# Investigating Fibrotic Pathways in Human Rotator Cuff Tendon Disease

A dissertation submitted to the University of Oxford in partial fulfilment of the requirements for the degree of

Doctor of Philosophy




Wataru Morita

Kellogg College

Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences

University of Oxford

Trinity Term 2018

Supervisors: Professor Andrew Carr, Associate Professor Stephanie Dakin and Associate Professor Sarah Snelling

## Declaration

I certify that this thesis is true and honest account of my own research, which was conducted ethically and the results obtained are genuine. Where I have drawn on the work, ideas, and results of others, this has been appropriately acknowledged in the thesis.

Wataru Morita

# Abstract

Tendon disease begins with inflammation and results in an incomplete repair with fibrosis. IL-1β is a pro-inflammatory cytokine related to tendinopathy and IL-4 is a pro-fibrotic cytokine implicated in torn tendons. TGF-β, BMPs and CTGF are key mediators of fibrosis and their expression is dysregulated in animal models of tendon injury and overuse. The aim of the thesis was to investigate the fibrotic pathways active in human tendon disease. TGF-β, BMP family and CTGF mRNA and protein expression were investigated in patient samples of healthy and diseased tendinopathic and torn supraspinatus tendons. Tendon stromal cells (TSCs) isolated from healthy and diseased (torn) tendons were treated with IL-1β and IL-4 to investigate mRNA expression of TGF-β, BMPs and CTGF. Cell signalling pathways driving cytokine-induced changes in their expression was determined. TSCs from healthy and diseased tendons were treated with TGF-β1 and BMP-2 to investigate how these treatments modulate the expression of extracellular matrix (ECM)-related genes. TGF-β1, BMPs and CTGF protein expression was reduced in diseased compared to healthy tendons, with marked suppression in tissues from torn tendons. IL-4 suppressed *TGFB1*, *BMP2* and *CTGF* mRNA in TSCs from diseased tendons indicating the regulatory role of the inflammatory milieu in disease development. IL-1β induced *TGFB1* and increased *BMP2* mRNA expression in TSCs from diseased tendons that was negated by ERK1/2 inhibition. The suppressive or inductive effects of TGF-β1 and BMP-2 treatments on ECM-related genes were similar in TSCs from healthy and diseased tendons. However, the cells from diseased compared to healthy tendons showed reduced responsiveness to TGF-β1 and BMP-2. The induced *TGFB1* and increased *BMP2* mRNA expression by with IL-1β is possibly a compensatory mechanism for their diminished baseline expression and cellular effects in diseased tendon tissues.

# Acknowledgements

I sincerely thank my supervisors, Professor Andrew Carr, Professor Stephanie Dakin, and Professor Sarah Snelling for their continuous support, guidance, encouragement and patience over the past 4 years. I am thankful for the support and friendship from the members of the Carr group and Professor Afsie Sabokbar. I would not have been able to complete this work without them. I would also like to thank Dr Eishi Kuroda and Dr Atsushi Tasaki for their support and encouragement.

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# Abbreviations

| | |
|---|---|
| ACL | Anterior cruciate ligament |
| ANOVA | Analysis of variance |
| AP-1 | Activator protein 1 |
| AZ | Arizona |
| BCA | Bicinchoninic acid |
| BMP | Bone morphogenetic protein |
| BSA | Bovine serum albumin |
| CA | California |
| cDNA | complementary deoxyribonucleic acid |
| COX | Cyclooxygenase |
| CS | Chondroitin sulphate |
| CTGF | Connective tissue growth factor |
| D | Dimensional |
| DAB | 3,3'-diaminobenzidne |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | deoxyribonucleotide triphosphate |
| DS | Dermatan sulphate |
| ECM | Extracellular matrix |

| | |
|---|---|
| EGF | Epidermal growth factor |
| EMT | Epithelial-to-mesenchymal transition |
| ERK | Extracellular signal-regulated kinase |
| FBS | Foetal bovine serum |
| GAG | Glycosaminoglycan |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| IFN-γ | Interferon gamma |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IMS | Industrial methylated spirit |
| IHC | Immunohistochemistry |
| IQR | Interquartile range |
| I-SMAD | Inhibitory SMAD |
| KS | Keratin sulphate |
| MA | Massachusetts |
| MAPK | Mitogen-activated protein kinases |
| Mass. | Massive |
| MD | Maryland |
| Med. | Medium |
| MMP | Matrix metalloproteinase |
| MO | Missouri |

| | |
|---|---|
| MRI | Magnetic resonance imaging |
| mRNA | Messenger ribonucleic acid |
| MSC | Mesenchymal stem cell |
| NF-κB | Nuclear factor-kappa B |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| OMB | Oxford musculoskeletal biobank |
| OSS | Oxford shoulder score |
| PAGE | Polyacrylamide gel electrophoresis |
| PARoT | Platelet rich plasma and rotator cuff tendons trial |
| PBS | Phosphate buffered saline |
| PGE2 | Prostaglandin E2 |
| Phospho- | Phosphorylated |
| PRP | Platelet-rich plasma |
| PS | Penicillin-streptomycin |
| PVDF | Polyvinylidene difluoride |
| ROI | Region of interest |
| R-SMAD | Regulatory SMAD |
| RT-qPCR | Real-time quantitative polymerase chain reaction |
| SARA | SMAD anchor for receptor activation |
| SAD | Subacromial decompression |
| SAPK/JNK | Stress-activated protein kinase/c-jun NH2-terminal kinase |

| | |
|---|---|
| SBS | Supraspinatus serial biopsy study |
| SD | Standard deviation |
| SDF | Superficial digital flexor |
| SDS | Sodium dodecyl sulphate |
| SECuRe | Scaffold evaluation for rotator cuff repair trial |
| SLRP | Small leucine-rich proteoglycans |
| SMAD | Small mothers against decapentaplegic |
| STAT | Signal transducer and activator of transcription |
| TGF-β | Transforming growth factor beta |
| TGF-β R | TGF-β receptor |
| TGIF | TGF-induced factor |
| Th | T-helper |
| TIMP | Tissue inhibitor of metalloproteinase |
| TNF-α | Tumour necrosis factor alpha |
| TSC | Tendon stromal cell |
| UK | United Kingdom |
| UKUFF | United Kingdom rotator cuff trial |
| WASP | Why are shoulders painful trial |
| WI | Wisconsin |

# Chapter 1 Introduction and Literature Review

## 1.1 Background

Musculoskeletal diseases are extremely common disorders that are becoming a global burden and a crucial component of health expenditure in many middle- to high-income countries [1, 2]. Pathologic conditions of the soft tissues such as tendon diseases are common causes of pain and disability impacting patients' function, activities of daily living and quality of life [3, 4]. Tendon diseases have especially been associated with overuse due to sports and work environment, and have become increasingly common along the growth in sports activity population and ageing society [3, 5]. Problems relating to the tendon comprise approximately 30 percent of all musculoskeletal complaints to a general practitioner [6], which the majority of the patients are treated by conventional methods of rest, physiotherapy, orthotics and supportive anti-inflammatory medications. However, only little evidence has shown the effectiveness of these treatments [7]. Hence, it is important to further our knowledge and understanding of tendon disease so that potential therapeutic targets could be identified for fundamental treatments.

## 1.2 The Tendon

A tendon is a fibrous, dense regular connective tissue that connects a muscle to the bone. It functions to transfer the force generated by muscle contraction efficiently to produce movement or to stabilise the skeleton aptly [8]. Tendons that take part in maintaining the posture are referred to as "positional tendons" and known to be inextensible such as the rotator cuff, hamstring and tibialis anterior tendons. "Energy-storing tendons" such as the patella and

Achilles tendons are characterised by the ability to stretch and recoil that enables storage and release of the energy for more efficient locomotion [9]. The morphology of a tendon could vary in shape and size, which is subject to their functional role. Flexor tendons such as the Achilles that are prone to uniaxial tensile loads are long and round, whereas the rotator cuff tendon that bears complex loading in different directions is short and flat [10]. The differences are defined by the extracellular matrix (ECM), which is composed of predominantly collagens.

### 1.2.1 Molecular Composition

Tendons are known to be hypo-cellular and vascular. The major component is the ECM, which is surrounded by water that takes up 65 percent of the total weight. This gives the tendon its gross white appearance [11]. The fibrous component that includes the abundant collagen and little elastin accounts for 30 percent. Ground substances such as proteoglycans and glycoproteins make up the remaining content [11]. The biomechanical properties of a tendon are defined by their structural organisation and composition of the ECM. These may vary even in the same tendon according to the local differences of applied forces, which is known as tendon anisotropy [12, 13].

#### 1.2.1.1 Collagen Fibres

Collagens are the most abundant type of protein in humans. Several different types of collagens have been defined, which their composition may vary within a tendon between mid-portion of a tendon and myotendinous or tendon-bone junctions. Tendons are composed of predominantly type I with few type III collagens. Type II collagen may be found in cartilaginous areas such as the tendon-bone junction, and types IV and V in the basement membrane. Types IX, X and XI collagens have also been identified in the tendon.

In the tendon, predominantly type I collagen fibres are aligned parallel to the mechanical axis in an organised hierarchical structure, which defines its mechanical property [14-16]. A collagen molecule, also referred to as tropocollagen, is a triple helix of 3 polypeptide chains that is composed of two alpha 1 chains and one alpha 2 chain with a length of 300 nm and 1.5 nm diameter. A tropocollagen is first synthesised as a polypeptide chain referred to as pre-pro-collagen at the ribosome, and then transferred to the rough endoplasmic reticulum for posttranslational modification to become pro-collagen. At the Golgi apparatus, pro-collagens are connected by hydrogen bonds to form the right-handed triple helix structure of a tropocollagen. Tropocollagens align themselves along the mechanical axis and then bundle together to form fibrils of 50 to 200 nm diameter, the basic unit for force transmission with a characteristic 67 nm banding pattern by forming inter-molecular crosslinks. The crosslinks give the tendon its mechanical strength and biological stability [17]. This process is referred to as fibrillogenesis [18]. During embryogenesis, fibrillogenesis takes part inside the cell at a protrusion of plasma membrane, namely fibripositor, and then the fibrils are deposited to the extracellular space subsequently. At the postnatal stage, fibrillogenesis ensues by accreting the pro-collagen to the extracellular collagen fibrils. Fibrils make up the fibre with a diameter ranging from 50 to 100 μm, which is the basic unit of the tendon and could be observed under light-microscopy. Fibres are bundled together as subfascicles and then to fascicles (diameter of 20 to 200 μm), both of which are surrounded by a thin reticular network of connective tissue, the endotenon. A fascicle is the smallest unit of the collagenous structure that the mechanical property could be examined [11]. The endotenon contains the neurovascular structure that contributes to the healing response of the tendon [19], and also functions as a flexible connection to the surrounding tissues and a gliding structure of the (sub)fascicles [20]. Multiple fascicles make up the tendon, which is surrounded by the epitenon, a fine connective tissue sheath [21]. Loose areolar connective tissue, namely paratenon, is at the tendon's most outside.

The paratenon is an important source of vascularization and the synovial cells at the inner surface provide lubrication of the tendon against the surrounding structures [22] (Figure 1-1).

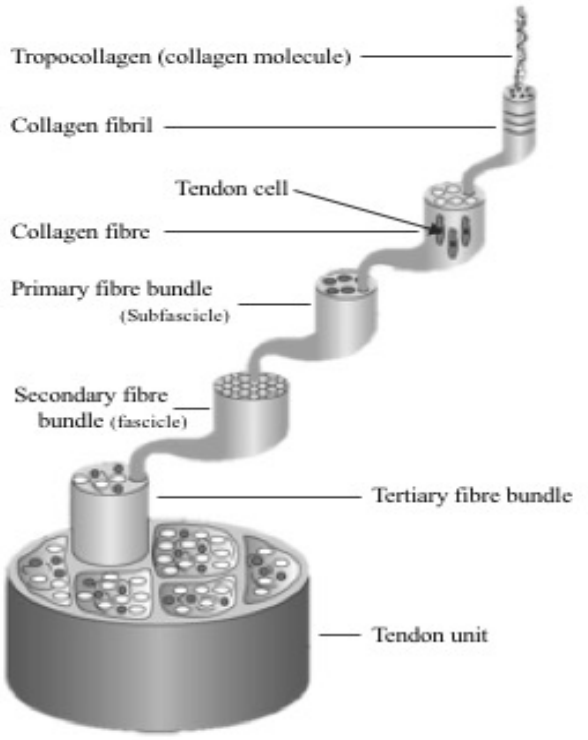



**FIGURE 1-1: Diagram showing the tendon hierarchical structure.** Left shoulder, posterior view. The diagram was adapted from Liu *et al*. (2008) [14].

### 1.2.1.2 Proteoglycans

Proteoglycans only make up 1 percent of the dry weight of a tendon, but play key roles in mediating the biomechanical property of a tendon by regulating ECM assembly and providing the ECM its high-water content [11]. Synthesis of proteoglycans start from the rough endoplasmic reticulum and is completed at the Golgi apparatus. Proteoglycans possess a central protein skeleton with multiple covalently linked glycosaminoglycan (GAG) chains such as chondroitin sulphate (CS), keratin sulphate (KS) and dermatan sulphate (DS). These GAG

chains define the function of the proteoglycan in fibrillogenesis, provide a site for the cytokines to bind to, and attract water with their negative charges [23]. Proteoglycans are categorised into 2 groups; small leucine-rich proteoglycans (SLRPs) or large aggregating proteoglycans, also referred to as lecticans.

SLRPs are characterised by the leucine-rich repeats on the small horseshoe-shaped core protein with a couple of CS or DS, or few KS attached. Their main function is to coordinate the whole process of matrix assembly, especially fibrillogenesis during turnover and development [14]. SLRPs such as decorin, lumican, biglycan and fibromodulin are known to bind to collagen fibrils and modulate lateral formation, spacing and the final size of fibres by interacting with cells, growth factors and ECM proteins such as fibronectin, so that the structure of the ECM could be optimised [14, 23]. During the course of fibrillogenesis, SLRPs stabilise and align the short and immature fibrils to grow linear and then to lateral while the crosslinks are being formed. They also bear and transmit the mechanical load between fibrils during this process. SLRPs have a role as a reservoir for cytokines, and regulate not just cell-matrix interaction but also the activity of cells such as apoptosis, proliferation, differentiation and migration [23].

Decorin is the most abundant proteoglycan in the mid-substance of a tendon that accounts for 80 percent [24]. Decorin limits the lateral growth of fibrils during fibrillogenesis, and lubricates the gliding of fibrils during movement [23]. Decorin interacts with cells via transforming growth factor beta (TGF-β) and epidermal growth factor (EGF) in regulating inflammation and healing. It is also the major SLRP in cell-matrix interaction [23]. Biglycan is associated with fibrillogenesis of type I collagen, and also has a binding site for TGF-β [23]. Fibromodulin and lumican take part in the thickening of the fibrils. Fibromodulin is involved at the early stages of fibrillogenesis, whereas lumican limits aberrant lateral growth at the later stages [23]. Lumican may compensate for fibromodulin, but not the other way around [23].

Lecticans such as aggrecan and versican are bound to hyaluronan, a large GAG that is not bound to a core protein via a link protein, and form a large multi-molecular aggregate [25]. The abundant hydrophilic GAG chains of CS and KS attract water molecules [14], which work as a buffer for compressive and tensile forces and hence are more prominent in the fibrocartilaginous enthesis [26]. They also lubricate the gliding of collagen fibres, and create an environment for nutrient and gas diffusion, cell migration and osmotic pressure [11].

The composition of proteoglycans varies according to the function of a tendon and also within a tendon, reflecting tendon anisotropy. Positional tendons such as rotator cuff tendons, tendons running along bony tuberosities and tendons at the bone insertion have a distinct expression of proteoglycans compared to that of the tensional tendons, especially mid-portion. These regions are composed of proteoglycans of a high molecular weight such as aggrecans, as they attract water and protect the tendons from compression forces [12, 26].

#### 1.2.1.3 Other Extracellular Matrix Proteins

Elastin is an elastic fibre that accounts for 1 to 2 percent of dry weight of a tendon and is arranged along the collagen fibres. Elastin gives the tendon its flexibility and prevents the tendon from excessive stretching [11]. It is also speculated to have a role in recoiling of collagen fibres [27]. Glycoproteins are composed of a small glycidic fraction attached to a large protein. Fibronectin interacts with integrins to mediate fibrillogenesis, and also plays roles in cell adhesion and migration with other glycoproteins such as undulin, thrombospondin, tenomodulin and tenascin-C [18, 23, 28, 29].

#### 1.2.1.4 Cells

The majority of the cells that compose the cellular component of the tendon are fibroblast type cells, also commonly referred to as tendon cells or tenocytes [22]. Tendon cells lie on or in between the collagen fibrils of the ECM, and are characterised by a spindle shape with an elongated nucleus and thin cytoplasm. They mainly take part in mechanoreception [30]. Other cells such as chondrocytes, synovial and vascular cells that include endothelial and smooth muscle cells could be found at the enthesis, tendon sheath and paratenon, and arteriole of the tendon that lie in the endotenon and epitenon, respectively. Although very few, the presence of epithelial cells in the basic membrane that surrounds the tendon has been reported as well [31].

Tendon cells synthesise ECM proteins such as collagens, proteoglycans and glycoproteins. A well-developed rough endoplasmic reticulum and Golgi apparatus could be noted by electron microscopy in a tendon cell. Tendon cells also play part in degradation of the ECM by phagocytosis/pinocytosis and production of lysosomal or cytoplasmic degradative enzymes such as matrix metalloproteinases (MMPs). It is known that tendon cells modulate the ECM according to the changes in mechanical and physiological environment not just by regulating the synthesis of matrix proteins but also by sensing the mechanical strains through the ECM and changes in cell shape. Cells adhere to and sense the changes through the ECM by receptors such as integrins, and translate the mechanical cues to intracellular signals in order to adapt to the environment by responding adequately [12].

There are no specific cell markers of tendon cells. Hence a combination of various genes and proteins are used as a reference of tendon cells or tenogenic differentiation. Scleraxis is a transcription factor playing an important role in tendon development and differentiation [32]. Scleraxis regulates the expression of a glycoprotein tenomodulin [33], which is another tendon marker necessary for tendon maturation and cell proliferation [34]. Tenascin-C was originally

discovered at the myotendinous junction [35], and is currently known to function as adaptors and modulators of interactions between cells and the matrix during development, remodelling and healing [36, 37]. Tendons of adult humans and mature horses have limited remodelling, slow tendon turnover and lack responses to mechanical stimuli unlike those of small animal models that adapt to loading [38, 39]. Hence, the expression of tenascin-C is generally low in uninjured adult human tendons and expressed in conditions with increased cell activity, proliferation, migration and differentiation such as inflammation and wound repair [36]. Decorin, the most abundant proteoglycan in the mid-substance of a tendon has also been used as a marker for tenogenic differentiation [24].

#### 1.2.1.5 Nerve and Blood Supplies

The nerves that innervate the tendon are mostly afferent [40]. They originate from the nearby cutaneous or deep nerve trunks, and the majority end at the tendon surface after branching from the plexus in the paratenon [41]. All 4 types of receptors are found in the tendon; type I Ruffini corpuscles, type II Vater Paccinian corpuscles, type III Golgi tendon organs and type IV free nerve endings, which sense stretch, movement, pressure and pain, respectively.

Healthy tendons are not highly vascular and could even be absent in some parts of the tendon [42]. The hypovascularity attributes to the low rate of metabolism, and the oxygen consumption rate of tendons have been reported to be 10- and 7.7-fold lower than that of liver and skeletal muscles, respectively [43]. Blood supply of the parenchymal part of the tendon branches from the vessels of the nearby perimysium and periosteum, forms a plexus at the paratenon or the synovial sheath, and enters the tendon by penetrating the epitenon and run along the endotenon forming vascular networks [11, 41].

## 1.3 Tendon Disease

Tendon disease is commonly described by the term "tendinopathy," which represents a clinical condition that is characterized by tendon swelling and pain resulting in impaired function [44]. However, it does not specify the aetiology of the disease.

Historically, this clinical entity was described as "tendinitis" owing to its clinical presentation, which implies that the pathobiology of the disease is inflammation. However, numerous randomised controlled clinical trials have shown no therapeutic effects of anti-inflammatory drugs such as steroids and non-steroidal anti-inflammatory drugs (NSAIDs) in patients with tendinopathy [7]. Tissue samples from these patients also suggested that the pathobiology is devoid of inflammation with no or little sign of infiltration of leukocytes [45]. The tissues showed features of degeneration; disorganised and thinned collagen fibres, increased proteoglycans and GAGs, fatty infiltration, chondral metaplasia, scattered vascular ingrowth, and hyperplasia of cells with changes in their morphology [41, 46-49]. With regards to these histological findings the term "tendinosis" replaced tendinitis, which indicates a failed healing process illustrated by scar tissue that disrupts and replaces the original structure [50]. It is now known that a typical inflammatory response occurs at the initial stage of tendinopathy, and although it may subside, inflammation may persist thereafter [51, 52].

Tendon disease may start as acute tendinopathy that is transient with features of acute inflammation, especially in the younger populations. This has been classified as stage I by Neer in rotator cuff tendon disease [53]. Chronic with persistent and/or repetitive inflammation in the tendon with disrupted tissue is referred to as chronic tendinopathy (Neer stage II), which eventually leads to a complete tear (Neer stage III). Nirschl *et al*. have reported that patients with tennis elbow follow similar pathologic stages from temporary inflammation (stage I) to chronic tendinopathy (stages II and III), and then tendon rupture (stage IV) [54]. The term "tendinopathy" has been used to encompass the whole spectrum of tendon disease as well as

the condition described by Neer stages I and II. To avoid confusion, the term “tendinopathy” was used to describe chronic tendinopathy (Neer stage II) unless otherwise stated, and “tendon disease” to describe the whole spectrum of tendon disease hereafter.

#### 1.3.1.1 Aetiology of Tendon Disease

Tendon disease could be caused by either extrinsic, intrinsic factors or a combination of both. The former includes excessive and/or repetitive load due to sports, trauma, overload due to environmental factors and training errors [55]. Even stresses within physiological limits may accumulate micro-trauma that leads to tendon damage [56]. Intrinsic factors include ageing, genetic factors and medication such as fluoroquinolone compromise the healing capacity of a tendon and increase the risk of tendinopathy when prone to an extrinsic factor. These extrinsic and intrinsic factors initiate and/or alter inflammation predisposing to tendon disease. Amongst the variety of different causes, ageing and overuse in sports are the most significant risks.

#### 1.3.1.2 Epidemiology of Tendon Disease

Patients with tendon disease have increased along the ageing society with an extended health-span and a growth in the number of senior recreational athletes [57]. The rotator cuff tendon is one of the most frequently affected tendons alongside the Achilles and patella tendons, all of which are frequently related to sports, overuse and ageing [58, 59]. Yamamoto *et al*. reported that more than 25 percent of the general population in the seventh decade have a torn rotator cuff, which is end-stage of tendon disease [60]. The prevalence dramatically increases along ageing to 50 percent for people who are in their 80’s. Other tendons such as posterior tibialis, gluteus, extensor and flexor carpi radialis, and tendons of the hand may get affected as well.

Impaired tendon function alters the kinematics of the adjacent joints and may predispose to diseases of the joints such as osteoarthritis [10].

#### 1.3.1.3 Rotator Cuff Tendon Disease

##### 1.3.1.3.1 The Shoulder Joint and Rotator Cuff

The shoulder joint is the most mobile joint in the body that allows freedom of upper limb movements for activities of daily living such as carrying objects and performing tasks that require the arm to be raised above the head.

The shoulder girdle consists of 3 bones which form the 3 articular joints of glenohumeral, acromioclavicular, sternoclavicular, and a functional scapulothoracic joint. The spheroidal humeral head and the glenoid fossa of the scapula compose the ball-and-socket synovial joint of the glenohumeral joint that permits the greatest arc of movement.

A group of 4 muscles; subscapularis, supraspinatus, infraspinatus, and teres minor, which are collectively referred to as the rotator cuff muscles, plays an important role in controlling stability of the shoulder glenohumeral joint (Figure 1-2). Rotator cuff muscles provide dynamic stabilising forces that compress the humeral head on to the glenoid fossa which keep the head centred on the glenoid during movement. The forces are essential to prevent the humeral head from translating excessively or dislocating during shoulder motion. Dysfunction of the rotator cuff due to either a single muscle or the whole group may result in clinical problems requiring treatment.

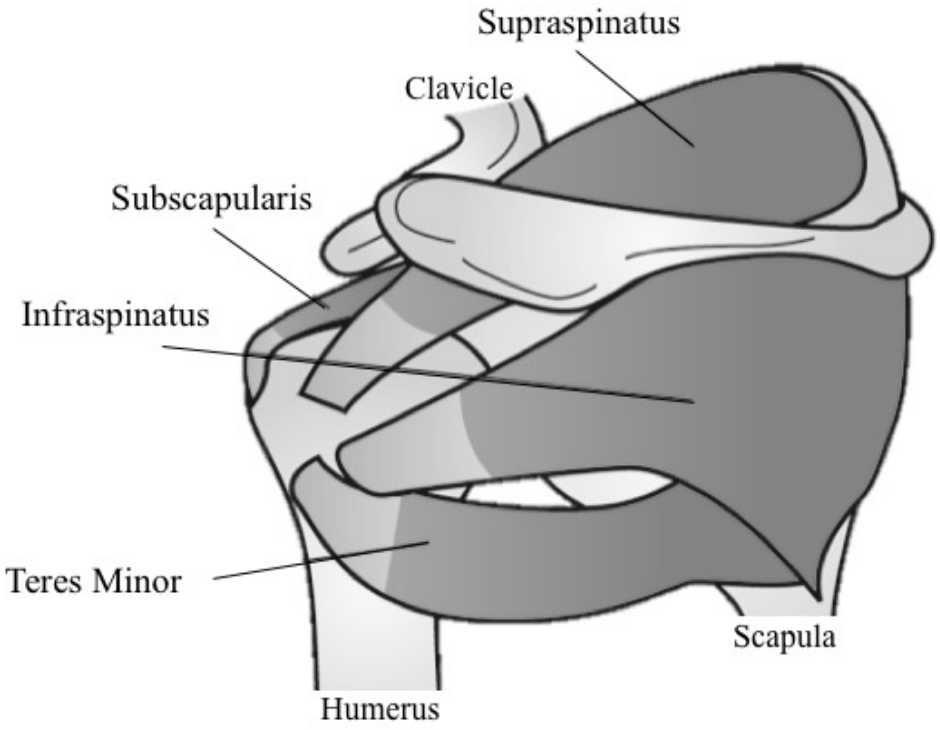



**FIGURE 1-2: Diagram showing the 4 composing muscles of the rotator cuff.** Left shoulder, posterior view. The diagram was adapted from the webpage of Matsuhisa Seikotsuin (https://matsuhisa-seikotsuin.com/2018/05/07/腱板損傷/)

#### 1.3.1.3.2 Overview, Clinical Presentation and Diagnosis

Rotator cuff tendon disease is the leading cause of shoulder pain, which is a common complaint in the general population with a prevalence of 16 to 34 percent [61, 62]. The aetiology is multifactorial as with other tendon disease, with ageing and overhead activities as notable risk factors [63, 64]. Supraspinatus tendon disease is the most common, but any component of the rotator cuff muscles could also be affected.

Patients with a rotator cuff tendon disease suffer from shoulder pain that could be accompanied by restricted function and weakness of the shoulder. History of the illness ranges from acute traumatic episodes to chronic gradual progression without an apparent onset. Pain

could occur at rest, sleep and during movement. The impaired function depends on the affected tendon and may be diagnosed through physical examination.

Physical examination of the shoulder may show pain and tenderness at the footprint of the rotator cuff at the greater tuberosity. Painful arc sign or positive impingement tests by Neer and Hawkins tests could be positive when the rotator cuff tendon impinges with the acromion. Manual muscle tests are performed to provoke pain and to evaluate the strength of the rotator cuff. Imaging studies are used auxiliary for diagnosis. Plain radiographs may show superior transition of the humeral head in patients with a torn tendon, but the change may be subtle in the early stages. Ultrasound and magnetic resonance imaging (MRI) are now the standard imaging modalities to assess the rotator cuff. Ultrasound imaging could be performed in the clinic, and it has the strength in the ability to assess the cuff dynamically. MRI enables a physician to assess the injured tendon from multiple views, image the whole shoulder joint and the surrounding tissues, and to evaluate the changes in the diseased tendon.

#### 1.3.1.3.3 Treatment of tendon disease

Standard treatments are predominantly supportive. A common treatment starts conservatively with physiotherapy and anti-inflammatory medications. Mechanical stimuli to the tendon by exercise and stretching promotes remodelling of the tendon by increasing collagen synthesis [11]. Exercises to improve ranges of motion and strengthen rotator cuff and scapular muscles have been shown to be effective even in patients with completely torn tendons [65]. Anti-inflammatory drugs such as steroids and NSAIDs could be beneficial for pain control at the early phase of tendinopathy [66]. However, long-term usage of steroids may predispose to tendon rupture [67, 68], and negative effects of NSAIDs on biomechanical properties in healing tendons have been reported [69]. Injection of hyaluronan, a component of lecticans have been practiced in several countries for pain management with its minimum adverse effects [70, 71].

Application of autologous blood or platelet rich plasma (PRP) was expected to augment tendon healing by supplementing cells and growth factors. However, Carr *et al.* reported that PRP does not improve clinical outcome in patients with chronic tendinopathy, but may have negative effects on tendon healing by reducing cellularity, vascularity and increasing apoptosis in tendon tissues [72].

Chronic tendinopathy is commonly treated surgically in refractory patients by removal of bone spurs and debridement of the affected soft tissues. However, a randomised placebo-controlled trial by Beard *et al*. reported no additional benefits of these surgical procedures [73]. Surgical repair for a torn tendon is indicated for younger patients especially with a sport-related or traumatic tear and patients with persistent symptoms. The principle of the surgery is to fix and restore the tendon-to-bone attach at the tendon footprint. However, the postoperative re-tear rate remains high at around 40 percent irrespective of the surgical method performed, and a failed repair results in negative patient outcomes [74]. This is related to the higher age of patients who go under surgery, and the relative indication for operation [75]. Torn tendons alter the mechanical loading of the shoulder joint, lose their biological and mechanical properties along ageing and over time after the injury [76].

#### 1.3.1.3.4 Histology of Rotator Cuff Tendon Disease

Histopathology of tendinopathic and torn tendon tissues is characterised by increased cellularity and disorganised ECM with changes in its composition. Cells become larger and their morphology changes from a spindle to a rounder shape in diseased tendons [46]. Collagens lose their matrix organisation and orientation with separation of the fibres. Type II and type III collagens are known to be increased as well as proteoglycans such as aggrecan, biglycan, decorin and GAG contents such as hyaluronan. Morphological changes such as calcification, chondral metaplasia, amyloid deposition and fatty infiltration have been detected in diseased

tendons, which could coexist [77-79]. Scattered and increased vascular growth is seen in tendinopathic and torn tendon tissues, but tendon tissues with larger tears may have reduced vascularity [41, 46-49, 80]. These features have been commonly referred to as fibrosis or degeneration

## 1.4 Fibrosis

Tissue fibrosis is a common pathological condition that results from chronic inflammation [81]. Cytokines and growth factors released by inflammation modulate cell phenotype, and in chronic inflammation where inflammation persists, the altered fibroblasts continuously produce excessive and inappropriate ECM proteins that disrupt its original structure [82, 83]. The synthesised fibrotic tissue is functionally inferior compared to the healthy tissue, and hence its accumulation results in an inappropriate function.

Fibrosis in other organs such as the kidney, liver, and lung have been investigated extensively [84]. Chronic kidney disease is a common disease characterised by fibrosis, which is caused by clinical and subclinical insults to the kidney such as infection, mechanical obstruction, autoimmune disease, hypertension and diabetes [85]. Chronic liver disease progresses from hepatitis, cirrhosis and finally to carcinogenesis. Alcoholism and sustained viral infection are known as its major aetiologies. Pulmonary fibrosis is caused by infection, drugs, foreign bodies or dysregulated autoimmunity imposing chronic inflammation. These insults trigger inflammation, activate the resident fibroblasts, and recruit leukocytes and fibroblasts by releasing inflammatory mediators and growth factors. Persistence of these triggers that does not resolve but promotes inflammation and subsequent deposition of inadequate ECM proteins results in progressive fibrosis [86].

In the tendon, continuous and/or excessive loading beyond the capacity of its mechanical property or turnover, namely overload may damage its ECM and initiate an inflammatory response for healing. The repetitive loading may become relatively excessive for a tendon due to a decline in cell activities and function by intrinsic factors such as ageing, driving an inflammatory response similarly [3]. Inadequate repair and removal of the damaged tissues prolong inflammation, which promote ECM synthesis lacking the organised structure. The biological and mechanical properties of the tendon never recover to the innate level, and becomes more prone to rupture [41].

### 1.4.1 Mechanism of Fibrosis in the Tendon

Resident tendon cells sense overload or damage to the ECM and initiate a local inflammatory response by releasing cytokines and growth factors. [87, 88]. Growth factors that are embedded in the ECM are released as well [89]. Cytokines and growth factors promote cell migration and proliferation, and the recruited fibroblasts along leukocytes and platelets secrete cytokines and growth factors likewise [90]. Especially, macrophages are known to play the main role in orchestrating the whole process [52, 91]. Resident and recruited fibroblasts adapt to the inflammatory milieu in response to these cues and change their morphology and phenotype to promote proliferation, migration, and synthesis of ECM proteins [89]. These metabolically active fibroblasts are referred to as myofibroblasts (or tenoblasts in correspondence to tenocytes) [30].

TGF-β plays the key role in regulating the synthesis of ECM proteins in fibroblasts. Initially, myofibroblasts synthesise predominantly type III collagens with small fibrils and no crosslinks. These collagens are typically laid randomly. Recruited fibroblasts are more proliferative and synthesise more immature collagens compared to resident tendon cells [92,

93]. The number and metabolic activities of recruited cells especially fibroblasts normally regress with time. Hence, synthesis of collagens such as type I collagen increases, and these mature collagens are aligned according to the tensile force as the previously laid disorganised ECM is degraded by MMPs [11, 41]. This process is known as remodelling. However, tendons often fail to remodel due to intrinsic factors such as ageing, resulting in an excessive deposition of poorly ordered matrix [48, 90]. The incomplete repair of the ECM prolongs the aforementioned responses without transiting to remodelling that results in chronic inflammation, and drives fibrosis and organ destruction [94].

#### 1.4.1.1 Cytokines Involved in Tendon Fibrosis

Various inflammatory cytokines are implicated in the pathobiology of tendon disease [95]. A systematic review of the literature to define the key cytokines responsible for tendon disease showed that interleukin (IL)-1 beta (IL-1β), IL-6, IL-10 and tumour necrosis factor alpha (TNF-α) are the most frequently investigated cytokines in relation to tendon disease or injury healing [96]. However, majority of the studies had implicated the roles of these cytokines in tendon disease or healing based on previous literature. Only a limited number of studies used hypothesis-free methods. Studies using tissue samples collected from patients with rotator cuff tendon disease have shown distinct inflammatory signatures at different stages of tendon disease; tissues of early stage disease (chronic rotator cuff tendinopathy) is characterized by expression of IL-1β, TNF-α and interferon gamma (IFN-γ) target genes and proteins; and tissues of end-stage disease (large to massive rotator cuff tear) show signs of STAT-6 signalling pathway activation that is triggered by IL-4 and IL-13 [52]. Hence, cytokines IL-1β, IL-6, IL-10, TNF-α, IL-4 and IL-13 and their implicated roles in tendon pathobiology are briefly described below.

**IL-1β**

IL-1β is a pro-inflammatory cytokine that activates mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK) 1/2, p38 and stress-activated protein kinase/c-jun NH2-terminal kinase (SAPK/JNK) for intracellular signalling [97]. IL-1β also utilises the nuclear factor-kappa B (NF-κB) signalling pathway [97, 98].

Protein and messenger ribonucleic acid (mRNA) expression of IL-1β in clinical samples of torn rotator cuff tendons was inconsistent between the 2 currently available studies [99, 100]. IL-1β protein expression was unchanged in Achilles tendon tissues 2 weeks after a repair surgery [91] and in tissues from tendinopathic tissues [101]. The role of IL-1β in human tendon disease development is yet to be determined. Meanwhile, it has been shown by numerous studies that IL-1β mRNA and protein expression tend to increase at the early stages of tendon injury or in response to exercises in animal models [96].

IL-1β secreted in response to injury or mechanical loading changes the cytoskeleton polymerization and morphology of the resident tendon cells so that they may adjust to the altered biomechanical property and behaviour of the damaged tendon [102]. IL-1β promotes inflammation, ECM degradation [103] and inhibits ECM synthesis by tendon cells [104]. IL-1β stimulates the expression of inflammatory mediators such as cyclooxygenase (COX) 2 and its downstream product prostaglandin E2 (PGE2) [105], which induce apoptosis of resident tendon cells and the expression of ECM degrading enzymes such as MMPs (MMP1,3 and 13) [103, 106]. IL-1β suppresses the synthesis of type I collagens [107, 108], but promotes the release of inflammatory cytokines such as IL-6 and growth factors such as TGF-β [106, 109, 110] so that scar tissues could be formed to replace the damaged tissues [103, 105, 106, 111].

These interactions conduct proliferation of tendon cells [105, 112] and subsequent differentiation towards myofibroblasts [113].

**IL-6**

IL-6 is belongs to the T-helper (Th) 2 cytokine group with IL-4, IL-10 and IL-13 [88] and possess multifunctional roles as an immunoregulatory cytokine. It signals through the signal transducer and activator of transcription (STAT) 3 and also MAPK pathways [114, 115]. IL-6 is released from leukocytes (lymphocytes) and tendon cells in response to cytokines such as IL-1β and TNF-α [103, 106, 116]. It may be both pro- and anti-inflammatory [117, 118] playing a key role in mediating inflammation, tendon disease and healing [117, 119]. IL-6 has been implied to be involved in human tendon disease and homeostasis with studies showing increased mRNA and protein expression in torn tendons [96, 120] and increased protein expression after prolonged running in healthy Achilles tendons [121, 122]. IL-6 knockout mice showed that IL-6 deficiency results in an inferior mechanical property and repair of a tendon[123, 124].

IL-6 is induced from 2 hours after an injury in animal models to promote the healing process [125], and is sustained up to 4 weeks unlike the short-lasting pro-inflammatory cytokines of IL-1β and TNF-α [96]. IL-6 promotes inflammation in the early stages by further producing pro-inflammatory cytokines, promoting cell infiltration and activity of immunoregulatory macrophages [117], and guides subsequent release of TGF-β1 [126]. Continuous expression of IL-6 shifts its function towards anti-inflammatory and regenerative roles in the later stages [91]. IL-6 diminishes the activities of pro-inflammatory cytokines by suppressing their expression, the expression of their receptors, producing receptor antagonists

and the anti-inflammatory cytokine IL-10 [103, 127-129]. The activities of IL-6 is regulated by IL-6 itself by inducing the inhibitors of its STAT3 cell signalling pathway [103].

IL-6 promotes proliferation and migration of tendon cells [88]. It has also been suggested to take part in collagen synthesis [130]. However, an *in vitro* experiment treating rat tendon stromal cells (TSCs) from healthy Achilles tendons with IL-6 showed that IL-6 suppresses type I and III collagen mRNA and protein expression [131]. IL-6 treatment did not have any effects on human TSCs on type I collagen expression either [103]. The effects of IL-6 on tendon cells in relation to ECM synthesis remains unclear.

**IL-10**

IL-10 is the most typical and effective anti-inflammatory cytokine which belongs to the Th2 cytokine group [88]. It signals through the STAT-3 pathway. IL-10 acts on immunoregulatory monocytes and macrophages to inhibit the production of pro-inflammatory cytokines such as IL-1β and TNF-α, as well as other regulatory cytokines such as IL-6 [132-135]. It also promotes the secretion of pro-inflammatory cytokine antagonists [132]. With these anti-inflammatory effects, IL-10 has been regarded to be expressed in the later phase of tendon healing, following the expression of pro-inflammatory cytokines upon injury. Ackermann *et al*. showed that protein expression of IL-10 is increased in the human Achilles tendon 2 weeks after a rupture repair surgery, when the inflammatory phase was on the course of resolving with subsided expression of pro-inflammatory cytokines of IL-1β and TNF-α [91]. They inferred that IL-10 plays a role in the regenerative or remodelling process of tendon healing as it suppresses inflammation [91]. However, *IL10* mRNA expression could be increased in ruptured supraspinatus and Achilles tendons in humans [99, 120], and also in rat Achilles tendons immediately after a collagenase-induced injury [136] and 4 weeks post-op of a repair surgery

after transection [137]. Exercise or loading of the tendon did not induce IL-10 expression in both humans [138] and rats [96]. Moreover, expression of mature collagens (type I collagen) are known to increase along tendon remodelling [88, 135], but IL-10 treatment on TSCs from healthy tendons *in vitro* did not have any effects on type I collagen protein expression [103]. Nevertheless, IL-10 limits excessive immune reactions, and the induced expression of IL-10 by pro-inflammatory cytokines are regarded to act as a self-limiting mechanism of inflammation [88].

**TNF-α**

TNF-α is a pro-inflammatory cytokine that signals through the MAPK and NF-κB pathways similar to IL-1β [139]. Hosaka *et al*. suggested TNF-α as the key cytokine in the pathobiology of tendon disease through studies using equine tendon cells and tendinopathic tissues [140, 141]. However, mRNA and protein expression of TNF-α tended not to change in tendon tissues sampled from patients with rotator cuff and Achilles tendinopathy and tear [96]. Tendon injury induced *TNF* mRNA in rats and dogs from 2 hours to 9 days post-op, whilst protein expression increased after 4 days after the intervention [96].

TNF-α modulates cell morphology, promotes ECM degradation by increasing the production of matrix degrading enzymes such as MMPs and suppresses the synthesis of type I collagens in human TSCs [103]. TNF-α does not function solely to induce apoptosis but in concert with environmental factors and co-stimuli such as other cytokines [88], possibly to limit ECM synthesis especially in the early stage of tendon injury or healing [142]. TNF-α recruits inflammatory cells locally [140] and stimulates the secretion of other cytokines such as IL-1β, IL-6, IL-8 and IL-10 as well as itself as an auto-amplification loop [88, 103].

TNF-α and IL-1β are both pro-inflammatory cytokines with similar effects on tendon cells, but Manning *et al*. reported that the up-regulation *IL1B* mRNA is significantly marked compared to that of *TNF* mRNA in response to tendon injury in a canine flexor tendon model [143]. Phan *et al*. using rat pulmonary artery endothelial cells showed that TNF-α and IL-1β treatments *in vitro* both induce TGF-β1 activity and *TGFB1* mRNA expression, which the magnitude of the effects is greater in the treatment by the latter [144]. Likewise, Kelly *et al*. summarised that IL-1β is more inductive of TGF-β, myofibroblastic differentiation of resident stromal cells and fibrosis compared to TNF-α in rodent lungs after a transgene transfer using adenovirus vectors [145].

**IL-4 and IL-13**

IL-4 and IL-13 are Th2 cytokines that are structurally and functionally related, sharing sequence homology and receptor subunits [146]. They both signal through the STAT-6 pathway primarily [146]. IL-4 and IL-13 have been suggested to take an important role in the initiation and maintenance of fibrosis in studies of fibrotic diseases such as pulmonary fibrosis and scleroderma [147-149]. Chronic inflammation promotes ECM synthesis, especially type I, III collagens and fibronectin from fibroblasts [150, 151] by activating TGF-β1 [149, 152, 153]. IL-13 rather than IL-4 appeared to be the dominant effector cytokine in these diseases [147, 154], but IL-4 has been investigated more extensively in studies on tendon disease. The effect of IL-4 on driving macrophages to a phenotype (M2) reducing pro-inflammatory cytokines and inflammatory responses has been focused with regards to improving tendon healing [155, 156]. Nonetheless, how IL-4 takes part in tendon disease and whether its pathobiology is similar to that of other fibrotic diseases is not fully elucidated. Courneya *et al*. reported that IL-4 promotes cell proliferation but has no effects on gene and protein expression of collagens [157].

#### 1.4.1.2 Cells Involved in Tendon Fibrosis

Number of cells increases in the damaged tendon in response to cytokines and growth factors through proliferation of intrinsic resident tendon cells, and migration and proliferation of extrinsic cells from surrounding tissues such as the synovium. Fibrocytes, which are circulating cells of mesenchymal origin are also recruited and activated by cytokines and growth factors to become fibroblasts [158]. Epithelial-to-mesenchymal transition (EMT) is a mechanism to form fibroblasts from epithelial cells to recruit cells in association with inflammation [137]. The recruited fibroblasts become more metabolically active as myofibroblasts and change their morphology to an ovoid shape with larger nuclei accordingly [46, 159]. TGF-β and its closely associated growth factors such as bone morphogenetic proteins (BMPs) and connective tissue growth factor (CTGF) are the key mediators of these processes [160].

#### 1.4.1.3 Intrinsic Factors Involved in Tendon Fibrosis

Various factors such as ageing, genetic factors and medication contribute to the intrinsic changes in the tendon. The decline in the functional competence of tendon cells due to these factors predisposes the tendon to undergo changes in the ECM and influence its biomechanical behaviour.

Number of tendon cells decreases due to the decline in the number and proliferation of differentiating cells [27, 161, 162]. Organelles such as the rough endoplasmic reticulum and Golgi apparatus regress reflecting the decline in the cell activity and function [162, 163]. Synthesis of ECM and enzymatic proteins decrease, as well as the ability to respond to intrinsic and extrinsic stresses and loads [161, 162, 164]. Cell responsiveness to cytokines and growth factors reduce with impaired ability to resolve inflammation [165]. These changes result in a

slower and less effective turnover and healing, which make the tendon more susceptible to injury and diseases [161, 162].

Vascular flow declines amongst the decrease in capillaries [161]. Reduced blood flow leads to hypoxia and malnutrition, which changes the metabolic pathway for energy production from aerobic to anaerobic [3]. This is also detrimental for tendon turnover or healing [162].

### 1.4.2 Mediators of Fibrosis

Studies of fibrotic diseases have implicated TGF-β and its closely associated growth factors such as BMPs and CTGF as key mediators of fibrosis orchestrating various cellular processes [166, 167]. TGF-β and BMPs belong to the TGF-β superfamily along inhibins and activins. The ligands bind to serine/threonine kinase receptors, phosphorylate and activate its downstream mediators, the small mothers against decapentaplegics (SMADs). The receptors phosphorylate the regulatory-SMADs (R-SMADs); TGF-β phosphorylates SMADs 2 and 3; BMPs phosphorylate SMADs 1, 5 and 8. Then the R-SMADs bind to a co-regulatory SMAD (SMAD4) and translocate into the nucleus where they act as transcription factors [168]. SMADs 6 and 7 are inhibitory SMADs (I-SMADs) that suppress the activity of R-SMADs. CTGF belongs to the CTGF, cysteine rich protein (Cyr61) and nephroblastoma overexpressed gene (Nov) family. However, it is known as one of the key profibrotic mediators acting closely with TGF-β as its downstream mediator. Yet, the key fibrotic pathways in tendon disease are understudied.

#### 1.4.2.1 TGF-β

TGF-β is found to be in 3 isoforms (TGF-β1, TGF-β2 and TGF-β3) in the human. They all signal through the same receptors TGF-β receptor (TGF-β R) 1 and TGF-β R2 [167]. All 3 isoforms take part in regulating the ECM of the tendon [169, 170], of which TGF-β1 and TGF-β2 have been related to fibrosis. TGF-β3 is expressed at high levels in foetal tissues during embryogenesis [171-173], and also has been implicated to play a major role in scar-less foetal wound healing that heals [174, 175]. Multiple studies have attempted to dampen scar formation and improve tendon healing by inhibiting TGF-β1, TGF-β2 and/or delivering TGF-β3 to the repair site [176-178]. To date, the majority of research on tendon disease and healing have focused on TGF-β1 as a key mediator of fibrosis as it is the most common isoform expressed in adult musculoskeletal tissues. Hence, this thesis will focus on TGF-β1 as one of the key mediators of fibrosis in tendon disease.

TGF-β is released from resident and migrating cells in response to overload and injuries [179]. TGF-β that is bound to the ECM is also released in response to these changes as well as mechanical loads [180, 181]. TGF-β acts on these cells through autocrine and paracrine mechanisms, which triggers various biological processes that results in fibrosis [182]. It promotes differentiation, proliferation of resident tendon cells, recruitment of fibroblasts and inflammatory cells [166]. EMT is one of the conspicuous effects of TGF-β as previously described [183]. TGF-β plays the main role in inducing the often excessive ECM synthesis and deposition [184]. In the tendon, TGF-β induces the synthesis of collagens [185, 186] and also proteoglycans [179]. The composition of ECM, proteoglycans in particular is adjusted in response to changes in the sustained mechanical force by TGF-β regulation [187, 188]. TGF-β induces tissue inhibitor of metalloproteinases (TIMPs) that inhibit the actions of ECM

degrading enzymes MMPs, which also contribute to the excessive deposition of the ECM [166]. TGF-β also has apoptotic, angiogenic and anti-inflammatory effects [182, 189-192].

A systematic review showed that TGF-β expression is induced along the course of healing in animal models of tendon injury [193], and could be induced as well in a rodent flexor digitorum tendon after long-term overloading [194]. In tendon tissues of tendinopathic patients (rotator cuff, Achilles and patella), its mRNA expression was increased [138, 195, 196], but protein expression varied from decreased [197], increased [198] and unchanged [199] compared to that of healthy patients. Only 1 study has investigated TGF-β expression in torn tendon tissues in human, which showed unchanged and decreased changes in mRNA and protein expression, respectively in torn supraspinatus compared to healthy tendons [197].

Studies of fibrosis in other organs have attempted to dampen fibrotic development by suppressing TGF-β expression or inhibiting its effects based on many studies showing increased expression and activity related to fibrotic development. For example, in chronic kidney disease attempts to inhibit TGF-β expression, neutralise TGF-β or its receptors using antibodies and small molecule inhibitors, and block its activation have been attempted in animal models showing improvement of kidney structure and function [200]. However, these treatments have not been applied to clinics as suppressing TGF-β expression and activity may also diminish its anti-inflammatory effects inciting massive inflammation and adverse consequences. In the tendon, local administration of TGF-β inhibitors have been attempted to improve the fibrotic healing after tendon injury repair (e.g. chitosan [201], metformin [202], TGF-β R1 inhibitor [203], mannose-6-phosphate [204], N-acetyl-L-cysteine [205], etc.), but these treatments have not been applied to clinical practice either with animal studies showing their detrimental effects such as reduced mechanical property [206, 207].

#### 1.4.2.2 BMPs

BMPs possess multifunctional roles alike TGF-β from cell apoptosis, proliferation and differentiation, and also have induces ECM synthesis as well [208, 209]. BMP-2 and BMP-7 are the most studies BMPs in relation to human tendon disease [193], and recent animal studies have focused on the tenogenic effect of BMP-12 to improve tendon healing [210]. We have briefly described BMP-2, BMP-7 and BMP-12 and their implicated roles in tendon pathobiology below.

**BMP-2**

BMP-2 has been studied extensively in research on delayed union and non-union fractures as the main mediator of osteogenic differentiation [211]. BMP-2 injection to the tendon induced ectopic bone formation in a rabbit model [212], and it has been focused as an effective growth factor to improve tendon-to-bone integration by reproducing the fibrocartilaginous structure at the tendon-to-bone junction of a tendon graft in anterior cruciate ligament (ACL) reconstruction surgery and surgical repair of torn rotator cuff tendons. BMP-2 addition has been shown to improves biomechanical and histological properties of tendon healing in a rat supraspinatus tendon injury model [213], but the ideal method of BMP-2 delivery, dose and timing has not been established to date even in animal models.

The osteogenic effects of BMP-2 has been shown in a mesenchymal stem cell line [214], equine bone marrow-derived mesenchymal stem cells (MSCs) [215], rat, rabbit, dog, horse and human TSCs derived from healthy tendons [215-220]. BMP-2 has also been shown to induce chondrogenic differentiation [221, 222], but whether it induces adipogenic differentiation in tendon cells remains controversial [222, 223]. Studies using rodent and murine TSCs implied that BMP-2 suppresses tenogenic differentiation as *Tnmd* mRNA expression decreased and *Scx*

mRNA expression only slightly increased in response to BMP-2 treatment *in vitro* [222, 224]. Meanwhile, BMP-2 does not have any effects on cell proliferation [218, 225, 226] or cell morphology [215]. BMP-2 may have slight inductive [215, 225-227] or no effects [218] on collagen synthesis in TSCs.

BMP-2 mRNA and protein expression tend to be induced in response to tendon injury in rat models regardless of the tendon anatomy or injury method [193], but the only study using a murine tendon injury model showed that *Bmp2* mRNA expression is suppressed at 1, 2 and 4 weeks after Achilles tendon transection [228]. Strenuous treadmill running in rats induces *Bmp2* mRNA [229], as well as repetitive tensile loading of rat tendon cells *in vitro* [217]. This is similar to the results of the study by Sumanasinghe *et al*. applying uniaxial cyclic tensile strain to mesenchymal stem cells [230].

Oliva *et al*. reported that *BMP2* mRNA expression is unchanged in tendon tissues from patients with supraspinatus tendinopathy [196] whilst Rui *et al*. found that BMP-2 protein is up-regulated in tissues from patella tendinopathy patients [231]. The study by Goodier *et al*. is the only study that investigated the expression of BMP-2 in torn tendons, which showed unchanged *BMP2* mRNA expression in tendon tissues from supraspinatus tear patients [197].

**BMP-7**

BMP-7 has been focused especially in research on kidney fibrosis, with its inhibitory effects on the actions of TGF-β [184]. Yang *et al*. reported that BMP-7 with BMP-2 suppress renal interstitial fibrosis by regulating EMT [232]. However, whether BMP-7 acts positively or negatively on the tendon has not yet been determined.

BMP-7 is known to promote cell proliferation, differentiation and ECM synthesis in tendon cells [233]. Its mRNA and protein expression are increased after tendon injury in rodents

[228, 234, 235]. Only 1 study each has investigated its expression in human tendinopathic or torn tendons; Rui *et al*. showed that BMP-7 protein is increased in tendinopathic patella tendons [231], while Goodier *et al*. showed that *BMP7* mRNA is unchanged in torn supraspinatus tendons [197]. There are no animal tendon overuse models that have looked into the mRNA expression of *BMP7*, but BMP-7 protein expression has been shown to be induced by mechanical stimuli in fibroblasts from other tissues [236].

BMP-7 treatment *in vitro* induces cell proliferation, mRNA and protein expression of collagens, especially types I and III, and proteoglycans in human, rodent and bovine tendon cells [225-227, 237-240]. Numerous studies have reported the chondrogenic and tenogenic effects of BMP-7 on tendon cells, but whether it induces osteogenesis is still debatable. Klatte-Schulz *et al*. reported that BMP-7 treatment *in vitro* induces *SOX9* and *SCX* mRNA expression, which are chondrogenic and tenogenic markers, respectively but not osteogenic markers compared to vehicle control in human tendon cells derived from torn supraspinatus tendons [241]. Meanwhile, Forslund *et al*. showed inductive effects of BMP-7 on bone formation in rat tendon cells [242], and Schwarting *et al*. showed similar results of BMP-7 inducing osteocalcin expression in bovine tendon cells [243]. These results support the improved tendon-to-bone integration with BMP-7 application in anterior cruciate ligament reconstruction surgery in mice [244].

**BMP-12**

BMP-12 has gained interest in research related to tendon healing and disease within the last few years with its effect on inducing tenogenic differentiation. The focus started from the study by Wolfman *et al*. showing formation of tendon-like tissues after subcutaneous injection of BMP-12 in rats [245]. Since then, numerous studies have shown that BMP-12 treatment on MSCs induces tenogenic differentiation monitored by cell morphology, expression of tenogenic

markers such as scleraxis, tenomodulin, mohawk, decorin, and type I but not type III collagens [246-254]. Few groups have reported that BMP-12 treatment does not induce osteogenic differentiation [215, 247], but Shen *et al*. indicated a certain level of its osteogenic and chondrogenic differentiation potentials [255]. MSCs were obtained from a wide range of species from monkeys, horses, dogs, rats to humans. Additionally, MSCs were derived from various tissues such as the bone marrow, synovial membrane, adipose tissue, subacromial bursa and umbilical cord blood, which cell proliferation and tenogenic differentiation capacities may differ according to their origins [256]. BMP-12 treatment on human and equine tenon cells from the patella and superficial digital flexor (SDF), respectively has been shown to induce cell proliferation, synthesis of type I collagens but not osteogenic effects similarly [30, 215, 257].

BMP-12 is expressed in human healthy patella tendon tissues, suggesting its role in homeostasis and remodelling [30]. However, its mRNA nor protein expression has been investigated in tissues of patients with tendinopathic or torn tendons to date. In rodent tendon injury models, induced BMP-12 protein expression has been reported in an Achille tendon transection model [210] and a supraspinatus tendon detach and repair model [258]. Meanwhile, loading or unloading of the affected limb after Achilles tendon transection did not have substantial effects in *BMP12* mRNA expression [259].

In animal tendon injury models, numerous studies have attempted to improve tendon healing by adding BMP-12 alone or in combination with MSCs to the injury site. BMP-12 treatment alone improved mechanical properties, organisation of fibres and the size of the tendon callus regardless of the species or the anatomy [257, 260, 261]. However, the effects of BMP-12 treatment on adhesion, angiogenesis, inflammation, collagen organisation, cellular profile and proliferation may differ by delivery methods such as embedded sutures, collagen sponges and injections [262]. Application of MSCs pre-treated with BMP-12 augment tendon healing by promoting proliferation of tenogenic cells, ECM deposition and expression of

tenogenic genes [156, 249], but Gelberman *et al*. have indicated that these treatments may increase and prolong inflammation depending on the delivery method [263].

#### 1.4.2.3 CTGF

CTGF modulates the ECM as a downstream regulator of TGF-β [258]. It is normally induced by TGF-β from mainly fibroblasts when a local threshold level of active TGF-β is met [264, 265]. CTGF expression has been shown co-ordinately with TGF-β expression in tissues of injury healing and fibrosis, but may follow 2 to 14 days after that of TGF-β as well [264, 266]. Grotendorst *et al*. showed that administration of TGF-β above the threshold level induces *CTGF* mRNA expression immediately within 30 minutes *in vitro* in human skin fibroblasts [267].

CTGF possesses similar functions as TGF-β, and regulates TGF-β-induced effects on the ECM such as promoting cell recruitment, differentiation and proliferation, and regulating apoptosis, ECM synthesis and angiogenesis [264, 268]. Meanwhile, the effects of CTGF such as inducing collagen synthesis is regulated by TGF-β, whilst TGF-β also carries out those effects directly on cells [269-271]. Another difference between CTGF and TGF-β is that CTGF may act only on fibroblasts whereas TGF-β has effects on epithelial and epidermal cells as well [272]. CTGF is required for most of the profibrotic activities induced by TGF-β [273], which selectively mediates the effects of TGF-β by binding to its receptors and enhancing cell signalling activity of the canonical SMAD pathway [274, 275], and inducing TGF-β expression through a positive feedback loop [276]. These synergised interactions of CTGF and TGF-β promote sustained fibrotic responses. Qi *et al*. showed that CTGF treatment and inhibition suppress *SMAD7* and *SMAD2* mRNA expression, respectively in human kidney fibroblasts (proximal tubular cells) [277]. However, in a CTGF knockout mouse model, CTGF deletion

did not have substantial effects on cell signalling activities of the canonical SMAD pathway, which indicate that CTGF is not indispensable [278]. CTGF does not compensate for TGF-β, but works as its downstream mediator [279].

CTGF has been reported to be overexpressed in fibrotic disorders [265], and its expression level has been used as an effective biomarker of disease progression and severity in diseases such as kidney fibrosis and scleroderma [265, 280]. In chronic liver disease, increased CTGF expression positively correlated to increased TGF-β expression likewise [182].

In the tendon, CTGF is expressed in healthy tissues, suggesting a role in maintaining its basic biological properties, i.e. homeostasis [281]. CTGF expression is induced in response to mechanical loading following that of TGF-β [167, 268, 282] and work synergistically to enhance the effects of TGF-β as its downstream mediator [283]. In rodent models overloading of a tendon induces CTGF protein expression that correlates to fibrotic changes [95, 110, 194]. However, unloading of a tendon does not necessarily have opposite effects suppressing *CTGF* mRNA expression [284]. mRNA expression of *CTGF* is increased, decreased or unchanged in the course of tendon healing depending on the timing after the injury or animal species in rabbit and chicken models [281, 285]. Only mRNA expression has been studied in tissues of tendinopathic tendons that was from Achilles tendinopathy patients, with 1 study showing decreased [138] and the other unchanged [195] *CTGF* mRNA expression. Only 1 study has investigated CTGF expression in torn tendon tissues in human, which showed unchanged *CTGF* mRNA expression in torn supraspinatus compared to healthy tendons [197].

## 1.5 Aim and Objectives

The aim of this thesis was to investigate fibrotic pathways active in human rotator cuff tendon disease with a particular focus on TGF-β and BMPs as key fibrotic mediators.

Studies of fibrotic diseases have implicated TGF-β and its closely associated growth factors BMPs and CTGF as key mediators of fibrosis. Their expression has been shown to be temporally altered in animal tendon injury or overuse models, but the transition along human tendon disease development is currently unknown. The first objective was to define the fibrotic signature, i.e. mRNA and protein expression of TGF-β1, BMPs and CTGF in diseased tendon tissues collected from patients with tendinopathic and torn supraspinatus tendons in comparison to healthy tendon tissues. It was hypothesised that the mRNA and protein expression of TGF-β1, BMPs and CTGF would increase along disease progression from healthy to tendinopathy, and tear. This has been addressed in Chapter 3.

Emerging evidence supports the roles of cytokines in the onset and progression of tendon disease; tissue samples collected from patients with tendon disease have been shown to have an abnormal tissue inflammation signature and the expression of fibrotic mediators are regulated by the inflammatory milieu. The second objective was to investigate how inflammatory cytokines regulate the expression of TGF-β, BMPs and CTGF in human TSCs with particular focus on the difference between the cells derived from healthy and diseased tendons in an *in vitro* cell culture model. The hypothesis was that cytokine treatment of TSCs would regulate expression of TGF-β1, BMPs and CTGF. It was also hypothesised that the extent and direction of this regulation is dependent on the disease status of the tendon from which the cells are derived from. This has been addressed in Chapter 4.

The effects of TGF-β and BMPs on TSCs have been investigated in numerous *in vitro* studies using TSCs derived from healthy tendons of various species to discover their role in tendon disease pathobiology. Although TSCs are known to alter their morphology and phenotype under chronic inflammation, it remains unknown how TGF-β and BMPs regulate the expression of genes related to the ECM and cell differentiation in TSCs from diseased

compared to healthy human tendons. The third objective was to investigate if TGF-β and BMP treatments of TSCs from healthy and diseased tendons differentially regulate ECM and cell differentiation genes in an *in vitro* cell culture model. It was hypothesised that the treatment of TSCs by TGF-β or BMPs would regulate mRNA expression of genes related to the ECM and cell differentiation, and there would be differential expression between TSCs from healthy and diseased tendons. This has been addressed in Chapter 5.

Defining the role of inflammation and the effects of fibrotic mediators in the development of human tendon disease is essential in understanding its pathobiology and the management of tendon disease. The results of this study should help facilitate the identification of potential therapeutic targets or strategies to dampen fibrosis progression in tendon disease or enhance the quality of tissue repair.

# Chapter 2 Materials and Methods

## 2.1 Collection of Human Tendon Tissues from Patient Cohorts

All the studies in this project were controlled laboratory studies. Clinical tendon tissue samples for protein, mRNA expression analyses and cell extraction for *in vitro* cell culture experiments were obtained from the Oxford Musculoskeletal Biobank (OMB) or by utilising the resources from multiple clinical trials (supraspinatus serial biopsy study (SBS), Why Are Shoulders Painful (WASP), United Kingdom Rotator Cuff Trial (UKUFF) and Scaffold Evaluation for rotator Cuff Repair (SECuRe)).

All the studies for this research project were carried out in concordance with the United Kingdom Human Tissue Act and the Declaration of Helsinki. All patients gave informed consent prior to donating their tissue.

### 2.1.1 Details of Patient Cohorts

The OMB is a biobank system that stores donated clinical samples (tissues and blood) from patients under sufficient informed consent to be used for medical research in full compliance with ethical and regulatory requirements (Oxfordshire REC C 09/H0606/11).

Healthy and tendinopathic supraspinatus tendon tissues were obtained from patients in the SBS under ethical approval by the local research ethics committee (Oxfordshire REC B ref. 09/H0605/111). Healthy tissues were sampled from patients undergoing surgical treatment for shoulder instability. Tendinopathic tissues were biopsied from patients treated by subacromial decompression surgery (SAD) or steroid injection before the intervention.

WASP (South Central Oxford REC B ref. 14/SC/022) is a study based on the SBS and another clinical trial Platelet rich plasma And Rotator Cuff Tendons (PARoT) that investigated the effects of PRP in patients with chronic rotator cuff tendinopathy. Tendon biopsies were carried out post-intervention in tendinopathic patients who were treated either by SAD, steroid injection or PRP. Healthy tissues were sampled similar to the SBS concomitantly through this study.

UKUFF through a randomised control trial compared the clinical and cost effectiveness of open and arthroscopic repair for torn rotator cuff (supraspinatus) tendons in a multicentre setting. Torn tendon tissues were sampled during surgery alike the SBS under ethical approval by the multicentre research and ethics committee (UK NHS RECs ref 10/H0402/24) and all related local research and development committees.

SECuRe was designed to compare the tissue characteristics of the rotator cuff tendon following surgical repair and augmentation using different scaffolds, and was approved by the local research ethics committee (OUH R&D REC ref 15/WS/0266). Torn tendon tissues were obtained through the repair surgery alike the SBS and UKUFF prior to scaffold augmentation.

Details of the cohorts each study utilised are shown in Table 2-1.

**TABLE 2-1: Details of cohorts each study utilised**

| | Chapters 3 and 4 | | | | | Chapters 4 and 5 | |
|---|---|---|---|---|---|---|---|
| | Tissues for IHC | | | Tissues for RT-qPCR | | Cells for *in vitro* Experiments | |
| | Healthy | Diseased | | Healthy | Diseased | Healthy | Diseased |
| | | Tendinopathy | Tear | | Tear | | Tear |
| OMB | - | - | - | ✓ | ✓ | ✓ | ✓ |
| SBS | ✓ | ✓ | - | - | - | - | - |
| WASP | ✓ | - | - | - | - | - | - |
| UKUFF | - | - | ✓ | - | - | - | - |
| SECuRe | - | - | - | - | ✓ | - | ✓ |

### 2.1.2 Diagnosis, Inclusion and Exclusion Criteria

The diagnosis of tendinopathy was made clinically in patients complaining shoulder pain who had positive subacromial impingement, painful arc and external rotation resistance tests [286]. Structural integrity of the tendon was confirmed by high-definition ultrasound examination by orthopaedic surgeons who have been trained by a specific validated protocol to identify rotator cuff tears [287]. A torn rotator cuff tendon was diagnosed by ultrasound or magnetic resonance imaging, and confirmed during surgery macroscopically or arthroscopically. The size of the tear was measured intraoperatively in the longest diameter, and defined as small for tears less than 1 cm, medium for tears 1 to 3 cm and large for tears 3 to 5 cm. Tears greater than 5 cm were described as massive, which the torn edge of the tendon was commonly retracted [288].

The exclusion criteria for all patients were prior history of diseases and treatments of the affected shoulder; other shoulder diseases that do not relate to the rotator cuff such as osteoarthritis, idiopathic frozen shoulder and fracture; bilateral shoulder problems; systemic inflammatory diseases such as rheumatoid arthritis; and symptoms related to the cervical spine or neurological disorders. Healthy tendon tissues were confirmed ultrasonographically pre-op, and macroscopically or arthroscopically during surgery that they were not fibrotic.

All patients with tendinopathic or torn tendons were asked to complete the Oxford shoulder score (OSS) before the therapeutic intervention. The OSS is a validated patient-reported scoring system used for the assessment of shoulder pain and functions. It is composed of 12 questions, each scored from 0 to 4 with 0 representing the worst outcome (and most symptomatic), hence the total score would range from 0 to 48. There are 4 and 8 questions that relate to pain and function, respectively [289].

## 2.2 Sampling Technique

Healthy and tendinopathic supraspinatus tendon tissue samples were obtained by a validated biopsy technique under ultrasound guidance and local anaesthesia [290]. In short, a local anaesthetic was injected to the skin, the underlying tissue and subacromial bursa with ultrasound guidance. After the local anaesthetic had taken action (approximately 0.5 hours after the injection), a Bard Magnum Core Biopsy Instrument with a 14-gauge and 10 cm long core biopsy needle (Bard Biopsy Systems, Tempe, AZ) was introduced percutaneously to the supraspinatus tendon at approximately 5 to 10 mm posterior to the rotator interval in the longitudinal direction medial to lateral under ultrasound. There were no adverse patient effects reported for this procedure. Torn tissue samples were obtained from the edge of the torn tendon as part of the debridement procedure for both open and arthroscopic repair surgeries.

## 2.3 Tendon Preparation for mRNA Extraction

Tendons that had been snap frozen and stored at -80 °C were cut to 70 to 100 mg for preparation. Each tendon sample was disaggregated using a tissue homogeniser (IKA Ultra Turrax disperser T-8; Sigma-Aldrich, St. Louis, MO) in a ready-made solution containing phenol and quinidine thiocyanate (RNA Bee; AMS Biotechnology, Abingdon, UK) and processed according to the acid guanidinium thiocyanate-phenol-chloroform extraction method [291].

## 2.4 mRNA Purification and cDNA Synthesis

The extracted mRNA was isolated using a purification kit (RNeasy Minikit; Qiagen, Hilden, Germany) with treatment by DNase (DNase I; Thermo Scientific, Waltham, MA). The composition of deoxyribonucleic acid (DNA) digestion solution has been shown in Appendix B.1, page 169, Table A1. A spectrophotometer (NanoDrop 1000; Thermo Scientific) was used

to confirm the quality of the processed samples to be within 1.8 and 2.2 by the ratio of absorbance at 260 nm and 280 nm. The concentration ranged between 4 to 20 ng $\mu l^{-1}$. mRNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) and a ribonuclease inhibitor (RNasin Plus RNase Inhibitor; Promega, Madison, WI). The composition of the reverse transcriptase, deoxyribonucleotide triphosphate (dNTP) and random primers in the kit and ribonuclease inhibitor that were added to the mRNA has been detailed in Appendix B.1, page 170, Table A2. Synthesis of complementary DNA (cDNA) was performed using a thermal cycler (Veriti Thermal Cycler; Applied Biosystems) with a protocol that heated the samples at 25 °C for 10 minutes, 37 °C for 2 hours, 85 °C for 5 minutes and then cooled to 4 °C. The generated cDNA (20µl) was further diluted with nuclease-free water (100 µl) resulting in 0.5 to 2.5 ng $\mu l^{-1}$.

## 2.5 Real-Time quantitative PCR

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using a ViiA 7 Real-Time PCR System (Applied Biosystems) using SYBR Green Master Mix (Applied Biosystems) and validated primers from QuantiTect Primer Assays (Qiagen) or Primerdesign (Southampton, UK). The details of the primers used and their sequences are shown in Table 2-2. A total reaction volume of 10 µl consisted of 2 µl of diluted cDNA (1 to 5 ng of cDNA), 5 µl of Fast SYBR Green, 1 µl of primer and 2 µl of water. The PCR System went through 3 stages starting from holding the samples at 95 °C for denaturation, rapid cooling for annealing of primers and heating for extension by polymerase for 40 cycles. Finally, a melt curve was generating and analysed to verify the specificity of the test by confirming that only a single melting peak was produced [292]. SYBR Green has its merit in the low cost and that it only requires validated primers, but may be non-specific as it may intercalate into any double-stranded DNA (extracted mRNA were treated by DNase and primers designed to span exon-

exon junctions were used to mitigate this issue). Florescent probes such as Taqman are more specific as fluorescence is only detected when the probe binds to the intended target, but could be costly [293]. The threshold cycle ($C_T$) was set to 0.15 for all genes, and relative expression was calculated by the comparative $C_T$ method [294]. All of the experiments and measurements were performed in duplicates, and the average $C_T$ values were used for analysis with a coefficient of variation between duplicates less than 0.05.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as endogenous housekeepers. An ideal housekeeper should be sufficiently expressed in the tissue or cell of interest, and its expression be stable between samples and under different experimental procedures [295, 296]. GAPDH takes part in glucose metabolism [297], which numerous studies have shown its abundant and stable expression in healthy and diseased tendon tissues [99, 298]. It has been frequently used as housekeeping genes for RT-qPCR in *in vitro* cell culture experiments treating TSCs with IL-4 [157], IL-1β [107, 299–302], TGF-β1 [203, 275, 303-305] and BMP-2 [223, 226, 227]. Stable *GAPDH* mRNA expression has been reported in TSCs treated with growth factors such as BMP-12 *in vitro* [306]. β-actin is related to the cytoskeleton [307] and has also commonly been used as an endogenous housekeeper with studies reporting similar results in the expression of gene of interest in tendon tissues and cells when normalised to GAPDH [197, 298]. Most other *in vitro* cell culture experiments have used β-actin as a housekeeping gene for PCR when investigating the effects of IL-4 [157], IL-1β [106, 301], TGF-β1 [308-310] or BMP-2 [216, 222, 311] treatments on TSCs. The reliability of *ACTB* as another internal control gene along *GAPDH* was screened using data from validation experiments at the determined doses and durations for the main experiments. The $C_T$ values for *ACTB* and *GAPDH* were not significantly affected by cytokine or growth factor treatments. Moreover, there were significant correlations between the $C_T$ values of *ACTB* and *GAPDH* in each treatment groups (figures shown in Appendix C.1, page 176, Figure A3). For

gene expression analyses, data on the gene of interest were normalised to the geometric mean of these two housekeepers as a reference of the internal control in this thesis, as normalisation based on multiple genes over a single gene has been indicated to be more accurate in measuring gene expression levels [295, 296, 312].

**TABLE 2-2: Details of primers used**

| Gene description | Gene symbol | Manufacturer | Reference or forward/reverse primer sequence |
|---|---|---|---|
| Transforming growth factor beta 1 | *TGFB1* | QIAGEN | HS_TGFB1_1_SG (QT00000728) |
| Transforming growth factor beta receptor 1 | *TGFBR1* | QIAGEN | HS_TGFBR1_1_SG (QT00083412) |
| Transforming growth factor beta receptor 2 | *TGFBR2* | QIAGEN | HS_TGFBR2_1_SG (QT00014350) |
| Connective tissue growth factor | *CTGF* | QIAGEN | HS_CTGF_1_SG (QT00052899) |
| Bone morphogenetic protein 2 | *BMP2* | QIAGEN | HS_BMP2_1_SG (QT00012544) |
| Bone morphogenetic protein 7 | *BMP7* | QIAGEN | HS_ BMP7_1_SG (QT00068936) |
| Growth differentiation factor 7 | *GDF7 (BMP12)* | QIAGEN | HS_ BMP12_1_SG (QT00227507) |
| Serpine family E member 1 | *SERPINE1* | QIAGEN | HS_SERPINE1_1_SG (QT00062496) |
| Inhibitor of DNA binding 1 | *ID1* | QIAGEN | HS_ID1_1_SG (QT00230650) |
| Prostaglandin-endoperoxide synthase 2 | *PGS2 (COX2)* | Primerdesign | CAGGCTTCCATTGACCAGAG / TTTCTCCTGTAAGTTCTTCAAATGAT |
| Prostaglandin E synthase | *PTGES* | QIAGEN | HS_PTGES_1_SG (QT00208607) |
| TGFB induced factor homeobox 1 | *TGIF1* | QIAGEN | HS_TGIF1_1_SG (QT00217259) |
| Collagen type I alpha 1 chain | *COL1A1* | QIAGEN | HS_COL1A1_1_SG (QT00037793) |
| Collagen type III alpha 1 chain | *COL3A1* | QIAGEN | HS_COL3A1_1_SG (QT00058233) |
| Aggrecan | *ACAN* | QIAGEN | HS_ACAN_1_SG (QT00001365) |
| Biglycan | *BGN* | QIAGEN | HS_BGN_1_SG (QT00000483) |
| Decorin | *DCN* | QIAGEN | HS_DCN_1_SG (QT00032546) |
| Scleraxis | *SCX* | QIAGEN | HS_SCX_1_SG (QT01324659) |
| Tenascin-C | *TNC* | QIAGEN | HS_TNC_1_SG (QT00024409) |
| Tenomodulin | *TNMD* | QIAGEN | HS_TNMD_1_SG (QT01024590) |
| Actin, alpha 2, smooth muscle, aorta | *ACTA2* | QIAGEN | HS_ACTA2_1_SG (QT00088102) |
| SMAD family member 6 | *SMAD6* | QIAGEN | HS_SMAD6_1_SG (QT00025242) |
| SMAD family member 7 | *SMAD7* | QIAGEN | HS_SMAD7_1_SG (QT00076391) |
| Actin, beta | *ACTB* | QIAGEN | HS_ACTB_1_SG (QT00095431) |
| Glyceraldehyde-3-phosphate dehydrogenase | *GAPDH* | QIAGEN | HS_GAPDH_1_SG (QT00079247) |

## 2.6 Tissue Preparation for Immunohistochemistry

Tissues for histopathological analysis were immediately fixed in 10 percent buffered formalin and left for the fixative to infiltrate sufficiently (for at least 24 hours, 1 mm $h^{-1}$). Fixed tissues were processed using a tissue processor (ASP300 S; Leica, Milton Keynes, UK) and embedded in paraffin wax. Sections 5 µm thick were sliced using a manual rotary microtome (RM2125 RTS; Leica), collected onto glass slides, dried at 60 °C for 30 minutes, 37 °C for 60 minutes and stored until for immunohistochemistry.

Tissue sections were baked at 60 °C for 60 minutes and then placed in to an automated pre-treatment system (PT Link; Dako, Carpinteria, CA) that allows de-paraffinisation, rehydration and antigen retrieval mediated by heat at high pH. An autostainer (Autostainer Link 48; Dako) was used for antibody staining and an antibody detection system (EnVision FLEX DAB+ Chromogen System; Dako) for visualisation of its binding to the antigen, which senses the primary mouse or rabbit antibody by a peroxidase-blocking reagent and visualises by 3,3'-diaminobenzidne (DAB). Haematoxylin was used for counterstain. The procedures were performed according to the manufacturer's instructions.

The details of the antibodies used and their working dilutions are shown in Table 2-2. The dilutions were determined based on the manufacturer's recommendations, but validated for use in sections of human tendon tissues. Universal negative control antibodies for mouse or rabbit (FLEX Negative Control Mouse or Rabbit; Dako) that consists of immunoglobulin (Ig) $G_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_3$ and IgM were used alternative to the primary antibody to test for nonspecific testing (figures shown in Appendix B.2, page 172, Figure A1). Sections of normal human spleen specimens (Zyagen Laboratories, San Diego, CA) were used as positive controls (figures shown in Appendix B.2, page 172, Figure A2).

After staining, slides were taken into graded industrial methylated spirit (IMS) (twice, 3 minutes each) for dehydration, then xylene (twice, 5 minutes each) for clearance, mounted

(DPX Mounting medium; Fisher Scientific, Waltham, MA) and dried for 2 hours prior to image acquisition.

**TABLE 2-3: Details of antibodies used for IHC**

| Antibody | Species | Isotype | Dilution | Manufacturer | Reference |
|---|---|---|---|---|---|
| TGF-β1 | mouse | $IgG_1$ | 1:50 | Abcam | ab64715 |
| TGF-β R1 | rabbit | IgG | 1:50 | Abcam | ab31013 |
| TGF-β R2 | mouse | $IgG_1$ | 1:50 | Abcam | ab78419 |
| BMP-2 | rabbit | IgG | 1:100 | LsBio | LS-B12549 |
| BMP-7 | rabbit | polyclonal | 1:200 | LsBio | LS-C156837 |
| BMP-12 | rabbit | IgG | 1:1000 | Abcam | ab189928 |
| CTGF | rabbit | polyclonal | 1:100 | LsBio | LS-B3284 |
| ERK1/2 (phospho-) | rabbit | polyclonal | 1:1000 | Abcam | ab214362 |

## 2.7 Image Acquisition for Immunohistochemistry

Ten to 20 images of each stained slide were captured using an inverted brightfield microscope (LSM 700; Carl Zeiss MicroImaging, Jena, Germany) and an imaging software (Axiovision Rel. 4.8; Carl Zeiss) at 100x magnification with oil immersion. Attention was paid so that the images did not overlap.

## 2.8 Quantitative Analysis of Immunopositive Staining

The number of DAB and haematoxylin stained cells in each image was counted using an image processing program (ImageJ 1.47v; National Institutes of Health, Bethesda, MD). The algorithm used by ImageJ uses colour deconvolution to divide the images into red, green and blue channels. Stains were quantified using the stain-specific channels, with a manually set threshold for each dataset [313-315]. Quantitative analysis of immunopositive cells by IHC has been commonly carried out by calculating the percentage of DAB positive cells relative to the

total number of cell nuclei imaged by haematoxylin, which has been shown to have good clinical correlations [313-315]. The percentage of DAB staining area relative to cell number in each image has also been measured to quantify protein expression with good to excellent inter- and intra-rater reliability [313]. This method has also been shown to have an excellent correlation with percentage positive cells in cellular proteins [67]. Data are shown as percentage immunopositive cells for Chapter 3 due to the predominant cellular location of immunopositive staining. The details of the macros used to perform quantitative analysis of immunopositive staining are shown in Appendix B.2, page 173.

## 2.9 Isolation of Tendon-derived Stromal Cells

Human TSCs were isolated by explant culture; tendon tissues were incised into pieces approximately 1 $mm^3$ large and cultured in media containing 50 percent foetal bovine serum (FBS) (Labtech, Heathfield, UK), Dulbecco's Modified Eagle Medium/Nutrient Mixture (DMEM F-12) (Lonza, Verviers, Belgium or Fisher Scientific) and 1 percent penicillin-streptomycin (PS) (Lonza) at 37 °C and 5 percent $CO_2$. After confirming by light microscopy that the cells had migrated out from the tissue after 7 to 10 days, culture media were changed to 10 percent FBS, DMEM F-12 and 1 percent PS until the cells were sub-confluent (approximately 80 to 90 percent confluent) for further passage or frozen storage [316]. TSCs from healthy tendons reached sub-confluence in approximately 1 week whereas it took 2 to 3 weeks for TSCs from diseased tendons to become sub-confluent (quantitative data not obtained). Culture media were changed twice a week. Cells cultured up to passage 2 or 3 were used for the main experiments [275]. Passage 3 and 4 cells were used for pilot and dose- and duration-finding validation experiments.

## 2.10 Preparation of Cytokines and Cell Treatments

Human recombinant cytokines and growth factors were reconstituted in sterile filtered phosphate buffered saline (PBS) containing 0.1 percent bovine serum albumin (BSA) (Sigma Aldrich) as per manufacturer instructions. ERK1/2 inhibitor PD98059 (Merck Millipore, Darmstadt, Germany) was reconstituted in dimethyl sulfoxide (DMSO) (Sigma Aldrich). Cytokines were added to serum-free DMEM F-12 and 1 percent PS at the following concentrations based on previous literature [106, 107, 300, 302, 317, 318] and validation experiments (data shown in Appendix C.3, pages 180-183, Figures A7-A12): IL-1β (Merck Millipore), 5 ng $ml^{-1}$ and IL-4 (Biolegend, San Diego, CA), 10 ng $ml^{-1}$. Growth factors were added to either serum-free or 1 percent FBS, DMEM F-12 and 1 percent PS at the following concentrations based on previous literature [216, 222, 223, 225-227, 305, 308, 311, 319, 320] and validation experiments likewise (data shown in Appendix C.4, pages 200-202, Figures A29-A32): TGF-β1 (Merck Millipore), 10 ng $ml^{-1}$ and BMP-2 (Merck Millipore), 100 ng $ml^{-1}$. Although the concentrations for the validation experiments were based on previous literature, there were no clear dose responses to cell treatments in any of the cytokines or growth factors. This is possibly due to the supraphysiologic levels of treatments in *in vitro* experiments even at their lowest concentrations [321-324]. TSCs were serum starved overnight before cytokine or growth factor treatment. For ERK1/2 inhibition experiments, cells were pre-treated with PD98059, 8 μg $ml^{-1}$ (30 μM) for 1 hour before cytokine treatment based on validation experiments (data shown in Appendix C.3, pages 184-186, Figures A13-15). Vehicle only treatments served as experimental controls.

### 2.11 mRNA Extraction, Purification and cDNA Synthesis

mRNA was isolated from TSCs harvested in Buffer RLT (Qiagen). The purification of mRNA and cDNA synthesis processes have been described previously (Section 2.4 mRNA Purification and cDNA Synthesis, page 54).

### 2.12 Preparation of Protein Lysates for Western Blotting

TSCs were treated with 1 mM sodium orthovanadate (Sigma-Aldrich) for phosphatase inhibition and then scraped in a cell lysis buffer that contains 0.5 mM sodium orthovanadate, 32 mM Tris base (Fisher Scientific), 50 mM (5 percent) glycerol (Sigma-Aldrich), 34.7 mM (1 percent) sodium dodecyl sulphate (SDS) (Fisher Scientific) and a protease inhibitor cocktail (0.5 percent, Protease Inhibitor Cocktail Set III; Merck Millipore).

### 2.13 Electrophoresis and Western Blotting

Protein separation was performed by electrophoresis by loading approximately 2.5 μg of protein per well to 10 percent polyacrylamide gel electrophoresis (PAGE) gels (Mini-Protean TGX Precast Gels; BioRad, Hercules, CA). The amounts of proteins loaded were measured by a bicinchoninic acid (BCA) assay (the method has been detailed in Appendix B.4, page 175). After electrophoresis the proteins were transferred by using a Western blot transfer system (Trans-Blot Turbo Transfer System; BioRad) on to a 0.2 μm polyvinylidene difluoride (PVDF) membrane (Trans-Blot Turbo Transfer Pack; BioRad). Molecular weight was indicated by a pre-stained protein standard (Precision Plus Protein Dual Color Standards; BioRad). The details of the antibodies used and their working dilutions are shown in Table 2-4. β-actin or GAPDH were used as endogenous housekeepers. Anti-rabbit IgG antibody was used as the secondary antibody (Cell Signaling Technology, Danvers, MA). Proteins were detected by

enhanced chemiluminescence (Amersham ECL Western Blotting Analysis System; GE Healthcare Life Sciences, Little Chalfont, UK) and visualised using an imaging system with its dedicated software (Alliance 6.7 system and NineAlliance 4.7 17.00; uvitec, Cambridge, UK) [325].

**TABLE 2-4: Details of antibodies used for Western blotting**

| Antibody | Species | Isotype | Dilution | Manufacturer | Reference |
|---|---|---|---|---|---|
| Phospho- ERK1/2 | rabbit | IgG | 1:1000 | Cell Signaling | 4370 |
| Total ERK1/2 | rabbit | IgG | 1:1000 | Cell Signaling | 4695 |
| Phospho- NF-κB (p65) | rabbit | IgG | 1:1000 | Cell Signaling | 3033 |
| Total NF-κB (p65) | rabbit | IgG | 1:1000 | Cell Signaling | 8242 |
| Phospho- p38 | rabbit | IgG | 1:750 | Cell Signaling | 4511 |
| Total p38 | rabbit | IgG | 1:1000 | Cell Signaling | 8690 |
| Phospho- SAPK/JNK | rabbit | IgG | 1:1000 | Cell Signaling | 4668 |
| Total SAPK/JNK | rabbit | polyclonal | 1:1000 | Cell Signaling | 9252 |
| Phospho- STAT-6 | rabbit | polyclonal | 1:1000 | Cell Signaling | 9361 |
| Total STAT-6 | rabbit | polyclonal | 1:1000 | Cell Signaling | 9362 |
| Phospho- Smad2/3 | rabbit | IgG | 1:750 | Cell Signaling | 8828 |
| Total Smad2/3 | rabbit | IgG | 1:1000 | Cell Signaling | 8685 |
| Phospho- Smad1/5 | rabbit | IgG | 1:1000 | Cell Signaling | 9516 |
| Total Smad1 | rabbit | IgG | 1:1000 | Cell Signaling | 6944 |
| Phospho- c-Jun (Ser63) | rabbit | IgG | 1:750 | Cell Signaling | 2361 |
| Total c-Jun | rabbit | IgG | 1:1000 | Cell Signaling | 9165 |
| β-actin | rabbit | IgG | 1:750 | Cell Signaling | 4970 |
| GAPDH | rabbit | IgG | 1:1000 | Cell Signaling | 5174 |
| Anti-rabbit IgG | - | polyclonal | 1:1000 | Cell Signaling | 7074 |

## 2.14 Semi-quantitative Image Analysis of Western Blots

The intensity of the protein bands acquired were semi-quantified by ImageJ. The Gel Analyzer command was used to generate profile plots of the intensity of the bands. The areas of each region of interest (ROI) for each band on the plots were measured as values that represent intensities. The data were normalised to the mean of the control group and the relative values of the phosphorylated proteins of interest to their respective total proteins or housekeepers were calculated.

## 2.15 Cell Viability Assay

Alamar Blue (Invitrogen, Waltham, MA), which is a dye that detects the activity of electron transport chains, was diluted 1:10 in culture media and added to each well before and after cytokine treatment and incubated for 1.5 hours before measuring the fluorescence using a microplate reader (FLUOstar Optima; BMG Labtech, Waltham, MA). The excitation and emission wavelengths for fluorescence measurement were 544 nm and 590 nm, respectively. Measurements were performed in technical duplicates. The effects of cytokine or growth factor treatments were evaluated by the ratio of the obtained values post- and pre- treatments, which were normalised to the mean of the control.

## 2.16 Statistical Analysis

Statistical analysis was operated by Prism 5 for Mac OS X Version 5.0b (GraphPad Software, La Jolla, CA). D'Agostino-Pearson omnibus normality test was carried out to determine normal distribution for each data. Differences in patient demographics were compared by analysis of variance (ANOVA) or unpaired t test when the data were normally distributed. Kruskall Wallis or Mann Whitney tests were used when the data were not normally distributed or the numbers were small (less than 8). Differences in mRNA and protein expression between patient cohorts or treatments were compared by Kruskall Wallis or Mann Whitney tests as the data were not always normally distributed or the numbers were small (less than 8). Tukey's or Dunn's multiple comparisons tests were used for post-hoc analyses. Statistical significance was set at $p<0.05$.

# Chapter 3 Fibrotic Signature of Tendon Disease

## 3.1 Introduction

### 3.1.1 Background

Studies of fibrotic diseases in other organs have implicated TGF-β and its closely related growth factors such as BMPs and CTGF as key mediators of fibrosis [166], and proposed them as effective biomarkers for fibrotic changes [326]. These fibrotic mediators have also been a focus in the pathobiology of tendon disease. A systematic review on the expression of these mediators in tendon disease showed that TGF-β has been commonly studied as the key mediator of fibrosis, and CTGF has gained interest as its downstream regulator [193]. The most studied BMPs were BMP-2 and BMP-7 [193], and animal studies showing evidence for the tenogenic effect of BMP-12 are accumulating within the last few years [255, 327, 328]. To date numerous studies have shown that the expression of TGF-β, BMPs and CTGF is altered in animal models of tendon healing and overuse [193]. However, how the development and progression of tendon disease in humans relate to the expression of TGF-β, BMPs and CTGF is yet to be elucidated.

### 3.1.2 Aim, Objectives and Hypotheses

The aim of this study was to define the fibrotic signature of tendon disease using tissues obtained from tendon biopsies of human patients.

The primary objective was to investigate the mRNA and protein expression of TGF-β1, BMP-2, BMP-7, BMP-12 and CTGF in diseased tendon tissues collected from patients with tendinopathic and torn supraspinatus tendons in comparison to healthy tendon tissues.

The hypothesis was that the mRNA and protein expression of TGF-β1, BMPs and CTGF would increase along disease progression from healthy to tendinopathy, and tear.

## 3.2 Materials and Methods

### 3.2.1 Patient Cohort

Surplus hamstring tendon were obtained from patients undergoing autograft ACL reconstruction surgery under ethical approval from the OMB and utilised as the healthy cohort of the mRNA expression analysis. Torn supraspinatus tendon tissues for mRNA expression analysis were obtained from OMB and through SECuRe study. Healthy and tendinopathic supraspinatus tendon tissues for protein expression analysis were obtained through the SBS and WASP studies. Torn supraspinatus tendon tissues for protein expression analysis were obtained through UKUFF trial (cf. Chapter 2, Table 2-1).

### 3.2.2 Sampling and Processing of Tissues

Tendon tissues were either snap frozen for mRNA expression analysis or immediately fixed in 10 percent buffered formalin for protein expression analysis. The details of the processing of the tissues are described in Chapter 2.

### 3.2.3 mRNA and Protein Expression Analyses

The processing of tissues of mRNA extraction, purification, conversion to cDNA, quantitative analysis of synthesised cDNA by RT-qPCR and histopathological procedures of tissue preparation, immunohistochemistry (IHC), image acquisition and analysis are described in Chapter 2. The details of the primers and antibodies used are also listed in Chapter 2.

### 3.2.4 Statistical Analysis

Statistical analysis was performed using Prism 5 for Mac OS X Version 5.0b (GraphPad Software Inc., La Jolla, CA). D'Agostino and Pearson normality test was carried out to determine normal distribution for each data. Differences in patient demographics were compared by analysis of variance (ANOVA) or unpaired t test when the data were normally distributed. Kruskall Wallis or Mann Whitney tests were used when the data were not normally distributed or the numbers were small (less than 8). Differences in mRNA and protein expression between patient cohorts were compared by Kruskall Wallis or Mann Whitney tests as the data were not always normally distributed or the numbers were small (less than 8). Tukey's or Dunn's multiple comparisons tests were used for post-hoc analyses. Statistical significance was set at $p<0.05$.

## 3.3 Results

### 3.3.1 Patient Demographics

#### 3.3.1.1 mRNA Expression Analysis

Healthy tissues were obtained from the hamstring tendon of 5 male and 1 female patient undergoing ACL reconstruction surgery. Torn supraspinatus tendon tissues were obtained from 9 male and 2 female patients undergoing surgical repair (Table 3-1). Patients with torn tendons were significantly older than the patients with healthy tendons ($p=0.0006$), with larger tears relating to increased age ($p=0.0091$). There were no significant differences in the ratio of males and females between patients with healthy and torn tendons.

**TABLE 3-1: Summary of patient demographics for mRNA expression analysis.** Median (IQR) shown for age as the numbers were small. * indicates significant difference to the healthy group. # indicates significant difference between small to medium and large to massive tears. IQR, interquartile range; med., medium; mass., massive. */#=$p<0.05$, **/##=$p<0.01$, ***/###=$p<0.001$.

| | Healthy | Tear (all) | Tear (small-med.) | Tear (large-mass.) |
|---|---|---|---|---|
| Number of patients | 6 | 11 | 4 | 7 |
| Age; median (IQR) years | 25 (20.75/34.25) | 55 (51/65) *** | 47 (35/47) ## | 65 (55/65) ## |
| Sex; male/female, n | 5/1 | 9/2 | 4/0 | 5/2 |

#### 3.3.1.2 Protein Expression Analysis

Tissues collected from healthy (n=15), tendinopathic (n=23) and torn (n=31) supraspinatus tendons were used to investigate the differences in the protein expression by IHC (Table 3-2). There were significant differences in the mean patient age of the three cohorts; patients with healthy tendons were significantly younger than patients with tendinopathic ($p<0.0001$) and torn tendons ($p<0.0001$). Moreover, patients with large to massive tears were significantly older than the patients with small to medium tears ($p=0.0464$). There were no significant differences in the ratio of males and females between patients with healthy, tendinopathic and torn tendons.

However, there were more females in the small to medium and more males in the large to massive tear groups (p=0.0064).

OSS of patients with healthy tendons were not available. The total OSS was significantly lower in patients with torn compared to patients with tendinopathic tendons (p=0.0033). The function element of the OSS was also significantly lower in patients with torn compared to tendinopathic tendons (p=0.0025). There were no significant differences by the size of the tear.

**TABLE 3-2: Summary of patient demographics and Oxford Shoulder Score for protein expression analysis.** Mean (SD) shown for age and OSS as the data were normally distributed. * indicates significant difference to the healthy group. § indicates significant difference to the tendinopathic group. # indicates significant difference between small to medium and large to massive tears. SD, standard deviation; OSS, Oxford Shoulder Score (scale of 0-48, pain element 0-16, 0=least symptomatic); NA, not available; med., medium; mass., massive. */§/#=p<0.05, **/§§/##=p<0.01, ***/§§§=p<0.001, ****=p<0.0001.

| | | Healthy | Tendinopathy | Tear (all) | Tear (small-med.) | Tear (large-mass.) |
|---|---|---|---|---|---|---|
| Number of patients | | 15 | 22 | 31 | 16 | 15 |
| Age; mean (SD) years | | 26.2 (4.48) | 52.36 (10.25) **** | 61.52 (6.97) ****§§§ | 59.13 (7.40) # | 64.07 (5.64) # |
| Sex; male/female, n | | 14/1 | 15/7 | 18/13 | 5/11 ## | 13/2 ## |
| OSS; mean (SD) | Total | NA | 30.29 (6.83) | 23.9 (7.61) §§ | 24.44 (7.75) | 23.33 (7.68) |
| | Function element | NA | 24.76 (5.92) | 19.45 (5.88) §§ | 19.81 (5.94) | 19.07 (5.99) |

### 3.3.2 mRNA Expression of *TGFB1* and *TGFBR1* is Increased in Torn Tendons

mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2*, *BMP7*, *BMP12* and *CTGF* in healthy and torn tendon tissues was analysed by RT-qPCR (the expression in tendinopathic tendons could not be investigated as the tissues obtained by biopsies were too small for analysis). Genes of interest were normalised to the geometric mean of the expression values of *ACTB* and *GAPDH* as a reference of the internal control using the comparative $C_T$ method (Figure 3-3). The data relative to *ACTB* and *GAPDH* alone are shown in Appendix C.2, pages 177-178,

Tables A3-4 and Figures A4-5. The results for *BMP7* and *BMP12* are not presented due to the low-level expression in tissues from both healthy and torn tendons.

There were 2.67- and 2-fold increased expression of *TGFB1* and *TGFBR1* mRNA in torn compared to healthy tendons, respectively. The mRNA expression of *TGFBR2*, *BMP2*, and *CTGF* did not differ between healthy and torn tendons. There were no significant differences in the investigated mRNA expression by the size of the tear.

**TABLE 3-3: Data on relative mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* in healthy and torn (small to medium and large to massive) tendon tissues by RT-qPCR.** Levels of mRNA expression were quantified by RT-qPCR and normalised to the internal control. Median (IQR) shown as the data were not normally distributed. * indicates significant difference to the healthy group. med., medium; mass., massive; IQR, interquartile range. *=p<0.05.

| | Healthy | Tear (all) | Tear (small-med.) | Tear (large-mass.) |
|---|---|---|---|---|
| *TGFB1* | 0.02 (0.01/0.03) | 0.04 (0.02/0.06) * | 0.03 (0.01/0.04) | 0.05 (0.02/0.06) |
| *TGFBR1* | 0.04 (0.02/0.06) | 0.07 (0.04/0.1) * | 0.07 (0.05/0.08) | 0.09 (0.04/0.11) |
| *TGFBR2* | 0.09 (0.06/0.16) | 0.07 (0.05/0.13) | 0.05 (0.04/0.10) | 0.08 (0.06/0.14) |
| *BMP2* | 0.01 (0.01/0.01) | 0.00 (0.00/0.02) | 0.00 (0.00/0.01) | 0.01 (0.00/0.02) |
| *CTGF* | 0.28 (0.14/0.36) | 0.28 (0.08/0.45) | 0.14 (0.05/0.44) | 0.44 (0.22/0.45) |

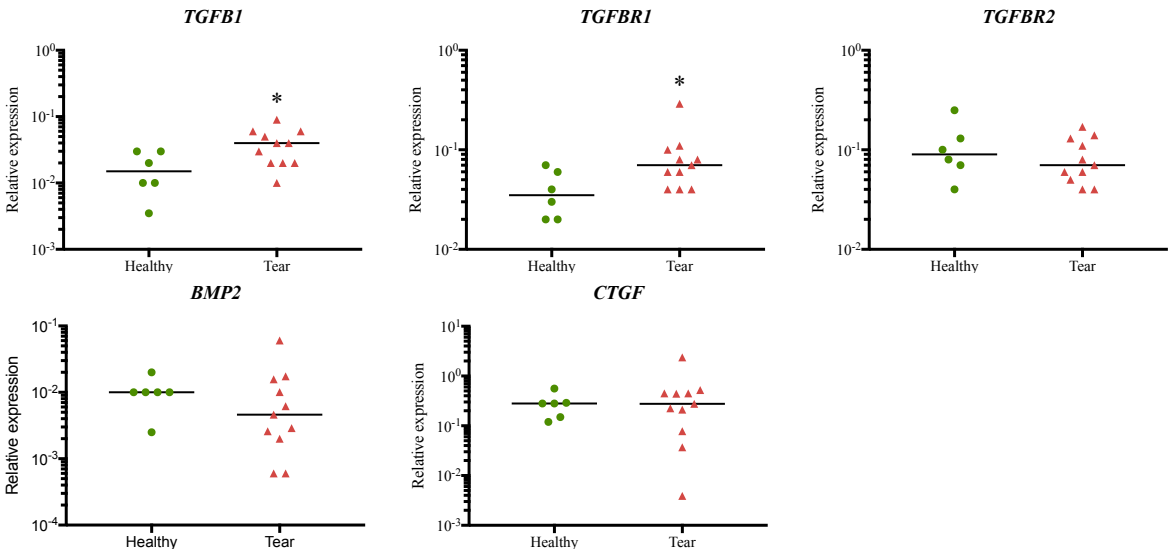



**FIGURE 3-1:** Relative **mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* in healthy and torn tendon tissues.** Levels of mRNA expression in tissues from healthy (n=6) and torn tendons (n=11) were quantified by RT-qPCR and normalised to the internal control. Bars shown represent median. * indicates significant difference to the healthy group. *=p<0.05.

### 3.3.3 Protein Expression of TGF-β1, its receptors, BMPs and CTGF is Down-regulated in Torn Tendons

Protein expression of TGF-β1, TGF-β R1, TGF-β R2, BMP-2, BMP-7, BMP-12 and CTGF in healthy, tendinopathic and torn tendon tissues was studied by IHC. Representative images are shown in Figure 3-2. The antibodies utilised can detect cellular (intracellular and membrane) and extracellular secreted protein. However, the acquired images showed predominantly cellular staining for all studied proteins. Staining of secreted proteins within the ECM was rarely seen in healthy and torn tendons for all studied proteins. In tendinopathic tendons extracellular CTGF was noted in some tissues and hence a representative image with extracellular staining is shown in Appendix C.2, page 179, Figure A6.

Quantitative analysis of immunopositive staining showed significant differences in the expression of the investigated proteins between healthy, tendinopathic and torn tendons (Table 3-3 and Figure 3-2). There were 2.94- and 5.73-fold decreased expression of TGF-β1 and TGF-β R2 in tendinopathic compared to healthy tendons ($p=0.0002$ and 0.0016, respectively). The expression of TGF-β R1 did not differ between tendinopathic and healthy tendons. The expression of TGF-β1, TGF-β R1 and TGF-β R2 was significantly reduced to 4.33-, 16.67- and 11.47-fold, respectively in torn compared to healthy tendons ($p<0.0001$, 0.0018 and 0.0002, respectively). There were no significant differences in the expression of BMP-2, BMP-7, BMP-12 and CTGF between tendinopathic and healthy tendons, but a marked reduction of 2.3-, 14.93-, 6.67- and 7.14-fold, respectively was seen in torn compared to healthy tendons ($p=0.0028$, 0.0004, 0.0003 and 0.0267, respectively). There were no significant differences in the expression of all of the investigated proteins by the size of the tear.

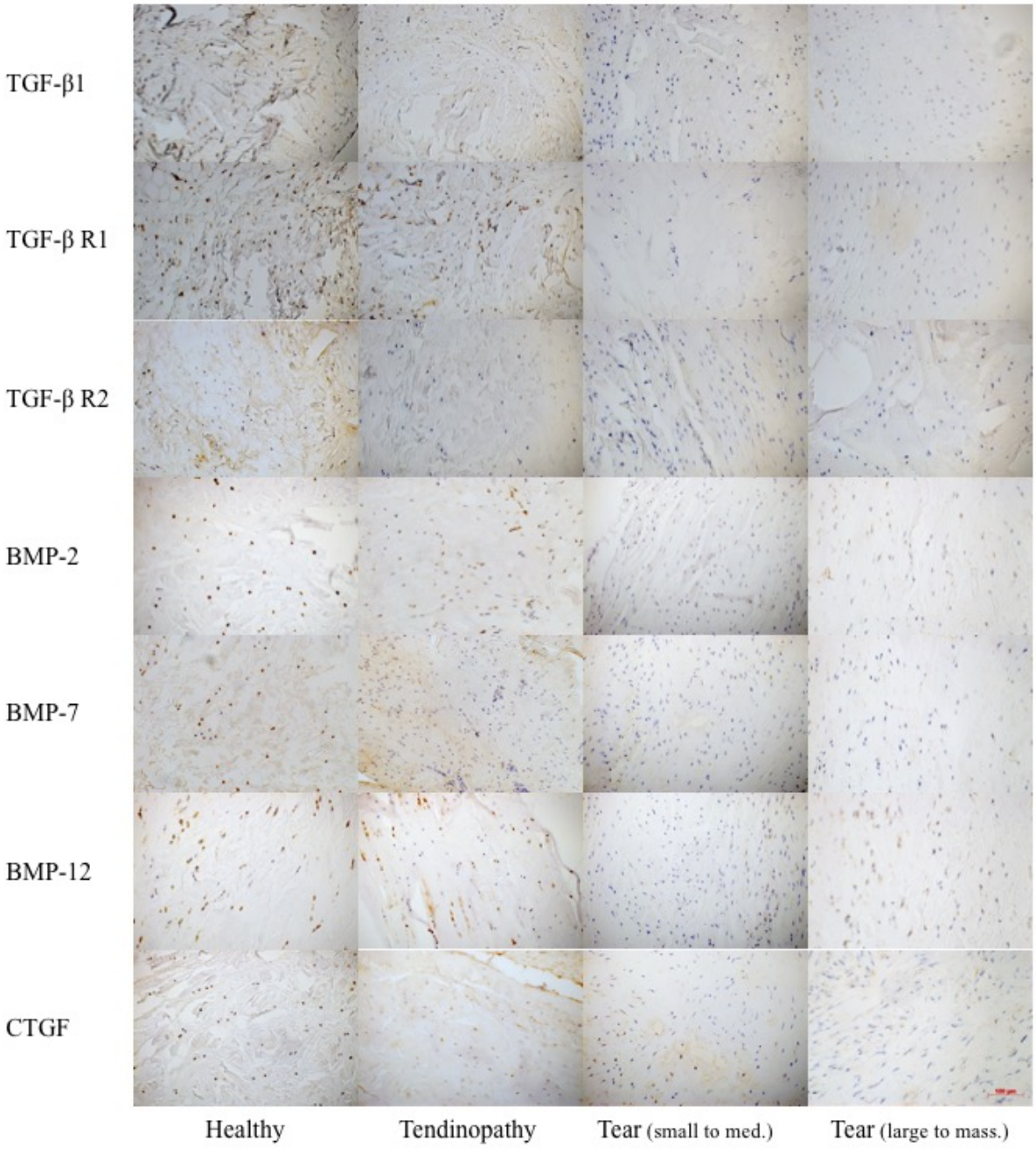



**FIGURE 3-2: Immunohistochemistry showing expression of fibrotic mediators in healthy and diseased (tendinopathic, torn; small to medium and large to massive) tendon tissues.** Representative images are shown with haematoxylin (blue) for nuclei and DAB immunopositive staining (brown) for TGF-β1, TGF-β R1, TGF-β R2, BMP-2, BMP-7, BMP-12 or CTGF. Scale bar = 100μm. med., medium; mass., massive.

**TABLE 3-4: Quantified data on protein expression of TGF-β1, TGF-β R1, TGF-β R2, BMP-2, BMP-7, BMP-12 and CTGF in healthy and diseased (tendinopathic and torn; small to medium and large to massive) tendon tissues by immunohistochemistry.** Median (IQR) shown for proteins of interest as the data were not normally distributed or numbers were small. * indicates significant difference to the healthy group. med., medium; mass., massive; IQR, interquartile range. *=p<0.05, **=p<0.01, ***=p<0.001.

| | Healthy | Tendinopathy | Tear (all) | Tear (small-med.) | Tear (large-mass.) |
|---|---|---|---|---|---|
| TGF-β1 | 0.91 (0.62/1) | 0.31 (0.17/0.5) * | 0.21 (0.06/0.39) ** | 0.28 (0.10/0.57) | 0.14 (0.05/0.42) |
| TGF-β R1 | 1 (1/1) | 0.95 (0.77/1) | 0.06 (0.04/0.29) *** | 0.20 (0.04/0.51) | 0.06 (0.03/0.15) |
| TGF-β R2 | 0.86 (0.64/0.95) | 0.15 (0/0.22) ** | 0.075 (0/0.31) ** | 0.08 (0/0.24) | 0.07 (0.02/0.23) |
| BMP-2 | 1 (1/1) | 1 (1/1) | 0.40 (0.06/0.88) ** | 0.69 (0.09/0.93) | 0.17 (0/0.76) |
| BMP-7 | 1 (1/1) | 0.72 (0.40/1) | 0.069 (0/0.26) *** | 0.10 (0/0.23) | 0.04 (0/0.47) |
| BMP-12 | 1 (1/1) | 0.69 (0.38/0.78) | 0.15 (0.03/0.71) *** | 0.3 (0.08/0.72) | 0.06 (0/0.45) |
| CTGF | 1 (0.59/1) | 1 (0.43/1) | 0.14 (0/0.48) * | 0.28 (0.10/1) | 0.11 (0/0.2) |

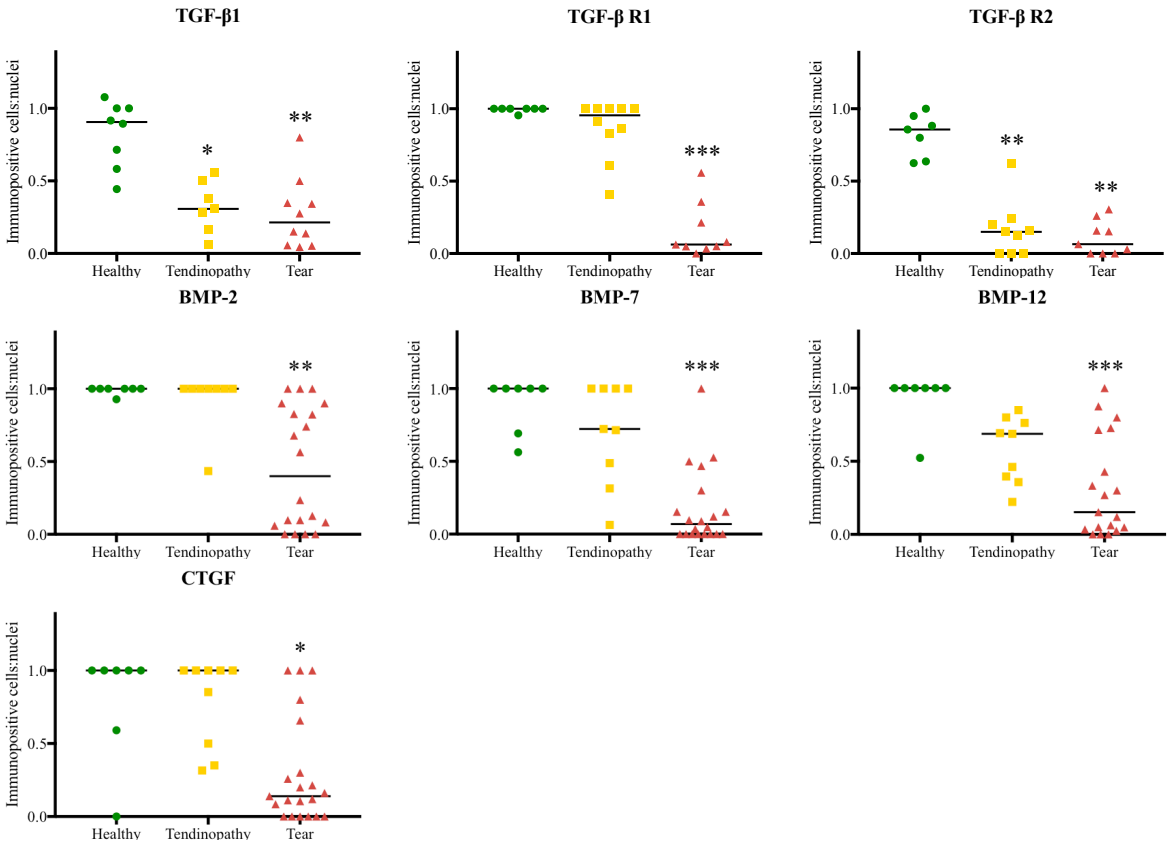



**FIGURE 3-3: Protein expression of TGF-β1, TGF-β R1, TGF-β R2, BMP-2, BMP-7, BMP-12 and CTGF in healthy and diseased (tendinopathic and torn) tendon tissues.** Levels of protein expression in tissues from healthy, tendinopathic and torn tendons were quantified as the number of immunopositive cells to the number of nuclei by immunohistochemistry. Bars shown represent median. * indicates significant difference to the healthy group. *=p<0.05, **=p<0.01, ***=p<0.001.

## 3.4 Discussion

This study demonstrated that the protein expression of TGF-β1, TGF-β R1, TGF-β R2, BMP-2, BMP-7, BMP-12 and CTGF is down-regulated along disease progression, with a marked suppression in torn compared to healthy tendon tissues contrary to the hypothesis. In contrast, *TGFB1* and *TGFBR1* mRNA were increased in torn tendon tissues.

### Expression of TGF-β, BMPs and CTGF in Fibrotic Diseases of Other Organs

The marked suppressed protein expression of TGF-β1, BMPs and CTGF was contrary to the hypothesis that their expression would increase along disease progression from healthy to tendinopathy, and tear. Studies of fibrotic diseases in other organs such as the lung [329, 330], liver [331] and kidney [332, 333] have shown increased expression of TGF-β and CTGF along disease progression. The protein expression of BMP-7 in the diseased compared to healthy tissues differed between organs; Koli *et al.* reported that *BMP7* mRNA expression was unchanged in idiopathic pulmonary fibrosis patients [334], Tacke *et al.* showed increased BMP-7 protein expression in cirrhotic human liver [335], whereas numerous studies have related suppressed expression of BMP-7 protein in renal fibrosis [336]. In this study, these fibrotic mediators were expressed in the healthy tendons, and their expression declined with the progression of disease from tendinopathy to tear. The results indicate that there is a tissue-specific mechanism regulating the expression of TGF-β1, BMPs and CTGF.

Progression of fibrosis results in impaired function of the affected organs. Tendon disease differs from fibrotic diseases of other organs as the functional load diminishes at its end-stage of tear. Historically the clinical diagnosis of tendinopathy where the mechanical loading remains, has been distinguished as either acute, that is transient with features of acute inflammation (Neer stage I) or chronic with persistent and/or repetitive inflammation resulting

in fibrosis (Neer stage II) [53], which are not distinct. Comparison of healthy, acute (stage I) and chronic (stage II) tendinopathy may be more relevant when considering the implications from fibrotic diseases of other organs.

**Expression of TGF-β, BMPs and CTGF in Human Tendon Disease**

The expression of TGF-β, BMPs and CTGF in torn human tendons have not been thoroughly investigated. Goodier *et al.* previously showed suppression of TGF-β1, TGF-β R1 and TGF-β R2 proteins in torn supraspinatus tendon tissues [197]. Meanwhile, they reported a significantly decreased *TGFBR1*, increased *TGFBR2* and unchanged *TGFB1* mRNA expression in the torn compared to healthy tendons [197]. The changes in mRNA expression did not match the findings from this study. RT-qPCR is an established method to quantify mRNA in biological specimen, but it is known that imbalance in sample size may predispose to errors [337]. The number of investigated healthy and torn tendons were similar in the study by Goodier *et al.* [197], but the number of torn tendons (n=11) were substantially greater than that of healthy tendons (n=6) in this study. This was due to an intention to perform a subgroup comparison by the size of the tear. Despite these discrepancies, it remains convincing to consider a role of TGF-β and its receptors in the pathogenesis of tendon disease.

In tendinopathic tendons of the supraspinatus, patella and Achilles, protein expression of TGF-β1 has been reported to be decreased [197], increased [198] and unchanged [199], respectively. Two studies have shown increased *TGFB1* mRNA expression in tendinopathic compared to healthy Achilles tendon tissues [138, 195], but whether the up-regulation relates to the anatomy or pathobiology driven changes is unknown. Only 1 study each investigated mRNA and protein expression of BMPs in tendinopathic tendons. mRNA expression of *BMP2* was unchanged whereas increase in the expression of *BMP4* and *BMP6* was noted in

tendinopathic supraspinatus tendons [196]. Protein expression of BMP-2, BMP-4 and BMP-7 was up-regulated in patella tendinopathy [231]. Substantial roles of the numerous processes occurring after mRNA production from post-transcriptional modification, translation and degradation in the regulation of protein expression have been emphasised recently [338]. mRNA and protein expression do not necessarily correlate.

Only mRNA expression of *CTGF* has been studied in tendinopathic tendons, which all studies (2 studies) sampled tissues from the Achilles tendon [138, 195]. The 2 studies used similar methods, but the results were inconsistent, with 1 study showing decreased [138] and the other unchanged *CTGF* mRNA expression [195]. In this study, CTGF protein was stained in the ECM for 4 out of 5 patients, but there were no correlations between sex, age, total OSS or OSS function element (data shown in Appendix C.2, page 179, Table A5). These results may represent the heterogeneity of clinical samples of tendinopathic tendons although tissue specimens were obtained from clinically well-phenotyped patients. Tendinopathic patients have been known to be younger in the earlier (Neer stage I) compared to chronic (Neer stage II) stage, and although there was no significant difference in the age of the patients with positive and negative ECM staining of CTGF, tissue samples with heterogeneous cellular and molecular biology with different endotypes may have been grouped together in the tendinopathy cohort for this study. Another possibility is that the ECM staining is non-specific and there are no correlations between the location of the staining and these patient factors.

**Expression of TGF-β, BMPs and CTGF in Animal Tendon Injury Models**

mRNA and protein expression of TGF-β, BMPs and CTGF has been studied in numerous animal models of tendon injury, where tendons have either been repaired or left for spontaneous healing. Although the timing after the intervention when tissue samples were harvested for

investigation differed between studies, both mRNA and protein expression of TGF-β and its receptors was increased along the course of healing [193]. Interestingly, there were no studies that reported suppressed protein expression of TGF-β, its receptors, BMPs and CTGF over the investigated periods. The majority of the studies used the Achilles transection model in rodents [193], 2 studies created a partial injury to the patella mechanically or using collagenase [235, 339], and the injured digital flexors were repaired surgically [281, 285, 340]. The Achilles tendon may regenerate even after its total removal [341], and hence it could be anticipated that mechanical loading of the tendon was not completely removed in these animal studies reviewed. The study by Dahlgren *et al*. is the only large animal model of tendon injury healing studying TGF-β expression. This used a collagenase-induced lesion in equine flexor digitorum superficialis tendons. mRNA and protein expression of TGF-β1 was increased similar to the rodent models [342]. As changes in the mechanical loading of the tendon affects the expression of TGF-β [104], it is conceivable that these animal models do not replicate tendon tears in humans. It would be of interest to investigate how the expression of these mediators changes by removing the tensile force in animal models for a lengthy period alike human tendon tear.

**Expression of TGF-β, BMPs and CTGF and OSS**

In this study, significant down-regulation in the expression of all the investigated proteins (TGF-β1, TGF-β R1, TGF-β R2, BMP-2, BMP-7, BMP-12 and CTGF) compared to healthy tissues were indicated in torn tissues, but statistically significant differences between healthy and tendinopathic tissues were noted only in TGF-β1 and TGF-β R2. Meanwhile, there were no significant correlations between all the investigated proteins except TGF-β R1 and both the total OSS and its function element (data shown in Appendix C.2, page 179, Table A5). OSS is a validated and reliable scale that represents patient well-being [289]. Both total OSS and its

function elements significantly decreased along disease progression from healthy, tendinopathy to tear in the investigated patients. However, protein expression of these fibrotic mediators would not be markers of disease progression. Still, it may be plausible to deem that the protein expression of TGF-β1, TGF-β R1, TGF-β R2, BMP-2, BMP-7 and BMP-12 decline as the tendon disease protract and progress from tendinopathy to tear.

**Limitations of the Study**

The number of samples were based on availability rather than performing a power calculation to determine necessary sample size for both mRNA and protein expression analyses due to the difficulties in obtaining abundant clinical tissue specimen. Ethical feasibility and the nature of acute and transient inflammation in acute tendinopathy (Neer stage I) limited the investigation to possibly predominantly chronic tendinopathy (Neer stage II) patients in the tendinopathy cohort. The tissues utilised for the healthy cohort for mRNA expression analysis were from the hamstring tendon, who were significantly younger than patients with tendinopathic or torn tendons. Some previous studies have sampled 'healthy' tissues form a macroscopically intact region of the same tendon [138, 194, 195] or from cadaveric tissues samples [199] for mRNA expression analysis of similar fibrotic mediators. However, whether molecular and cellular biology of macroscopically intact tendons from affected tendons are comparable to that of healthy tendons have not been thoroughly validated, nor have the effects of post-mortem changes. Miller *et al.* have shown inflammatory cell infiltration that is consistent with tendinopathy in anatomically distinct and macroscopically, arthroscopically or ultrasonographically intact subscapularis tendons of patients with supraspinatus tears [343]. The hamstring tendon which, like the rotator cuff, is a positional tendon has also been used as an ethically feasible source to obtain 'healthy' tendon samples [197, 343]. Nevertheless, the

differences in the anatomy and age of the donors should be considered when interpreting the results of the mRNA and protein expression analysis.

## 3.5 Conclusions

The protein expression of TGF-β, BMPs and CTGF was down-regulated in diseased tendons, most prominently in torn tendons. The size of the tear was not a significant factor in their expression. Diminished expression of these mediators has not been reported in other fibrotic diseases, which indicate that the pathobiology of fibrosis is specific to the tendon. Further investigation in elucidating its mechanism would be warranted to determine whether the marked reduction is due to a functional depression to produce these growth factors or a protective mechanism to prevent development of further abnormal tissue since their dysregulation results in fibrosis.

# Chapter 4 Differential Expression of TGF-β and BMPs in Stromal Cells from Diseased and Healthy Tendons

## 4.1 Introduction

### 4.1.1 Background

The previous chapter suggested that the protein expression of TGF-β1, BMPs and CTGF decline from healthy, tendinopathy to tear, with a marked suppression in torn tendons that represent end-stage disease. Emerging evidence supports the regulatory roles of cytokines in the development and progression of tendon disease [4, 344]. Studies using tissue samples collected from patients with tendon disease have identified an abnormal tissue inflammation signature that transits from expression of IL-1β target genes and proteins in early tendon disease of tendinopathy, to IL-4 target genes and proteins along disease progression to tear [52, 53]. Animal studies demonstrate that stromal cells derived from injured tendons show an altered expression of genes related to the ECM (*Mmp13*) in response to IL-1β treatment *in vitro* [92]. However, how cytokines regulate the expression of fibrotic mediators and whether there is a difference between cells derived from healthy and diseased tendons have not been investigated thus far.

### 4.1.2 Aim, Objectives and Hypotheses

The aim of this study was to elucidate the mechanism by which inflammatory cytokines regulate the expression of TGF-β, BMPs and CTGF in tendons using human clinical samples by an *in vitro* cell culture model.

It was hypothesised that inflammatory cytokine treatment of TSCs would regulate the expression of TGF-β1, BMPs and CTGF. It was also hypothesised that the extent and direction of this regulation, and the signalling pathways activated is dependent on the disease status of the tendon from which the cells are derived from.

In the first part of the study, TSCs derived from healthy and torn tendons that represent end-stage disease (diseased) were treated with IL-4. Differences in mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2*, *BMP7*, *BMP12* and *CTGF* between the two cell cohorts were investigated. It was anticipated that the tissue inflammation signature characterised by IL-4 activity may relate to the marked suppressed expression of TGF-β1, BMPs and CTGF in torn tendons.

In the second part, a similar experiment was carried out to investigate how TSCs from healthy and diseased tendons respond to IL-1β, a cytokine involved in early tendon disease and acute inflammation. Investigating how TSCs from diseased (torn) tendons respond to IL-1β was thought to be beneficial as torn tendons are frequently treated surgically, which induces acute inflammation and hence IL-1β. TSCs from healthy and diseased tendons were treated with IL-1β and differences in mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2*, *BMP7*, *BMP12* and *CTGF* between the two cell cohorts were compared. Cell signalling pathways responsible for the differences in mRNA expression between TSCs from healthy and diseased tendons if there were any were also defined and confirmed using a small molecule inhibitor of that pathway.

## 4.2 Materials and Methods

### 4.2.1 Patient Cohort

Surplus hamstring tendon tissues of patients undergoing autograft ACL reconstruction surgery were obtained under ethical approval from the OMB and utilised as the healthy cohort. Diseased tendon tissues were obtained from patients with a large to massive supraspinatus tendon tear from OMB and through the SECuRe study. Healthy and torn supraspinatus tendon tissues for protein expression analysis by IHC were obtained through the WASP and UKUFF studies, respectively.

### 4.2.2 Tendon Stromal Cell Isolation and Culture

Human TSCs were isolated by explant culture as described in Chapter 2. TSCs cultured up to passage 2 were used for experiments. TSCs were seeded in 24-well plates at a density of 30,000 cells $ml^{-1}$ (0.5 ml) and cultured to sub-confluence for the mRNA expression study. For analysis of cell signalling pathway activation by Western blotting, TSCs were seeded in 6-well plates at a density of 60,000 cells $ml^{-1}$ (2 ml) and cultured to sub-confluence.

### 4.2.3 Cell Treatment

Following serum starving overnight, TSCs were treated with human recombinant IL-4 (10 ng $ml^{-1}$) or IL-1β (5 ng $ml^{-1}$) in serum-free medium or vehicle control (serum-free medium) for 24 hours and 30 minutes for the mRNA expression and cell signalling pathway activity analysis experiments, respectively. The treatment duration and dosages were determined by validation experiments (data shown in Appendix C.3, pages 180-183, Figures A7-A12). For the inhibition experiment, TSCs were pre-treated with a small molecule inhibitor of the ERK1/2 pathway

(PD98059, 8 μg $ml^{-1}$ (30 μM)) for 1 hour before cytokine treatment based on validation experiments (data shown in Appendix C.3, pages 184-186, Figures A13-A15). Vehicle only treatments served as experimental controls.

### 4.2.4 mRNA Expression and Cell Signalling Pathway Activity Analysis

The processing of cell lysates of mRNA extraction, purification, conversion to cDNA, quantitative analysis of synthesised cDNA by RT-qPCR, preparation of protein lysates, protein separation by electrophoresis, Western blotting, image acquisition and analysis are described in Chapter 2. Details of the primers and antibodies used are also listed in Chapter 2.

### 4.2.5 Immunohistochemistry

Histopathological procedures from tissue preparation, IHC, image acquisition and analysis have been described in Chapter 2. Details of the antibodies used are also listed in Chapter 2.

### 4.2.6 Statistical Analysis

Statistical analysis was performed using Prism 5 for Mac OS X Version 5.0b (GraphPad Software Inc., La Jolla, CA). D'Agostino and Pearson normality test was carried out to determine normal distribution for each data. Differences in patient demographics were compared using parametric tests. Differences in mRNA expression between treatments were compared by Mann Whitney test as the data were not always normally distributed. Differences in protein expression between healthy and diseased tendon tissues were also compared by Mann Whitney test as the numbers were small (less than 8). Statistical significance was set at $p<0.05$.

## 4.3 Results

### 4.3.1 Patient Demographics

Experiments were performed using TSCs isolated from tendons of healthy donors (n=10) and diseased patient donors (n=10) (Table 4-1). Donors of healthy tendons were significantly younger than that of diseased tendons ($p<0.0001$). There was no significant difference in the ratio of males and females in the 2 patient groups.

**TABLE 4-1: Summary of patient demographics for mRNA expression analysis.** Mean (SD) shown for age as the data were normally distributed. * indicates significant difference to the healthy group. SD, standard deviation. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

| | Healthy | Diseased |
|---|---|---|
| Number of patients | 10 | 10 |
| Age; mean (SD) years | 24.6 (7.73) | 62.7 (7.83) **** |
| Sex; male/female, n | 6/4 | 7/3 |

### 4.3.2 Effects of IL-4 on Expression of Fibrotic Mediators

#### 4.3.2.1 IL-4 suppressed mRNA Expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* & *CTGF* in TSCs from diseased tendons

TSCs derived from healthy and diseased tendons from 10 patients each were treated with IL-4 or vehicle control to determine the effects of IL-4 on mRNA expression of fibrotic mediators (*TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2*, *BMP7*, *BMP12* and *CTGF*) by RT-qPCR. Genes of interest were normalised to the geometric mean of the expression values of *ACTB* and *GAPDH* as a reference of the internal control using the comparative $C_T$ method (Figure 4-1). The results for individual housekeeping genes are shown in Appendix C.3, pages 188-189, Figures A18-19. The results for *BMP7* and *BMP12* are not presented due to the low-level expression in TSCs from both healthy and diseased tendons regardless of treatment.

IL-4 treatment suppressed *TGFB1*, *TGFBR1*, *TGFBR2* and *BMP*2 mRNA expression compared to vehicle control in TSCs from diseased tendons ($p=0.0219$, 0.0147, 0.0024 and 0.0301, respectively). IL-4 treatment suppressed *CTGF* mRNA expression compared to vehicle control in TSCs from healthy and diseased tendons ($p<0.0001$ and 0.0018, respectively). IL-4 treatment induced *TGFB1*, *TGFBR1* and *BMP*2 mRNA expression compared to vehicle control in 2 donors of the healthy cohort (these 2 donors can be noticed as outliers in Figure 4-1). Graphs with the data points for each donor linked are shown in Appendix C.3, page 187, Figure A17. However, age or sex of these 2 donors did not significantly differ from those of other donors.

STAT-6 signalling pathway activities by IL-4 treatment measured by Western blotting did not differ between TSCs from healthy and diseased tendons (data shown in Appendix C.3, page 192, Figure A22).

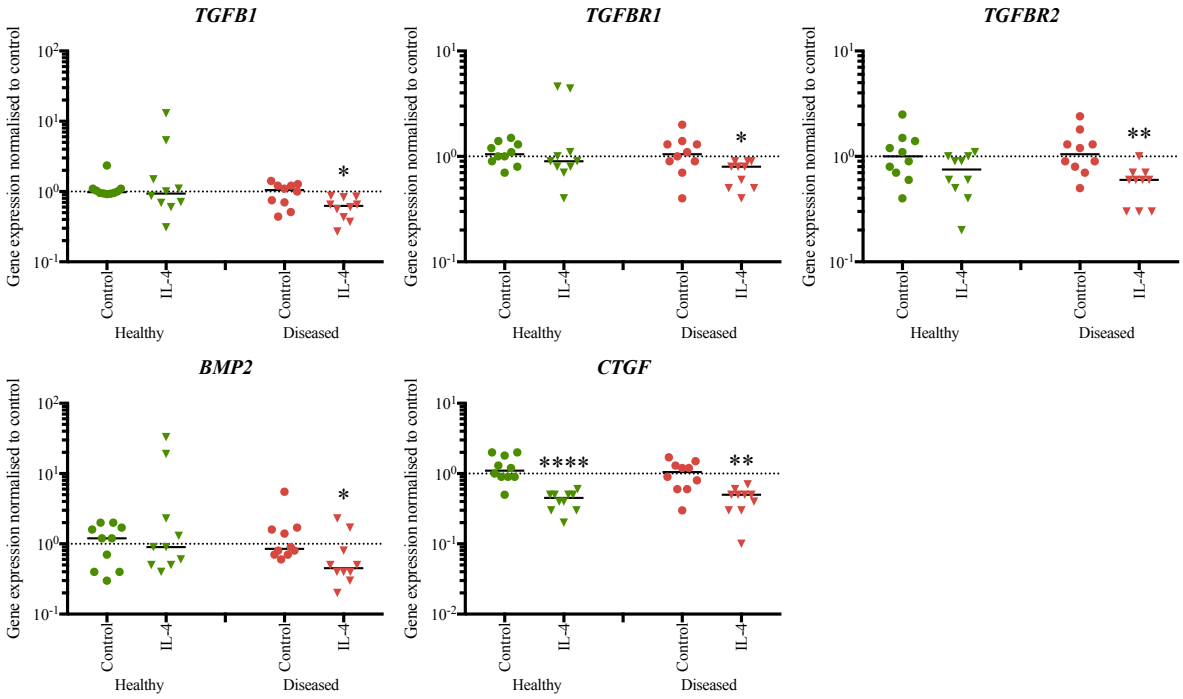



**FIGURE 4-1: mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* in cells derived from healthy and diseased tendons after treatment by IL-4 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=10) and diseased tendons (n=10) were quantified by RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. *=p<0.05, **=p<0.01, ***=p<0.001.

### 4.3.3 Effects of IL-1β on Expression of Fibrotic Mediators

#### 4.3.3.1 Increased ERK1/2 signalling pathway activity implicated in TSCs from diseased tendons

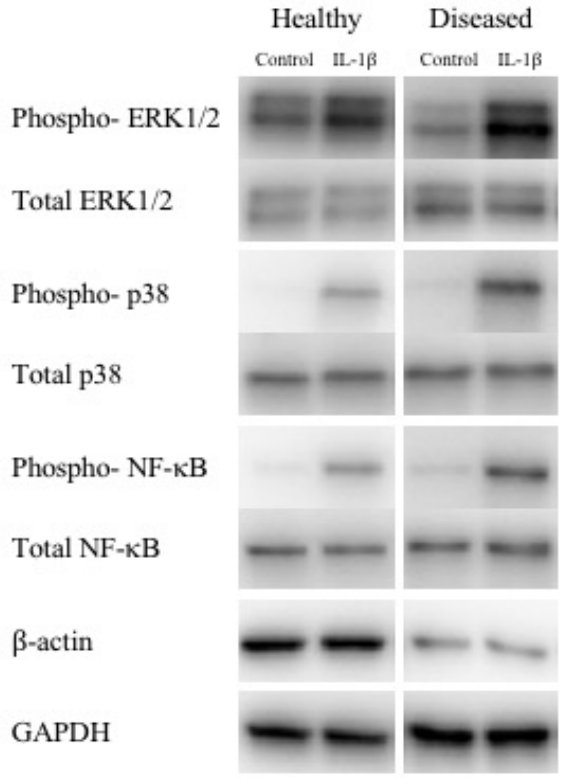
Pilot experiments evaluating mRNA expression of fibrotic mediators indicated TSCs from healthy and diseased tendons have differential responsiveness to IL-1β treatment *in vitro*; IL-1β treatment induced *TGFB1* mRNA expression compared to vehicle control in TSCs from diseased but not healthy tendons (data shown in Appendix C.3, page 186, Figure A16).

Consequently, cell signalling pathway activation of the 4 major pathways of IL-1β (ERK1/2, p38, SAPK/JNK and NF-κB) were investigated to determine the pathway responsible

for the altered mRNA expression observed in TSCs from diseased compared to healthy tendons. TSCs from healthy and diseased tendons from 3 patients each were treated with IL-1β and cell signalling pathway activities were measured by Western blotting. Expression levels of phosphorylated proteins were normalised to that of their respective total proteins or housekeepers β-actin and GAPDH. Representative images of the Western blots are shown in Figure 4-2-A (all obtained images are shown in Appendix C.3, pages 195-196, Figure A25). Expression levels of phosphorylated relative to their respective total proteins are presented in Figure 4-2-B. The results relative to β-actin and GAPDH followed similar patterns (data shown in Appendix C.3, page 193, Figure A23).

IL-1β treatment induced expression of phosphorylated ERK1/2, p38 and NF-κB in TSCs from healthy and diseased tendons. IL-1β treatment showed stronger induction of signalling activities in TSCs from diseased compared to healthy tendons, with 2.30-, 2.15- and 1.30-fold increases for ERK1/2, p38 and NF-κB, respectively. There were no significant differences in the expression levels of the total proteins of interest between TSCs from healthy and diseased tendons (data shown in Appendix C.3, page 193, Figure A23). The results of SAPK/JNK are not presented due to the low-level expression of its phosphorylated protein in human TSCs (data shown in Appendix C. 3, page 197, Figure A26).

(A)




(B)

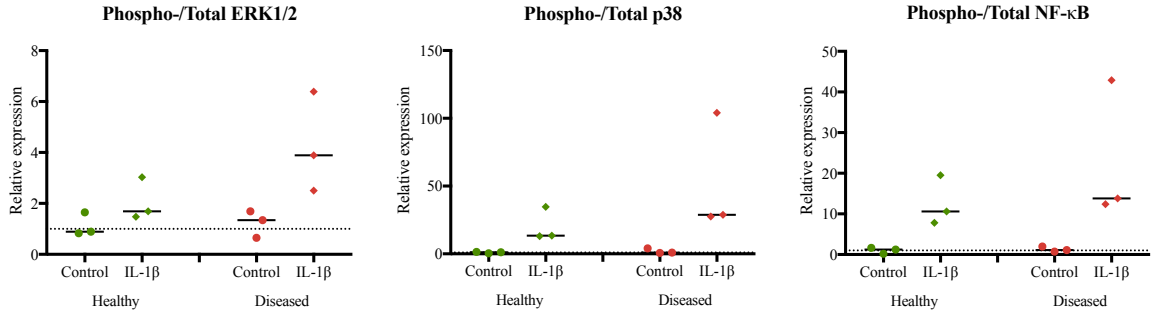



**FIGURE 4-2: Western blotting showing cell signalling activities of ERK1/2, p38, and NF-κB pathways in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control). (**A) Representative images of Western blotting for phospho- and total ERK1/2, p38, NF-κB, and housekeepers β-actin and GAPDH in tendon stromal cells from healthy and diseased tendons after treatment by IL-1β or vehicle control. (B) Quantitative analysis of phospho- ERK1/2, p38, and NF-κB expression in tendon stromal cells from healthy (n=3) and diseased tendons (n=3). Levels of cell signalling activities were quantified by measuring the intensities of Western blots and normalised to their respective total protein. Bars shown represent median.

#### 4.3.3.2 IL-1β induced mRNA expression of *TGFB1* and *TGFBR2* only in TSCs from diseased tendons

IL-1β treatment induced *TGFB1* mRNA expression compared to vehicle control in TSCs from diseased tendons only ($p<0.0001$) (Figure 4-3). IL-1β treatment did not significantly modulate *TGFB1* in TSCs from healthy tendons and *TGFBR2* mRNA expression in TSCs from both healthy and diseased tendons.

The effects of IL-1β treatment on inducing *TGFBR1* or *BMP2*, and suppressing *CTGF* mRNA expression did not differ between TSCs from healthy and diseased tendons; IL-1β treatment induced *TGFBR1* ($p<0.0001$ and $p=0.0011$, respectively), *BMP2* ($p<0.0001$ and $p<0.0001$, respectively) and suppressed *CTGF* ($p=0.0002$ and $p<0.0001$, respectively) mRNA expression compared to vehicle control.

#### 4.3.3.3 Inhibition of the ERK1/2 signalling pathway suppressed IL-1β-induced mRNA expression of *TGFB1* and *TGFBR2* in TSCs from diseased tendons

To determine if the ERK1/2 signalling pathway drives the altered IL-1β-induced mRNA expression in TSCs from diseased tendons, cells of both cohorts were treated with a small molecule inhibitor of the ERK1/2 pathway (PD98059).

Inhibition of the ERK1/2 pathway completely suppressed the effects of IL-1β treatment on inducing *TGFB1* expression in TSCs from diseased tendons ($p=0.0021$). Inhibition of the ERK1/2 pathway did not significantly modulate IL-1β-induced *TGFB1* mRNA expression in TSCs from healthy tendons, and IL-1β-induced *TGFBR1*, *TGFBR2* or *CTGF* mRNA expression in TSCs from both cohorts.

#### 4.3.3.4 Inhibition of the ERK1/2 signalling pathway partially suppressed IL-1β-induced mRNA expression of *BMP2* and NF-κB target genes in TSCs from diseased tendons

Inhibition of the ERK1/2 pathway partially suppressed the effects of IL-1β treatment on inducing *BMP2* mRNA expression in TSCs from diseased tendons (p=0.0019). BMP-2 is a target gene of NF-κB pathway. Inhibition of the ERK1/2 pathway had similar effects on IL-1β-induced mRNA expression of other NF-κB target genes *PTGES* and *COX2* in TSCs from diseased tendons (p=0.0005 and 0.0232, respectively). Inhibition of the ERK1/2 pathway did not significantly modulate IL-1β induced *BMP2*, *PTGES* or *COX2* mRNA expression in TSCs from healthy tendons.

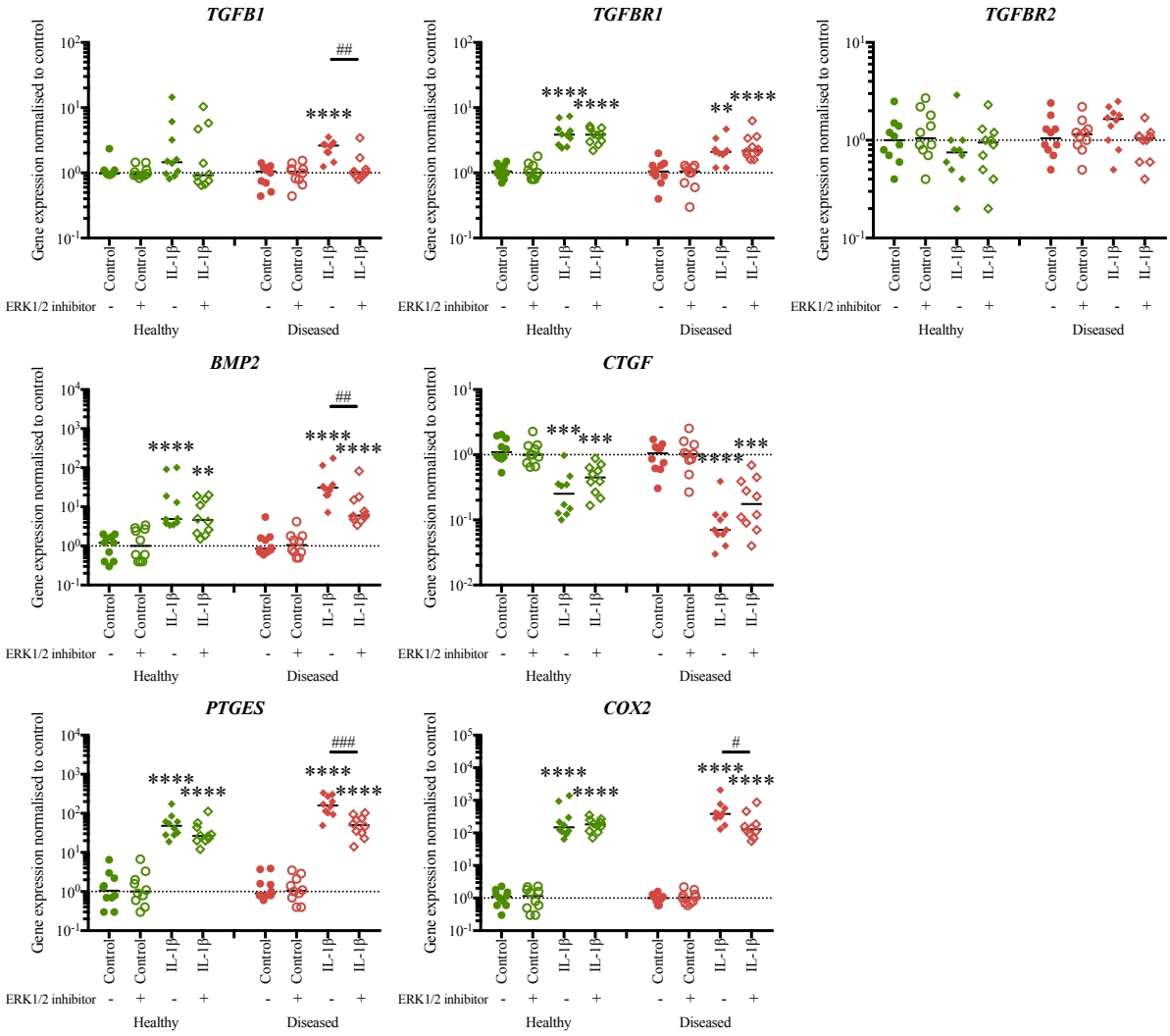



**FIGURE 4-3: mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2*, *CTGF*, *PTGES* and *COX2* in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control) with or without an ERK1/2 inhibitor.** Levels of gene expression in tendon stromal cells from healthy (n=10) and diseased tendons (n=10) were quantified by RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control without the ERK1/2 inhibitor ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between with and without the ERK1/2 inhibitor. */#=p<0.05, **/##=p<0.01, ***/###=p<0.001, ****/####=p<0.0001.

#### 4.3.3.5 Inhibition of the ERK1/2 signalling pathway increased IL-1β-induced expression of canonical TGF-β target genes in TSCs from healthy and diseased tendons

Inhibition of the ERK1/2 pathway completely suppressed the effects of IL-1β treatment on inducing *TGFB1* mRNA expression in TSCs from diseased tendons. Subsequently, the consequences of ERK1/2 inhibition on the signalling activities of canonical TGF-β and BMP pathways were investigated by measuring the mRNA expression of their target genes *SERPINE1* [345] and *ID1* [346], respectively (Figure 4-4).

IL-1β treatment induced *SERPINE1* mRNA expression compared to vehicle control in TSCs from healthy tendons only ($p=0.0330$). Inhibition of the ERK1/2 pathway up-regulated *SERPINE1* mRNA expression compared to IL-1β treatment alone in TSCs from healthy and diseased tendons ($p=0.0048$ and 0.0005, respectively). The effects of the ERK1/2 inhibitor on up-regulating IL-1β-induced *SERPINE1* mRNA expression was statistically significant compared to vehicle control in TSCs from healthy and diseased tendons ($p<0.0001$ and $p=0.0031$, respectively).

IL-1β treatment induced *ID1* mRNA expression compared to vehicle control in TSCs from healthy tendons only ($p=0.0063$). Inhibition of the ERK1/2 pathway down-regulated *ID1* mRNA expression compared to IL-1β treatment alone in TSCs from healthy and diseased tendons ($p=0.0004$ and $p<0.0001$, respectively). The effects of the ERK1/2 inhibitor on down-regulating IL-1β-induced *ID1* mRNA expression was statistically significant compared to vehicle control in TSCs from healthy and diseased tendons ($p=0.0192$ and $p=0.0001$, respectively).

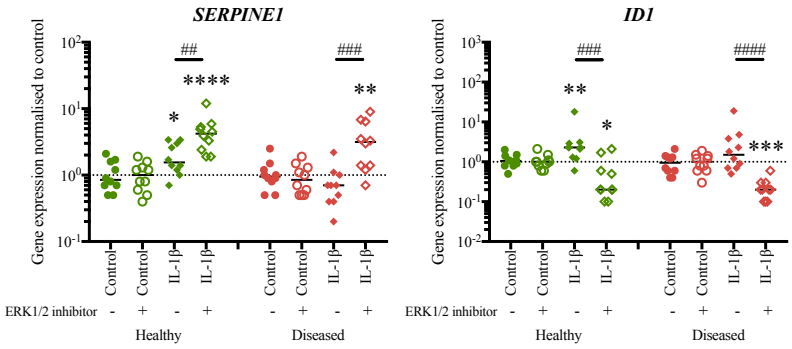



**FIGURE 4-4: mRNA expression of *SERPINE1* and *ID1* in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control) with or without an ERK1/2 inhibitor.** Levels of gene expression in tendon stromal cells from healthy (n=10) and diseased tendons (n=10) were quantified by RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control without the ERK1/2 inhibitor ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between with and without the ERK1/2 inhibitor. */#=p<0.05, **/##=p<0.01, ***/###=p<0.001, ****/####=p<0.0001.

#### 4.3.3.6 Role of TGIF implicated in the suppressed TGF-β signalling pathway activity by IL-1β in TSCs from diseased tendons

TGF-induced factor (TGIF) is an active transcriptional co-repressor of SMAD2, a key transducer of the canonical TGF-β signalling pathway. TGIF is induced by the activation of ERK1/2 in fibroblasts [347]. mRNA expression of *TGIF1* was measured with the hypothesis that the increased ERK1/2 signalling pathway activity by IL-1β treatment in TSCs from diseased tendons would result in an up-regulation of *TGIF1* mRNA expression and subsequent suppression of TGF-β signalling pathway activities (Figure 4-5).

IL-1β treatment induced *TGIF1* mRNA expression compared to vehicle control in TSCs from healthy and diseased tendons ($p<0.0001$ and $p<0.0001$, respectively). Inhibition of the ERK1/2 pathway down-regulated *TGIF1* mRNA expression compared to IL-1β treatment alone in TSCs from diseased tendons only ($p=0.0184$). Inhibition of the ERK1/2 pathway did not significantly modulate IL-1β induced *TGIF1* mRNA expression in TSCs from healthy tendons.

These results indicate that *TGIF1* mRNA expression induced by IL-1β treatment is up-regulated in TSCs from diseased tendons through the increased ERK1/2 signalling pathway activity.

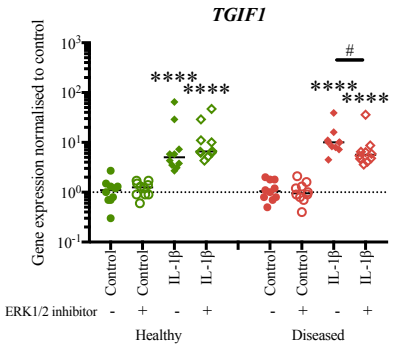


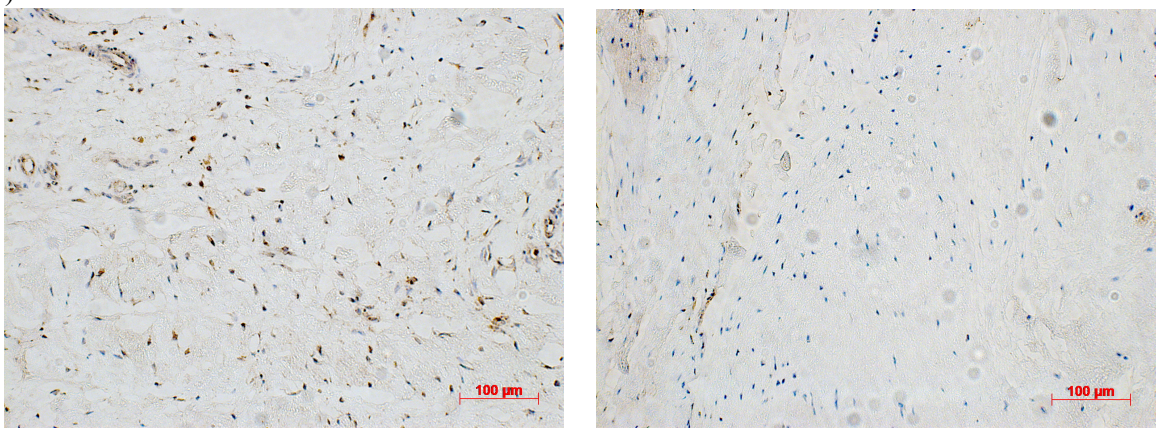

**FIGURE 4-5: mRNA expression of *TGIF1* in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control) with or without an ERK1/2 inhibitor.** Levels of gene expression in tendon stromal cells from healthy (n=10) and diseased tendons (n=10) were quantified by RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control without the ERK1/2 inhibitor ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between with and without the ERK1/2 inhibitor. */#=p<0.05, **/##=p<0.01, ***/###=p<0.001, ****/#####=p<0.0001.

### 4.3.4 Protein Levels of Phosphorylated ERK1/2 is Suppressed in Diseased Tendon Tissues

The ERK1/2 pathway was shown to be a key pathway driving the altered mRNA expression in response to IL-1β treatment in TSCs from diseased tendons. Hence, differences in the expression of active (phosphorylated) ERK1/2 between supraspinatus tendon tissues collected form healthy (n=7) and diseased (large to massive tear, n=6) patients were investigated by IHC. Donors of healthy tendons were significantly younger than that of diseased tendons (p=0.0012). There was no significant difference in the ratio of males and females in the two groups of patients.

Representative images of phosphorylated ERK1/2 staining are shown in Figure 4-6-A (data including tendinopathic and torn (small to medium tear) tissues are shown in Appendix C. 3, pages 198-199, Figure A28). Quantitative analysis of immunopositive staining showed a 3.64-fold decreased expression of phosphorylated ERK1/2 in diseased compared to healthy tendons (p=0.0047) (Figure 4-6-B).

(A)




(B)

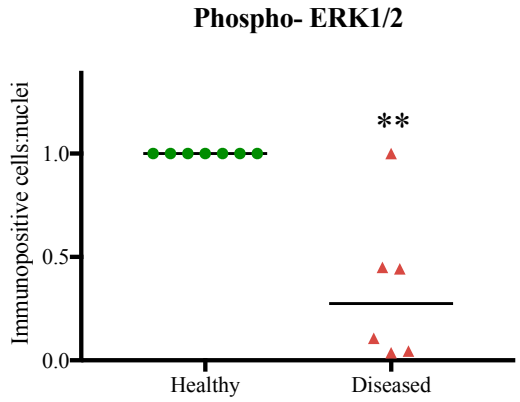



**FIGURE 4-6: Immunohistochemistry showing expression of phosphorylated (phospho-) ERK1/2 in healthy and diseased (torn) tendon tissues.** (A) Representative images of healthy and diseased supraspinatus tendon sections with haematoxylin (blue) for nuclei and DAB (brown) for immunopositive staining. Scale bar =100 µm. (B) Quantitative analysis of phospho- ERK1/2 immunohistochemical staining in healthy (n=7) and diseased (n=6) tendon tissues. Levels of protein expression were quantified as the number of immunopositive cells to the number of nuclei. Bars shown represent median. * indicates significant difference to the healthy group. *=p<0.05, **=p<0.01.

## 4.4 Discussion

The aim of the study was to elucidate the mechanism by which inflammatory cytokines regulate the expression of TGF-β, BMPs and CTGF in human TSCs in relation to tendon disease. As hypothesised, the results of this study showed that TSCs derived from diseased tendons exhibit dysregulated expression of fibrotic mediators in response to inflammatory cytokine treatments *in vitro*. IL-4 treatment suppressed *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2*, and *CTGF* mRNA expression compared to vehicle control in TSCs from diseased tendons.

IL-1β treatment induced *TGFB1* and *TGFBR2* mRNA expression compared to vehicle control in TSCs from diseased tendons only. IL-1β treatment induced mRNA expression of NF-κB target genes such as *BMP2* in TSCs from healthy and diseased tendons. Increased ERK1/2 signalling pathway activity was implicated in TSCs from diseased tendons; inhibition of the ERK1/2 pathway completely suppressed the effects of IL-1β treatment on inducing *TGFB1* mRNA expression, and partially suppressed IL-1β-induced mRNA expression of NF-κB target genes in TSCs from diseased tendons.

**IL-4 suppresses mRNA expression of fibrotic mediators in TSCs from diseased tendons**

IL-4 is known to have proliferative [157] and anti-inflammatory effects on (mesenchymal) stromal cells [156, 348]. However, in TSCs from healthy and diseased tendons IL-4 treatment suppressed the expression of fibrotic mediators that promote cell proliferation, ECM synthesis and, in the case of TGF-β1 could also be anti-inflammatory. The suppressive effects of IL-4 treatment on *TGFB1*, *TGFBR1*, *TGFBR2* and *BMP2* mRNA expression were statistically significant compared to vehicle control in TSCs from diseased tendons. Similar effects of IL-4 treatment on *TGFB1* mRNA expression have been shown in fibroblasts and stromal cells derived from rat synovium *in vitro* [349].

The activation of STAT-6 signalling pathway in response to IL-4 treatment was confirmed by Western blotting but how another cytokine IL-13 that also activates this pathway contributes to the expression of the fibrotic mediators was not explored. IL-4 and IL-13 are secreted by similar leukocytes and share many functional activities [123]. Studies using IL-4 knock-out mice have shown that IL-4 is indispensable for tendon development [124] but IL-4 deficiency could be made up for by the compensatory up-regulation of IL-13 in injury healing in a developed tendon [123]. The differential mechanisms of IL-4 and IL-13 are yet to be defined but there are possibilities that the two cytokines interact, or the role of IL-13 increases especially after development. Future work should investigate how TSCs from healthy and diseased tendons respond to IL-13 in the mRNA expression of TGF-β and BMPs, especially with regards to the effects of IL-4 on suppressing these genes.

The results from this study imply that the tissue inflammation signature may take part in regulating the expression of fibrotic mediators. However, whether this represents diminished capacity to express these mediators or is a protective mechanism to prevent perpetuation of fibrosis is yet to be determined. Nevertheless, consideration of the inflammatory milieu as a whole with IL-4 and possibly IL-13 as the potential regulators of the tissue inflammation signature should be pursued. How mRNA expression of fibrotic mediators translates to their protein expression have not been investigated and future work should also address this.

**IL-1β induced mRNA expression of *TGFB1* and *BMP2* in TSCs from diseased tendons through increased ERK1/2 signalling pathway activity**

It is noteworthy that TSCs from diseased compared to healthy tendons show increased ERK1/2 signalling pathway activity, which drives the altered mRNA expression of fibrotic mediators (*TGFB1* and *BMP2*). Similar findings have been reported in animal studies of rat supraspinatus

tendinopathy models [46]. Furthermore, Chen *et al.* reported the Erk1/2 mediated up-regulation of *Tgfb1* mRNA expression by low-level laser therapy in rat Achilles TSCs [350]. However, IHC data in this study showed that the level of phosphorylated ERK1/2 is significantly suppressed in diseased compared to healthy tendon tissues. This is in agreement with the lower overall expression levels of phosphorylated ERK1/2 in TSCs from diseased tendons detected by Western blotting in this study (the intensities of the blots in response to treatment by vehicle control tended to be lower in TSCs from diseased compared to healthy tendons macroscopically. Images shown in Appendix C.3, pages 195-196, Figure A25). These findings along with the results from the previous chapter suggest that the expression of fibrotic mediators and their regulatory mechanism are generally suppressed. The increased ERK1/2 signalling pathway activity in TSCs from diseased tendons could be to compensate for the diminished basal ERK1/2 activity in unstimulated cells and tissues that results in a secondary up-regulation of *TGFB1* and *BMP2* mRNA expression. It could also be discussed as a protective mechanism to prevent further development of abnormal tissue as their dysregulation results in fibrosis. Investigating how TSCs from diseased tendons respond to TGF-β and BMPs may further elucidate the underlying pathobiology of tendon disease.

It has been widely shown that *BMP2* is a NF-κB pathway target gene alike *COX2* and *PTGES* [351-354]. IL-1β treatment induced the mRNA expression of NF-κB target genes in TSCs from healthy and diseased cells. In TSCs from diseased tendons, the effects of IL-1β treatment on inducing these genes were up-regulated due to the increased induction of ERK1/2 signalling pathway activity. Opposingly, ERK1/2 signalling pathway has been reported to negatively regulate NF-κB dependent gene expression in a monocytic cell line (THP-1) [355]. These differences suggest that TSCs from diseased tendons have an altered cell signalling mechanism in response to ERK1/2-activating cytokines, resulting in dysregulated expression of their target genes. Moreover, Carter *et al.* have also indicated the role of p38 in the regulation

of NF-κB-driven transcription in conjunction with ERK1/2 signalling pathway activity [355]. Whether and how p38 signalling pathway contributes to human tendon disease is yet to be explored.

The increase in NF-κB signalling pathway activity was less prominent compared to that of the ERK1/2 or p38 signalling pathways in TSCs from diseased tendons. Furthermore, inhibition of the ERK1/2 pathway did not significantly modulate the levels of phosphorylated NF-κB by Western blotting in TSCs from diseased tendons (data shown in Appendix C.3, pages 194-195, Figure A24). Hence, a mechanism downstream of NF-κB phosphorylation that regulates its target gene expression was considered. Activator protein 1 (AP-1) is a transcription factor induced by MAPKs such as ERK1/2, p38 and JNK [356]. It exists as either a homo- or a heterodimer of proteins of the Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fos-related antigen 1and 2 (Fra1 and Fra2)) families. c-Jun interacts with NF-κB to regulate target gene expression [357, 358]. The activation of c-Jun by Western blot was investigated with an anticipation that the increased activity of ERK1/2 signalling pathway would induce c-Jun activity and result in up-regulated NF-κB target gene expression. However, substantial levels of phosphorylated c-Jun could not be detected by Western blotting in TSCs from both healthy and diseased tendons, especially in the latter (images shown in Appendix C.3, page 198, Figure A27). This suggests minor activity of SAPK/JNK in human TSCs at least at baseline and in response to IL-1β treatment *in vitro*.

TGIF is an active transcriptional corepressor of SMAD2 that inhibits the activity of the canonical SMAD signalling pathway of TGF-β [359]. TGIF expression is regulated by the ERK1/2 signalling pathway via the activator EGF [347, 360]. In this thesis IL-1β treatment activated the ERK1/2 signalling pathway and induced subsequent *TGIF1* mRNA expression in TSCs from healthy and diseased tendons. In TSCs from diseased tendons, further up-regulation

of *TGIF1* mRNA expression was indicated with increased ERK1/2 signalling pathway activity. Previous studies using epithelial cell lines (HKC and HaCaT) have shown that regulation of TGIF expression by the ERK1/2 signalling pathway is at the post-translational level [347, 361]. Conversely, the results from this study showed that the ERK1/2 signalling pathway also regulates TGIF expression at the transcriptional level at least in TSCs from diseased tendons. IL-1β treatment did not induce *SERPINE1* mRNA expression, a target gene of the canonical SMAD signalling pathway by TGF-β compared to vehicle control in TSCs from diseased tendons. However, IL-1β treatment with the ERK1/2 inhibitor induced *SERPINE1* and suppressed *TGIF1* mRNA expression in TSCs from diseased tendons to a level similar to that of TSCs from healthy tendons. These results suggest that the suppressed *SERPINE1* mRNA expression by IL-1β treatment in TSCs from diseased tendons is due to the induced *TGIF1* mRNA expression driven by increased ERK1/2 signalling pathway activation. The increased ERK1/2 signalling pathway activation in response to IL-1β treatment in TSCs from diseased tendons up-regulates *TGFB1* mRNA expression but down-regulates mRNA expression of canonical SMAD signalling pathway target genes. The regulation of the TGF-β axis is complex.

**IL-4 and IL-1β suppressed mRNA expression of *CTGF* in TSCs from healthy and diseased tendons**

A study using a rat flexor digitorum tendinopathy model suggested that IL-1β induces CTGF protein expression by activation of substance P [110]. However, studies using a rabbit flexor digitorum tendon injury model have shown that IL-1β mRNA and protein expression is induced but *CTGF* mRNA expression does not change in tissues of injured tendons [285, 362]. *In vitro* experiments treating different cells such as rat mesangial cells, human dermal fibroblasts, human healthy and diseased (dihydropyridine-induced gingival overgrowth) gingival

fibroblasts with IL-1β all had opposing results in *CTGF* mRNA expression [363-365]. The effects of IL-1β on *CTGF* mRNA expression is thus cell and context specific, and the interpretation of the results from this study should not be expanded beyond the comparison of human TSCs from healthy and diseased tendons.

CTGF acts as a downstream regulator of TGF-β by controlling the binding of TGF-β to its receptor [274, 366]. With regards to the induced *TGFB1* mRNA expression by IL-1β treatment in TSCs from diseased tendons, it could be hypothesised that *CTGF* mRNA expression in response to IL-1β treatment is suppressed simultaneously so that further activation of TGF-β is prevented. It is also possible that IL-1β treatment increases relative *TGFB1* compared to *CTGF* mRNA expression in TSCs from diseased compared to healthy tendons such that TGF-β activity prevails over that of CTGF. Future work should investigate whether such a protective mechanism exists by measuring how TSCs from healthy and diseased tendons respond to TGF-β, BMPs and CTGF.

**Limitations of the Study**

The cells utilised for the experiments were explanted from human tendons and include tendon cells (tenocytes), tendon stem or progenitor cells, and cells associated with the vasculature. These tendon stromal cells have distinctive differentiation potentials and cell morphology *in vitro* with further variation introduced by the nature of the method used to explant cells from clinical specimens [367]. Hence, the cells were termed collectively as "stromal" cells. The *in vitro* experiments were carried out in 2-Dimensional (D) conditions in the absence of immune populations and biomechanical environment that do not replicate *in vivo* settings. It is known that fibroblasts exhibit differences in cell proliferation, gene expression and cell signalling pathway activities according to their environment such as 2-D or 3-D [368-371].

The two cytokines used for this study (IL-4 and IL-1β) were selected based on a previous study that used well phenotyped clinical samples and showed distinct inflammatory signatures at different stages of tendon disease [52]; it indicated that tissues of early stage disease (chronic rotator cuff tendinopathy) is characterized by NF-κB and IFN pathways whereas end-stage disease (large to massive rotator cuff tear) show signs of STAT-6 pathway activation. TNF-α and IL-13 also activate NF-κB and STAT-6 pathways, respectively while IFN-γ activates IFN pathway. Furthermore, numerous studies have investigated the roles of IL-6 and IL-10 in tendon disease and healing [96]. In studies of fibrotic diseases of other organs, IL-6 has been implicated as the core mediator of fibrosis in relation to the aberrant expression of TGF-β [372]. How these cytokines regulate the development and progression of tendon disease has not been fully understood. Therefore, the results of this study are not conclusive and the involvement of other cytokines should not be eliminated.

Roles of other signalling pathways, such as p38 in response to IL-1β, or the reason of low-level expression of phosphorylated SAPK/JNK in human TSCs was not fully interrogated. c-Jun is involved in the SAPK/JNK pathway whereas the ERK1/2 pathway regulates AP-1 activity by inducing the expression of c-Fos, another protein of the AP-1 complex [373]. The relevance of AP-1 in the crosstalk between ERK1/2 and NF-κB pathways in TSCs from diseased tendons will require further investigation of its other constituent proteins (e.g. c-Fos).

Numerous studies have indicated changes in the activity of ERK1/2 and NF-κB signalling pathways in aging and senescence [374, 375]. Donors of diseased tendons were significantly older than that of healthy tendons in this study, and it is widely known that prevalence and severity of tendon disease increase with age. Hence, the abnormal findings of TSCs from diseased tendons may be confounded by age-related changes to cell phenotype.

## 4.5 Conclusions

Tendon stromal cells from diseased tendons show a dysregulated response in the expression of TGF-β and BMPs following treatment with inflammatory cytokines. IL-4 suppressed *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* & *CTGF* mRNA expression in TSCs from diseased tendons. IL-1β induced *TGFB1* and increased *BMP2* mRNA expression in TSCs from diseased tendons. These IL-1β-induced effects were negated by ERK1/2 inhibition, which indicates the involvement of the ERK1/2 signalling pathway in relation to end-stage tendon disease. The results from IL-1β treatment in TSCs from diseased tendons suggest that the suppressed regulation of fibrotic mediators in diseased tendon tissues in relation to the tissue inflammation signature could be a protective mechanism to prevent aberrant of fibrosis. However, the pathobiology of tendon disease remains to be established as it could also be a result of impaired ability to express TGF-β and BMPs. Hence, future research should investigate how TSCs from healthy and diseased tendons respond to TGF-β and BMPs treatments.

# Chapter 5 The *in vitro* Effects of TGF-β1 and BMP-2 on Fibrotic Phenotype of Tendon Stromal Cells

## 5.1 Introduction

### 5.1.1 Background

TGF-β and BMPs are widely accepted as key mediators of fibrosis by regulating synthesis of ECM, guiding cell differentiation, migration and promoting cell proliferation [172]. The previous chapter showed that cytokine-induced expression of TGF-β and BMPs differ between TSCs derived from diseased and healthy tendons. Cytokine treatment with IL-4 suppressed *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* mRNA expression in TSCs from diseased tendons. IL-1β treatment induced *TGFB1* mRNA expression in TSCs from diseased tendons only. IL-1β-induced *BMP2* mRNA expression was significantly increased in TSCs from diseased compared to healthy tendons. Animal studies demonstrate that TSCs from injured tendons show altered expression of genes related to the ECM in response to TGF-β1 treatment [308]. Cell signalling activity of the canonical SMAD pathway in response to BMP-2 treatment was also increased in animal TSCs from injured rodent tendons [216]. However, it remains unknown how TGF-β and BMPs regulate the expression of genes related to the ECM and if their cell signalling pathway activities differ in human TSCs derived from healthy and diseased tendons.

### 5.1.2 Aim, Objectives and Hypotheses

The aim of this study was to investigate if TGF-β and BMP treatments of TSCs from healthy and diseased tendons differentially regulate ECM, tenogenic and myofibroblastic genes in an *in vitro* cell culture model.

It was hypothesised that the treatment of TSCs by TGF-β or BMPs would regulate mRNA expression of genes related to the ECM, and there would be a differential expression between TSCs from healthy and diseased tendons. It was also hypothesised that TSCs from diseased tendons would exhibit less tenogenic and more fibrotic (myofibroblastic) genes in response to TGF-β or BMP treatment as tendon cells are known to change their morphology and phenotype becoming metabolically active along the development of fibrosis [30].

In the first part of the study, TSCs from healthy and torn tendons that represent end-stage disease (diseased) were treated with TGF-β1. Differences in mRNA expression of collagens (*COL1A1* and *COL3A1*) and proteoglycans (*ACAN*, *DCN* and *BGN*), as well as genes related to tenogenic (*TNC*, *TNMD* and *SCX*) and myofibroblastic (*ACTA2*) differentiation between the two cell cohorts were investigated. Differences in canonical SMAD signalling pathway activities between TSCs from healthy and diseased tendons were also investigated. It was hypothesised that the levels of canonical SMAD signalling pathway activities would relate to the differences in mRNA expression between TSCs from healthy and diseased tendons.

In the second part of the study, TSCs from healthy and diseased tendons were treated with BMP-2. Differences in mRNA expression of collagens (*COL1A1* and *COL3A1*), proteoglycans (*ACAN*, *DCN* and *BGN*) and genes related to tenogenic and myofibroblastic differentiation between the two cell cohorts were investigated. Differences in canonical SMAD signalling pathway activities between TSCs from healthy and diseased tendons were also investigated.

## 5.2 Materials and Methods

### 5.2.1 Patient Cohort

Surplus hamstring tendon tissues of the patients undergoing autograft ACL reconstruction surgery were obtained under ethical approval from the OMB and utilised as the healthy cohort. Diseased tendon tissues were obtained from patients with a large to massive supraspinatus tendon tear from OMB and through the SECuRe study.

### 5.2.2 Tendon Stromal Cell Isolation and Culture

Human TSCs were isolated by explant culture as described in Chapter 2. TSCs cultured up to passage 3 were used for experiments. TSCs were seeded in 24-well plates at a density of 30,000 cells $ml^{-1}$ (0.5 ml) and cultured to sub-confluence for the mRNA expression study. For analysis of cell signalling pathway activation by Western blotting, TSCs were seeded in 6-well plates at a density of 60,000 cells $ml^{-1}$ (2 ml) and cultured to sub-confluence.

### 5.2.3 Cell Treatment

Following serum starving overnight, TSCs were treated with human recombinant TGF-β1 (10 ng $ml^{-1}$) or BMP-2 (100 ng $ml^{-1}$) in either 1 percent serum or serum-free medium, or vehicle control (1 percent serum or serum-free medium) for 3 days and 1 hour for the mRNA and cell signalling pathway activity analysis experiments, respectively. Culture media were changed twice a week. The treatment duration and dosages were determined by validation experiments (data shown in Appendix C.4, pages 200-202, Figures A29-A32). Expression of the same genes were also investigated at 14 days to study changes over time. Vehicle only treatments served as experimental controls.

### 5.2.4 mRNA and Protein Expression Analyses

The processing of cell lysates of mRNA extraction, purification, conversion to cDNA, quantitative analysis of synthesised cDNA by reverse transcription polymerase chain reaction (RT-qPCR), preparation of protein lysates, protein separation by electrophoresis, Western blotting, image acquisition and analysis are described in Chapter 2. Details of the primers and antibodies used are also listed in Chapter 2.

### 5.2.5 Statistical Analysis

Statistical analysis was performed using Prism 5 for Mac OS X Version 5.0b (GraphPad Software Inc., La Jolla, CA). D'Agostino and Pearson normality test was carried out to determine normal distribution for each data. Differences in patient demographics were compared using the unpaired t test. Differences in mRNA expression between treatments or TSCs from healthy and diseased cells were compared by Mann Whitney test as the data were not always normally distributed. Differences in protein expression between treatments or TSCs from healthy and diseased cells were compared by Mann Whitney as the numbers were small (less than 8). Statistical significance was set at $p<0.05$.

## 5.3 Results

### 5.3.1 Patient Demographics

Experiments were performed using TSCs isolated from healthy donors (n=8) and diseased patient donors (n=8) (Table 5-1). Donors of healthy tendons were significantly younger than that of diseased tendons ($p<0.0001$). There was no significant difference in the ratio of males and females in the 2 patient groups.

**TABLE 5-1: Summary of patient demographics for mRNA expression analysis.** Mean (SD) shown for age as the data were normally distributed. * indicates significant difference to the healthy group. SD, standard deviation. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

| | Healthy | Diseased |
|---|---|---|
| Number of patients | 8 | 8 |
| Age; mean (SD) years | 23.44 (5.59) | 56.63 (7.65) **** |
| Sex; male/female, n | 5/3 | 5/3 |

### 5.3.2 Effects of TGF-β1 treatment on expression of ECM and Cell Differentiation Genes

TSCs derived from healthy (n=8) and diseased (n=8) tendons were treated with TGF-β1 (10ng $ml^{-1}$) or vehicle control to determine the effects of TGF-β1 treatment on mRNA expression of ECM related genes (*COL1A1*, *COL3A1*, *ACAN*, *DCN* and *BGN*) and cell differentiation (*TNC*, *TNMD*, *SCX* and *ACTA2*) by RT-qPCR. Genes of interest were normalised to the geometric mean of the expression values of *ACTB* and *GAPDH* as a reference of the internal control using the comparative $C_T$ method (Figure 4-1). (Figures 5-1 and 5-2). The results for individual housekeeping genes are shown in Appendix C.4, pages 203-204, Figures A33-34. The results for *TNMD* and *SCX* are not presented due to the low-level expression in both TSCs from healthy and diseased tendons regardless of treatment.

#### 5.3.2.1 Effects of TGF-β1 treatment on expression of ECM genes in TSCs from healthy and diseased tendons

At the primary timepoint of day 3, TGF-β1 treatment suppressed *ACAN* mRNA expression compared to vehicle control in TSCs from healthy and diseased tendons (p=0.0002 and p=0.0064, respectively). The effects of TGF-β1 treatment on suppressing *ACAN* were 6.75-fold more profound in TSCs from healthy compared to diseased tendons (p=0.0023). TGF-β1 treatment suppressed *DCN*, and induced *COL1A1*, *COL3A1* and *BGN* compared to vehicle control in TSCs from healthy (p=0.0002, p=0.0002, p=0.0003 and 0.0002, respectively) and diseased tendons (p=0.0095, p=0.0002, p=0.0070 and p=0.0001, respectively). The effects of TGF-β1 treatment on suppressing *DCN* and inducing *COL1A1*, *COL3A1* or *BGN* mRNA expression did not differ between TSCs from healthy and diseased tendons.

At day 14, the effects of TGF-β1 treatment on suppressing *ACAN* expression in TSCs from healthy and diseased tendons decreased relative to day 3 in both cohorts. TGF-β1 treatment suppressed *ACAN* compared to vehicle control in TSCs from healthy (p=0.0002) but not in diseased tendons. The suppressive effects of TGF-β1 treatment on *ACAN* were 4.13-fold more marked in TSCs from healthy compared to diseased tendons (p=0.0155).

At day 14, the effects of TGF-β1 treatment on suppressing *DCN* in TSCs from healthy and diseased tendons significantly increased relative to day 3 in both cohorts (p=0.0002 and 0.0012, respectively). mRNA expression of *DCN* did not differ between TSCs from healthy and diseased tendons after TGF-β1 treatment for 14 days. The effects of TGF-β1 treatment on inducing *COL1A1A* mRNA continued at day 14 in TSCs from both cohorts.

The effects of TGF-β1 treatment on inducing *COL3A1* mRNA expression significantly decreased at day 14 relative to day 3 in TSCs from healthy and diseased tendons (p=0.0281 and

0.0037, respectively). At day 14, the effects of TGF-β1 treatment on inducing *COL3A1* mRNA remained in TSCs from healthy tendons (p=0.0070) but were diminished in TSCs from diseased tendons.

The effects of TGF-β1 treatment on inducing *BGN* mRNA expression in TSCs from healthy and diseased tendons significantly increased at day 14 relative to day 3 in both cohorts (p=0.0002 and 0.0054, respectively). Interestingly, TGF-β1-induced *BGN* mRNA was 1.41-fold greater in TSCs from healthy compared to diseased tendons at day 14 (p=0.0193).

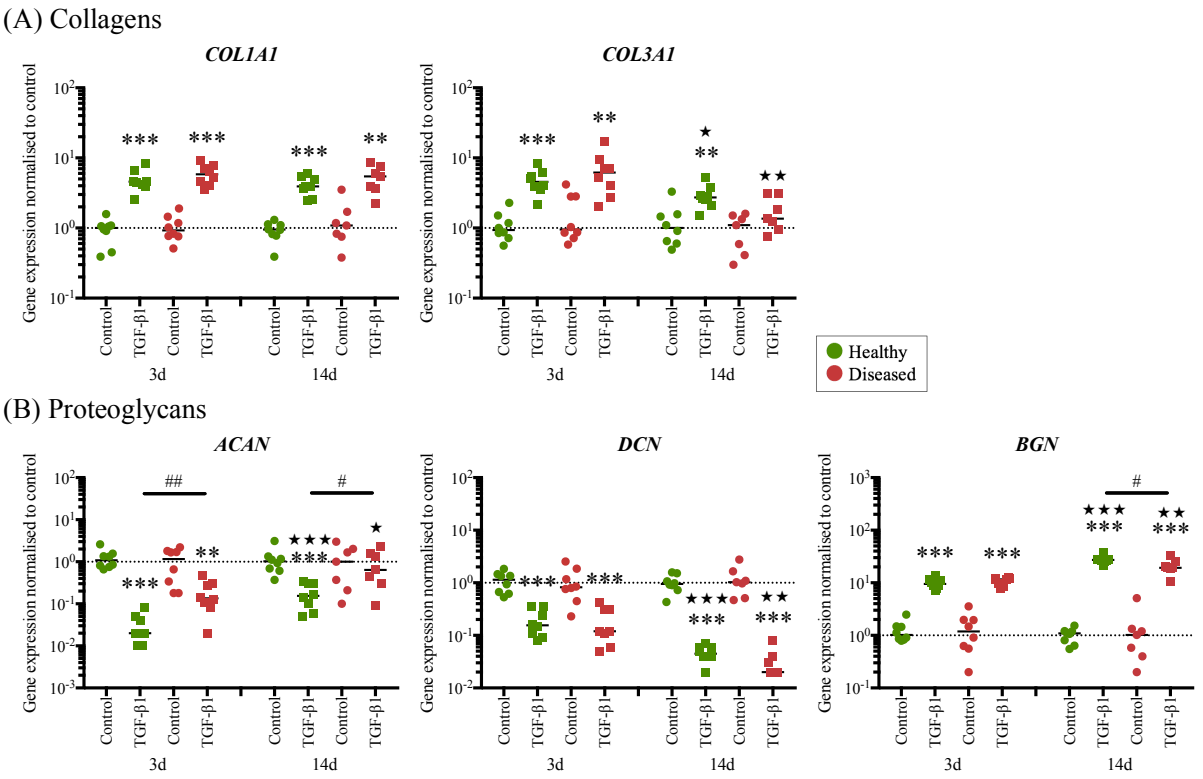



**FIGURE 5-1: mRNA expression of *COL1A1*, *COL3A1*, *ACAN*, *DCN* and *BGN* in cells derived from healthy and diseased tendons after treatment by TGF-β1 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) were quantified RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between the cells from healthy and diseased tendons. ★ indicates significant difference between day 3 and day 14. d, day. */#/★=p<0.05, **/##/★★=p<0.01, ***/★★★=p<0.001.

#### 5.3.2.2 Effects of TGF-β1 treatment on cell differentiation genes in TSCs from healthy and diseased tendons

At day 3, TGF-β1 treatment did not significantly modulate *TNC* mRNA expression in TSCs from healthy and diseased tendons. TGF-β1 treatment induced mRNA expression of *ACTA2* compared to vehicle control in TSCs from healthy and diseased tendons (p=0.0002 and p=0.0002, respectively). The effects of TGF-β1 treatment on inducing *ACTA2* mRNA expression were 2.56-fold more marked in TSCs from diseased compared to healthy tendons (p=0.0070).

TGF-β1 treatment did not have any significant effects on *TNC* mRNA expression in TSCs from healthy and diseased tendons at day 3. At day 14, TGF-β1 treatment induced *TNC* mRNA expression compared to vehicle control in TSCs from healthy but not from diseased tendons (p=0.0354).

The effects of TGF-β1 on inducing *ACTA2* mRNA expression in TSCs from healthy and diseased tendons significantly decreased at day 14 relative to day 3 in both cohorts (p=0.0011 and p=0.0022, respectively). The significant difference of *ACTA2* mRNA expression by TGF-β1 treatment between TSCs from healthy and diseased tendons at day 3 had diminished at day 14.

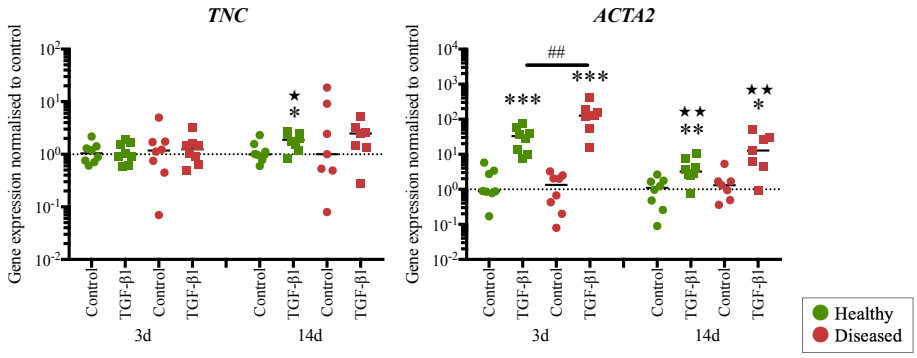



**FIGURE 5-2: mRNA expression of *TNC* and *ACTA2* in cells derived from healthy and diseased tendons after treatment by TGF-β1 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) were quantified RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between tendon stromal cells from healthy and diseased tendons. ★ indicates significant difference between day 3 and day 14. d, day. */★/#=p<0.05, **/★★/##=p<0.01, ***=p<0.001.

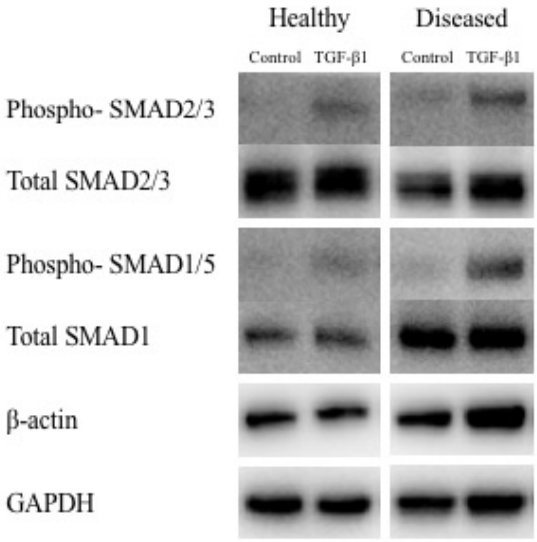
### 5.3.2.3 Increased canonical SMAD signalling pathway activities by TGF-β1 implicated in TSCs from diseased tendons

Cell signalling pathway activation of the canonical SMAD pathways in response to TGF-β1 treatment was investigated. TSCs from healthy (n=5) and diseased (n=5) tendons were treated with TGF-β1 and cell signalling activities were measured by Western blotting. Levels of phosphorylated proteins were normalised to that of their respective total proteins or housekeepers β-actin and GAPDH. Representative images of the Western blots are shown in Figure 5-3 (all obtained images are shown in Appendix C.4, page 211, Figure A41). Levels of phosphorylated relative to their respective total proteins are also presented in Figure 5-3. The results relative to β-actin and GAPDH followed similar patterns (data shown in Appendix C.4, page 207, Figure A37).

TGF-β1 treatment induced phosphorylation of SMAD2/3, the canonical regulatory (R-) SMAD for TGF-β1, in TSCs from healthy and diseased tendons (p=0.0079 and p=0.0079, respectively). TGF-β1 showed 2.07-fold marked induction of SMAD2/3 signalling activity in

TSCs from diseased compared to healthy tendons (p=0.0317). TGF-β1 treatment also induced phosphorylation of SMAD1/5, the canonical R-SMADs of BMPs, in TSCs from healthy and diseased tendons. This was statistically significant in TSCs from diseased tendons only (p=0.0079).

(A)




(B)

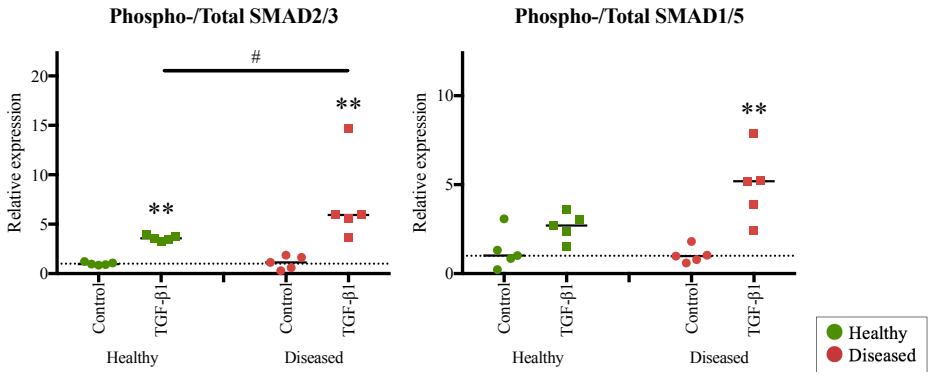



**FIGURE 5-3: Western blotting showing cell signalling activities of SMAD2/3 and SMAD1/5 pathways in tendon stromal cells derived from healthy and diseased tendons after treatment by TGF-β1 or vehicle control (control).** (A) Representative images of Western blotting for phosphorylated (phospho-) and total SMADs, and housekeepers β-actin and GAPDH in tendon stromal cells from healthy and diseased tendons after treatment by TGF-β1 or vehicle control. (B) Quantitative analysis of phospho- SMAD2/3 and SMAD1/5 in tendon stromal cells from healthy (n=5) and diseased tendons (n=5). Levels of cell signalling activity were quantified by measuring the intensities of Western blots and normalised to the respective total protein. Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between tendon stromal cells from healthy and diseased tendons. */#=p<0.05, **=p<0.01.

#### 5.3.2.4 Effects of TGF-β1 treatment on inhibitory SMAD genes in TSCs from healthy and diseased tendons

mRNA expression of inhibitory I-SMADs was investigated to establish whether changes in their expression was mediating the differences observed in TGF-β1-induced activation of canonical SMAD signalling pathways between TSCs from healthy and diseased tendons (Figure 5-4).

At day 3, TGF-β1 treatment suppressed mRNA expression of *SMAD6* compared to vehicle control in TSCs from healthy and diseased tendons (p=0.0019 and p=0.0002, respectively). TGF-β1 treatment induced mRNA expression of *SMAD7* compared to vehicle control in TSCs from healthy and diseased tendons (p=0.0002 and 0.0261, respectively). There were no significant differences in the TGF-β1-induced expression of *SMAD6* or *SMAD7* mRNA between TSCs from healthy and diseased tendons at day 3 or day 14.

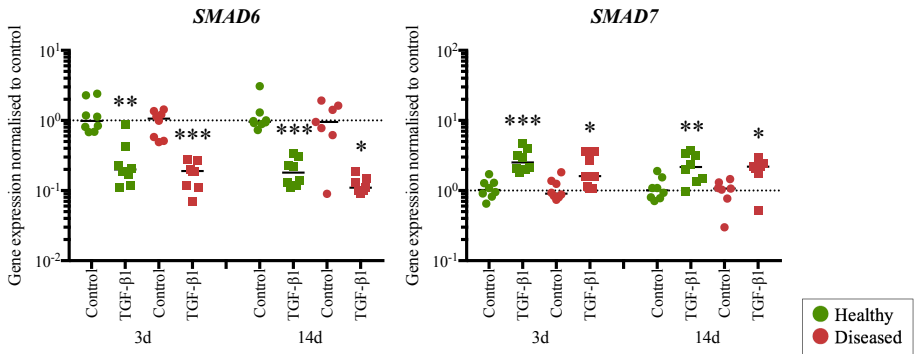



**FIGURE 5-4: mRNA expression of *SMAD6* and *SMAD7* in cells derived from healthy and diseased tendons after treatment by TGF-β1 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) were quantified RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. *=p<0.05, **=p<0.01, ***=p<0.001.

### 5.3.3 Effects of BMP-2 treatment on expression of ECM and Cell Differentiation Genes

TSCs derived from healthy (n=8) and diseased (n=8) tendons were treated with BMP-2 (100ng $ml^{-1}$) or vehicle control to determine the effects of BMP-2 on genes related to the ECM (*COL1A1*, *COL3A1*, *ACAN*, *DCN* and *BGN*) and cell differentiation (*TNC*, *TNMD*, *SCX* and *ACTA2*). Gene expression was normalised to housekeeping genes *ACTB* and *GAPDH* and the mean of the values calculated by the comparative $C_T$ method for the 2 housekeepers were used for analysis (Figures 5-5 and 5-6). The results for individual housekeeping genes are shown in Appendix C.4, pages 205-206, Figures A35-36. The results for *TNMD* and *SCX* are not presented due to the low-level expression in both healthy and diseased cells regardless of treatment.

#### 5.3.3.1 Effects of BMP-2 treatment on ECM genes in TSCs from healthy and diseased tendons

At day 3, BMP-2 treatment induced *COL1A1* and *ACAN* mRNA compared to vehicle control in TSCs from healthy (p=0.0045 and 0.0002, respectively) and diseased tendons (p=0.0207 and 0.0019, respectively). BMP-2 treatment induced *BGN* compared to vehicle control in TSCs from healthy tendons only (p=0.0070). BMP-2 treatment did not significantly modulate *COL3A1* or *DCN* in TSCs from healthy and diseased tendons.

At day 14, the effects of BMP-2 treatment on inducing *COL1A1* mRNA expression were maintained relative to day 3 in TSCs from both cohorts. The effects of BMP-2 on inducing *ACAN* and *BGN* mRNA expression significantly increased relative to day 3 in TSCs from healthy tendons only (p=0.0006 and p=0.0003, respectively).

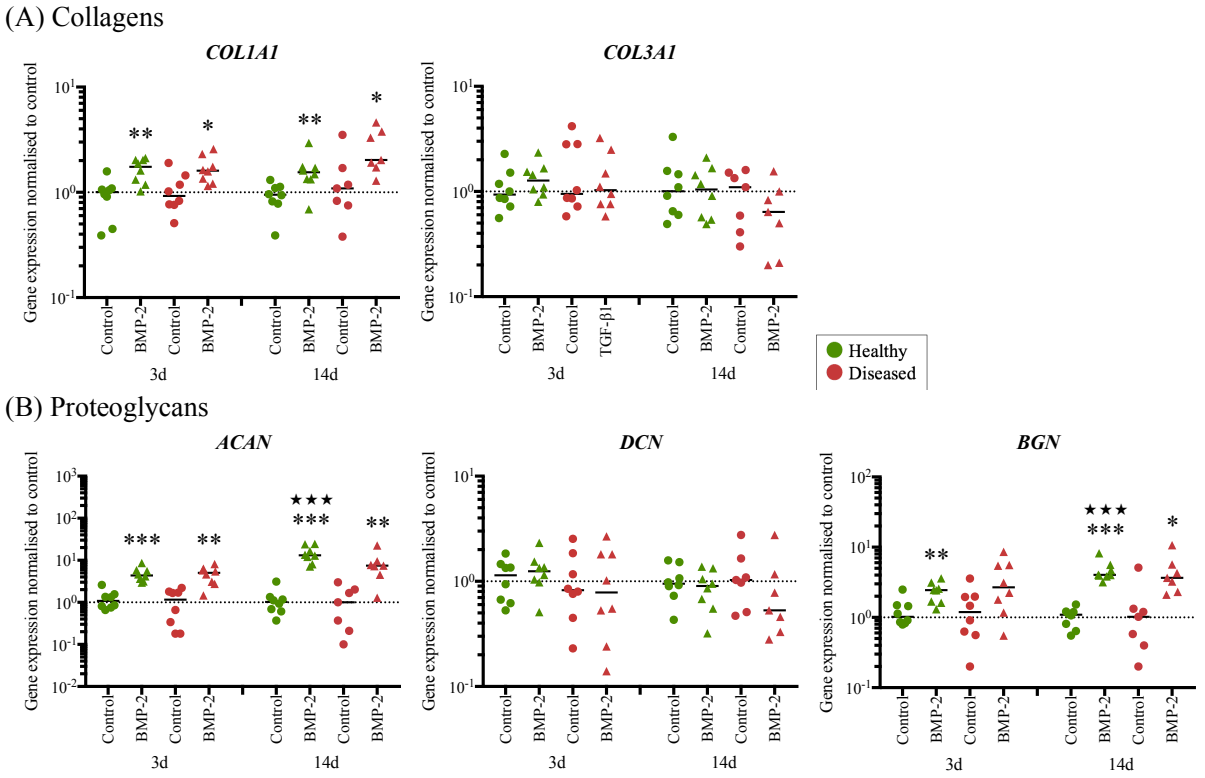



**FIGURE 5-5: mRNA expression of *COL1A1*, *COL3A1*, *ACAN*, *DCN* and *BGN* in cells derived from healthy and diseased tendons after treatment by BMP-2 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) were quantified RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. ★ indicates significant difference between day 3 and day 14. d, day. */★=p<0.05, **/★★=p<0.01, ***/★★★=p<0.001.

### 5.3.3.2 Effects of BMP-2 treatment on cell differentiation genes in TSCs from healthy and diseased tendons

At day 3, BMP-2 treatment induced *ACTA2* mRNA expression compared to vehicle control in TSCs from diseased tendons only (p=0.0379). The effect of BMP-2 treatment on inducing *ACTA2* mRNA in TSCs from diseased tendons had diminished over time at day 14.

BMP-2 treatment did not have any significant effects on *TNC* mRNA expression in TSCs from healthy and diseased tendons at day 3.

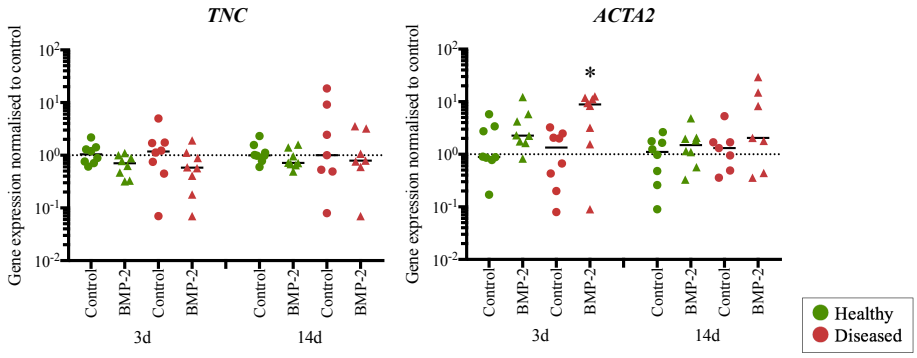


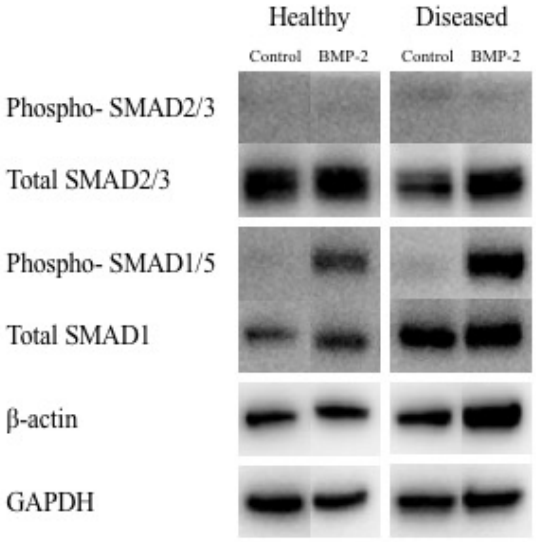

**FIGURE 5-6: mRNA expression of *TNC* and *ACTA2* in cells derived from healthy and diseased tendons after treatment by BMP-2 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) were quantified RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. *=p<0.05.

#### 5.3.3.3 Increased canonical SMAD pathway activity by BMP-2 implicated in TSCs from diseased tendons

The activation of SMAD signalling pathways induced by BMP-2 treatment was investigated in TSCs from healthy and diseased tendons (Figure 5-7 and Appendix C.4, page 208, Figure A38).

BMP-2 treatment induced phosphorylation of the canonical BMP R-SMAD, SMAD1/5, in TSCs from healthy and diseased tendons (p=0.0317 and p=0.0079, respectively). BMP-2 showed a trend of marked induction of signalling activity in TSCs from diseased compared to healthy tendons (p=0.0556). BMP-2 treatment did not induce expression of phosphorylated SMAD2/3, the canonical R-SMADs of TGF-β.

(A)




(B)

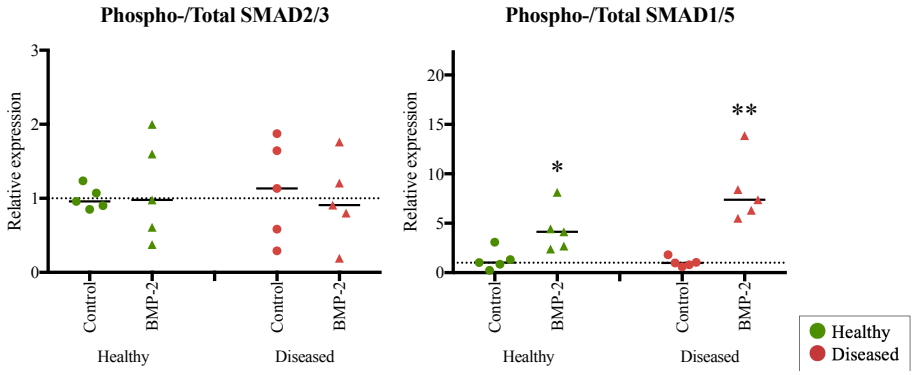



**FIGURE 5-7: Western blotting showing cell signalling activities of SMAD2/3 and SMAD1/5 pathways in tendon stromal cells derived from healthy and diseased tendons after treatment by BMP-2 or vehicle control (control).** (A) Representative images of Western blotting for phosphorylated (phospho-) and total SMADs, and housekeepers β-actin and GAPDH in tendon stromal cells from healthy and diseased tendons after treatment by BMP-2 or vehicle control. (B) Quantitative analysis of phospho- SMAD2/3 and SMAD1/5 in tendon stromal cells from healthy (n=5) and diseased tendons (n=5). Levels of cell signalling activity were quantified by measuring the intensities of Western blots and normalised to the respective total protein. Bars shown represent median. * indicates significant difference to the respective vehicle control. *=$p<0.05$, **=$p<0.01$.

### 5.3.3.4 Effects of BMP-2 treatment on inhibitory SMAD genes in TSCs from healthy and diseased tendons

mRNA expression of I-SMADs was investigated similarly to the TGF-β1 experiment (Figure 5-8).

BMP-2 treatment induced mRNA expression of *SMAD6* and *SMAD7* compared to vehicle control in TSCs from healthy (p=0.0030 and p=0.0030, respectively) and diseased tendons (p=0.0002 and p=0.0019, respectively). There were no significant differences in the BMP-2-induced expression of *SMAD6* or *SMAD7* mRNA between TSCs from healthy and diseased tendons at day 3 or day 14.

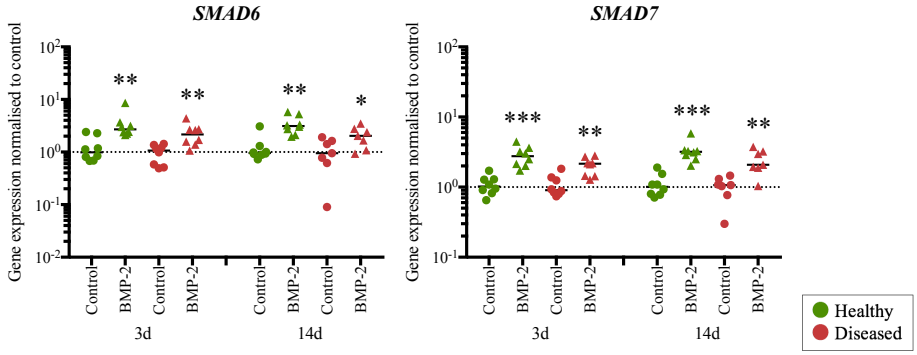



**FIGURE 5-8: mRNA expression of *SMAD6* and *SMAD7* in cells derived from healthy and diseased tendons after treatment by BMP-2 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) were quantified RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. d, day. *=p<0.05, **=p<0.01, ***=p<0.001.

## 5.4 Discussion

The aim of this study was to investigate if TGF-β and BMP treatments of TSCs from healthy and diseased tendons differentially regulate the expression of ECM, tenogenic and myofibroblastic genes in an *in vitro* cell culture model. TGF-β1 and BMP-2 treatments elicited similar gene expression effects in TSCs from both healthy and diseased tendons. The magnitude of the suppressive or inductive effects tended to be lower in TSCs from diseased compared to healthy tendons. TGF-β1 treatment induced expression of genes related to tenogenic differentiation in TSCs from healthy tendons, whereas BMP-2 treatment did not have any effects on *TNC* mRNA expression. TGF-β1 and BMP-2 treatments induced expression of genes related to myofibroblastic differentiation, which was more prominent in TSCs from diseased compared to healthy tendons. Cell signalling pathway activities of the respective canonical SMAD pathways of TGF-β1 and BMP-2 treatments were increased in TSCs from diseased compared to healthy tendons. However, neither the TGF-β1- nor BMP-2-elicited reduction in mRNA expression of inhibitory SMADs differed between TSCs from healthy and diseased tendons.

**Effects of TGF-β1 and BMP-2 treatments on ECM genes are reduced in TSCs from diseased tendons**

Numerous studies have investigated abnormal expression of collagens along development of tendon disease and during fibrotic healing [45, 376-378]. It is noteworthy that this thesis showed that mRNA expression of *COL1A1* and *COL3A1* induced by TGF-β1 and *COL1A1* induced by BMP-2 treatment does not differ between TSCs from healthy and diseased tendons. mRNA expression of the most abundant SLRP *DCN* followed a similar pattern after TGF-β1 treatment, although its expression has been reported to be dysregulated in tendon tissues of

rotator cuff disease [45]. Moreover, BMP-2 did not significantly modulate mRNA expression of *COL3A1* or *DCN*.

TGF-β1 treatment suppressed *ACAN* mRNA expression in TSCs from healthy and diseased tendons, the suppressive effect was less marked in the latter. Numerous studies have shown that diseased rotator cuff tendon tissues are characterised by increased expression of aggrecan [379-382]. The current study showed that the effect of TGF-β1 on suppressing *ACAN* is diminished in TSCs from diseased tendons. This could be postulated as a consequence of impaired ability of TSCs from diseased tendons to suppress *ACAN* mRNA expression in response to TGF-β1 treatment. Meanwhile, both TGF-β1 and BMP-2 treatments induced *BGN* mRNA, an effect that increased over time in TSCs from healthy tendons. Biglycan has been associated with fibrillogenesis of type 1 collagens [23], and its expression has been shown to be increased in diseased rotator cuff tendon tissues [380]. The lower expression of *BGN* in response to fibrotic mediators in TSCs from diseased tendons may suggest the reduced ability of TSCs from diseased tendons to synthesise healthy ECM, or that its expression is reduced at end-stage disease which may have been elevated earlier in the disease process. Furthermore, the changes of mRNA expression in tissues may do not necessarily correlate to how cells respond to certain growth factors by *in vitro* experiments.

No study to date has compared the expression of ECM-related genes in response to TGF-β1 in human TSCs from healthy and diseased tendons. An animal study of tendon healing using a rat patella tendon injury (defect) model has shown that *DCN* and *BGN* mRNA expression after TGF-β1 treatment is increased and decreased respectively in TSCs from healing compared to healthy tendons [308]. The discrepancy between these results and the findings from this study suggests that caution should be applied when applying findings from *in vivo* models of tendon healing in rats to the understanding of human tendon disease pathobiology. Rui *et al.* reported decreased *Dcn* and unchanged *Bgn* mRNA expression in rat

TSCs from healthy tendons by BMP-2 treatment for the same dose and duration as this experiment *in vitro* [222]. Differences in species may also contribute to different results even in TSCs from healthy tendons treated under similar conditions *in vitro*.

**TGF-β1 and BMP-2 treatments reduce tenogenic and increase myofibroblastic differentiation in TSCs from diseased tendons**

mRNA expression of *ACTA2* was investigated to assess myofibroblastic differentiation of tendon cells (tendon fibroblasts/tenocytes) that may contribute to the development of fibrosis in tendon disease [383, 384]. TGF-β1 induced *ACTA2* mRNA in TSCs from healthy and diseased tendons, which was more profound in the cells from diseased tendons. This study confirms previous literature demonstrating TGF-β1 induces *ACTA2* mRNA and furthermore implicates BMP-2 as a regulator of *ACTA2* mRNA expression in TSCs from diseased tendons. It can be postulated that TSCs from diseased tendons are more prone to myofibroblastic differentiation.

TGF-β1 and BMP-2 induced *ACTA2* mRNA expression was higher at day 3 compared to day 14. Meanwhile, TGF-β1 treatment induced *TNC* mRNA expression in TSCs from healthy tendons after 14 days of treatments. This may suggest that cells react differently according to the duration of TGF-β1 or BMP-2 treatment, with fibrotic responses occurring in the earlier phase that converge with sustained exposure to fibrotic cytokines in TSCs from healthy tendons. This is noteworthy as studies that investigate the effects of cytokines and growth factors in a time-dependent manner are limited [88]. The diminished *TNC* mRNA expression by both treatments at day 14 in TSCs from diseased tendons are convincing that diseased tendons have impaired tenogenic abilities.

**Increased canonical SMAD signalling pathway activity by TGF-β1 and BMP-2 that is not regulated by inhibitory SMADs implicated in TSCs from diseased tendons**

TGF-β1 and BMP-2 treatments induced their respective canonical SMAD signalling pathway activities, which were more profound in TSCs from diseased compared to healthy tendons. The result of BMP-2 parallel that of Lui *et al.* using a rat tendon injury model reporting increased phosphorylated Smad1/5/8 expression by BMP-2 treatment in TSCs from healing compared to healthy tendons [216].

TGF-β1 treatment significantly activated the canonical SMAD pathway of BMP-2 as in TSCs from diseased tendons. Previous studies have shown the regulatory role of TGF-β1 in *ACTA2* mRNA expression through its R-SMADs [385], however this study showed that *ACTA2* mRNA is also induced by BMP-2 in TSCs from diseased tendons. TGF-β1 may also activate the R-SMADs of BMPs [386], and although further mechanistic studies inhibiting their actions are necessary to confirm, the increased phosphorylation of SMAD1/5/8 in TSCs from diseased tendons could contribute to the increased *ACTA2* mRNA expression noted in this study. However, the mRNA expression data on ECM-related genes suggested that the increased cell signalling of SMAD pathways does not necessarily directly correlate to the expression of their target genes.

Phosphorylation of R-SMADs are known to take place at the C-terminal and linker regions [387]. The activated TGF-β or BMP receptors directly phosphorylate the C-terminal region of the R-SMADs. The linker region is activated secondary to the cell signalling activity of non-canonical pathways such as mitogen-activated protein kinase (MAPK; ERK1/2, p38 and SAPK/JNK) pathways [388]. The antibodies used for this study target the C-terminus and hence, the increased signalling pathway activation of R-SMADs by TGF-β1 and BMP-2 treatments represent the activities of the canonical signalling pathways. I-SMADs (SMAD6 and SMAD7)

act negatively in phosphorylation and nuclear translocation of R-SMADs [389-392]. However, there were no significant differences in mRNA expression of both I-SMADs by TGF-β1 or BMP-2 treatment between TSCs from healthy and diseased tendons. As other mechanisms such as SMAD anchor for receptor activation (SARA), a cytoplasmic protein that bridges non-activated R-SMADs to receptors for phosphorylation [393], and cytoskeletal proteins such as microtubule filaments have been shown to take part in the localisation and phosphorylation of R-SMADs [394], it would be of interest to further investigate the actions of these proteins to define the mechanism of increased canonical signalling pathway activity in TSC from diseased tendons.

The involved MAPKs in the non-canonical pathway signalling may differ between cell types [388], but Hough *et al.* have shown substantial activities of the Erk1/2 signalling pathway in response to TGF-β treatment in fibroblasts [395]. Using the same protein lysates, ERK1/2 pathway signalling activities in response to TGF-β1 and BMP-2 treatments in TSCs from healthy and diseased tendons were investigated (data shown in Appendix C.4, page 209, Figure A39). It was anticipated that ERK1/2 pathway signalling activities in response to TGF-β1 and BMP-2 treatments would be increased in TSCs from diseased tendons similar to when treated by IL-1β (Chapter 4). However, there were no significant differences in the extent of ERK1/2 activation in response to TGF-β1 and BMP-2 treatments between TSCs from healthy and diseased tendons. One reason for not detecting changes in ERK1/2 signalling pathway activity between TSCs from healthy and diseased tendons could be that the timing of harvesting the protein lysates was too soon as the activation of the ERK1/2 signalling pathway is known to be induced later than the phosphorylation of the C-terminal region of R-SMADs [396]. Erk1/2 mediates phosphorylation of the linker region [395], and regulates the expression of TGF-β and BMP target genes at the transcription level in primary human fibroblasts [397]. Other MAPK pathways such as p38 and SAPK/JNK [273] may also interact with SMAD pathways, which

add complexity in the regulation of these signalling cascades [395, 398]. The results of this study imply non-canonical signalling pathway activities do not have a substantial role in TSCs from diseased tendons, but their potential roles should not be eliminated as this study was not designed to investigate these mechanisms as the primary objective.

**Limitations of the Study**

Human TSCs were obtained and treated *in vitro* like in the previous chapter in a 2-D condition. This related to the undetected mRNA expression of *TNMD* and *SCX*, as the expression of tendon-selective genes are known to be lost once plated in a 2-D system [381]. Cellular responses to fibrotic mediators (TGF-β1) *in vitro* may differ due to factors such as cell type, density, media and culture conditions [399]. Validation experiments were performed in TSCs from healthy tendons to determine the dose and duration of TGF-β1 and BMP-2 treatments that show greatest changes in mRNA expression of their target genes. Temporal changes were also investigated after 14 days of treatments, but this timepoint was set arbitrarily. More detailed investigation by sampling at more timepoints during sustained treatment may have been more informative of changes over time. TSCs from healthy and diseased tendons were treated similarly, but based on the results showing increased *TGFB1* and *BMP2* mRNA expression by IL-1β (Chapter 4) and diminished mRNA expression of proteoglycans by TGF-β1 and BMP-2 treatments in TSCs from diseased tendons, it would be of interest to investigate the effect of higher concentrations of TGF-β1 and BMP-2 for TSCs from diseased tendons. If the size of the effects on expression of genes related to ECM and tenogenic differentiation increases to a level similar to that of TSCs from healthy tendons at lower concentrations of TGF-β1 and BMP-2, it may indicate that the increased mRNA expression of *TGFB1* and *BMP2* by IL-1β treatment is to compensate for the diminished expression of their target genes in TSCs from diseased

tendons. Moreover, TSCs from diseased tendons were derived from only torn tendons that represent end-stage disease. TSCs from earlier stage of tendon disease such as tendinopathy may exhibit different responses to TGF-β1 and BMP-2 treatments compared to that of TSCs from healthy and torn tendons.

TGF-β1 and BMP-2 were chosen based on the results of the previous chapter. TGF-β1 is known to have fibrotic effects whereas BMP-2 has been more related to osteogenesis and chondrogenesis. Animal studies on tendon injury and healing have indicated positive roles of other BMPs such as BMP-7 [243], BMP-12 and BMP-13 in tendon healing [245, 400-403]. BMP-7 and BMP-12 expression was suppressed in diseased tendon tissues (Chapter 3) and IL-1β and IL-4 treatments did not induce their mRNA expression in vitro in TSCs from both healthy and diseased tendons (Chapter 4). However, it would be of interest to investigate how these BMPs act on human TSCs with view for potential clinical therapeutic application.

## 5.5 Conclusions

The suppressive or inductive effects of TGF-β1 and BMP-2 treatments on ECM-related genes were similar in TSCs from healthy and diseased tendons. However, the magnitudes of the treatment effects were reduced in TSCs derived from diseased compared to healthy tendons (especially proteoglycans). TGF-β1 and BMP-2 treatments induced myofibroblastic differentiation in TSCs from diseased tendons. TGF-β1 and BMP-2 treatments induced their respective canonical SMAD pathway activities, which were more marked in TSCs from diseased compared to healthy tendons. However, there were no significant differences in TGF-β1- or BMP-2-induced expression of *SMAD6* or *SMAD7* mRNA between TSCs from healthy and diseased tendons.

These results suggest a decline in the functional competence of TSCs from diseased tendons to express genes related to the ECM, especially proteoglycans in response to TGF-β1 and BMP-2 treatments. The increased myofibroblastic differentiation and cell signalling activities of the canonical SMAD pathways in TSCs from diseased tendons could be considered as a compensatory mechanism for the decreased cell activity, function and responsiveness to regulatory stimuli.

# Chapter 6 Discussion

## 6.1 Key Findings

The overarching aim of this thesis was to improve understanding of how TGF-β and BMPs contribute to the development of fibrosis in human tendon disease.

TGF-β and BMPs are known as key mediators of fibrosis and CTGF is a downstream regulator of TGF-β. Their increased expression in tissues has been acknowledged as one the main features of fibrosis in studies from other organs. In this thesis, there was a marked reduction of TGF-β1, TGF-β R1, TGF-β R2, BMPs and CTGF protein expression in torn supraspinatus tendons that represent end-stage disease compared to healthy supraspinatus tendons.

Emerging evidence suggests the roles of inflammation in the onset and progression of tendon disease. Results from Chapter 4 showed that TSCs from torn supraspinatus tendons (diseased tendons) show a dysregulated response in the expression of TGF-β, BMPs and CTGF following treatments with inflammatory cytokines. Tissues from torn supraspinatus tendon patients have previously been shown to have an altered tissue inflammation signature, expressing IL-4 target genes and proteins [52]. In this thesis, IL-4 treatment suppressed *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* mRNA expression in TSCs from diseased tendons. These results imply regulatory roles of the tissue inflammatory milieu in the expression of fibrotic mediators in the human tendon.

IL-1β treatment induced *TGFB1* and increased *BMP2* mRNA expression in TSCs from diseased but not healthy tendons through increased ERK1/2 signalling pathway activity. This could be a compensatory mechanism for the diminished effects of TGF-β and BMP-2 on

mRNA expression of proteoglycans (*ACAN* and *BGN*) in TSCs from diseased compared to healthy tendons. There were no significant differences in mRNA expression of collagens (*COL1A1* and *COL3A1*) between TSCs from the 2 cohorts. These results also imply the importance of dysregulated proteoglycan expression in the fibrotic extracellular matrix. TGF-β1 and BMP-2 induced the myofibroblastic gene *ACTA2* expression in TSCs from diseased compared to healthy tendons. There was also a tendency of suppressed tenogenic gene *TNC* expression especially in TSCs from diseased tendons.

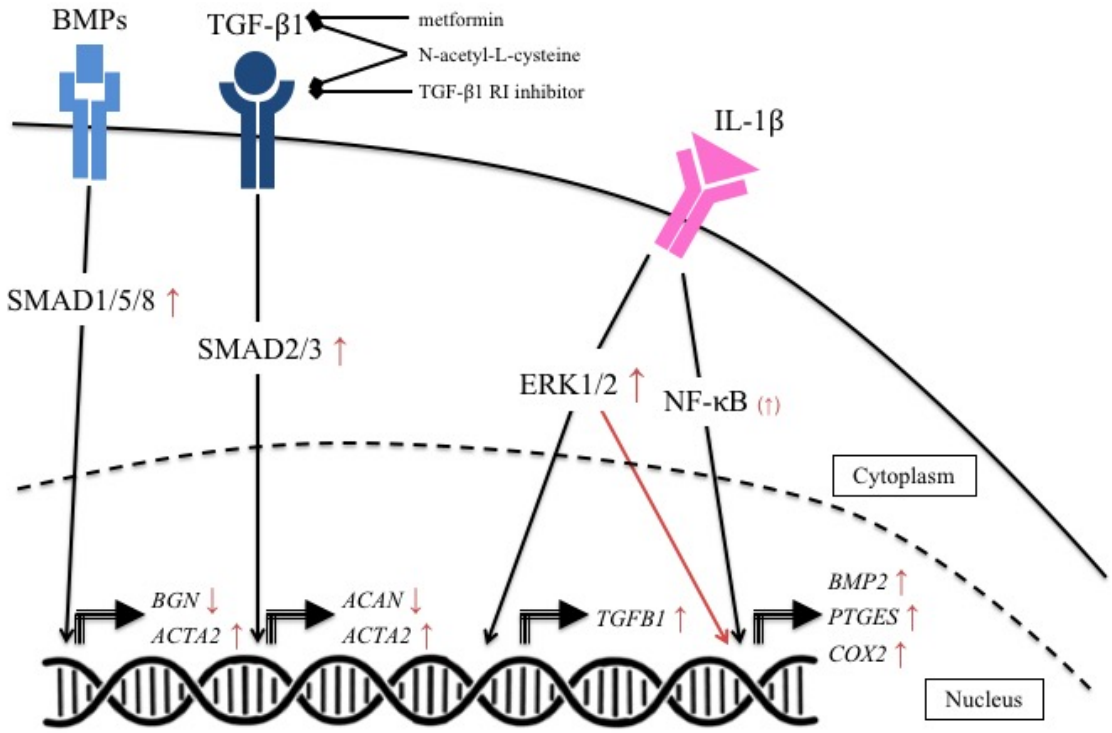



**FIGURE 6-1: Diagram describing the fibrotic pathways in tendon disease.** Only the genes that the expression in response IL-1β, TGF-β1 or BMP-2 differed between tendon stromal cells from healthy and diseased tendons have been shown. Arrows with rhomboid arrow-heads indicate suppressive effects. Red arrows indicate the significant findings of TSCs from diseased compared to healthy tendons. IL-1β treatment induced *TGFB1* mRNA expression in tendon stromal cells from diseased tendons through increased ERK1/2 signalling pathway activity. IL-1β treatment induced mRNA expression of NF-κB target genes *BMP2*, *PTGES* and *COX2* in tendon stromal cells from both healthy and diseased tendons, which was increased in the cells from diseased tendons in relation to increased ERK1/2 signalling pathway activity as well. TGF-β1 and BMP-2 treatments activated their respective canonical SMAD signalling pathways, which were marked in TSCs from diseased compared to healthy tendons. The magnitude of the effects of TGF-β1 and BMP-2 on *ACAN* and *BGN* mRNA expression, respectively was reduced in TSCs from diseased compared to healthy tendons. TGF-β1 and BMP-2 treatments induced myofibroblastic differentiation in TSCs from diseased tendons. Numerous studies have attempted to dampen fibrotic development and improve healing in tendon disease by suppressing TGF-β expression or inhibiting its activity (e.g. metformin, N-acetyl-L-cysteine, TGF-β RI inhibitor).

## 6.2 Implications of the Study

### Expression of TGF-β and BMPs is tissue specific

TGF-β is known as a central mediator in the pathogenesis of fibrosis by regulating ECM synthesis and remodelling, cell proliferation and differentiation. Numerous animal studies have shown that overexpression of TGF-β results in excessive and abnormal deposition of ECM, and mechanical and chemical injuries induce fibrosis in relation to the expression of TGF-β in the affected organs [172, 273, 404]. Increased expression of TGF-β as well as abnormal expression of BMPs have been correlated to the onset and progression of disease clinically in fibrotic diseases of the lung, kidney, liver, rheumatoid arthritis and scleroderma. [273, 404]. Arteriosclerosis has also been defined to have a fibrotic pathology [273] with studies indicating increased TGF-β expression and/or activities [399, 405]. However, similar to the expression pattern of TGF-β1 in tendon disease in this thesis, decreased levels of TGF-β and its receptors in plasma and vascular tissues have been shown in its advanced stages [399, 406]. These data further demonstrate that the expression and effects of TGF-β are context dependent.

### Expression of TGF-β and BMPs implicated in tendon homeostasis

TGF-β is a pleiotropic cytokine that acts as a fibrotic mediator, but also regulates homeostasis, the normal control of tissues [273]. Cumulative loading of the flexor digitorum tendon in a rat model induced TGF-β expression in the tendon [194] while unloading of the patella tendon (for 2 and 6 weeks) in a rat model also resulted in up-regulation of TGF-β expression [104]. Sudden changes in the mechanical force transmitted in the tendon leads to increased TGF-β release [189], whilst gradual loss may result in opposing results. Maeda *et al.* reported that the expression of a tenogenic differentiation marker scleraxis that is regulated by TGF-β is

suppressed by slowly removing the tensile loading of an Achilles tendon for 6 months in a mouse model of tendon injury [189]. Moreover, immobilisation of a tendon for 3 weeks in humans resulted in a decreased collagen synthesis rate, which TGF-β is also known to take part in [407]. These studies indicate the active roles of TGF-β in response to changes in mechanical loading within physiological limits, i.e. homeostasis. Active expression of TGF-β and its closely related fibrotic mediators has been shown in the results of Chapter 3, which was suppressed in torn supraspinatus tendon tissues that follow a chronic course. Moreover, tissue expression of phosphorylated ERK1/2 that was shown to regulate *TGFB1* mRNA expression in TSCs from diseased tendons was also significantly suppressed in diseased (torn) tendons.

**Patient ageing may affect the expression and effects of TGF-β and BMPs in TSCs**

Patient donors of torn (diseased) supraspinatus tendon tissues were significantly older than the donors of healthy hamstring tendons. Ageing is associated with increased cell senescence [408], but studies focusing on the effects or levels of senescence in human TSCs is limited [409]. Similar to the results of this thesis, age-related decreases in the protein expression of TGF-β and cellular responses to TGF-β in the production of collagens have been shown in tissues and cells derived from equine digital flexor tendons [410]. Studies on equine chondrocytes have also shown age-related decline in response to TGF-β1 treatment in the synthesis of proteoglycans [411]. In the human, there was a decline in both the local expression of TGF-β and BMPs, and cellular response to these growth factors in chondrocytes from aged patients [412-414]. Hence, the abnormal findings of TSCs from diseased tendons may be confounded by age-related changes to cell phenotype.

**Expression and effects of TGF-β and BMPs are diminished in end-stage human tendon disease**

IL-1β induced *TGFB1* and increased *BMP2* mRNA expression in TSCs from diseased tendons through increased ERK1/2 signalling pathway activity. Although the direction of suppressive or stimulatory effects of TGF-β1 and BMP-2 on mRNA expression of genes related to the ECM (especially proteoglycans) were similar in TSCs from healthy and diseased tendons, the size of the effects was less marked in TSCs from the diseased tendons. As the basal expression of TGF-β and BMPs were diminished in diseased tendon tissues, the induced *TGFB1* and *BMP2* mRNA by IL-1β treatment in TSCs from diseased tendons could act as a compensatory mechanism for the marked suppression. The enhanced ERK1/2 cell signalling activity in response to IL-1β treatment in TSCs from diseased tendons could also be considered as a compensatory mechanism for its marked suppressed basal expression shown in Chapter 4.

TSCs from diseased compared to healthy tendons have been shown to be "hyper-responsive" to inflammatory stimuli [301]. The results from Chapter 4 showed that TSCs from diseased tendons have increased responses to IL-1β in the mRNA expression of fibrotic mediators such as *TGFB1* and *BMP2*. However, this was a gene-specific response as the expression of ECM-related genes by TGF-β1 and BMP-2 treatments were decreased in TSCs from diseased compared to healthy tendons.

In animal models of tendon injury or overload, basal expression of phosphorylated Erk1/2 is not suppressed unlike in diseased human tendon tissues. Hence, the nature of increased ERK1/2 signalling pathway activity by IL-1β treatment in TSCs from human diseased tendons *in vitro* is likely to differ from the pathobiology of increased Erk1/2 signalling pathway activity noted in tendons from a rat model of supraspinatus tendinopathy [46]. These results emphasise the limitations of applying the knowledge from animal studies comparing the

effects of injury or overloading in healthy tendons to human disease as the basal expression of growth factors and cell signalling molecules may differ.

## 6.3 Clinical Relevance

### Application of TGF-β and BMPs as a potential therapeutic method to improve tendon healing

Numerous studies have attempted to dampen fibrotic development and improve healing in tendon disease by suppressing TGF-β expression or inhibiting its activity (e.g. TGF-β RI inhibitor [203], metformin [202, 415], N-acetyl-L-cysteine [205, 416], etc.). However, no promising results have been achieved, with many studies reporting detrimental effects of TGF-β blockade such as reduced tissue strength [206, 207]. The results from Chapter 4 also suggested that suppression of *TGFB1* mRNA expression by inhibiting its regulatory ERK1/2 signalling pathway may actually result in increased activity of TGF-β shown by *SERPINE1* mRNA expression, which was more evident in TSCs from diseased tendons. In the treatment of arteriosclerosis, therapies inhibiting TGF-β activity did not show positive results while treatments that increase TGF-β levels were shown to have favourable outcomes [172, 399]. The findings from this thesis suggest suppressed expression of TGF-β1 and BMPs in end-stage human tendon disease of large to massive tear, therefore treatments that increase TGF-β levels could be considered as a potential therapeutic method to improve healing of a chronic tendon tear especially in relation to surgical repair. Kim *et al.* in an animal study used TGF-β1 whilst repairing a torn rodent supraspinatus tendon and reported fibrotic repair with reduced mechanical properties [417], but the underlying pathobiology in human disease and animal models are likely to differ. Moreover, animal tendon injury or overuse models currently available only represent acute injury or tendinopathy, which may possess more classical fibrotic phenotype. Therefore, whether to dampen or stimulate TGF-β expression and/or its activities may differ along disease progression or the healing process. To date, there are no studies that

have used TGF-β addition treatment in combination with rotator cuff repair surgeries in humans [418].

**Redefining tendon disease as a fibrotic and not a degenerative disease**

Histopathological features of tendon disease have commonly been described as "degeneration (degenerative)," which is a term that denotes a passive process devoid of inflammation [7]. Emerging evidence suggests the role of inflammation in the development and progression of tendon disease [344]. This thesis supports a role of inflammation in tendon disease pathobiology and has shown that expression of fibrotic mediators that regulate ECM synthesis and cell differentiation is dysregulated in TSCs from diseased cells in response to inflammatory cytokines.

Inflammation is a physiological process that enables cells to adapt to abnormal conditions and restore cell and tissue homeostasis [83]. Persistence of inflammatory triggers results in chronic inflammation and it is proposed that stromal cells primed by inflammatory cues continuously produce inappropriate and often excessive matrix proteins leading to fibrosis. This is typified by chronic diseases in other organs such as the liver, kidney and lung [84]. The histopathology of these diseases is similar to that of tendon "degeneration", but has been termed as "fibrosis". Fibrosis implies excessive and abnormal matrix formation as a result of dysregulated and persistent inflammation [84].

In tendon disease, cumulative mechanical loading and damage of the tendon may lead to inflammatory triggers that alter the phenotype of tendon resident stromal and immunoregulatory cells, resulting in the distinctive persistent inflammatory signature seen in cells from diseased tendons compared to cells from healthy tendons [52, 301]. The altered production of ECM due to the decline in both cellular response to and the local expression of

the growth factors in end-stage tendon disease may not be homologous to the pathobiology of fibrotic diseases of other organs represented by increased activity and expression of fibrotic mediators. Tendon disease differs from other fibrotic diseases as the functional load diminishes when it progresses to tear, while the affected organs are continuously loaded even at the end-stage of the disease. Earlier stages of tendon disease where the loading is sustained may resemble the pathobiology of fibrosis in other organs. Nevertheless, we have shown that inflammatory cytokines regulate ECM in tendon disease. Therefore, using the term "fibrosis" may be more suitable in describing tendon disease, as this reflects the underlying chronic and dysregulated inflammation seen in diseased tendons. The fibrotic nature of tendon disease indicates that the therapeutic strategies to moderate disease should include targeting the dysregulated mechanism of inflammation or subsequent processes. This has potential to facilitate the identification of therapeutic targets to enhance the quality of repair or dampen disease progression.

## 6.4 Limitations

Multiple experiments were carried out to address the aim to define the pathobiology of fibrosis in human tendon disease as a mechanism mediated by TGF-β and BMPs. In Chapter 3 clinical tissue samples of healthy and diseased tendons were obtained, and their mRNA and protein expression were analysed to investigate whether TGF-β and BMPs are implicated in the development of tendon disease. Protein expression analysis showed marked suppression, but no substantial changes in mRNA expression were noted in tissues from diseased tendons. The *in vitro* experiments showed altered mRNA expression but how mRNA expression of fibrotic mediators translates to their protein expression was not investigated. Numerous substantial processes occur after mRNA production from post-transcriptional modification, translation and degradation in the regulation of protein expression [338]. Furthermore, there is also a risk of missing localised changes due to the homogenisation of the tissue samples [419]. The results from Chapter 4 implied that the suppressed expression of TGF-β and BMP proteins in large to massive torn supraspinatus tendon tissues may relate to the inflammation signature expressing IL-4 target genes and proteins [52]. The *in vitro* experiment showed that IL-4 treatment significantly suppresses *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* mRNA expression in TSCs from diseased tendons. However, there are limitations of *in vitro* experiments that do not necessarily reproduce the processes that occur *in vivo* or during disease development. Cell culture do not replicate the *in vivo* environment by various factors such as structural, multicellular, metabolic and immunological differences that relate to disease or provide mechanical forces that is also known to contribute to tendon pathobiology. Whether the suppressed mRNA expression of the fibrotic mediators by cytokines *in vitro* is applicable to human disease pathobiology requires further investigation.

**Using tissues from anatomically distinct supraspinatus and hamstring tendons**

Diseased tendon tissues were obtained from patients with rotator cuff disease (supraspinatus tendinopathy and tear), whereas intact hamstring tissues from younger patient donors were utilised for the healthy cohort in some studies. As discussed previously, there is a possibility that the results from this thesis are confounded by age-related changes to cell phenotype. The limitations of using tissues from anatomically distinct tendons have been discussed in the experimental chapters as well. Tendon disease also affects the Achilles and patella tendons, which are energy storing compared to positional rotator cuff tendons. Rotator cuff tendon disease was focused on with regards to the clinical course that progress from tendinopathy to tear [53] and the ethical feasibility to obtain clinical samples due to the number of patients and the surgeries performed. Whether the findings from this project could be applied to the understanding of diseases of other tendons is another area that needs to be studied further. Dakin *et al.* reported that although there are common cellular mechanisms of chronic inflammation between the supraspinatus and Achilles tendon diseases, there are some differences in torn supraspinatus and Achilles tendons. Torn Achilles but not supraspinatus tendon tissues show findings of acute inflammation and vascularisation [420]. Furthermore, the timing of tissue collection in terms of disease chronicity is an important factor to be considered when using clinical tissue samples. Hence, the possibility that the expression of fibrotic mediators had dampened by the time the tissues were obtained should be taken into account of for the perceived reduced expression as well.

**Roles of other TGF-β isoforms and BMPs should be considered**

Out of the numerous BMPs, the expression of BMP-2, BMP-7 were chosen based on previous literature as the most investigated BMPs in relation to tendon disease or healing in animal

models [193]. BMP-12 was also investigated as numerous studies using animal tendon injury models have reported its ability to induce tenogenic differentiation [245, 255, 327, 328]. Studies have shown that other BMPs such as BMP-4 and BMP-6 may relate to human tendon disease [196, 231], and hence this project should not eliminate the possibilities of their contribution in the pathobiology. Moreover, other isoforms of TGF-β, TGF-β2 and TGF-β3 were not referred to throughout the thesis. All 3 isoforms activate similar cell signalling pathways resulting in similar effects, but TGF-β1 is known to be the most common type expressed in relation to musculoskeletal tissues in adults that also plays a key role in fibrotic healing (TGF-β3 has been indicated to take major roles in embryonic microenvironment) [171, 172]. Only the two receptors TGF-β R1 and TGF-β R2 were studied as TGF-β R3 does not function in signalling activities [172, 421, 422]. The differences in the roles of the different TGF-β receptors should be considered.

A potential therapeutic strategy of applying TGF-β and BMPs or increasing their activity to improve tendon healing was indicated. However, the details such as the timing, dosage, delivery method and whether to use a single growth factor or in combination are beyond the scope of this thesis.

## 6.5 Future Work

This thesis addressed the differences in the local expression of TGF-β and BMPs, their regulation by cytokines and their effects on genes related to ECM and cell differentiation between tissues and TSCs from healthy and diseased (torn) tendons. Increased mRNA expression of ERK1/2 and NF-κB signalling pathway target genes in TSCs in relation to end-stage tendon disease was highlighted. However, how other MAPKs such as p38 and SAPK/JNK are involved and interact in the expression of their target genes are yet to be discovered. A transcription factor AP-1 was focused on as a protein that regulates the crosstalk between the MAPK and NF-κB pathways, but further investigation of its constituent proteins other than c-Jun such as c-Fos would be necessary to elucidate the underlying mechanism of the dysregulated expression of TGF-β and BMPs in relation to tendon disease.

Erk1/2 signalling pathway activation has been reported to be important in EMT [423], which is a process mediated by TGF-β where epithelial cells undergo transition to cells of the mesenchymal lineage such as fibroblasts and myofibroblasts [424]. As TGF-β1 and BMP-2 induced the myofibroblastic gene *ACTA2* expression in TSCs from diseased tendons, investigating how the increased ERK1/2 signalling pathway activity in response to IL-1β shown in TSCs from diseased tendons relates to EMT and the expression of *ACTA2* would be another aspect of fibrosis that would be interesting to focus on.

**Defining the pathobiology of fibrosis in tendon tear**

Numerous studies have reported that the size of a tear may increase with time in full thickness rotator cuff tears [425-427]. TSCs from large to massive tear supraspinatus tendons that represent end-stage disease over a chronic course were studied in comparison to TSCs from healthy tendons in this thesis. However, how the highlighted findings develop along tear

progression was not investigated. As torn supraspinatus tendons are known to have distinct features of inflammation according to the size of the tear [52], it is possible that the pathobiology of TSCs from more acute small to medium tear differs from that of TSCs from end-stage large to massive tear tendons. Defining the temporal changes should indicate the appropriate timing for intervention. Moreover, the possibility that the results from this thesis are confounded by age-related changes to cell phenotype has been discussed. Since rotator cuff tears are common in the older generation [75], it would be more ideal to sample the untorn "healthy" tendon tissues from patients of similar age. These untorn supraspinatus tendon tissues may be obtained from patients with a comminuted proximal humerus fracture treated surgically under the PROFHER-2 (PROximal Fracture of the Humerus Evaluation by Randomisation 2) trial (HTA 16/73/03). PROFHER-2 is a randomised control trial comparing the clinical and cost effectiveness of shoulder replacement surgery and non-surgical treatment for comminuted proximal humerus fractures in patients aged 65 and over in a multicentre setting. The patients of the surgery cohort will undergo a reverse shoulder arthroplasty, which the supraspinatus tendon will be resected and discarded.

**Creating an animal model of chronic tendon tear**

The results of the thesis showing decreased local expression of TGF-β and BMPs in tissues and cellular response to these growth factors in TSCs from end-stage torn tendons suggest a potential therapeutic method to improve tendon healing of rotator cuff repair surgeries by applying TGF-β and BMPs. As no study has used TGF-β addition treatments in combination with a rotator cuff repair surgery in humans [418], preliminary validation studies using animal models would be warranted for future clinical application. However, currently available small animal tendon injury models do not reproduce the nature of chronic tendon tear in humans, which the mechanical loading of the affected tendon is completely lost. It has been reported

that rat Achilles and rotator cuff tendons regenerate even after their complete removal [341, 428]. Uchida *et al*. attempted to stress-deprive the patella tendon in a rat by drawing the patella and tibia together using a flexible wire, however the patella tendon remained in continuity between the 2 bones [104]. Ovine infraspinatus and equine SDF tendon full-thickness partial-width transection models have been proposed as animal models with minimal tendon repair [13, 429]. Studies using these animal models indicated that TSCs derived from unloaded (transected) regions and also overloaded (continuous) regions show changes in their metabolism after the partial transection [13, 429]. However, these cells were harvested 4 to 6 weeks after the intervention, which may only reflect acute changes. It has been shown in torn human Achilles tendons that the histological and molecular characteristics of its tissues may differ according to the chronicity of the tear [120]. Moreover, the mechanobiology of a tendon is known to relate to its associated musculature and bones [430], and chronic tendon tears such as a rotator cuff tendon tear are characterised by changes in their associated muscles (such as muscle atrophy and fatty infiltration) resulting in negative functional outcomes after repair surgery [431, 432]. The distant pathological effects of these partial transections on the entire tendon have been indicated [13], however whether these models induce similar changes to the musculature like in rat models of massive rotator cuff tears [428] is yet to be studied. Whether these models recapitulate the chronic tendon ruptures in humans is still to be determined, and establishing an animal model of chronic tendon tear would be more indicative for clinical application.

## 6.6 Conclusions

To conclude, this thesis showed that the local expression of TGF-β and BMPs in supraspinatus tendon tissues decline along disease progression. Furthermore, mRNA expression of these fibrotic mediators by cytokine regulation is dysregulated in TSCs from diseased compared to healthy tendons. In addition, cellular response to these fibrotic mediators is diminished in TSCs from diseased compared to healthy tendons.

The regulatory roles of the tissue inflammatory milieu in the expression of TGF-β and BMPs was indicated; IL-4 treatment significantly suppressed their mRNA expression in TSCs from diseased tendons. Meanwhile, IL-1β treatment *in vitro* induced *TGFB1* and increased *BMP2* mRNA expression in relation to increased ERK1/2 signalling pathway activity in TSCs from diseased tendons only.

Effects of TGF-β1 and BMP-2 treatments on ECM genes (especially proteoglycans) were reduced in TSCs from diseased compared to healthy tendons *in vitro*. TGF-β1 and BMP-2 treatments *in vitro* induced myofibroblastic differentiation (*ACTA2* mRNA expression) with increased activation of their respective canonical SMAD pathways in TSCs from diseased tendons. The increased responsiveness of TSCs from diseased tendons to inflammatory stimuli could be considered as a compensatory mechanism for the diminished expression and effects of TGF-β and BMPs. Although further research is necessary to understand the underlying mechanism, these findings demonstrate that the pathobiology of human tendon disease in relation to fibrosis is tendon specific and its end-stage is represented by reduced expression and cellular responses to TGF-β and BMPs.

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# Appendices

## Appendix A. Publications associated with this thesis

- Profibrotic mediators in tendon disease: a systematic review. Morita W, Snelling SJ, Dakin SG, Carr AJ. Arthritis Res Ther. 2016 Nov 18;18(1):269.
- Cytokines in tendon disease: A Systematic Review. Morita W, Dakin SG, Snelling SJ, Carr AJ. Bone Joint Res. 2017 Dec;6(12):656-64.

# Appendix B. Laboratory Protocols

## B.1 Details of mRNA extraction, Purification and cDNA Synthesis

### mRNA Extraction and Purification Protocol by RNeasy Minikit

1. Add and mix 350 µl of 70 percent ethanol to the cell lysates.
2. Transfer the mixed cell lysates to the spin columns and centrifuge at 10,000 rpm for 1 minute. Discard the flow-through.
3. Add 350 µl of Buffer RW1 and centrifuge at 10,000 rpm for 2 minutes. Discard the flow-through.
4. Add 80 µl of DNase I as solved as below (Table 8-1) and leave for 15 minutes at room temperature.
5. Add 350 µl of Buffer RW1 and centrifuge at 10,000 rpm for 1 minute. Discard the flow-through.
6. Add 500 µl of Buffer RPE and centrifuge at 10,000 rpm for 1 minute. Discard the flow-through.
7. Add 500 µl of Buffer RPE and centrifuge at 12,000 rpm for 1 minute. Discard the flow-through. Centrifuge at 12,000 rpm for 1 minute again to dry up the columns.
8. Place the columns into collection Eppendorf tubes, add 30 µl of RNase-free water and centrifuge at 10,000 rpm for 1 minute.
9. Transfer the flow-through in the collection tubes back to their respective columns and centrifuge at 10,000 rpm for 1 minute.

**TABLE A1: Composition of DNA digestion solution.**

| | |
|---|---|
| DNase I | 3 µl |
| 10x buffer | 8 µl |
| ETPC treated water | 69 µl |

## Composition of cDNA Reverse Transcription Kit mixed with mRNA

**TABLE A2: Composition of cDNA reverse transcription kit**

| | |
|---|---|
| 10x RT Buffer | 2 µl |
| 10x RT Random Primers | 2 µl |
| dNTP Mix | 0.8 µl |
| Reverse Transcriptase | 1 µl |
| RNasin Plus | 0.2 µl |
| mRNA | 14 µl |

## B.2 Details of Immunohistochemistry

### De-paraffinisation and Target Retrieval Protocols

De-paraffinisation, rehydration and antigen retrieval were carried out by PT Link.

1. Dilute EnVision FLEX TRS antigen retrieval fluid [DM828] (30 ml) to 50x by adding 1470 ml of deionised-distilled $H_2O$ ($ddH_2O$).
2. Fill the tank with the diluted EnVision FLEX TRS antigen retrieval fluid.
3. Place the section slides into the racks and insert the racks in the tanks.
4. The PT Link automatically carries out the following steps that takes approximately 90 minutes; (1) preheat to 65 °C, (2) incubate at 97 °C for 20 minutes and (3) cool to 65 °C.
5. Immerse the rack in Wash buffer [DM831] (75 ml) diluted to 20x by adding 1425 ml of $ddH_2O$ for 5 to 10 minutes (up to 2 to 3 hours) at room temperature.

### Immunostaining

Immunostaining was carried out by Autostainer Link 48 with EnVision™ FLEX DAB+ Chromogen System. Autostainer automatically applies the following agents in the following order: (1) peroxidase blocker [SM801], (2) primary antibody (diluted in antibody diluent [DM830]), (3) mouse or rabbit linker [SM804/805], (4) peroxidase (FLEX HRP) [SM802], (5) DAB chromogen [DM827] (in substrate buffer [SM803]) and (6) haematoxylin [SM806]. The section slides are rinsed by buffer each time the agents have been applied, and by $ddH_2O$ at the end.

### Negative Control Images

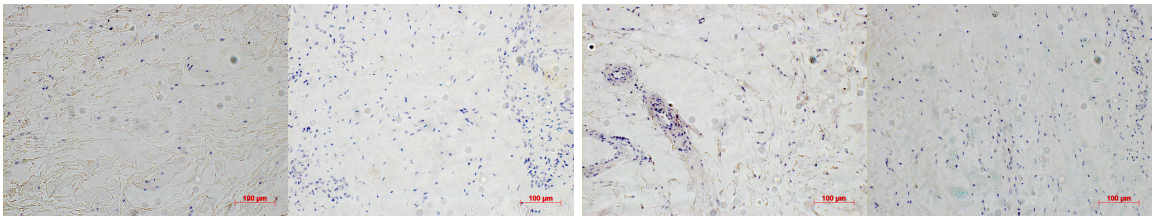



**FIGURE A1: Immunohistochemistry showing rabbit and mouse negative control in healthy and diseased (torn; large to medium) tendon tissues.** Representative images of healthy and torn rotator cuff tendon sections with haematoxylin (blue) for nuclei and DAB (brown) for immunopositive staining. Scale bar = 100 µm.

## Positive Control Images

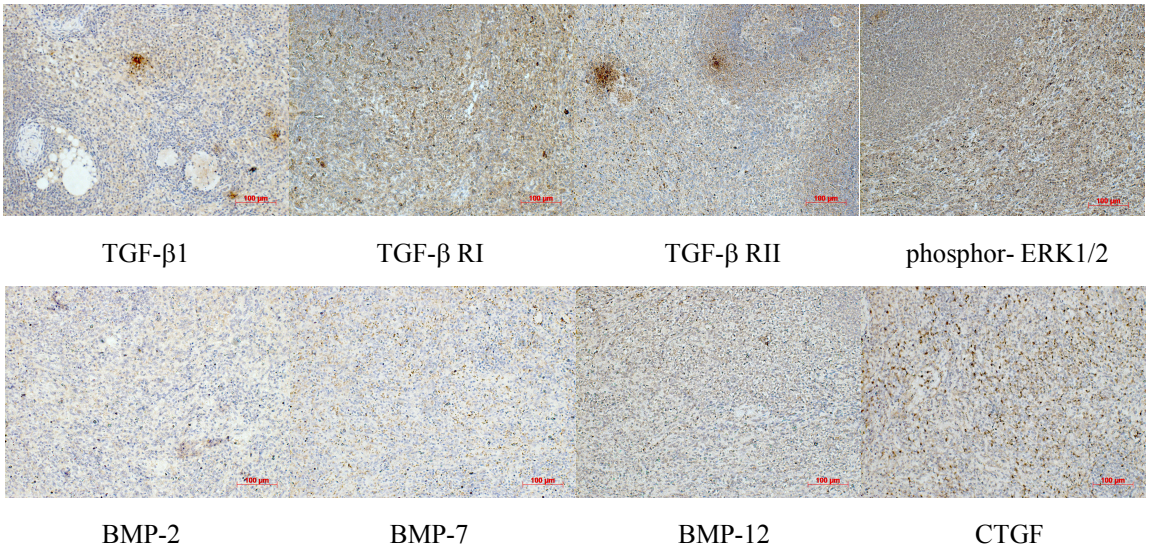



**FIGURE A2: Immunohistochemistry showing staining of fibrotic mediators in human spleen tissues (positive control).** Representative images of human spleen sections with haematoxylin (blue) for nuclei and DAB (brown) for immunopositive staining. Scale bar =100 µm.

## Immunohistochemistry Image Analysis Macros

- Nuclei Count

```
imgName=getTitle(); run("Colour Deconvolution", "vectors=[H&E 2]");
selectWindow(imgName+"-(Colour_1)"); setAutoThreshold("Default");
//run("Threshold..."); setThreshold(0, *); run("Convert to Mask");run("Fill Holes");
run("Despeckle"); run("Remove Outliers...", "radius=10 threshold=50 which=Dark");
run("Analyze Particles...", "size=10-Infinity circularity=0.00-1.00 show=Nothing
display summarize");
```

* manually set threshold for each dataset

- Immunopositive Cell Count

```
imgName=getTitle(); run("Colour Deconvolution", "vectors=[H DAB]");
selectWindow(imgName+"-(Colour_2)"); setAutoThreshold("Default");
//run("Threshold..."); setThreshold(0, *); run("Convert to Mask"); run("Remove
Outliers...", "radius=10  threshold=50 which=Dark"); run("Analyze Particles...",
"size=10-Infinity circularity=0.00-1.00 show=Nothing display summarize");
```

* manually set threshold for each dataset

## B.3 Details of Western Blotting Protocol

1. Prepare samples by mixing same amounts of protein lysate and 20 mM dithiothreitol (Sigma Aldrich) in 2x Laemmli sample buffer (BioRad).
2. Heat samples for 5 minutes at 95 °C.
3. Cool and centrifuge samples.
4. Load 30 µl of sample and 5 µl of protein standard marker to gels.
5. Separate the proteins in samples by electrophoresis (Mini-Protean Cell System; BioRad) at 100 volts for approximately 90 minutes with 1x SDS electrophoresis buffer that contains 16.5 mM Tris base (Fisher Scientific), 20 mM glycine (Sigma Aldrich) and 0.05 percent SDS (Fisher Scientific) in $ddH_2O$.
6. Transfer the separated proteins from gels to membranes by Trans-Blot Turbo Transfer System according to the manufacturer's instructions.
7. Block the membrane with 10 percent skimmed milk, 2 percent BSA (Sigma Aldrich) in Tris buffered saline (20 mM Tris base (Fisher Scientific), 20 mM Tris HCl (Fisher Scientific) and 50 mM NaCl (Fisher Scientific) in $ddH_2O$) with 0.1 percent Triton x-100 (Sigma Aldrich) (TBST) by shaking for 1 hour at room temperature.
8. Apply primary antibody in 5 percent BSA, 1 percent TWEEN 20 (Fischer Scientific) and TBST after washing with TBST and shake at 4 °C overnight.
9. Apply secondary antibody similarly and shake at 4 °C for 1 to 2 hours.
10. Wash with TBST.

## B.4 BCA Protein Assay

Protein quantification was carried out using a bicinchoninic acid (BCA) protein assay kit (BCA Protein Assay Kit; Merck Millipore) according to manufacturer's instructions. BSA standards (1500, 1000, 750, 500, 250, 125, 100, 50, 25, 5, 0 μg $ml^{-1}$) were prepared using the same cell lysis buffer for protein extraction as above. Protein samples (10 μl) were pipetted to 96-well plates and BCA working reagents (200 μl) were added. After the plate was incubated at 37 °C for approximately 60 minutes, absorbance at 562 nm was measured using FLUOstar OPTIMA Microplate Reader. The absorbance for each sample was corrected by subtracting the value of the blank standards. The amount of protein in each sample was calculated by using a standard curve generated by the measurements of the BSA standards.

# Appendix C. Additional Data

## C.1 Correlation of $C_T$ values between *ACTB* and *GAPDH*

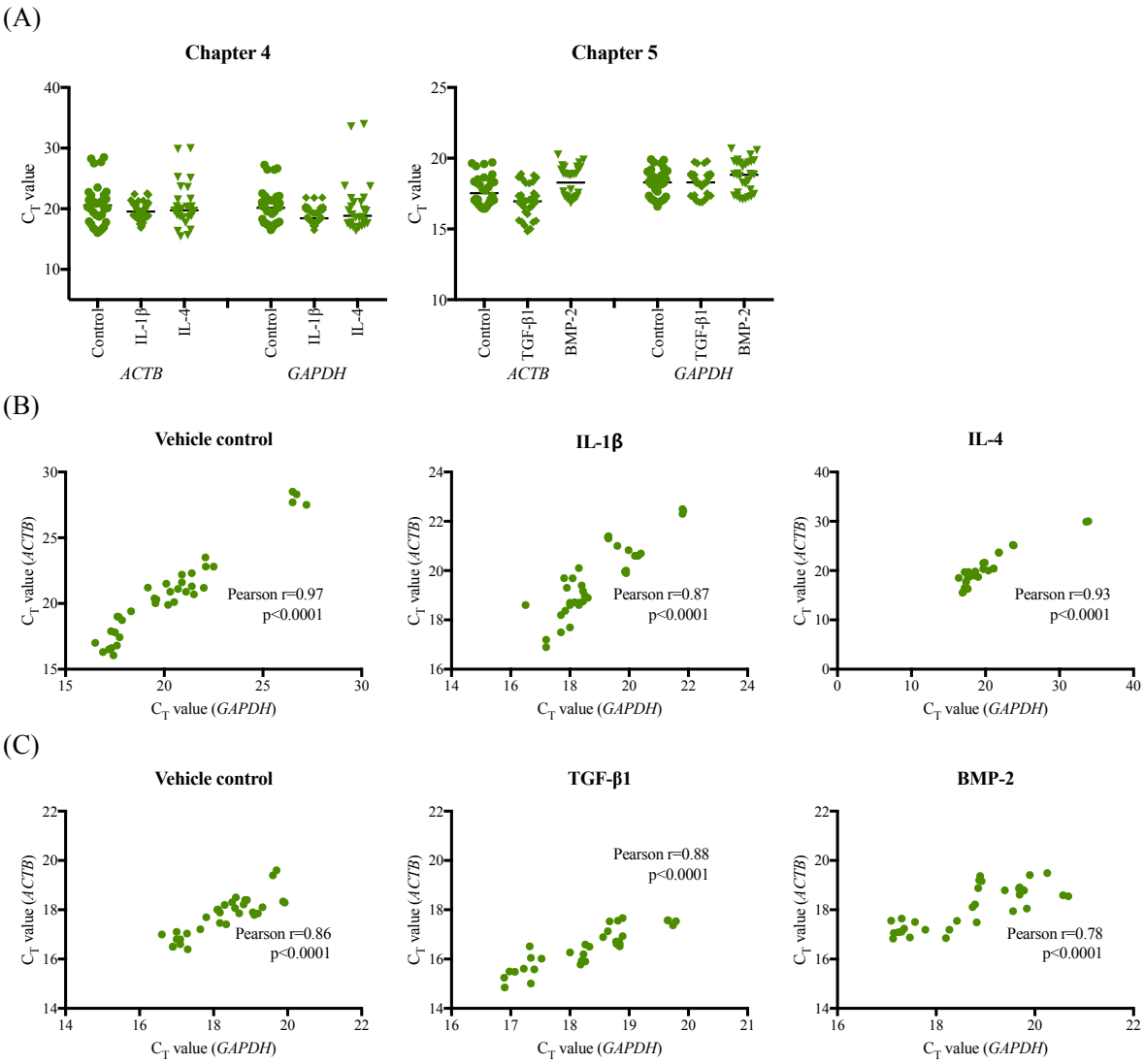



**FIGURE A3: Data of $C_T$ values of *ACTB* and GAPDH in each treatment groups.** (A) Comparison of $C_T$ values between each treatment groups. (B) Correlation of the raw $C_T$ values between *ACTB* and *GAPDH* in each treatment groups for Chapter 4 and (C) Chapter 5 were analysed by Pearson correlation (r).

## C.2 Fibrotic Signature of Tendon Disease

### mRNA Expression Data

- Data Normalised to β-actin

**TABLE A3: Data on relative mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* in healthy and torn (small to medium and large to massive) tendon tissues by RT-qPCR.** Levels of mRNA expression were quantified by RT-qPCR and normalised to β-actin. Median (IQR) shown as the data were not normally distributed. * indicates significant difference to the healthy group. med., medium; mass., massive; IQR, interquartile range. *=p<0.05.

| | Healthy | Tear (all) | Tear (small-med.) | Tear (large-mass.) |
|---|---|---|---|---|
| *TGFB1* | 0.01 (0.02/0.03) | 0.04 (0.02/0.05) | 0.03 (0.02/0.05) | 0.04 (0.02/0.06) |
| *TGFBR1* | 0.05 (0.05/0.07) | 0.06 (0.05/0.09) | 0.07 (0.05/0.08) | 0.06 (0.05/0.09) |
| *TGF-BR2* | 0.16 (0.13/0.21) | 0.07 (0.05/0.09) * | 0.05 (0.05/0.08) | 0.08 (0.07/0.13) |
| *BMP2* | 0.02 (0.01/0.02) | 0.01 (0.00/0.01) | 0.00 (0.00/0.01) | 0.01 (0.00/0.02) |
| *CTGF* | 0.40 (0.24/0.59) | 0.24 (0.09/0.59) | 0.15 (0.06/0.38) | 0.33 (0.12/0.61) |

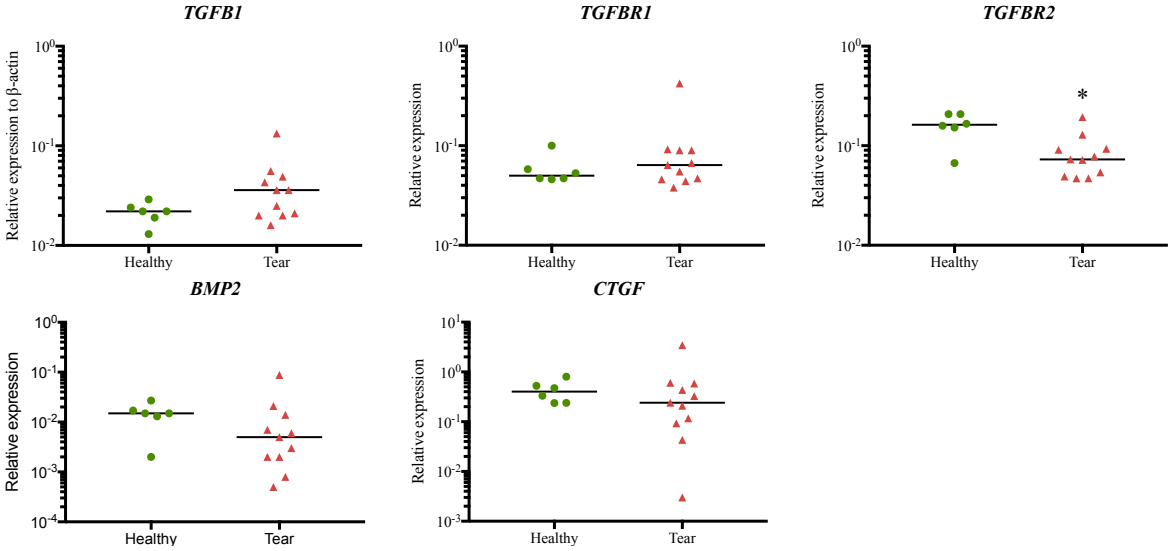



**FIGURE A4: Relative mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* in healthy and torn tendon tissues.** Levels of mRNA expression in tissues from healthy (n=6) and torn tendons (n=11) were quantified by RT-qPCR and normalised to β-actin. Bars shown represent median. * indicates significant difference to the healthy group. *=p<0.05.

- Data Normalised to GAPDH

**TABLE A4: Data on relative mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* in healthy and torn (small to medium and large to massive) tendon tissues by RT-qPCR.** Levels of mRNA expression were quantified by RT-qPCR and normalised to GAPDH. Median (IQR) shown as the data were not normally distributed. * indicates significant difference to the healthy group. med., medium; mass., massive; IQR, interquartile range. *=p<0.05.

| | Healthy | Tear (all) | Tear (small-med.) | Tear (large-mass.) |
|---|---|---|---|---|
| *TGFB1* | 0.01 (0.00/0.03) | 0.03 (0.02/0.07) * | 0.03 (0.01/0.05) | 0.06 (0.02/0.08) |
| *TGFBR1* | 0.03 (0.01/0.06) | 0.08 (0.04/0.12) * | 0.06 (0.05/0.09) | 0.12 (0.03/0.15) |
| *TGF-BR2* | 0.07 (0.03/0.16) | 0.07 (0.04/0.13) | 0.04 (0.03/0.11) | 0.09 (0.05/0.24) |
| *BMP2* | 0.01 (0.00/0.01) | 0.01 (0.00/0.01) | 0.00 (0.00/0.02) | 0.01 (0.00/0.01) |
| *CTGF* | 0.21 (0.06/0.39) | 0.34 (0.07/0.60) | 0.14 (0.04/0.52) | 0.34 (0.32/0.60) |

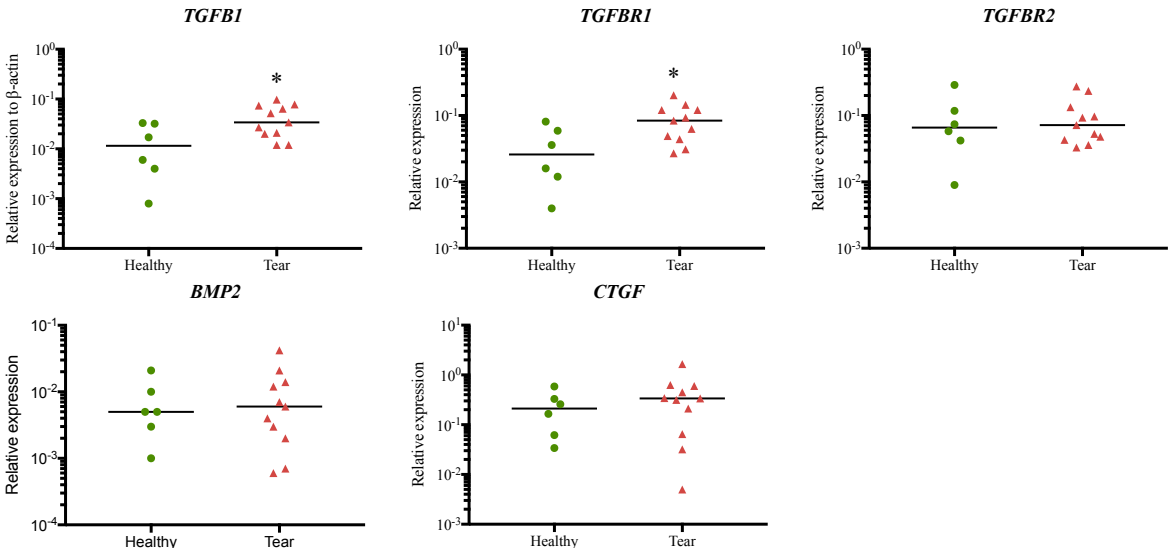



**FIGURE A5: mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* in healthy and torn tendon tissues.** Levels of mRNA expression in tissues from healthy (n=6) and torn tendons (n=11) were quantified by RT-qPCR and normalised to GAPDH. Bars shown represent median. * indicates significant difference to the healthy group. *=p<0.05.

## Immunohistochemical staining of CTGF in ECM

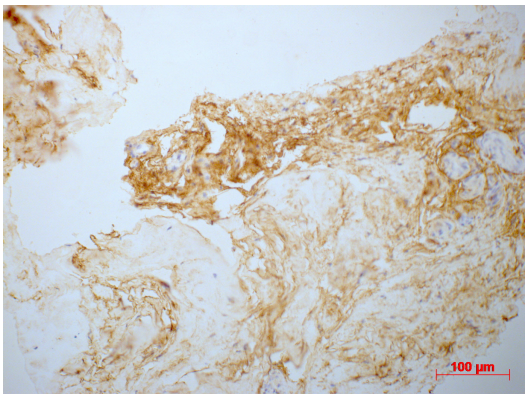



**FIGURE A6: Immunohistochemistry showing expression of CTGF in ECM of tendinopathic tendon tissues.** A representative image of tendinopathic rotator cuff tendon section is shown with haematoxylin (blue) for nuclei and DAB (brown) for CTGF. Scale bar =100 µm.

## Patient Demographics for CTGF Protein Expression based on ECM staining

**TABLE A5: Summary of patient demographics and Oxford Shoulder Score for CTGF protein expression analysis based on negative and positive ECM staining.** Median (IQR) shown for age and OSS as the data were small. -ve, negative; +ve, positive; ECM, extracellular matrix; IQR, interquartile range; OSS, Oxford Shoulder Score (scale of 0-48, function element 0-32, 0=least symptomatic).

| | | -ve ECM staining | +ve ECM staining |
|---|---|---|---|
| Number of patients | | 5 | 4 |
| Age; median (IQR) years | | 44 (42.5/54.5) | 50 (43.75/57.75) |
| Sex; male/female, n | | 4/1 | 3/1 |
| OSS; mean (IQR) | Total | 34 (31.75/34.75) | 27.5 (19.25/35.75) |
| | Function element | 28 (24.5/30) | 21.5 (16/30) |

## Correlation between Protein Expression of Fibrotic Mediators and OSS

**TABLE A6: Correlation between protein expression of TGF-β1, TGF-β R1, TGF-β R2, BMP-2, BMP-7, BMP-12 and CTGF and Oxford Shoulder Score.** Correlation coefficients were calculated by Pearson correlation or Spearman's rho when the data were parametric or non-parametric, respectively. * indicates statistical significance. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

| PROTEIN | TGF-β1 | TGF-β R1 | TGF-β R2 | BMP-2 | BMP-7 | BMP-12 | CTGF |
|---|---|---|---|---|---|---|---|
| OSS, total | -0.3957 | 0.6337** | 0.3381 | 0.2292 | 0.2085 | 0.2363 | -0.146 |
| OSS, function element | -0.421 | 0.6471** | 0.2667 | 0.1666 | 0.2022 | 0.1499 | 0.0512 |

## C.3 Differential Expression of TGF-β and BMPs in Stromal Cells from Healthy and Diseased Tendons

### Validation Experiments

- Validation Experiments for IL-4

<Duration validation>

Following serum-starving overnight, TSCs from healthy tendons cultured up to passage 3 (n=5) were seeded in 24-well plates, grown to sub-confluence and treated with human recombinant IL-4 (10 ng $ml^{-1}$) or vehicle control for 3, 6, 12 or 24 hours.

The effects of IL-4 treatment on *TGFB1* and *BMP2* mRNA expression were inconclusive. The trend of IL-4 treatment on suppressing *CTGF* mRNA expression increased over time.

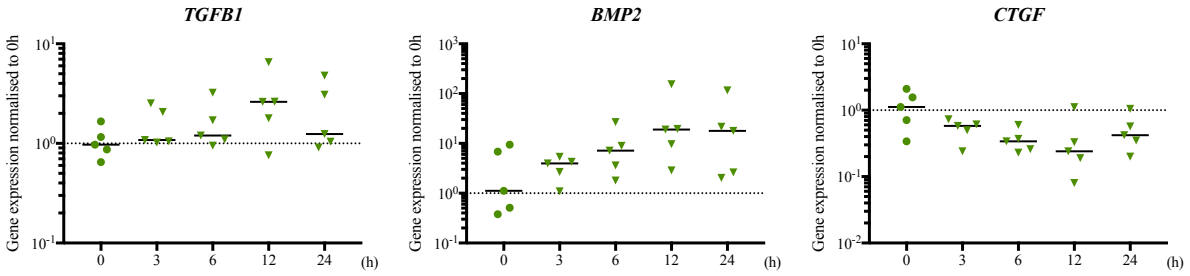



**FIGURE A7: mRNA expression of *TGFB1*, *BMP2* and *CTGF* in cells derived from healthy tendons after treatment by IL-4 or vehicle control (control) for 3, 6, 12 and 24 hours.** Levels of gene expression in tendon stromal cells from healthy tendons (n=5) were quantified by RT-qPCR, normalised to the internal control and then to the median of 0 hours. h, hour(s).

<Dose Validation>

Following serum-starving overnight, TSCs from healthy tendons cultured up to passage 3 (n=3) were seeded in 24-well plates, grown to sub-confluence and treated with human recombinant IL-4 (0.1, 1, 10 or 50 ng $ml^{-1}$) or vehicle control for 24 hours.

There were no significant differences in IL-4-induced *TGFB1*, *BMP2*, and *CTGF* mRNA expression by different doses.

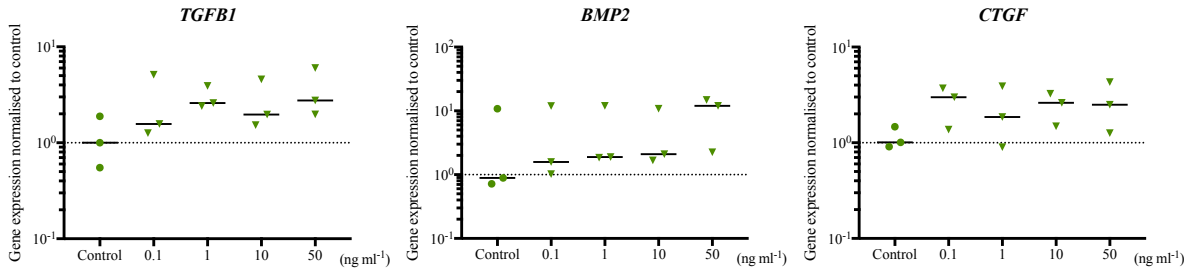



**FIGURE A8: mRNA expression of *TGFB1*, *BMP2* and *CTGF* in cells derived from healthy tendons after treatment with different doses of IL-4 or vehicle control (control) for 24 hours.** Levels of gene expression in tendon stromal cells from healthy tendons (n=3) were quantified by RT-qPCR, normalised to the internal control and then to the median of vehicle control ('control' on y axis). Bars shown represent median.

<Cell Viability>

Following serum-starving overnight, TSCs from healthy and diseased tendons cultured up to passages 2 to 3 (n=5 and n=5, respectively) were seeded in 96-well plates at a density of 30,000 cells $ml^{-1}$ (0.1 ml), grown to sub-confluence and treated with human recombinant IL-4 (10 ng $ml^{-1}$) or vehicle control for 12, 24 or 48 hours.

IL-4 treatment did not significantly modulate cell viability in TSCs from healthy and diseased tendons at any time points.

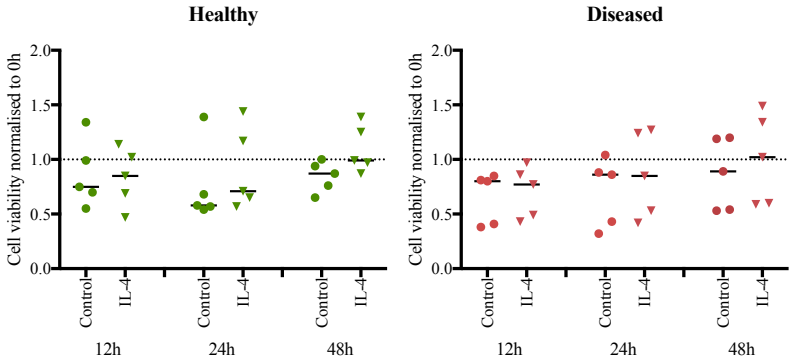



**FIGURE A9: Cell viability in cells derived from healthy and diseased tendons after treatment by IL-4 or vehicle control (control) for 12, 24 and 48 hours.** Cell viability was quantified in tendon stromal cells from healthy (n=5) and diseased tendons (n=5) by Alamar Blue assay and normalised to the median value of 0 hours. Bars shown represent median. h, hours.

- Validation Experiments for IL-1β

<Duration validation>

Following serum-starving overnight, TSCs from healthy tendons cultured up to passage 3 (n=5) were seeded in 24-well plates, grown to sub-confluence and treated with human recombinant IL-1β (5 ng $ml^{-1}$) or vehicle control for 3, 6, 12 or 24 hours.

IL-1β treatment tended to induce *TGFB1* mRNA expression compared to vehicle control. IL-1β treatment induced *BMP2* and suppressed *CTGF* mRNA expression compared to vehicle control, which became significant after 12 hours of treatment.

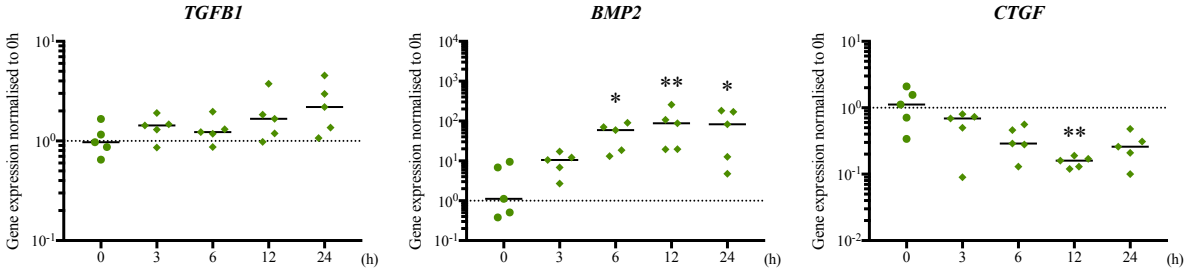



**FIGURE A10: mRNA expression of *TGFB1*, *BMP2* and *CTGF* in cells derived from healthy tendons after treatment by IL-1β or vehicle control (control) for 3, 6, 12 and 24 hours.** Levels of gene expression in tendon stromal cells from healthy tendons (n=5) were quantified by RT-qPCR, normalised to the internal control and then to the median of 0 hours. Bars shown represent median. * indicates significant difference to 0 hours. h, hour(s). *=p<0.05, **=p<0.01.

<Dose Validation>

Following serum-starving overnight, TSCs from healthy tendons cultured up to passage 3 (n=3) were seeded in 24-well plates, grown to sub-confluence and treated with human recombinant IL-1β (0.1, 1, 5 or 50 ng $ml^{-1}$) or vehicle control for 24 hours.

IL-1β treatment did not significantly modulate *TGFB1* and *BMP2* mRNA expression compared to vehicle control regardless of the dose. IL-1β treatment significantly suppressed *CTGF* mRNA expression compared to vehicle control at 50 ng $ml^{-1}$. Moreover, IL-1β treatment tended to induce *BMP2* mRNA expression compared to vehicle control.

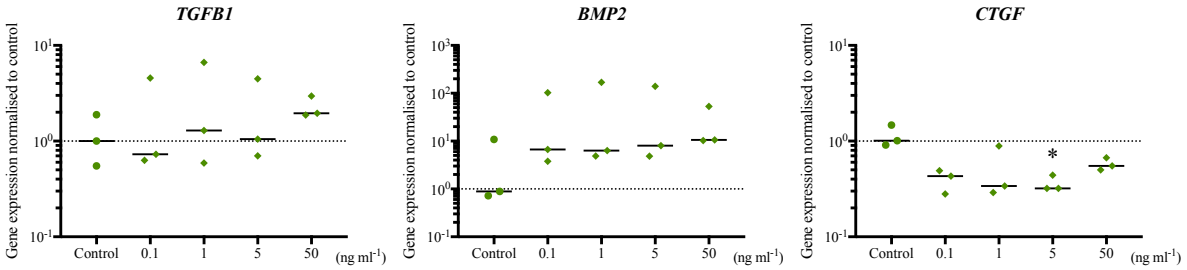



**FIGURE A11: mRNA expression of *TGFB1*, *BMP2* and *CTGF* in cells derived from healthy tendons after IL-1β treatment with different doses for 24 hours.** Levels of gene expression in tendon stromal cells from healthy tendons (n=3) were quantified by RT-qPCR, normalised to the internal control and then to the median of vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to vehicle control (control). *=p<0.05.

<Cell Viability>

Following serum-starving overnight, TSCs from healthy and diseased tendons cultured up to passages 2 to 3 (n=5 and n=5, respectively) were seeded in 96-well plates at a density of 30,000 cells ml-1 (0.1 ml), grown to sub-confluence and treated with human recombinant IL-1β (5 ng $ml^{-1}$) or vehicle control for 12, 24 or 48 hours.

IL-1β treatment significantly increased cell viability compared to vehicle control in TSCs from healthy tendons at 48 hours (p=0.0397). IL-1β treatment did not significantly modulate cell viability in TSCs from healthy cells at 12 and 24 hours. IL-1β treatment did not significantly modulate cell viability in TSCs from diseased tendons at any time points.

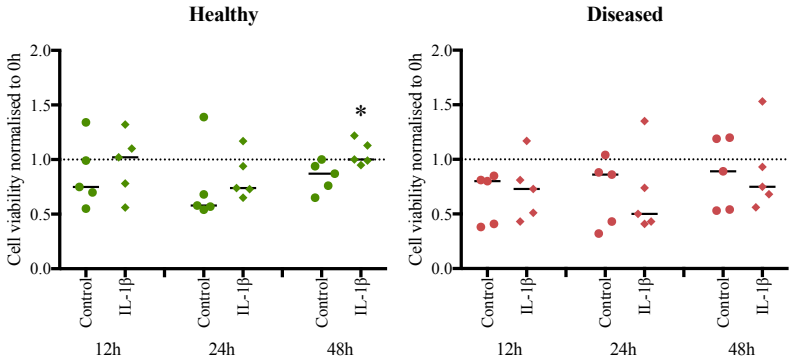



**FIGURE A12: Cell viability in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control) for 12, 24 and 48 hours.** Cell viability was quantified tendon stromal cells from healthy (n=5) and diseased tendons (n=5) by Alamar Blue assay and normalised to the median value of 0 hours. Bars shown represent median. * indicates significant difference to the respective vehicle control. h, hour(s). *=p<0.05.

- Validation Experiments for ERK1/2 Inhibitor

<Duration validation>

Following serum-starving overnight, TSCs from diseased tendons cultured up to passage 3 (n=2) were seeded in 6-well plates, grown to sub-confluence and treated with human recombinant IL-1β (5 ng $ml^{-1}$) or vehicle control with or without an ERK1/2 inhibitor (PD98059, 30 μM) for 30 minutes.

Pre-treatments with an ERK1/2 inhibitor for 1 and 4 hours both suppressed IL-1β-induced phosphorylated ERK1/2 expression.

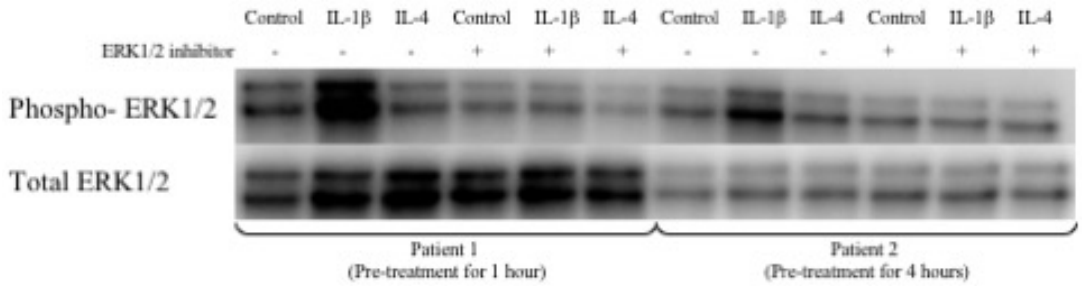



**FIGURE A13: Representative images of Western blotting for phosphorylated (phospho-) and total ERK1/2 in cells derived from diseased tendons after treatment by IL-1β or vehicle control (control) with or without pre-treatments by an ERK1/2 inhibitor for 1 and 4 hours.**

<Dose validation>

Following serum-starving overnight, TSCs from diseased tendons cultured up to passage 4 (n=4) were seeded in 24-well plates, grown to sub-confluence and treated with human recombinant IL-1β (5 ng $ml^{-1}$) or vehicle control with or without an ERK1/2 inhibitor (PD98059, 30, 60 or 90 μM) for 30 minutes.

Pre-treatment with an ERK1/2 inhibitor with the lowest dose suppressed IL-1β-induced *BMP2* and *PTGES* mRNA expression in TSCs from diseased tendons. The effects of the

ERK1/2 inhibitor on suppressing IL-1β-induced *BMP2* and *PTGES* mRNA expression in TSCs from diseased tendons did not change with different doses of the ERK1/2 inhibitor.

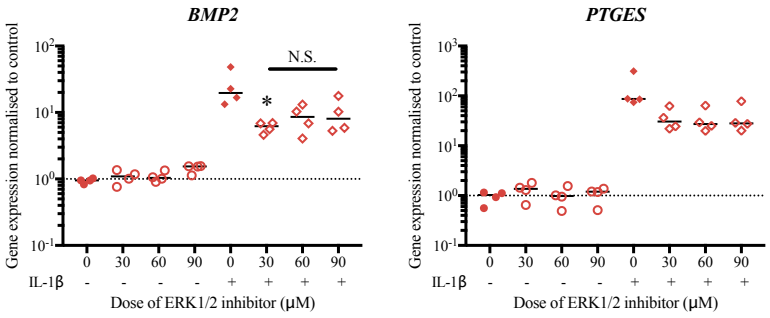



**FIGURE A14: mRNA expression of *BMP2* and *PTGES* in cells derived from diseased tendons after treatment by IL-1β or vehicle control (control) with different doses of the ERK1/2 inhibitor.** Levels of gene expression in tendon stromal cells from diseased tendons (n=4) were quantified by RT-qPCR, normalised to the internal control and then to the median of the respective 0μM, ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective 0μM. N.S., no significance. *=p<0.05.

<Cell Viability>

Following serum-starving overnight, TSCs from healthy and diseased tendons cultured up to passages 2 to 3 (n=5 and n=5, respectively) were seeded in 24-well plates at a density of 30,000 cells ml-1 (0.5 ml), grown to sub-confluence and treated with human recombinant IL-1β (5 ng $ml^{-1}$) or vehicle control with or without ERK1/2 inhibitor for 12, 24 or 48 hours.

IL-1β treatment had a proliferative effect on TSCs from healthy tendons after 48 hours of treatment (p=0.0397), which was not modulated by ERK1/2 inhibition. IL-1β or ERK1/2 inhibitor treatment did not significantly modulate cell viability in TSCs from diseased cells at any time points.

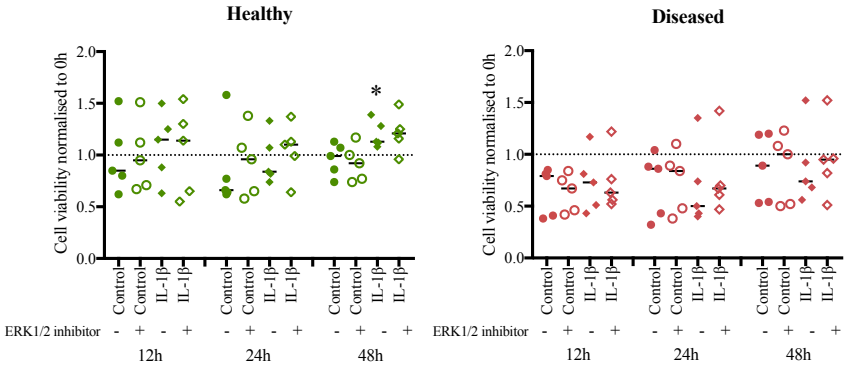



**FIGURE A15: Cell viability in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control) with or without an ERK1/2 inhibitor for 12, 24 and 48 hours.** Cell viability was quantified in tendon stromal cells from healthy (n=5) and diseased tendons (n=5) by Alamar Blue assay and normalised to the median value of 0 hours. Bars shown represent median. h, hours. * indicates significant difference to the respective vehicle control. *=$p<0.05$

## Pilot Experiment

Following serum-starving overnight, TSCs from healthy and diseased tendons cultured up to passage 3 (n=5) were seeded in 24-well plates, grown to sub-confluence and treated with human recombinant IL-1β (5ng $ml^{-1}$) or vehicle control for 24 hours.

There was a trend of increased *TGFB1* mRNA expression by IL-1β treatment compared to vehicle control in TSCs from diseased tendons. IL-1β treatment did not significantly modulate *BMP2* but there was a trend of decreased *CTGF* mRNA expression by IL-1β treatment in TSCs from healthy and diseased cells.

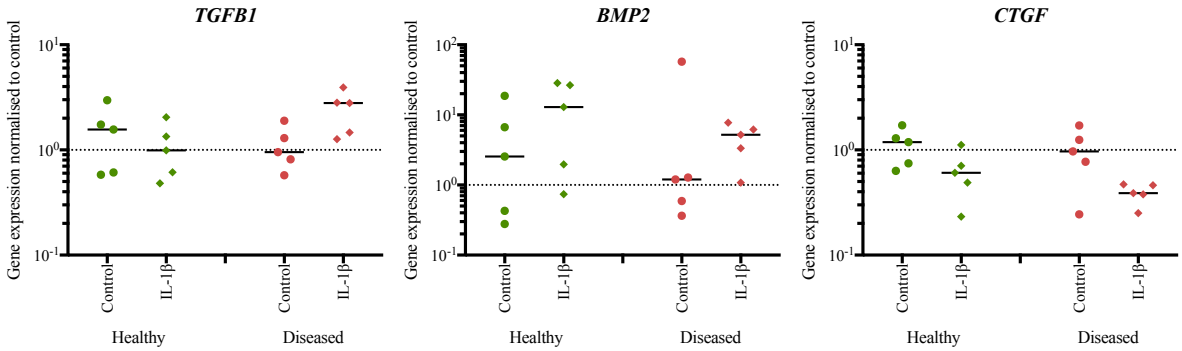



**FIGURE A16: mRNA expression of *TGFB1*, *BMP2* and *CTGF* in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=5) and diseased tendons (n=5) were quantified by RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median.

## Gene Expression Data

- Effects of IL-4 on Expression of Fibrotic Mediators

<Graphs with data points from each donor linked >

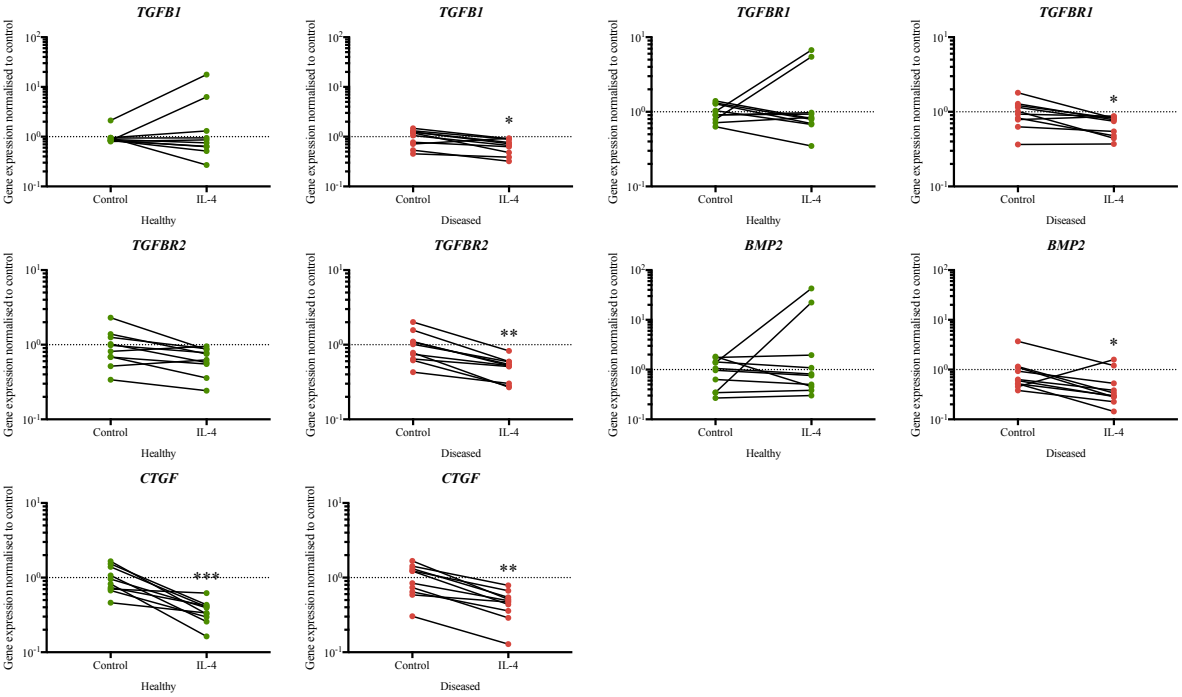



**FIGURE A17: mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* in cells derived from healthy and diseased tendons after treatment by IL-4 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=10) and diseased tendons (n=10) were quantified by RT-qPCR, normalised to the internal control and then to the median of vehicle control ('control' on y axis). Lines connect the data points from each donor. * indicates significant difference to the respective vehicle control. *=p<0.05, **=p<0.01, ***=p<0.001.

<Data Normalised to β-actin>

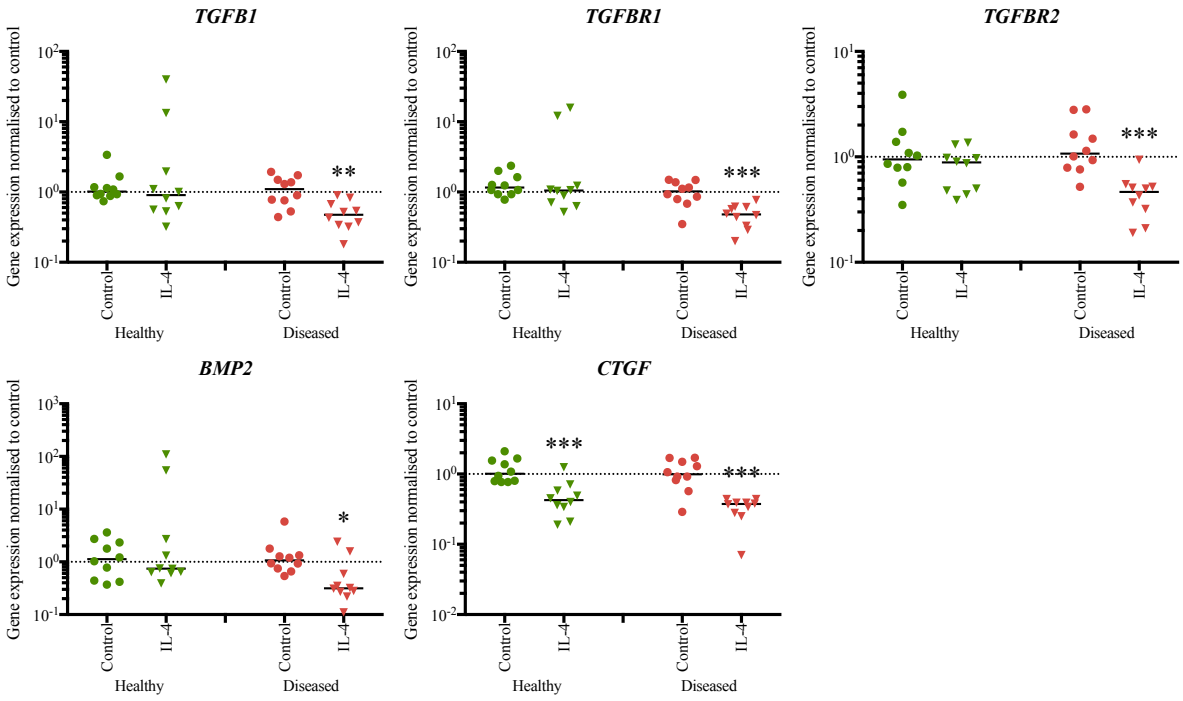



**FIGURE A18: mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* in cells derived from healthy and diseased tendons after treatment by IL-4 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=10) and diseased tendons (n=10) were quantified by RT-qPCR, normalised to β-actin and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. *=p<0.05, **=p<0.01, ***=p<0.001.

<Data Normalised to GAPDH>

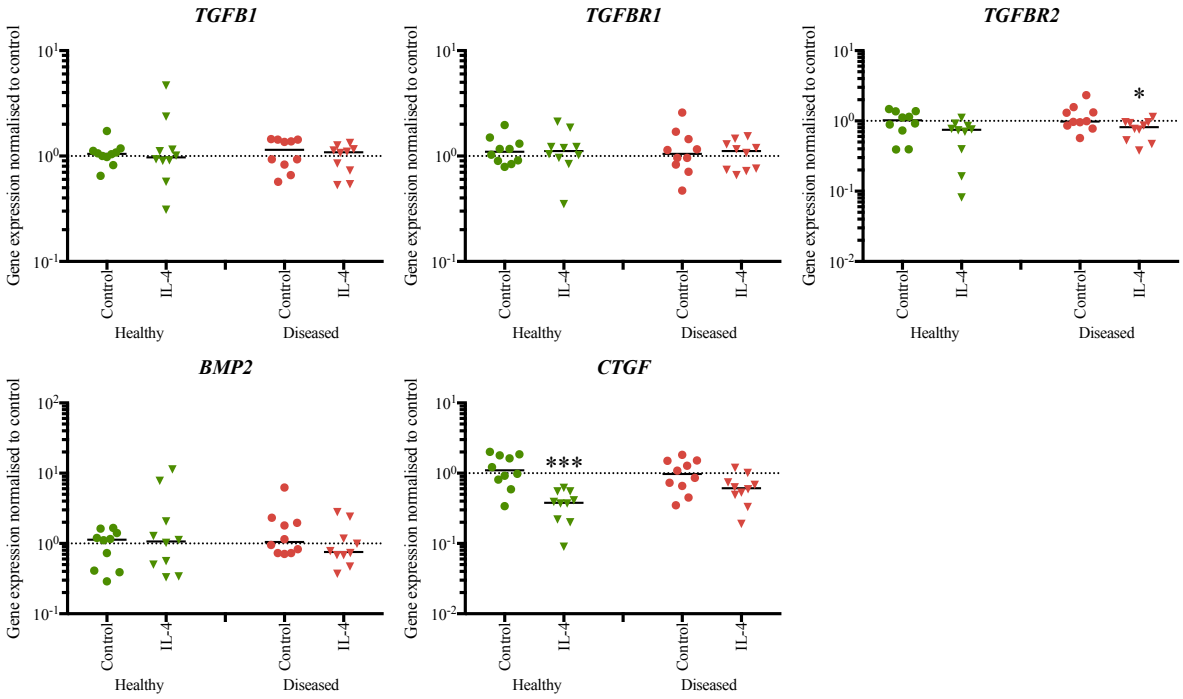



**FIGURE A19: mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2* and *CTGF* in cells derived from healthy and diseased tendons after treatment by IL-4 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=10) and diseased tendons (n=10) were quantified by RT-qPCR, normalised to GAPDH and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. *=p<0.05, **=p<0.01, ***=p<0.001.

- Effects of IL-1β on Expression of Fibrotic Mediators

<Data Normalised to β-actin>

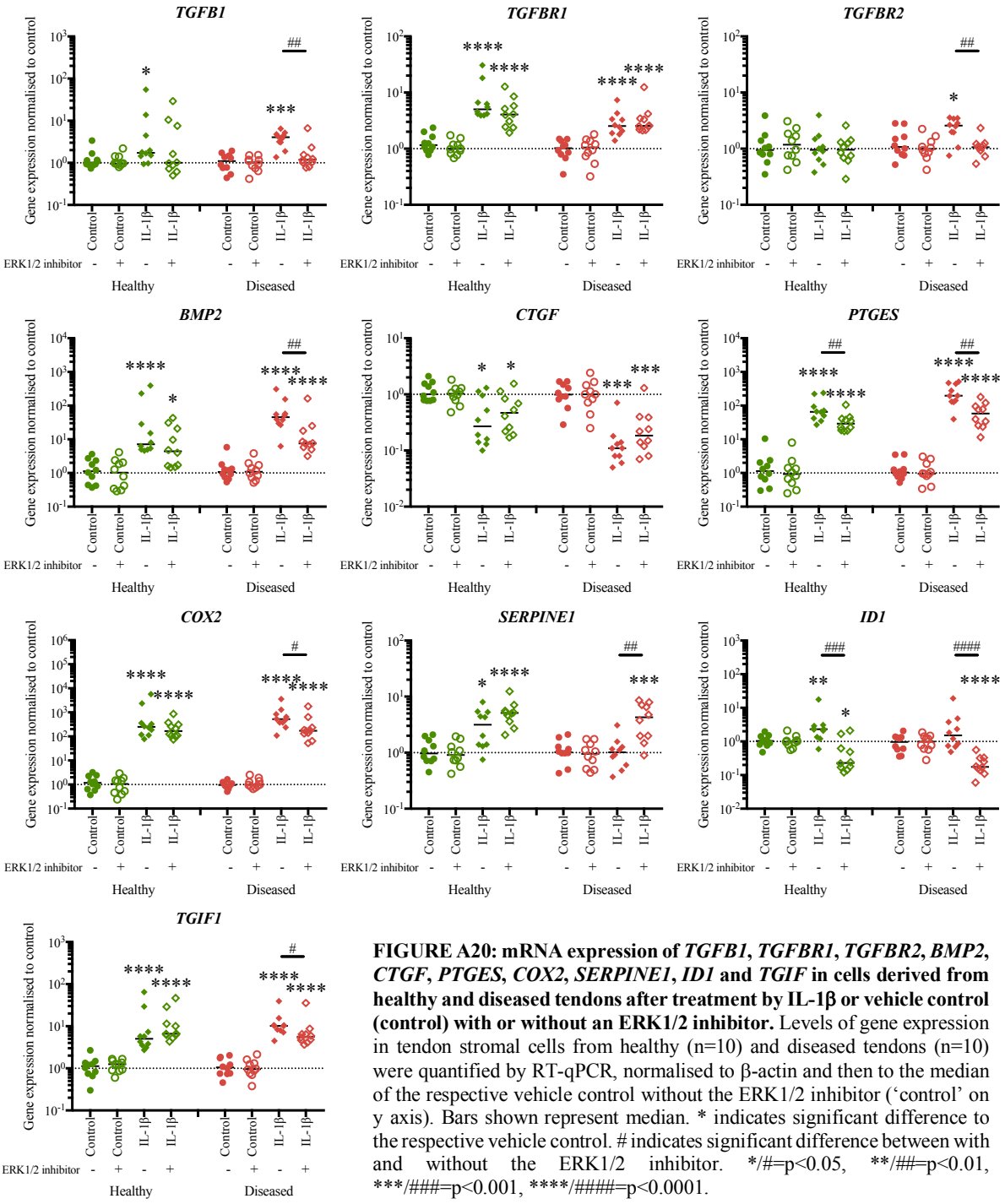



**FIGURE A20: mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2*, *CTGF*, *PTGES*, *COX2*, *SERPINE1*, *ID1* and *TGIF* in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control) with or without an ERK1/2 inhibitor.** Levels of gene expression in tendon stromal cells from healthy (n=10) and diseased tendons (n=10) were quantified by RT-qPCR, normalised to β-actin and then to the median of the respective vehicle control without the ERK1/2 inhibitor ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between with and without the ERK1/2 inhibitor. */#=p<0.05, **/##=p<0.01, ***/###=p<0.001, ****/####=p<0.0001.

<Data Normalised to GAPDH>

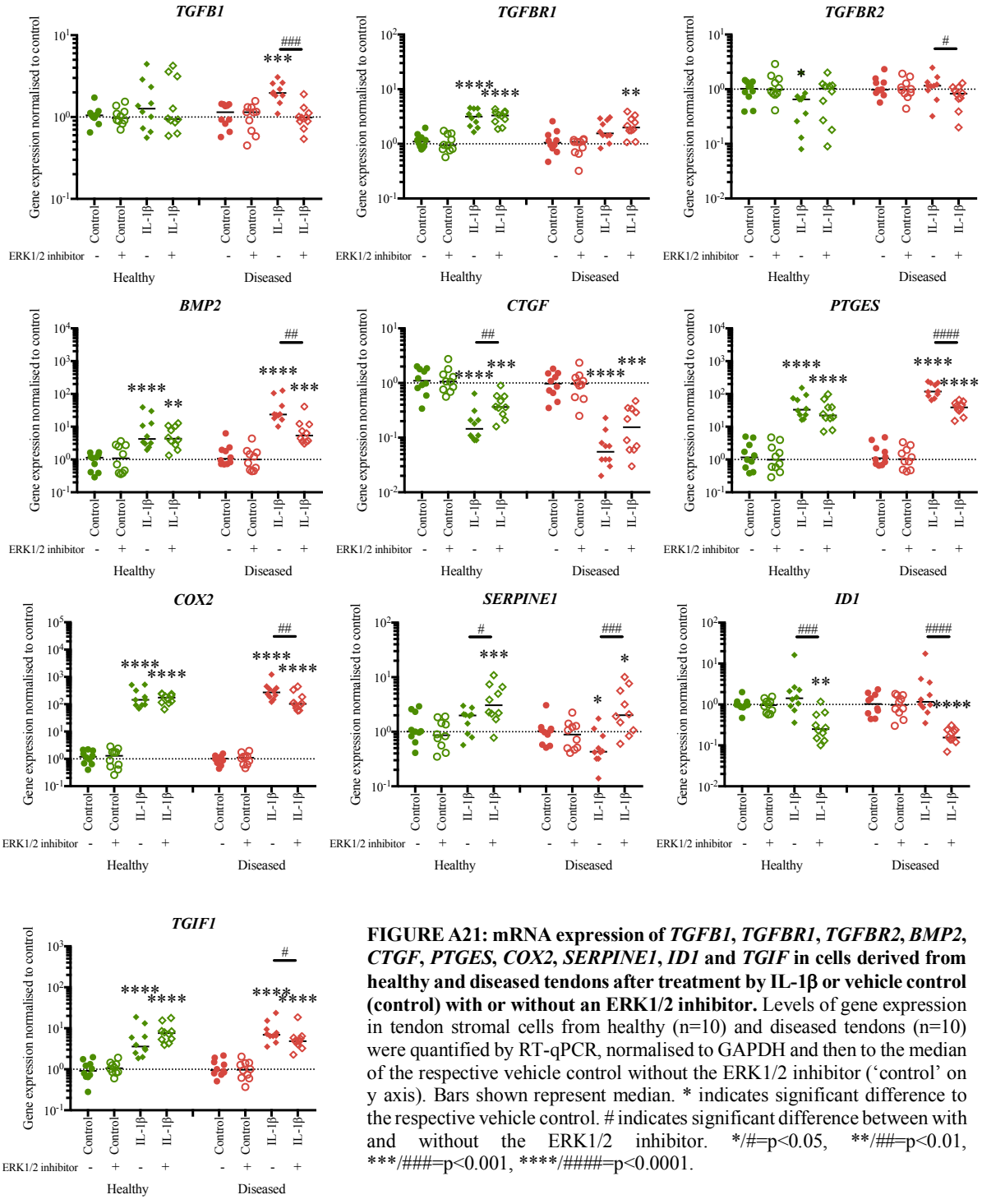


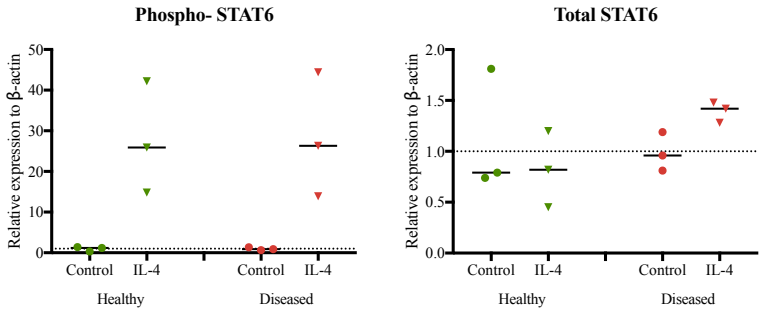

**FIGURE A21: mRNA expression of *TGFB1*, *TGFBR1*, *TGFBR2*, *BMP2*, *CTGF*, *PTGES*, *COX2*, *SERPINE1*, *ID1* and *TGIF* in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control) with or without an ERK1/2 inhibitor.** Levels of gene expression in tendon stromal cells from healthy (n=10) and diseased tendons (n=10) were quantified by RT-qPCR, normalised to GAPDH and then to the median of the respective vehicle control without the ERK1/2 inhibitor ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between with and without the ERK1/2 inhibitor. */#=p<0.05, **/##=p<0.01, ***/###=p<0.001, ****/####=p<0.0001.

## Protein Expression Data

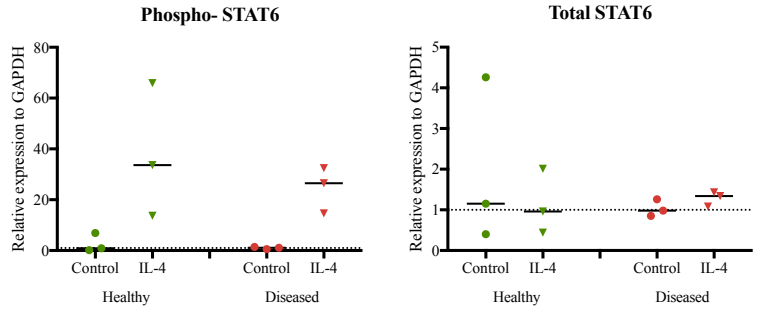
- Results of Semi-quantitative Analysis

<Cell Signalling Activity by IL-4 treatment>

(A)




(B)




(C)

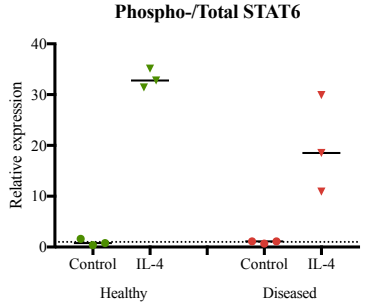



**FIGURE A22: Quantitative analysis of phosphorylated (phospho-) and total STAT-6 expression in cells derived from healthy and diseased tendons after treatment by IL-4 or vehicle control (control).** Levels of cell signalling activities were quantified in tendon stromal cells from healthy (n=3) and diseased tendons (n=3) by measuring the intensities of Western blots and normalised to (A) β-actin, (B) GAPDH or (C) their respective total protein. Bars shown represent median.

<Cell Signalling Activity by IL-1β treatment>

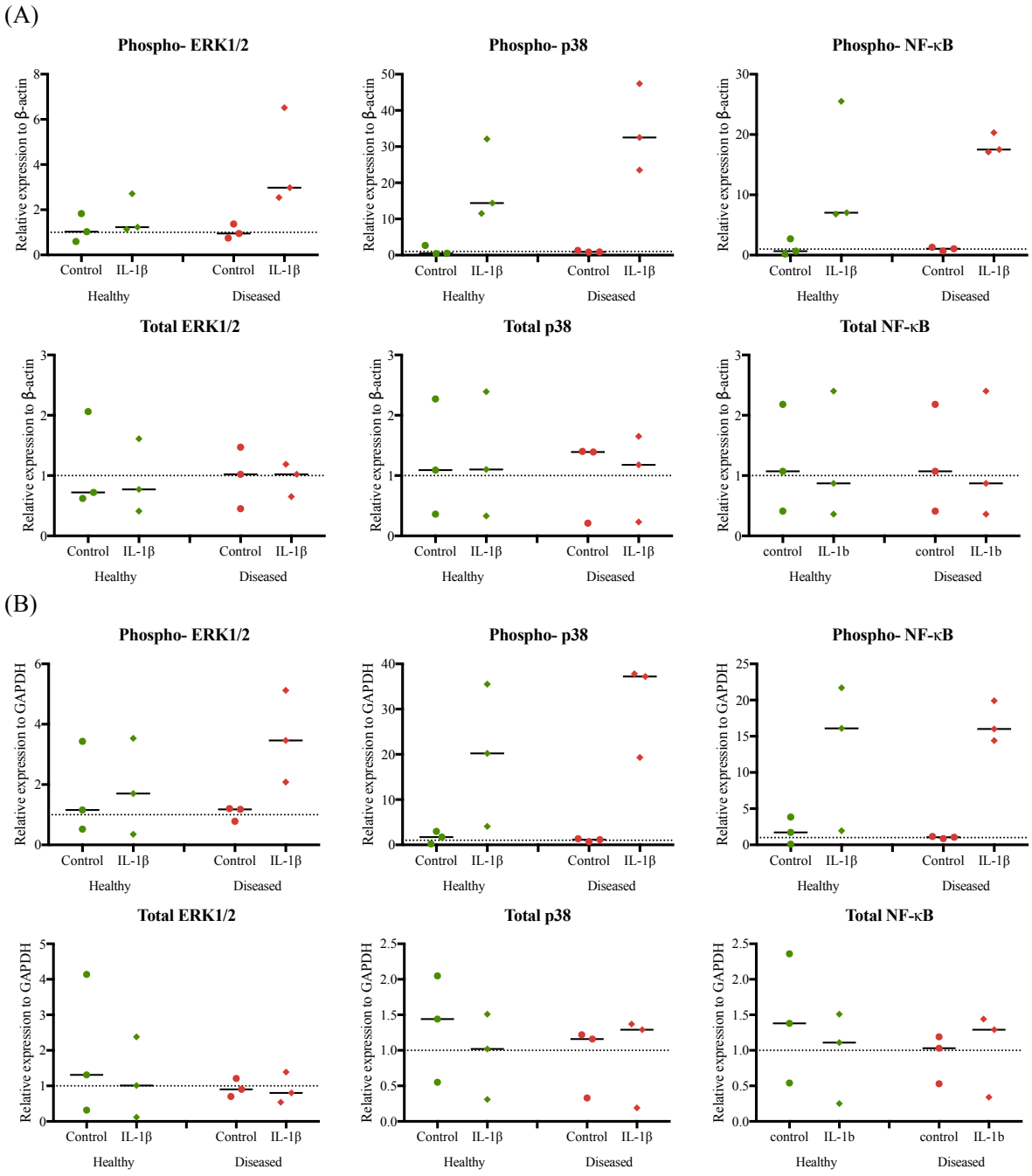
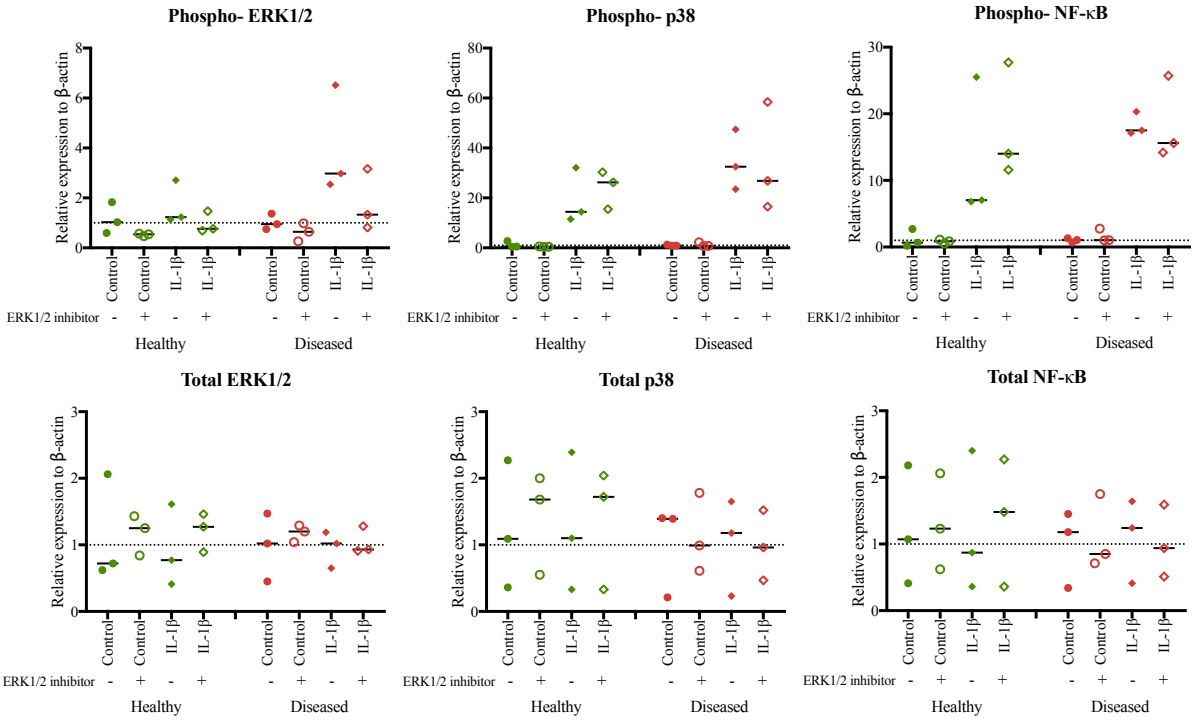


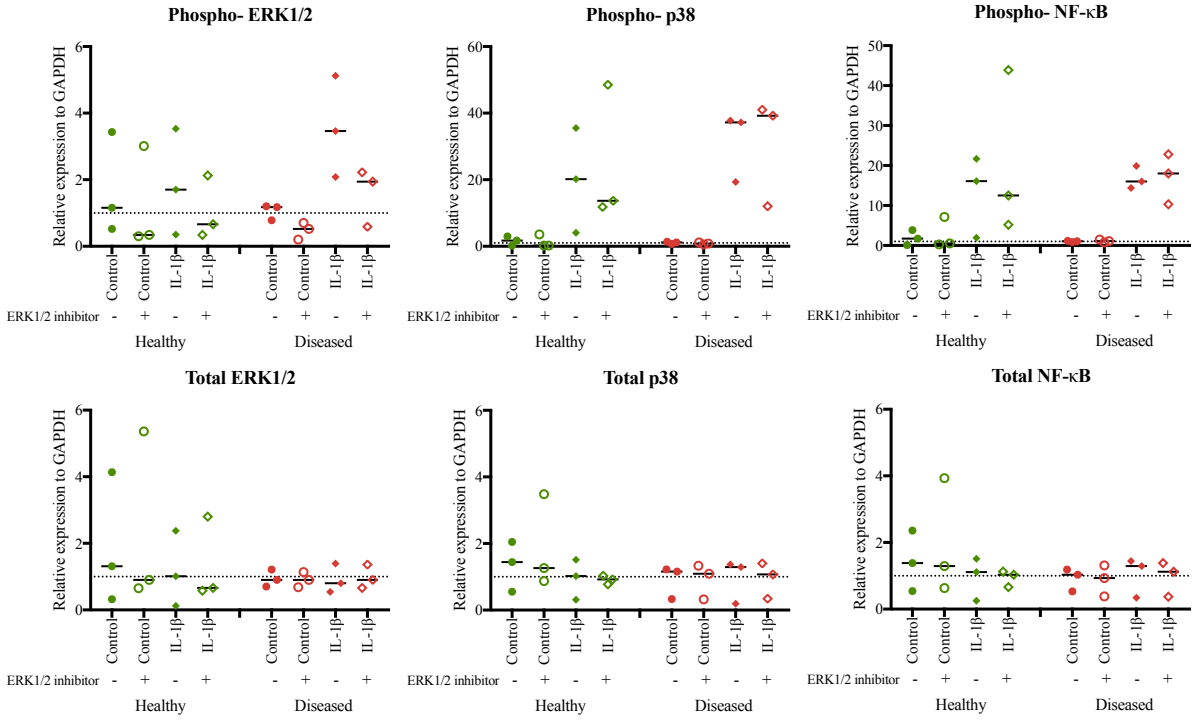

**FIGURE A23: Quantitative analysis of phosphorylated (phospho-) and total ERK1/2, p38 and NF-κB expression in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control).** Levels of cell signalling activities were quantified in tendon stromal cells from healthy (n=3) and diseased tendons (n=3) by measuring the intensities of Western blots and normalised to (A) β-actin or (B) GAPDH. Bars shown represent median.

<Cell Signalling Activity by IL-1β treatment with and without ERK1/2 inhibitor>

(A)




(B)



(C)

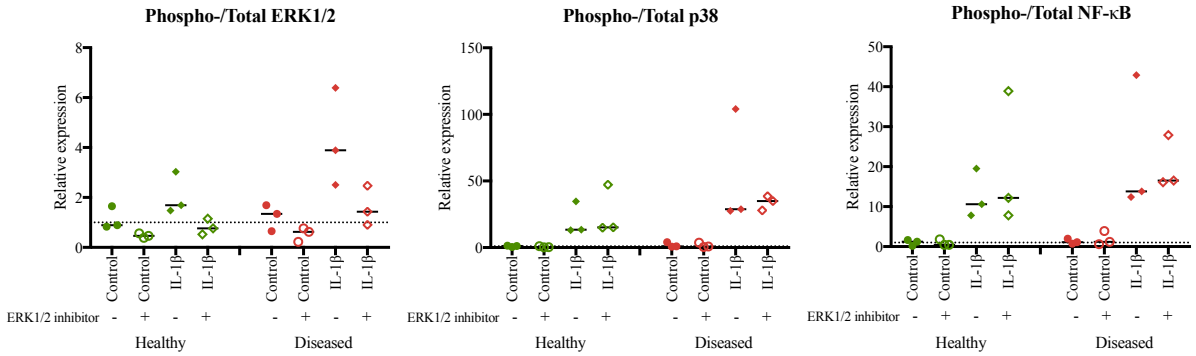
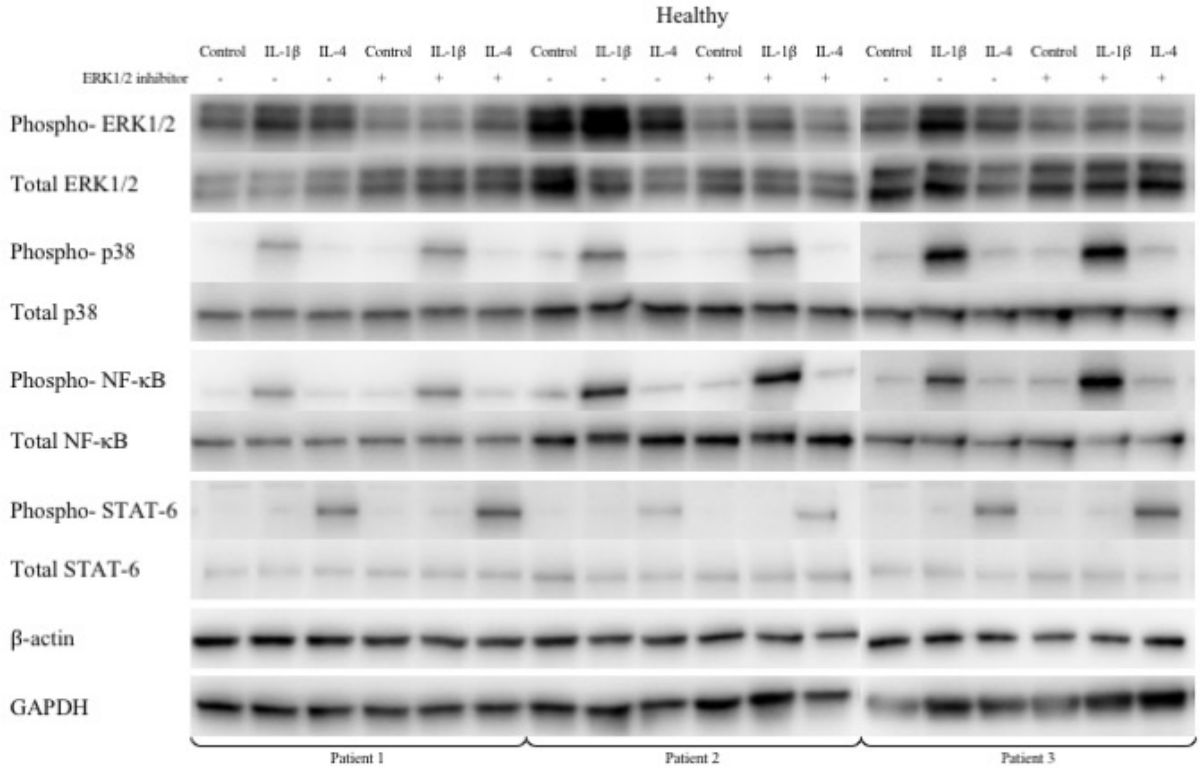



**FIGURE A24: Quantitative analysis of phosphorylated (phospho-) and total ERK1/2, p38 and NF-κB in cells derived from healthy and diseased tendons after treatment by IL-1β or vehicle control (control) with or without an ERK1/2 inhibitor.** Levels of cell signalling activities were quantified in tendon stromal cells from healthy (n=3) and diseased tendons (n=3) by measuring the intensities of Western blots and normalised to (A) β-actin, (B) GAPDH or (C) their respective total protein. Bars shown represent median.

- Images of Western Blots

<Phosphorylated & Total ERK1/2, p38, NF-κB, STAT-6, β-actin & GAPDH>

(A)



(B)

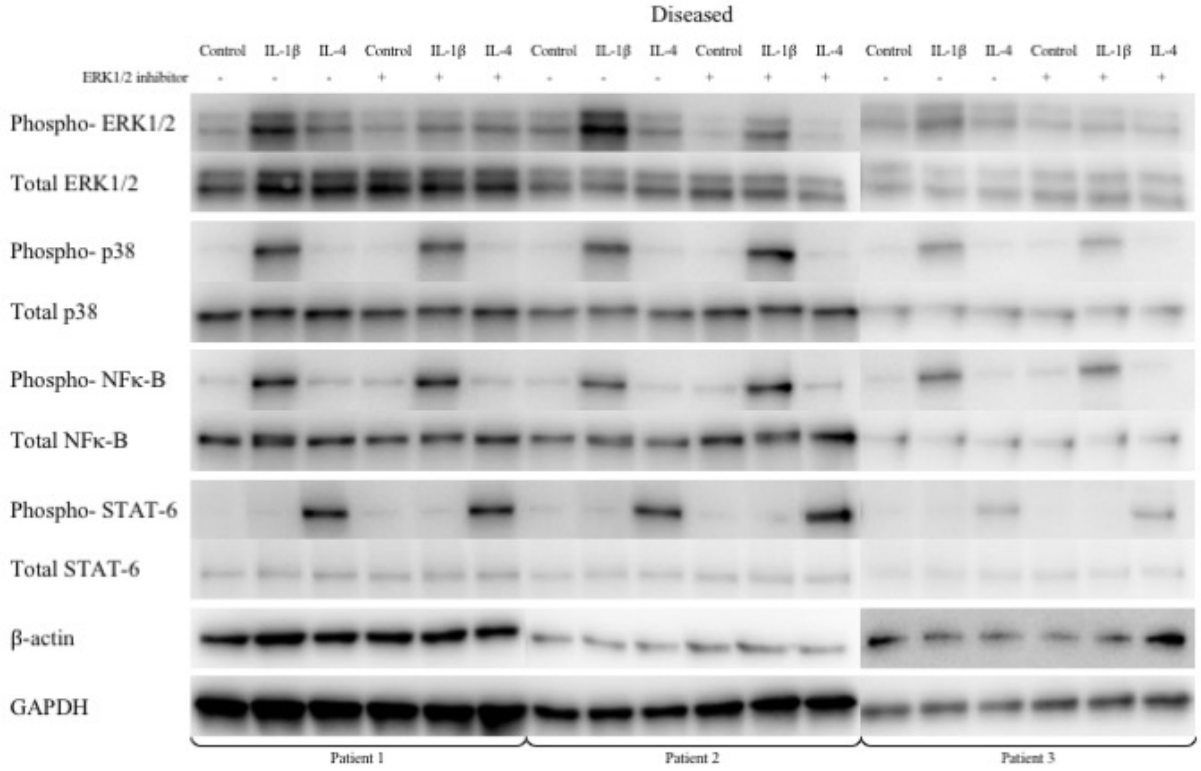



**FIGURE A25: Images of Western blotting for all investigated proteins in cells derived from (A) healthy and (B) diseased tendons after treatment by IL-1β, IL-4 or vehicle control (control) with or without an ERK1/2 inhibitor.**

<Phosphorylated & Total SAPK/JNK>

Substantial expression of phosphorylated SAPK/JNK could not be detected by Western blotting in TSCs from healthy and diseased tendons after treatment by IL-1β. Western blots using control cell extracts (SAPK/JNK Control Cell Extracts #9253, Cell Signaling) demonstrate that the antibodies used and their dilutions were appropriate.

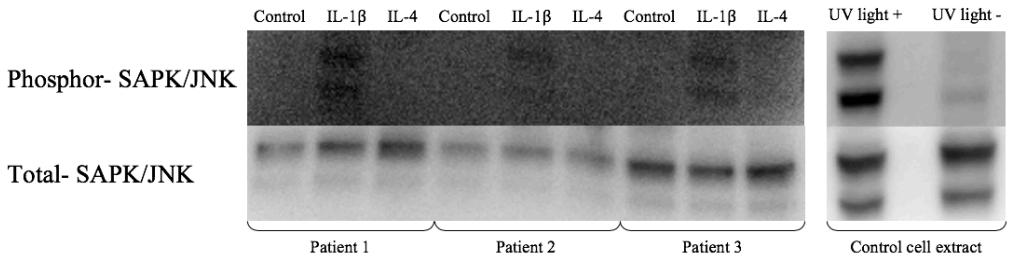
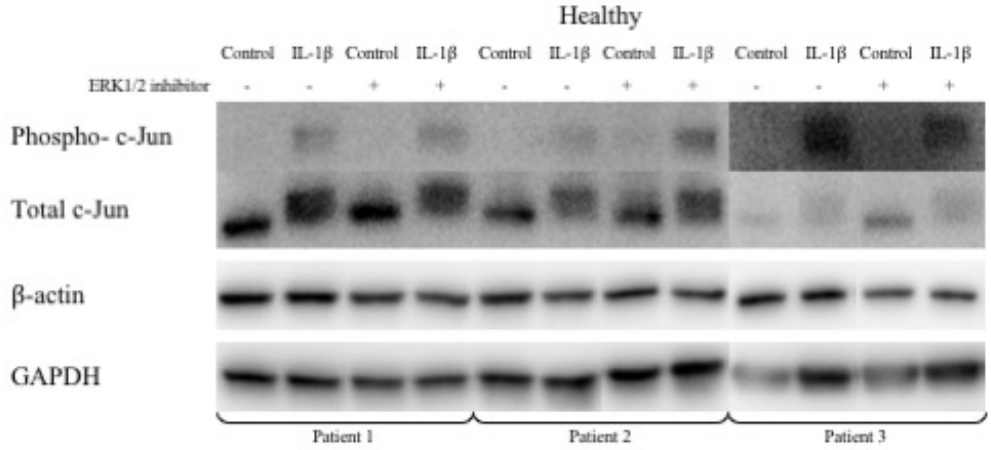



**FIGURE A26: Representative images of Western blotting for phosphorylated (phospho-) and total SAPK/JNK in cells derived from healthy and diseased tendons after treatment by IL-1β, IL-4 or vehicle control (control), and control cell extracts.**

<Phosphorylated & Total c-Jun>

(A)




(B)

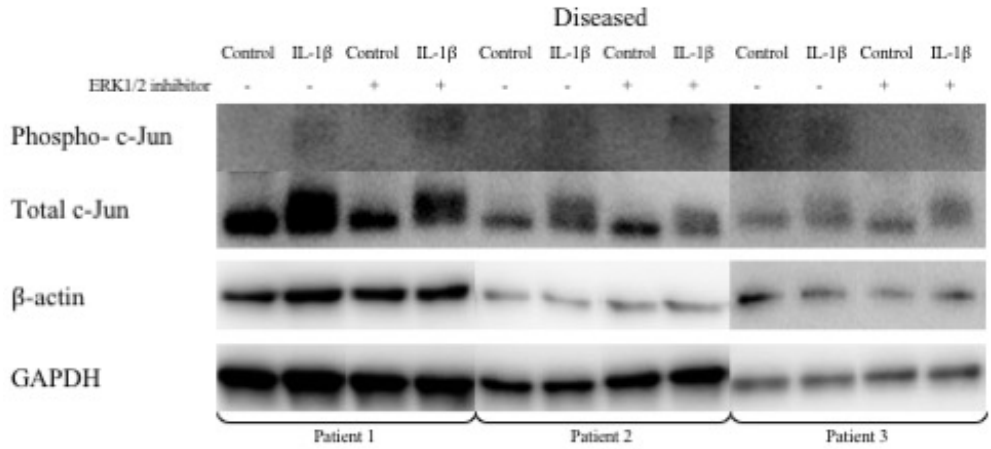


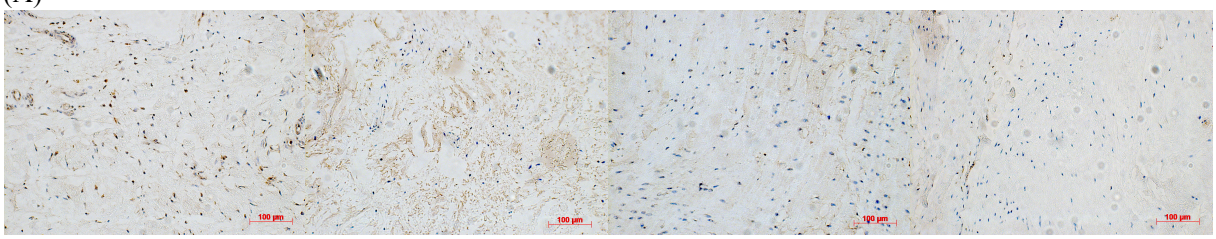

**FIGURE A27: Images of Western blotting for phosphorylated (phospho-) and total c-Jun and housekeepers β-actin and GAPDH in all cells from (A) healthy and (B) diseased tendons after treatment by IL-1β or vehicle control (control) with or without an ERK1/2 inhibitor.**

## Images of Immunohistochemistry

Protein expression of activated (phosphorylated) ERK1/2 in supraspinatus tendon tissues collected from tendinopathic (n=7) and torn (small to medium tear) (n=7) supraspinatus tendons was investigated as well. Donors of both tissues were significantly older than that of healthy tissues (p=0.0006 and p=0.0006, respectively). There were no significant differences in the ratio of males and females in between the groups of patients.

Quantitative analysis of immunopositive staining showed 1.48- and 2-fold decreased expression of phosphorylated ERK1/2 in tendinopathic and torn (small to medium) compared to healthy tendon tissues, respectively (p=0.0403 and 0.0403, respectively).

(A)



Healthy Tendinopathy Tear (small to med.) Tear (large to mass.)

(B)

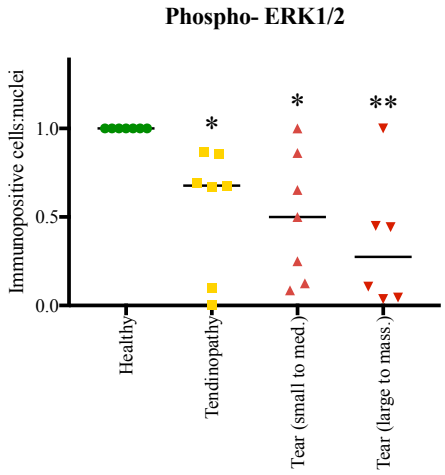



**FIGURE A28: Immunohistochemistry showing expression of phosphorylated (phospho-) ERK1/2 in healthy and diseased (tendinopathic, torn; small to medium and large to massive) tendon tissues.** (A) Representative images of healthy and diseased (tendinopathic, torn; small to medium and large to massive) supraspinatus tendon sections with haematoxylin (blue) for nuclei and DAB (brown) for immunopositive staining. Scale bar = 100 µm. (B) Quantitative analysis of phospho- ERK1/2 immunohistochemical staining in healthy (n=7), tendinopathic (n=7) and torn; small to medium (n=7) and large to massive (n=6) tendon tissues. Levels of protein expression were quantified as the number of immunopositive cells to the number of nuclei. Bars shown represent median. * indicates significant difference to the healthy group. med., medium; mass., massive. *=p<0.05, **=p<0.01.

## C.4 The *in vitro* Effects of TGF-β1 and BMP-2 on Fibrotic Phenotype of Tendon Stromal Cells

### Validation Experiments

- TGF-β1

<Duration validation>

Following serum-starving overnight, TSCs from healthy tendons cultured up to passage 4 (n=4) were seeded in 24-well plates, grown to sub-confluence and treated with human recombinant TGF-β1 (10 ng ml$^{-1}$) or vehicle control for 0.5, 1, 3 or 7 days.

TGF-β1 treatment induced *COL1A1* and *COL3A1* mRNA expression compared to vehicle control after 1 and 0.5 days, respectively. TGF-β1 treatment induced TGF-β target gene *SERPINE1* mRNA expression compared to vehicle control after 0.5 days.

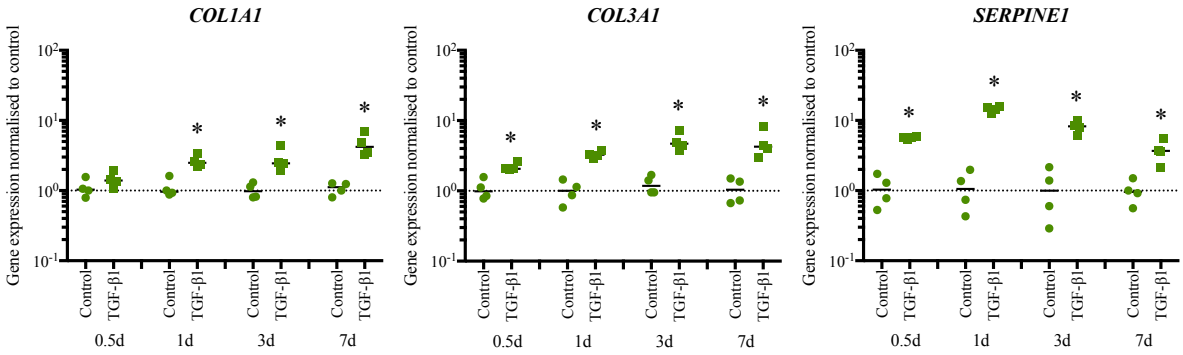



**FIGURE A29: mRNA expression of *COL1A1*, *COL3A1* and *SERPINE1* in cells derived from healthy tendons after treatment by TGF-β1 or vehicle control (control) for 0.5, 1, 3 or 7 days.** Levels of gene expression in tendon stromal cells from healthy tendons (n=4) were quantified by RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. d, day (s). *=p<0.05.

<Dose Validation>

Following serum-starving overnight, TSCs from healthy tendons cultured up to passage 4 (n=4) were seeded in 24-well plates, grown to sub-confluence and treated with human recombinant TGF-β1 (1, 10 or 50 ng ml$^{-1}$) or vehicle control for 3 days.

All doses of TGF-β1 treatment induced mRNA expression of *COL1A1*, *COL3A1* and its target gene *SERPINE1*.

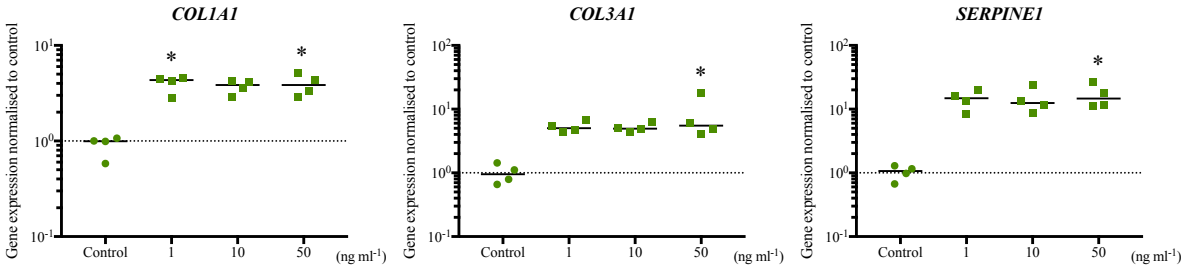



**FIGURE A30: mRNA expression of *COL1A1*, *COL3A1* and *SERPINE1* in cells derived from healthy tendons after treatment by different doses of TGF-β1 or vehicle control (control) for 3 days.** Levels of gene expression in tendon stromal cells from healthy tendons (n=4) were quantified by RT-qPCR, normalised to the internal control and then to the median of vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to vehicle control. *=p<0.05.

- BMP-2

<Duration validation>

Following serum-starving overnight, TSCs from healthy tendons cultured up to passage 4 (n=4) were seeded in 24-well plates, grown to sub-confluence and treated with human recombinant BMP-2 (100 ng $ml^{-1}$) or vehicle control for 0.5, 1, 3 or 7 days.

BMP-2 treatment did not have any effects on *COL1A1* or *COL3A1* mRNA expression compared to vehicle control. BMP-2 treatment induced BMP target gene *ID1* mRNA expression compared to vehicle control after 3 days.

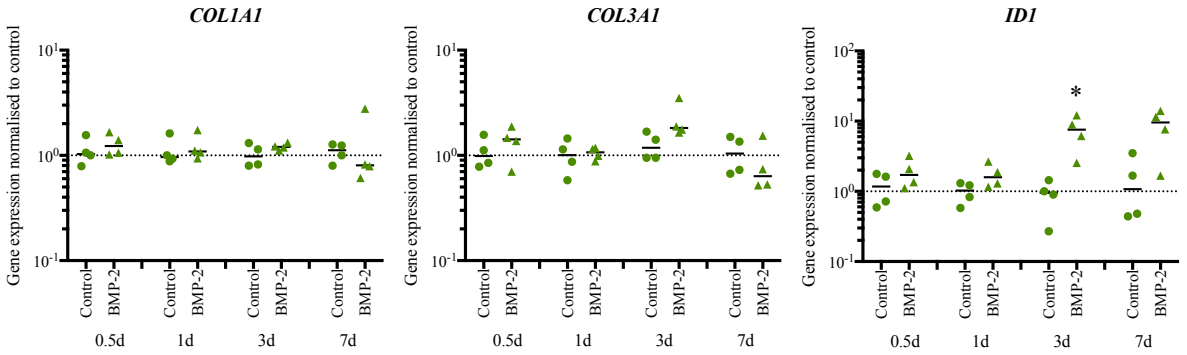



**FIGURE A31: mRNA expression of *COL1A1*, *COL3A1* and *ID1* in cells derived from healthy tendons after treatment by BMP-2 or vehicle control (control) for 0.5, 1, 3 or 7 days.** Levels of gene expression in tendon stromal cells from healthy tendons (n=4) were quantified by RT-qPCR, normalised to the internal control and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. d, day (s). *=p<0.05.

<Dose Validation>

Following serum-starving overnight, TSCs from healthy tendons cultured up to passage 4 (n=4) were seeded in 24-well plates, grown to sub-confluence and treated with human recombinant TGF-β1 (10, 100 or 250 ng ml$^{-1}$) or vehicle control for 3 days.

BMP-2 treatment did not have any effects in mRNA expression of *COL1A1* or *COL3A1* compared to vehicle control regardless of the dose, but significantly induced BMP target gene *ID1* mRNA expression compared to vehicle control when greater than 100 ng ml$^{-1}$.

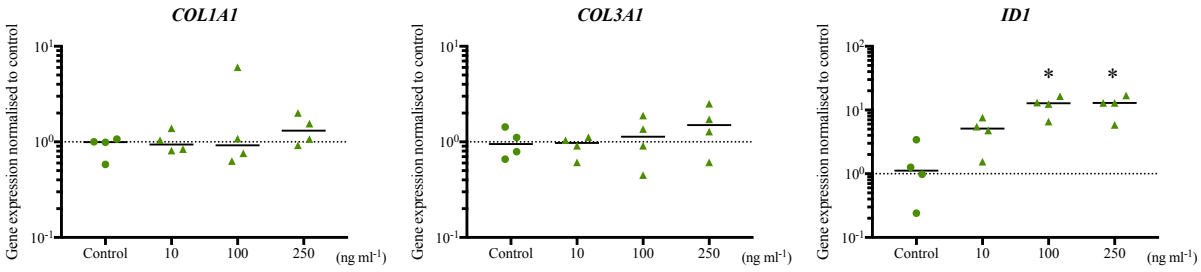



**FIGURE A32: mRNA expression of *COL1A1*, *COL3A1* and *ID1* in cells derived from healthy tendons after treatment by different doses of BMP-2 or vehicle control (control) for 3 days.** Levels of gene expression in tendon stromal cells from healthy tendons (n=4) were quantified by RT-qPCR, normalised to the internal control and then to the median of vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to vehicle control. *=p<0.05.

## Gene Expression Data

- Effects of TGF-β1 on mRNA Expression of ECM and Cell Differentiation Genes

<Data normalised to β-actin>

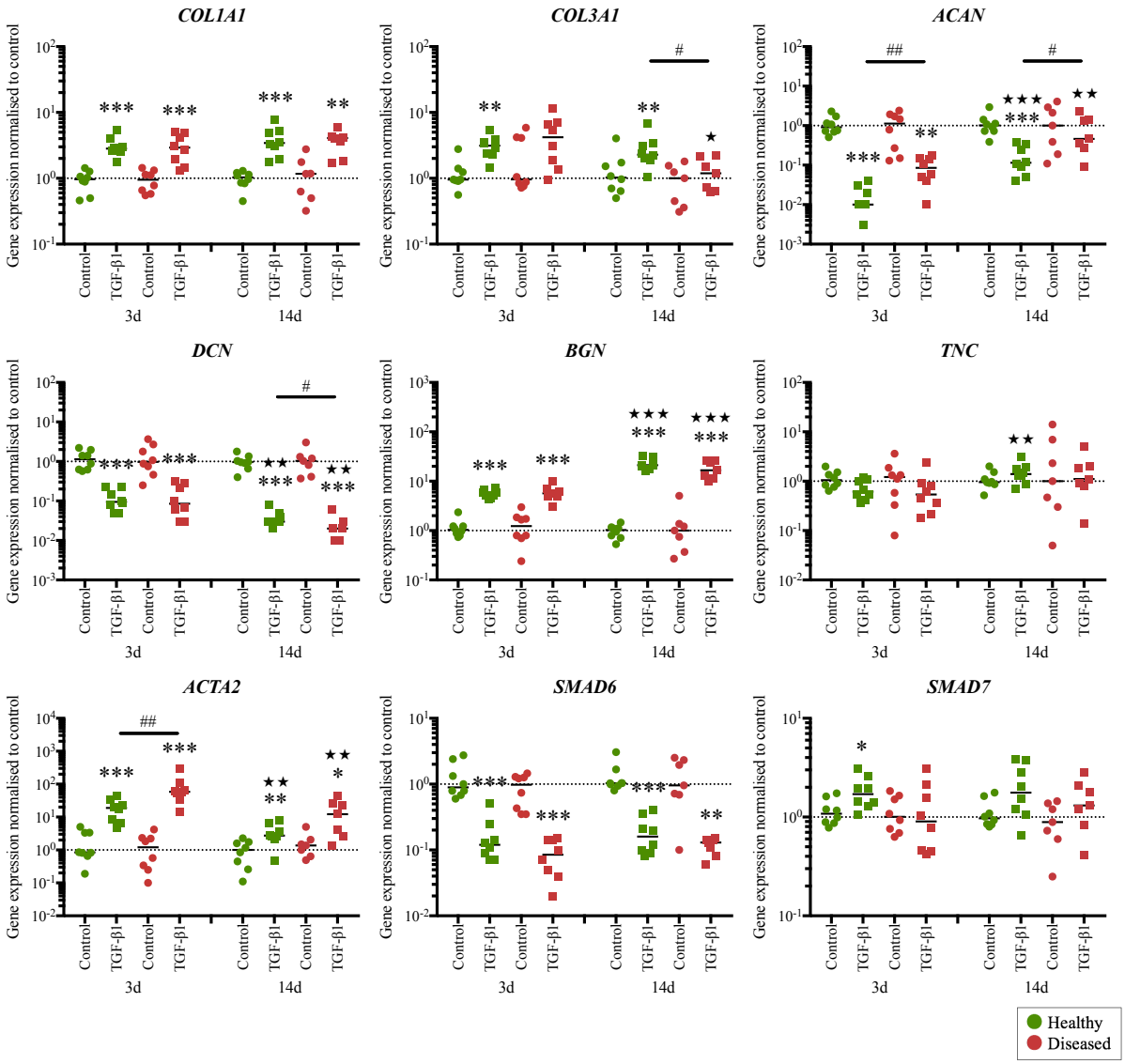



**FIGURE A33: mRNA expression of *COL1A1*, *COL3A1*, *ACAN*, *DCN*, *BGN*, *TNC*, *ACTA2*, *SMAD6* and *SMAD7* in cells derived from healthy and diseased tendons after treatment by TGF-β1 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) were quantified RT-qPCR, normalised to β-actin and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between tendon stromal cells from healthy and diseased tendons. ★ indicates significant difference between day 3 and day 14. d, day. */#/★=p<0.05, **/##/★★=p<0.01, ***/★★★=p<0.001.

<Data normalised to GAPDH>

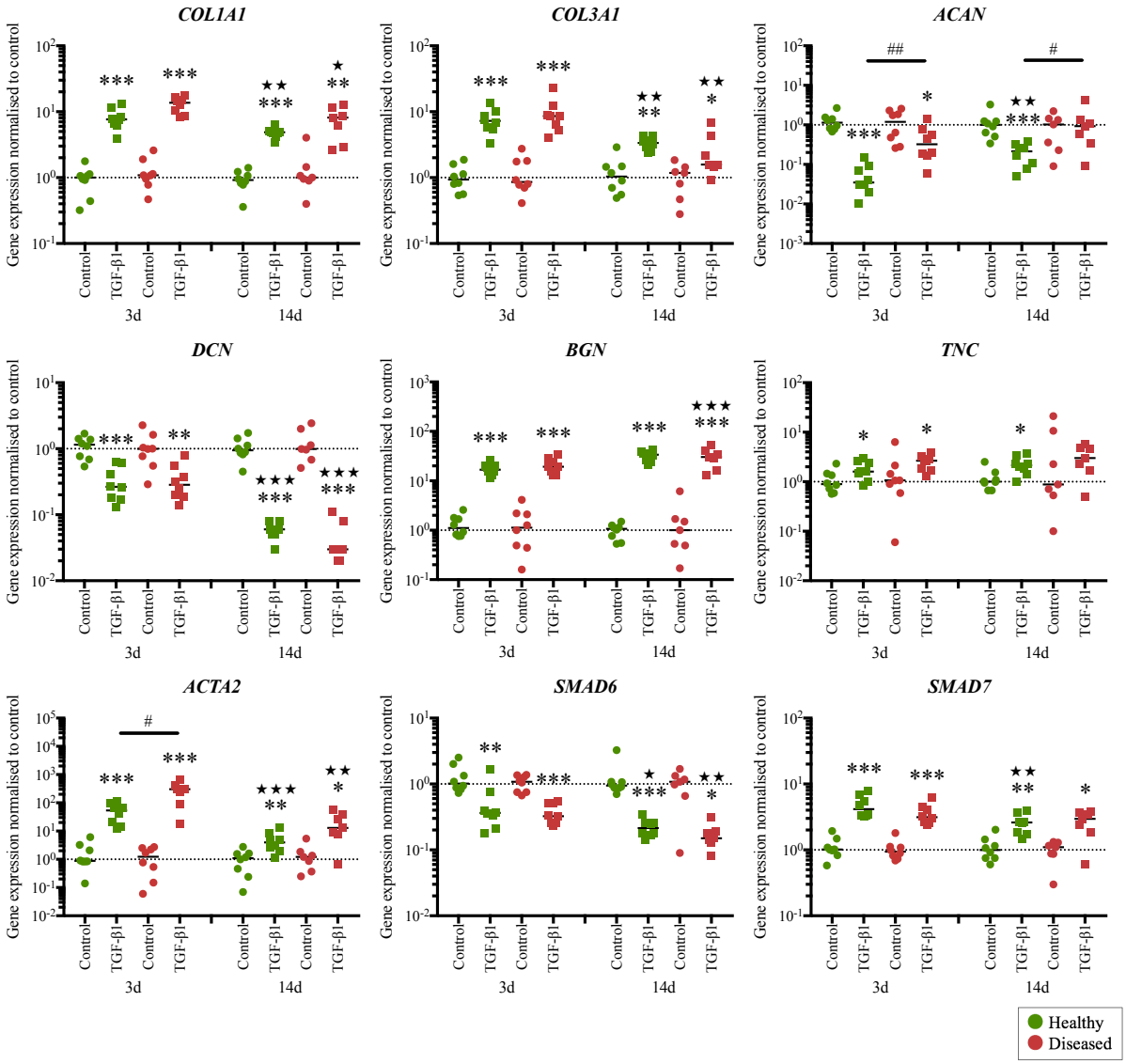



**FIGURE A34: mRNA expression of *COL1A1*, *COL3A1*, *ACAN*, *DCN*, *BGN*, *TNC*, *ACTA2*, *SMAD6* and *SMAD7* in cells derived from healthy and diseased tendons after treatment by TGF-β1 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) were quantified RT-qPCR, normalised to GAPDH and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between tendon stromal cells from healthy and diseased tendons. ★ indicates significant difference between day 3 and day 14. d, day. */#/★=p<0.05, **/##/★★=p<0.01, ***/★★★=p<0.001.

- Effects of BMP-2 on mRNA Expression of ECM and Cell Differentiation Genes

<Data normalised to β-actin>

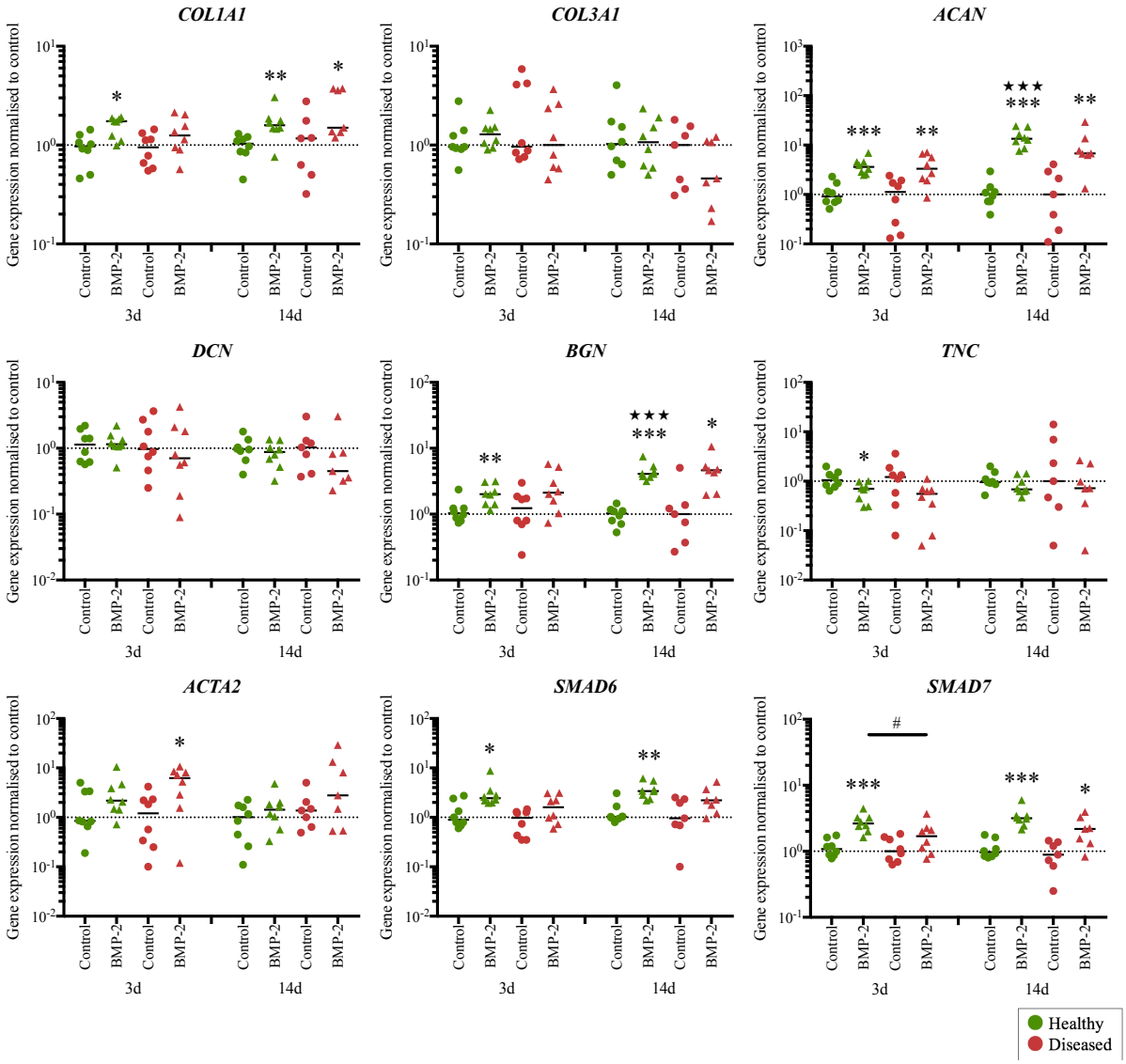



**FIGURE A35: mRNA expression of *COL1A1*, *COL3A1*, *ACAN*, *DCN*, *BGN*, *TNC*, *ACTA2*, *SMAD6* and *SMAD7* in cells derived from healthy and diseased tendons after treatment by BMP-2 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) were quantified RT-qPCR, normalised to β-actin and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between tendon stromal cells from healthy and diseased tendons. ★ indicates significant difference between day 3 and day 14. d, day. */#/★=p<0.05, **/★★=p<0.01, ***/★★★=p<0.001

<Data normalised to GAPDH>

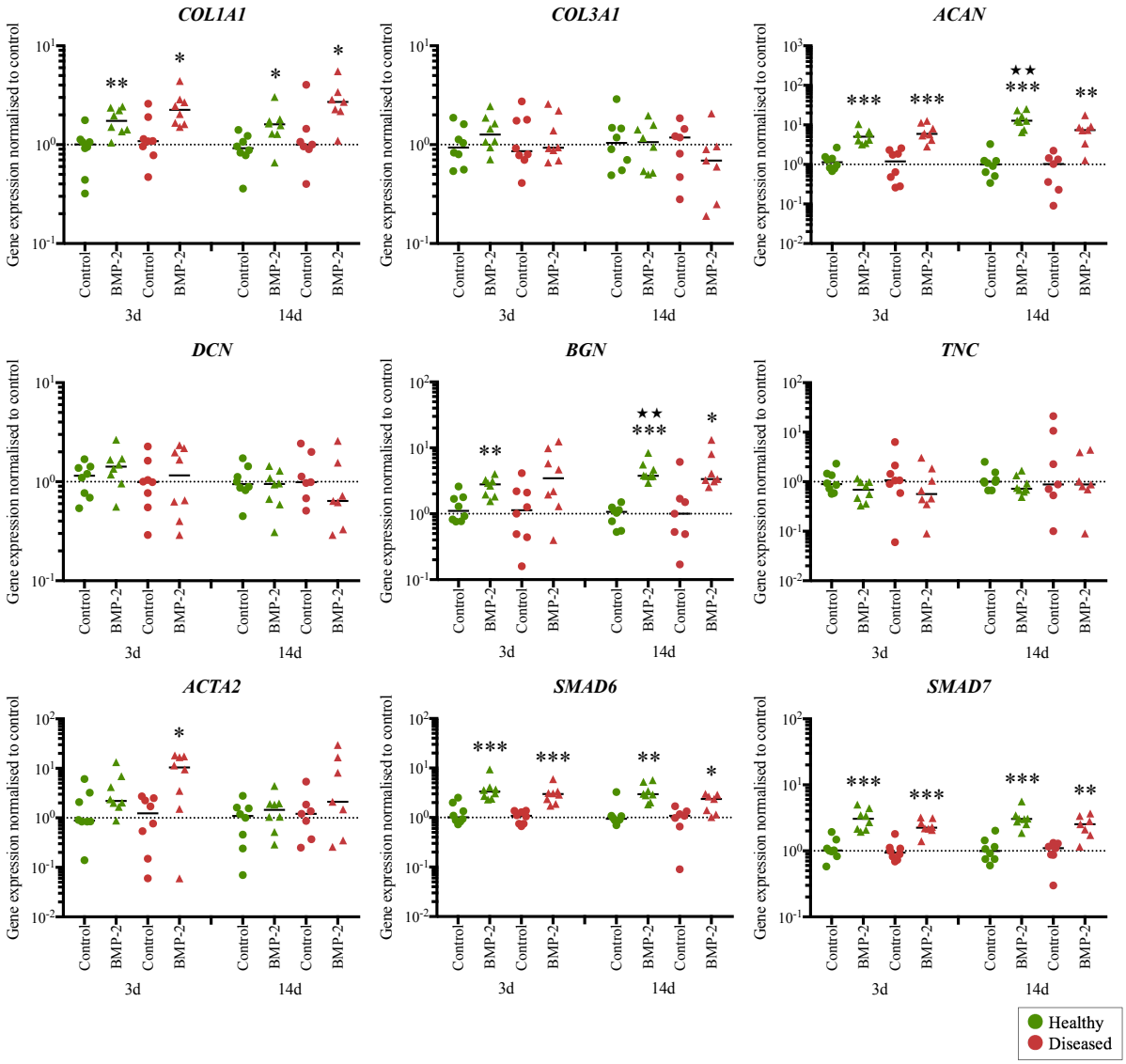


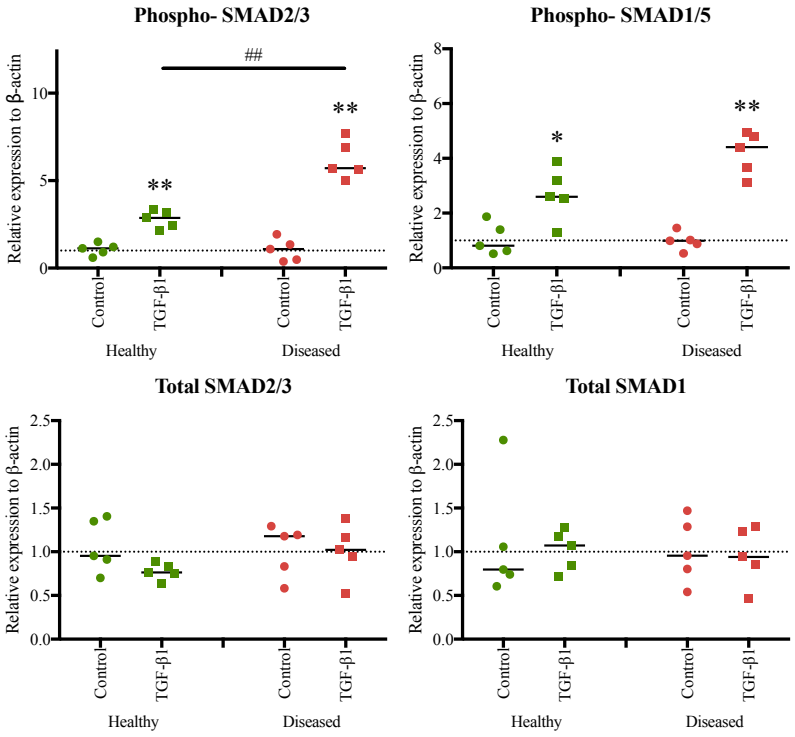

**FIGURE A36: mRNA expression of *COL1A1*, *COL3A1*, *ACAN*, *DCN*, *BGN*, *TNC*, *ACTA2*, *SMAD6* and *SMAD7* in cells derived from healthy and diseased tendons after treatment by BMP-2 or vehicle control (control).** Levels of gene expression in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) were quantified RT-qPCR, normalised to GAPDH and then to the median of the respective vehicle control ('control' on y axis). Bars shown represent median. * indicates significant difference to the respective vehicle control. ★ indicates significant difference between day 3 and day 14. d, day. */★=p<0.05, **/★★=p<0.01, ***=p<0.001

## Protein Expression Data

- Results of Semi-quantitative Analysis

<Cell Signalling Pathway Activity by TGF-β1 Treatment>

(A)




(B)

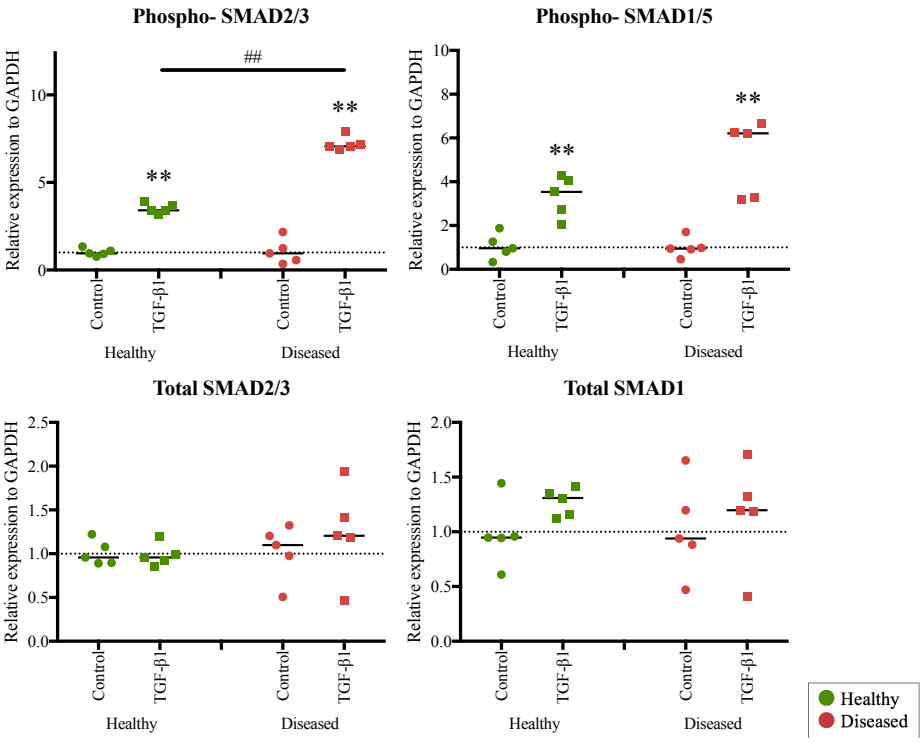
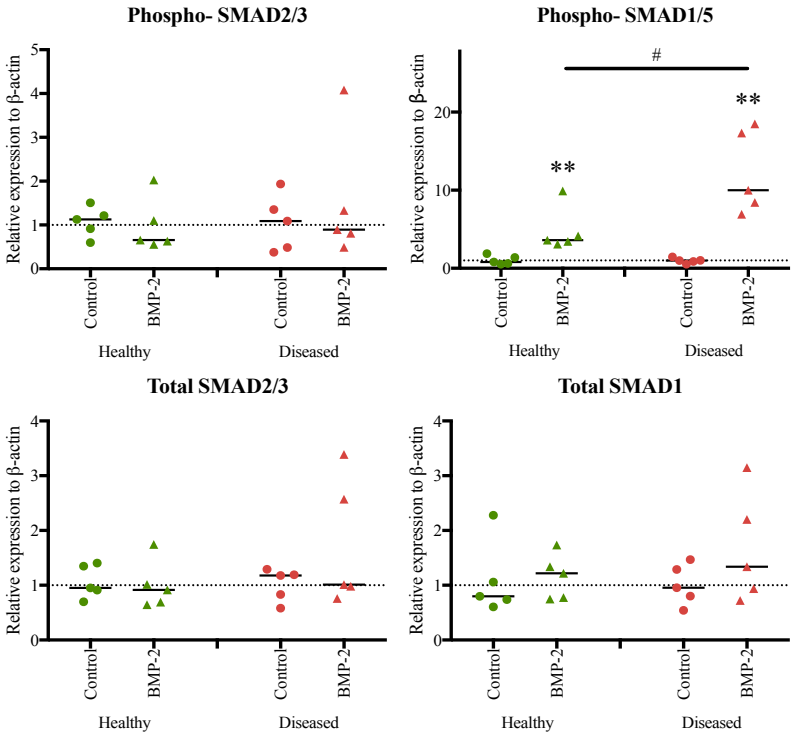



**FIGURE A37: Expression of phosphorylated (phospho-) and total SMAD2/3, phosphorylated (phospho-) SMAD1/5 and total SMAD1 in cells derived from healthy and diseased tendons after treatment by TGF-β1 or vehicle control (control).** Levels of protein expression in tendon stromal cells from healthy (n=5) and diseased tendons (n=5) were quantified by measuring the intensities of Western blots and normalised to (A) β-actin or (B) GAPDH. Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between tendon stromal cells from healthy and diseased tendons. */#=p<0.05, **/##=p<0.01

<Cell Signalling Pathway Activity by BMP-2 Treatment>

(A)




(B)

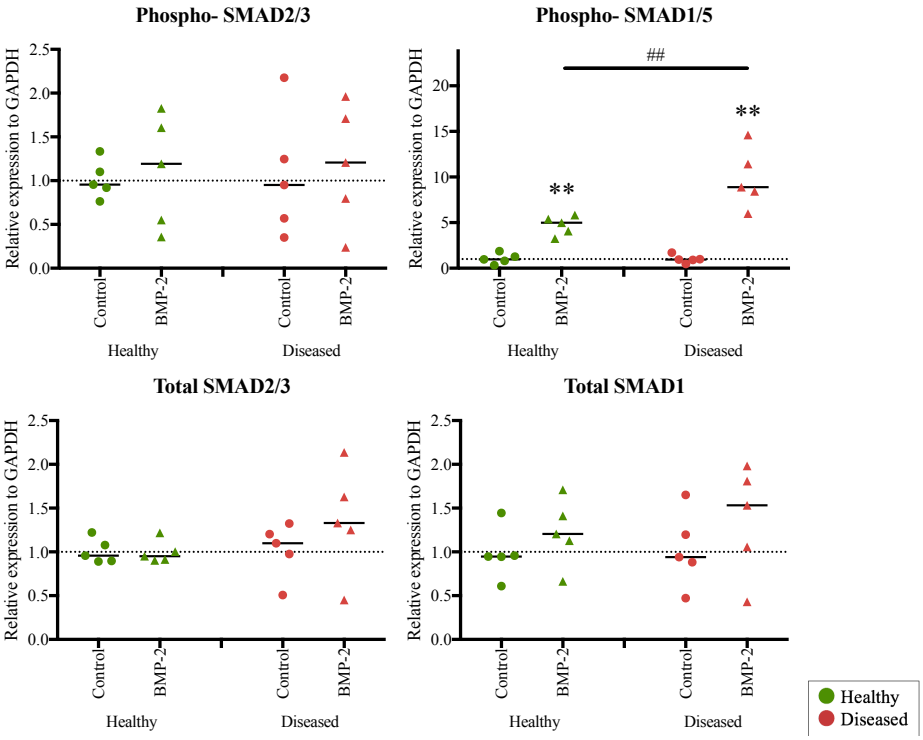
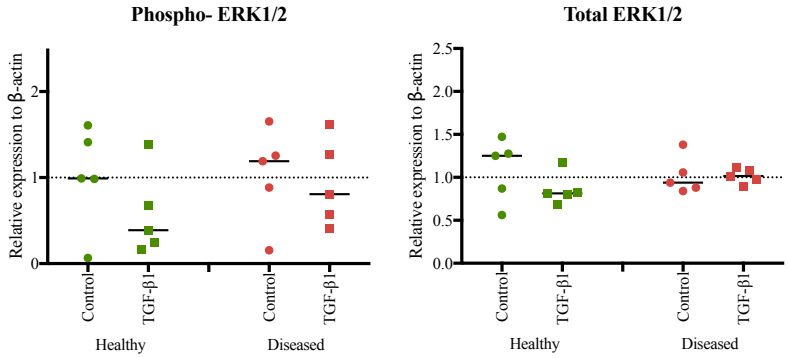


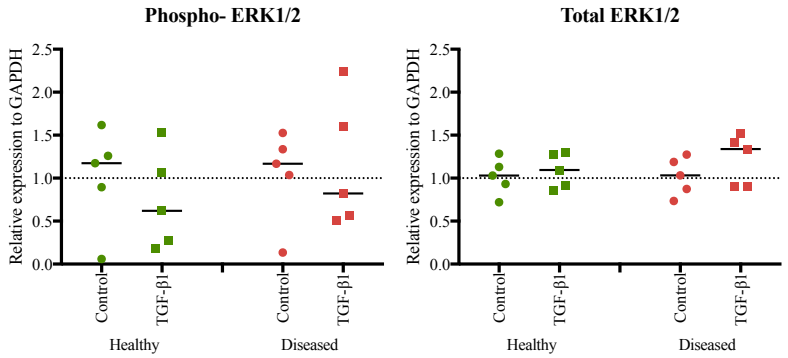

**FIGURE A38: Expression of phosphorylated (phospho-) and total SMAD2/3, phosphorylated (phospho-) SMAD1/5 and total SMAD1 in cells derived from healthy and diseased tendons after treatment by BMP-2 or vehicle control (control).** Levels of protein expression in tendon stromal cells from healthy (n=5) and diseased tendons (n=5) were quantified by measuring the intensities of Western blots and normalised to (A) β-actin or (B) GAPDH. Bars shown represent median. * indicates significant difference to the respective vehicle control. # indicates significant difference between tendon stromal cells from healthy and diseased tendons. */#=p<0.05, **/##=p<0.01

<ERK1/2 Signalling Pathway Activity by TGF-β1 Treatment>

(A)




(B)




(C)

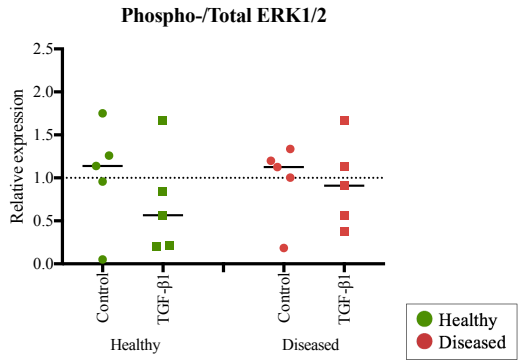
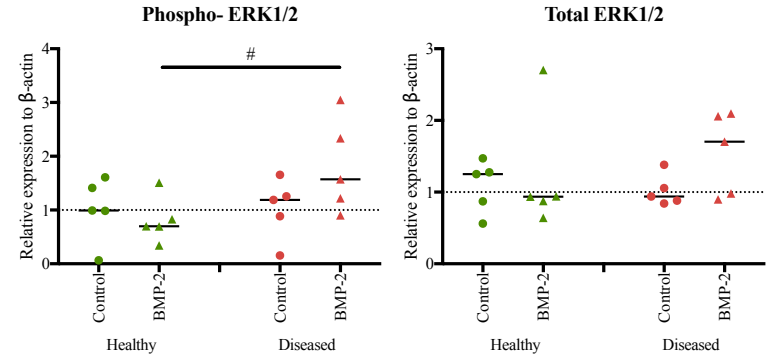


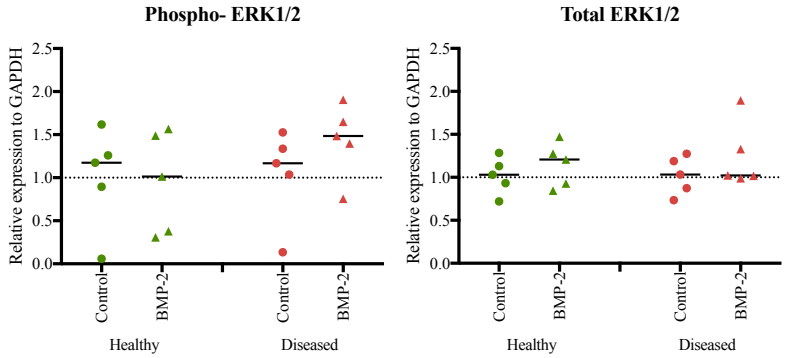

**FIGURE A39: Expression of phosphorylated (phospho-) and total ERK1/2 in cells derived from healthy and diseased tendons after treatment by TGF-β1 or vehicle control (control).** Levels of protein expression in tendon stromal cells from healthy (n=5) and diseased tendons (n=5) were quantified by measuring the intensities of Western blots and normalised to (A) β-actin, (B) GAPDH or (C) their respective total protein. Bars shown represent median.

<ERK1/2 Signalling Pathway Activity by BMP-2 Treatment>

(A)




(B)




(C)

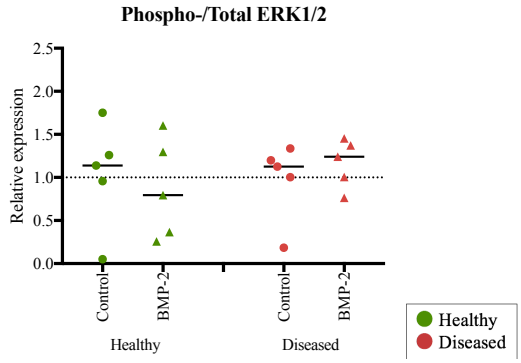
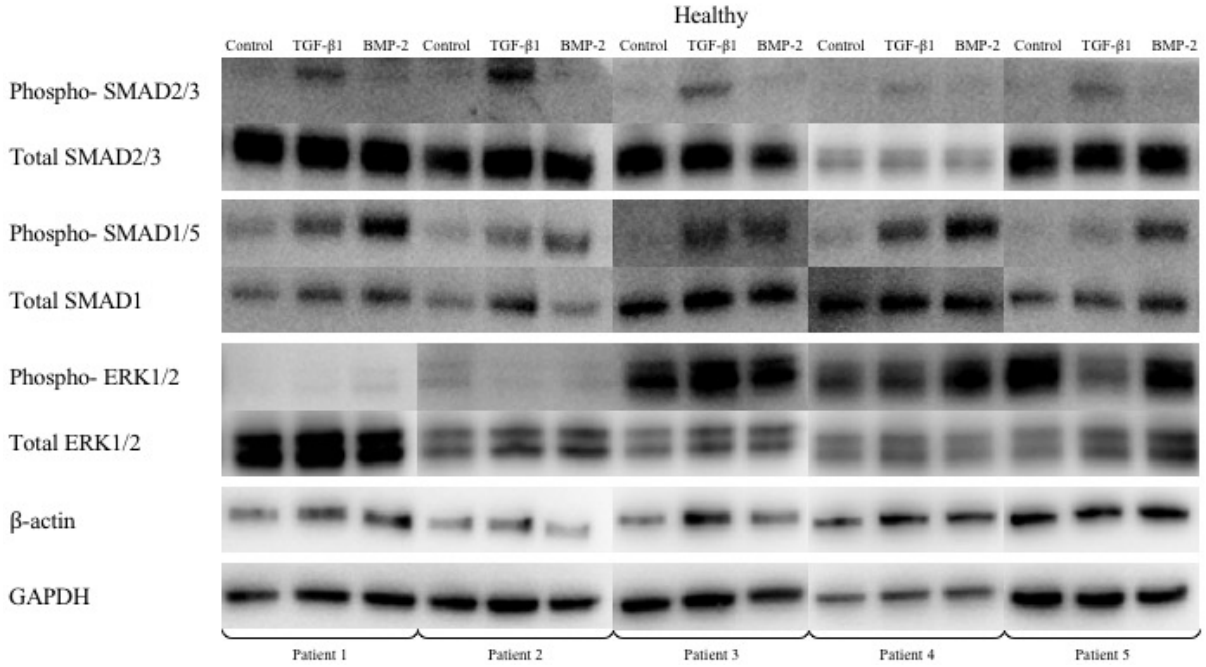



**FIGURE A40: Expression of phosphorylated (phospho-) and total ERK1/2 in cells derived from healthy and diseased tendons after treatment by BMP-2 or vehicle control (control).** Levels of protein expression in tendon stromal cells from healthy (n=5) and diseased tendons (n=5) were quantified by measuring the intensities of Western blots and normalised to (A) β-actin, (B) GAPDH or (C) their respective total protein. Bars shown represent median. # indicates significant difference between tendon stromal cells from healthy and diseased tendons. #=$p<0.05$

- Images of Western Blots

(A)




(B)

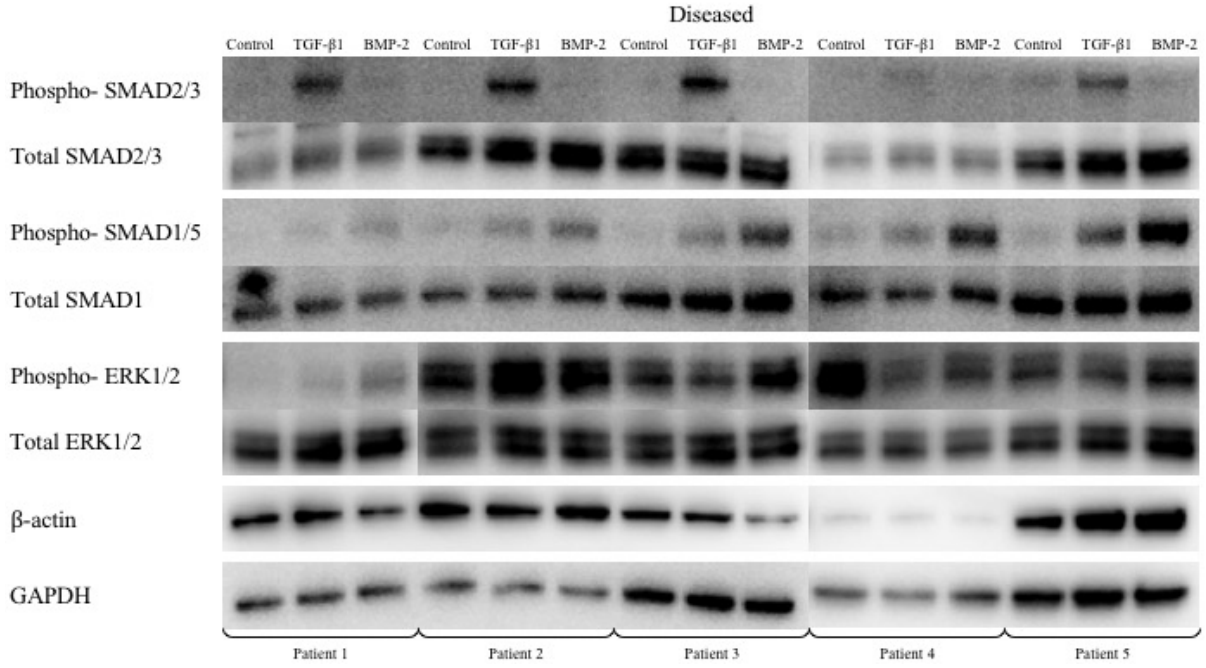



**FIGURE A41: Images of Western blotting of all investigated proteins in cells from (A) healthy and (B) diseased tendons after treatment by TGF-β1, BMP-2 or vehicle control (control).**

## Cell Viability Assay

Following serum-starving overnight, TSCs from healthy and diseased tendons cultured up to passages 3 (n=8 and n=8, respectively) were seeded in 24-well plates at a density of 30,000 cells ml-1 (0.5 ml), grown to sub-confluence and treated with human recombinant TGF-β1 (10 ng $ml^{-1}$) or vehicle control for 3 or 14 days. The experiment with BMP-2 treatment was carried out similar to that of TGF-β1 treatment.

At day 3, TGF-β1 treatment had proliferative effects on TSCs from diseased tendons only (p=0.0493). However, there were no significant differences in cell viability by TGF-β1 treatment between TSCs from healthy and diseased tendons. At day 14, TGF-β1 treatment had proliferative effects on TSCs from both healthy and diseased tendons (p=0.0262 and p=0.0006, respectively)

At day 3, BMP-2 treatment did not have any significant effects on cell viability in TSCs from both healthy and diseased tendons. At day 14, BMP-2 treatment had a proliferative effect on TSCs from diseased tendons only (p=0.0105).

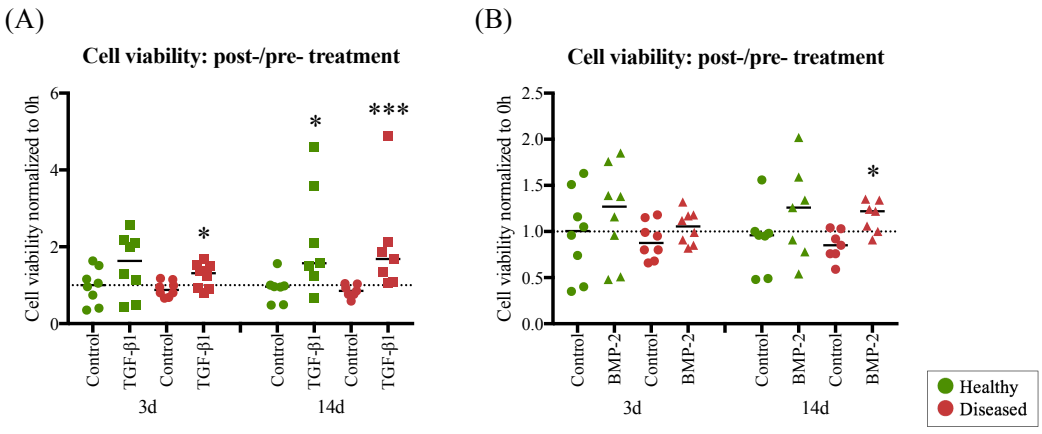



**FIGURE A42: Cell viability in cells derived from healthy and diseased tendons after treatment by (A) TGF-β1, (B) BMP-2 or vehicle control (control) for 3 or 14 days.** Cell viability was quantified in tendon stromal cells from healthy (n=8) and diseased tendons (n=8) by Alamar Blue assay and normalised to the median value of 0 hours. Bars shown represent median. * indicates significant difference to the respective treatment by vehicle control. *=p<0.05, **=p<0.01, ***=p<0.001.