure 4.6 Quantitative PCR results. A: COL1A2 expression in 6% stimulated group is significantly higher than other groups. B: COL3A1 expression in 9% stimulated group is significantly higher than other groups. Three percent and 6% group had much lower COL3A1 expression compared to the other groups. C–E: Three percent stimulated group has significantly high expression on MMP1, MMP3 and MMP12, respectively. Result is expressed as the mean±S.D (*p<0.05, **p<0.01, ***p<0.001)
4.5 Discussion

A previous study has suggested that engineered neo-tendons in a non-loading environment result in loose, disorganized and less collagen-rich matrices (Jiang, Liu et al. 2011). Static mechanical loading has been shown to improve the fiber arrangement of engineered tendon constructs, but did not produce a compacted tissue structure in vitro (Chen, Willers et al. 2007). Engineered neo-tendons with PMS had a well-organized fiber arrangement and structure and enhanced tensile strength, suggesting that dynamic mechanical loading is preferable for engineered tendon formation (Jiang, Liu et al. 2011). Although PMS has been recognized as a critical regulator for tendon formation (Brown and Carson et al. 1999), the optimal PMS parameters are yet to be defined.

In this study we designed a uni-axial bioreactor system with a programmable stimulation regimen. Achilles tendons from rabbits were used to evaluate the impact of different PMS conditions on tendon tissue. Our result showed that 3% cyclic tensile strain in the bioreactor did not prevent matrix deterioration, whilst at 6% cyclic tensile strain structural integrity and cellular function was maintained. At 9% cyclic tensile strain there was massive rupture of the collagen bundles. However, these results are for an already mature tendon and the loading regimes may be different for engineered constructs at various stages of growth. Furthermore, the frequency of loading may also have an important role in maintaining tendon homeostasis and this need to be better qualified by in vivo measurements of Achilles tendon loading. Nevertheless, the current
results demonstrate that PMS can be achieved in this bioreactor, and that an optimal cyclic tensile loading (around 6% cyclic tensile strain) is required to maintain healthy tendon homeostasis, at least over the time period of the cultures used in this study.

It has been well known that tensile mechanical loading is essential for tendon integrity, and loading deprivation can cause tendinopathy-like morphology such as disorientated collagen fibers and rounded cell nuclei (Hannafin, Arnoczky et al. 1995). Yet the specific effect of different loading conditions on tendon has not been well elucidated. To study the effect of PMS on tendon/ligament, both in vitro and in vivo models have been used. The Flexcell® system has been applied to examine the in vitro gene expression profiles of tenocytes under tensile or shear force (Skutek, van Griensven et al. 2001; Wang, Jia et al. 2003; Yang, Im et al. 2005). These systems can precisely control the mechanical regimen and monitor the cell behaviors, but only use a monolayer of cells, not an organized tissue. Cells isolated from their extracellular niche and cultivated in a monolayer may not necessarily replicate their original behavior in vivo. In vivo models, such as Achilles tendon in rats or rabbit, have been commonly used as study models. Tendon loading is mainly induced by electrical stimulation on muscle (Nakama, King et al. 2005; Asundi, King et al. 2008), treadmill training on animal and partial tendon transaction (Smith, Sakurai et al. 2008). However, mechanical stimulation on the tendon varies between individuals, making precise quantification of load next to impossible. In this study, we have demonstrated a bioreactor system that not only can be used as an device for tendon/ligament engineering, but also serves as an ex vivo culture model for the study of tendon biology. Although the biomechanics of Achilles tendon is more
complicated under *in vivo* environment (West, Juncosa *et al.* 2004), by applying programmable mechanical stimulation, the effect of different cyclic tensile strain on tendon tissue could be systematically analyzed on a tissue level.

The present study has shown for the first time that only a narrow range of PMS can stimulate the anabolic effect of tendon tissue. This suggests a proposed model describing the effect of mechanical loading, from under-load to over-load, on tendon homeostasis. This model is illustrated in Figure 4.7 in which the black curve presents the typical mechanical response caused by increasing tensile strain in tendon. The breakage of collagen fibers increases with increasing strain, leading to eventual rupture at the failure point. The red curve, on the other hand, proposes the biological repair response of the tendon induced by tensile strain. Mechanical loading is required for the biological activity of tenocytes, yet the regime needs to be within a certain range to promote matrix product and net tissue repair. By combining these two curves, three crossover areas are seen, which can be divided according to their metabolic status into: A (anabolic) zone and C (catabolic) zones. In the A zone, where the tensile loading is within a certain range, the biological repair response caused by PMS exceeds the induced damage. In the C zone, there is either too little load to stimulate matrix production or too much mechanical damage caused by excess tensile loading. In our study the 3% strain stimulated group (under-loaded) was located in C zone. When the tendons were subjected to insufficient tensile strain, early tendinopathologic changes (including increased cellular number, type III collagen expression and disorientated collagen fibers) could be observed. These morphological changes may be attributed by
the up-regulation of MMPs expression in tendon under 3% tensile loading. MMPs are zinc-dependent endopeptidases that able to degrade all the components of the extracellular matrix. MMP-1 is primarily responsible for the cleavage of collagen, including type I, II and III (Krane, Byrne et al. 1996). MMP-3 and MMP-12 are other members of the MMP family that have a wide range of substrate specificities. Moreover, MMP-12 is able to activate MMP-3, and MMP-3 has the ability to activate other MMPs such as MMP-1, MMP-7 and MMP-9 (Knauper, Lopez-Otin et al. 1996; Chen 2004). Studies have shown that decreased mechanical force on tendon fiber would lead to the deprivation of cytoskeletal tension in tenocytes and alter the mechano-transduction in the cells (Arnoczky 2008). Insufficient loading of tenocyte can initiate the degenerative cascade by expressing a pattern of catabolic gene, including MMPs (Lavagnino, Arnoczky et al. 2003; Arnoczky, Tian et al. 2004; Lavagnino, Arnoczky et al. 2006; Arnoczky, Lavagnino et al. 2008; Egerbacher, Arnoczky et al. 2008). In this study, we have confirmed that under the underloading condition, the phenotype of the tenocytes changed from spindle to round, and the expression levels of catabolic genes, such as MMP-1, 2, and 12, were upregulated. The elevated catabolic factors may contribute to the further micro-damage of the collagen fiber. Conversely, although the tendons in the 6% strain stimulated group were subjected to more mechanical damage, the repair mechanism was able to maintain tissue homeostasis (i.e. there is a narrow ‘net anabolic window’ between two net catabolic zones). In the 9% group (overloaded), mechanical damage overwhelmed the tendon tissue repair and caused partial rupture, which is consistent with our proposed model.
The identification of an optimal PMS range for tendon will not only guide us to a better tissue-engineering pathway for tendon regeneration, but also provide an insight into tendon homeostasis under both physiological and pathological conditions. Clinically, chronic tendinopathy of Achilles tendon is usually conservatively managed by eccentric overload training. The calf muscle is eccentrically loaded with the knee held straight or the soleus muscle is maximally loaded with the knee bent (Alfredson, Pietila et al. 1998). Although positive impacts of eccentric overload training on tendinopathy treatment have been recorded, the outcomes have varied widely (Stanish, Rubinovich et al. 1986; Alfredson, Pietila et al. 1998; Silbernagel, Thomee et al. 2001; Alfredson and Lorentzon 2003; Fahlstrom, Jonsson et al. 2003; Roos, Engstrom et al. 2004; Shalabi, Kristoffersen-Wilberg et al. 2004). In the study of Alfredson et al. (Alfredson, Pietila et al. 1998) the overall improvement in pain score was up to 94%, though in the study of Silbernagel et al. reported only 29% (Alfredson and Lorentzon). Although the mechanism of eccentric overload training on tendinopathy management remains unclear, the present study has provided a possible explanation of the mechanism of eccentric loading on chronic tendinopathy. The high variation of clinical outcomes might be due to poor control of the loading regimen. Given that there is only a narrow range of tensile strain where eccentric exercise could provide an anabolic effect on tendon, clinical treatment protocols will need to accurately control the magnitude of load applied through the injured tendon.

Finally, it is noted that nutrient supply might be altered by advective transport associated with mechanical stimulation. Although a previous study on canine flexor
tendons suggested that mechanical stimulation did not alter small nutrient uptake compared to static culture, and the tendon repair improvements derived from passive motion is not related to an increase of nutrient transport (Hannafin and Arnoczky 1994), under different loading regimes, or in different tendon types, this may not be the case. Moreover, the transport of large molecules, such as growth factors and matrix components, may be affected by mechanical stimulation, as has been shown in studies in related tissues (Bonassar, Grodzinsky et al. 2000; Zhang, Gardiner et al. 2007). Therefore, the tendon stimulated by dynamic mechanical loading in this study might be attributed to the enhanced nutrient transport, compared to the static control. Consequently it is difficult to isolate the direct effect of mechanical stimulation on tendon response from potential enhanced advective transport processes. Further study would be required to measure the nutrition transport in tendon under PMS.
4.6 Conclusion

We have developed and validated a uniaxial bioreactor system. The results in our study showed tendons subjected to 6% of cyclic tensile strain were able to maintain their structural integrity and cellular function. Our data suggested that tendon is a mechano-sensing tissue where the net anabolic action of cyclic tensile strain only occurs over a narrow range. In our study, mechanical underloading (3% cyclic tensile strain) and overloading (9% cyclic tensile strain) produced a net negative impact on the rabbit Achilles tendon tissue.
Chapter 5

Cyclic mechanical stimulation rescues rabbit Achilles tendon in a bioreactor system

This chapter is based on the paper

*Cyclic mechanical stimulation rescues rabbit Achilles tendon from degeneration in a bioreactor system*

Tao Wang, Zhen Lin, Ming Ni, Robert E Day, Bruce Gardiner, Euphemie Landao-Bassonga, Jonas Rubenson, Thomas B. Kirk, David W. Smith, David G. Lloyd, Yan Wang, Qiujian Zheng, Ming H Zheng

(Submitted to Tissue Engineering Part A)
CHAPTER 5 (RESULTS): CYCLIC MECHANICAL STIMULATION RESCUES RABBIT ACHILLES TENDON FROM DEGENERATION IN A BIOREACTOR SYSTEM

5.1 Abstract

Mechanical stimulation has been identified as an essential factor for maintaining tendon homeostasis, and various studies have reported that Achilles tendons subjected to long-term *in vivo* immobilization display pathological changes while mechanical properties degrade. Our previous study showed that 6% strain cyclic mechanical stimulation is able to maintain the tendon homeostasis in the bioreactor system. Therefore, in this study, we hypothesize that 6% strain cyclic mechanical stimulation can rescue morphological and mechanical degeneration similar to tendinopathy *ex vivo*. New Zealand white rabbits and divided into 4 groups: native control group, 6 days and 12 days loading deprivation groups, and stimulation group. In loading deprivation groups, the Achilles tendons were cultured without loading for either 6 days or 12 days. In stimulation group, tendons were subject to 6% cyclic strain at 0.25Hz for 8 h/d for 6 days after 6 days no loading culture. Tendons in both loading deprivation groups developed progressive histological degradation, increased tenocytes apoptosis and weakened mechanical properties after loading deprivation. However, in stimulation group, the histological and mechanical properties substantially improved. Our results indicate that a 6% strain cyclic mechanical stimulation was able to rescue early-stage pathological changes from loading deprivation and restore Achilles tendon *ex vivo*. 
5.2 Introduction

Clinical findings have demonstrated tendon degeneration and compromised biomechanical properties occurs after load deprivation, including in situations ranging from bedridden patients to astronauts (Reeves, Maganaris et al. 2005). Studies have reported that early-stage pathological alterations in Achilles tendon following long term immobilization in both human and rat (Larsen, Forwood et al. 1987; Nakagawa, Totsuka et al. 1989; Kannus, Jozsa et al. 1997; Trudel, Koike et al. 2007). The previous study (Chapter 4) observed that rabbit Achilles tendon after load deprivation exhibited pathological changes that included: (i) wavy and ruptured collagen fibers, (ii) rounded cell morphology and (iii) increased cell apoptosis (Wang, Lin et al. 2013). Accumulated evidence, including Chapter 4, shows that in order to maintain normal tendon homeostasis, mechanical stimulation is required (Arnoczky, Lavagnino et al. 2007; Egerbacher, Arnoczky et al. 2008; Smith, Sakurai et al. 2008; Thornton, Shao et al. 2008; Wang, Gardiner et al. 2013). The key question is can functional tendon be restored after pathological changes due to unloading?

In the previous study, appearing in Chapter 4, it was found that a relatively narrow range of mechanical stimulation is able to maintain rabbit Achilles tendon in the bioreactor, whereas too little strain results in protease degradation while too much strain results in mechanically induced fatigue damage. For Achilles rabbit tendon it was found that a 6% strain set in a cyclic mechanical stimulation device is able to maintain tendon integrity in a bioreactor system. This current study examined if it was possible to rescue
the early pathological changes caused by 6 days of loading deprivation, similar to that observed in tendinopathy, by then applying 6% strain cyclic loading for a further 6 days. The positive effects of mechanical loading following load deprivation reported here have implications for all kinds of situations involving tendon degeneration, ranging from physical treatment following immobilization to the tendon healing process following surgery, and even to planning the most effective exercises for astronauts.

5.3 Material and method

5.3.1 Tissue preparation and programmable mechanical stimulation

Full-length Achilles tendons, including the bone-tendon and tendon-muscle junction were dissected from the hindlimbs of 15 weeks old female New Zealand white rabbits (*Oryctolagus cuniculus*). Animals were obtained immediately post-euthanasia. By-products from tendon samples were washed by 1x phosphate buffered saline (PBS) 3 times, and submerged in serum free Dulbecco’s modified Eagle’s medium (DMEM/F-12; GIBCO, Invitrogen, Auckland, New Zealand) supplemented with 50 μg/mL Gentamicin (Invitrogen, Auckland, New Zealand) before further processing.

In total, 72 Achilles tendons from 36 rabbits were evenly allocated to 4 groups (see Figure 1). In the native control group 18 Achilles tendons were dissected from the rabbits and stored for further experiments. In the 6 and 12 day load deprivation groups (LD6, LD12), 18 tendons each were cultured in the growth medium (DMEM/F-12, 10%
fetal bovine serum and 50 µg/ml Gentamicin) on a petri dish without mechanical loading at 37°C in a humid environment with 5% CO2 for either 6 or 12 days. In the stimulation group an additional 18 rabbit Achilles tendon were cultured on petri dish without mechanical loading for 6 days, followed by 6 days in the bioreactor chambers undergoing 6% cyclic tensile loading.

Figure 5.1 Flowchart of experimental plan and rabbit Achilles tendon allocation (LD: loading deprivation)

In the bioreactor chambers, the Achilles tendons were secured to the tissue hook using surgical sutures, as shown in Figure 5.2A, and then placed into the bioreactor chamber, which was in turn placed in a standard incubator at 37°C in a humid environment with
5% CO₂ (Figure 5.2B). During the dynamic culture process, the tendons were subjected to 6% cyclic strain mechanical stimulation at 0.25Hz for 8 hours followed by 16 hours rest (7200 cycles/day) for total 6 days (see Figure 5.2C).

Figure 5. 2 (A) Rabbit Achilles tendon was fixed in a bioreactor culture chamber; (B) Bioreactor setup in the incubator; (C) mechanical stimulation regime applied in the stimulation group; (D) Biomechanical testing with a custom cryogrips. The arrow indicates the rupture of Achilles tendon happened at the middle portion.

In each group, 6 samples were fixed in 4% paraformaldehyde overnight for histological analysis, 6 samples were directly subjected to RNA extraction, and the rest were wrapped by PBS soaked gauze and stored at -20°C for further mechanical testing.
5.3.2 Histological preparation and assessment

Refer to 2.5

5.3.3 TUNEL assay

Refer to 2.6

5.3.4 Biomechanics testing

To examine the effect of mechanical stimulation on the biomechanical properties of tendon, we performed mechanical testing using a modified dual frozen technique (Chen, Yu et al. 2011). Nine tendons from each group were used for the testing. The samples were thawed at room temperature on the day of testing. Both bone-tendon and tendon-muscle ends were placed in a pair of custom-designed cry grips. To prevent tissue sliding, both ends were snap-frozen by liquid nitrogen. A thermometer was used to monitor the temperature of the tissue between two cryogrips, to avoid the testing substrate freezing. The grips were then fixed into the Instron mechanical testing system (model 5566, Instron, China), and the middle portion of the Achilles tendon were subjected to tensile loading to failure (Chen, Yu et al. 2011). The samples were preloaded at 2N, then gradually pulled at a speed of 2mm/second until it reach the ultimate force and dropped to failure force. Failure force was preprogrammed as 70% of ultimate force. During the experiment, the samples were closely monitored. To confirm that each tested tendon was broken at the middle portion but not the point near the grip.
(Figure 2A). A force versus displacement curve was recorded, and the ultimate load was recorded. Mean stiffness was calculated based on the linear region of the loading curve.

5.3.5 Real-time polymerase chain reaction

Refer to 2.3 and 2.4

5.3.6 Immunohistochemistry

Refer to 2.6

5.3.7 Statistical analyses

Refer to 2.8

5.4 Results

5.4.1 Cyclic mechanical stimulation improved the histological structure and cell viability of cultured tendon

The Achilles tendons cultured under loading deprivation exhibit minor wavy and loose collagen structure (Figure 5.3B), compared to native tendon (Figure 5.3A), more gaps between the collagen fiber were observed, and the tenocytes started to lose their
elongated morphology (Figure 5.3F). When the culture period extended to 12 days, disorientated fibers with progressive extracellular matrix (ECM) disruption and rounded cell nuclei were observed (Figure 5.3C, G). These morphological changes are consistent with the pathology of tendinopathy. Interestingly, when 6% strain cyclic mechanical loading was applied on the Achilles tendon after 6 days loading deprivation culture, the tendon morphology seems to be similar to native tendon. Collagen fibers were found to be straight and well organized (Figure 5.3D), with no gap was observed between collagen fibers, while long spindle-shape cell morphology was successfully restored (Figure 5.3H).

Using the quantitative scoring system (Figure 5.4), the 6 days loading deprivation group
expressed a slight abnormality in fiber arrangement, i.e. fiber structure, cell roundness, and a moderate increase of cell density. In addition, the overall score further increased as the loading deprivation culture period was extended to 12 days, whereby every aspect increased significantly except for cell density. For the stimulation group, the tendon successfully regained normality, in both the overall score and the individual scores with no significant differences observed when compared to native tendon.

Figure 5. Histological score of three groups. (A) Overall histology score, and the 6% rescue group is significantly better than the other groups; (B) fiber arrangement; (C) fiber structure; (D) cell roundness; (E) cell density. Results are expressed as the mean±SEM. One way ANOVA significance values were *p < 0.05, **p < 0.01, and ***p < 0.001

In regard to cell apoptosis, the 6 day loading deprivation group exhibited numerous
positive TUNEL stained tenocytes, as can be observed under the microscope image (Figure 5.5B), while for the 12 day loading deprivation group, large numbers of apoptotic cells were observed (Figure 5.5C). However, there were only a few apoptotic cells detected in the stimulation group (Figure 5.5D), similar to that seen in the native tendon (Figure 5.5A). Quantification of these observations showed that cell apoptosis rate was around 30.6% for the 6 days loading deprivation group, which further increased to 91.3% in the 12 days loading deprivation group. However, the stimulation group’s tissue had a 17.8% cell apoptosis rate that was significantly lower than either load deprivation groups (Figure 5.5E), and only slightly higher than the native group.
Figure 5. 5TUNEL assay (A) Apoptotic cell was rarely detected in native Achilles tendon tissue (B) In 6 days loading deprivation group, around 30% of tenocytes were under apoptosis, pointed by arrows; (C) In 12 days loading deprivation group, almost all the tenocytes were undergoing apoptosis; (D) apoptotic tenocytes significantly decreased compared to the other experimental groups; (E) Quantitative cell apoptosis rate. Results are expressed as the mean±SEM. One way ANOVA significance values were *p < 0.05, **p < 0.01, and ***p < 0.001 Scale bar is 20mm.
5.4.2 Cyclic mechanical stimulation improved mechanical properties of cultured tendon

As the force transferring tissue, mechanical properties are the fundamental characteristic of tendon, although the pathological changes in the tendon microstructure were observed, the impact on the overall mechanical properties is still unclear. Therefore, mechanical testing was performed to examine the strength of the Achilles tendon after the *ex vivo* culture. In the mechanical tests, all samples failed at the mid-tendon region, which was the region of interest in this study (Figure 5.2D). As shown in Figure 5.6A, the average ultimate tensile force after 6 days of loading deprivation was 613.6±49.7 N, which was significantly higher than 12 days loading deprivation group (449.8±55.9 N) but lower than stimulation group (773.3±89.6 N). However, all the culture groups had a significantly lower ultimate tensile force compared to the native Achilles tendon (896.1 ±96.1 N).

The mean stiffness (Figure 5.6B) of the stimulation group was 165.6±39.1 N/mm, which was significantly higher than 6 days and 12 days loading deprivation groups (99.2 ±22.4 N/mm, 52.5 ±12.8 N/mm), while there was no statistical difference compared to the native controls (193.1±26.8 N/mm).
Figure 5. 6 (A) The maximum load of Achilles tendon tissue. The maximum loads decreased with extended cultured time in static cultured group, however, after 6 days static culture, 6% stimulation group is able to restored part of mechanical properties. Compared to native control tissue, the maximum loads in cultured group are significantly lower. (B) The mean stiffness of Achilles tendon tissue. The mean stiffness of static cultured group dropped dramatically with extended culture period. However, in 6% rescue group, no significant difference was observed compared to native control tendon. Results are expressed as the mean±SD. One way ANOVA significance values were *p < 0.05, **p < 0.01, and ***p < 0.001

The force versus displacement curves of native Achilles tendons, 6 days loading deprivation group and stimulation group are similar (Figure 5.7). In these the force increased dramatically for the first 4mm displacement, and then moderately increased, which was followed by a sudden drop in the tensile force as the tendons began to fail. However, in the 12 days loading deprivation group, the force climbed moderately for the first 8mm and slowly decreased to 70% of ultimate force, at which point the tests were terminated.
Figure 5.7 Force versus displacement curves for the native Achilles tendon (Control), rescue group (0% 6d+6% 6d), 6 days static cultured and 12 days static cultured Achilles tendon. 6% rescued cultured tendon displays similar mechanical character with native Achilles tendon. After 12 days static culture, Achilles tendon lost its original mechanical character became less stiff and soft.

5.4.3 Rescue effect of cyclic mechanical stimulation on ECM remodeling gene expression

Although the histological and mechanical changes have been identified, the underlying molecular mechanism still remains unclear. The homeostasis of tendon required the balance of the ECM generation and degradation. To estimate the generative process, the gene expression of COL1A1, COL3A1, TGFβ were tested, whiles MMP1, MMP3, TIMP1, and TIMP2 were considered to be the predominant cytokine in tendon remodeling. All the gene expression level was examined using RT-PCR. As shown in Figure. 5.8, although there was a slight decrease on COL1A1 expression of the loading
deprivation groups compared to native control, no significant difference was detected; however, \textit{COL1A1} expression increased dramatically in the stimulation group. A similar trend was observed in \textit{TGFb} and \textit{MMP3} expression. For the first 6 days no loading culture, \textit{MMP 1} was highly expressed, yet it dropped with extended culture period. In the stimulation group, \textit{MMP 1} was less expressed compared to 6 days loading deprivation group, however, no statistical difference was detected compared to the 12 day loading deprivation group. On the other hand, \textit{TIMPs} expressions of culture tendon were significant higher than the native control except for the 6 day loading deprivation group on \textit{TIMP2}. Extensive increase on \textit{TIMPs} expressions were detected on stimulation group, especially \textit{TIMP1}. However, no significant difference between stimulation group and 12 days loading deprivation groups on \textit{TIMP2}.

Figure 5.8 Comparison of gene expression fold change normalized by native tissue gene expression amount in the 6 day and 12 day loading deprivation group, the stimulation group and the native control group, the gene expression level is normalized against 36B4. Result are expressed as the mean±SEM. One way ANOVA significance values were *\(p < 0.05\), **\(p < 0.01\), and ***\(p < 0.001\)
5.4.4 Cyclic mechanical stimulation decreased Type III collagen expression of cultured tendon

Increased collagen type III content is an index of tendon degeneration and tendinopathy. Tendon cultured under loading deprivation condition for 6 days started to express moderate collagen type III turnover and several positive stained tenocytes were detected (as pointed by arrow in Figure 5.9B). Furthermore, almost all the tenocytes in the 12 days loading deprivation group were expressing type III collagen, and substantial type III collagen was observed histologically (Figure 5.9C). Interestingly, in the stimulation group, collagen type III expression was only minimally elevated, but at a level (Figure 5.9D) similar to native tendon (Figure 5.9A).

Figure 5.9 Type III collagen immunohistochemistry, positive staining is indicated by arrows (A) Type III collagen rarely detected in native Achilles tendon tissue (B) In 6 days loading deprivation group, type III collagen was upregulated, positive staining were pointed by arrow; (C) In 12 days loading deprivation group with massive increased type III collagen expression ; (D) No obvious type III collagen expression detected in 6% rescue group. Scale bar is 20μm.
5.5 Discussion

It is well known that mechanical stimulation plays a fundamental role in maintaining tendon homeostasis, and that the absence of mechanical stimulation can cause morphological changes such as rounded cell nuclei and disorientated collagen fibers associated with tissue degeneration (Hannafin, Arnoczky et al. 1995; Wang, Lin et al. 2013). “Tendon exercise” such as eccentric overload training or concentric training seems to be effective in patient rehabilitation, however, the mechanism still remains unclear (Kingma, de Knikker et al. 2007). In Chapter 3, a bioreactor system was developed that could be used to mimic the in vivo tensile mechanical loading on Achilles tendon and in Chapter 4 it was verified that cyclic mechanical stimulation indeed was essential for the maintenance of the tissue integrity of tendon. We also suggested that a narrow range of mechanical stimulation was required to create a net anabolic effect in Achilles tendon, and identified that 6% strain cyclic mechanical stimulation was optimal to maintain Achilles tendon homeostasis in the bioreactor system (Wang, Lin et al. 2013). However, the therapeutic effect of mechanical stimulation on early-stage of pathological change in Achilles tendon was yet to be observed.

In this study, this dynamic bioreactor culture system was combined with the unloaded culture condition to examine the effect of cyclic mechanical stimulation on deteriorated tissue caused by loading deprivation. Loading deprivation was achieved by culturing the rabbit Achilles tendons freely on the petri dish with adequate growth medium. After
6 days of loading deprivation, early-stage degenerative changes were observed, including wavy collagen fiber, increased type III collagen expression, and slightly rounded nuclei. After 12 days of culture without loading, further severe pathological changes such as disorientated collagen fiber pattern, ruptured fibers, rounded nuclei and substantial increased type III collagen expression developed evenly throughout the tendon. The deterioration of the tendon appeared to be no different at the center and the edge of the tissue, suggesting that inadequate nutrition penetration was not the sole cause of the damage. In the stimulation group, the tendons were transferred into the bioreactor system after 6 days loading deprivation, and 6% strain cyclic mechanical strain stimulation was applied for a further 6 days. The stimulation tendons successfully regained their original morphology, exhibiting as straight, well-organized collagen fibers and cells with elongated nuclei, whilst the type III collagen expression had reduced to the normal level. The result suggested that the previously identified optimal cyclic tensile loading (6%) was able to repair the early pathological change of tendon caused by load deprivation.

Programmed cell death (apoptosis) has been identified as a common feature of tendon degeneration and injury (Yuan, Wang et al. 2003; Hosaka, Teraoka et al. 2005; Lian, Scott et al. 2007). I previously showed that mechanical loading was essential for tenocyte viability, where increased cell apoptosis was observed in the tendon tissues that has no mechanical loading (Wang, Lin et al. 2013). In this study, the cell apoptosis rate in the 6 and 12 days loading deprivation groups were 30.6±3.9% and 91.3±3.5% respectively, which might explain why in the 12 day loading deprivation group, no
increased cell density was detected. When the tendon developed early degeneration, cell proliferation increased, presumably to activate repair. However, in the absence of mechanical stimulation, the observed high cell apoptosis rate inhibited this repair cascade then reducing the cell population to a “normal” level. The stimulation group, on the other hand, expressed an apoptosis rate of 17.8±3.9%, which was close to that of healthy tendon tissue, suggesting that 6% strain cyclic mechanical stimulation eliminated cell apoptosis in early-stage degradation and may thus contribute to the restoration of the homeostasis of tendon.

The microstructural organization of the tendon’s extracellular matrix, particularly collagen type I, determines the mechanical properties of tendon tissue. Although the disturbed collagen structure and increased collagen type III turnover were observed in loading deprivation culture groups, this was rescued by 6% strain cyclic loading stimulation. Critically mechanical testing confirmed that the mechanical stimulation restored normal mechanical properties of the Achilles tendon.

The biomechanical characteristics of tendon have not been well documented in literature as failure often occurs at sites other than the middle portion of tendon, such as muscle-tendon junction, tendon-bone junction or surgical repair site (Thermann, Frerichs et al. 2001; Matsumoto, Trudel et al. 2003; Gebauer, Beil et al. 2007; Chen, Yu et al. 2011). To ensure the efficacy of the testing, a pair of modified cryogrips and the freezing technique using liquid nitrogen was adopted in the study to avoid sliding of the
tissue during testing. During the mechanical loading, the tissue was closely monitored to ensure it broke at the middle portion rather than the ends near the grips. The peak load of native control tissue tested for the whole Achilles tendon in this study was 896.1 ±96.1 N, which is comparable to the findings reported in the study by Trudel et al. (Trudel, Koike et al. 2007). The results of biomechanical testing showed significantly reduced mechanical strength and stiffness of Achilles tendon cultured for 6 days and 12 days load deprivation. The mechanical characteristic could be better described from the force versus displacement curves. Due to ruptured and disorientated collagen fibers, the force increased more slowly in 12 days loading deprivation group compared to the other groups, for a given strain. However, in the stimulation group, the rabbit Achilles tendons regained their mechanical properties to levels close to that of normal tissue. This was evidenced by there being no significant difference in the mean stiffness of the stimulation group compared to that of the native control group, and the force-displacement curve in stimulation group appearing similar to that of the native control, apart from a lower ultimate tensile strength. Thus, the testing demonstrated a positive effect of mechanical stimulation in preventing further tendon degeneration, and indeed in promoting tendon healing.

The homeostasis of tendon requires constant remodeling of the collagen through the generation of new collagen in response to the degradation of collagen by proteases and mechanical loading. Matrix metalloproteinase (MMPs) are zinc-dependent endopeptidases that are able to degrade all the components of the extracellular matrix. MMP-1 is primarily responsible for cleavage the collagen type I, the dominant collagen
of tendon (Knauper, Lopez-Otin et al. 1996; Krane, Byrne et al. 1996). MMP-3 is another subset of the MMP family with a wide range of substrate specificity. Moreover, it has the ability to activate other MMPs such as MMP-1, MMP-7 and MMP-9 (Ye, Eriksson et al. 1996). TIMPs, on the other hand, are the inhibitors of MMPs. The ratio of MMPs to TIMPs is the key to understanding the rate of protease degradation, which then impacts on tendon homeostasis. Over expression of MMPs or under expression of TIMPs will cause the pathogenesis of tendon degradation (Riley, Curry et al. 2002; Lo, Marchuk et al. 2004; Jones, Corps et al. 2006). In our study, in the first 6 days of loading deprivation culture, MMP1 and MMP3 were significantly up-regulated, while TIMP1 and 2 were down-regulated. The shift in balance between MMPs and TIMPs may explain the early-stage pathological changes observed in the tendon. However, in the stimulation group, the up-regulation of TIMP1 and down-regulation of MMP1 may have suppressed the protease degeneration of collagen fibers. Upon mechanical stimulation, expression of TGF-β and COL1A2 promoted the formation of collagen fiber. The RT-PCR results are consistent with our observation that 6% cyclic tensile strain mechanical stimulation was optimal for creating a net anabolic effect that could reverse the early-stage degenerated changes.

Immobilization is a common postoperative treatment for bone fracture and tendon injury. For example, fracture of the lower end of the tibia or rupture of the Achilles tendon commonly require immobilization for up to 6 weeks (Cetti, Henriksen et al. 1994) (Ruedi and Allgower 1979), resulting in the deprivation of physical dynamic loading of the tendon. Early-stage structural degradation of Achilles tendon has been reported after
long-term immobilization in both humans and rats (Larsen, Forwood et al. 1987; Nakagawa, Totsuka et al. 1989; Kannus, Jozsa et al. 1997; Trudel, Koike et al. 2007). However, clinical rehabilitation guideline for Achilles tendon weakness after a period of immobilization has not been well established. In this study, we have demonstrated that loading deprivation in a bioreactor tendon culture led to tendon structural deterioration compromised mechanical properties and elevated expression levels of catabolic markers. By applying 6% strain cyclic mechanical stimulation, the histomorphology and biomechanical properties of rabbit Achilles tendon were successfully restored. However, all characteristics of the preferred cyclic strain profiles need to be defined, i.e. magnitude, frequency and dosage, and these may vary between species. Interestingly, a recent review suggests that about 5% strain may be the value that affects human Achilles tendons in vivo (Obst, Newsham-West et al.). Even though all strain profile characteristics are to be elucidated, the findings of this current study suggest that precisely calibrated mechanical stimulation is able to reverse early-stage tendon degradation. It has been reported that compared to long episodes of early loading, short periods of mechanical stimulation during the inflammatory phase could improve tendon healing with histological signs of repeated damage in the form of increased bleeding (Enwemeka, Spielholz et al. 1988; Eliasson, Andersson et al. 2011). Taken together, accurate and controllable mechanical stimulation on injury tendon may be considered as a potential therapeutic approach for patient rehabilitation. A specially designed orthotic device could be developed to apply an optimal titration of strain on tendon tissue and facilitate tendon repair.
5.6 Conclusion

In summary, our findings suggest that loading deprivation induced degenerated pathogenesis in rabbit Achilles tendon could be rescued by 6% cyclic tensile loading stimulation. The findings suggest that loading deprivation, such as during long-term immobilization, should be avoided when possible to reduce the complication of tendon weakness in patients. In addition, the benefit of dynamic loading may be an inspiration to develop a rehabilitation device for tendon weakness after long-term immobilization.
Chapter 6

General Discussion and Future Directions
CHAPTER 6 GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 General discussion

Tendon is a soft connective tissue between muscle and bone and functions to transfer forces. Tendons are also responsible for storing elastic energy. Tendons are subjected to high mechanical loading during daily activities and must undergo continuous repair to maintain its functional properties. Owing to its specific mechanical function, it is unsurprising that its biomechanical environment is essential for maintaining tendon homeostasis.

In 1995, Hannafin et al. demonstrated that even without mechanical loading, tendons undergo tendinopathy-like morphological changes characterized by disorientated collagen fibers and rounded cell nuclei (Hannafin, Arnoczky et al. 1995). Manipulation of loading on the tendon is able to regulate tendon metabolism and activate self-repair mechanisms. Eccentric overload is one of the known effective interventions for patients with chronic Achilles tendinopathy. By maximizing the activation of soleus or gastrocnemius muscles, mechanical loading is able to be applied on tendon (Kingma, de Knikker et al. 2007). Traditionally, immobilization is a standard recommendation post-surgical tendon repair. However, studies have shown that short periods of mechanical stimulation during the inflammatory phase could improve tendon healing (Enwemeka, Spielholz et al. 1988; Eliasson, Andersson et al. 2011). This is also consistent with the results present in this thesis (Chapter 5). Various studies on tendon
engineering suggest that mechanical stimulation is able to increase cell proliferation, collagen deposition and improve the mechanical properties of neo-tendon tissue (Altman, Lu et al. 2002; Juncosa-Melvin, Shearn et al. 2006; Webb, Hitchcock et al. 2006; Androjna, Spragg et al. 2007; Nirmalanandhan, Rao et al. 2008; Nirmalanandhan, Shearn et al. 2008; Butler, Hunter et al. 2009; Nguyen, Liang et al. 2009; Chen, Yin et al. 2010; Doroski, Levenston et al. 2010; Saber, Zhang et al. 2010; Issa, Engebretson et al. 2011; Woon, Kraus et al. 2011). Previous evidence suggests that mechanical stimulation is able to confer beneficial effects on tendon regeneration, although the reported range of improvements is varied. Silbernagel et al. showed that in 12 weeks of eccentric training, the overall improvement on pain relief was 29% (Silbernagel, Thomee et al. 2001), however, in a study by Alfredson et al., 94% improvement was reported (Alfredson, Pietila et al. 1998). Owing to limited understanding of the biomechanical environment in tendon tissue and the tissues’ repair and remodeling response, the explanation of the present results within a physiological framework was difficult. It is well known that sudden and excessive loading on the tendon can cause tear or rupture and loading deprivation can cause tendon degradation. However, the physiology of loading and underloading is rarely studied. Therefore, the investigation of various loading conditions on the tendon is essential for understanding tendon biology and the development of treatments for tendon repair.

6.1.1 The custom-made bioreactor (Chapter 3)

In the past decade, various methodologies have been employed to study the effect of mechanical loading on tendon such as in vitro monolayer cellular models and in vivo
animal models. In the cellular studies, the Flexcell® system has been widely used (Skutek, van Griensven et al. 2001; Wang, Jia et al. 2003; Yang, Im et al. 2005). Although precise control and monitoring systems are allowed at the cellular level, the application is on a monolayer of cells, not an organized tissue. Cells isolated from their extracellular niche and cultivated in a monolayer may not necessarily replicate their behavior in vivo. In vivo models, on the other hand, allow the systemic study of the biomechanical environment of the tendon. However, mechanical stimulation on the tendon varies between individuals, making precise quantification of load difficult. Therefore, in order to study the effect of various mechanical stimulations on tendon, a bioreactor that can provide a sterilized and programmable biomechanical environment for tendon culture is necessary.

In the first study, we adopted a relatively simple “incubator-based” design, in which the culture conditions such as temperature, humidity and CO₂ are controlled by the incubator. An actuation system and a culture chamber are the key components of the bioreactor system, as they provide the mechanical output and the sterilized culture environment, respectively. A step motor-ball screw transmission system (SMBS) was adopted as the actuator in our bioreactor. Compared to the other actuator systems such as the pneumatic actuators(Juncosa-Melvin, Shearn et al. 2006; Nirmalanandhan, Rao et al. 2008; Nirmalanandhan, Shearn et al. 2008) and linear motors(Nguyen, Liang et al. 2009; Doroski, Levenston et al. 2010), SMBS has the advantages of high resolution, highloading capacity and good resistance to humid and warm environment(Liu, Hsu et al. 2001; Altman, Lu et al. 2002; Webb, Hitchcock et al. 2006; Androjna, Spragg et al.)
AISI 316 stainless steel, which has good machinability and resistance to chemicals and high temperature, was used for the construction of the culture chamber. The labyrinth channel integrated in the chamber ensured sufficient air exchange without inducing contamination, and the glass covers allowed for visual monitoring. Several commercial bioreactor systems have been developed such as The Bose-ElectroForce BioDynamic system and the LigaGen system. Compared to these products, our system has an advantage in that it provides two methods for tissue fixation, tissue clamp and tissue hook. Tissue clamps have been found to be more effective at stabilizing membrane-like tissue, while tissue hooks are designed for fixing thick and irregular-shaped tissue.

In the present studies, full-length rabbit Achilles tendons, including the bone-tendon and tendon-muscle junction, were used as an Achilles tendon tissue model and cultured in the bioreactor system. Tissue hooks were found to be able to provide excellent fixation without slippage, which was essential for this study. Slippage can cause inconsistent mechanical loading and can comprise the results.

6.1.2 Mechanical stimulation in bioreactor system (Chapter 4 and 5)

The present studies utilized the novel approach of applying mechanical stimulation on fresh-harvest rabbit Achilles tendon in a bioreactor system. The application of the bioreactor in evaluating the effect of mechanical stimulation on tendon has several advantages:
Unlike the monolayer cellular study, the tenocytes were in their native niche embedded in the collagen matrix. During mechanical stimulation, tenocytes were not only subjected to tensile strain, but also compression from adjacent collagen fibers (Caliari, Weisgerber et al. 2012). Therefore, mechanical stimulation applied by the bioreactor system can be better replicated in in vivo loading environments compared to a monolayer system.

Unlike animal studies such as treadmill training, the bioreactor system used in this study is able to provide accurate, programmable and reproducible mechanical stimulation, allowing the systematic study of different intensity loading on the tendon.

The use of a bioreactor system allows the chemical environment in the culture to be manipulated. Therefore, it offers the potential for systematically studying the effects of nutrients, growth factors and cytokines under different loading regimes.

6.1.3 Effect of various mechanical stimulation on tendon homeostasis (Chapter 4)

Full-length rabbit Achilles tendons were dissected and cultured under different mechanical environments: 0% 3% 6% and 9% strain at 0.25Hz 8h/day for 6 days. Histology, immunohistochemistry, TUNEL assay and RT-PCR were carried out to evaluate tissue integrity.

Histologically, tendons subjected to 6% strain mechanical loading exhibited similar morphology as native tendon. However, loading deprivation group, 3% strain and 9%
strain groups showed pathological changes.

- **Loading deprivation**

  Tendon exhibited minor pathological changes including loose collagen fiber and a moderately increased cell apoptosis rate after 6 days of loading deprivation culture (Figure 4.2 B). The morphology further degenerated when the culture period was extended to 2 weeks. Typical tendinopathy morphologies were observed such as massive cellular apoptosis, disorientated collagen fiber and rounded tenocyte nuclei (Figure 4.2C, 5.4C). The severe pathological changes are initiated by high cell death. The remaining cells then fail to maintain the tissue integrity.

- **3% strain**

  In the 3% strain group, wavy collagen fiber, minor rounded cell nuclei, loose ECM structure and increased collagen type III synthesis were observed (Figure 4.3B, 5.5C). The elevated MMPs expression and downregulation of COL1A2 are consistent with these pathological changes. In 2007, Arnoczky et al. hypothesized that pathological development of tendinopathy maybe the result of under stimulation rather than overloading (Arnoczky, Lavagnino et al. 2007). Our study provided further evidence in support of this theory. It is suggested in this thesis that increase protease activity acting on underloaded collagen and reduced anabolic activity of tenocytes due to reduced mechanical stimulus, despite a reduced mechanical damage, can explain the observed pathological changes in this 3% strain group.
● 6% strain

In the 6% loading group, tendons were able to maintain their normal morphology, type III collagen content and apoptosis rate. RT-PCT results showed an increased expression of COL1A2. The rate of micro-damage caused by mechanical loading is matched by the rate of repair such that the structural integrity was able to be maintained.

● 9% strain

In this group, an acute injury model was successfully established. Tendon subjected to excessive loading caused rupture of collagen fiber (i.e. mechanical damage) and increased cell apoptosis close to the injury site, which are the typical morphology of acute injury on tendon. Type III collagen was highly expressed in RT-PCR results and was confirmed by immunohistochemistry.

Although pathological changes were detected in three of four loading conditions, the underlying causes are different. From the different loading groups and the pathological changes and protein expressions, we proposed a hypothetical model for tendon damage and repair, as shown in Figure 4.7. An increased intensity of mechanical stimulation increases the mechanical damage. Protease (chemical) damage was active in the low strain groups. Only at intermediate strains of 6% was the repair able to match the rate of mechanical and proteolytic damage, and hence the ability to maintain structural integrity.
Therefore, the conclusion drawn from this study is that a narrow range of mechanical stimulation in this bioreactor system was able to maintain tendon homeostasis; with mechanical underloading and overloading both detrimental to tendon.

6.1.4 Rescue of degenerated tendon with mechanical stimulation.

In the previous study, we demonstrated that 6% strain mechanical stimulation was able to maintain the tendon homeostasis in the bioreactor system. Following this, the next hypothesis was whether or not 6% strain can also rescue tendon following load deprivation. Therefore, in this study, rabbit Achilles tendon was first cultured without loading for 6 days, and then followed by either an additional 6 days without load or 6 days at 6% strain at 0.25Hz 8hr/day for. The tendons then were analyzed by biomechanical testing and histological assessment.

Compared to native rabbit Achilles tendons, tendons under static culture for 6 days and 12 days showed 30% and 50% reduction in their mechanical properties, respectively. However, the rescue group showed significant improvement compared to these static culture groups. Indeed tendons in the rescue group exhibited similar morphology, apoptosis rate and collagen type III content to native tendons. RT-PCR results showed, application of 6% strain mechanical stimulation significantly reduced MMPs expression and increases COL1A2 and TIMPs expression, which was consistent with a suppression of tissue catabolic processes and an increase in those associated with tissue repair.
Immobilization is a common postoperative treatment of bone fracture and tendon injuries (Ruedi and Allgower 1979; Cetti, Henriksen et al. 1994). In various studies, early-stage degradation in Achilles tendon was reported after long-term immobilization in both humans and rats (Larsen, Forwood et al. 1987; Nakagawa, Totsuka et al. 1989; Kannus, Jozsa et al. 1997; Trudel, Koike et al. 2007). However, there is no clinical rehabilitation guideline for Achilles tendon weakness after a period of immobilization. The results of this study suggests that proper mechanical loading may activate a self-repair mechanism to repair early stage degenerative pathological changes in tendon.

### 6.2 Limitation of this work

It is important to mention several limitations of the work described in this PhD thesis.

#### 6.2.1 Chapter 3

In chapter 3, the bioreactor designed is only able to provide some basic function; there are still several limitations in this design, with the benefit of hindsight could be improved.

1. **Failure warning**: although the bioreactor system can provide preprogrammed automatic mechanical stimulation on the tissue sample, some accidents like power failure and maloperation might still happen. A complete computer monitoring system would be ideal, but even a basic warning light should be installed and preprogrammed in the system to report the error.
2. **Program interface**: The operational system was programmed using the basic PLC language, which might not be familiar to the other users. A more user-friendly ‘common’ programming language should be designed for the convenience of the other users.

3. **Bioreactor connection**: in the present design, the bioreactor is connected to the control box through a cable, which required specific incubator model with a cable hole back panel. However, a cable hole is not a standard configuration in all incubators; therefore, this bioreactor can only be used in some special incubator.

6.2.2 Chapter 4

1. **Mechanical stimulation accuracy**: Achilles tendons from different individuals show variations in length. Although the tissue hook of the bioreactor can provide an effective way to stabilize the tendon and the stimulation distant are fixed at 23mm, some error might still be induced.

2. **Medium infiltration**: During the *in vitro* culture, nutrient delivery is depended on medium infiltration. In loading deprivation group, medium infiltration might not sufficient for the whole tendon. Although the histological section exhibited consistent morphology in the center and the edge of the tendon, the nutrient transport still needs to be considered when interpreting the results.

3. **Statistical analyze**: In the histological scoring, scores should have been analyzed using a nonparametric statistical procedure as the data is ordinal, not continuous.
6.2.3 Chapter 5

Biomechanical testing: Achilles tendons from different individuals show variations in initial mechanical properties. Although in this experiment, the Achilles tendon from same individual we distributed in different groups to minimize error, it is still needed to be mentioned; especially as the sample size was relatively small.

6.3 Future directions

6.3.1 Gene profile of tenocyte subjected to 2D or 3D mechanical stimulation

As demonstrated by the results in this thesis, tendons are able to sense and respond to mechanical loading. Although they are mainly subjected to tensile strain on the macroscale (i.e. the whole tissue scale), the micromechanical environment is much more complicated. Tendon is a dense connective tissue that comprised by well-organized collagen fiber with tenocyte adhered on it. Subjected to overall tensile strain, tenocytes not only receive tensile but also the shear force and compression from the surrounding collagen fibers and proteoglycans as well as potential fluid shear stress (Figure 7.1) (Khan and Scott 2009). However, the in vitro studies that evaluate the effect of mechanical stimulation on tenocyte or fibroblast mostly focus on tensile strain only. The Flexcell® system was commonly used in the studies of in vitro gene expression profiles of tenocytes under mechanical stimulation (Skutek, van Griensven et al. 2001; Wang,
Jia et al. 2003; Yang, Im et al. 2005). These systems can precisely control the mechanical regimen and monitor the cell behaviors, but only can be used for 2D monolayer cell culture, not the organized tissue. Cells isolated from their extracellular niche and cultured in a monolayer may not necessarily replicate their original behavior in vivo. The recent study of Ni et al. successfully generated a scaffold-free engineered tendon from tendon-derived stem cell by stimulation with connective tissue growth factor (Ni, Rui et al. 2013). This model allows the better study on the effect of mechanical stimulation on tenocyte. As the structure of scaffold-free tendon is more similar to native tendon, when compared to monolayer cell cultures, the in vivo mechanical stimulation can be better mimicked in our bioreactor system using this scaffold-free tendon model. The gene profile of tenocyte subjected to 2D and 3D mechanical stimulation can lead to the better understanding how tenocyte respond to in vivo mechanical stimulation.

Figure 7.1 Tenocyte undergoing (A,B) shear and (C) compression during tendon subjected to tensile strain. Figures taken from Khan et al. (Khan and Scott 2009)
6.3.2 The effect of mechanical stimulation on tendon nutrient infiltration

Nutrient supply during *in vitro* culture of tendon mainly depends on passive infiltration. However, during mechanical stimulation, advective nutrient transport would be induced. Although Hannafin *et al.* reported that mechanical stimulation did not alter small nutrient uptake, compared to static culture, and that the tendon repair improvements derived from passive motion is not related to an increase of nutrient transport (Hannafin and Arnoczky 1994), under different loading regimes, or in different tendon types, this may not be the case. Moreover, the transport of large molecules, such as growth factors and matrix components, may be affected by mechanical stimulation, as has been shown in studies in cartilage (Bonassar, Grodzinsky *et al.* 2000; Zhang, Gardiner *et al.* 2007). Although unlike cartilage, the *in vivo* nutrient supply is dependent on limited blood vessels instead of synovial fluid, the nutrient infiltration *in vitro* is essentially the same as cartilage, especially for large scale animal engineered tendon generation. Therefore, rabbit Achilles tendon culture in a bioreactor system can be used as a nutrient transport model for study. Different sizes of dye that are compatible to target cytokines can be used in the bioreactor system (Day, Megson *et al.* 2005). Nutrient delivery could then be assessed based on the dye infiltration. Furthermore, nutrient delivery is often a key obstacle in tissue engineering. If the nutrient delivery induced by tensile cyclic strain in the bioreactor is not sufficient, medium circulation system could be designed based on the current model of bioreactor to enhance nutrient infiltration.
6.3.3 The effect of mechanical stimulation on stem cell tenogenic differentiation

Stem cells have been suggested to have great potential in regenerative medicine application due to its self-renewal capacity, hypoinmunogenicity and multi-lineage differentiation ability (Uccelli, Moretta et al. 2006). However, the complexity and reliability of manipulating stem cell to differentiate into a target cell type limits the clinical application. In vitro induction of stem cell differentiation is commonly achieved by chemical modulations. Recent studies suggested that mechanical stimulation acted as an important regulator in stem cell differentiation induced by cytokines (Rui, Lui et al. 2011), and might be essential for lineage selection and cell differentiation. Khayat et al. suggested low frequency mechanical stimulation inhibited MSC adipogenic differentiation (Khayat, Rosenzweig et al. 2012), and a similar result was reported by Sen et al. (Sen, Xie et al. 2008). Continuous cyclic mechanical tension has been indicated to inhibit osteogenic differentiation of MSC (Shi, Li et al. 2011), whilst short term cyclic tensile strain has been reported to stimulate osteogenic differentiation (Rui, Lui et al. 2011). Furthermore, dynamic compression can induce the chondrogenic differentiation of MSC (Huang, Farrell et al. 2010; Kupcsik, Stoddart et al. 2010). The study of Chen et al. reported that static mechanical stimulation was able to stimulate tenogenic differentiation of human embryonic stem cells (Chen, Song et al. 2009). Although the mechanical stimulation has been suggested as a key regulator of stem cell differentiates into musculoskeletal cell type, tenogenic differentiation induced by mechanical loading is relatively rarely recorded. As a mechano-transduction tissue, it is reasonable to speculate that dynamic tensile strain is able to simulate the tenogenic differentiation of stem cells, although the magnitude, frequency and duration remain unclear. The present study showed that 6% strain, 0.25Hz for 8h/day was able to
increase collagen type I expression of dissected rabbit Achilles tendon, which was one of the tenogenic market of tenocytes. Therefore, the mechanical stimulation regime used in this study should be tested for tenogenic differentiation of stem cells.

6.3.4 Therapeutic guideline

One commonly used conservative treatment for chronic Achilles tendinopathy is eccentric overload. The basic principle is to maximize the activation of the calf or soleus muscle using body weight, so generate a tensile strain on Achilles tendon (Alfredson, Pietila et al. 1998). The treatment is instructed to be continued unless the patient experience disabling pain, however, if the patient can perform the exercise without experiencing any pain, extra weight is required (Alfredson, Pietila et al. 1998). The treatment is normally performed 2 times daily, 7 days/week for 12 weeks, and for each treatment including 3 sets of loading, 15 repetitions each set (Alfredson, Pietila et al. 1998). Several studies reported positive effect on symptoms relief (Stanish, Rubinovich et al. 1986; Alfredson, Pietila et al. 1998; Silbernagel, Thomee et al. 2001; Alfredson and Lorentzon 2003; Fahlstrom, Jonsson et al. 2003; Roos, Engstrom et al. 2004; Shalabi, Kristoffersen-Wilberg et al. 2004) however, the study of Woodley et al. showed no effect (Woodley, Newsham-West et al. 2007) and Rompe et al. reported an inferior results (Rompe, Furia et al. 2008). The present study indicated that there was only a narrow range of tensile strain stimulation that could achieve a net anabolic or even therapeutic effect, which was 6% strain in the rabbit Achilles tendon model. Overloading and underloading would trigger degenerative cascade through different pathways. This study provides a cue to explain the clinical outcome variety from the
eccentric overloading training. As the treatment is based on the sense of patients, the
strain applied on Achilles tendon may not be sufficient to achieve the optimal clinical
outcome; moreover, the treatment is not consistence with different patients. Although
rabbit tissue is different from human tissue, similar principles can be applied in human.
Physiological soleus and calf muscle force during gait, running and jumping can be
measured by the inverse dynamics algorithm, and then the physiological strain on
Achilles tendon can be calculated. Base on the physiological strain range of Achilles
tendon, more experiments are required to evaluate the optimal loading regime for the
rehabilitation program. However, even knowing the optimal loading strain on Achilles
tendon, the monitoring of tendon strain is still a big challenge, which requires various
expertise from sport science.

6.3.5 Robot-driven orthosis

Immobilization is a standard postoperative procedure after tendon surgery to eliminate
the loading on the surgical site then prevent the rerupture during recovery period;
however, several studies have reported that the immobilization clinically caused
degenerative changes in tendon tissue (Amiel, Woo et al. 1982; Loitz, Zernicke et al.
1989). Recent study reported that short-episode of mechanical stimulation at the
inflammatory stage could improve healing with histological signs of repeated damage in
the form of increased bleeding (Enwemeka, Spielholz et al. 1988; Eliasson, Andersson
et al. 2011). Therefore, early intervention seems to be beneficial for the tendon healing;
however, self-initiative training might be difficult to control and might damage the
surgical site. Robot-driven orthosis, in the other hand, can control the activity level and
protect the surgical site from overloading. Although no robot-driven orthosis is currently being used for tendon rehabilitation, a clinical trial on patient suffered from multiple sclerosis and stroke seems to be promising (Husemann, Muller et al. 2007; Lo and Triche 2008; Schwartz, Sajin et al. 2012; Straudi, Benedetti et al. 2013). Our studies indicate that proper mechanical stimulation has therapeutic effect; therefore, robot-driven orthosis could be developed based on this model as a postoperative rehabilitation. The combination of surgical treatment and conservative treatment at the early inflammatory phase might provide a better clinical outcome.

6.3.6 Tendon engineering

Tendon’s primary function is force transmission. It operates in a varying load environment, both on short timescales (e.g. walking, running) and on longer timescales (e.g. changes in body size with age). In fact, it has been shown that after 4 weeks in a load-free culture environment tenocytes lost their native elongated morphology, became increasingly rounded, and the collagen fiber became more crimped (Hannafin, Arnoczky et al. 1995). Unsurprisingly, given its functional role, a suitable mechanical stimulus is vital for engineered tendon/ligament formation. Cyclic stretching has been shown to produce an up to 9 folds increase in the cell number of an engineered tendon compared with a static culture over a 2 week period (Abousleiman, Reyes et al. 2009). Furthermore, mechanical stimulation could help guide collagen fiber formation, i.e. along the direction of loading, which is able to enhance or optimize the mechanical properties including stiffness, elastic modulus, maximum tensile stress and maximum force (Juncosa-Melvin, Shearn et al. 2006; Saber, Zhang et al. 2010; Woon, Kraus et al.)
However, most mechanical stimulation applied on the engineered tendon cultured is lack of systemic optimization, the magnitude and frequency varied from studies. Some studies used up to 10% strain for tendon stimulation, which might exceed the tolerance of native tendon (our study indicated 9% strain could cause partial tear of rabbit Achilles tendon). In the present study, rabbit Achilles tendon was used as a model to evaluation the effect of different mechanical stimulation on tendon homeostasis. Knowing the fact that 6% strain was able to create anabolic effect and increased tendon formation, the further application of this study is to combine the optimal mechanical stimulation and the engineered tendon culture to create a better tendon construct for implantation.

6.4 Conclusion

In the present PhD project, a functional bioreactor that can provide programmable mechanical stimulation and sterilized culture environment was designed and constructed. The application of this bioreactor allowed the evaluation on the effect of different mechanical stimulation on rabbit Achilles tendon homeostasis. It was observed that only a narrow range of mechanical stimulation can maintain the tendon integrity, mechanical underloading and overloading were both detrimental to tendon tissue. It was observed that successful restoration of the tendon’s structural integrity and mechanical properties was available by the application of 6% tensile cyclic loading from early degenerative load deprivation model. The current results suggest that mechanical stimulation may provide therapeutic benefits.
REFERENCE


Lian, O., A. Scott, et al. (2007). "Excessive apoptosis in patellar tendinopathy in


Mikic, B., B. J. Schalet, et al. (2001). "GDF-5 deficiency in mice alters the


1455-1461.


APPENDIX
Bioreactor Design for Tendon/Ligament Engineering

Tao Wang, BS,1 Bruce S. Gardiner, PhD,2 Zhen Lin, PhD,1,3 Jonas Rubenson, PhD,4 Thomas B. Kirk, PhD,5 Allan Wang, PhD,6 Jiake Xu, PhD,7 David W. Smith, PhD,2 David G. Lloyd, PhD,4,8 and Ming H. Zheng, MD, PhD, FRCPA1

Tendon and ligament injury is a worldwide health problem, but the treatment options remain limited. Tendon and ligament engineering might provide an alternative tissue source for the surgical replacement of injured tendon. A bioreactor provides a controllable environment enabling the systematic study of specific biological, biochemical, and biomechanical requirements to design and manufacture engineered tendon/ligament tissue. Furthermore, the tendon/ligament bioreactor system can provide a suitable culture environment, which mimics the dynamics of the in vivo environment for tendon/ligament maturation. For clinical settings, bioreactors also have the advantages of less-contamination risk, high reproducibility of cell propagation by minimizing manual operation, and a consistent end product. In this review, we identify the key components, design preferences, and criteria that are required for the development of an ideal bioreactor for engineering tendons and ligaments.

Introduction

Tendons and ligaments have an important function in transferring force from muscle to bone or bone to bone. Tendons also help store elastic energy while walking, increasing locomotion efficiency. However, sudden, excessive strain of ligaments and tendons from athletic or recreational activities can cause acute traumatic injury to these tissues, which can range from small, partial tears to complete ruptures. Furthermore, repetitive loadings over long periods of time can lead to similar tissue injuries, when the fatigue damage within the tendon exceeds the capacity of the tissue to repair itself. In the United States, injuries to tendons and ligaments represent about half of the 33 million musculoskeletal injuries.1 Each year more than 33,000 tendon reconstructions occur in the United States, costing $30 billion USD2,3 and in Australia, $250 million is spent annually just on rotator cuff repair.4 However, despite the high prevalence of tendon injury and associated tendinopathy worldwide, treatment options remain poorly defined.

Autograft and allograft transplantations are the common surgical treatments for tendon and ligaments that are injured or degenerated. However, the risks of damage to the donor site from which the autografts are taken, and the potential immune reactions for allografts are the major concerns.5–7 A promising translational approach to the treatment of tendon/ligament injury or degeneration is through the use of engineered autologous grafts made available through the development of bioreactors that generate tendon/ligament tissue in vitro. One common view is that the key to a successful bioreactor is being able to recreate, in vitro, the cell microenvironments that are experienced by cells in vivo. The cell microenvironments can be defined using cellular morphological information with data from molecular biology, biochemistry, and biomechanics. This review aims to clarify the requirements for a successful bioreactor that may be used for tendon/ligament engineering, and to provide an overview of the range of components found in tendon/ligament bioreactors, including custom-made and commercial products. We will also discuss the studies that have involved the application of tendon/ligament bioreactors.

Key Elements for Tendon/Ligament Formation and Regeneration

Tendons and ligaments are force-transferring tissues from muscle to bone and bone to bone, respectively. They consist of collagens, cells, proteoglycans, elastin, glycolipids, and water. Although roughly 65%–70% of the total weight is

1Centre for Orthopaedic Translational Research, School of Surgery, University of Western Australia, Crawley, Australia.
2School of Computer Science and Software Engineering, University of Western Australia, Crawley, Australia.
3Division of Orthopaedic Surgery, Department of Surgery, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong, China.
4School of Sport Science, Exercise and Health, University of Western Australia, Crawley, Australia.
5Department of Mechanical Engineering, Curtin University, Bentley, Australia.
6Sir Charles Gairdner Hospital, Perth, Australia.
7School of Pathology and Laboratory Medicine, University of Western Australia, Crawley, Australia.
8Centre for Musculoskeletal Research, Griffith Health Institute, Griffith University, Gold Coast, Australia.
been shown to result in increasing ECM deposition rate, a lower initial cell-seeding density, high seeding density has cell sources, tenocytes and tissue, respectively. Preclinical and early clinical studies using tissue using a patented technique developed by our with fibroblasts isolated from biopsied anterior cruciate ligament. 25 patients with recalcitrant lateral epicondylitis were treated in recent clinical trial of autologous tenocyte therapy, a total of have also been shown to form tendon tissue, although the complexity of cell manipulation required. Dermal fibroblasts (depending on the policy of different countries) and the markers and histopathologic correlations. Being native skin-derived fibroblasts is suppressed with a lack of tenocyte expression, fibronectin-binding interactions, cell proliferation, and collagen production. Recent studies demonstrated that tendon and ligament formation was impaired in TGFβ2 and TGFβ3 knockout mouse embryos, reinforce the importance of TGFβs in tendon development and homeostasis. Both synthetic and natural biomaterials are commonly used in tendon/ligament engineering. Synthetic polymer scaffolds have the advantage of reproducible mechanical and chemical properties, and they are relatively easy to fabricate into different sizes. However, their rapid degradation rate and potential risk of releasing acidic byproducts or toxic polymers during degradation have limited their application in clinical trials. Given these disadvantages, more researchers have turned their focus on exploring natural biomaterials. Being the main component of native tendon, collagen type I is the most obvious choice of material. Although the biocompatibility is excellent, the poor mechanical properties of reconstituted type I collagen scaffolds has limited their further development as a load-bearing material. Silk fibroin, on the other hand, has similar biocompatibility as collagen scaffolds and comparable mechanical properties as native tendon/ligament. In several in vivo studies, silk fibroin-based engineered ligaments have been proven their ability to restore the function of injured ligament. Another option is the decellularised tendon/ligament construct. Although the mechanical and biological properties are a better match to native tissue than any other currently available scaffolds, donor cells may remain in the allograft, even with strict sterilization and cleaning, and thus they can potentially cause inflammatory responses.

After choosing an appropriate cell source and scaffold type, the tissue needs to be encouraged to develop the properties of native tissue by providing an appropriate biochemical and biomechanical environment to stimulate ECM synthesis. Regarding the biochemical environment, several growth factors have been found to play an important role in tendon/ligament formation and healing. These include insulin-like growth factor-I, vascular endothelial growth factor, platelet-derived growth factor, basic fibroblast growth factor, transforming growth factor (TGFβ), and growth differentiation factor 5 (GDF-5). The roles of TGFβ and GDF-5 seem to be particularly prominent. TGFβ remains active throughout tendon/ligament healing, and is able to regulate cell migration, proteinase expression, fibronectin-binding interactions, cell proliferation, and collagen production. Recent studies demonstrated that tendon and ligament formation was impaired in TGFβ2 and TGFβ3 knockout mouse embryos, reinforce the importance of TGFβs in tendon development and homeostasis. CDF-5 regulates cell growth and differentiation, with a lack of CDF-5 causing delayed tendon healing, irregular collagen type I fibrils, and weakened fibril mechanical properties.
How these various biochemical factors should be introduced into the tissue bioreactor system to shape the chemical environment is an extremely challenging and open question. Specifically, at what concentrations, in what combinations, and at what sequence or timing should they be made available? Studies have shown that the expression of these factors in tendon repair changes over periods of days. Presumably, as the engineered tissue matures, cells begin to control their own biochemical environment, and the role of the bioreactor is to now provide the building block nutrients and expected systemic signals, along with a mechanical stimulus. The early stage in the tissue-engineered tendon is likely to be the most critical in establishing tendon development along a pathway to resemble native tendon. Finding the correct combination of factors is daunting due to the complexity arising from the multitude of possible combinations and interactions. Systematically, varying experimental conditions, coupled with computational modeling of transport processes and signaling molecule pathways leading to cell responses, provide the only conceivable means to both understanding and efficiently optimizing the tissue bioreactor system. Tendon’s primary function is mechanical. It operates in a varying load environment, both on short timescales (e.g., walking and running) and on longer timescales (e.g., changes in body size with age). Tendon responds to its mechanical environment through changes in ECM biosynthesis and degradation. Unsurprisingly, given its functional role, a suitable mechanical stimulus is vital for tendon/ligament homeostasis. In fact, it has been shown that after 4 weeks in a load-free culture environment, tenocytes lose their native elongated morphology, become increasingly rounded, and the collagen fiber becomes more crimped. In cell-free reconstructed collagen fibril network systems, a tensile load has been shown to be protective to degradation by MMPs. Furthermore, cyclic stretching has been shown to produce an up to nine-fold increase in the cell number of an engineered tendon compared with a static culture over a 2-week period. Finally, an appropriate mechanical environment could help guide collagen fiber formation, that is, along the direction of loading, which is able to enhance or optimize the mechanical properties, including stiffness, elastic modulus, maximum tensile stress, and maximum force.

Rather than being two separate signals, there is a crosstalk between mechanical and chemical signals. Recent studies have shown that gradual and temporary loss of tensile loading leads to reversible loss of Scleraxis (Scx) expression, which is a transcription factor specific for tenocytes and their progenitors. In addition, it has been shown that TGFβ directly induced the expression of Scx in cultured tenocytes isolated from mice. In Scx−/− mice, a disordered limb tendon phenotype was observed, and a similar phenomenon happened in TGFβ type II receptor gene knockout (Tgfrb2−/−) mice with dramatic loss of Scx expression.

Providing a suitable biomechanical signal is clearly an important component for the success of tendon/ligament engineering. Generating a suitable mechanical signal within the bioreactor system is critically important for tendon/ligament tissue engineering.

### Bioreactor Design for Tendon/Ligament Engineering

Despite the increasing appreciation of tendon/ligament biology and function, conventional culture methods do not seem to meet the biochemical and biomechanical requirements to generate bioengineered tendon/ligament in vitro. A bioreactor system that subjects the cell culture to dynamic loading, mimicking the physiological conditions of tendon/ligament in vivo, while allowing cellular proliferation, differentiation, and matrix production in a mechanical environment, may provide a solution.

Bioreactors for tendon/ligament engineering are different to the systems that have been used in various other tissue-engineering fields in the past decades, that is, systems for the muscle, liver, and bone. Compared to other bioreactors, the main task of the bioreactor for tendon/ligament engineering is to provide the proper biomechanical and biochemical environment specific to tendon/ligament formation. To achieve this, certain basic components are required, that is, the actuating system and the culture chamber, which can provide the construct’s mechanical stimulation and controlled culture environment, respectively. Furthermore, the bioreactor may also include a medium circulation system, monitoring system, feedback system, and a medium analysis system, depending on the operational requirements (Fig. 1). With these facts in mind, several custom-made bioreactors have been developed for tendon/ligament engineering (see Table 1), and the aforementioned components of these are now are discussed in detail.

### Actuator and Culture Chamber Design

The actuating system is the main component for providing different mechanical stimulation to engineered tissue. Pneumatic actuators, linear motors, and step motor-ball screws (SMBSs) are the most common actuators used in tendon/ligament bioreactors. They have several advantages, including the ease of maintenance, cleanliness, low cost, and high power-to-weight ratio. Unfortunately, by using air as a medium, pneumatic actuators are subject to high friction. The sensitivity and response to an input signal are relatively slow because of the dead band and dead time caused by stiction and air compressibility. Due to these nonlinearities, it is difficult to achieve accurate position control with pneumatic actuators. Typically, the accuracy of pneumatic actuators is ~±0.1 mm, which is not insignificant when typical tendon bioreactors require <5% strain on 1–5-cm tissues. Compared with pneumatic actuators, electrical actuators are more expensive, but the level of accuracy in their positional control is much higher. A direct-drive linear motor is able to provide high-speed/high-accuracy linear motion by eliminating mechanical transmission, and the accuracy is the highest of all three actuators, that is, ~±1 μm. SMBS transmission systems are based on a ball-screw linkage and a crank-slider mechanism. In general, SMBSs transfer the rotation of the crank to a reciprocating motion of the screw. By choosing different ball screws, the optimal speed range and output force can be selected using Equation (1), that is,

\[
T = \frac{F l}{2 m v}
\]

where \(T\) is torque applied to screw; \(F\) is linear force; \(l\) is ball screw lead; and \(v\) is ball screw efficiency. Although SMBS is not as accurate as a linear motor, a positional accuracy of around ±5 μm can still be achieved. In a multichamber-shared
loading system, SMBS is widely used given its high accuracy and relative high loading capacity. In independent loading multichamber systems, linear motors are more popular due to their small size and relatively simple mechanical arrangement.

In addition to the actuator, the connection between the mechanical input and the tissue is vital and often presents a major challenge. During dynamic loading of the tissue in culture, an even distribution of force throughout the entire sample is critical, otherwise tissue integrity is compromised by overloading the mechanical connection regions and/or by inhomogeneous mechanical stimulation. Different strategies of applying mechanical loads have been adopted based on different construct dimensions. In a study by Chen et al., cell-seeded knitted silk–collagen sponge scaffolds were fixed on stainless rings and connected to sample hooks. The bioreactor system by Juncosa-Melvin et al. applied two posts to fix the construct, punching through the scaffold as shown in Figure 2. However, nonuniform construct deformation is clearly apparent. Tensile force is focused on the side of constructs, which causes uneven distribution of mechanical stimulation. Although tissue clamps are the most popular and relatively effective method for holding tendon constructs, the potential for damage at the clamping region needs to be considered. The method of reproducibly applying uniform loads to soft tissue without tissue damage or slippage is a critical problem in need of a satisfactory solution. It is our opinion that a robust clamping region should be designed along with the artificial bioscaffold to ensure the proper connection between the sample and the mechanical load.

The culture chamber is an essential part of whole bioreactor system. The high humidity of culturing conditions (99% humidity, 37°C, and 5% CO₂) and chemistry of the culture medium are corrosive to many materials. Corrosion products may in turn be toxic to the tissue. Therefore, noncorrosive and autoclavable materials such as stainless steel, polymethylmethacrylate, polyoxymethylene, polycarbonate, glass, and silicon are preferred and widely used in culture chamber design.

The chamber structure is an important consideration in the bioreactor design, with most currently available culture...
chambers divided into two groups: integrated \(^4_{2,83}\) and separated chambers.\(^2,3,19–27,35,76,82\) In integrated chambers, multiple samples are cultured while sharing the same culture medium. Conversely, separated chambers can provide separate culture environments for each sample. Although the complexity of design and manufacturing costs may be higher in a separated chamber system, reduced cross-contamination and the option of independent environmental control are distinct advantages.

During tissue culture, sufficient air exchange within the culture chamber is critical. Air exchange in conventional cell culture incubators is through the integrated hydrophobic filter of the culture flask and the gap between the leak and the culture dish/well plate. Therefore, the ideal design for the culture chamber should be similar. Like conventional cell culture, an unsealed chamber bioreactor connects to the outside environment through various ways,\(^4_{2,22,27,35}\) such as the hydrophobic filter leak\(^4_{2,22,27,35}\) and the labyrinth channel (Fig. 3).\(^8_{2}\) In a study by Webb et al.,\(^4_{2}\) a modified tissue culture flask was used as an integrated culture chamber, and could culture up to four samples simultaneously. Although there are potential risks of cross-contamination from different samples and toxicity from autoclaving the culture flask, the integrated hydrophobic filter leak can ensure adequate gas exchange without inducing contamination. In the bioreactor system used in Parent et al.’s research,\(^8_{2}\) a labyrinth channel was added to improve the air exchange and eliminate contamination as shown as Figure 3. However, in closed chambers, air exchange mostly depends on medium circulation, which is now discussed in the following section.

### Environmental Control and Medium Circulation Systems

Although to the best of our knowledge, no contamination has been reported in any bioreactor study, transportation of the bioreactor and opening of chamber for medium exchange every 3 days, especially for multichamber systems, are still a potential contamination risk. Therefore, a medium circulation system can be introduced to improve the efficiency and minimize these risks. In addition to the advantages of reduced contamination, a circulating medium may be better able to infiltrate into cultured tissue. For example, perfusion bioreactors used in bone engineering circulate the culture medium for better nutrient delivery and subsequent improved cell numbers.\(^8_{4–89}\) Similarly, human umbilical vein cultured under a circulating medium had approximately three times the cellular number as those cultured using a quiescent medium.\(^23\)

---

**Table 1. Components of Custom-Made Bioreactor Systems**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Actuating system</th>
<th>Culture chamber</th>
<th>Monitor system</th>
<th>Feedback system</th>
<th>Medium circulation system</th>
<th>Medium analysis system</th>
</tr>
</thead>
<tbody>
<tr>
<td>76</td>
<td>Biaxial</td>
<td>Multiple</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>42</td>
<td>Uniaxial</td>
<td>Single chamber, multiple samples</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>19,20,21,3</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>Displacement</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>35</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>Force</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>22</td>
<td>Uniaxial</td>
<td>Single</td>
<td>Force</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>23</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>83</td>
<td>Uniaxial</td>
<td>Single chamber, multiple samples</td>
<td>Displacement Force</td>
<td>×</td>
<td>×</td>
<td>✓</td>
</tr>
<tr>
<td>2</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>27</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>Displacement</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>82</td>
<td>Uniaxial</td>
<td>Multiple</td>
<td>Displacement Force</td>
<td>✓</td>
<td>×</td>
<td>×</td>
</tr>
</tbody>
</table>

---

**FIG. 2.** Stem cell-seeded Collagen sponge deformation during mechanical stimulation. Modified from reference 19. Color images available online at www.liebertpub.com/teb

**FIG. 3.** Schematic drawings of air exchange through the labyrinth channel in the culture chamber. Modified from reference 82. Color images available online at www.liebertpub.com/teb
A traditional incubator-based bioreactor and/or independent bioreactor can be used for tendon/ligament engineering. In an incubator bioreactor system, air exchange is through the hydrophobic filter leak of the medium reservoir, and proper CO₂ and temperature level is controlled by the incubator. Then, by circulating the culture medium, suitable conditions can be applied to engineered tendon/ligament, as shown in Figure 4. However, an independent bioreactor system, as shown Figure 5, does not rely on an incubator to control the culture environment. The percentage of different gases (PO₂, CO₂, and N₂) are controlled by air valves, and the mixed gas is humidified first, and then passed into a medium heater. Waste gas emission is transported through a filter in the case of contamination. The prepared, warm culture medium is circulated through the bioreactor culture chamber. For certain tissues such as cartilage, some specific culture conditions are required. For example, under a low-oxygen environment, engineered cartilage displays faster matrix glycosaminoglycan deposition rate and better cellular morphology, but with less dedifferentiation. The environmental chamber allows researchers to manipulate different culture conditions, thereby enabling a systematic study of cell growth and differentiation into functional tissue.

**Monitoring and Feedback Systems**

The biomechanical properties of the final engineered tendon/ligament should be a central concern of bioreactor design, as the tendon/ligament’s functional role in the body is primarily mechanical. The maximum load and elastic modulus are essential mechanical properties to evaluate the suitability of engineered tendon/ligament. However, in most studies, mechanical tests are performed only at the end of tissue culture. For example, the stiffness of the constructs at different time points during culture is rarely recorded or assessed. The correlation between stiffness and tissue maturation may provide a better understanding about how the cell differentiates into functional tissue, and for this reason, online stiffness monitoring is likely to be invaluable.

In tendon/ligament bioreactors, force measurement is enabled using sensors called load cells, which are located differently in various bioreactor designs. Load cells located between the actuator and sample clamps, shown in Figure 6A, requires high manufacturing accuracy, as the friction between the shaft and culture chamber can induce error. For fragile materials such as biological tissues, this friction might be higher than the applied load. Conversely, a load cell placed at the end of the culture chamber, shown in Figure 6B, can minimize the friction between the actuator and culture chamber and also the fluid resistance during stimulation. To acquire accurate data, load cell selection should be based on the initial mechanical properties of the constructs. Working with the sensitivity of the load cell, extra attention is needed when manipulating the construct so as to avoid causing damage to the load cell through overloading.

In addition to force monitoring, tissue displacement is another important variable that requires monitoring. Linear variable differential transformers and optical decoders are commonly used position sensors. Although the motion of actuating systems is preprogrammed, overload of the actuator and manual misoperation can cause desynchronization between the program and actual stimulation. The real-time displacement monitor can produce a full record of stimulation position, which then allows researchers to track if there are any unusual features in the results. Another function of the position sensor is to provide feedback of the displacement information to the actuator control system to correct for any desynchronization. Critically, by monitoring load and displacement, real-time stiffness during engineered tendon/ligament culture can be estimated.

Lastly, imaging the construct might be a potentially important monitoring method that can be adopted in bioreactor systems. Two imaging modalities, the confocal microscope and optical coherence tomography, are potential candidates. However, sample deformation induced by mechanical stimulation can cause image shift resulting in poor focus or loss of image. Imaging dynamic sample is extremely difficult in conventional bioreactors, as features of interest (e.g., cells) soon leave the field of view. Techniques need to be developed to move the sample and microscope together. This can become difficult, especially, when doing in situ imaging of a tendon tissue culture.

**Commercial Bioreactor Systems for Tendon/Ligament Engineering**

Recently, commercial tendon-/ligament-engineering bioreactor systems have become available. As discussed above, these basic principles are applied to customized bioreactors; however, with greater design input and advanced manufacturing techniques, commercial products are able to provide more accurate and complex environments for
FIG. 5. Schematic diagram of an independent bioreactor system. Suitable temperature, humidity, and CO2 level of the culture medium are controlled by the environmental chamber. Cold culture medium is pumped to the environmental chamber for heating and then circulated through the culture chamber. The waste valve is closed normally, and it will open during medium exchange. Color images available online at www.liebertpub.com/teb

FIG. 6. Load cell position in different bioreactor systems. (A) Load cell is located between the actuator and sample clamps. (B) A separate load cell placed at the end of culture chamber system. Color images available online at www.liebertpub.com/teb
<table>
<thead>
<tr>
<th>First author</th>
<th>Bioreactor type (Company)</th>
<th>Parameters of mechanical stimulation</th>
<th>Scaffold material (dimensions)</th>
<th>Cell source</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Altman (2002)</td>
<td>Custom-made step motor bioreactor with environmental chamber</td>
<td>Cyclic stretching 2-mm, 90° rotation, 0.0167 Hz, 21 days</td>
<td>Collagen type I gels, <em>Rambys mariali</em> silk worm silk fiber matrices (length 30 mm)</td>
<td>Human bone marrow stromal cells (<em>hMSC</em>)</td>
<td>Elongation of hMSCs, cross-section cell density ↑</td>
<td>76</td>
</tr>
<tr>
<td>Juncosa-Melvin (2006)</td>
<td>Custom-made pneumatic cylinder bioreactor with LVDT for displacement monitoring</td>
<td>Cyclic stretching 2.4% strain, 0.0033 Hz, 8 h/day for 2 weeks</td>
<td>Type I collagen sponge (22 ± 0.8 mm × 9 ± 0.8 mm × 3 ± 0.1 mm)</td>
<td>Rabbit MSCs</td>
<td>Maximum force ↑, linear stiffness ↑, maximum stress ↑, linear modulus ↑</td>
<td>19</td>
</tr>
<tr>
<td>Webb (2006)</td>
<td>Custom-made step motor bioreactor</td>
<td>Cyclic stretching 10% strain, 0.25 Hz, 8 h/day for 7 days</td>
<td>Polyurethane construct (20 × 10 × 2 mm)</td>
<td>Human tracheal fibroblasts</td>
<td>Type 1 collagen ↑, TGF-β ↑, CTGF, Elastin ↑, alpha ↑, Procollagen ↑, Fibronectin ↑, MMP-1 ↑, elastic modulus ↑ Cell density ↑, stiffness ↑</td>
<td>42</td>
</tr>
<tr>
<td>Androja (2007)</td>
<td>Custom-made bioreactor with load-displacement measure system</td>
<td>Cyclic stretching 9% strain, twice daily for 30 min each period separated by 8-h rest for 2 weeks</td>
<td>Small intestine submucosa (3 × 5 × 95 μm)</td>
<td>Dog tenocytes</td>
<td>The stimulation pattern of 2.4% strain, 3000 cycles/day, 1 Hz has the best effect on increasing stiffness.</td>
<td>35</td>
</tr>
<tr>
<td>Nirmalanandhan (2008)</td>
<td>Custom-made pneumatic cylinder bioreactor</td>
<td>Cyclic stretching 2.4% and 1.2% strain, 1 Hz, stimulation period: 8 h/day, 100 and 3000 cycles/day for 12 days</td>
<td>Type I collagen sponge</td>
<td>Rabbit iliac-crest MSCs</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>Nirmalanandhan (2008)</td>
<td>Custom-made pneumatic cylinder bioreactor</td>
<td>Cyclic stretching 2.4% strain, 0.0033 Hz, 8 h/day for 12 days</td>
<td>Type I purified bovine collagen gel, type I collagen sponges (length: 11 and 51 mm)</td>
<td>Rabbit MSCs</td>
<td>Stiffness ↑</td>
<td>20</td>
</tr>
<tr>
<td>Nguyen (2009)</td>
<td>Custom-made linear actuator bioreactor with load cell for force measurement</td>
<td>Preloaded with 0.05 N, cyclic stretching 10%, 0.5 Hz, 2 h stimulation—2 h rest—2 h stimulation—3 h rest for 3 days</td>
<td>Porcine small intestine submucosa (length: 2 cm and width: 1 cm)</td>
<td>Rabbit MCL fibroblasts</td>
<td>Improved fiber orientation, fiber angular dispersion ↑, better organized collagen fiber, elongated cell morphology.</td>
<td>22</td>
</tr>
<tr>
<td>Abousleiman (2009)</td>
<td>Custom-made linear actuator bioreactor with medium circulation system</td>
<td>Cyclic stretching 2% strain, 1 h/day, 0.0167 Hz for 1 and 2 weeks</td>
<td>Human umbilical veins (wall thickness: 0.75 mm, outer diameter: 6.75 ± 0.25 mm, length: 8.5 cm)</td>
<td>Wistar Rat bone marrow MSCs</td>
<td>Better cell distribution, more elongated cell morphology, cell proliferation ↑, ultimate stress ↑, elastic modulus ↑</td>
<td>23</td>
</tr>
<tr>
<td>Butler (2009)</td>
<td>Custom-made pneumatic cylinder bioreactor with LVDT for displacement monitoring</td>
<td>Cyclic stretching 2.4% strain, 0.0033 Hz, 8 h/day for 2 weeks</td>
<td>Type I collagen sponge (94% pore volume, 62-mm mean pore diameter)</td>
<td>Mouse mesenchymal stem cell</td>
<td>Type 1 collagen ↑, linear stiffness ↑</td>
<td>83</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>First author</th>
<th>Bioreactor type (Company)</th>
<th>Parameters of mechanical stimulation</th>
<th>Scaffold material (dimensions)</th>
<th>Cell source</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chen (2010)</td>
<td>Custom-made step motor bioreactor</td>
<td>Cyclic stretching 10% strain, 2 h/day, 1 Hz for 14 days</td>
<td>Knitted silk-collagen sponge scaffold (5 x 0.5 x 0.2 cm)</td>
<td>Human embryonic stem cell</td>
<td>Collagen I, Collagen III, Ephad↑, Sox↑, Sox9↑, Myosin↑, Integrin α1↑, Integrin α2↑, Integrin β1↑, Collagen content↑, Collagen diameter↑, better cell alignment↑</td>
<td>2</td>
</tr>
<tr>
<td>Doroski (2010)</td>
<td>Custom-made linear motor bioreactor</td>
<td>Cyclic stretching 10% strain (5% offset, 5% amplitude), 1 Hz, 3 h/day, 1, 7, 14, and 21 days</td>
<td>Poly(ethylene glycol)-based hydrogel material oligo(poly(ethylene glycol)fumarate) (12.5 x 9.5 x 1.6 mm)</td>
<td>MSCs (PT-2510; Lonza)</td>
<td>Collagen I↑, Collagen III↑, TNC↑, Tenascin-C↑</td>
<td>27</td>
</tr>
<tr>
<td>Saber (2010)</td>
<td>Ligagen L30-4C (Tissue Growth Technologies)</td>
<td>Cyclic stretching 1.25 N, 1 cycle/min, 1 h/day for 5 days</td>
<td>Acellular rabbit hindpaw tendon (length: 5cm)</td>
<td>Rabbit tenocytes</td>
<td>Ultimate tensile stress↑ (closed to fleshly harvested tendon), elastic modulus↑, tensile strength↑</td>
<td>36</td>
</tr>
<tr>
<td>Issa (2011)</td>
<td>Custom-made bioreactor</td>
<td>Cyclic stretching 2% strain, 1 h/day, 0.0167 Hz for 1 and 2 weeks.</td>
<td>Human umbilical veins (wall thickness: 0.41 mm) with Wharton’s jelly matrix as central portion (total thickness: 0.75 mm)</td>
<td>Wistar rat bone marrow MSCs</td>
<td>Cell proliferation↑, tensile strength↑</td>
<td>28</td>
</tr>
<tr>
<td>Woon (2011)</td>
<td>Ligagen L30-1C &amp; Ligagen L30-4C (Tissue Growth Technologies)</td>
<td>L30-1C: dynamic loading, 10 N, 1 h/day, 0.0167 Hz for 5 days L30-4C: dynamic loading, 0.625 N, 1.25 N, 2.5 N, 1 h/day, 0.0167 Hz for 3, 5, and 8 days</td>
<td>Acellular human flexor tendon scaffolds</td>
<td>Human dermal fibroblasts</td>
<td>Ultimate tensile stress↑, elastic modulus↑</td>
<td>31</td>
</tr>
</tbody>
</table>

LVDT, linear variable differential transformers; ↑, up regulation; ↓, down regulation.
tendon/ligament culture. To our knowledge, two relatively complete commercial bioreactor systems have been developed recently: The Bose® ElectroForce® BioDynamic® system and the LigaGen system.

The Bose ElectroForce BioDynamic test instrument provides an accurate programmable uniaxial stretching stimulation and a controllable medium circulation environment to engineered tendon/ligament, which theoretically can be adjusted to mimic the in vivo biomechanical environment. With a load cell and optional laser micrometer, this bioreactor system is able to monitor the force/strain curve of engineered tendon/ligament during the culture period. Two different force and displacement ranges are available. Single-chamber and multiple-chamber systems with shared or independent loading are optional. As there are culture chambers compatible to the Bose testing devices such as ElectroForce 3200, biomechanical tests can be done at different time points without disruption of the tissue culture (www.bose-electroforce.com).

The LigaGen system is a lightweight (<3 kg) incubator-compatible bioreactor. It is capable of applying a maximum force of 40 N to the tissue sample and simulating complex, and presumably more physiologically realistic, loading patterns. Two systems are available from LigaGen. L30-1× is a single-culture model, which has a 23-ml internal-volume chamber for single-tissue culture, and the L30-4C is a multi-culture model with an 80-ml chamber for shared dynamic culture on two or four samples. The standard medium circulation system can reduce contamination risk during medium exchange. Rather than being a comprehensive system, the bioreactor is extensible to suit individual needs, by adding various components to the (universal) basic model as required. With an accessory tissue-monitoring sensor, this system is able to achieve real-time measurements of the sample stiffness during culture. If flow control is necessary, extra control systems can be installed (www.tissuemgrowth.com/).

However, there are some disadvantages in the commercial bioreactors. First, the fixation mechanism to stabilize the tendon tissue in the bioreactor cannot be adjusted. Tissue clamps provided in the commercial bioreactor systems can become less effective when it comes to the use of a cylindrical scaffold. Secondly, capacities of the chamber and mechanical input are limited, and they can only host small-animal tissues. This may restrict clinical development. Moreover, some ligaments, such as the anterior cruciate ligament, are not only subjected to tensile force, but also to rotational loading, and none of the commercial bioreactor systems provide the addition of torsional loading. Lastly, full-scale commercial bioreactor systems are expensive, and for most of the time, not all of the functions they provide are commonly used in every study.

**Ideal Bioreactors for Tendon/Ligament Engineering**

The ideal bioreactor should be able culture tendon- and ligament-like constructs, which are well-organized, cell-seeded assemblies of collagen bundles with mechanical properties functionally similar to the native tissue. Autologous cell-seeded constructs are biologically compatible, and able to provide mechanical support similar to native tissue, and consequently, are widely studied in tendon/ligament engineering. However, induction of cell-directed collagen fiber reorganization and assembly of collagen bundles are two important impediments to this approach. Mechanical tensile loads can help provide the necessary signals to cells to increase collagen synthesis, spatially organize of the collagen along the primary stress direction, and stabilize collagen from collagenase degradation. Moreover, proper mechanical stimulation can upregulate different proteoglycans, such as decorin, biglycan, fibromodulin, and fibronectin, which help the cells organize the parallel collagen fibrils forming bundles.

The host body is in many ways the ultimate bioreactor for all engineered tissues. The study of Juncosa-Melvin et al. indicated that the maximum force of engineered patellar tendons increased more than 3000 times after 2 weeks of implantation in rabbits, and to date, none of the bioreactors have been able to accomplish this outcome. Therefore, an ideal bioreactor should aim to mimic the dynamic biochemical and biophysical environments in vivo. In musculoskeletal tissue engineering, various bioreactors have been developed. For instance, muscle tissues are not only subjected to mechanical stretching, but also able to receive the electrical impulses to simulate inputs from the central nervous system, and mechanical and electrical stimulation bioreactors have been developed based on mimicking the in vivo environment. Compared to muscle, the in vivo environment is comparatively less complex in tendon/ligament and appears to require only passive mechanical input.

Summarizing, the ideal tendon-/ligament-engineering bioreactor that enables systemic research should integrate all aforementioned components (Fig. 1). The bioreactor should have culture chambers and an actuating system, but also be fitted with a medium circulation system, an environmental system, a monitoring system, a feedback system, and a waste medium analysis system. This bioreactor should first be able to provide not only a multiple, suitably sized, and sterilized chambers for tissue culture, but also accurate and programmable mechanical stimulation. Tensile strain and rotation are needed to mimic different in vivo tendon/ligament loads. Second, the circulated medium should infiltrate into tissue better than a static medium configuration, and the circulation system needs to reduce the risks of contamination during medium exchange and drug delivery. Moreover, environmental control, such as PO2, CO2, and pH level, allows researchers to explore the impact of different culture conditions on tissue maturation. Third, a monitoring system should provide the real-time status of cultured tendon/ligament, such as force and displacement, and based on these data, adjustment of mechanical stimulation by use of a feedback system. Fourth, through analysis of the waste medium, nutrient consumption needs to be evaluated, which may enable the changing of the medium base at different stages as required, rather than fixed, regular medium exchange every 3 days. Finally and importantly, the best patterns of mechanical stimulation for culturing engineered tendon/ligaments need to be defined.

**Investigation of Evidence**

Since the first 3D engineered tendon/ligament bioreactor system published by Altman et al. in 2002, the effect of mechanical stimulation on the engineered tendon/ligament has drawn a lot of research attention. In the past decade,
dynamic loading of culture in bioreactor systems has been proven to have been a significant development in producing an engineered tendon/ligament. Indeed, various studies have been performed using bioreactor systems and have met with considerably success, and these are summarized in Table 2. Compared with static culture, the tissue produced using dynamic mechanical stimulation has a better cell morphology, including elongated cellular morphology and increased cell density. The mechanical properties of engineered tendon/ligament, such as tensile strength and elastic modulus, are also greatly improved by cyclic loading of the tissue culture, as is the microstructure of the extracellular matrix, such as collagen fiber alignment. Gene expression is also positively influenced by cyclic mechanical stimulation. For instance, collagen I expression under dynamic loading is three times higher than static culture in 2 weeks. Although cyclic stretching has been proven to be an effective way to stimulate the engineered tendon/ligament culture, the optimal stimulation pattern is still unknown. Nirmalanandhan et al. revealed that a 2.4% strain cycle consisting of 3000 cycles per day produced the best linear stiffness in rabbit MSCs seeded in type I collagen sponge. However, from the perspective of Butler et al., the stimulation pattern should be adjusted based on maturation of the engineered tendon/ligament, with higher dose loading applied at the later stage of tissue culture.

Conclusion

The goal should be to create a construct with similar microstructure and mechanical properties, as native tissue, using bioscaffolds and autologous tenocytes. However, a tendon-like unoriented collagen structure cannot be achieved without a mechanical stimulus within the culture environment. Traditional culture techniques do not provide this mechanical stimulation. The use of a bioreactor system is able to bridge the gap between in vitro and in vivo systems by creating suitable biochemical and biomechanical environments. Although it is clearly very difficult to reproduce the in vivo microenvironmental conditions exactly within the bioreactor, the goal is to mimic the in vivo biomechanical condition as closely as possible. The essential components of a tendon-/ligament-engineering bioreactor are the actuating system and culture chamber, which are responsible for the mechanical stimulation and providing sterilized environment for tissue culture. For better manipulation of the culture environment, accurate stimulation and more precise reporting of mechanical maturation of engineered tendon/ligament, an environmental control system, medium circulation system, monitor, and feedback system need to be included. As bioreactors nowadays are becoming more focused on preclinical research, using these clinically in the future still presents a substantial challenge. Tissue engineering requires substantial system optimization to achieve a reproducible functional engineered tendon/ligament consistently. This process is difficult and expensive in animal models, let alone in humans. However, there are additional problems in moving from small-animal models to humans related to the physical size of human tissue samples. Issues of nutrient transport, cell source, and spatial heterogeneity of scaffold properties and cell stimulation become more prominent in these larger tissues. However, all the evidence suggests that by using bioreactors as described above, it will be possible to successfully produce engineered tendons and ligaments. When this occurs, we will be able to manufacture basic multichamber bioreactors with accurate and appropriate patterns of mechanical stimulation, which are affordable, and have low maintenance costs that can be used in commercial settings.

Acknowledgments

This work is supported by the Australia Research Council Linkage Grant (LP110100581). We would like to thank our fellow group members, in particular Ms. Euphemia Landao and Mr. Robert Day for helpful discussion.

Disclosure Statement

No competing financial interests exist.

References


Address correspondence to: Ming H. Zheng, MD, PhD, FRCPath, FRCPA
Centre for Orthopaedic Research
School of Surgery
The University of Western Australia
M Block, QE2 Medical Centre
Nedlands, WA 6009
Australia
E-mail: minghao.zheng@uwa.edu.au

David G. Lloyd, PhD
Centre for Musculoskeletal Research
Griffith Health Institute
Clinical Science 1 (G02) Room 2.40
Griffith University
Gold Coast Campus, QLD 4222
Australia
E-mail: david.lloyd@griffith.edu.au

David W. Smith, PhD
School of Computer Science and Software Engineering
The University of Western Australia (M002)
35 Stirling Highway
Crawley, WA 6009
Australia
E-mail: david.smith@uwa.edu.au

Received: May 17, 2012
Accepted: September 25, 2012
Online Publication Date: November 19, 2012
Programmable Mechanical Stimulation Influences Tendon Homeostasis in a Bioreactor System

Tao Wang,1 Zhen Lin,1,2 Robert E. Day,3 Bruce Gardiner,4 Euphemie Landao-Bassonga,1 Jonas Rubenson,5 Thomas B. Kirk,6 David W. Smith,4 David G. Lloyd,7 Gerard Hardisty,8 Allan Wang,9 Qiujian Zheng,2 Ming H. Zheng1

1Centre for Orthopaedic Translational Research, School of Surgery, University of Western Australia, M Block, QEII Medical Centre, Nedlands, Crawley, Western Australia 6009, Australia; telephone: 61-8-93464050; fax: 61-8-93463210; e-mail: minghao.zheng@uwa.edu.au
2Division of Orthopaedic Surgery, Department of Surgery, Guangdong General Hospital, Guangdong Academy of Medicine Science, Guangzhou, Guangdong, China
3Department of Medical Engineering and Physics, Royal Perth Hospital, Perth, Western Australia, Australia
4School of Computer Science and Software Engineering, University of Western Australia (M002), Crawley, Western Australia, Australia
5School of Sport Science, Exercise and Health, Musculoskeletal Tissue Mechanics and Muscle Energetics University of Western Australia (M408), Crawley, Western Australia, Australia
6Curtin University, Bentley, Chancellory, Western Australia, Australia
7Centre for Musculoskeletal Research, Griffith Health Institute, Griffith University, Gold Coast Campus, Queensland, Australia
8St John of God Medical Clinic, Perth, Subiaco, Western Australia, Australia
9Sir Charles Gairdner Hospital, Perth, Nedlands, Western Australia, Australia

ABSTRACT: Identification of functional programmable mechanical stimulation (PMS) on tendon not only provides the insight of the tendon homeostasis under physical/pathological condition, but also guides a better engineering strategy for tendon regeneration. The aims of the study are to design a bioreactor system with PMS to mimic the in vivo loading conditions, and to define the impact of different cyclic tensile strain on tendon. Rabbit Achilles tendons were loaded in the bioreactor with/without cyclic tensile loading (0.25 Hz for 8 h/day, 0–9% for 6 days). Tendons without loading lost its structure integrity as evidenced by disoriented collagen fiber, increased type III collagen expression, and increased cell apoptosis. Tendons with 3% of cyclic tensile loading had moderate matrix deterioration and elevated expression levels of MMP-1, 3, and 12, whilst exceeded loading regime of 9% caused massive rupture of collagen bundle. However, 6% of cyclic tensile strain was able to maintain the structural integrity and cellular function. Our data indicated that an optimal PMS is required to maintain the tendon homeostasis and there is only a narrow range of tensile strain that can induce the anabolic action. The clinical impact of this study is that optimized eccentric training program is needed to achieve maximum beneficial effects on chronic tendinopathy management.

(C) 2012 Wiley Periodicals, Inc.

KEYWORDS: bioreactor; programmable mechanical stimulation; tendon; collagen

Introduction

Tendinopathy is common in both young athletes and the elderly and is thought to be due to overuse and/or overloading. Tendinopathy causes significant pain and can lead to chronic degenerative changes in the tendon
matrix (Smith, 2000), and potentially tendon rupture (Lea and Smith, 1972; Leppilahit and Orava, 1998). Achilles tendinopathy is one of the most common tendinopathies and has the greatest potential to restrict the activities in daily living. Treatment outcomes are often poor. These poor results are largely due to the underlying physiological processes within tendons, which are not well understood.

Tissue engineering methods such as cell-seeded bio scaffolds have great potential in the repair of damaged tendons. However, these methods are still very much in their infancy, due to the lack of an integrated understanding of the key conditions required for successful tendinopathy intervention and repair. Recent tendon research has focused on the development of tendon tissue engineering, with the aim of constructing a bio-substitute material for tendon/ligament repair (Chen et al., 2007, 2008; Juncosa-Melvin et al., 2006; Nirmalanandhan et al., 2008a; Peach et al., 2012; Saber et al., 2010; Saho et al., 2006; Stoll et al., 2010; Wang et al., 2012; Webb et al., 2006; Woon et al., 2012). This strategy involves an integration of technologies from cell biology, material science, and mechanical engineering.

Engineered bioreactor systems have been demonstrated to be effective tools for constructing various biological tissues and organs such as muscle (Dennis et al., 2009), liver (Catapano et al., 2010), and bone (Rauh et al., 2011, 2012). Several bioreactor systems have been developed specifically for engineered tendon/ligament formation (Altman et al., 2002; Issa et al., 2011; Juncosa-Melvin et al., 2006; Nirmalanandhan et al., 2008a; Webb et al., 2006). Owing to force transmission being the primary function role of tendon/ligament, mechanical stimulation plays an essential role in the maintenance of tendon structure and function (Hannafin et al., 1995). Achilles tendons endure tensile loading in vivo during daily physical activities. More complex structures, like the anterior cruciate ligament, undergo both tensile and torsion loading during weight-bearing knee flexion (Li et al., 2005). To restore tendon function after injury, engineered tendon constructs should be compatible with the in vivo environment both biologically and mechanically (Calve et al., 2010). Cell scaffold constructs may be “exercised” in an artificial mechanical environment before implantation. Indeed, programmable mechanical stimulation (PMS) is considered to be one of the key components for tendon/ligament forming bioreactor system (Altman et al., 2002; Issa et al., 2011; Juncosa-Melvin et al., 2006; Nirmalanandhan et al., 2008a; Webb et al., 2006). In this study, a bioreactor system was designed to apply different cyclic tensile strain on isolated rabbit Achilles tendon to establish an ex vivo model. The present aim is to evaluate the effect of different mechanical stimulation on tendon tissue.

The mechanical stimulation of engineered tendon constructs can result in different structures. Non-loading of engineered neo-tendons results in loose, disorganized matrices with reduced collagen quantity (Jiang et al., 2011). Static mechanical stress has been shown to improve the fiber arrangement of engineered tendon constructs, but did not produce a compacted tendon-like collagen structure in vitro (Chen et al., 2007). More promisingly, engineered tendon under cyclic tensile strain exhibited a well-organized collagen fiber arrangement and structure and enhanced tensile strength, suggesting that PMS is preferable for engineered tendon formation (Jiang et al., 2011). Even though the importance of providing PMS in tendon bioreactor has been recognized previously (Brown and Carson, 1999; Wang et al., 2012), the optimal cyclic tensile stimulation parameters have not been defined. It is generally assumed that mimicking the dynamic mechanical loading created by daily physical activity will give the optimal loading regime in tendon bioreactor system, but this is not yet proven.

The goals of this study were: (1) to develop and validate a bioreactor system; (2) to investigate the effect of different mechanical stimulation regimes on tendon integrity. We hypothesized that there is an optimum range of mechanical stimulation required for tendon homeostasis and that under or over-stimulation would reduce tendon integrity.

Materials and Methods

Bioreactor Design Criteria

To develop a functional bioreactor, several design criteria were formulated. Firstly, the bioreactor must be able to process more than three individual cultures simultaneously with transparent covers allowing visual monitoring. Secondly, the extension accuracy should be better than ±100 μm with the load varying as required to produce the set strain (the minimum expected load was 20 N per sample). Third, a construct fixation method must prevent slippage during stimulation. Moreover, as the bioreactor system is operated in an incubator, all of the electrical and mechanical components have to be able to function in a warm, humid environment. Finally, but not least, the parts involved with the tissue culture should be autoclavable.

To achieve these design criteria two structural materials were used, AISI 316 stainless steel and 6063 aluminum. The main bioreactor components are the culture chamber, tissue clamps and hooks made of stainless steel (which can be easily sterilized) and frame. The frame was made of aluminum to reduce the weight and 2 mm thick glass was used for transparent cover. The actuator was a motor-ball screw transmission design using a step motor and precision ball screw to maximize the load output and positional accuracy. A programmable logic control was chosen, rather than direct computer control, to reduce cost and allow reliable operation in uncertain experimental environments.

Tissue Harvest and Distribution

Achilles tendons were dissected from the hindlimbs of female New Zealand White rabbits (Oryctolagus cuniculus).
aged between 14 and 15 weeks with body weight 3–5 kg. The tissue was briefly rinsed in 70% ethanol, washed in 1× phosphate-buffered saline (PBS) three times, and submerged in serum free Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (GIBCO, Invitrogen, Auckland, New Zealand) supplemented with 50 μg/mL Gentamicin before further processing.

In total 24 Achilles tendons from 12 rabbits were allocated to three groups (Fig. 1A): a loading deprivation group (8), a dynamic culture (cyclic tensile strain) group (12), and a control group (4). In the loading deprivation group, the Achilles tendons were cultured ex vivo in growth medium (DMEM/F-12, 10% fetal bovine serum and 50 μg/mL Gentamicin) without mechanical loading for either 6 days (four tendons) or 2 weeks (four tendons). In the dynamic strain group, the tendons were cultured under 3%, 6%, or 9% cyclic tensile strain for 6 days (8 h per day, 0.25 Hz, Fig. 1A). The stimulating cycle was as demonstrated in Figure 1B. This regimen was based on previously published studies and data on rabbit activity levels (West et al., 2004). The samples were then fixed in 4% paraformaldehyde overnight for histological analysis or directly subjected to RNA extraction. The control group was made up of four tendons directly dissected from the animal and immediately fixed.

**Histological Preparation and Assessment**

For histological assessment, the tissue samples were fixed in 4% paraformaldehyde overnight, dehydrated through graded alcohol, cleaned with xylene and embedded in paraffin blocks. Histological sections (5 μm) were cut on a microtome and stained with Haematoxylin & Eosin. Blind general histological scoring was performed by three individuals as previously described (Chen et al., 2011). Four elements (cell density, cell roundness, fiber structure, and fiber arrangement) were assessed using scoring scale of 0–3, where 0 is normal and 3 severely abnormal. The average score of each parameter was used for comparison.

**TUNEL Assay**

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay (Cell Death Detection-AP Kit, Roche, Switzerland) was performed to identify apoptotic cells by labeling the nuclear DNA fragments. Paraffin-embedded sections were dewaxed and rehydrated using graded ethanol (100%, 95%, 80%, and 70%). The tissue was digested with 20 μg/mL proteinase K (Qiagen, Hilden, Germany) for 15 min, incubated with TUNEL reaction mixture for 60 min and the convert-AP (alkaline phosphatase) reagent for another 30 min, according to the manufacturer’s instruction. After rinsing with PBS, the sections were visualized with Diaminobenzidine (DAB) kit (DAKO, Glostrup, Denmark) for 5 min, which stained the positive cells brown; and counterstained with hematoxylin.

**Immunostaining for Type III Collagen**

To evaluate the extracellular matrix (ECM) turnover in tendons with and without dynamic tensile strain, protein expression of type III collagen was evaluated. Paraffin-embedded sections were dewaxed and rehydrated. The endogenous peroxidase was blocked with 3% hydrogen peroxide (H2O2) for 10 min, and the sections were then...
incubated with 5% fetal bovine serum in 1× PBS for 30 min, followed by monoclonal anti-collagen type III (036K4782, Sigma, St Louis, MO; 1:100) in a damp chamber overnight at 4°C. Biotinylated goat antimouse IgG at a dilution of 1:200 (Sigma) was used as secondary type III antibody. PBS was used to wash the sections between solutions and reagents. Sections were developed to visualize the positive staining using the DAB kit, and counterstained with hematoxylin solution.

**Quantitative Real-Time Polymerase Chain Reaction (Q-PCR)**

The effect of mechanical stimulation on collagen gene expression was examined in Achilles tendon after the programmed dynamic training. Approximately 200 mg of tendon tissue was dissected from the middle region, and the total ribonucleic acid (RNA) was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA). The RNA concentration was quantified and using SmartSpec™ 3000 (Bio-Rad, Hercules, CA), and A260/A280 was measured to evaluate the RNA quality. The RNA (500 ng) was then reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using SuperScript® III First-Strand Synthesis System (Invitrogen, USA) according to the manufacturer’s instructions.

The cDNA was amplified and quantified by Q-PCR. For this reaction, 5 μL of iTaq® SYBR® Green Supermix (Bio-Rad), 1 μL of cDNA, 0.5 μL of each forward and reverse primer and 3 μL of DEPC water were combined and mixed. The real-time PCR was performed as previously described (Lin et al., 2008). The primer sequences are listed in Table I.

**Statistical Analyses**

All statistics have been analyzed using the prism 5.03 software package (GraphPad Software, Inc., San Diego, CA). Data are presented as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA). P values less than 0.05 were considered significant.

**Results**

**Bioreactor for Tendon Engineering**

By constructing a bioreactor to the design criteria, we have generated a bioreactor that is able to simultaneously apply pre-programmed uniaxial stimulation on a maximum of six tendons, cultured separately in their own chamber (to prevent cross contamination) (Fig. 2A). As Achilles tendons require only tensile stimulation (Louis-Ugbo et al., 2004), system performance was tested with rabbit Achilles tendons. The system was actuated by a step motor-ball screw transmission system (SMSB) using VEXTA step motor (PX245M-01AA, Oriental Motor, Kabushiki Kaisha, Tokyo, Japan) and precision ball screw (SG0601, KSS Co., Ltd., Ojiya, Niigata, Japan), which produced 198 N peak force and a positional accuracy of ±5 μm. Using programmable logic controllers (MELSEC FX1S, Mitsubishi, Marunouchi, Chiyoda-ku, Tokyo, Japan), as shown as Figure 2A, displacement, frequency, and stimulation period are all programmable. The culture chamber contains six independent vessels with individual capacity of 20 mL (width = 2 cm, length = 9 cm). Individual transparent glass covers minimized the risk of cross contamination between tested samples, and provided a window for monitoring each tendon (Fig. 3). This system provides two sample stabilization methods: tissue clamps (Fig. 2C) and hooks (Fig. 2D). Soft and flat scaffolds, like a collagen and silk construct, can be fixed using the tissue clamps, whereas robust and slippery tissue, such as bone/tendon and muscle/tendon junctions, can be tied to the hooks using suture. The whole system was placed inside a water-jacketed incubator (Forma Scientific, Marietta, OH) at 37°C with 5% CO2, and 95% air.

Using the final bioreactor design, Achilles tendons isolated from rabbits were placed into the system to optimize of condition of dynamic culture. Cyclic tensile strain at 3% 6%, and 9% (0.25 Hz for 8 h per day for 6 days) were applied, to a total of 12 rabbit Achilles tendons. Overall culture period was over 3 weeks with no contamination found during the culture period. Clear vision was achieved through transparent glass cover. Both the tissue clamp and tissue hook worked effectively with no slippage observed.

**Histological Examination of Tendon Undergoing Different Cyclic Tensile Strains**

Compared with the native tendon (Fig. 4A), the collagen fiber arrangement changed from parallel to moderately wavy after 6 days in the absence of applied loading. Cell density was increased with slightly rounded nuclei. After 2 weeks without loading, progressive ECM disruption was observed.

### Table I. Rabbit specific primer sequences for the genes used.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Forward (5′–3′)</th>
<th>Reverse (3′–5′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1A</td>
<td>TTGCCCTTCTTGTAGTATTGC</td>
<td>CTCCTTTGCCCTACATTTTA</td>
</tr>
<tr>
<td>COL3A</td>
<td>AAGCCCGGAGCCAGAAAAATG</td>
<td>TGTTGGAACAGAAAATCA</td>
</tr>
<tr>
<td>MMP-1</td>
<td>GGCTAGCTGTCGCTTACCTT</td>
<td>CAGGTCCATCAAAGGGAGAA</td>
</tr>
<tr>
<td>MMP-3</td>
<td>GCTTGTGCTAGCCTACAC</td>
<td>AGGCTCAAGGCCGAGAAGA</td>
</tr>
<tr>
<td>MMP-12</td>
<td>ATGCCAGGGGAGACAGGATG</td>
<td>AAAAGCATGGGGCTATGACACC</td>
</tr>
<tr>
<td>36B4</td>
<td>ACCAAAATGTTTCATCGTG</td>
<td>CAGGGTGTGTTCCTCAGATGC</td>
</tr>
</tbody>
</table>

Biotechnology and Bioengineering, Vol. 110, No. 5, May, 2013
Collagen fiber arrangement had no discernible pattern. Fragmentation of collagen fibers and rounded tenocyte nuclei were observed microscopically (Fig. 4C). Histological assessment showed a time dependent increasing grading in fiber arrangement, fiber structure and cell roundness, indicating progressive divergence from the native (control) tissue. Cell density was also increased after 6 days without loading (Fig. 4D–G).

In the dynamic culture (cyclic tensile strain) group, histology revealed that the 3% strain stimulated group had slightly disrupted ECM structure and significant cellular morphology changes (Fig. 5B). These tendon samples graded 1.5 in fiber arrangement, 1.1 in fiber structure and 1.3 in cell roundness (Fig. 5E–G). However, in the tendons that underwent 6% strain, the ECM orientation and cellular morphology appeared normal (Fig. 5A and C). That is, there was no statistical difference of the histology scoring between the 6% group and native tissue (Fig. 5E–H). In the 9% strain stimulated group, the tendon samples were found to be partially torn and graded 1.45 in fiber structure and 0.8 in fiber arrangement (Fig. 5E and F). Increased cell number and slightly rounded tenocyte nuclei were observed around the rupture site (Fig. 5D, G, and H).

Impact of Cyclic Tensile Strain on Apoptosis of Tenocytes

To investigate the impact of mechanical force on the apoptosis of tenocytes, we used the TUNEL assay to examine the cellular apoptosis rate in specimens. In the native tendons, the average cell apoptosis fraction (as a proportion of total cell number) was less than 10% (Fig. 6A and G). The apoptosis fraction progressively increased over time when the tissues were cultured without loading (35% in 6 days and 95% in 2 weeks) (Fig. 6G). Cell viability in 3% and 6% stimulated Achilles tendon was similar to that in native tendon (Fig. 6D and E) without statistical significance (Fig. 6G), whilst cell apoptosis rate increased to 45% when the stimulation strain reach 9% (Fig. 6F and G).

Type III Collagen Turnover in Achilles Tendon Under Dynamic Culture

To investigate the ECM turnover in tendon undergoing different cyclic tensile strain, type III collagen immunohistochemistry was done. No positive type III collagen was observed in the core of healthy tendon using
Figure 4. H&E staining assessment and histological scoring of loading deprivation culture group. A: Native Achilles tendon structure (day 0). B: Achilles tendon histology after 6 days loading deprivation culture with minor wavy collagen structure. C: Achilles tendon histology after 2 weeks culture displays disorganized collagen structure with round tenocytes. D: Fiber arrangement, scale of 0–3, 0 represents compacted and parallel, and 3 represents no identifiable pattern. E: Fiber structure, scale of 0–3, 0 represents continue, long fiber, and 3 represents severe fragmented. F: Cell roundness, scale of 0–3, 0 represents long spindle shape, and 3 represents severely rounding. G: Cell density, scale of 0–3, 0 represents normal pattern, and 3 represents severely increase. Result are expressed as the mean ± SD (P<0.05, ***P<0.01, ****P<0.001). Scale bar = 50 μm.
Figure 5. H&E staining assessment and histological scoring of 6 days dynamic culture group. A: Native Achilles tendon structure. B: Three percent stimulated group with wavy collagen structure and minor rounded tenocytes. C: Six percent stimulated group with similar morphology compared with the native group. D: Nine percent stimulated group with severe tear collagen structure, and rounded nuclei around the tear region (arrow). E: Fiber arrangement, scale of 0–3, 0 represents compacted and parallel, and 3 represents no identifiable pattern. F: Fiber structure, scale of 0–3, 0 represents continue, long fiber, and 3 represents severely fragmented. G: Cell roundness, scale of 0–3, 0 represents long spindle shape, and 3 represents severely rounding. H: Cell density, scale of 0–3, 0 represents normal pattern, and 3 represents severely increase. Result is expressed as the mean ± SD (\( P < 0.05, \quad **P < 0.01, \quad ***P < 0.001 \)). Scale bar = 50 μm.
Figure 6. TUNEL assay. A: Native Achilles tendon with less than 10% cell apoptosis. B: Six days loading deprivation group has increased apoptotic tenocytes pointed out by arrows. C: In 2 weeks loading deprivation group, almost all tenocytes are under apoptosis. D: Three percent stimulated group with minor increased apoptotic cells (arrow). E: Six percent stimulated group with similar cell apoptosis rate as native sample. F: Nine percent stimulated group with massive cell apoptosis around the damaged site (pointed by arrows). G: Quantified cell apoptosis fraction of each group. Result are expressed as the mean ± SD (\(P < 0.05, **P < 0.01, ***P < 0.001\)). Scale bar = 50 μm.
immunochemistry (Fig. 7A). After 6 days of either no loading or 3% strain, the Achilles tendons expressed only minor and moderate collagen type III respectively (Fig. 7B and C), whilst relatively higher positive staining were found in the 9% strain group (Fig. 7E). No type III collagen expression was observed in the 6% cyclic strain stimulated group (Fig. 7D), which was similar to the native tendon.

Impact of Cyclic Tensile Strain on ECM Remodeling Gene Expression

Q-PCR was carried out to examine the gene expression of type I (COL1A1), type III collagen (COL3A1), and MMP-1, 3, 12 (matrix metalloproteinases). As shown in Figure 8, the tissues subjected to 6% cyclic tensile strain had the highest expression of COL1A1 (Fig. 8A), whereas 3% stimulated group had the lowest expression. Type III collagen (COL3A1) was suppressed by minor and moderate loading (3% and 6%), yet upregulated in the 9% strain stimulated group (Fig. 8B). Gene expression of MMP1, 3, and 12 were highly upregulated by 3% strain stimulation compared to the other groups.

Discussion

A previous study has suggested that engineered neo-tendons in a non-loading environment result in loose, disorganized and less collagen-rich matrices (Jiang et al., 2011). Static mechanical loading has been shown to improve the fiber arrangement of engineered tendon constructs, but did not produce a compacted tissue structure in vitro (Chen et al., 2007). Engineered neo-tendons with PMS had a well-organized fiber arrangement and structure and enhanced tensile strength, suggesting that dynamic mechanical loading is preferable for engineered tendon formation (Jiang et al., 2011). Although PMS has been recognized as a critical regulator for tendon formation (Brown and Carson, 1999), the optimal PMS parameters are yet to be defined.

In this study we designed a uni-axial bioreactor system with a programmable stimulation regimen. Achilles tendons from rabbits were used to evaluate the impact of different PMS conditions on tendon tissue. Our result showed that 3% cyclic tensile strain in the bioreactor did not prevent matrix deterioration, whilst at 6% cyclic tensile strain structural integrity and cellular function was maintained. At 9% cyclic tensile strain there was massive rupture of the collagen bundles. However, these results are for an already
A mature tendon and the loading regimes may be different for engineered constructs at various stages of growth. Furthermore, the frequency of loading may also have an important role in maintaining tendon homeostasis and this need to be better qualified by in vivo measurements of Achilles tendon loading. Nevertheless, the current results demonstrate that PMS can be achieved in this bioreactor, and that an optimal cyclic tensile loading (around 6% cyclic tensile strain) is required to maintain healthy tendon homeostasis, at least over the time period of the cultures used in this study.

In recent years, many studies of tendon engineering have been conducted using custom-made bioreactor system, and the results have been promising (Abousleiman et al., 2009; Androjna et al., 2007; Chen et al., 2010; Doroski et al., 2010; Issa et al., 2011; Juncosa-Melvin et al., 2006; Nguyen et al., 2009; Nirmalanandhan et al., 2008a,b; Saber et al., 2010; Webb et al., 2006; Woon et al., 2011). Several commercial tendon engineering bioreactors are available now, such as Bose® ElectroForce®, BioDynamic® and LigaGen system. To develop a functional bioreactor system, several factors needed to be considered, including material, mechanical design, tissue fixation method, and operating environment (Wang et al., 2012). Compared with commercial bioreactors in Bose and LigaGen, the current design has several advantages. Firstly, our device can condition multiple samples simultaneously but separately. It can be easily cleaned and sterilized in standard autoclaves. Being constructed from AISI 316 stainless steel it can go through many sterilization cycles without corrosion or degradation. The device can produce a maximum force of 198 N, which is sufficient for most tendon scaffold and tendon tissue from small scale animals such as are commonly used in tissue engineering research. Most commercial bioreactor systems have problems in holding thick and slippery tissue during PMS, whereas our novel tissue hook design demonstrated excellent tissue fixation. Finally, the actuation system is designed to operate inside a standard incubator. It is able to function in a humid environment and the horizontal design is easy to move and will only occupy a small space in the incubator. As the tendon’s main function is mechanical, this bioreactor system is able to provide high-accuracy PMS.

Figure 8. Quantitative PCR results. A: COL1A1 expression in 6% stimulated group is significantly higher than other groups. B: COL3A1 expression in 9% stimulated group is significantly higher than other groups. Three percent and 6% group had much lower COL3A1 expression compared to the other groups. C-E: Three percent stimulated group has significantly high expression on MMP1, MMP3 and MMP12, respectively. Result is expressed as the mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001). Scale bar = 50 μm.
enabling different loading regimes including displacement, frequency and stimulation period. Once programmed it is able to operate reliably for weeks without computer support.

It has been well known that tensile mechanical loading is essential for tendon integrity, and loading deprivation can cause tendinopathy-like morphology such as disorientated collagen fibers and rounded cell nuclei (Hannafin et al., 1995). Yet the specific effect of different loading conditions on tendon has not been well elucidated. To study the effect of PMS on tendon/ligament, both in vitro and in vivo models have been used. The Flexcell® system has been applied to examine the in vitro gene expression profiles of tenocytes under tensile or shear force (Skutek et al., 2001; Wang et al., 2003; Yang et al., 2005). These systems can precisely control the mechanical regimen and monitor the cell behaviors, but only use a monolayer of cells, not an organized tissue. Cells isolated from their extracellular niche and cultivated in a monolayer may not necessarily replicate their original behavior in vivo. In vivo models, such as Achilles tendon in rats or rabbit, have been commonly used as study models. Tendon loading is mainly induced by electrical stimulation on muscle (Asundi et al., 2008; Nakama et al., 2005), treadmill training on animal and partial tendon transaction (Smith et al., 2008). However, mechanical stimulation on the tendon varies between individuals, making precise quantification of load next to impossible. In this study, we have demonstrated a bioreactor system that not only can be used as a device for tendon/ligament engineering, but also serves as an ex vivo culture model for the study of tendon biology. Although the biomechanics of Achilles tendon is more complicated under in vivo environment (West et al., 2004), by applying PMS, the effect of different cyclic tensile strain on tendon tissue could be systematically analyzed on a tissue level.

The present study has shown for the first time that only a narrow range of PMS can stimulate the anabolic effect of tendon tissue. This suggests a proposed model describing the effect of mechanical loading, from under-load to overload, on tendon homeostasis. This model is illustrated in Figure 9 in which the black curve presents the typical mechanical response caused by increasing tensile strain in tendon. The breakage of collagen fibers increases with increasing strain, leading to eventual rupture at the failure point. The red curve, on the other hand, proposes the biological repair response of the tendon induced by tensile strain. Mechanical loading is required for the biological activity of tenocytes, yet the regime needs to be within a certain range to promote matrix product and net tissue repair. By combining these two curves, three crossover areas are seen, which can be divided according to their metabolic status into: A (anabolic) zone and C (catabolic) zones. In the A zone, where the tensile loading is within a certain range, the biological repair response caused by PMS exceeds the induced damage. In the C zone, there is either too little load to stimulate matrix production or too much mechanical damage caused by excess tensile loading. In our study the 3% strain stimulated group (under-loaded) was located in C zone. When the tendons were subjected to insufficient tensile strain, early tendinopathologic changes (including increased cellular number, type III collagen expression and disorientated collagen fibers) could be observed. These morphological changes may be attributed by the upregulation of MMPs expression in tendon under 3% tensile loading. MMPs are zinc-dependent endopeptidases that able to degrade all the components of the ECM. MMP-1 is primarily responsible for the cleavage of collagen, including type I, II, and III (Krane et al., 1996). MMP-3 and MMP-12 are other members of the MMP family that have a wide range of substrate specificities. Moreover, MMP-12 is able to activate MMP-3, and MMP-3 has the ability to activate other MMPs such as MMP-1, MMP-7, and MMP-9 (Chen, 2004; Knauper et al., 1996). Studies have shown that decreased mechanical force on tendon fiber would lead to the deprivation of cytoskeletal tension in tenocytes and alter the mechanotransduction in the cells (Arnoczky et al., 2008). Insufficient loading of tenocyte can initiate the degenerative cascade by expressing a pattern of catabolic gene, including MMPs (Arnoczky et al., 2004, 2008; Egerbacher et al., 2008; Lavagnino et al., 2003, 2006). In this study, we have confirmed that under the underloading condition, the phenotype of the tenocytes changed from spindle to round, and the expression levels of catabolic genes, such as MMP-1, 2, and 12, were upregulated. The elevated catabolic factors may contribute to the further micro-damage of the collagen fiber. Conversely, although the tendons in the 6% strain stimulated group were subjected to more mechanical damage, the repair mechanism was able to maintain tissue homeostasis (i.e., there is a narrow “net anabolic window” between two net catabolic zones). In the 9% group (overloaded), mechanical damage overwhelmed the tendon tissue repair and caused partial rupture, which is consistent with our proposed model.

The identification of an optimal PMS range for tendon will not only guide us to a better tissue-engineering pathway.

Wang et al.: Programmable Mechanical Stimulation
Biotechnology and Bioengineering
for tendon regeneration, but also provide an insight into tendon homeostasis under both physiological and pathological conditions. Clinically, chronic tendinopathy of Achilles tendon is usually conservatively managed by eccentric overload training. The calf muscle is eccentrically loaded with the knee held straight or the soleus muscle is maximally loaded with the knee bent (Alfredson et al., 1998). Although positive impacts of eccentric overload training on tendinopathy treatment have been recorded, the outcomes have varied widely (Alfredson and Lorentzon, 2003; Alfredson et al., 1998; Fahlstrom et al., 2003; Roos et al., 2004; Shalabi et al., 2004; Silbernagel et al., 2001; Stanish et al., 1986). In the study of Alfredson et al. (1998) the overall improvement in pain score was up to 94%, though in the study of Silbernagel et al. reported only 29% (Silbernagel et al., 2001). Although the mechanism of eccentric overload training on tendinopathy pathomechanical remains unclear, the present study has provided a possible explanation of the mechanism of eccentric loading on chronic tendinopathy. The high variation of clinical outcomes might be due to poor control of the loading regimen. Given that there is only a narrow range of tensile strain where eccentric exercise could provide an anabolic effect on tendon, clinical treatment protocols will need to accurately control the magnitude of load applied through the injured tendon.

Finally, it is noted that nutrient supply might be altered by advective transport associated with mechanical stimulation. Although a previous study on canine flexor tendons suggested that mechanical stimulation did not alter small nutrient uptake compared to static culture, and the tendon repair improvements derived from passive motion is not related to an increase of nutrient transport (Hannafin and Arnoczky, 1994), under different loading regimes, or in different tendon types, this may not be the case. Moreover, the transport of large molecules, such as growth factors and matrix components, may be affected by mechanical stimulation, as has been shown in studies in related tissues (Bonassar et al., 2000; Zhang et al., 2007). Therefore, the tendon stimulated by dynamic mechanical loading in this study might be attributed to the enhanced nutrient transport, compared to the static control. Consequently it is difficult to isolate the direct effect of mechanical stimulation on tendon response from potential enhanced advective transport processes. Further study would be required to measure the nutrition transport in tendon under PMS.

References


Wang et al.: Programmable Mechanical Stimulation

Biotechnology and Bioengineering